

Doctorat de l'Université de Toulouse

préparé à Toulouse INP

Le sevrage du chiot, un stress alimentaire et social :
rôle du microbiote digestif et programmation nutritionnelle

Thèse présentée et soutenue, le 22 septembre 2025 par
Ilyas BENDAHMANE

École doctorale

SEVAB - Sciences Ecologiques, Vétérinaires, Agronomiques et Bioingenieries

Spécialité

Infectiologie, Physiopathologie, Toxicologie, Génétique et Nutrition

Unité de recherche

NeoCare

Thèse dirigée par

Annabelle Meynadier et Hanna Mila

Composition du jury

M. Frédéric Barbut, Rapporteur, Université Paris Cité. Unité de recherche

M. Laurent Naudon, Rapporteur, Université Paris-Saclay, INRAE

M. Bruno Guiard, Examineur, Université Toulouse III, CBI

Mme Stéphanie Blanquet, Examinatrice, Université Clermont Auvergne, INRAE

M. Dominique Peeters, Examineur, Faculté de Médecine Vétérinaire de Liège

Mme Annabelle Meynadier, Directrice de thèse, École Nationale Vétérinaire de Toulouse

Mme Hanna Mila, Co-directrice de thèse, École Nationale Vétérinaire de Toulouse

Avant-propos

« La vraie science est une ignorance qui se sait » **Michel de Montaigne**

Cette phrase résume avec justesse l'humilité que requiert toute démarche scientifique. À travers cette thèse, j'ai tenté, modestement, de dépeussier une infime parcelle de cette ignorance, d'explorer un recoin du savoir encore flou, dans l'espoir d'y apporter un peu de clarté. Car faire de la recherche, c'est avant tout accepter de se confronter à ce que l'on ne sait pas, pour mieux comprendre, pas à pas, ce que l'on pourrait savoir demain.

Remerciements

D'abord et avant tout, je souhaite commencer par remercier mes parents, véritables piliers de ma vie. Merci pour votre soutien sans faille, vos encouragements à toute épreuve et vos innombrables prières (qui, je pense, ont parfois eu plus d'effet que mes analyses statistiques). À mon père **Abdellatif Bendahmane**, pour son soutien financier et ses conseils de vie toujours pleins de sagesse... même s'ils commençaient souvent par "Moi, à ton âge...". Et à ma mère **Amina El Mehdi**, pour m'avoir appris des valeurs précieuses. Merci d'avoir cru en moi, et de m'avoir répété que « le savoir, c'est la vraie richesse » (juste après la santé et un bon couscous du vendredi).

Un proverbe arabe dit : « *Respecte énormément l'enseignant. L'enseignant a failli être un prophète.* » Ces mots résonnent profondément en moi, car je mesure aujourd'hui pleinement l'impact et la valeur de celles et ceux qui m'ont transmis leur savoir, leur passion, et leur confiance.

Je souhaite tout d'abord exprimer ma plus profonde gratitude à **Hamid Amarouch**, qui m'a accompagné dès les tout premiers pas de mon parcours au Maroc. Son soutien initial m'a permis d'avancer avec confiance.

Mes remerciements les plus chaleureux vont également à **Isabelle Castant-Laurelle**, dont l'engagement, la générosité et la passion pour l'enseignement ont été décisifs dans mon orientation. Elle m'a accueillie et soutenue depuis mes premiers jours à l'Université Paul Sabatier, et sans elle, je ne serais certainement pas là où je suis aujourd'hui. Elle a su me transmettre non seulement des connaissances, mais aussi l'amour du métier.

À **Bruno Guiard**, je souhaite adresser toute ma reconnaissance. Son enthousiasme contagieux, son immense savoir et sa capacité à rendre les neurosciences passionnantes ont profondément marqué mon parcours. Il est une source d'inspiration constante, et je le remercie sincèrement pour l'énergie positive qu'il m'a transmise tout au long de ces années.

Enfin, mes pensées les plus sincères et reconnaissantes vont à mes deux chères directrices de thèse, **Annabelle Meynadier** et **Hanna Mila**, qui ont cru en moi et ont joué un rôle essentiel dans mon développement personnel et scientifique.

À **Annabelle Meynadier**, je dis merci pour sa brillante intelligence, son dynamisme infatigable, son soutien sans faille et sa bienveillance constante. Elle a su me guider et m'inspirer chaque jour.

À **Hanna Mila**, je suis profondément reconnaissant pour la richesse de ses enseignements, sa patience, sa générosité, et cette bienveillance remarquable qui m'a soutenue dans les moments clés. Elle a su me transmettre un savoir-faire précieux, accompagné d'une énergie toujours positive.

À **Philippe Valet, Cedric Dray, Laure Verret, Etienne Chatelut, Romain Madelaine et Sébastien Bullich**. À chacun et chacune d’entre vous, merci d’avoir fait de ce parcours bien plus qu’un simple chemin académique. Vous avez contribué à faire de moi la personne et le chercheur que je suis aujourd’hui.

Je tiens à adresser mes remerciements les plus chaleureux aux membres du jury, **Bruno Guiard, Frederic Barbut, Laurent Naudon, Stéphanie Blanquet et Dominique Peeters** pour le temps qu’ils ont bien voulu consacrer à l’évaluation de mon travail. Je remercie tout particulièrement **Frédéric Barbut et Laurent Naudon** d’avoir accepté d’assumer le rôle de rapporteurs. Je mesure pleinement l’investissement que représente la relecture attentive d’un manuscrit de thèse, et je suis profondément reconnaissant.

Je tiens à adresser un grand merci à tous les membres de mon comité de suivi de thèse — **Olivier Zemb, Laurence Liaubet, Nuria Mach, Narjis Kraimi et Charlotte Paes** — pour la qualité de nos échanges, leurs conseils précieux, leurs remarques toujours pertinentes et bienveillantes, ainsi que pour leur accompagnement tout au long de ces années. Leur regard extérieur m’a permis de prendre du recul, d’approfondir mes réflexions, et d’enrichir considérablement ce travail.

Un immense merci à toute l’équipe **NeoCare** : Hanna Mila (la cheffe), **Aurélien Grellet** (le leader), **Laurie Milesie, Anaïs Hurteau, Célia Boudou, Muhammad Zaid Tahir, Coline Cebe, Julia Lundi Sato, Ugo Beaujardin, Chloé Chauvel, Rayan Ragonneau et Camille Arias**.

Merci pour la bonne humeur constante, les moments partagés, les échanges (scientifiques ou complètement improbables), et pour certains... ces fameuses nuits de surveillance des mises bas, où l’on a partagé fatigue, stress, café... et parfois même quelques sueurs froides face à des comportements maternels très protecteurs (clin d’œil à Hanna, qui a été témoin de mon moment de solitude et de quasi-sacrifice héroïque).

C’était un privilège de faire partie de cette équipe, et en tant que deuxième plus vieux membre après **Hanna Mila**, je suis fier de passer le flambeau. Comme **Laurie Milesie** partira avant ma soutenance, le titre de “**doyenne bis**” reviendra officiellement à **Anaïs Hurteau** — toutes mes félicitations pour cette promotion inattendue !

Travailler à vos côtés, c’était un vrai privilège, et clairement, le meilleur environnement possible, avec les meilleures conditions humaines et professionnelles. Si toutes les équipes pouvaient ressembler à celle-là, on signerait les yeux fermés (enfin... sauf face à une chienne en plein travail).

Je tiens à remercier chaleureusement **Sylvie Chastant**, une personne **brillante**, dont la gentillesse et l’énergie communicative ont grandement enrichi ma première année à NeoCare. Merci pour ta bienveillance, ton esprit d’équipe et ta bonne humeur — une qualité rare et précieuse.

Un grand merci également à **Quentin Garrigues**, le doctorant NeoCare de la promo d’avant, qui m’a transmis ses conseils, ses astuces... et surtout **ses cartes mentales de géographie** (c’est bien connu, il n’y

a pas un seul recoin de la planète qu'il ne connaisse pas). Grâce à toi, mes débuts ont été beaucoup plus simples — sauf quand il fallait te suivre dans une discussion sur la capitale de Sri-Lanka !

Merci aussi à **Amélie Mugnier** pour sa gentillesse, sa disponibilité, sa bonne humeur et ses conseils toujours avisés, qui m'ont beaucoup aidé au début de la thèse, notamment dans l'organisation du projet.

Je tiens à remercier chaleureusement **tous les membres de l'équipe GenPhySE** pour leur accueil et leur bienveillance tout au long de ces trois années. Un grand merci à **Marie-Luce Chemit** pour sa précieuse aide dans la gestion des manipulations de laboratoire, sa disponibilité et sa rigueur, qui ont grandement facilité le bon déroulement de la partie expérimentale. Un grand merci à **Annabelle Meynadier, Tiphaine Blanchard, Quentin le Graverand** pour la bienveillance. C'était un plaisir d'avoir travaillé avec vous.

Je tiens à exprimer ma profonde gratitude à la **Fondation VISIO** pour le financement généreux de ce travail de recherche. Sans leur soutien, ce projet n'aurait pas pu voir le jour. Je remercie également chaleureusement l'**École Nationale Vétérinaire de Toulouse** pour m'avoir accueillie durant ces trois années. J'ai pu y bénéficier d'un environnement scientifique stimulant, ainsi que d'un accompagnement de grande qualité, qui ont grandement contribué à la réalisation de cette thèse.

Je tiens à adresser mes plus sincères remerciements aux étudiants que j'ai eu le plaisir d'encadrer au cours de ces dernières années. Merci à **Emma Vellard, Alizée Ammar** et **Lucy Montagnier** pour leur rigueur, leur curiosité scientifique et leur sens de l'engagement. Merci également à **Anaïs Deloste, Kenza Kezimana**, et **Milan Rotter** pour leur investissement lors de leurs stages ; vos projets respectifs ont été essentiels dans l'avancée de mes recherches. J'ai beaucoup appris à vos côtés, et je suis très reconnaissant d'avoir partagé avec vous cette belle aventure scientifique.

Un grand merci à mes amis **Achraf Boulfrad, Fatma Khelifi** et **Soukaina**, qui ont été bien plus que de simples camarades d'études... Ce sont mes complices de galère, de fou rire, et parfois de désespoir académique. Merci à mon cher ami et frère **Achraf Boulfrad**, pour tous les moments passés ensemble : nos aventures parisiennes dignes d'un court-métrage, nos discussions interminables (et parfois très confuses) sur la physiologie, l'anatomie, la pharmacologie, et sur la vie en général. Avec toi, même apprendre l'innervation du côlon devenait presque fun. Merci à **Fatma Khelifi**, la toute première personne que j'ai rencontré à Toulouse. Une amie en or, une sœur, une partenaire de révision... et de rigolade surtout. Merci pour tous les bons moments à la fac, les fous rires incontrôlables (et pas toujours appropriés en amphithéâtre). Et bien sûr, merci à **Soukaina** — ou plutôt Soukiiina (oui, je rajoute toujours quelques "i", c'est plus affectueux... ou agaçant, ça dépend des jours). Elle, c'est celle à qui je répétais sans cesse que Toulouse

c'est mieux que Bordeaux, juste pour l'énervier (ça marche à tous les coups). Merci pour ton amitié, ton humour, et ta capacité à me supporter malgré mes provocations.

Et bien sûr, un immense merci à **Zahra Fahim**, une amie en or que j'ai rencontrée il y a maintenant 7 ans — oui, 7 ans d'amitié solide comme les pyramides, de rires, de soutien et de thé (toujours bien sucré, comme en Égypte !). Zahra, merci pour ta présence constante et ta sagesse de grande sœur.

Un grand merci aussi à son mari, **Hussein Allam**, un homme remarquable qui, lors d'un séjour à Barcelone, m'a appris le nom de chaque plante, arbre, buisson et herbe du coin. Grâce à lui, je peux aujourd'hui me balader comme un expert en botanique méditerranéenne (même si j'ai oublié la moitié... mais c'est l'intention qui compte).

Et enfin, une dédicace spéciale à la petite **Zainab**, leur fille, adorable et drôle. À elle seule, elle mérite une médaille pour sa capacité à faire fondre tout le monde avec un simple sourire.

Un énorme merci à **Kaoutar El Khalifa**, mon amie d'enfance depuis maintenant 13 ans — une amitié qui a survécu à l'adolescence, aux examens, aux crises existentielles... Brillante, inspirante, et aujourd'hui dentiste (oui, je sais, personne n'est parfait). On se voit un peu moins depuis qu'elle pratique en chirurgie dentaire... disons que c'est peut-être aussi parce que j'ai une peur irrationnelle des dentistes (même quand ce sont mes amis proches). Mais ça ne change rien : Kaoutar, tu restes l'une des personnes les plus précieuses pour moi (tant que tu es loin de ma cavité buccale).

Merci à **M hammed Chaoui Roqai** et **Nadia el Jaouhari** pour les deux années enrichissantes passées à leurs côtés à l'EHEB de Casablanca. Ce fut un point de départ solide et inspirant, qui a éveillé en moi le goût d'apprendre et m'a donné l'élan nécessaire pour poursuivre ma quête de connaissance.

Événement scientifique EHEB 2018

Cet événement a été couronné par la remise d'un prix d'excellence au meilleur poster 2e année EHEB et 3e année EHEB.

Les gagnants sont :

*2ème année EHEB : Mr Ilyas Bendahmane Thème » Virus HPV »

*3ème année EHEB : Mlle Marwa El Mennouni Thème » La maladie coeliaque »

Félicitations à nos gagnants – futurs professionnels et peut être chercheurs scientifiques .



Je garde un souvenir particulièrement marquant de l'événement scientifique EHEB 2018, où j'ai eu l'honneur de recevoir le prix du meilleur poster. À l'époque, la direction écrivait : « Félicitations à nos gagnants — futurs professionnels et peut-être chercheurs scientifiques ». <https://www.old.eheb.ma/blog/evenement-scientifique-eheb-2018>. Huit ans plus tard, je mesure avec émotion à quel point cette prédiction s'est réalisée.

À ma sœur **Rihame Bendahmane**, la doyenne du groupe (oui oui, la vieille de la famille — c'est elle qui a le plus de souvenirs... et le plus de remarques à faire). Merci d'avoir couvert toutes mes bêtises d'enfance sans jamais les divulguer (ou presque). Ta loyauté vaut de l'or... surtout quand elle m'évite des ennuis !

À **Ayoub Bendahmane**, mon petit frère de 23 ans — même si tu n'es plus si "petit". Motivé comme jamais, avec un sens du business inné depuis qu'il est tout petit. Toujours prêt à lancer une idée, un projet, ou au moins un plan pour arrondir les fins de mois.

À **Raounaq Bendahmane**, la petite perle de 8 ans, déjà ambitieuse, brillante, et avec une répartie qui ferait pâlir un adulte. Elle a réponse à tout, des rêves plein la tête, et parfois des arguments plus solides que les miens.

Et enfin, à **Amjad Bendahmane**, notre petit miracle de 1 an, la surprise de fin du scénario familial — adorable, drôle, et capable de captiver toute la maison juste en pointant du doigt un biscuit. Il ne parle pas encore, mais il dirige déjà la maison comme un chef.

Un immense merci à toute la grande **famille Bendahmane** et la **famille El Mehdi**, réunies sous l'aile bienveillante de Mii « **Khadija Regragui** » notre mémoire vivante et le cœur battant de plusieurs générations.

Un clin d'œil complice à la brigade des fous rires – **Hanane, Nada Rihame, Sahar, Achraf, Inès, Widad, Ayoub, Aya, Rayan, Adam, Oumnia** et **Nizar** – avec qui j'ai vécu des moments inoubliables. Entre les soirées Loups-Garous pleines de trahisons amicales, les tricheries dans le jeu de 'Sota Mskhota', et nos débats improvisés sur qui triche le plus... Comment ne pas mentionner l'**épisode mémorable de Widad** – cascadeuse officielle de la famille – et cette fameuse journée où, après un accident de moto, elle nous a regardés avec un air de "Vous êtes qui déjà ?". Grâce à notre sang-froid avec **Sahar et Nada**, cette mission sauvetage a tourné au miracle collectif. Un moment de stress... transformé en souvenir d'équipe drôle « Très drôle ».

Enfin, avec émotion, je dédie cette thèse à ceux qui nous ont quittés trop tôt, mais qui continuent d'habiter chaque étape de ma vie : **Mehdi Kechabi**, parti à 27 ans, et les jeunes **Mohammed Amine El Mehdi, Aimran El Mehdi**, et **Yassmina El Mehdi**.

Et je garde le meilleur pour la fin. Je souhaite exprimer toute ma gratitude à ma femme, **Khadija Ajahiou Zinoun**, pour son soutien indéfectible tout au long de ces deux dernières années. Ta présence à mes côtés, en particulier durant les moments de doute et de fatigue, a été essentielle. Merci pour ta patience face aux longues journées, aux soirées absorbées par l'écriture, aux absences mentales lorsque j'étais plongé dans mes analyses. Merci pour tes mots réconfortants, ton écoute, ton calme, et surtout ta foi constante en moi, même lorsque moi-même j'en doutais. Ta force m'a soutenu, et ta présence a rendu ce chemin possible. Cette thèse, je la dédie en partie à toi, car sans toi, elle n'aurait pas eu la même profondeur, ni la même lumière.

Abréviations

SSN/SNW : Sevrage social et nutritionnel

1SPS/1WPW : 1 semaine post-sevrage

SB : *Saccharomyces var.boulardii* CNCM I-1079

AGCC/SCFA : Acides gras à chaînes courtes

CPV : Parvovirus canin

CCV : Coronavirus canin

MICI/IBD : Maladie inflammatoire chronique de l'intestin

PPQ : Prise de poids quotidien.

PP : Prise de poids

TDAH : Trouble déficitaire de l'attention avec hyperactivité

ACP/PCA : Analyse en composantes principales

AFE/EFA : Analyse Factorielle Exploratoire

AFC/CFA : Analyse Factorielle Confirmatoire

CI/ICC : Corrélation intra-classe

Résumé

Le sevrage est une étape critique du développement du chiot, marquée par une double séparation : sociale (mère et fratrie) et nutritionnelle (lait maternel). Cette période du sevrage social et nutritionnel (SSN) s'accompagne d'une forte vulnérabilité physiologique. La diarrhée y est l'un des troubles les plus fréquents, mais ses facteurs de risque restent encore mal identifiés chez le chiot. Parmi les pistes prometteuses, l'axe santé-microbiote-cerveau, qui relie la santé digestive, le microbiote et le comportement, semble particulièrement pertinent pour mieux comprendre ce risque digestif au sevrage. Cette thèse visait donc (i) à explorer les liens entre comportement, microbiote intestinal et risque de diarrhée chez le chiot au moment du sevrage, et (ii) à évaluer l'effet d'une supplémentation maternelle en probiotiques sur cet axe. Pour le premier objectif, 113 chiots nés par voie naturelle de 18 chiennes Golden Retriever ont été suivis dans une même maternité, de la naissance jusqu'à une semaine après le sevrage. Pour le second, une autre cohorte de 17 chiennes de quatre races (Golden Retriever, Labrador Retriever, Berger Blanc Suisse, Berger Australien) et leurs 105 chiots, suivis dans un élevage de l'ovulation jusqu'à 56 jours de vie. Une synthèse bibliographique a d'abord mis en évidence l'importance du microbiote et du comportement dans la vulnérabilité digestive, sur la base de modèles animaux proches du chiot. Une première étude a ensuite évalué les associations entre tempérament et santé digestive à une semaine post-sevrage. Deux profils comportementaux ont été identifiés : audacieux et timide. Les chiots audacieux comparés aux timides présentaient un taux d'ingestion et de croissance plus faible, un microbiote enrichi en bactéries pro-inflammatoires (*Campylobacter*, *Prevotellaceae sagatella* et un genre inconnu) et appauvri en bactéries lactiques (*Lactobacillus*, *Ligilactobacillus*, *Enterococcus*). En revanche, le lien avec le risque de troubles digestifs n'a pas été clairement établi. Nous avons également mis en évidence l'existence d'un entérotipe à risque accru de diarrhée, enrichi en bactéries pro-inflammatoires (*Campylobacter*, *Helicobacter*, *Mucispirillum*, *Sutterella*) comparé à l'entérotipe sain. De plus, l'abondance précoce augmentée d'*Enterococcus* pourrait constituer un marqueur de cet entérotipe à risque. Enfin, la supplémentation maternelle en *Saccharomyces boulardii* durant la gestation et la lactation a permis de réduire cette abondance des *Enterococcus* et d'améliorer la consistance fécale des chiots à la fin de la période néonatale. Ces résultats apportent de nouvelles connaissances sur l'association du microbiote avec le risque de diarrhée au sevrage, et sur le potentiel de la supplémentation maternelle en probiotiques à prévenir ce risque. D'autres études sur une large population de chiots sont nécessaires pour confirmer cette découverte préliminaire et permettre de lutter plus efficacement contre la diarrhée au sevrage.

Mots clés : Chiot, Diarrhée du sevrage, Microbiote intestinal, Tempérament, Programmation nutritionnelle, Levure.

Abstract

Weaning is a critical stage in a puppy's development, marked by both social (from the mother and littermates) and nutritional (from maternal milk) separation. This period, referred to as social and nutritional weaning (SNW), is accompanied by high physiological vulnerability. Diarrhea is one of the most frequently observed disorders during this time, yet the associated risk factors remain poorly identified in puppies. Among the emerging hypotheses, the health–microbiota–brain axis—which links digestive health, gut microbiota, and behavior—appears particularly promising for better understanding digestive risk at weaning. This thesis therefore aimed (i) to explore the relationship between behavior, gut microbiota, and diarrhea risk in puppies at the time of weaning, and (ii) to evaluate the impact of maternal probiotic supplementation on this axis. For the first objective, 113 puppies born naturally from 18 Golden Retriever dams were followed in the same maternity unit, from birth to one-week post-weaning. For the second, a separate cohort of 17 dams from four breeds (Golden Retriever, Labrador Retriever, White Swiss Shepherd, and Australian Shepherd) and their 105 puppies were monitored in a single breeding facility, from ovulation to 56 days of age. A literature review first highlighted the importance of the microbiota and behavior in digestive vulnerability, based on animal models physiologically close to puppies. A first study then assessed the associations between temperament and digestive health at one-week post-weaning. Two behavioral profiles were identified: bold and shy. Compared to shy puppies, bold individuals exhibited lower food intake and growth rates, a microbiota enriched in pro-inflammatory bacteria (*Campylobacter*, *Prevotellaceae sagatella*, and an unknown genus), and depleted in lactic acid bacteria (*Lactobacillus*, *Ligilactobacillus*, and *Enterococcus*). However, the link to digestive risk was not clearly established. We also identified the presence of an enterotype associated with a higher risk of diarrhea, characterized by an enrichment in pro-inflammatory bacteria (*Campylobacter*, *Helicobacter*, *Mucispirillum*, and *Sutterella*) compared to a “healthy” enterotype. Additionally, early-life abundance of *Enterococcus* may serve as a potential marker for this at-risk enterotype. Finally, maternal supplementation with *Saccharomyces boulardii* during gestation and lactation reduced *Enterococcus* abundance and improved fecal consistency at the end of the neonatal period.

These findings provide new insights into the association between gut microbiota and weaning-related diarrhea risk and highlight the potential of maternal probiotic supplementation as a preventive strategy. Further studies on larger puppy populations are needed to confirm these preliminary findings and improve prevention of weaning-associated diarrhea.

Keywords: Puppy, weaning diarrhea, gut microbiota, temperament, nutritional programming, yeast.

Table des matières

Avant-propos	1
Remerciements	3
Abréviations	9
Résumé	10
Abstract	11
Valorisation scientifique	14
Chapitre 1 : Introduction générale	17
Chapitre 2 : Contexte scientifique	19
Le sevrage chez le chiot : un stress nutritionnel et social	19
Chapitre 3 : Objectifs et hypothèses de travail	41
Chapitre 4 : Validation de la méthode d'évaluation du score fécal via images photographiques	43
Article 1 : Use of stool photographs for fecal scoring and diagnosis of diarrhea in the puppy: validation of the method	45
Chapitre 5 : Étude du lien entre le risque de troubles digestives - microbiote et comportement	60
Article 2 : Two distinct temperaments in puppies one-week post-weaning: associations with stress, nutrition and fecal quality	62
Article 3 : Association between fecal microbiota and temperament traits in Golden Retriever puppies one-week post-weaning	90
Article 4 : Early-life gut microbiota as a potential indicator of weaning-enterotypes associated with diarrhea susceptibility in puppies	117
Annexes Chapitre 5	142
Chapitre 6 : Modulation de la santé des chiots à travers la programmation nutritionnelle	172
Article 5 : Effect of maternal oral supplementation with <i>Saccharomyces boulardii</i> I-1079 on the microbiota and fecal score of puppies during the first 8 weeks of Life	174
Article 6 : Maternal oral supplementation with <i>Saccharomyces boulardii</i> I-1079 during gestation and early lactation impacts the early growth rate and metabolic profile of newborn puppies	193
Annexes Chapitre 6	207
Chapitre 7 : Discussion générale	211
Chapitre 8 : Conclusion générale	223
Annexes de thèse	224

Poster 1 : Early dynamics of the gut microbiota in golden retriever puppies during the first 12 hours of life : A preliminary study	224
Références	227

Valorisation scientifique

Publications scientifiques

Ilyas Bendahmane, Quentin Garrigues, Emmanuelle Apper, Amelie Mugnier, Ljubica Svilar, Jean-Charles Martin, Sylvie Chastant, Annabelle Meynadier, Hanna Mila. Maternal oral supplementation with *Saccharomyces boulardii* I-1079 during gestation and early lactation impacts the early growth rate and metabolic profile of newborn puppies. **Front. Nutr.** 2025 ; 12:1500600. doi: 10.3389/fnut.2025.1500600

Ilyas Bendahmane, Milla Salonen, Océane Richard, Lucy Montagnier, Kenza. Kezimana, Faouzi Lyazrhi, Hanna Mila, Annabelle Meynadier. Two distinct temperaments in puppies one-week post-weaning: Association with stress, nutrition and fecal quality. Soumis dans **J. Animal behavior**

Chloé Chauvel*, Ilyas Bendahmane*, Emma Vellard, Faouzi Lyazrhi, Annabelle Meynadier, Hanna Mila, Aurélien Grellet. Use of stool photographs for fecal scoring and diagnosis of diarrhea in the puppy: validation of the method. Soumis dans **J. Preventive Veterinary Medicine**

Ilyas Bendahmane, Annabelle Meynadier, Hanna Mila. Association between fecal microbiota and temperament traits in Golden Retriever puppies one-week post-weaning. À soumettre dans **J. Applied Animal Behavior Science**

Ilyas Bendahmane, Faouzi Lyazrhi, Annabelle Meynadier, Hanna Mila. Early-life microbiota as a potential indicator of weaning-enterotypes associated with diarrhea susceptibility in puppies. À soumettre dans **J. Microbial Pathogenesis**

Ilyas Bendahmane, Faouzi Lyazrhi, Annabelle Meynadier, Hanna Mila. Effect of maternal oral supplementation with *Saccharomyces boulardii* I-1079 on the Microbiota and fecal score of puppies during the first 8 week of life. À soumettre dans **J. Probiotics and Antimicrobial Proteins**

Ilyas Bendahmane, Aurélien Grellet, Annabelle Meynadier, Hanna Mila. Digestive and behavioral disorders in puppies at weaning and prevention tools. À soumettre dans **J. Animal Health Research Review**

* = Co-premiers auteurs

Présentations orales : Congrès internationaux

Relationship between temperament and fecal microbiota in Golden Retriever puppies one-week post-weaning. **International Scientific Conference on Probiotics, Prebiotics, Gut Microbiota and Health (IPC2025)**, Athènes, Grèce, 24-26 Juin 2025 – Emerging Talent Award – 2ème place

Maternal Oral Supplementation with *S. boulardii* I-1079 during gestation impacts the newborn puppies' metabolome and growth. <https://amu.hal.science/hal-04890248v1>. **European Society of Veterinary and Comparative Nutrition (ESVCN)**, Belfast, Irlande, 11-15 Septembre 2024

Maternal Oral Supplementation with *S. boulardii* I-1079 during gestation impacts the newborn puppies' metabolome and growth. **European Veterinary Society For Small Animal Reproduction (EVSSAR)**, 27-29 Juin 2024

Effect of maternal supplementation with live yeast *S. boulardii* I-1079 on the gut microbiota of newborn puppies. <https://doi.org/10.1111/rda.14437>. **European Symposium on Animal Reproduction (ESAR)**, Nantes, France, 21-23 Septembre 2023

Présentations orales : Vulgarisation scientifique

- **MT180** : vulgarisation de mes travaux de recherche (<https://www.youtube.com/watch?v=sHKFM-8ctKc&t=6s>)
- **Soirée NeoCare** : Comment la supplémentation maternelle peut influencer la santé du chiot nouveau-né ?

Présentation de Poster:

Evolution of puppy behavior around t7/15/25 3:29:00 PM the adoption period: Study on a cohort of 62 Golden Retriever puppies. https://www.ecbb.uzh.ch/assets/pdf/Conference_book.pdf **European Conference on Behavioural Biology (ECBB)**, Zurich, Suisse, 16-19 July 2024.

Early dynamics of the gut microbiota in Golden Retriever puppies during the first 12 hours of life: A preliminary study. **International Scientific Conference on Probiotics, Prebiotics, Gut Microbiota and Health (IPC2025)**, Athènes, Grèce, 24-26 Juin 2025.

Early dynamics of the gut microbiota in Golden Retriever puppies during the first 12 hours of life: A preliminary study. **European Society for Small Animal Reproduction (EVSSAR)**, Porto, Portugal, 03-05 Juillet 2025.

Enseignements :

Cours + TP (3h) : santé, bien-être animal et éthologie, 3ème année Agro-bachelor à l'école d'ingénieurs purpan. Mars 2023

Cours + TP (3h) : santé, bien-être animal et éthologie, 3ème année Agro-bachelor à l'école d'ingénieurs purpan. Mars 2024

Cours + TP (3h) : santé, bien-être animal et éthologie, 3ème année Agro-bachelor à l'école d'ingénieurs purpan. Mars 2025

Encadrement de thèses vétérinaires

Emma Vellard. Caractérisation de l'état de santé du chiot de race Golden Retriever au cours de l'adoption et évaluation de la méthode de notation du score fécal. **École Nationale Vétérinaire de Toulouse**, France 2024

Alizée Ammar. Développement d'une grille d'évaluation du comportement d'anxiété chez le chiot sur l'exemple de chiots Golden Retriever entre 3 semaines et 3 mois d'âge. **École Nationale Vétérinaire de Toulouse**, France 2024

Lucie Montagnier. Évolution du comportement après le sevrage chez le chiot Golden Retriever : Étude longitudinale. **École Nationale Vétérinaire de Toulouse**, France, En cours

Encadrement de stages

Kenza Kezimana. Détermination des profils comportementaux chez les chiots à l'âge de 2 mois lors d'une situation inconnue. M1 Innovation pharmacologique et métiers du médicament. **Université Toulouse III Paul Sabatier**, Toulouse France 2023.

Milan Rotter. Installation du microbiote intestinal au cours des 24 premières heures de vie chez le chiot. 2ème année BUT, **Université Toulouse III Paul Sabatier**, Toulouse France 2024.

Anais Deloste. Évaluation de la concentration du cortisol pileaire chez les chiots, durant la période du sevrage. 2ème année BUT, **Université Toulouse III Paul Sabatier**, Toulouse France 2024.

Chapitre 1

Introduction générale

Chez les mammifères, le sevrage constitue une phase critique du développement, marquée par une transition d'un régime mixte, composé de lait maternel et d'aliments solides, vers une alimentation exclusivement solide. Chez certaines espèces comme le chiot (Romaniuk et al., 2025), le porcelet (Cheng et al., 2023) et le veau (Nielsen et al., 2023), ce changement s'accompagne également d'une séparation sociale avec la mère. La double rupture, à la fois nutritionnelle et sociale, induit un stress important qui perturbe l'homéostasie physiologique (Moeser et al., 2017). Le système digestif, encore immature (Pohl et al., 2015), doit alors s'adapter rapidement à de nouvelles contraintes alimentaires et microbiennes, ce qui favorise l'émergence de troubles de santé.

Étant largement sous-étudiée chez le chiot, cette période de vulnérabilité mérite plus d'attention. Il devient essentiel d'étudier plus en profondeur les troubles de santé affectant les chiots en période du sevrage. Parmi ces troubles, la diarrhée représente un problème à la fois fréquent, multifactoriel et encore largement sous-estimé (Meineri et al., 2023; Grellet, 2016; Grellet et al., 2014). Les conséquences de la diarrhée du sevrage sont multiples. Cliniquement, elle peut entraîner une déshydratation, altérer le fonctionnement du système immunitaire, voire provoquer la mort du chiot dans les cas les plus sévères (Dinler Ay, 2022; Münnich & Küchenmeister, 2014; Schoeman et al., 2007). Pour les éleveurs, elle représente une contrainte économique et logistique : soins vétérinaires, gestion des animaux malades, dégradation potentielle de la réputation. Pour les futurs propriétaires, c'est une source d'inquiétude et de stress parfois couteuse qui peut compromettre la socialisation du chiot et l'établissement d'un lien affectif avec son propriétaire.

Certaines catégories de chiots sont encore plus concernées, notamment ceux destinés à devenir chiens guides ou chiens d'assistance. Chez l'espèce canine, la période du sevrage coïncide avec celle de la socialisation, une fenêtre développementale cruciale pour l'apprentissage, l'adaptation émotionnelle et l'interaction avec l'humain (Serpell et al., 2016). Un état de santé altéré chez les futurs chiens d'assistance peut les disqualifier définitivement du programme d'aide aux personnes (Caron-Lormier et al., 2016). Les conséquences dépassent alors le cadre vétérinaire : elles sont humaines et sociales, car ces chiens jouent un rôle essentiel dans l'autonomie, la sécurité et la qualité de vie de personnes en situation de handicap (déficiences visuelles, troubles moteurs, troubles du spectre autistique...).

Chez d'autres espèces animales comme les porcelets, les rongeurs et les primates non humains, l'impact du sevrage sur la santé digestive a fait l'objet de nombreuses recherches, mettant en évidence le rôle central du

microbiote (Ren et al., 2022; Xie et al., 2024; Zheng et al., 2023), ainsi que les liens entre santé digestive et comportement (Dong et al., 2017; Gottlieb et al., 2018). En revanche, chez le chiot, à notre connaissance, aucune étude n'a jusqu'à présent exploré l'association du comportement et du microbiote digestif avec la diarrhée du sevrage, ni les stratégies préventives qui pourraient en découler.

Le présent travail vise donc à mieux comprendre les facteurs associés à l'apparition de la diarrhée du sevrage chez le chiot et à explorer des pistes de prévention. Plus précisément, ce travail s'intéresse aux liens entre le comportement des chiots, la composition de leur microbiote intestinal et le risque de troubles digestifs en post-sevrage et la possibilité d'identification de potentiels marqueurs microbiens en amont de ces troubles. Il examine également l'effet d'une intervention nutritionnelle périnatale, à travers la supplémentation maternelle en probiotiques, comme stratégie de prévention des troubles digestifs.

Chapitre 2

Contexte scientifique

Le sevrage chez le chiot : un stress nutritionnel et social

I. Introduction

Le mot "sevrage" vient du verbe "sevrer" issu du mot latin "*separare*", ce qui signifie "séparer" (Buchi & Schweickard, 2014). Historiquement, ce terme désigne la période durant laquelle un individu réduit progressivement sa dépendance alimentaire, passant de l'allaitement à une alimentation autonome. Cette transition est connue sous le nom de période du sevrage. Cependant, sur le plan étymologique, le terme "sevrer" renvoie plutôt à un acte unique de rupture, l'instant précis où l'individu est séparé de sa source de dépendance alimentaire. Cette distinction entre l'acte de séparation "sevrage" et la période d'adaptation "préparation au sevrage" mérite d'être clarifiée.

Chez les chiots comme chez les autres mammifères, le sevrage est une étape cruciale dans leur développement physiologique et social. Cette étape est marquée par d'importants changements physiologiques et comportementaux. Au moment du sevrage, les chiots passent d'un mélange d'alimentation lactée et d'alimentation semi-solide à une alimentation solide uniquement, tout en s'adaptant à un nouvel environnement social. Ce processus est souvent associé à un stress émotionnel accru (Elliot & Scott, 1961a) et des problèmes digestifs (Grellet et al., 2012), qui peuvent impacter directement le bien-être des chiots. Ces troubles perturbent non seulement le développement physique des chiots, mais peuvent également entraîner des comportements inadaptés, tels qu'une hypersensibilité et une anxiété accrue. Si ces troubles ne sont pas correctement pris en charge, ils peuvent se transformer en problèmes comportementaux durables, pouvant affecter gravement la relation entre le chiot et son propriétaire. De même pour les troubles digestifs qui peuvent devenir chroniques. Ces problèmes de santé rencontrés par les chiots durant le sevrage peuvent avoir des répercussions économiques et sociales importantes, d'abord pour le propriétaire, car ces troubles de santé entraînent des consultations vétérinaires fréquentes et coûteuses (entre 33 et 230€ par consultation selon les données de CareCredit ; <https://www.carecredit.com/vetmed/costs/>), ainsi que des coûts liés à des traitements prolongés ou à des programmes de soutien comportemental. Dans les cas les plus graves, cela peut mener à des solutions extrêmes comme l'abandon ou même l'euthanasie du chien (Masson & Gaultier, 2018).

Ceci est problématique également pour les éleveurs canins et les structures de formation des chiens d'utilité. Les chiots ayant souffert de maladies pendant cette période peuvent montrer des retards dans leur apprentissage ou présenter des comportements inadéquats, les rendant inadaptés aux fonctions spécifiques pour lesquelles ils sont élevés, tels que l'assistance aux personnes handicapées, le sauvetage ou la garde. Il a été démontré que dans les programmes de formation de chiens guides, 30% des chiots sélectionnés pour la formation n'atteignent pas la qualification complète dû à des problèmes de tempérament et de santé (Bray et al., 2017; Trybocka, 2010). Il est donc essentiel d'assurer un maximum de confort et de soins aux chiots durant cette phase critique pour minimiser ces impacts négatifs et optimiser leur apprentissage, ce qui permet également de réduire les pertes économiques associées.

Bien que la période du sevrage représente une étape cruciale dans le développement du chiot, peu d'études offrent une vision globale de ses implications sur la santé de l'animal. Les travaux disponibles abordent généralement des aspects fragmentés du processus. Ainsi, cette synthèse a pour objectif de faire un état des connaissances actuelles sur le sevrage chez le chiot, en abordant sa définition, les évolutions physiologiques du comportement, la maturation de la fonction digestive, ainsi que le risque accru de diarrhée durant cette période. Cette revue se propose également d'examiner les stratégies de prévention et d'intervention des troubles digestifs au sevrage disponibles dans la littérature scientifique.

II. La définition du sevrage chez le chiot

Après la naissance, 3 phases alimentaires se succèdent pour amener le chiot à une alimentation solide. D'abord, une **phase exclusivement lactée** qui commence dès les premières minutes de vie et qui dure généralement jusqu'à l'âge de 3-4 semaines. Durant cette période, le lien avec la mère est également vital pour la régulation de la température corporelle, la miction et la défécation (stimulées par la mère) ou encore l'apprentissage des premiers comportements sociaux. Ensuite vient la **phase de transition alimentaire** qui commence vers l'âge de 3-4 semaines, en parallèle de l'apparition des premières dents. À ce stade, les chiots continuent à téter leur mère, mais des aliments semi-solides, souvent sous forme de bouillie, leur sont progressivement introduits. Ce changement permet à leur système digestif de s'adapter aux nouvelles textures et types de nourriture tout en continuant à bénéficier du lait maternel. Ce processus de diversification alimentaire permet également de préparer le chiot à l'autonomie alimentaire. Et enfin, la **phase du sevrage** qui intervient au moment de l'adoption (en moyenne entre 8-10 semaines de vie) ; (Enquête NeoCare "Sevrage," 2018). Durant cette phase, le chiot est entièrement sevré du lait maternel et dépend entièrement de l'alimentation solide pour couvrir ses besoins nutritionnels. Cette étape marque donc à la fois une séparation avec le lait maternel, mais également une séparation physique et émotionnelle avec

la mère et la fratrie. C'est ce moment de séparation suivi de l'adoption par de nouveaux propriétaires que l'on appelle le **sevrage social et nutritionnel (SSN)** (Figure1).

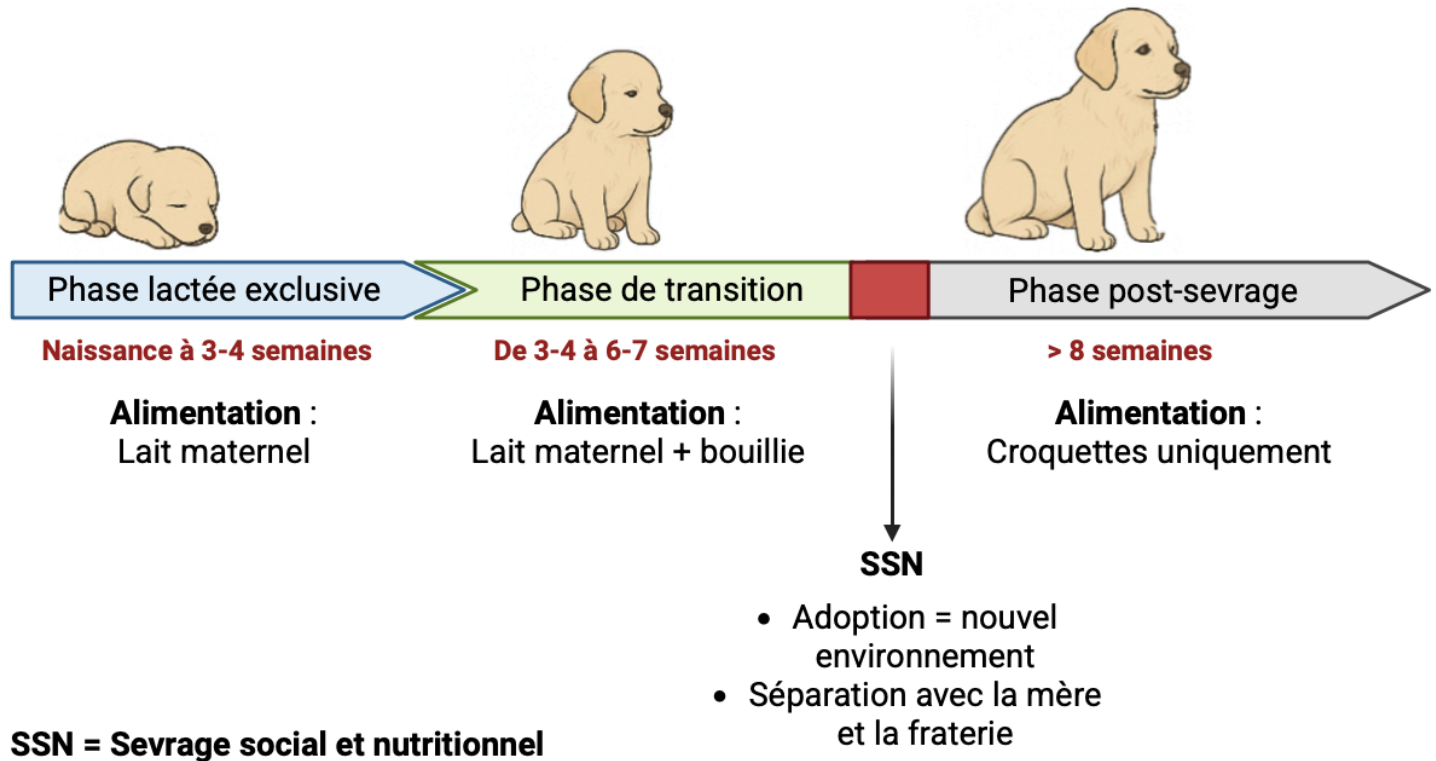


Figure 1 : Evolution de l'alimentation du chiot de la naissance jusqu'au sevrage.

III. Evolution du comportement et de la fonction digestive chez les chiots de la naissance jusqu'au sevrage

Les deux premiers mois de vie des chiots sont marqués par une grande évolution de leur physiologie et de leur anatomie. Durant cette période, les chiots, encore immatures à la naissance, subissent des transformations rapides pour atteindre une autonomie progressive. Cette phase est également caractérisée par une grande vulnérabilité, pouvant les prédisposer à certaines maladies, notamment au moment du sevrage. Pour mieux comprendre cette vulnérabilité, il apparaît indispensable de connaître le développement des différentes fonctions de l'organisme entre la naissance et le sevrage. Étant donné que cette transition est principalement marquée par des changements d'ordre alimentaire ainsi que par des modifications socio-

comportementales, nous avons choisi, dans le cadre de cette étude bibliographique, de centrer notre attention sur l'étude du développement comportemental et du système digestif.

Effectivement, les systèmes digestif et nerveux, en particulier, sont extrêmement sous-développés à la naissance. Le système digestif ne peut initialement digérer que le lait maternel, tandis que le système nerveux, encore immature, limite leurs capacités sensorielles et motrices. C'est au fil des semaines que ces systèmes se développent et s'adaptent pour répondre aux exigences d'une vie plus autonome

A) Evolution du comportement

Chez le chiot, le développement du système nerveux commence avant la naissance puis, évolue rapidement après (Gross et al., 2010). Pendant cette phase, le système nerveux des individus est très adaptable et dépend largement des facteurs environnementaux (Chapillon et al., 2002). La période néonatale est particulièrement cruciale pour le développement neuropsychologique des chiots (Foyer et al., 2013). Les différences dans les soins maternels ou les conditions d'élevage peuvent significativement impacter le développement neurologique et son expression comportementale (Caldji et al., 1998; Guardini et al., 2016).

Dans le prolongement de cette maturation cérébrale, s'instaure la phase de socialisation. Cette phase est importante pour l'apprentissage et le développement comportemental et émotionnel des chiots. La phase de socialisation débute généralement autour de la troisième semaine de vie et s'étend jusqu'à environ la 12^{ème} semaine de vie. Durant cette période, les chiots apprennent à interagir avec leurs congénères, les humains et d'autres stimuli environnementaux. Toute perturbation durant cette période accroît le risque de troubles comportementaux comme la peur et l'agressivité à l'âge adulte (Howell et al., 2015).

C'est à ce moment que les fondations de leur comportement social futur sont établies, ce qui influence directement leur capacité à s'adapter à des situations nouvelles et à gérer le stress à l'âge adulte (Appleby et al., 2002). Chez le chiot, le comportement d'interaction avec la fratrie (léchage, reniflements) et le jeu social commencent au 14-21^{ème} jour après la naissance. Tandis que les interactions agonistiques (postures de domination, menaces, morsures et soumission) apparaissent au 35^{ème} jour (Lund & Vestergaard, 1998). Le comportement de peur, quant à lui, apparaît dès l'âge de 40-55 jours post-naissance. Ce comportement est exprimé par 26 à 78% des chiots selon la race (Morrow et al., 2015).

Cette phase de socialisation coïncide en partie avec le sevrage, qui correspond à une transition importante où le chiot est séparé de sa mère et de sa fratrie tout en subissant une modification de son régime alimentaire. Cette double transition, à la fois sociale et nutritionnelle, est une source potentielle de détresse émotionnelle. En effet, la séparation du chiot avec sa mère et sa fratrie est souvent accompagnée d'une anxiété marquée.

Les chiots manifestent cette anxiété par des pleurs excessifs (Gaultier et al., 2008), une tachypnée (respiration accélérée), et une tachycardie (accélération du rythme cardiaque) (Elliot & Scott, 1961b).

L'évolution de ce comportement lié à la séparation a été étudié chez des Beagles entre 21 et 84 jours de vie. Elliot et al. (1961) a montré que cette détresse émotionnelle atteint son pic entre le 49 et 56^{ème} jours après la naissance qui correspond à l'âge où les chiots sont sevrés. Une autre étude récente a montré que le taux de cortisol pileire (marqueur de stress chronique) a augmenté significativement après le sevrage (Pan et al., 2025) soulignant ainsi le stress émotionnel important induit par cet événement chez le chiot. Ce stress de séparation est une réponse adaptative normale à ce changement environnemental. Toutefois, le chiot est généralement capable d'y faire face grâce à sa résilience. À l'inverse, certains chiots présentent des difficultés d'adaptation, ce qui peut entraîner des répercussions sur leur santé. Ces individus plus sensibles méritent une attention particulière et des stratégies de prise en charge adaptées

Lorsque les troubles comportementaux ne sont pas correctement pris en charge chez les chiots, cela peut entraîner des conséquences graves, non seulement pour l'animal, mais également pour ses propriétaires. Les troubles comportementaux les plus fréquemment diagnostiqués chez les chiens incluent l'anxiété générale, l'anxiété de séparation, et le trouble déficitaire de l'attention avec hyperactivité (TDAH) (Lindsay, 2001). Une étude menée par Pirrone et al. révèle que 65% des propriétaires signalent des problèmes de comportement chez leurs chiots, principalement liés à la peur et à l'anxiété (Pirrone et al., 2015). Comme mentionné plus haut, la persistance de ces troubles conduit parfois à des décisions drastiques, comme l'abandon ou l'euthanasie des chiots. Par exemple, Masson et al. ont constaté que 54% des propriétaires ayant des chiens souffrant de troubles liés au TDAH ont envisagé l'abandon ou l'euthanasie (Masson & Gaultier, 2018). Bien que l'impact de la séparation sur le comportement soit documenté, aucune étude ne s'est intéressée spécifiquement au lien entre le comportement et la santé des chiots lors du sevrage.

B) Le développement du tube digestif, impact du sevrage

Le système digestif suit une évolution rapide au cours des deux premiers mois de vie. En effet, la longueur des intestins augmente de manière significative d'environ 80 cm à la naissance à environ 240 cm à 63 jours, soit une semaine après le sevrage (Paulsen et al., 2003). Ces changements ne sont pas limités à l'allongement des intestins : les villosités intestinales proximales subissent également une transformation. Avant le sevrage, à 42 jours, leur longueur moyenne est d'environ 826 µm, mais elle diminue à 766 µm après le sevrage (63 jours). Parallèlement, la profondeur des cryptes (proximal, médial et distal) augmente de manière marquée, passant de 818 µm à 1062 µm à la même période (Paulsen et al., 2003). Il a été rapporté que cette augmentation de la profondeur des cryptes au moment du sevrage permet de maintenir une absorption efficace des nutriments (Pluske et al., 1996), suggérant une modification morphologique

commune à plusieurs espèces en réponse aux nouvelles conditions nutritionnelles et environnementales du sevrage. Cependant, au-delà de leur fonction adaptative, certains de ces changements pourraient pareillement contribuer à une plus grande fragilité digestive. Il a été démontré chez les porcelets, mais pas le chien, que les changements morphologiques au niveau des intestins, notamment la diminution de la longueur des villosités intestinales, peuvent aussi contribuer à l'apparition de la diarrhée pendant la période du sevrage (Nabuurs et al., 1993).

Au cours du développement postnatal, le système digestif du chiot subit une série de modifications enzymatiques visant à s'adapter à la transition alimentaire associée au sevrage. On observe une diminution progressive de l'activité de la lactase, enzyme essentielle à la digestion du lactose contenu dans le lait maternel (Henning, 1986), concomitante à une augmentation progressive de l'activité des enzymes pancréatiques, notamment l'amylase et la lipase, dans le contenu de l'intestin grêle et au niveau du tissu pancréatique (Buddington et al., 2003). En parallèle, les activités protéolytiques, telles que celles de la pepsine et de la chymotrypsine, augmentent également entre l'âge de 63 jours et l'âge adulte chez le chien. Ces modifications enzymatiques reflètent l'adaptation du système digestif à une alimentation plus complexe, riche en glucides complexes, protéines et lipides, et soulignent l'importance de cette période du sevrage dans le développement fonctionnel du tractus digestif du chiot.

Les acides biliaires jouent un rôle essentiel dans la digestion des graisses, en particulier après le sevrage, lorsque l'alimentation devient plus lipidique. L'étude de Blake et al montre une augmentation de la concentration fécale des acides biliaires primaires à partir de la 3-4^{ème} semaine, puis les concentrations rediminuent significativement à partir de la 10^{ème} semaine de vie. En revanche, les concentrations d'acides biliaires secondaires restent très faibles jusqu'à la 7^{ème} semaine, puis augmentent et restent stables jusqu'à l'âge adulte (Blake et al., 2020). Ces acides biliaires sont essentiels à la physiologie intestinale, avec des rôles anti-inflammatoires et inhibiteurs de la croissance des bactéries entéropathogènes (*Clostridium difficile*, *Clostridium perfringens*, *Escherichia coli*). Ainsi, une perturbation de la maturation du système digestif, notamment de la production ou de la transformation des acides biliaires, pourrait altérer l'homéostasie intestinale et favoriser l'émergence de troubles digestifs, tels que la diarrhée.

C) Evolution du microbiote intestinal pendant le sevrage

Le microbiote intestinal, considéré aujourd'hui comme un organe à part entière, évolue fortement au cours des premières semaines de vie du chiot, en lien étroit avec la maturation digestive et les transitions alimentaires (**Figure 2**). Chez le chien, une chute transitoire de la richesse bactérienne est observée dès le 2^e jour de vie, suivie d'une augmentation progressive jusqu'à 28 à 42 jours (Garrigues et al., 2023; Guard et al., 2017).

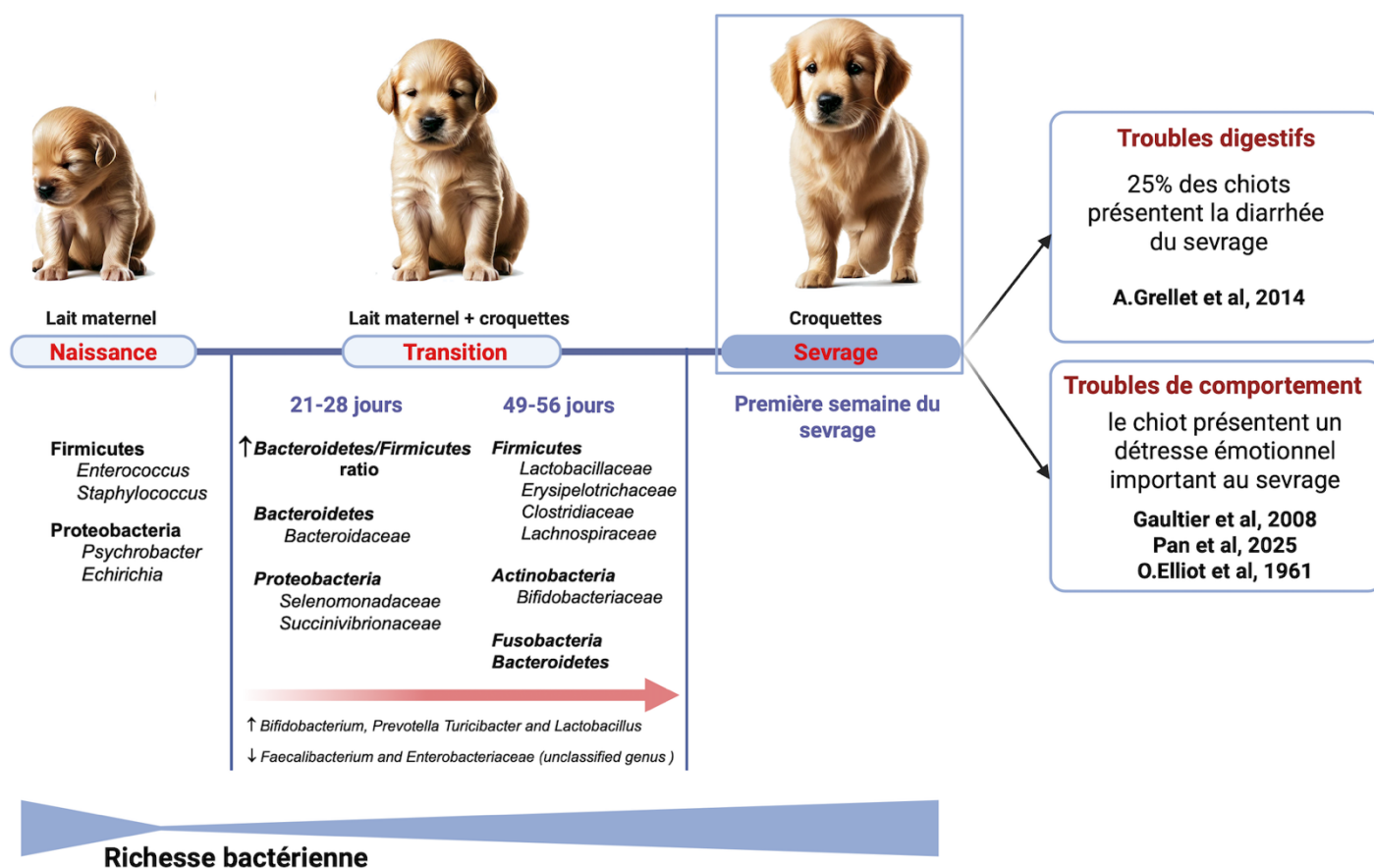


Figure 2 : Evolution du microbiote intestinal chez le chiot de la naissance jusqu’au sevrage et les troubles de santé liés à cet événement. Les taxa situés au-dessus de la flèche rouge correspondent aux taxa les plus abondantes à chaque stade (21–28 jours et 49–56 jours). Les taxa situés sous la flèche rouge indiquent les variations observées entre 21 et 56 jours, issues d’une analyse comparative entre ces deux points temporels.

Durant la période de transition alimentaire (21–56 jours), le microbiote intestinal se réorganise progressivement sous l’influence du lait maternel et de l’introduction d’aliments solides. Le lait maternel, riche en *Firmicutes* (notamment *Lactobacillaceae*), soutient une dominance de ce phylum en début de période (Martín et al., 2010; Vilson et al., 2018a). À partir de 21 jours, une augmentation du ratio *Bacteroidetes/Firmicutes* est observée (Guardini et al., 2016), en lien avec l’introduction des croquettes riches en glucides.

Cette évolution favorise l’expansion de bactéries capables de métaboliser les polysaccharides alimentaires, comme les *Bacteroidetes*, qui produisent des acides gras à chaîne courte aux rôles immunomodulateurs et anti-inflammatoires (Vital et al., 2014; Ríos-Covián et al., 2016). Parallèlement, des changements

spécifiques sont notés : augmentation des *Bifidobacterium*, *Prevotella*, *Lactobacillus* et *Turicibacter*, et diminution de *Faecalibacterium* et des *Enterobacteriaceae* non classés (Ephraïm et al., 2020). Ces modifications reflètent l'adaptation du microbiote intestinal à une alimentation postnatale de plus en plus complexe.

L'impact des premiers jours suivant le sevrage complet sur le microbiote intestinal des chiots n'a jamais été étudié. En revanche, chez le porcelet, Li et al ont démontré une modification du microbiote intestinal au cours des 3 premiers jours suivant le sevrage. Une augmentation de l'abondance des *Firmicutes* (*Lachnospiraceae*, *Negativicutes* et *Selenomonadales*), *Proteobacteria* (*Campylobacterales*) et une diminution de l'abondance des *Bacteroidetes* (*Porphyromonadaceae*, *Alloprevotella*, *Barnesiella*) et *Firmicutes* (*Oscillibacter*) a été notée (Li et al., 2018). Étant donné le rôle d'*Alloprevotella* et *Oscillibacter* dans l'inflammation (Downes et al., 2013; Gophna et al., 2017), ainsi que l'association des *Campylobacterales* aux troubles gastrointestinaux (Y. Xie et al., 2011), il est probable que ces modifications du microbiote intestinal au moment du sevrage contribuent au moins en partie à l'apparition de la diarrhée du sevrage.

IV. Diarrhée du sevrage chez le chiot : pathophysiologie et étiologie

La diarrhée se définit comme une augmentation de la fréquence, de la fluidité ou du volume des selles ("American Gastroenterological Association Medical Position Statement" 1999). Elle est le plus souvent caractérisée selon sa durée (aiguë ou chronique) et son mécanisme physiopathologique. La diarrhée constitue l'un des problèmes de santé les plus fréquents chez le chiot au moment du sevrage, avec une prévalence atteignant 25 % selon Grellet et al., (2014).

A) Physiopathologie de la diarrhée

La diarrhée peut résulter de 4 grandes catégories de mécanismes physiopathologiques (Marks, 2013a) (**Figure 3**):

Diarrhée osmotique : Elle survient lorsqu'une quantité excessive de solutés osmotiquement actifs, mal absorbés (ex. : nutriments non digérés), reste dans la lumière intestinale et attire passivement l'eau. C'est le cas par exemple lors d'insuffisance pancréatique exocrine. La fermentation de ces résidus par le microbiote intestinal peut aggraver l'effet osmotique. La diarrhée cesse généralement à l'arrêt de l'ingestion du soluté en cause. Ce type de diarrhée se caractérise par un **écart osmotique fécal élevé** ($> 50 \text{ mOsm/kg}$) c'est-à-dire une différence marquée entre l'osmolalité totale des selles et la concentration en électrolytes fécaux.

Diarrhée sécrétoire : Elle résulte d'un dysfonctionnement du transport ionique à travers l'épithélium intestinal, avec augmentation de la sécrétion de chlorure et/ou diminution de l'absorption de NaCl. Ce mécanisme est souvent induit par des médiateurs intracellulaires (AMPc, GMPc, Ca^{2+} , kinases) activés par des toxines bactériennes, des médiateurs inflammatoires ou des acides biliaires. La présence accrue de chlorure (et d'autres ions) dans la lumière intestinale crée un gradient osmotique qui attire passivement l'eau, entraînant une accumulation de liquide dans l'intestin. Ce mécanisme explique la consistance très liquide des selles, indépendamment de l'ingestion alimentaire. La diarrhée sécrétoire persiste même à jeun et présente un écart osmotique faible.

Augmentation de la perméabilité de la muqueuse intestinale : Déclenchée par des lésions inflammatoires, érosives ou néoplasiques de la muqueuse intestinale (par exemple MICI, lymphome) peuvent entraîner un passage accru de liquides, électrolytes, protéines et cellules sanguines dans la lumière digestive.

Troubles de la motricité intestinale : Des altérations de la motricité, notamment la suppression des contractions phasiques et l'activation de contractions géantes migrantes (CGM), peuvent provoquer un transit intestinal excessivement rapide. Cela empêche l'absorption adéquate des nutriments et des sécrétions, augmentant ainsi la charge osmotique colique. Ces anomalies sont observées notamment dans les entéropathies inflammatoires.

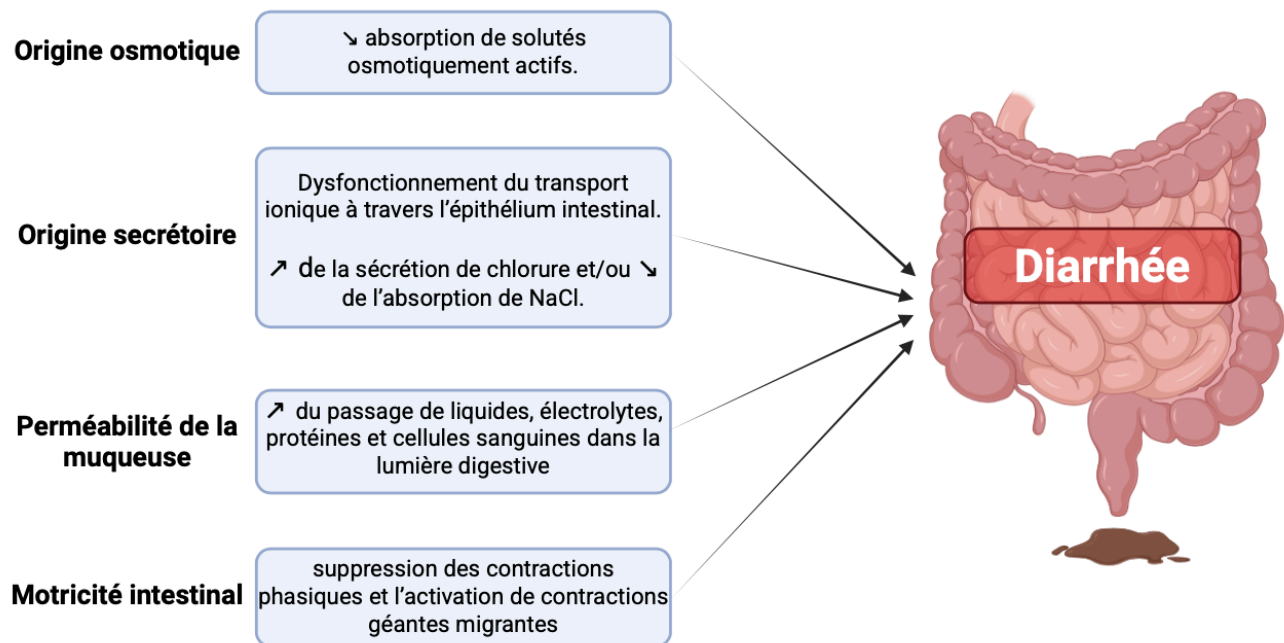


Figure 3 : Principaux mécanismes physiopathologiques de la diarrhée. Flèche (↘) signifie diminution et flèche (↗) signifie augmentation [d'après la revue de (Marks, 2013a)].

B) Étiologie de la diarrhée du sevrage

Plusieurs facteurs peuvent être à l'origine de ce trouble (Grellet, 2016), ils sont résumés dans la **Figure 4**. Ces facteurs peuvent être divisés en 3 catégories : les facteurs viraux, parasitaires et alimentaires. Parmi les agents viraux, le parvovirus canin (CPV) et le coronavirus canin (CCV) sont les plus fréquemment impliqués dans les cas de diarrhée chez le jeune, le premier touchant jusqu'à 58 % des chiots infectés (Godsall et al., 2010) et le second étant détecté chez 10 à 20 % des individus (Grellet et al., 2017).

En ce qui concerne les parasites, les Giardias sont les plus fréquemment retrouvés dans les selles du chiot, avec une prévalence d'environ 13 %, dont 75 % des cas concernent des chiots (López-Arias et al., 2019; Yu et al., 2018). Les coccidies sont également fréquentes, avec une prévalence estimée à 6 % (López-Arias et al., 2019; Yu et al., 2018). Ces deux agents sont associés à la diarrhée chez le chiot.

Enfin, les facteurs alimentaires sont également impliqués. Par exemple, un repas trop volumineux, un régime déséquilibré (notamment trop riche en acides gras) ou une transition alimentaire brutale d'un régime à un autre peuvent perturber la digestion et provoquer une diarrhée chez le chiot (Gerbaux, 2002; Marks, 2013b, Liao et al., 2023).

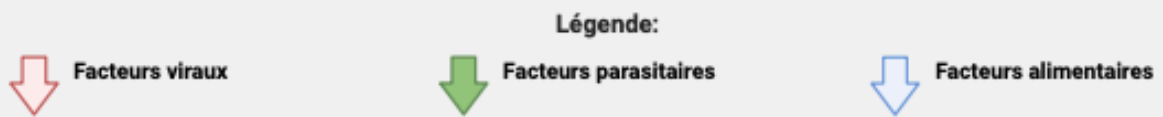
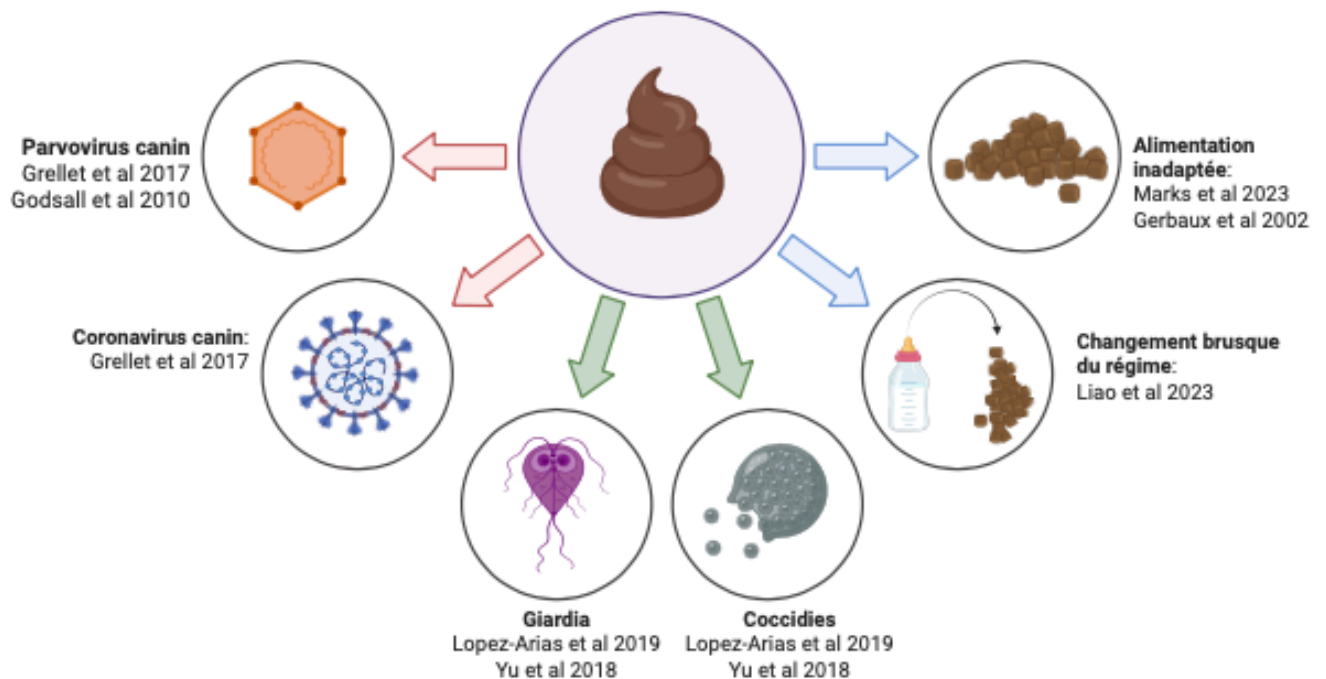


Figure 4 : Étiologie générale de la diarrhée du sevrage chez le chiot.

V. Rôle de l'axe intestin-cerveau dans les troubles digestifs

A) Définition de l'axe intestin-cerveau

L'axe intestin-cerveau désigne les interactions bidirectionnelles entre le système nerveux central et le tractus gastro-intestinal. Ces interactions impliquent le système nerveux entérique, le système immunitaire, le système endocrinien, ainsi que le microbiote intestinal (Cryan & Dinan, 2012; Mayer et al., 2015). Ce réseau complexe permet à l'intestin d'influencer le comportement et réciproquement, au cerveau d'agir sur la fonction digestive (Carabotti et al., 2015; Foster et al., 2017).

B) Mise en évidence de l'axe intestin cerveau au sevrage

De nombreuses études ont démontré l'influence du microbiote intestinal sur le comportement. Chez la souris, des modèles de troubles neurodéveloppementaux ont montré une altération du microbiote accompagnée de troubles comportementaux (Hsiao et al., 2013). Par exemple, chez les souriceaux modèles d'autisme, une abondance relative plus élevée de *Bacteroidial* et *Clostridial* a été observée, tandis que *Clostridiales* (*Ruminococcaceae*), *Erysipelotrichales* (*Erysipelotrichaceae*), *Burkholderiales* (*Beta proteobacteria*) ont été moins abondantes. L'administration de *Bacteroides fragilis* après le sevrage a corrigé certains de ces troubles comportementaux. Chez le porcelet, Jiang et al. (2020) ont démontré une association négative entre le niveau de cortisol salivaire et à l'abondance du phylum *Lentisphaerae*, et une association positive avec le phylum *Bacteroidetes* et au genre *Prevotellaceae* groupe *NK3B31*. Ces travaux démontrent l'existence d'un dialogue entre microbiote, intestin et cerveau dans des modèles animaux jeunes, notamment autour de la période du sevrage.

C) Rôle potentiel de l'axe intestin cerveau dans la diarrhée du sevrage

Le stress psychologique est un facteur bien connu d'exacerbation des troubles gastro-intestinaux (Fava & Pavan, 1976). Le sevrage constitue un événement particulièrement stressant chez les chiots (Elliot & Scott, 1961a), mais son effet sur la diarrhée n'a jamais été étudié dans cette espèce.

Chez les souriceaux l'étude de Dong et al. (2017) a démontré qu'un niveau de stress élevé au sevrage a été associé à une diarrhée plus sévère et à un niveau plus élevé de la sérotonine plasmatique.

Par ailleurs, des altérations du microbiote intestinal ont été associées à la diarrhée dans plusieurs espèces. Chez le chien adulte, des différences significatives de composition bactérienne ont été observées entre chiens sains et diarrhéiques (Guard et al., 2015 ; Suchodolski et al., 2012). Chez le porcelet, une association entre la diminution de l'abondance des *Bacteroidetes* et la diarrhée du sevrage a été soulignée (Ren et al.,

2022; F. Xie et al., 2024; Zheng et al., 2023) ainsi qu'une augmentation de l'abondance des *Proteobacteria* *Campylobacter* (Zheng et al., 2023).

À notre connaissance, aucune étude n'a encore exploré le lien entre comportement, microbiote intestinal et diarrhée chez le chiot au moment du sevrage. Ce champ mérite une attention particulière, car il pourrait contribuer à une meilleure compréhension des troubles digestifs chez le chiot.

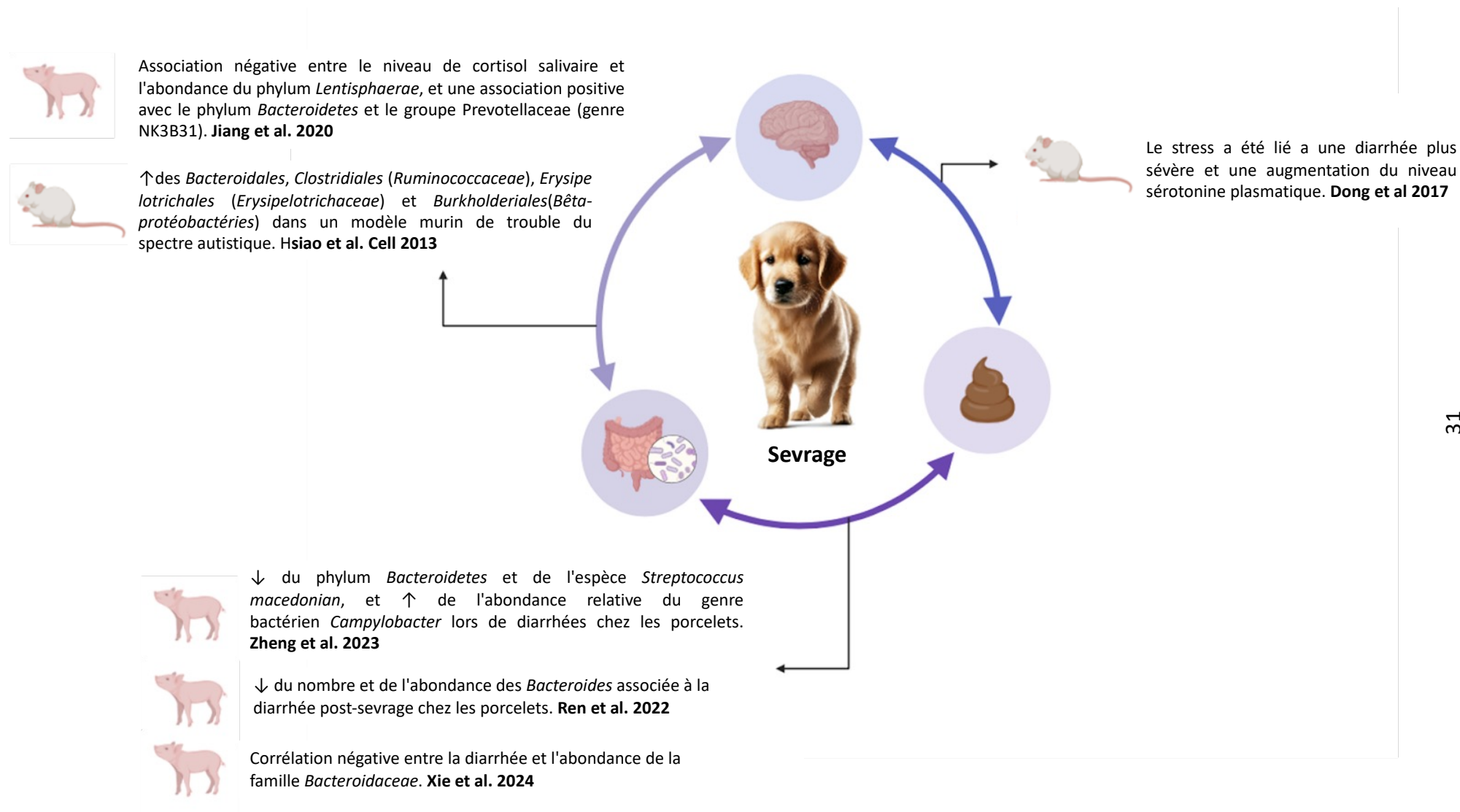


Figure 5 : Les différentes interactions entre le microbiote intestinal, le cerveau et la santé digestive au moment du sevrage – multi espèces.

VI. Prévention de la diarrhée du sevrage via utilisation de probiotiques

Plusieurs stratégies ont été proposées pour réduire le risque de diarrhée du sevrage chez différentes espèces animales, mais les études spécifiques menées chez le chien restent encore très limitées. Agir sur le microbiote digestif est l'une des méthodes les plus prometteuses. Dans cette étude, nous nous limiterons aux probiotiques.

A) Supplémentation des jeunes en probiotiques

La supplémentation en probiotiques au cours du sevrage a suscité un intérêt croissant en raison de son potentiel à améliorer la santé digestive et immunitaire des individus. Les probiotiques sont définis comme « des micro-organismes vivants qui, lorsqu'ils sont administrés en quantité adéquate, confèrent un bénéfice pour la santé de l'hôte » (Gibson et al., 2017). Aujourd'hui, les principales catégories de probiotiques utilisées incluent principalement des bactéries et des levures, telles que *Enterococcus faecium*, *Bacillus subtilis*, *Lactobacillus acidophilus*, et *Saccharomyces cerevisiae*. Ces souches ont montré des effets significatifs sur l'amélioration des fonctions digestives, la modulation du microbiote intestinal, et la prévention des troubles gastro-intestinaux chez différentes espèces animales, incluant le porc et l'humain (Table 1).

Bien que de nombreuses études aient été menées chez des espèces comme le porcelet et l'humain, les recherches spécifiques sur les effets des probiotiques chez le chien, notamment durant le sevrage, restent relativement limitées.

Une étude réalisée par Benyacoub et al. a examiné l'effet de *Enterococcus faecium* administré à des chiots du sevrage jusqu'à 1 an d'âge (Benyacoub et al., 2003). Les chiots ont reçu une supplémentation quotidiennement avec une souche spécifique d'*Enterococcus faecium*. Les résultats ont montré une augmentation significative des niveaux d'IgA sériques et fécaux, indiquant un renforcement des défenses immunitaires. Cependant, le risque de diarrhée n'a pas été évalué dans cette étude. Chez le porcelet cette supplémentation a permis d'augmenter la prise de poids quotidien, de diminuer l'incidence de la diarrhée post-sevrage, de modifier positivement le microbiote intestinal et module la maturation du système digestif en augmentant la longueur des villosités, le ratio surface des villosités/circonférence des cryptes ce qui optimise l'absorption des nutriments (Hu et al., 2015; Rieger et al., 2015; Y. B. Wang et al., 2016; Zeyner & Boldt, 2006).

Les effets de la supplémentation par les *Bacillus* sur la santé au moment du sevrage ont également été étudiés chez le porcelet. La supplémentation des porcelets par *Bacillus subtilis* et *Bacillus licheniformis* a diminué l'incidence de la diarrhée, stimulé système immunitaire intestinal, augmenté du poids et modifié positivement le microbiote intestinal (Alexopoulos et al., 2004; Kantas et al., 2015; Kyriakis et al., 1999; Scharek et al., 2007; Taras et al., 2005). En plus de cela, la supplémentation par *Bacillus subtilis* et *Bacillus licheniformis* a montré une diminution de la mortalité chez les porcelets 43-44. La supplémentation avec les *Bacillus* pourrait se révéler intéressante chez le chien, mais cela reste à démontrer.

Les *Lactobacillus* quant à eux, ont également montré leurs effets bénéfiques sur la santé digestive des enfants et des porcelets, notamment en augmentant l'abondance des bactéries bénéfiques comme *Lactobacillus* et *Bifidobacterium*, en réduisant l'incidence de la diarrhée post-sevrage et réduisant les réactions allergiques (Chorell et al., 2013; Grześkowiak et al., 2012; Nocerino et al., 2019; West et al., 2008, 2008, 2009). De même que pour *Bacillus*, il manque actuellement des données sur les chiots au sevrage pour évaluer si ces souches sont bénéfiques pour la santé du chiot.

Chez le porcelet, la levure, en particulier *Saccharomyces cerevisiae*, semble jouer un rôle clé dans la réduction des troubles digestifs post-sevrage. Elle a été associée à une amélioration de la réponse immunitaire et à une augmentation de l'épaisseur des villosités intestinales, ce qui optimise l'absorption des nutriments et réduit la mortalité liée à la diarrhée (Kyriakis et al., 1999; Trevisi et al., 2017).

Table 1 : Résumé des effets de la supplémentation directe par probiotiques sur la santé digestive au moment du sevrage – multi espèces.

Espèce	Probiotique		Moment de supplémentation	Effets
Chiot		<i>Enterococcus faecium</i> (Benyacoub et al., 2003)	Du sevrage à 1 an d'âge	↗ les IgA et IgG au sérum et IgA fécal des chiots
Porcelet	Bactérie	<i>Enterococcus faecium</i> (Zeyner & Boldt, 2006)	De la naissance au sevrage	↘ la diarrhée du sevrage et ↗ PPQ ^a
		<i>Enterococcus faecium</i> (Rieger et al., 2015)	Pendant le sevrage	↗ la longueur des villosités, le ratio surface des villosités/circonférence des cryptes, et le nombre de lymphocytes intra-épithéliaux.
		<i>Enterococcus faecium</i> (Y. B. Wang et al., 2016)	1er, 3ème et 5ème jour post naissance	↗ le poids pendant la phase d'alitement par 31% et le sevrage 321%, ↘ l'incidence de la diarrhée du sevrage de 71%, ↗ l'abondance relative des Bacteroidetes, Firmicutes (<i>Lactobacillale</i>) et ↘ les <i>Proteobacteria</i> .
		<i>Enterococcus faecalis</i> LAB31 (Hu et al., 2015)	Du 26ème jour au 54ème jour post naissance	↗ la PPQ ^a et l'efficacité de conversion alimentaire, ↘ l'incidence de la diarrhée, la diversité bactérienne, le nombre relatif des <i>Lactobacillus</i> .
		<i>Bacillus toyonensis</i> (Kantas et al., 2015)	Du 26ème au 70ème jours post naissance	↗ le poids, ↘ le score de diarrhée, ↘ le nombre de bactéries pathogènes (<i>E. coli</i> , <i>Enterobacteriaceae</i> , <i>Campylobacter jejuni</i> , <i>Clostridium perfringens</i>), et ↗ les bactéries lactiques.
		<i>Bacillus cereus</i> var. toyoi (Taras et al., 2005)	Les mères (du 90ème avant mise-bas jusqu'au 28ème post-partum), porcelets (du 15ème au 56ème jour post-partum)	↗ la PPQ ^a et le rapport gain/alimentation à 6 et 8 semaines post-sevrage, ↘ l'incidence de la diarrhée du sevrage de 59%.
		<i>Bacillus cereus</i> var. toyoi (Scharek et al., 2007)	Chez les mères du 25ème jour après insémination. Chez les	↗ les cellules T CD8+ intra-épithéliales, les cellules T gamma-delta dans l'épithélium intestinal, ↗ les lymphocytes CD25+ et cellules T gamma-delta dans la lamina propria.

			porcelets à partir du 15ème jour post-partum	
		<i>Bacillus licheniformis</i> (Kyriakis et al., 1999)	Pendant le sevrage	↘ l'incidence de la diarrhée et de sa sévérité, ↘ la mortalité.
		<i>Bacillus licheniformis</i> et <i>B. subtilis</i> (Alexopoulos et al., 2004)	Pendant le sevrage	↘ de la diarrhée du sevrage et la mortalité, ↗ la PP ^b
		<i>Brevibacillus brevis</i> (Che et al., 2016)	Pendant le sevrage	↗ le poids, les IgG dans le sérum, l'activité cellulase, protéase, amylase, hémicellulase et l'expression enzymatique sanguine.
		<i>Enterococci, lactobacilli, Bifidobacteria</i> (Mair et al., 2010)	Pendant le sevrage	↗ les villosités jéjunales, des cellules caliciformes neutres, et de l'expression génétique de CDK4 et TGFbeta.
		<i>Lactococcus lactis</i> exprimant l'EGF (Kang et al., 2010)	Au 21ème jour post naissance	↗ la hauteur des villosités jéjunales et duodénales, et de la longueur intestinale, ↗ la prolifération des cellules intestinales.
Enfants		<i>Lactobacillus paracasei</i> ssp. paracasei F19 (Chorell et al., 2013)	De 4 à 13 mois d'âge	↗ le niveau sanguin de la putrescine et diminue l'acide palmitoléique.
		<i>Lactobacillus</i> F19 (West et al., 2009)	De 4 à 13 mois	↘ l'incidence cumulée de l'eczéma à 13 mois, ↗ le rapport ARNm IFN-gamma/IL4.
		<i>Lactobacillus rhamnosus</i> LPR et <i>Bifidobacterium longum</i> BL999 (Grześkowiak et al., 2012)	Chez la mère : de 2 mois avant l'accouchement à 2 mois de lactation. Chez l'enfant : pendant le sevrage	↗ l'abondance des <i>Lactobacillus-Enterococcus</i> et ↘ l'abondance <i>Bifidobacteria</i> .

		<i>Lactobacillus rhamnosus</i> GG (Nocerino et al., 2019)	4 à 6 ans	↘ l'apparition de troubles gastro-intestinaux fonctionnels chez les enfants ayant des antécédents d'allergie au lait de vache.
		<i>Lactobacillus</i> F19 (West et al., 2008)	De 4 à 13 mois	↘ le nombre de jours de traitement par antibiotiques. ↗ la concentration des anticorps anti diphtheria toxin.
		<i>Bifidobacterium lactis</i> Bb-12 ou <i>Lactobacillus</i> GG (Isolauri et al., 2000)	4.6 mois post-naissance	Améliore l'état de la peau (score SCORAD) des enfants avec de l'eczéma, diminue les CD4 solubles.
		<i>Bifidobacterium lactis</i> Bb-12 (Kirjavainen et al., 2002)	Pendant le sevrage	↘ le nombre des bacteroides and E coli dans les fèces des enfants pendant le sevrage.
Porcelet	Levure	<i>Saccharomyces cerevisiae boulardii</i> (Collier et al., 2011)	Pendant le sevrage	↘ la mortalité induite par LPS de 20%, ↗ le nombre de cellules blanches, lymphocytes et neutrophiles, ↘ le cortisol sanguin.
		<i>Saccharomyces cerevisiae</i> CNCM I-4407 (Trevisi et al., 2017)	Pendant le sevrage	↗ la profondeur des cryptes intestinales et réduit les cellules apoptotiques après infection par E. coli F4ac.

Le signe (↗) signifie une augmentation et (↘) signifie une diminution. PPQ^a : prise de poids quotidien. PP^b : prise de poids.

B) Programmation nutritionnelle

Une des stratégies proposées pour moduler la santé des individus au moment du sevrage est à travers la nutrition maternelle, un processus connu sous le nom de « programmation nutritionnelle ». Ce concept suggère que les variations dans la qualité ou la quantité des nutriments consommés pendant la gestation et la lactation peuvent influencer le développement fœtal et avoir des effets à court, moyen et long terme sur la santé de la progéniture (Langley-Evans, 2009). L'utilisation de probiotiques pendant la gestation et la lactation est une approche largement étudiée pour améliorer la santé des jeunes au moment du sevrage (Table 2).

Chez le chien, certaines études ont mis en évidence les effets bénéfiques de la supplémentation probiotique maternelle sur les chiots. Par exemple, la supplémentation des mères avec *Saccharomyces cerevisiae boulardii* pendant le dernier trimestre de la gestation a permis de réduire le nombre de chiots présentant un faible poids à la naissance, tout en homogénéisant leur taux de croissance entre 21 et 56 jours d'âge. En plus de ces effets sur la croissance, une diminution de l'inflammation chez les chiots a également été observée après vaccination des chiots à l'âge de 56 jours (Garrigues et al., 2024), ce qui indique une amélioration globale de la santé des chiots et du fonctionnement de leur système immunitaire au moment du sevrage. Les mêmes effets de la supplémentation maternelle par la levure sur la modulation de la réponse immunitaire, et l'augmentation du poids ont été notés chez le porcelet en péri-sevrage (Lu et al., 2019; Salak-Johnson et al., 2022; Xia et al., 2022).

Dans une autre étude, la supplémentation des chiennes pendant le dernier trimestre de gestation avec *Lactobacillus acidophilus*, *Enterococcus faecium* en plus des prebiotiques (Mannan-oligosaccharides et Fructose-oligosaccharides) a montré ses effets bénéfiques chez les chiots à 9 semaines d'âge. En effet, le traitement de la mère, que ce soit pendant les 4 dernières semaines de gestation ou la dernière semaine de gestation, a réduit l'incidence des gastroentérites chez les chiots (Melandri et al., 2020). Dans une seconde étude, la supplémentation des chiennes pendant le dernier trimestre de gestation par *Lactobacillus johnsonii* NCC533 (La1) n'a montré aucun effet significatif sur le microbiote intestinal des chiots, ni sur leurs niveaux d'IgA sériques et fécaux (Vilson et al., 2018b). Cependant, ces résultats suggèrent que les effets des probiotiques peuvent varier en fonction des souches utilisées.

Plusieurs mécanismes d'action ont été proposés pour expliquer l'effet de la supplémentation maternelle en probiotiques sur la santé de la progéniture. Parmi eux, la modulation du microbiote intestinal et vaginal de la mère qui joue potentiellement un rôle clé dans la transmission des micro-organismes bénéfiques au moment de la naissance, influençant ainsi la colonisation microbienne initiale des jeunes. Il a également été

suggéré que les probiotiques peuvent moduler le métabolome de la mère pendant la gestation. Une altération de la composition du colostrum et du lait maternel a été également notée (Farshim et al., 2016). Par ailleurs, certaines études ont démontré une colonisation de la progéniture par le probiotique donné à la mère (Taras et al., 2005), cela peut potentiellement s'expliquer par une contamination de l'environnement par les probiotiques via les matières fécales maternelles, mais également par le léchage du chiot par la mère, ajoutant des voies indirectes, mais potentiellement significative pour moduler la santé de la progéniture.

Table 2 : Effet de la supplémentation maternelle sur la santé de la progéniture de la naissance au sevrage – multi espèces.

Espèce	Supplémentation maternelle		Période	Effets sur la progéniture
Chien	Probiotiques	<i>Saccharomyces boulardii</i> (Garrigues et al., 2024)	Dernier trimestre	↗Le taux de croissance 21-56 jours. ↘Le nombre de petits poids de naissance et le taux d'inflammation.
	Probiotiques	<i>Lactobacillus johnsonii</i> NCC533 (La1) (Vilson et al., 2018b)	Dernier trimestre	Aucun effet sur le microbiote intestinal et les niveaux d'IgA et IgE au sérum et IgA fécal.
	Probiotiques + Prebiotiques	<i>E. faecium</i> , <i>L. acidophilus</i> + MOS, FOS (Melandri et al., 2020)	Dernier trimestre	↘ La diarrhée chez les chiots (de la naissance jusqu'à 9 semaines d'âge).
Porc	Probiotiques	<i>Saccharomyces cerevisiae</i> var. <i>boulardii</i> (Salak-Johnson et al., 2022)	84 ème jours de gestation	Améliore la réponse immunitaire innée et adaptative de la progéniture.
	Probiotiques	<i>Saccharomyces cerevisiae</i> (Xia et al., 2022)	60 ème jours de gestation	↗ IgA et IgG au sérum des porcelet à 14 et 28 jours après sevrage.
	Probiotiques	<i>Saccharomyces cerevisiae</i> (Lu et al., 2019)	Toute la période de gestation	↗ Le poids des porcelets 21 et 42 jours après sevrage. ↗ La digestibilité des nutriments.
	Probiotiques	<i>Saccharomyces cerevisiae</i> + <i>Lactobacillus Plantarum</i> (K. Wang et al., 2021)	Toute la période de gestation	↗ L'abondance des <i>Bacteroidetes</i> et <i>Bifidobacterium</i> et améliore la capacité antioxydante des intestins.
	Postbiotiques dérivés de la levure	Levure (Xu et al., 2023)	90 ème jours de gestation	↗ Le poids des porcelets au moment du sevrage. ↘ La mortalité et la diarrhée.
	Probiotiques	<i>Bacillus altitudinis</i> WIT588 (Crespo-Piazuelo et al., 2022)	100 jours de gestation	↗ Le poids à 105 et 127 jours de vie. ↗ La capacité d'absorption de l'intestin grêle.

Le signe (↗) signifie une augmentation et (↘) signifie une diminution.

VII. Conclusion

Pour conclure, les études disponibles chez le chien montrent que le sevrage s'accompagne de changements physiologiques majeurs : modifications anatomiques de l'intestin, modifications de l'activité enzymatique digestive et des profils d'acides biliaires, essentiels à la digestion et à l'absorption des nutriments. Cette phase critique du développement s'est révélée être liée à l'émergence de troubles digestifs, en particulier de diarrhée, chez le chiot. Plusieurs facteurs prédisposant à la diarrhée du sevrage ont été bien documentés chez d'autres espèces modèles telles que le porcelet et la souris. Parmi eux, le comportement et la composition du microbiote intestinal ont été identifiés comme des éléments liés au risque des troubles digestifs. En revanche, chez le chiot, le lien de ces facteurs avec la diarrhée n'a jamais été exploré. De plus, plusieurs travaux ont mis en évidence l'effet bénéfique d'interventions par supplémentation probiotique, que ce soit directement chez le jeune ou de manière indirecte par supplémentation maternelle pendant la gestation et la lactation. Ces approches de programmation nutritionnelle périnatale ont permis de réduire significativement l'incidence des troubles digestifs dans la descendance.

Chapitre 3

Objectifs et hypothèses de travail

3.1 Objectif général

Le chapitre précédent (**Chapitre 2**) a permis de souligner que la diarrhée du sevrage est un phénomène fréquent chez le chiot. Il a également synthétisé les connaissances actuelles acquises chez d'autres espèces animales proches physiologiquement du chiot, mettant en lumière l'implication du microbiote intestinal et du comportement dans ce risque de diarrhée post-sevrage. Toutefois, les données spécifiques au chiot concernant l'implication de ces facteurs restent limitées. C'est dans ce contexte que s'inscrit le travail présenté dans cette thèse dont l'objectif est d'évaluer le lien entre le **comportement**, le **microbiote digestif** et le **risque de diarrhée au sevrage** chez les chiots, ainsi que les possibilités de sa **prévention** par la **programmation nutritionnelle périnatale**, à travers la supplémentation maternelle en probiotiques.

3.2 Hypothèses de travail

Pour répondre à ces objectifs, quatre hypothèses de travail ont été formulées :

Hypothèse 1 : Le comportement des chiots est associé au risque de troubles digestifs en post-sevrage.

Hypothèse 2 : La composition du microbiote fécal des chiots est associée à leur comportement et au risque de troubles digestifs en post-sevrage.

Hypothèse 3 : La supplémentation maternelle en probiotiques pendant la gestation et la lactation est un outil de prévention de diarrhée.

3.3 Démarche expérimentale

Afin de tester ces hypothèses, deux essais expérimentaux complémentaires ont été mis en place, chacun structuré pour répondre à des objectifs spécifiques.

Le premier essai a été conduit sur un effectif de 113 chiots Golden Retriever, issus de 18 mères hébergées dans une même maternité. Cet essai visait principalement à explorer les associations entre comportement, microbiote et le risque des troubles digestifs post-sevrage.

Ce protocole a permis de répondre aux deux premières hypothèses à travers 3 articles présentés dans le **Chapitre 5** :

- **L'Article 2** explore l'Hypothèse 1 en analysant la relation entre les profils comportementaux des chiots et la santé digestive en post-sevrage.
- **L'Article 3** examine la première partie de l'Hypothèse 2 en évaluant les liens entre les caractéristiques du microbiote fécal (diversité et composition) et les données comportementales.
- **L'Article 4** examine la deuxième partie de l'Hypothèse 2 en évaluant l'association entre microbiote fécal et le risque des troubles digestifs observés en post-sevrage.

Le second essai a été conçu pour évaluer l'impact de la supplémentation maternelle en probiotiques pendant la gestation et la lactation sur le microbiote et la santé précoce des chiots. Il s'inscrit dans une démarche de programmation nutritionnelle périnatale. Cet essai a été réalisé sur 17 femelles gestantes appartenant à quatre races : Berger Australien, Golden Retriever, Labrador Retriever et Berger Blanc Suisse, ainsi que leurs portées, représentant un total de 105 chiots. Il visait à répondre à l'Hypothèse 3, à travers deux articles scientifiques présentés dans le **Chapitre 6** :

- **L'Article 5** étudie l'effet de la supplémentation maternelle en probiotiques sur l'évolution du microbiote intestinal et l'incidence des troubles digestifs chez les chiots.
- **L'Article 6** évalue l'impact de cette stratégie sur la santé générale des chiots et leurs profils métaboliques dès la naissance.

Chapitre 4

Validation de la méthode d'évaluation du score fécal via images photographiques

4.1 Introduction

Dans la première partie de cette thèse, la santé digestive a été suivie chez les chiots, pendant la première semaine après le sevrage, par les adoptants des chiots. Aussi, il était indispensable de pouvoir suivre la santé des chiots de manière standardisée au sein du nouveau foyer. Dans ce but, les adoptants ont été demandés d'enregistrer la prise alimentaire des chiots en pesant les croquettes distribuées et les croquettes restantes sur la même balance fournie pour tous. Les visites de contrôle ont été programmées une semaine après le départ de la maternité pour évaluer la santé générale ainsi que peser les chiots. Pour évaluer la fonction digestive, les adoptants ont été demandés de réaliser des photos des selles lors de la première semaine après l'adoption et de les envoyer par e-mail. Cependant, l'interprétation des selles sur l'image photographique n'a jamais encore été validée pour l'espèce canine.

Ce chapitre a pour objectif de valider une méthode de caractérisation des selles de jeunes chiots à partir d'images photographiques. Cette approche vise à proposer un outil reproductible et applicable à grande échelle. Afin de répondre à cet objectif, nous nous sommes appuyés sur un protocole d'évaluation standardisé des caractéristiques fécales, développé par (Grellet et al., 2012) permettant une notation objective des différents critères d'aspect des selles.

Cependant, avant d'appliquer cette méthode dans les futurs chapitres, une étape indispensable était de valider sa pertinence. Plus précisément, il s'agissait de s'assurer que l'évaluation des selles à partir d'images photographiques permettait d'obtenir des résultats comparables à ceux obtenus par une observation directe des selles réelles. Ce travail de validation méthodologique constitue l'objet principal de ce chapitre.

Pour cela, nous avons mis en place une étude comparative permettant d'évaluer la concordance entre l'analyse d'images de selles et l'observation directe, en s'appuyant sur un protocole rigoureux d'évaluation réalisé par deux observateurs formés et dont les notations se sont révélées concordantes. Différents critères visuels ont été pris en compte, notamment la consistance et la couleur qui sont des critères importants à prendre en compte. L'analyse statistique a ensuite permis de démontrer une bonne concordance entre les notations effectuées sur les selles réelles et celles réalisées à partir de photographies. Ceci souligne la fidélité et la reproductibilité de la méthode basée sur les images.

Cette validation est un prérequis fondamental pour les études présentées dans les chapitres suivants, où l'évaluation des selles à partir d'images constitue une composante méthodologique centrale. Ce chapitre vise donc à démontrer la robustesse de cette approche, tout en apportant une réflexion sur ses limites. La mise en place d'un outil d'évaluation fiable et facilement mobilisable ouvre la voie à une standardisation de la description des selles dans des études cliniques et épidémiologiques chez le chiot.

Article 1 : Use of stool photographs for fecal scoring and diagnosis of diarrhea in the puppy: validation of the method

Chloé Chauvel ^{1*}, Ilyas Bendahmane ^{1,2*}, Emma Vellard ¹, Faouzi Lyazrhi ³, Annabelle Meynadier ²,

Hanna Mila ¹, Aurélien Grellet ¹

¹Neocare, Université de Toulouse, ENVT, Toulouse, France

²GenPhySE, Université de Toulouse, INRAE, ENVT, F-31326, Castanet Tolosan, France

³Biostatistiques, Université de Toulouse, ENVT, Toulouse, France

* Co-first authors

Key words: fecal score, puppy, dog, feces, diarrhea, diagnosis, photograph, method validation

Abstract

Background- Diarrhea is a common issue in puppies and is associated with poor daily weight gain and increased risk of mortality. Abnormal stool consistency can be objectively assessed using a fecal scoring scale. Such a tool has previously been validated in puppies around weaning. Although designed for use with fresh feces, obtaining such samples may be logistically demanding. Photography can provide a simple and efficient method for collecting large quantities of data for research projects. This study aimed to validate the use of the puppy fecal grid on stool images and assess the reliability of this method for diagnosing diarrhea.

Methods- In a first experiment, 40 fresh stools were collected from a cohort of 25 Golden Retriever puppies and scored by a first observer with a 13-point fecal scale. Feces were then photographed and pictures were later assessed by a second operator. Agreement between the two scoring methods was evaluated using a Bland-Altman plot and weighted kappa coefficient. Validity of the method for diarrhea diagnosis was also studied. In a second experiment, four observers scored 98 stool photographs in similar conditions. Intraclass correlation coefficient was calculated to assess inter-observer agreement. A scoring training session was later provided by one observer considered as a reference, and all observers re-scored photographs. Influence of the training session on intraclass correlation coefficient was studied. Intra-observer reproducibility was also explored for reference observer with a Bland-Altman plot and weighted kappa coefficient.

Results- Our findings showed excellent agreement between the two methods (weighted kappa coefficient of 0.83 (95% CI [0.75, 0.91])). Validity of the photograph method for diarrhea diagnosis was deemed satisfactory. Sensitivity and specificity were both at 84.6%, with positive and negative

predictive values at 73.3% and 91.7%, respectively. Our study showed good inter-observer and intra-observer reproducibility, with no effect of prior training on scoring.

Conclusions- The puppy fecal scoring scale employed in the present study can reliably be used on stool photographs to assess fecal consistency and objectify diarrhea in puppies. It is a promising solution for monitoring dog transit at veterinary practices and for future research studies.

Background

Gastrointestinal disorders are pervasive in dogs. A study showed that 29.5% of dog consultations at English primary-care veterinary practices involved digestive disorders, making the digestive tract the most prevalent organ system affected after the integument. Digestive disorders are particularly problematic in puppies, leading to common diarrhea in this population¹⁻³. Diarrhea in weaning puppies, observed in about one out of four individuals, can have many origins, such as parasitic, viral, nutritional, or environmental ones². It is associated with poor daily weight gain and increased risk of mortality⁴, being a threat to animal welfare, economic performance of breeding facilities and well-being of dog owners. Objective evaluation of diarrhea is useful both in practices and for research projects for standardizing diagnosis, monitoring clinical evolution and assessing the efficacy of treatments. It can be achieved using a fecal scoring system⁵⁻⁷. A puppy scale, designed to adapt to specific stool consistency of puppies around weaning, has previously been published⁴. As young puppies generally have less molded stools than adults, the use of a specific scale prevents over-estimating cases of diarrhea. This scale also enables a more refined assessment of stool consistency, through a greater number of categories than adult scales, so as to more accurately monitor small changes in transit around weaning⁴.

Stool consistency scoring tools are intended for direct observation of fresh stools^{4,8}. However, accessing fresh feces can be complicated by possible reluctance for fecal collection^{9,10}. Additionally, transportation raises the question of sample preservation, and the effect of temperature variations and mechanical constraints on stool consistency assessment, which has yet to be studied. Furthermore, it is impossible to collect liquid stools, in which case an in situ assessment is necessary. Transporting and storing large numbers of fecal samples represents a major human and time cost for research projects⁹. Logistical constraints may restrict repeated access to stool samples, posing challenges for monitoring chronic cases or longitudinal research studies. Currently, no viable solution exists.

Photography offers a simple and accessible way to capture the appearance of a stool immediately after bowel movement. Photographs could thus provide a front-line tool for veterinary practitioners to identify cases of diarrhea, particularly during teleconsultations. Indeed, veterinary telemedicine is becoming an increasing widespread practice¹¹⁻¹³. Dog owners could send multiple pictures over time for remote assessment, giving veterinarians a useful tool for triage and a broader view of a puppy's

health status between face-to-face consultations. Photograph-based monitoring could also help with clinical follow-up and evaluation of treatment effectiveness by objectively comparing stool consistency over time. In research projects, fecal scoring on photographs would facilitate the inclusion of a large number of cases from distant areas, promoting geographical diversity for greater representativeness. It would provide a rigorous method for objectively monitoring animal transit, while minimizing financial and human costs. However, image-based evaluation may introduce bias compared to direct observation of fresh stools, necessitating validation of this method.

The present study aimed to validate the reliability of Grellet *et al.* fecal scoring scale (FSS)⁴ using stool photographs. The accuracy of this new method in diagnosing diarrhea in puppies was also investigated. Two different experiments were carried out: one to study agreement with fresh stool reference method and one to assess reproducibility of the new method.

Methods

1. Evaluation of variability between direct and photographic assessment of stools

1.1. Variability of fecal score between direct and photographic assessment

This study was evaluated by a local ethical committee (CEEA SSA N°115).

In this first experiment, feces from 25 two-month-old (ranging from 7 to 11 weeks of life) clinically healthy Golden Retriever puppies from the Toulouse Gold Cohort were assessed¹⁴. These puppies were born and raised in a dedicated nursery as part of a French research program and benefited from regular veterinary care. They were fed the same diet as part of diversification and received the same antiparasitic treatments and vaccination protocol. They were not yet separated from their mother at the time of the study. A first operator (Op1) evaluated 40 randomly selected fresh stools immediately after spontaneous defecation between April 3rd and May 1st, 2024. The stools were scored according to their consistency and shape using the 13-point puppy FSS, ranging from 1 (completely liquid) to 13 (dry and hard feces)⁴. Briefly, scores below 5 are indicative of diarrhea in large-breed puppies while scores above 5 are compatible with a healthy animal⁴. After scoring, pictures of the assessed feces were taken by Obs1 with a smartphone camera, thereby creating a library of 40 photographs.

To avoid recall bias¹⁵, a second operator (Op2) scored each stool photograph of the aforementioned library by displaying it on a computer and applying the same FSS, in order to compare both scoring methods. In order to minimize inter-observer variability and ensure similar scoring, the two operators scored together approximately 500 photographed stools of varying consistency before conducting the study.

All statistical analyses were performed using R software (version 4.2.2)^{16,17}. To assess agreement between the scoring method on a fresh stool (FS) and a photographed stool (PS), a Bland and Altman diagram was created using ‘BlandAltmanLeh’ package. In Bland-Altman plot, mean difference between the two measurement methods is called bias (b). Assuming a normal distribution of differences, approximately 95% of the differences are expected to be between $b \pm 2sd$, where sd is the standard deviation of the differences. The limits of this range are called limits of agreement (LoAs). 95% confidence intervals of the b and LoAs are also shown in the Bland and Altman diagram¹⁸. Precise normality of differences is not required for this test, and thus only visual assessment was performed¹⁹. For the present study, maximum acceptable b and LoAs were set at ± 1 and ± 3 points of the FSS, respectively. Dots located outside the 95% confidence intervals of LoAs were considered outliers and were removed from the data for further analysis. Weighted kappa coefficient was then calculated using ‘psych’ package. Kappa coefficient interpretation grid is shown in Table 1²⁰.

Table 1 - Interpretation of Kappa coefficient according to Landis and Koch

Kappa coefficient	Interpretation
< 0	Poor agreement
0-0.20	Slight agreement
0.21-0.40	Fair agreement
0.41-0.60	Moderate agreement
0.61-0.8	Substantial agreement
0.81-1.00	Almost perfect agreement

1.2. Variability in detection of diarrhea between direct evaluation and photographic assessment

A pathological threshold was previously set for this FSS, based on its link with reduced daily weight gain. For large breeds, this threshold was set at 5/13⁴. The 80 fecal scores (40 stools assessed by the two methods) were thus converted into binary data. Fecal scores of 5 or less were considered as diarrhea (D+), whereas scores of 6 or more were considered as normal feces (D-).

FS scoring was considered the reference method for detecting diarrhea; false positives and negatives were identified among PS scores. Validity of the studied method was then assessed by calculating sensibility, specificity, and positive and negative predictive values.

2. Reproducibility of photographic stool assessment

2.1. Inter-observer agreement

A previously available library of 98 photographs of stools coming from puppies of various breeds and ages was used for this study. Pictures were carefully chosen to ensure a selection of different consistencies representative of the entire FSS. Four observers (Obs1 to Obs4), all laboratory staff, three veterinarians and one non-veterinarian researcher, took part in this second experiment. Obs4 was an experienced user of the FSS, while the three other observers had never used it before. Each observer scored independently this photo library in Toulouse, France around the same period, using the FSS described above. . No further explanation was provided to the observers in addition to the FSS prior to the scoring session (S1).

For each picture, the difference between Obs1, Obs2 and Obs3 score and the Obs4 (reference observer) score was calculated. Inter-operator reproducibility was assessed by calculating intraclass correlation coefficient (ICC) using ‘irr’ package. Two-way random-effects, absolute agreement, single measurement model was used. ICC interpretation grid is shown in Table 2²¹.

Table 2 – Interpretation of Intraclass correlation coefficient according to Koo and Li

ICC	Interpretation
< 0.5	Poor reliability
0.5-0.75	Moderate reliability
0.75-0.9	Good reliability
> 0.9	Excellent reliability

2.2. Influence of a training session on inter-observer agreement

Two months after S1 described above, Obs4 provided the three other observers with training on the practical application of the FSS. During this training, Obs4 explained in more detail the differences in consistency described in the FSS, warned about the bias associated with stool color and the presence of mucus, and answered the observers’ questions, using practical case studies illustrated with pictures. Immediately after the training, each observer scored for the second time the same 98 photographs of stools during a second scoring session (S2).

Post-training ICC (ICC2) was calculated in the same way as for the pre-training ICC (ICC1). The influence of training on inter-observer agreement was assessed based on the 95% confidence interval overlap of ICCs.

2.3. Intra-observer agreement

Spatio-temporal reproducibility of the PS method was assessed by comparing the fecal scores assigned by Obs4 during S1 and S2. Indeed, Obs4 was the only observer not impacted by the training on FSS use.

For each picture, the difference between S1 and S2 scores was calculated. To assess intra-observer agreement, a Bland and Altman diagram was created in the same way as in the between-method agreement experiment. The same maximum acceptable b and LoAs were used as above. The normality test, removal of outliers and calculation of weighted kappa coefficient were done as previously.

Results

1. Evaluation of variability between direct and photographic assessment of stools

Fecal scores of the 40 fresh stools (reference method) ranged from 3 to 10, with a median value of 7. PS scores ranged from 2 to 10, with a median value of 6 (Fig 1A). All fecal scores are shown in an additional file (see Additional file 1). Differences between PS and FS scores followed a normal distribution (Fig 1B). Fecal scores evaluated on PS were equal to those on FS in 37.5% of cases (15/40), and 1 point lower in 40.0% of cases (16/40). According to the Bland-Altman diagram for between-method agreement, the b of the method was -0.55 (95% CI [-0.90, -0.20]) (Fig 1C). Lower and upper LoAs were -2.68 (95% CI [-3.28, -2.08]) and 1.58 (95% CI [0.98, 2.18]), respectively. Differences between PS and FS scores were randomly distributed on either side of the b line for all score values, indicating that there was no variation in b according to fecal score value. One outlier was identified (stool 29) and removed from the dataset for further analysis. Weighted kappa coefficient ($n=39$) was 0.83 (95% CI [0.75, 0.91]).

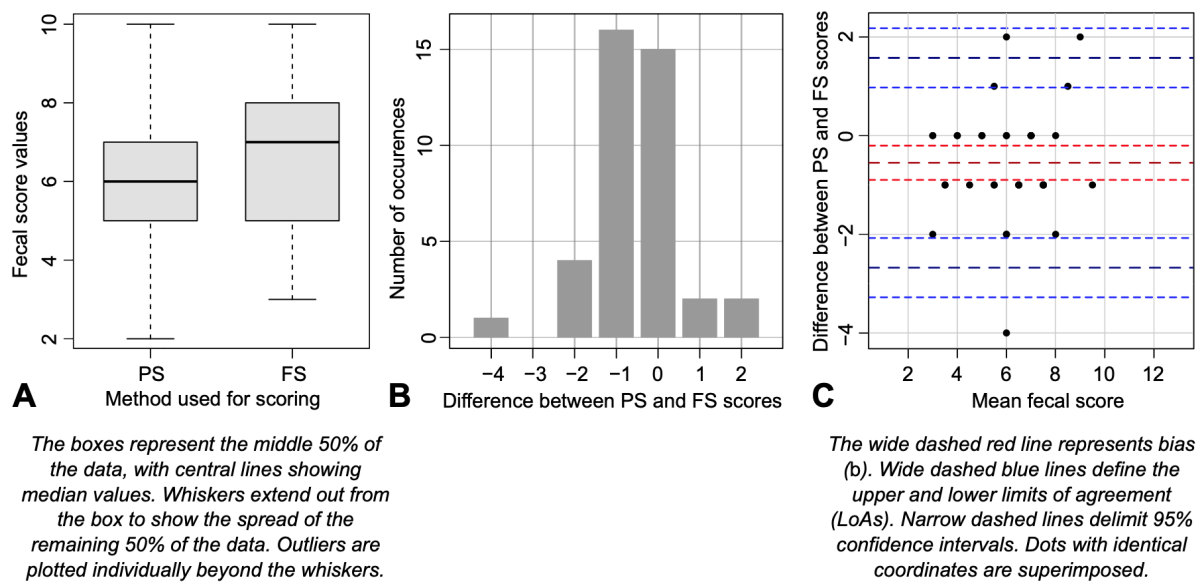


Fig 1 – Between-method agreement. The box plot represents the fecal scores obtained on the 40 stools according to the scoring method: on photographed stools (PS) and on fresh stools (FS) (1A). The histogram represents the differences between PS and FS scores (1B). And the dot-plot is the Bland-Altman diagram: differences between the scores are shown in relation with the average score obtained using both methods (1C).

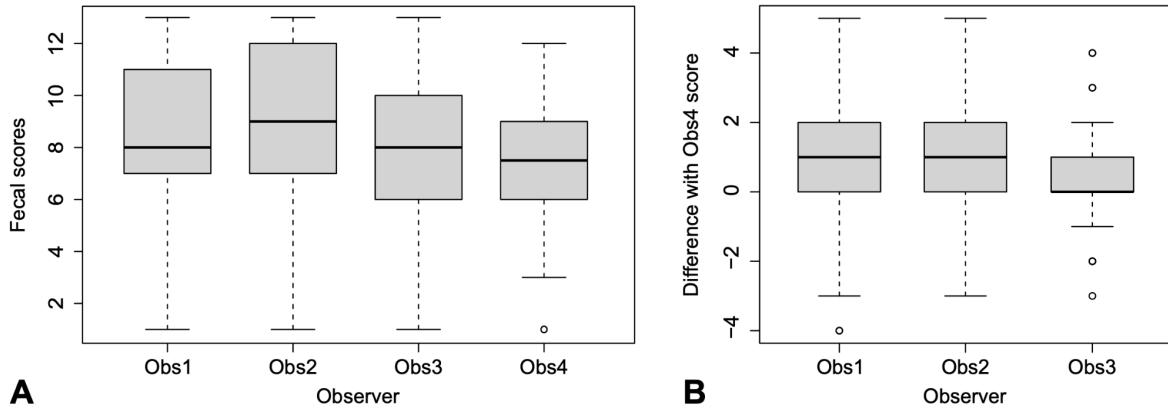
Prevalence of diarrhea diagnosed on FS was 33.3% (13/39; Table 3). When compared to the results on PS, the number of false positives and negatives were 4 and 2, respectively. Sensitivity and specificity of the PS method were 84.6% for both indicators. Positive and negative predictive values were 73.3% and 91.7%, respectively.

Table 3 – Number of stools scored as D+ and D- according to the method used

		FS method		Total
		D+	D-	
PS method	D+	11	4	15
	D-	2	22	24
Total		13	26	39

2. Reproducibility of photographic stool assessment

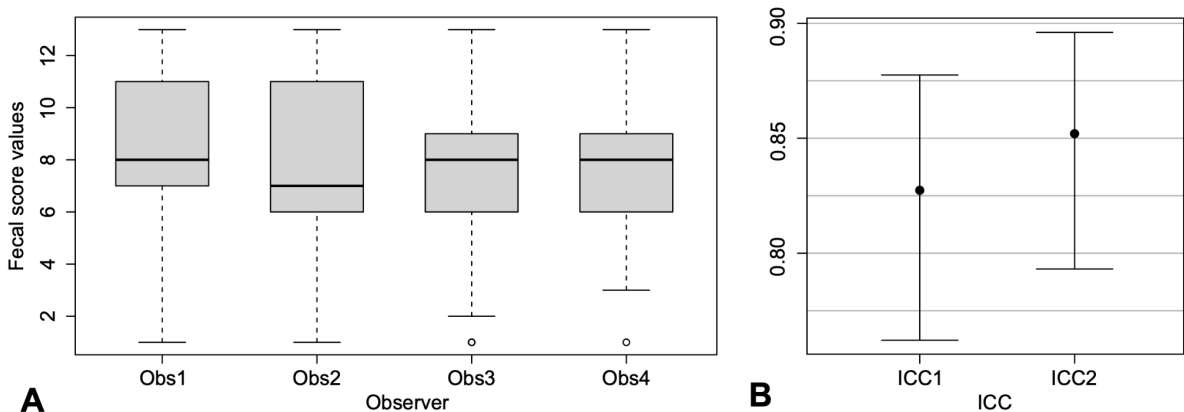
The 98 stools studied during S1 had fecal scores ranging from 1 to 12 according to the reference observer (Obs4; Fig 2A). Median fecal scores attributed by Obs1, Obs2, Obs3 and Obs4 during S1 were 8, 9, 8 and 7.5, respectively (see Additional file 1 for all data). Fecal scores attributed by Obs1, Obs2 and Obs3 during S1 were equal to those attributed by Obs4 in 26.5% (26/98), 28.6% (28/98) and 40.8% (40/98) of cases, respectively. Mean differences with Obs4 scores for Obs1, Obs2 and Obs3 scores were 0.7 ± 1.7 , 1.1 ± 1.7 and 0.28 ± 1.2 , respectively (Fig 2B). ICC1 was 0.83 (95% CI [0.76, 0.88]).



The boxes represent the middle 50% of the data, with central lines showing median values. Whiskers extend out from the box to show the spread of the remaining 50% of the data. Outliers are plotted individually beyond the whiskers.

Fig 2 – Inter-observer agreement before a training session. The box and whisker plots represent the fecal scores obtained on the 98 stool photographs according to the observer (Obs1 to Obs4) (2A) and the differences between the fecal scores assigned by non-trained observers (Obs1 to Obs3) and those assigned by the reference observer (Obs4) (2B).

Median fecal scores attributed by Obs1, Obs2, Obs3 and Obs4 during S2 were 8, 7, 8 and 8, respectively (Fig 3A). ICC2 was 0.85 (95% CI [0.79, 0.90]). Overlap of ICC1 and ICC2 95% confidence intervals was marked (Fig 3B), and the two ICCs were therefore considered not significantly different.



The boxes represent the middle 50% of the data, with central lines showing median values. Whiskers extend out from the box to show the spread of the remaining 50% of the data. Outliers are plotted individually beyond the whiskers.

Central dots represent values and error bars their 95% confidence intervals.

Fig 3 – Influence of a training session on inter-observer agreement. The box and whisker plot represents the fecal scores obtained on the 98 stool photographs during the post-training evaluation according to the observer (Obs1 to Obs4) (3A). The dot-plot represents the intraclass correlation coefficients (ICCs) before (ICC1) and after (ICC2) the training session according to their 95% confidence intervals (3B).

Median Obs4 fecal scores assigned during S1 and S2 were 7.5 and 8, respectively (Fig 4A). Differences between S1 and S2 scores attributed by Obs4 followed a normal distribution (Fig 4B). Fecal scores attributed by Obs4 during S2 were equal to those attributed during S1 in 59.2% of cases (58/98). They were one point lower in 17.3% of cases (17/98) and one point higher in 15.3% of cases (15/98). According to the Bland-Altman diagram for intra-observer agreement, the bias was -0.08 (95% CI [-0.26, 0.09]). Lower and upper LoAs were -1.79 (95% CI [-2.09, -1.48]) and 1.62 (95% CI [1.32, 1.92]), respectively. Differences between S1 and S2 scores were randomly distributed on either side of the bias line for all score values, indicating that there was no variation in bias according to fecal score value. Five outliers were identified (stools 12, 13, 27, 43 and 72) and removed from the dataset for further analysis. Weighted kappa coefficient (n=93) was 0.95 (95% CI [0.93, 0.97]).

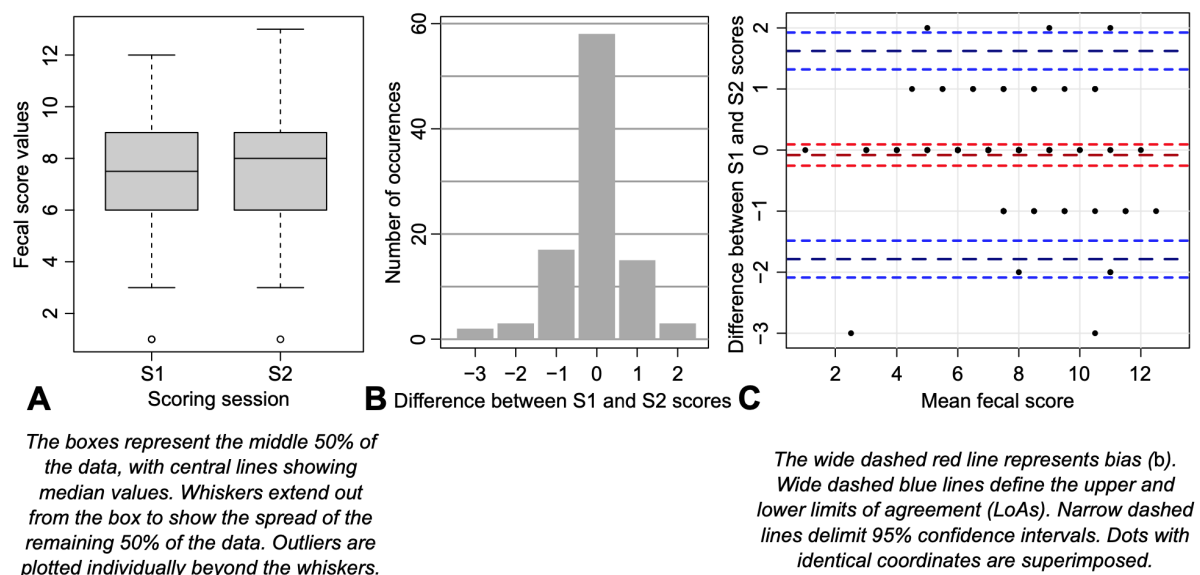


Fig 4 – Intra-observer agreement. The box and whisker plot shows the fecal scores attributed to the 98 stool photographs by the reference observer (Obs4) according to the scoring session (S1 and S2) (4A). The histogram represents the differences between the S1 and S2 scores attributed by Obs4 (4B). And the dot-plot is the Bland-Altman diagram differences between the scores are shown in relation with the average score obtained during the two sessions (4C).

Discussion

Photographic versus direct assessment of stools

This study highlighted the potential for using photograph-based scoring in daily medical practice and research projects to remotely evaluate fecal scores in puppies. Indeed, in the corresponding Bland and Altman plot, b fell within the maximum acceptable bias of ± 1 . The lower and upper LoAs were both within the acceptable LoAs range of ± 3 . Additionally, the weighted kappa coefficient indicated almost

perfect agreement according to Landis and Koch²⁰. However, the 95% confidence interval of the lower LoA extended below -3, representing a limitation of our study. Further studies with a larger sample size could help clarify the potential range of the lower LoA.

To minimize bias associated with visual memory, assessments in our study were conducted by two different operators. Recall bias is a well-documented phenomenon. For instance, a study demonstrated that pathologists could recall slides viewed four weeks earlier, with recall rates sufficient to influence intra-observer study design [9]. Therefore, we opted to use two observers to evaluate agreement between the reference and the photograph-based scoring methods. While this approach may add inter-observer variability, representing a potential study limitation, the two observers collaborated closely beforehand to reduce its impact.

The prevalence of diarrhea in our population was 33.3%, consistent with the reported prevalence in the general French puppy population around the weaning period. Indeed, previous research showed that diarrhea is the most frequently reported health issue among French breeders after puppy sale, with a prevalence of 32%²². Our findings demonstrated that photograph-based evaluation is an effective method for diagnosing diarrhea in puppies. Both sensitivity and specificity of this method were satisfactory for utilization in practice. The negative predictive value indicated excellent performance, while the positive predictive value was lower, but still acceptable. This aligns with the significant negative bias observed in the Bland-Altman analysis, where observers tended to slightly underestimate PS compared with FS, leading to some false positives in diarrhea diagnosis. While a high NPV is crucial in fecal scoring tests to ensure that no sick puppy is left untreated and that all diarrhea cases are accounted for in research studies, a lower PPV can be considered acceptable as diarrhea can easily be re-evaluated through direct fresh stool scoring.

A limitation of this study is that it only included Golden Retriever dogs, introducing a potential breed bias. Generalization to other breeds remains to be investigated. In addition, since the stools were randomly selected in this study, the entire scoring scale was not represented; no fecal score below 3 and above 10 were included. Future studies should address these missing scores to confirm their agreement with reference method. Nevertheless, the Bland-Altman diagram showed no difference in rating variation depending on whether the score was rather low or high. This suggests that the results obtained can reasonably be extrapolated to extreme scores. And as the tendency is to underestimate the score with picture reading, a score below 3 will most likely be identified as diarrhea.

Intra and inter-observer reproducibility

We demonstrated that the photograph-based scoring method exhibited good intra-observer and inter-observer reliability. Space-time intra-observer reproducibility was assessed using a Bland-Altman analysis. The 95% confidence interval of observed b included zero, indicating no significant difference

in scoring for Obs4 between S1 and S2. The lower and upper LoAs were both well within the maximum LoA range of ± 3 . The weighted kappa coefficient indicated almost perfect agreement according to Landis and Koch²⁰. These results confirmed excellent space-time reproducibility.

Our study also showed good inter-observer reproducibility. Using only the scoring grid without prior training, the ICC corresponded to the ‘good reliability’ category defined by Koo and Li²¹. A training session further improved the ICC, though the increase was not statistically significant, as 95% confidence intervals for ICC1 and ICC2 overlapped substantially. Thus, the FSS proved easy to use without requiring additional training.

However, only one non-veterinary observer took part in this study. Veterinarians have been found to assign more homogeneous scores than lay people with different fecal score grids⁶. Inter-observer reproducibility may therefore be overestimated in our study, and further studies involving a larger number of observers with different backgrounds are necessary to extend with confidence the use of the FSS to lay people in future research projects.

Perspectives

Taken together, our results suggest that fecal consistency assessment and diarrhea identification can reliably be performed using a photograph-based method. This aligns with findings in infants and young children, for whom a specific stool scale was developed and shown to have good agreement with fresh stool scoring^{8,23}.

For research studies, photography is a simple, low-cost solution for collecting visual data on a large number of stools, enabling deferred scoring across time and space. This approach could facilitate large multicenter studies and the creation of international databases in the future to better study transit-related factors. Strong inter-operator agreement would allow multiple veterinarians from different research centers to be included in study protocols, potentially accelerating research advancements in canine gut health. Beyond fecal scoring, stool photographs could also support the assessment of additional features, such as its color, mucus presence or traces of blood, further aiding in describing the animals’ state of health^{24,25}.

For veterinary practitioners, using a stool consistency scale can be useful to monitor changes in transit time²⁶. Photography could enable them to objectify more quickly diarrhea cases through first-line remote assessment. Stool photographs could thus be integrated into health monitoring software, enabling closer surveillance by veterinarians through visual comparison of stools over time. However, it is worth remembering that remote fecal scoring should remain a mere complementary tool and should not replace a face-to-face physical examination to identify the cause of diarrhea. Evaluating puppy’s weight gain curve still remains essential for identifying serious health concerns.

Conclusion

In conclusion, the puppy fecal scoring scale developed by Grellet *et al.* can be reliably applied to stool photographs. This easy-to-use technique requires no prior training, making it accessible to all practitioners and researchers. It delivers results consistent with those obtained from fresh stools, enabling accurate diagnosis of diarrhea in puppies. This method offers a promising solution for monitoring canine transit in veterinary practices or research projects. Additionally, the use of digital photographs opens up the possibility of integrating artificial intelligence into fecal score interpretation in the future. This could significantly reduce the workload of research teams and accelerate advancements in the fields of veterinary gastroenterology and pediatrics.

Competing interests

Authors report no conflict of interest in this study.

Acknowledgment

The authors would like to express their gratitude the Visio Foundation²⁷ and Occitanie Region for their financial support on this project. Special thanks are extended to all NeoCare team members and volunteers who ensure the smooth running of the Toulouse Gold Cohort, as well as to all adopters and foster families involved in the associated projects.

Author contributions

The authors contributed to the project as follows: Conceptualisation – IB, EV, AG, HM; Scoring – IB, EV, AH, HM; Data registration – IB, EV; Data Analysis – CC, EV, FL, IB; Original writing – CC; Review and editing – AG, AM, HM, FL, IB. All authors read and approved the final manuscript.

Funding

This research project was financially supported by the Occitania Region and VISIO Foundation²⁷.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article (and its additional file).

Ethics, Consent to Participate, and Consent to Publish declarations

This study was approved by local ethics committee *Animal Health and Science Ethics Committee* (“Comité d’éthique science et santé animales”, CEEA SSA N°115). Some stool pictures were taken

from client-owned animals as part of a previous study with informed consent from their owner. Other animals involved in this study were owned by Ecole Nationale Vétérinaire de Toulouse.

Consent to participate and consent to publish: not applicable.

References

1. Sævik, B. K., Skancke, E. M. & Trangerud, C. A longitudinal study on diarrhoea and vomiting in young dogs of four large breeds. *Acta Vet Scand* **54**, 8 (2012).
2. Grellet, A. *et al.* Risk factors of weaning diarrhea in puppies housed in breeding kennels. *Prev Vet Med* **117**, 260–265 (2014).
3. Pugh, C. A. *et al.* Incidence rates and risk factor analyses for owner reported vomiting and diarrhoea in Labrador Retrievers – findings from the Dogslife Cohort. *Prev Vet Med* **140**, 19–29 (2017).
4. Grellet, A. *et al.* Validation of a fecal scoring scale in puppies during the weaning period. *Prev Vet Med* **106**, 315–323 (2012).
5. Westermarck, E. Chronic Diarrhea in Dogs: What Do We Actually Know About It? *Top Companion Anim Med* **31**, 78–84 (2016).
6. Cavett, C. L. *et al.* Consistency of faecal scoring using two canine faecal scoring systems. *J Small Anim Pract* **62**, 167–173 (2021).
7. Rolfe, V. E., Adams, C. A., Butterwick, R. F. & Batt, R. M. Relationship between faecal character and intestinal transit time in normal dogs and diet-sensitive dogs. *J Small Anim Pract* **43**, 290–294 (2002).
8. Vandenplas, Y. *et al.* Development of the Brussels Infant and Toddler Stool Scale ('BITSS'): protocol of the study. *BMJ Open* **7**, e014620 (2017).
9. Vandeputte, D., Tito, R. Y., Vanleeuwen, R., Falony, G. & Raes, J. Practical considerations for large-scale gut microbiome studies. *FEMS Microbiol Rev* **41**, S154–S167 (2017).
10. McSweeney, B. *et al.* In search of stool donors: a multicenter study of prior knowledge, perceptions, motivators, and deterrents among potential donors for fecal microbiota transplantation. *Gut Microbes* **11**, 51–62 (2020).
11. Smiley, A. Asynchronous Veterinary Telemedicine. *Vet Clin North Am Small Anim Pract* **52**, 1135–1140 (2022).
12. Gyles, C. Veterinary telemedicine. *Can Vet J* **60**, 119–122 (2019).
13. Kastelic, J. & Ogilvie, T. Veterinary Telemedicine is not only here to stay, it's poised to grow and likely exponentially. *Can Vet J* **62**, 1277–1279 (2021).
14. Accueil. *Toulouse GOLD Cohort* <https://toulousegoldcohort.envt.fr/>.

15. Campbell, W. S. *et al.* Visual memory effects on intraoperator study design: determining a minimum time gap between case reviews to reduce recall bias. *Am J Clin Pathol* **143**, 412–418 (2015).
16. Team, R. R: A language and environment for statistical computing. *MSOR connections* (2014).
17. R: The R Project for Statistical Computing. <https://www.r-project.org/>.
18. Martin Bland, J. & Altman, Douglas G. STATISTICAL METHODS FOR ASSESSING AGREEMENT BETWEEN TWO METHODS OF CLINICAL MEASUREMENT. *The Lancet* **327**, 307–310 (1986).
19. Bland, J. M. & Altman, D. G. Measuring agreement in method comparison studies. *Stat Methods Med Res* **8**, 135–160 (1999).
20. Landis, J. R. & Koch, G. G. The measurement of observer agreement for categorical data. *Biometrics* **33**, 159–174 (1977).
21. Koo, T. K. & Li, M. Y. A Guideline of Selecting and Reporting Intraclass Correlation Coefficients for Reliability Research. *J Chiropr Med* **15**, 155–163 (2016).
22. NeoCare. Relationship between breeder and buyer: NeoCare survey results. <https://neocare.pro/wp-content/uploads/2023/05/Enquete-NeoCare-17-Relationnel.pdf> (2020).
23. Aman, B. A., Levy, E. I., Hofman, B., Vandenplas, Y. & Huysentruyt, K. Real Time Versus Photographic Assessment of Stool Consistency Using the Brussels Infant and Toddler Stool Scale: Are They Telling Us the Same? *Pediatr Gastroenterol Hepatol Nutr* **24**, 38–44 (2021).
24. McKenzie, E. *et al.* Prevalence of diarrhea and enteropathogens in racing sled dogs. *J Vet Intern Med* **24**, 97–103 (2010).
25. Mortier, F., Strohmeier, K., Hartmann, K. & Unterer, S. Acute haemorrhagic diarrhoea syndrome in dogs: 108 cases. *Vet Rec* **176**, 627–627 (2015).
26. Lewis, S. J. & and Heaton, K. W. Stool Form Scale as a Useful Guide to Intestinal Transit Time. *Scand J Gastroenterol* **32**, 920–924 (1997).
27. Fondation VISIO. <https://ifi.fondation-visio.org/>.

Chapitre 5

Étude du lien entre le risque de troubles digestives - microbiote et comportement

5.1 Introduction

La période du sevrage représente une phase critique dans le développement des chiots, au cours de laquelle ils sont particulièrement vulnérables à divers troubles, en particulier digestifs (Grellet et al., 2014). Dans ce contexte, l'identification des facteurs de risque et des marqueurs précoces constitue un enjeu majeur en médecine préventive canine. Ce chapitre s'inscrit dans cette démarche, en explorant les liens entre ce risque de troubles digestifs, microbiote fécal et le comportement chez le chiot, à travers les deux premières hypothèses soulevées dans le **Chapitre 3**.

Dans cette étude, nous avons démontré l'existence de deux profils comportementaux distincts chez les chiots à une semaine post-sevrage, correspondant à deux tempéraments : Audacieux (Bold) et Timides (Shy). Ce résultat nous a amenés à recentrer notre réflexion sur le rôle de ces différences de tempérament dans la santé globale du chiot afin de répondre à la première hypothèse selon laquelle : La santé digestive des chiots en post-sevrage est associée à leur tempérament.

En effet, les chiots avec un tempérament audacieux montraient un état de stress physiologique plus élevé et une santé générale – mesurée par le taux de croissance et la prise alimentaire – plus détériorée sur la première semaine post-sevrage. En revanche, s'agissant plus spécifiquement du risque des troubles digestifs – mesuré notamment par le score fécal – les résultats n'ont pas permis de démontrer une association claire avec le tempérament. Cette étude (**Article 2**) a ainsi posé les bases d'une réflexion plus large sur l'interdépendance entre tempérament, microbiote et le risque de troubles digestifs.

Forts de ces premiers résultats, nous avons poursuivi l'exploration en nous appuyant sur un cadre conceptuel bien établi dans la littérature scientifique : l'axe microbiote-intestin-cerveau (gut-brain axis). Ce concept décrit les interactions bidirectionnelles entre le système nerveux central, le comportement (y compris les traits de tempérament) et le microbiote intestinal. Plusieurs études, chez l'homme comme chez l'animal, ont mis en évidence des profils microbiens distincts selon les traits de personnalité (Choudhury et al., 2022; Kim et al., 2018; Rapp et al., 2023)

Dans ce contexte, nous avons cherché à déterminer si les deux profils de tempérament observés chez les chiots s'accompagnaient de compositions microbiennes fécales différenciées afin d'évaluer la

première partie de notre deuxième hypothèse selon laquelle : La composition du microbiote fécal des chiots est associée à leur comportement. L'étude a effectivement révélé des différences significatives de microbiote entre les deux groupes de tempérament. En particulier, certains taxa bactériens se sont révélés discriminants : deux genres appartenant à la famille de *Prevotellaceae* (*Sagatella* et un genre inconnu) et *Campylobacter* ont été significativement plus abondantes chez les Audacieux, tandis que les *Lactobacillaceae* (*Lactobacillus* et *Ligilactobacillus*), *Anaerofilum*, *Enterococcus* et *Corynebacterium* ont été plus abondantes chez les Timides. Ces résultats confirment pour la première fois chez le chiot l'existence d'un lien entre tempérament et microbiote intestinal dès le plus jeune âge.

Enfin, la troisième étude (**Article 4**) nous a permis d'aborder la seconde partie de l'hypothèse 2, en évaluant l'association entre la composition du microbiote fécal des chiots et le risque de troubles digestifs en période de post-sevrage. D'autre part, nous avons également cherché à mettre en évidence l'existence de marqueurs microbiens précoces prédictifs de ces troubles digestifs au sevrage.

Dans cette étude, nous nous sommes intéressés plus spécifiquement au lien direct entre le microbiote et la survenue de diarrhée pendant la première semaine post-sevrage. L'analyse des données a mis en évidence deux entérotypes distincts, présentant des niveaux de risque digestif contrastés : un groupe qualifié de « Sain » (Healthy), caractérisé par une très faible prévalence de diarrhée, et un groupe dit « À risque » (At risk), associé à une forte prévalence de diarrhée. Sept genres bactériens (*Mucispirillum*, *Helicobacter*, *Campylobacter*, *Anaerobiospirillum*, *Anaerofilum*, un genre inconnu de *Butyricoccaceae* et *Suterella*) se sont révélés être associés à l'entérotipe à risque de diarrhée. De plus, nous avons montré que la présence d'*Enterococcus* dans le microbiote précoce (avant le sevrage) était associée de manière significative à l'entérotipe à risque de diarrhée. Ces bactéries pourraient ainsi constituer un marqueur précoce du risque de troubles digestifs, détectable avant même l'apparition de ces troubles. Cette découverte ouvre des perspectives intéressantes en termes de dépistage et de prévention, en permettant d'anticiper le risque digestif à partir de prélèvements simples réalisés au cours de la période néonatale.

En conclusion, ce chapitre illustre comment une approche multidisciplinaire, croisant les dimensions cliniques, microbiologiques et comportementales, permet de mieux comprendre les facteurs associés au risque des troubles digestifs chez le jeune chiot. La mise en évidence d'un lien entre microbiote précoce et troubles digestifs suggère qu'une évaluation précoce du microbiote pourrait servir d'outil de prédiction ou d'orientation pour des interventions ciblées.

Article 2 : Two distinct temperaments in puppies one-week post-weaning: associations with stress, nutrition and fecal quality

I. BENDAHMANE ^{1,2}, L. MONTAGNIER¹, M. SALONEN ³, O. RICHARD¹, K. KEZIMANA¹, F. LYAZRHI⁴, A. MEYNADIER ², H. MILA ¹

¹NeoCare, ENVT, Université de Toulouse, France.

²GenPhySE, Université de Toulouse, INRAE, ENVT, F-31326, Castanet Tolosan, France.

³Department of Biology, University of Turku, 20014 Turun yliopisto, Finland.

⁴École Vétérinaire de Toulouse, Unité pédagogique Biostatistique, 23 chemin des Capelles, F-31076 Toulouse, France

Correspondance : ilyas.bendahmane@envt.fr

Abstract

Social and nutritional weaning (SNW) at adoption is challenging for puppies. This critical period involves significant physiological, functional and behavioral adjustments that can impact puppies' health and well-being. This study investigated the relationship between temperament and health parameters of 103 Golden Retriever puppies during the first week of SNW. For each puppy, three behavioral evaluations (free exploration, sound exposure, and novel object interaction) were conducted the day before weaning (BW) and one-week post-weaning (1WPW). Following these evaluations, blood glucose levels, heart rate, respiratory rate, and rectal temperature were recorded. Stool photographs were taken at 24 hours, 5 and 7 days post-weaning, and at 1WPW puppies were weighed and owners completed a puppy's behavioral questionnaire. Principal Component Analysis on 31 quantitative parameters registered under behavioral evaluations allowed to identify two temperaments in puppies at 1WPW. Seventy-one puppies spent more time in close proximity to the evaluator, showing prolonged latency before interacting with novel objects, and displaying frequent orientation behaviors toward humans (named shy), whereas thirty-two puppies were marked by higher time of environmental exploration, higher time of interaction with novel object, and high locomotor activity with frequent Star-movements (named bold). Additionally, bold puppies trended toward higher hyperactivity/impulsivity scores ($P = 0.100$) according to the behavioral questionnaire. Bold puppies exhibited higher glucose levels ($P = 0.012$), heart rate ($P = 0.023$), and a trend toward increased respiratory rates ($P = 0.084$) compared with shy puppies. Bold puppies also showed a tendency toward reduced food intake during the first week post weaning ($P = 0.054$) and a tendency toward lower fecal scores ($P = 0.081$). These results highlight the relationship between the temperament in puppies and

their health after SNW and emphasize the importance of choosing a home adapted to the puppy behavioral profile to optimize its welfare.

Keywords: Weaning, Puppy, Temperament, Behavior, Fecal score, Weight, Stress, Dog.

Introduction

Dogs are one of the two most popular companion animals worldwide, with 66% of US and 25% of European households owning at least one dog (Megna, 2025; Tim, 2024). Mainly, they are adopted at a young age, typically at about 2 months, which is included in weaning. The term "weaning" is derived from the Latin word "*separare*", meaning "to separate" (Buchi & Schweickard, 2014). In puppies, this phase is marked by both social separation (from the dam, littermates, the breeder), and nutritional separation (with no more access to the maternal milk), collectively referred to as social and nutritional weaning (SNW). This period coincides with the critical socialization phase, essential for the adaptation to the new environment and learning tasks in puppies. Therefore, it is crucial for puppies to feel comfortable during this time to optimize their learning potential.

Although unavoidable from the developmental perspective, weaning can pose significant health challenges that may affect a puppy's development and overall well-being. Key health issues during weaning include digestive disorders (affecting approximately 30% of puppies; (Grellet et al., 2012), weight loss, and emotional stress (Dietz et al., 2018; Scott & Marston, 1950). In guide dog training programs, it has been shown that 30 to 50% of puppies fail to complete training due to temperament issues or health problems (Cobb et al., 2015). Thus, a better understanding of the causes of health issues during the post-weaning period is essential to promote healthy development and improve long-term well-being in dogs.

Puppies' temperament can influence an individual's behavior and responses to the environment. Temperament is often referred to as "coping style," "behavioral tendency," "strategy," "syndrome," "axis," or "construct" (Gosling, 2001; Sih et al., 2004). It has been studied in puppies during weaning using different behavioral tests (DemiRbaş et al., 2016), revealing significant inter-individual variation. While previous research has identified temperament dimensions like bold/shy types (DemiRbaş et al., 2016) or traits including playfulness, curiosity, and sociability (Svartberg & Forkman, 2002). To date, none of the studies investigated any relationship between those temperaments and the gastrointestinal health in the young dog.

It was hypothesized in the following study that the gastrointestinal health of puppies at weaning may be related to their temperament. Thus, this study aimed to (i) determine the temperament of puppies after weaning, (ii) to investigate the link between post weaning temperament, physiological stress, fecal

score, food intake and weight changes during the first week after weaning (iii) to identify a potential preweaning behavioral parameters predictive of post-weaning temperament.

Materials and methods

1. Ethical Note

The study was conducted at the National Veterinary School of Toulouse (ENVT, Toulouse, France). The use of animals and the procedures applied during the study were approved by the Ethics Committee of the University of Toulouse (SSA_2023_007).

Eighteen Golden Retriever female dogs were selected based on health criteria (absence of chronic diseases, common genetic diseases for the breed), behavioral criteria (only non-aggressive dams with adequate maternal behavior) and zootechnic criteria (multiparous dams, with mean litter size at 2 months post-partum of ≥ 5) and included in this study. From about the 5th-7th week of gestation, the female dogs were housed in a dedicated maternity room at the ENVT, and fed the same diet (Hill's Science Plan for puppy- Large Breed – Chicken, Topeka, USA). The food ration provided to the bitches was calculated based on their weight and Body Condition Score (BCS) and adjusted according to the weekly clinical assessment, according to the NRC recommendations (Nutrient Requirements of Dogs and Cats, 2006).

The bitches gave birth to 123 puppies via natural delivery, among which 7 were stillborn, 2 had a congenital malformation and 1 died between 0 and 2 days after birth. Ten puppies were excluded because of missing data. A total of 103 healthy puppies were included in this study.

The puppies were monitored from birth until the weaning (between 56 and 71 days), via daily weight recordings and weekly general clinical exams and socialization sessions (**Annexes du Chapitre 5 i - Supplementary data 1**). Dietary transition was initiated at 28 days of age by introducing kibbles (**Hill's Science Plan for puppy - Large Breed - Chicken**) three times daily in addition to maternal milk. After weaning, puppies received the same kibbles only, with the daily rations based on the puppies' weight as follows:

- 270 g/day for puppies weighing less than 7 kg.
- 300 g/day for puppies weighing between 7 kg and 9 kg.
- 330 g/day for puppies weighing over 9 kg.

Owners were not permitted to give any leftovers or treats to the puppies.

2. Behavioral Assessment

2.1 Behavioral test

All behavioral assessments were conducted by the same researcher and recorded using an Insta360 ONEX2 camera (Arashi Vision, Shenzhen, China) mounted on a 200 cm-high tripod with approximately 150 cm lateral extension (MANFROTTO Pied 420CSU, Cassola, Italy).

The day before weaning (**BW**), the animals were weighed using a calibrated scale and behavioral tests were conducted in a room unfamiliar for the puppies (dimensions: 270cm x 160cm) at the teaching small animal hospital of ENVIT. They were exposed to a series of different situations for approximately 7 minutes per puppy (**Table 1**).

One week later (1 week post weaning; 1WPW), the behavioral tests were repeated in a new room, also unfamiliar to the puppies (dimensions: 240cm x 190cm). They were exposed to another series of situations for approximately 11 minutes per puppy, as described by Riemer et al., (2013) except for the Back test, Staring test, and Table test, which were deemed too constraining for the puppies (**Table 1, Annexes du Chapitre 5 i - Supplementary data 2**).

Table 1 : Behavioral testing performed before and one week post-weaning.

Period	Behavioral test	Duration
Before weaning	Free exploration	2min
	Plush toy interaction test	2min
	Rope interaction	1min
	Walking on plastic bag test	1min
	Noise exposure test	30sec
Post weaning	Free exploration	2min
	Greeting test	1min
	Rope interaction	2min
	Leader-following test	1min
	Problem solving	2min
	Vet check test	30sec
	Noise exposure test	30sec
	Plush toy interaction test	2min

Three comparable sequences among all those filmed BW and 1WPW considered most relevant for assessing the general behavior of the puppies, were chosen to be analyzed:

- Free Exploration Test: The puppy is placed in an unfamiliar room for 2 minutes. The evaluator places the puppy at the center of the delimited area and then positions themselves at one edge of this area without interacting with the puppy. If the puppy escapes from the area, it is returned to the center.
- Noise Exposure Test: The puppy is exposed to an unfamiliar sound for 30 seconds. The evaluator and the puppy remain in the delimited area without interaction. The sound differs according to the day of the evaluation (machine gun noises on day before weaning and balloon pops one week post weaning day, sound level 80 dB-A).
- Plush toy Test: An interactive robotic plush toy designed in the shape of a dog, capable of movement and audio playback (JJ My Jumpin' Pug FurReal Friends, Hasbro, Pawtucket, Rhode Island, USA) is activated and placed at the center of the delimited area. The evaluator stands at the edge of the arena and does not interact with the puppy. The plush toy is reactivated after one minute.

Based on these three sequences, 31 behavioral variables were identified (**Table 2**)

Table 2: Parameters measured in behavioral tests. Exploration = sequence 1, noise exposure = sequence 2, Plush toy interaction = sequence 3.

Behavioral parameter	Details	Sequences
Exploration	Time spent exploring the environment (in s)	1
Movement_Latency	Latency before the first movement (in s)	1
Movement	Time spent moving (in s)	1, 2, 3 ^a
Sitting	Time spent sitting (in s)	1, 2, 3 ^a
Lying_down	Time spent lying down (in s)	1, 2, 3 ^a
Crying_Time	Time spent crying (in s)	1+2+3 ^b
Barking	Number of barks	1+2+3 ^b
Observation	Time spent observing the evaluator (in s)	1, 2, 3 ^a
Glance	Number of glances at the evaluator	1, 2, 3 ^a
Proximity	Time spent near the evaluator (in s)	1, 2, 3 ^a

Star movements	Number of back-and-forths between the toy and the evaluator	3
Ears	Number of times the puppy perks up its ears after a noise	2, 3
Origin	Number of head movements to locate the origin of the noise	2
Tail	Number of times the puppy wags its tail during the first ten seconds	3
Interaction	Time spent interacting with the toy (in s)	3
Environment	Time spent interacting with the environment (in s)	1
Micro-movements	The puppy makes micro movements towards the toy (yes/no)	3
Latency	Latency before interaction with the toy (in s)	3

(1, 2, 3) indicates that the parameter was quantified independently in each sequence.

(1+2+3) indicates that the parameter was quantified across all three sequences combined.

2.2 Behavioral surveys

Two behavioral questionnaires (Questionnaire 1 and Questionnaire 2) were created using the Sphinx IQ3 software (Le Sphinx, Chavanod, France). The questionnaires were completed by the puppy owners at 1WPW, in accordance with the General Data Protection Regulation (GDPR).

The first questionnaire focused on the characteristics of the adopter, the puppy's lifestyle, and the puppy's behavior on the first week after weaning. It included detailed questions about the owners' family situation, the puppy's housing, the method of transport to its new home, as well as its observed behavior upon arrival, during the first night, and during the first week in its new home (**Annexes du Chapitre 5 i - Supplementary data 3**).

The second questionnaire (**Annexes du Chapitre 5 i - Supplementary data 4**) was originally developed to assess the prevalence of problematic behaviors adapted from (Salonen et al., 2020). The questionnaire covered various domains, including general fear and hyperactivity-impulsivity/inattention. In the general fear section, owners first were asked to evaluate whether they feel the puppy is fearful, then asked to select all behavioral responses that describe their puppy and finally, if they classified their puppy as fearful, were asked to evaluate how often the puppy displays fear-related behavioral responses. The hyperactivity-impulsivity/inattention questionnaire was developed and validated (Vas et al., 2007). This questionnaire includes 13 statements and owners' rate how often the statements apply to their puppies on a 4-point Likert scale (never, rarely, often, very often).

3. Stress and Health Evaluation

3.1 Evaluation of physiological parameters of stress

Following the behavioral assessment, the heart rate (HR) and respiratory rate (RR) were measured. A blood sample was taken from the cephalic vein, and glycemia was immediately measured using a glucometer (FreeStyle Optium Neo; Abbott Diabetes Care, Rungis, France). Body temperature was measured with a digital rectal thermometer (Digivet, Génia, Saint-Hilaire-de-Chaléons, France).

3.2 Evaluation of global health

Global health status of the puppies at weaning was assessed using three main indicators: growth rate, ingestion rate, and fecal score. A decrease in any of these indicators was considered suggestive of poorer health status.

Puppies were weighed using the same calibrated scale as previously BW and 1WPW. The growth rate was then calculated as follows:

- Growth rate = $\frac{\text{Weight 1WPW (g)} - \text{Weight BW (g)}}{\text{Weight 1WPW (g)}} * 100$

During the first week after weaning, owners were asked to record the weight of kibbles left by their puppy after a maximum 30-minute-long meal, using a dedicated scale (Intelligent Pet Bowl XL, Eyenimal, Num'axes, Olivet, France). The daily food intake was then calculated separately for each puppy as a difference between the weight of kibbles distributed to the puppy per day and the weight of kibbles left after the three meals. The ingestion rate was then calculated using the following formula:

- Daily ingestion rate = $\frac{\text{Daily food intake (g)}}{\text{Puppy weight at weaning (g)}}$
- Total ingestion rate = $\frac{\text{Total food intake during the first week (g)}}{\text{Puppy weight at weaning (g)}}$

To evaluate the fecal scores of puppies during the first week post-weaning, owners were asked to take photographs of the puppies' stools at 24 hours, 5 and 7 days after weaning. As this schedule was not always respected, photos were regrouped in three periods: first photo: between 0 and 2nd post-weaning day; second photo: between 4th and 6th post-weaning day; third photo: between 7th and 9th post-weaning day.

Subsequently, fecal scoring was performed on the obtained photos as validated by Vellard et al. (Vellard, 2024), using scale developed for puppies by Grellet et al., (2012) ranging from 1 (very liquid stools) to 13 (well-formed but dry feces).

4. Data Analysis

All statistical analyses were performed using R software (version 4.2.1) (R Core Team., 2022). Behavioral parameter quantification was conducted using Boris Behavior version 8.20.3 (Department of Life Sciences and Systems Biology University of Torino - Italy) (Friard & Gamba, 2016).

4.1 Clustering analysis using K-means

K-means clustering was applied to behavioral variables collected at 1WPW. The optimal number of clusters was determined using the Average Silhouette Width method (Batool & Hennig, 2021), which performs k-means clustering across a range of k values (1 to 10 in our case). For cluster visualization, a “cluster plot” was generated by mapping subjects in two dimensions using multidimensional scaling.

4.2 PERMANOVA and principal component analysis (PCA)

To characterize the two clusters identified by the K-means analysis, a PERMANOVA test was conducted using the *adonis2* function on the first and second principal component scores, incorporating explanatory factors: Cluster Group (K-means), Mother, Sex, Batch, and Litter Size. Based on the results, only the first principal component (PC1) was selected for further analyses.

Behavioral variables contributing to PC1 were filtered based on thresholds of contribution ($>1\%$) and squared cosine ($\cos^2 > 0.1$). Subsequently, PCA was performed using the *prcomp* function to visualize the two clusters and identify the discriminating behavioral variables.

4.3 Questionnaire adaptation Protocol: Exclusion of Unsuitable Items, Confirmatory Factor Analysis, and Fearfulness Scoring

The questionnaires were originally developed to assess the behavior of adult dogs. To make these tools more suitable for young puppies, we altered the categorization of puppies after the data collection. First, other questionnaire sections beside general fear and hyperactivity-impulsivity/inattention were deemed unsuitable to assess the behavior of very young puppies and were excluded from the analyses. Second, some items in the (Vas et al., 2007) survey required knowledge of puppy’s behavior during training or trained behaviors. These items (4. “Leaves from place when should stay”, 7. “Seems that doesn’t listen”, 10. “Difficulties with complicated tasks”, 11. “Fails tasks as reacts hastily”) were excluded. For the remaining nine items, we fit a confirmatory factor analysis (CFA) to assess whether the original structure of two factors (hyperactivity/impulsivity and inattention) holds in this sample of young puppies. We ran the CFA model with robust maximum likelihood and full information maximum likelihood approach for missing data and assessed model fit by examining the standardized estimates and model fit indices. We finally extracted regression-based factor scores. Third, puppies were categorized in the fear section manually based on their behaviors (**Table 3**). However, some puppies

were rated differently based on the owner’s free text responses. For example, one owner estimated that their puppy shows fearful behaviors toward people sometimes, but commented that the puppy approaches familiar people, but withdraws from unfamiliar people. Therefore, this puppy was rated as showing high fear.

Table 3. Rating of fear responses in the questionnaire data.

Rating	Fear of unfamiliar people	Fear of unfamiliar dogs	Fear of unfamiliar situations/environments
No fear	keen to meet/ indifferent	keen to meet/ indifferent	calm/curious/able to eat and sleep
Low fear	rarely signs of fear (withdraws)	generally friendly but reciprocates aggressiveness, barks/growls	rarely signs of fear (e.g. stays close, does not want to explore)
Moderate fear	sometimes/often signs of fear (e.g. tail down, tense tail, stays close to owner)	sometimes/often signs of fear (e.g. withdraws, tail down, stays close)	sometimes/often signs of fear (e.g. stays close, does not want to explore) or trembles
High fear	almost always fearful (e.g. withdraws, tail down, stays close)	almost always fearful (e.g. withdraws, stays close, tail between legs)	always/almost always fearful (e.g. stays close, remains still) or pants or trembles often

4.4 Identifying pre-weaning predictors of post-weaning behavioral profiles

To identify BW behavioral variables that could potentially predict the post-weaning behavioral profiles, the dataset of BW variables was analyzed alongside the 1WPW behavioral group data. A Partial Least Squares Discriminant Analysis (PLS-DA) was performed using the “plsda” function from the MixOmics package (Rohart et al., 2017). The optimal number of principal components (ncomp) to maximize discrimination between the two groups was determined using the “tune.splsda” function. The

classification performance was then assessed using two metrics: the Balanced Error Rate (BER) and the Area Under the Receiver Operating Characteristic Curve (AUROC).

To further analyze the model, the Variable Importance in Projection (VIP) scores were computed for the selected principal components. This step identified the key BW behavioral variables contributing to the prediction of 1WPW temperament groups.

4.5 Relationship between behavioral profiles and growth, physiological parameters or predictive behavioral parameters

To examine the relationship between temperament types and various metrics, including growth rate, feed intake, fecal score, heart rate, respiratory rate, blood glucose, temperature, predictive behavioral parameters and hyperactivity-impulsivity—a variance comparison was performed using Fisher’s test “var.test”, followed by Student’s t-test “t.test”.

Additionally, a chi-squared test (chi2) was used to compare levels of fear across the two temperament types.

Results

Characteristics of the studied population

A total of 103 Golden Retriever puppies from six batches, representing 18 litters, were included in this study. The sex ratio (females/males) was 0.9, indicating a balanced distribution. The average birth weight was 412 ± 61 g, with a litter size of 7 ± 3 (ranging from 2 to 11 puppies).

During the first week post-weaning, the growth rate was $10 \pm 8\%$. Post-weaning food intake followed a progressive pattern, with ingestion rate values of 0.031 ± 0.009 g/g body weight at day 2, 0.033 ± 0.009 at day 3, 0.035 ± 0.009 at day 4, 0.036 ± 0.009 at day 5, 0.037 ± 0.008 at day 6, and 0.038 ± 0.008 at day 7, resulting in a total intake of 0.210 ± 0.042 g/g body weight. Fecal scores averaged 6.8 ± 2.1 before weaning, 7.4 ± 2.2 during the first two days post-weaning, 6.8 ± 2.1 between days 4 and 6, and 7.8 ± 2.3 between days 7 and 9.

Puppy weight, blood glucose level, rectal temperature, heart rate, and respiratory rate were measured before and one week after weaning; the corresponding values are summarized in **Table 4**.

Table 4. Health parameters before and 1 week after weaning (mean $\pm$ SD)

Health parameters	^a BW (mean SD)	^b 1WPW (mean SD)
Weight (g)	68451630	74881640
Blood glucose level (mg/dL)	13221	12017
Rectal temperature (°C)	38.40.4	38.60.3
Heart rate (bpm)	19824	18125
Respiratory rate (rpm)	6627	7642

^aBW = Before weaning; ^b1WPW = One week post-weaning

Puppy behavioral profile group one-week post-weaning

In order to determine the behavioral profiles in puppies at 1WPW, a cluster analysis was performed based on the 31 quantitative behavioral variables (**Table 2 ; Annexes Chapitre 5 i - Supplementary data 5**). According to the average silhouette score, the optimal number of clusters was 2 (**Figure 1A**), with good cluster separation observed on the cluster plot (**Figure 1B**).

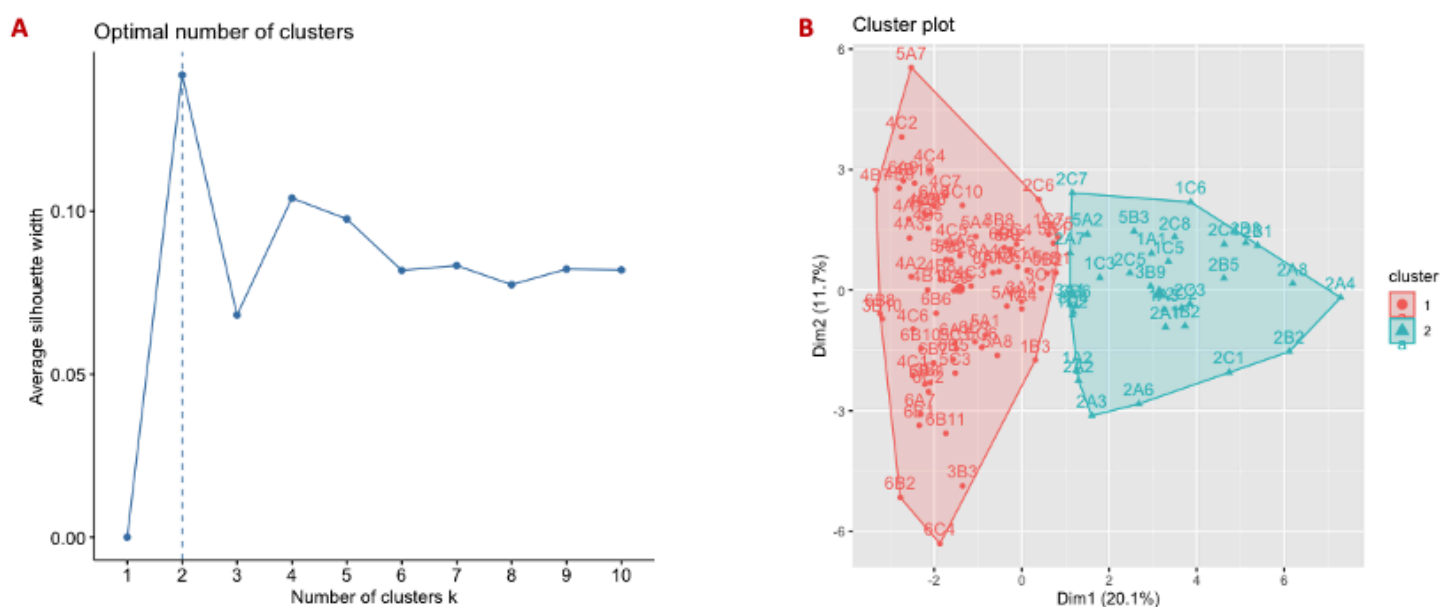


Figure 1: K-means clustering analysis of 1-week post weaning behavioral parameters. The optimal number of clusters is determined using (A) the silhouette method. (B) The clusters are visualized on the first and second dimensions.

According to the permutational multivariate analysis of variance on the first and second principal components, the cluster explained the largest proportion of the variance in PC1 ($R^2 = 72.9\%$; $P < 0.001$), while the mother, batch, and litter size explained only 1.4%, 8%, and 1.1%, respectively. The second principal component does not allow to distinguish between the two proposed clusters (**Annexes Chapitre 5 i -Supplementary data 6**).

Group 1 consisted of 32 puppies (14 males and 18 females), distributed across batches as follows: 8 from batch 1, 18 from batch 2, 4 from batch 3, none from batch 4, 2 from batch 5, and none from batch 6. Group 2 comprised 71 puppies (40 males and 31 females), with the following distribution: 4 from batch 1, 1 from batch 2, 6 from batch 3, 21 from batch 4, 15 from batch 5, and 24 from batch 6.

The first identified cluster (Group 1) was primarily defined by positive loadings on the following 7 behavioral variables: movement time across all three evaluations (Movement1, Movement2, Movement3), exploration time during the first evaluation (Exploration1), interaction time with the plush toy (Interaction3), star-shaped movements during the third evaluation (Star_movements3), and tail wagging when the plush toy was activated (Tail3). In contrast, Cluster 2 (Group2) was associated with different behavioral variables, marked by significant loadings on 7 variables: latency time before interacting with the plush toy (Latency3), the number of glances toward the evaluator during the first and third evaluations (Glance1, Glance3), the number of ear contractions during the second and third evaluations (Ears2, Ears3), proximity time to the evaluator during the third evaluation (Proximity3), lying down time (Lying_down3). The contribution of these behavioral parameters to the first principal component ranged from 2% to 12% (**Figure 2B**). The PCA analyses performed on the two principal components explained 53% of the variance (**Figure 2A**).

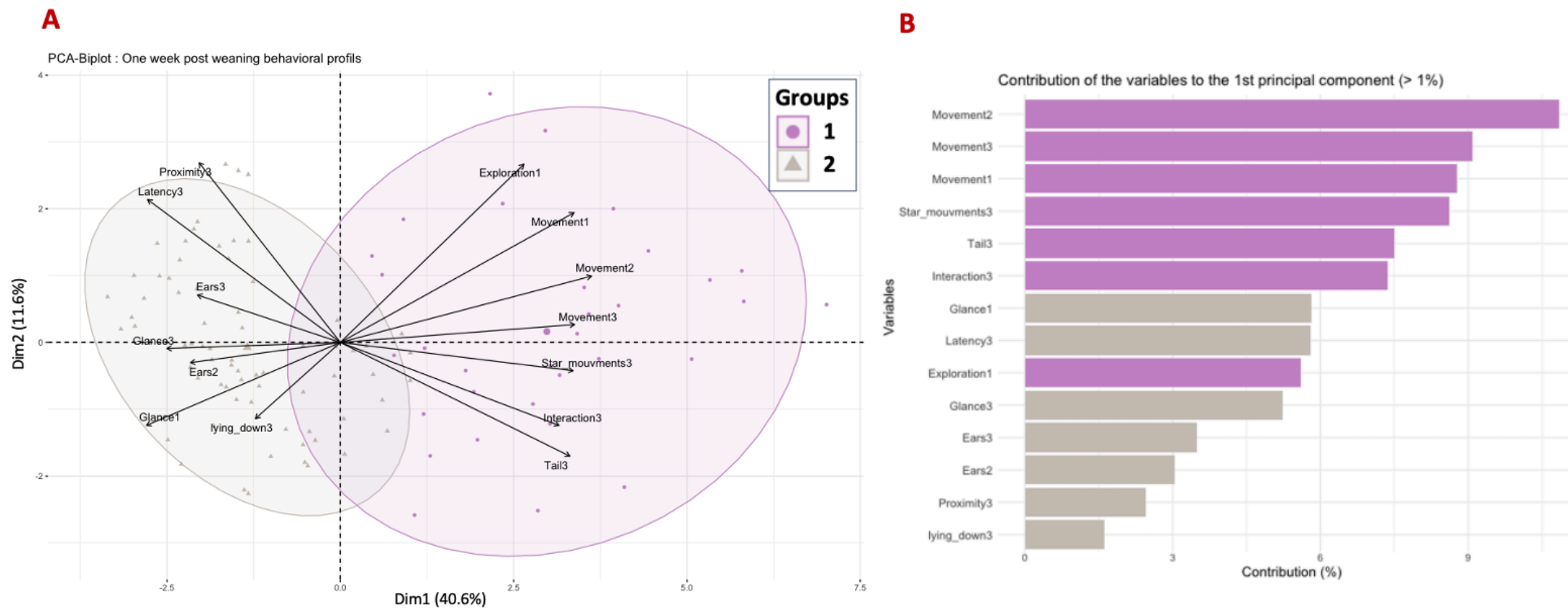


Figure 2 : (A) PCA of behavioral parameters of puppies one week after weaning. Only variables with $\cos^2 > 0.1$ was presented to discriminate the two groups. (B) Contribution plot of behavioral variables to the first principal component.

According to Student's t-tests (two-tailed), significant differences were observed in several behavioral variables between the two profiles. Puppies of the Group1 exhibited more behaviors related to environmental interaction (Latency3, Interaction3, Tail3 and Exploration1) and locomotion (Movement1-2-3, Star_movements3) compared to Group2 puppies. Conversely, the Group2 demonstrated greater interaction and proximity with the evaluator (Glance1-2, Observation2, Ears2-3, Proximity3), as well as more immobility (Lying_down1-3, Sitting3; **Table 5**)

Table 5. Behavioral variables discriminating two profiles of puppies at one-week post-weaning according to PCA Analysis (Group1=32 puppies; Group2=71 puppies). Only variables with $\cos^2 > 0.1$ was evaluated.

Behavioral category	Variables	Group 1 (mean	Group 2 (mean	t.test p-value
Environmental Interaction	Latency3 (sec)	1331	6854	<0.001
	Interaction3 (sec)	3428	59	<0.001
	Tail3 (count)	24	108	<0.001
	Exploration1(sec)	6833	3223	<0.001
Locomotion/Activity	Movement1(sec)	6532	2413	<0.001
	Movement2 (sec)	1610	44	<0.001
	Movement3 (sec)	4932	1111	<0.001

	Star_movements3 (count)	75	11	<0.001
Human Interaction	Glance1(count)	11	32	<0.001
	Glance3(count)	21	53	<0.001
	Ears2 (count)	2	42	<0.001
	Ears3 (count)	22	42	<0.001
	Proximity3 (sec)	3232	5543	0.003
Posture/Immobility	Lying_down3 (sec)	2626	4633	0.001

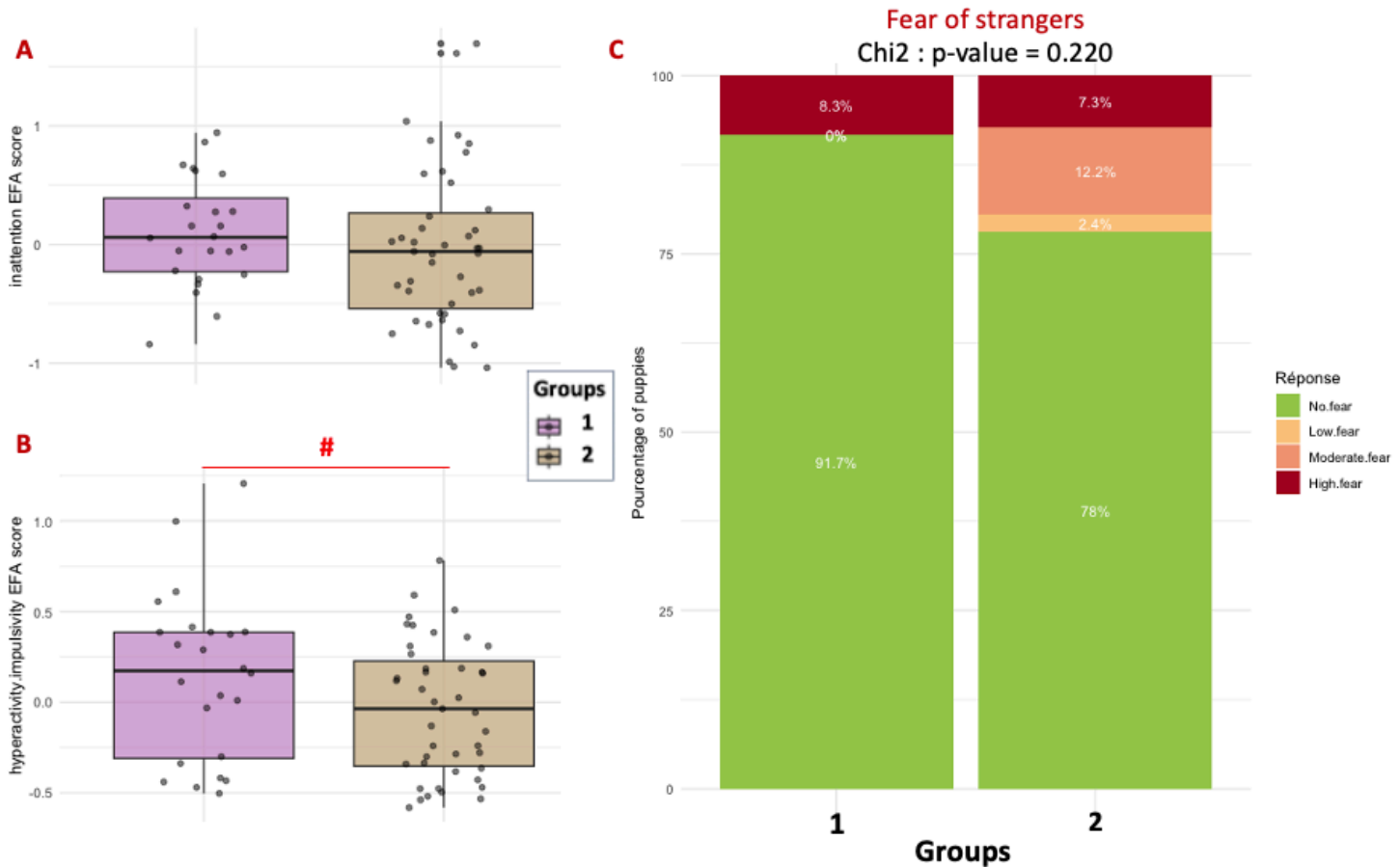
Behavioral profiles and inattention, hyperactivity and fear

Since the increased behavior parameters linked to puppies' activity observed during the behavioral evaluation may be associated with daily impulsivity and hyperactivity, we examined the relationship between these two profiles (**Group 1/Group 2**) and the CFA scores obtained from questionnaires completed by the owners at the end of the first week post-weaning. The results of the student's t-test show no significant difference in inattention ($P = 0.351$) (**Figure 3A**), while there is a trend toward higher hyperactivity/impulsivity scores in **Group1** individuals compared to **Group2** ones ($P = 0.100$) (**Figure 3B**).

Regarding the level of fear in puppies during the first week of weaning, the majority of puppies did not exhibit fear toward strangers (92% in **Group 1** vs. 79% in **Group 2**), whereas those displaying moderate to high fear accounted for 8% in Group 1 and 19% in Group 2. The Chi-square test revealed no significant difference between the two behavioral profile groups across the different fear categories ($p = 0.740$) (**Figure 3C**).

Similarly, 75% of puppies in **Group 1** and 56% in **Group 2** showed no fear of other dogs, while 19% of **Group 1** puppies and 32% of **Group 2** puppies exhibited moderate to high fear. No significant difference was observed between the groups (Chi-square test: $P = 0.637$) (**Figure 3D**).

Regarding fear of novel situations, 47% of **Group 1** puppies showed no fear compared to 70% in **Group 2**, while 28% of **Group 1** puppies exhibited moderate to high fear, compared to 16% in **Group 2** (**Figure 3E**; Chi-square test: $P = 0.637$).



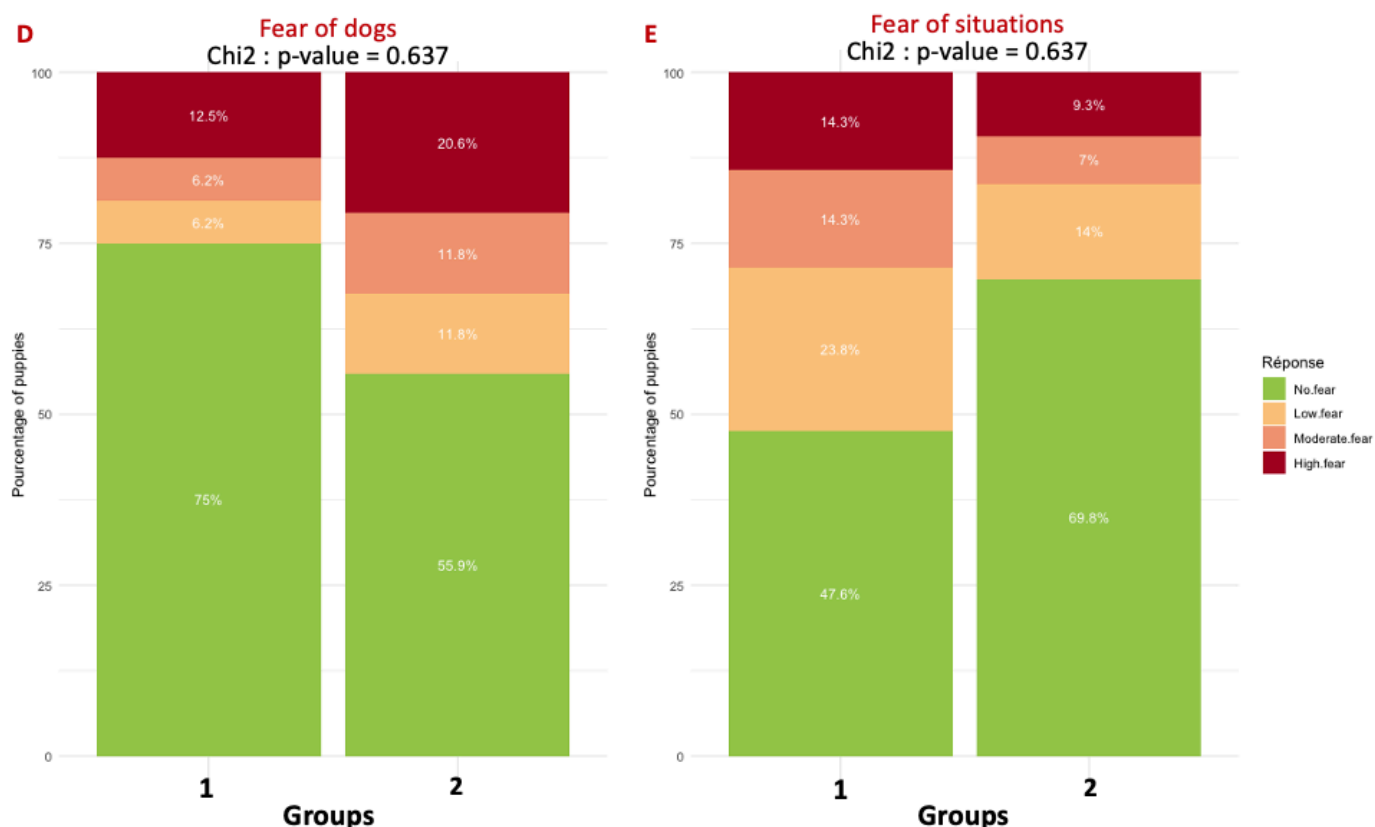


Figure 3 : Student t.test of EFA scores for (A) inattention and (B) hyperactivity/impulsivity based on temperament group. Chi-square test for the distribution of (C) strangers fear, (D) dogs fear and (E) situations fear categories by puppies' behavioral profiles. Significance levels: $0.05 < P \leq 0.1$ (#).

Behavioral profiles and physiological stress

According to univariate analysis, the following stress markers were significantly increased in Group 1 puppies at 1WPW compared with Group 2: glycemia (127 mg/dl vs. 117 mg/dl ; $P = 0.012$), heart rate (190 bpm vs. 178 bpm; $P = 0.023$), and a trend toward increased respiratory rate was observed (87 rpm vs. 72 rpm; $P = 0.084$). No difference was found in rectal temperature values between the two groups (Group 1: 38.7°C vs. 38.7°C in Group 2; $P = 0.990$) (**Figure 4**).

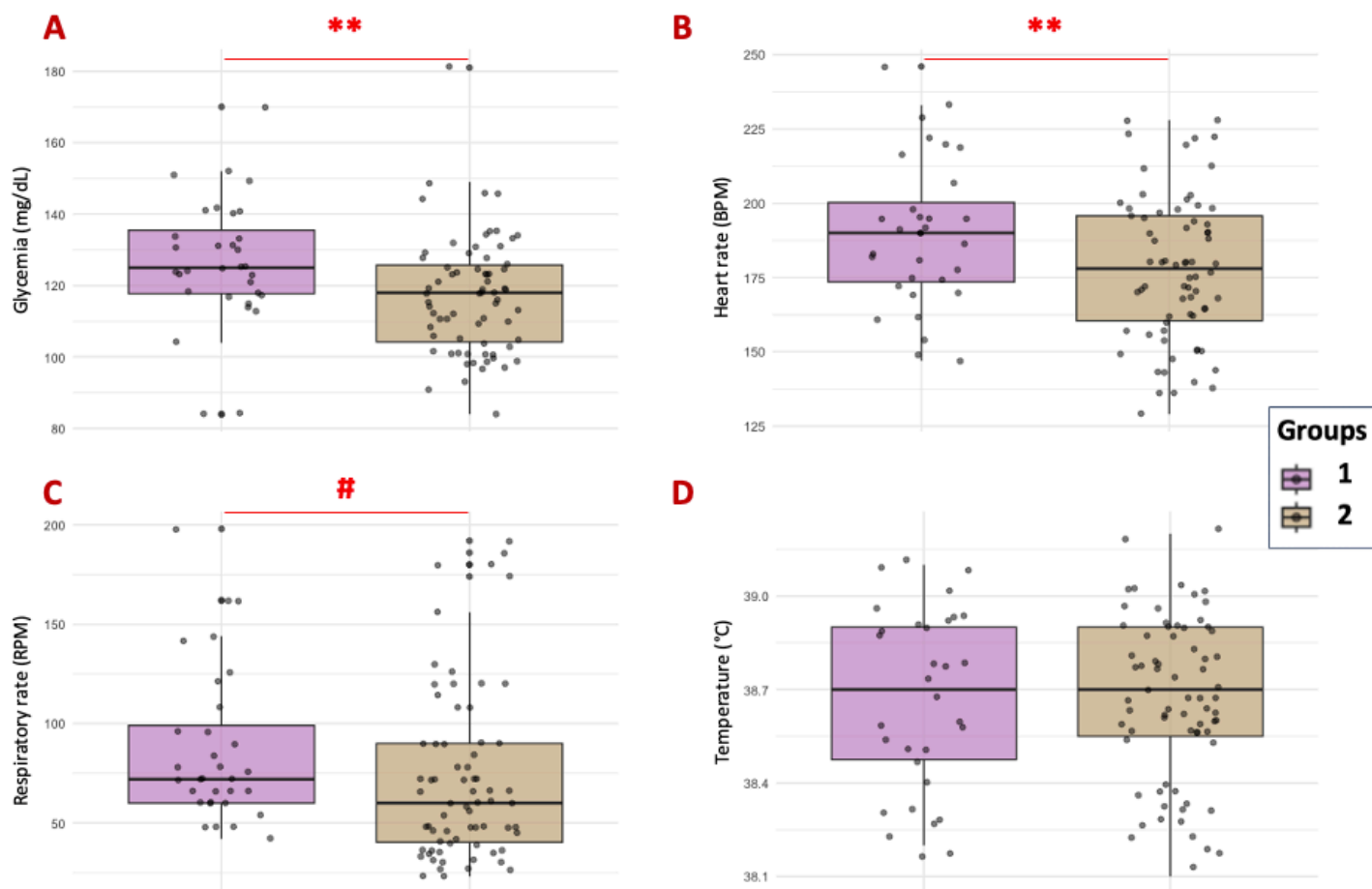


Figure 4: Student's t-test comparing one week post weaning behavioral groups for (A) Glycemia (B) Heart rate (C) Respiratory rate and (D) temperature. Data are presented as mean $\pm$ SD. $P < 0.05$, indicating a significant difference between Group1 and group2. Significance levels: $0.001 P \leq 0.01$ (**), $0.05 P \leq 0.1$ (#).

Behavioral profiles and health

According to the univariate analysis, puppies from Group 1 exhibited a lower growth rate during the first week after weaning compared to puppies from Group 2 (7.7% vs 11.2%; $P = 0.026$) (**Figure 5A**), and a tendency toward lower fecal scores during post-weaning days 7–9 (6.8 vs 8.0; $P = 0.081$) (**Figure 5B**). Moreover, Group 1 puppies tended to have reduced overall food intake during the first week after weaning (20% of puppy weight vs. 22%; $P = 0.054$). (**Figure 5C, 5D**)

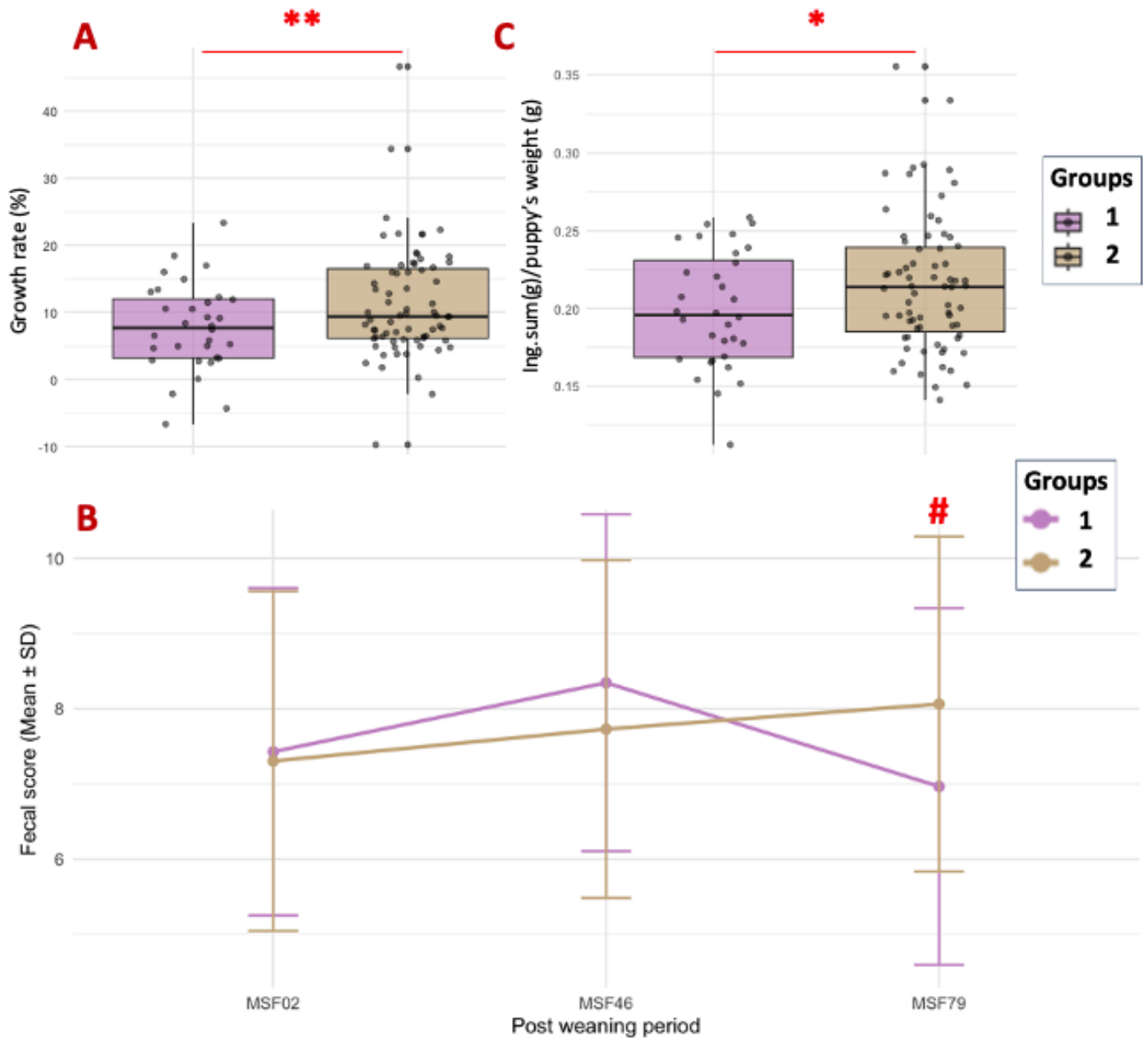


Figure 5 : Student's t-test comparing behavioral groups for : (A) Growth rate during the first week of weaning, (B) Fecal score between days 0–2 (MSF02; n = 92: 30 in Group1, 62 in Group2), days 4–6 (MSF46; n = 84: 27 in Group1, 57 in Group2), and days 7–9 (MSF79; n = 68: 15 in Group1, 53 in Group2) post-weaning, (C) Total kibble intake(g) during the first week of weaning/the puppy's weight at weaning. Data are presented as mean ± SD. Significance levels: $0.001 < P \leq 0.01$ (**), $0.01 < P \leq 0.05$ (*), $0.05 < P \leq 0.1$ (#).

Prediction of behavioral profiles

In order to predict the previously determined post weaning behavioral profiles of puppies, Partial Least Squares Discriminant Analysis (PLSDA) analysis was performed on 31 preweaning behavioral variables. The PLSDA achieved high accuracy in discriminating the two profiles ($\text{AUROC} \geq 0.98$) (Figure 6A, Figure 6B). The two principal components explained 15% of the variance and distinguished the two types of behavioral profiles with a low balanced error rate ($\text{BER} = 0.20$; Figure

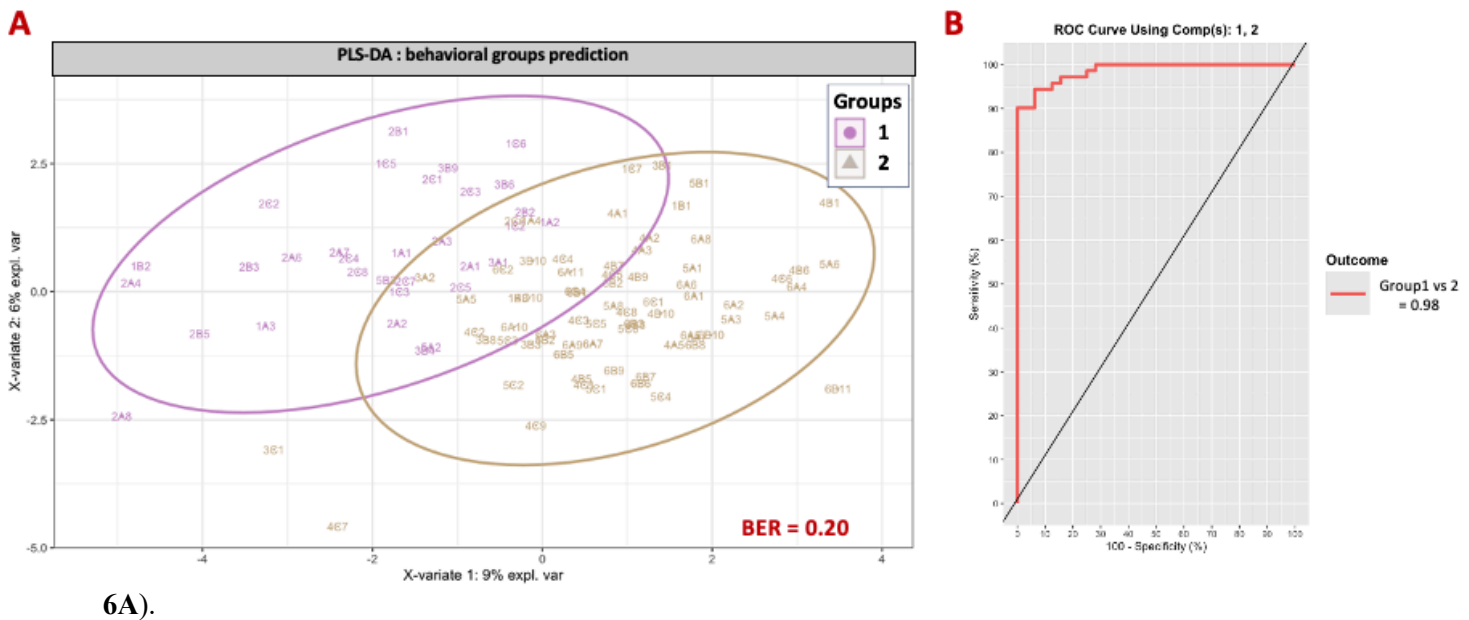


Figure 6 : (A) Partial least-squares-discriminant analysis (PLS-DA) of behavioral preweaning variables responsible for the discrimination of post weaning behavioral profiles. (B) ROC curve.

From the first two principal components, the VIP were calculated, and 10 variables with a score >1 were selected (Table 6). The loading scores of the selected variables ranged between 0.10 and 0.33.

The student's t-test revealed significant differences between Group 1 and Group 2 puppies in all the 10 predictive behavioral parameters (Table 6). BW, Group1 puppies spent less time in proximity to the evaluator compared with Group2 ($71 \text{ sec} \pm 31 \text{ sec}$ vs. $85 \text{ sec} \pm 23 \text{ sec}$; $P = 0.032$ during the first sequence and $50 \text{ sec} \pm 36 \text{ sec}$ vs. $80 \text{ sec} \pm 34 \text{ sec}$; $P < 0.001$ during the third sequence), they spent less time sitting ($39 \text{ sec} \pm 24 \text{ sec}$ vs. $54 \text{ sec} \pm 31 \text{ sec}$; $P = 0.007$), and had a shorter latency before interacting with the interactive toy ($53 \text{ sec} \pm 56 \text{ sec}$ vs. $91 \text{ sec} \pm 50 \text{ sec}$; $P = 0.001$). Conversely, the number of tail movements after the activation of the toy was higher in **Group 1** puppies compared to **Group 2** puppies (11 ± 7 vs. 5 ± 5 ; $P < 0.001$).

Table 6. Discriminant behavioral parameters evaluated one day before weaning allowing to predict puppy behavioral profile one week after weaning according to PLSDA analysis. Group1= 32 puppies; Group2= 71 puppies.

Behavioral category	Parameters	Group1 (mean $\pm$ SD)	Group2 (mean $\pm$ SD)	^a VIP	t.test p-value
Environmental Interaction	Latency3 (sec)	53 $\pm$ 56	91 $\pm$ 50	1.63	0.001
	Tail3 (count)	11 $\pm$ 7	5 $\pm$ 5	2.14	<0.001
Locomotion/Activity	Movement2 (sec)	8 $\pm$ 6	5 $\pm$ 4	1.31	0.026
Human interaction	Proximity1 (sec)	71 $\pm$ 31	85 $\pm$ 23	1.17	0.032
	Proximity3 (sec)	50 $\pm$ 36	80 $\pm$ 34	1.87	<0.001
	Glance2 (count)	1 $\pm$ 1	2 $\pm$ 2	1.16	0.006
	Ears2 (count)	2 $\pm$ 1	4 $\pm$ 2	2.05	<0.001
	Ears3 (count)	2 $\pm$ 2	4 $\pm$ 2	1.95	<0.001
	Origin2 (count)	2 $\pm$ 2	3 $\pm$ 2	1.29	0.004
Posture/Immobility	Sitting3 (sec)	39 $\pm$ 24	54 $\pm$ 31	1.21	0.007

^aVIP-Variable importance score

Discussion

While the weaning period (marked by simultaneous nutritional, environmental, and social transitions) represents a critical developmental challenge for dogs, our understanding of its immediate impacts

remains incomplete. Studies have increasingly focused on the early post-weaning period in puppies (Grellet et al., 2014; Hennessy et al., 2001; Pan et al., 2025), yet significant gaps persist in characterizing the associated health and behavioral consequences. Our study provides novel evidence identifying two distinct behavioral profiles in Golden Retriever puppies as early as 1WPW and establishes their potential links to health outcomes.

Since DemiRbaş et al., (2016) demonstrated that puppies begin to express their emotionality through behavior from the second week of life, the objective of this study was to evaluate the puppies' behavior after the first week of SNW using three tests designed to describe temperament: free exploration of an arena, response to an auditory stimulus, and interaction with a novel object (Hennessy et al., 2001). Two behavioral profiles were identified in the present study: the first (**Group 1**) was characterized by a longer time of environmental exploration, interaction with a novel object, and higher locomotor activity characteristic of a bold temperament. In contrast, the second profile (**Group 2**) was characterized by a longer latency before interacting with the novel object, observing the reference person, and immobility, indicative of a shy temperament. These behavioral patterns align precisely with the 'bold' and 'shy' temperament dimensions previously described in Kangal puppies at 5-6 weeks of age (DemiRbaş et al., 2016), where similar differences in activity levels and body postures were observed during open field tests and novel object interactions. Such temperamental dichotomies have been consistently identified in ruminants (Amin et al., 2022; Finkemeier et al., 2022), rodents (Eccard et al., 2022), fish (Sales et al., 2023) and carnivores (Jean-Joseph et al., 2025).

In order to understand the impact of temperament on physiological stress responses in puppies, we examined heart rate (HR), respiratory rate (RR), blood glucose levels, and body temperature. Interestingly, the bold temperament was associated with higher blood glucose and HR, as well as a trend toward increased RR compared to shy temperament (**Figure 4**). These findings suggest that bold temperament could be associated with a more pronounced physiological stress response, consistent with observations in other species. For example, in the porcine model, bold piglets exhibited increased heart rate during the human approach test (Brown, 2009). A similar mechanism has also been identified in spiders. Bold spiders, as measured by their rapid resumption of activity following an aversive stimulus, showed elevated HR (Shearer & Pruitt, 2014). The observed physiological changes align with known stress markers (Javorka et al., 2018; Moberg et al., 1994; Suess et al., 1980; Taelman et al., 2009), which may reflect either acute stress reactivity or metabolic adaptations linked to temperament. In bold individuals, these responses could stem from hypothalamic modulation, as demonstrated in bold green anole lizards, which exhibit upregulated neuroendocrine gene expression in the ventromedial hypothalamus compared to shy ones (Kabelik et al., 2021). It is also important to consider that elevated HR, RR, and blood glucose levels observed in bold puppies may partly result from increased locomotor activity, as bold individuals tend to explore more actively and engage with their environment more

frequently. Future studies should assess whether these physiological traits predict real-world outcomes (e.g., bold puppies excelling in high-stimulation roles like working dogs) or long-term health implications.

In addition, we observed a trend toward higher hyperactivity/impulsivity scores in the bold group compared to the shy group during the 1st week following the SNW (**Figure 3**). Indeed, the temperament has been shown to be associated with behavioral problems in puppies at this period (Hennessy et al., 2001). In a previous study, puppies that made quicker contact with the car and approached the horn more rapidly during the behavioral assessment—both indicative of boldness—were later identified as having higher behavioral problem scores. These scores, based on owner-completed questionnaires two weeks after adoption, covered nine items: responses to separation, timidity, excitability, fearfulness, elimination, escaping from the residence, destruction, integration into the household, and biting (Hennessy et al., 2001). And in humans, hyperactivity/impulsivity was positively correlated with the novelty-seeking score (a personality trait associated with exploratory activity in response to novel stimulation), with impulsive decision-making being characteristic of boldness (Salgado et al., 2009). The consequences of the temperament in case of behavioral problems may be decisive for the individual from the educational perspective as children with attention deficit hyperactivity disorder (ADHD) were shown to have significantly greater difficulty disengaging from distracting stimuli and completing tasks compared to children without ADHD (Ross & Randolph, 2016). In the context of working dogs, puppies must be able to complete tasks even in distracting environments, such as busy urban areas or parks. Thus, distraction in young puppies is a cause of exclusion from training programs (Arata et al., 2010). Therefore, it is essential to assess the relationship between a puppy's temperament, ADHD-like behavioral traits, and their ability to learn and perform tasks.

Understanding puppy behavior from the earliest stages of life is crucial for predicting their future temperament, optimizing their post-weaning environment, and anticipating potential health challenges. In this study, we demonstrate that pre-weaning behaviors such as movement time and tail wagging (distinguishing the bold temperament), proximity time to the evaluator, latency before interacting with a toy, and sitting duration (distinguishing the shy temperament) could predict the temperament of puppies in the first week following the SNW. However, further research is needed to assess the validity of these short-term predictions beyond the juvenile period and across diverse contexts.

In our study, we observed that boldness was associated with lower food intake, reduced growth rate, and a tendency toward lower fecal scores over the first week post SNW (**Figure 5**). Similarly, Gottlieb et al., (2018) reported a negative association between interaction with a novel object (an indicator of boldness) and the increased risk of diarrhea in macaques. This finding is particularly intriguing, as it forms part of the gut-brain relation (Aziz & Thompson, 1998) poorly studied in young canines.

In summary, our findings underscore the association between temperament, stress, and health parameters of puppies during the first week of SNW. Nevertheless, this study presents some limitations. Only puppies from one breed and from one breeding facility were included, limiting the extrapolation of our results. Although the number of included puppies was relatively high, the number of individuals with retarded growth, low food intake or fecal scores in each group was low, limiting our statistical power. Thus, further studies on a larger dog population are needed to explore the long-term associations between early temperament and health. In particular, investigating the role of the gut microbiota in this relationship may provide novel insights, as recent research in other species has highlighted the influence of microbiota composition on both behavior and digestive health. This opens promising perspectives for a better understanding of the gut-brain axis in weaning puppies.

Author contributions

Conceptualization: I.B., H.M., A.M., O.R.; Data curation: I.B., K.K., L.M.; Formal analysis: I.B., M.S.; Funding acquisition: H.M.; Investigation: I.B.; Methodology: I.B., H.M., A.M.; Project administration: I.B., H.M., A.M.; Visualization: I.B.; Writing Original draft: I.B.; Writing Review & editing: I.B., H.M., A.M., M.S., O.R., FL.

All co-authors revised and approved the manuscript.

Funding

The authors declare financial support was received for the research and publication of this article. This research was supported by the Occitania Pyrenees-Mediterranean Region and Visio Fondation, Bouchemaine, France.

Conflict of interest

We declare no conflicts of interest.

References

- Amin, B., Verbeek, L., Haigh, A., Griffin, L. L., & Ciuti, S. (2022). Risk-taking neonates do not pay a survival cost in a free-ranging large mammal, the fallow deer (*Dama dama*). *Royal Society Open Science*, 9(9), 220578. <https://doi.org/10.1098/rsos.220578>
- Arata, S., Momozawa, Y., Takeuchi, Y., & Mori, Y. (2010). Important Behavioral Traits for Predicting Guide Dog Qualification. *Journal of Veterinary Medical Science*, 72(5), 539–545. <https://doi.org/10.1292/jvms.09-0512>

- Aziz, Q., & Thompson, D. G. (1998). Brain-gut axis in health and disease. *Gastroenterology*, 114(3), 559–578. [https://doi.org/10.1016/S0016-5085\(98\)70540-2](https://doi.org/10.1016/S0016-5085(98)70540-2)
- Batool, F., & Hennig, C. (2021). Clustering with the Average Silhouette Width. *Computational Statistics & Data Analysis*, 158, 107190. <https://doi.org/10.1016/j.csda.2021.107190>
- Brown, J. (2009). *Effects of temperament and handling experience on the stress response and meat quality of pigs*. The University of Guelph.
- Buchi, É., & Schweickard, W. (Eds.). (2014). *Dictionnaire Étymologique Roman (DÉRom): Genèse, méthodes et résultats*. DE GRUYTER. <https://doi.org/10.1515/9783110313482>
- Cobb, M., Branson, N., McGreevy, P., Lill, A., & Bennett, P. (2015). The advent of canine performance science: Offering a sustainable future for working dogs. *Behavioural Processes*, 110, 96–104. <https://doi.org/10.1016/j.beproc.2014.10.012>
- DemiRbaş, S., Emre, Y., & Bahri, B. (2016). Investigation of behavioral developments of Kangal puppies using open field and barrier tests. *Ankara Üniversitesi Veteriner Fakültesi Dergisi*, 63(3), 215–222. https://doi.org/10.1501/Vetfak_00000002732
- Dietz, L., Arnold, A.-M. K., Goerlich-Jansson, V. C., & Vinke, C. M. (2018). The importance of early life experiences for the development of behavioural disorders in domestic dogs. *Behaviour*, 155(2–3), 83–114. <https://doi.org/10.1163/1568539X-00003486>
- Eccard, J. A., Herde, A., Schuster, A. C., Liesenjohann, T., Knopp, T., Heckel, G., & Dammhahn, M. (2022). Fitness, risk taking, and spatial behavior covary with boldness in experimental vole populations. *Ecology and Evolution*, 12(2), e8521. <https://doi.org/10.1002/ece3.8521>
- Finkemeier, M.-A., Krause, A., Tuchscherer, A., Puppe, B., & Langbein, J. (2022). Personality traits affect learning performance in dwarf goats (*Capra hircus*). *Frontiers in Veterinary Science*, 9, 916459. <https://doi.org/10.3389/fvets.2022.916459>
- Friard, O., & Gamba, M. (2016). BORIS: a free, versatile open-source event-logging software for video/audio coding and live observations. *Methods in Ecology and Evolution*, 7(11), 1325–1330. <https://doi.org/10.1111/2041-210X.12584>
- Gosling, S. D. (2001). From mice to men: What can we learn about personality from animal research? *Psychological Bulletin*, 127(1), 45–86. <https://doi.org/10.1037/0033-2909.127.1.45>
- Gottlieb, D. H., Del Rosso, L., Sheikhi, F., Gottlieb, A., McCowan, B., & Capitanio, J. P. (2018). Personality, environmental stressors, and diarrhea in *Rhesus macaques*: An interactionist perspective. *American Journal of Primatology*, 80(12), e22908. <https://doi.org/10.1002/ajp.22908>
- Grellet, A., Chastant-Maillard, S., Robin, C., Feugier, A., Boogaerts, C., Boucraut-Baralon, C., Grandjean, D., & Polack, B. (2014). Risk factors of weaning diarrhea in puppies housed in breeding kennels. *Preventive Veterinary Medicine*, 117(1), 260–265. <https://doi.org/10.1016/j.prevetmed.2014.07.016>

Grellet, A., Feugier, A., Chastant-Maillard, S., Carrez, B., Boucraut-Baralon, C., Casseleux, G., & Grandjean, D. (2012). Validation of a fecal scoring scale in puppies during the weaning period. *Preventive Veterinary Medicine*, 106(3–4), 315–323. <https://doi.org/10.1016/j.prevetmed.2012.03.012>

Hennessy, M. B., Voith, V. L., Mazzei, S. J., Buttram, J., Miller, D. D., & Linden, F. (2001). Behavior and cortisol levels of dogs in a public animal shelter, and an exploration of the ability of these measures to predict problem behavior after adoption. *Applied Animal Behaviour Science*, 73(3), 217–233. [https://doi.org/10.1016/S0168-1591\(01\)00139-3](https://doi.org/10.1016/S0168-1591(01)00139-3)

Javorka, M., El-Hamad, F., Czippelova, B., Turianikova, Z., Krohova, J., Lazarova, Z., & Baumert, M. (2018). Role of respiration in the cardiovascular response to orthostatic and mental stress. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 314(6), R761–R769. <https://doi.org/10.1152/ajpregu.00430.2017>

Jean-Joseph, H., Kortekaas, K., Range, F., & Kotrschal, K. (2025). Decreased risk-proneness with increasing age in equally raised and kept wolves and dogs. *PLOS ONE*, 20(1), e0313916. <https://doi.org/10.1371/journal.pone.0313916>

Kabelik, D., Julien, A. R., Ramirez, D., & O’Connell, L. A. (2021). Social boldness correlates with brain gene expression in male green anoles. *Hormones and Behavior*, 133, 105007. <https://doi.org/10.1016/j.yhbeh.2021.105007>

Megna, M. (2025, January 2). Pet Ownership Statistics 2025. *Forbes*. <https://www.forbes.com/advisor/pet-insurance/pet-ownership-statistics/>

Moberg, E., Kollind, M., Lins, P.-E., & Adamson, U. (1994). Acute mental stress impairs insulin sensitivity in IDDM patients. *Diabetologia*, 37(3), 247–251. <https://doi.org/10.1007/BF00398050>

Nutrient Requirements of Dogs and Cats (p. 10668). (2006). National Academies Press. <https://doi.org/10.17226/10668>

Pan, C., Xu, S., Zhang, W., Zhao, Y., Song, M., & Zhao, J. (2025). Investigating hair cortisol dynamics in German Shepherd Dogs throughout pregnancy, lactation, and weaning phases, and its potential impact on the hair cortisol of offspring. *Domestic Animal Endocrinology*, 92, 106921. <https://doi.org/10.1016/j.domaniend.2025.106921>

R Core Team. *R: a language and environment for statistical computing*. *R found stat Comput* (2022) Vienna, Austria.(2022).

Riemer, S., Müller, C., Virányi, Z., Huber, L., & Range, F. (2013). Choice of conflict resolution strategy is linked to sociability in dog puppies. *Applied Animal Behaviour Science*, 149(1–4), 36–44. <https://doi.org/10.1016/j.applanim.2013.09.006>

Rohart, F., Gautier, B., Singh, A., & Lê Cao, K.-A. (2017). mixOmics: An R package for ‘omics feature selection and multiple data integration. *PLOS Computational Biology*, 13(11), e1005752. <https://doi.org/10.1371/journal.pcbi.1005752>

Ross, P., & Randolph, J. (2016). Differences between Students with and without ADHD on Task Vigilance under Conditions of Distraction. *Journal of Educational Research and Practice*.

- Sales, E., Rogers, L., Freire, R., Luiz, O., & Kopf, R. K. (2023). Bold-shy personality traits of globally invasive, native and hatchery-reared fish. *Royal Society Open Science*, 10(8), 231035. <https://doi.org/10.1098/rsos.231035>
- Salgado, C. A. I., Bau, C. H. D., Grevet, E. H., Fischer, A. G., Victor, M. M., Kalil, K. L. S., Sousa, N. O., Garcia, C. R., & Belmonte-de-Abreu, P. (2009). Inattention and Hyperactivity Dimensions of ADHD Are Associated with Different Personality Profiles. *Psychopathology*, 42(2), 108–112. <https://doi.org/10.1159/000203343>
- Salonen, M., Sulkama, S., Mikkola, S., Puurunen, J., Hakanen, E., Tiira, K., Araujo, C., & Lohi, H. (2020). Prevalence, comorbidity, and breed differences in canine anxiety in 13,700 Finnish pet dogs. *Scientific Reports*, 10(1), 2962. <https://doi.org/10.1038/s41598-020-59837-z>
- Scott, J. P., & Marston, M.-Vesta. (1950). Critical Periods Affecting the Development of Normal and Mal-Adjustive Social Behavior of Puppies. *The Pedagogical Seminary and Journal of Genetic Psychology*, 77(1), 25–60. <https://doi.org/10.1080/08856559.1950.10533536>
- Shearer, T. A., & Pruitt, J. N. (2014). Individual differences in boldness positively correlate with heart rate in orb-weaving spiders of genus *Larinioides*. *Current Zoology*, 60(3), 387–391. <https://doi.org/10.1093/czoolo/60.3.387>
- Sih, A., Bell, A. M., Johnson, J. C., & Ziemba, R. E. (2004). Behavioral Syndromes: An Integrative Overview. *The Quarterly Review of Biology*, 79(3), 241–277. <https://doi.org/10.1086/422893>
- Suess, W. M., Alexander, A. B., Smith, D. D., Sweeney, H. W., & Marion, R. J. (1980). The Effects of Psychological Stress on Respiration: A Preliminary Study of Anxiety and Hyperventilation. *Psychophysiology*, 17(6), 535–540. <https://doi.org/10.1111/j.1469-8986.1980.tb02293.x>
- Svartberg, K., & Forkman, B. (2002). Personality traits in the domestic dog (*Canis familiaris*). *Applied Animal Behaviour Science*, 79(2), 133–155. [https://doi.org/10.1016/S0168-1591\(02\)00121-1](https://doi.org/10.1016/S0168-1591(02)00121-1)
- Taelman, J., Vandeput, S., Spaepen, A., & Van Huffel, S. (2009). Influence of Mental Stress on Heart Rate and Heart Rate Variability. In J. Vander Sloten, P. Verdonck, M. Nyssen, & J. Haueisen (Eds.), *4th European Conference of the International Federation for Medical and Biological Engineering* (Vol. 22, pp. 1366–1369). Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-540-89208-3_324
- Tim, W. (2024, July 29). European pets up from previous 2022 estimate; Cats still top. *European Pet Food Industry Federation*. [https://www.petfoodindustry.com/regions/europe/news/15680551/european-pets-up-from-previous-2022-estimate-cats-still-top#:~:text=The%20European%20Pet%20Food%20Industry%20Federation's%20\(FEDIAF\)%20estimated,year%2Dto%2Dyear%20comparisons.](https://www.petfoodindustry.com/regions/europe/news/15680551/european-pets-up-from-previous-2022-estimate-cats-still-top#:~:text=The%20European%20Pet%20Food%20Industry%20Federation's%20(FEDIAF)%20estimated,year%2Dto%2Dyear%20comparisons.)
- Vas, J., Topál, J., Péch, É., & Miklósi, Á. (2007a). Measuring attention deficit and activity in dogs: A new application and validation of a human ADHD questionnaire. *Applied Animal Behaviour Science*, 103(1–2), 105–117. <https://doi.org/10.1016/j.applanim.2006.03.017>

Vas, J., Topál, J., Péch, É., & Miklósi, Á. (2007b). Measuring attention deficit and activity in dogs: A new application and validation of a human ADHD questionnaire. *Applied Animal Behaviour Science*, 103(1–2), 105–117. <https://doi.org/10.1016/j.applanim.2006.03.017>

Vellard, E. (2024). *Caractérisation de l'état de santé du chiot de race Golden Retriever au cours de l'adoption et évaluation de la méthode de notation du score fécal*. National veterinary school of Toulouse.

Article 3 : Association between fecal microbiota and temperament traits in Golden Retriever puppies one-week post-weaning

I. Bendahmane ^{1,2}, A. Meynadier ², and H. Mila ¹

¹NeoCare, Université de Toulouse, ENVT, Toulouse, France

²GenPhySE, Université de Toulouse, INRAE, ENVT, Castanet Tolosan, France

Abstract

Weaning, defined as the physical separation from the mother, is a critical and stressful event in a puppy's life. It coincides with major behavioral, physiological, and microbial changes, making this a sensitive period for long-term health and temperament development. The gut microbiota (GM), known to influence host behavior through immune, metabolic, and neuroendocrine pathways, may play a key role in shaping emerging personality traits during this window. This study explored the relationship between gut microbial composition and temperament—specifically boldness and shyness—one-week post-weaning in Golden Retriever puppies. Seventy-three puppies from 18 dams across six breeding groups were included and housed under identical conditions until adoption (between 56 and 71 days of age). All received the same diet. At seven days post-weaning, fecal samples were collected via rectal swabbing for 16S rRNA gene sequencing. Puppies also underwent standardized behavioral tests, identifying two profiles: Bold ($n = 25$), marked by high exploration and object interaction; and Shy ($n = 48$), characterized by proximity to the evaluator, resting, and delayed novelty response. Microbiota analysis using ANCOM-BC revealed that Bold puppies had higher abundances of *Prevotellaceae* and *Campylobacteraceae*, and lower levels of *Enterococcaceae*, *Lactobacillaceae*, and *Pasteurellaceae*. At the genus level, *Segatella*, *Campylobacter*, and an unknown *Prevotellaceae* genus were more abundant in Bold puppies, while *Lactobacillus*, *Ligilactobacillus*, *Anaerofilum*, *Enterococcus*, and *Corynebacterium* were more abundant in Shy individuals. *Prevotellaceae* and its genera, particularly *Segatella*, along with two unknown genera were positively associated with exploration and movement. These findings suggest that gut microbial composition may be linked to early-life temperament in puppies. Further studies are needed to better understand how these microbes influence the brain. Integrative approaches, such as blood metabolome analysis, could help clarify the underlying mechanisms. Understanding this link may provide insights into early-life microbial influences on personality development in dogs and its eventual modulation.

Keywords : Temperament, Microbiome, Weaning, Puppy.

Introduction

Weaning is a critical and stressful event in a puppy's life, involving abrupt nutritional and social changes. During this transition, puppies are particularly vulnerable to health disorders, among which diarrhea is one of the most frequently reported issues, with a prevalence of up to 25% (1). Beyond gastrointestinal disturbances, this period also coincides with major physiological and behavioral developments, making it a sensitive window for long-term health programming (2).

Our previous research has demonstrated that temperament may play a role in health outcomes during the early post-weaning period. Specifically, bold temperament—a behavioral trait characterized by increased movement, exploration, and risk-taking—has been associated with decreased growth rates, altered feeding behaviors, and changes in fecal consistency at one week post weaning (1WPW) (**Bendahmane et al. – Article2 – Chapitre 5**). These findings suggest that behavioral traits might influence physiological responses to the weaning process, potentially via underlying biological pathways such as the gut microbiota.

The gut microbiota (GM) is a highly dynamic and complex microbial ecosystem that colonizes the gastrointestinal tract and plays a key role in host health and development. In early life, the GM undergoes major compositional shifts, especially during dietary transitions such as weaning (3). These microbial changes can affect not only digestive health but also immune and neurological functions (4). Indeed, the emerging concept of the gut-brain axis—a bidirectional communication network linking the GM and the central nervous system—provides a compelling framework to understand the interplay between microbial composition and host behavior. Through the production of neurotransmitters, hormones, cytokines, and modulation of the autonomic nervous system, the GM has been shown to influence cognitive and behavioral traits (5). Conversely, stress and behavioral states can modulate gut microbial composition (6).

While this axis has been explored in humans (7,8) and rodent models (4,9), its relevance in domestic animals such as dogs remains under-investigated. In particular, no studies to date have explored the potential interaction between the gut microbiota and temperament in puppies during the sensitive post-weaning period.

Therefore, the objective of this study was to investigate the potential link between GM and temperament, specifically along the bold-shy axis in Golden Retriever puppies at 1WPW. Understanding this interaction could shed light on mechanisms linking early-life behavioral traits to health outcomes and may help identify microbial signatures predictive of resilience or vulnerability in young dogs.

Material and methods

1. Location and ethics

This research study was carried out at the National Veterinary School of Toulouse (ENVT, Toulouse, France). The experimental procedures conducted during the study were reviewed and approved by the Ethics Committee of the University of Toulouse (approval number: SSA_2023_007).

2. Animals

A total of 113 Golden Retriever puppies, born naturally from 18 dams across 6 batches (3 dams per batch), were included in this study. At birth, each puppy was weighed and their sex was recorded. All puppies were born and raised under standardized conditions at the same maternity facility of the National Veterinary School of Toulouse, where they remained until 2 months of age. At the time of inclusion, none of the animals presented with chronic health conditions.

On the day prior to adoption (between 56 and 71 days of age), puppies were individually weighed using a calibrated veterinary scale. Since 4 weeks of age and until the end of the study (at one week post weaning: 1WPW) puppies were fed the same equilibrated dry food for growing dogs according to NRC recommendations (Hill's Science Plan for Puppy - Large Breed – Chicken, Topeka, USA) with the volume being adjusted to their body weight at a weekly basis.

At 1WPW, puppies were presented at the Toulouse Veterinary Teaching Hospital at ENVT for fecal microbiota and behavioral assessments. At the end of the behavioral assessments, they were reweighed and returned to their owners.

3. Behavior and fecal microbiota sampling and analysis

3.1. Temperament profiling

The temperament of the puppies was characterized as part of our previous study (**Bendahmane et al. – Article2 – Chapitre 5**). Briefly, a series of three behavioral test sequences: sequence1 (free exploration), sequence2 (noise exposure), and sequence3 (plush toy interaction), as outlined by Riemer et al. (10) were used to define the temperament profiles of puppies. According to the outcome, puppies were allocated in one of the following temperamental profiles: Bold and Shy.

Puppies assigned to the Bold profile generally displayed greater locomotor activity, increased exploratory behavior in unfamiliar settings and more frequent and prolonged interactions with novel stimuli. Conversely, Shy puppies were characterized by more passive or withdrawn behaviors, including increased latency to approach novel objects, longer durations of immobility or low posture, heightened attention to the human evaluator, and more frequent signs of vigilance or hesitation.

3.2. Sampling, DNA extraction and PCR amplification

Rectal swabs (ETM, COPAN, Brescia, Italy) were collected from all the 113 puppies at exactly 1WPW. Immediately after collection, the swabs were stored at -80°C until further processing.

To extract genomic DNA from the swabs, the Eppendorf tubes containing the swabs were placed in an ice tray and allowed to thaw for approximately 30 minutes. Subsequently, the Quick-DNA Fecal/Soil Microbe Kit (ZymoResearch, Irvine, CA, USA) was used for DNA extraction, following the manufacturer's protocol. Following extraction, the V3-V4 region of the 16S ribosomal RNA gene was amplified by two rounds of PCR as described by Lluh et al (11) using the universal primers 460F (ACGGRAGGCAGCAG) and 460R (TACCAGGGTATCTAATCCT) (12). Then Paired-end sequencing, with read lengths of 250 base pairs (bp) from each end, was conducted using the Illumina MiSeq Platform at the GeT-PlaGe INRAE Platform in Toulouse, France (13).

1.3 Bioinformatics

The resulting sequenced amplicons were analyzed using FROGS (version 3.2.3), following the recommended pipeline guidelines (11,12). The analysis began with read pair assembly using VSEARCH (16), where the amplicon size range was set between 380 and 500 bp. Clustering was performed with parameters set to a distance of 1 and the fastidious option (--distance 1 --fastidious). Chimeras were then removed, and only clusters present in at least 2 individuals and with a minimum relative abundance of 0.005% of the total number of sequences per sample were retained, as recommended by Bokulich et al. (17). Finally, taxonomic classification was conducted using the 16S SILVA database (v138.1) with a pintail score of 100 for quality filtering.

4. Statistical analysis

All statistical analyses were conducted using RStudio (version 2024.09.1+394) (18). First, the 113 individuals were filtered by excluding those with less than 10% of the total Amplicon Sequence Variants (ASVs). This resulted in the removal of 35 individuals from the study. These excluded individuals had a total number of sequences ranging between 0 and 112. Additionally, 5 individuals were excluded due to missing behavioral data. In total, 73 individuals with both microbiota and behavioral data were retained for subsequent analyses.

Alpha diversity was measured using the Shannon index. This index was calculated using vegan package (version 2.6-2) (19). Cofactor effects were tested by analysis of variance (ANOVA) using the following formula:

$$\text{Alpha diversity} \sim \text{Group} + \text{Sex} + \text{Batch} + \text{Weaning age} + \text{1WPW weight} + \text{nSEQ}$$

With nSEQ is the number of reads per individual.

Tukey post-hoc tests were used to assess significance.

Beta diversity was assessed using the Bray-Curtis distance. A PERMANOVA (permutational multivariate analysis of variance) analysis was performed with the `adonis2` function from the `vegan` package using the following model:

$$\text{Bray-Curtis dissimilarity} \sim \text{Group} + \text{Sex} + \text{Batch} + \text{Weaning age} + \text{1WPW weight} + \text{nSEQ}$$

To visualize the results, Non-Metric Multidimensional Scaling (NMDS) was used to represent the beta diversity, providing a graphical overview of the microbial community structure and its variation between temperament groups. For alpha diversity, boxplot was generated to illustrate the difference in microbial diversity (Shannon index) between bold and shy puppies.

Subsequently, the Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) was performed using the ANCOMBC R package (20) to identify differentially abundant microbial genera and families between the temperament groups defined by the variable "Temperament", while controlling for the effect of the batch. The model is represented by the following formula:

$$y = \beta_0 + \beta_1 \cdot \text{Temperament} + \beta_2 \cdot \text{batch} + \epsilon$$

where y being the vector of centered log-ratio (CLR) abundance for each taxa (genus and family), β_0 is the intercept (reference value), β_1 is the coefficient associated with the variable Temperament, β_2 is the coefficient associated with the variable batch, and ϵ is the residual error term.

A heatmap based on Pearson correlation was employed to explore the relationships between behavioral parameters characteristic of boldness/shyness, and the taxa of interest identified through ANCOM-BC analysis at the genus and family levels. This analysis was conducted on the batch-effect-corrected and CLR transformed microbiota data. The heatmap was generated using the `Heatmap` function from the `ComplexHeatmap` package (21), with clustering applied to both rows (bacterial taxa) and columns (behavioral parameters).

Results

Characteristics of the puppies included in the study

On average, 12 ± 2 puppies were included per batch, ranging between 9 and 15. Among the total 73 puppies retained for microbiota analysis, 35 were females and 38 were males. Their weight ranged respectively, from 275g to 573g at birth, from 3885g to 10690g at weaning and from 4400g to 10950g at 1WPW. The adoption took place between 56 to 71 days after birth.

According to the behavioral evaluation, 25 puppies were characterized as bold and 48 as shy. The variables that discriminated between these two temperaments are presented in **Table 1**.

Table 1. Behavioral variables discriminating two temperaments of puppies at one-week post-weaning. (Bold=32 puppies; Shy=71 puppies).

Behavioral category	Variables	Details	Bold (mean $\pm$ SD)	Shy (mean $\pm$ SD)	P-value
Environmental Interaction	Latency3 (sec)	Latency before interaction with the toy (in s)	11 $\pm$ 30	54 $\pm$ 54	<0.001
	Interaction3 (sec)	Time spent interacting with the toy (in s)	33 $\pm$ 28	7 $\pm$ 10	<0.001
	Tail3 (count)	Number of times the puppy wags its tail during the first ten seconds	24 $\pm$ 8	12 $\pm$ 8	<0.001
	Exploration1(sec)	Time spent exploring the environment (in s)	64 $\pm$ 34	31 $\pm$ 25	<0.001
Locomotion/Activity	Movement1 (sec)	Time spent moving (in s)	61 $\pm$ 33	23 $\pm$ 11	<0.001
	Movement2 (sec)		15 $\pm$ 11	5 $\pm$ 4	<0.001
	Movement3 (sec)		49 $\pm$ 32	13 $\pm$ 13	<0.001
	Back-and-forth	Number of back-and-forths	7 $\pm$ 4	1 $\pm$ 1	<0.001

	movements³ (count)	between the toy and the evaluator			
Human Interaction	Glance¹ (count)	Number of glances at the evaluator	1±1	3±2	<0.001
	Glance³ (count)		2±2	5±2	<0.001
	Ears² (count)	Number of times the puppy perks up its ears after a noise	2±2	4±2	<0.001
	Ears³ (count)		2±2	4±2	<0.001
	Proximity³ (sec)	Time spent near the evaluator (in s)	35±32	51±40	0.090
Posture/Immobility	Lying_down³ (sec)	Time spent lying down (in s)	23±24	44±32	0.007

1 = variable measured during the free exploration test of the room. 2 = variable measured during the noise exposure test. 3 = variable measured during the plush toy test.

No significant differences were observed between bold and shy puppies according to the zootechnical characteristics (**Table 2**).

Table2: Characteristics of puppies according to their temperament group (n= 73).

	Bold (mean±SD / Ratio)	Shy (mean±SD / Ratio)	P-value
TOTAL number of puppies	25	48	
Sex ratio (male/female)	0.8 (11 M. 14 F)	1.3 (27 M. 21 F)	0.454
Birth weight (g)	425±60 g	419±61 g	0.721
Weaning weight (g)	7165±1218 g	6894±1741g	0.440
Post weaning weight (g)	7745±1114 g	7668±1706 g	0.818
Weaning age (D)	60±3 D	61±5 D	0.241

M = male; F = female; D = days. p-values: t-test for continuous variables (birth weight, weaning weight, and weaning age); chi-square test for categorical variables (sex distribution and the distribution of puppy groups across batches).

Characterization of the gut microbiota at 1 week post weaning

A total of 1,341,132 sequences were observed in the GM at 1WPW, with 480 identified ASVs. These ASVs were distributed across 7 phyla, 47 families and 170 genera. At the phylum level, the GM was predominantly composed of *Bacillota* (64.4% of the total sequences), followed by *Actinomycetota*, *Bacteroidota*, *Fusobacteriota*, *Pseudomonadota* (**Figure 1A**). At the family level, *Peptostreptococcaceae*, *Lachnospiraceae* accounted for 39.6% of the total sequences (**Figure 1B**). At the genus level 19 genera over 170 presented a relative abundance higher than 1%, with *Peptoclostridium*(previously known as *Clostridium*) and *Blautia* being the most dominant, together accounting for almost one-third (29.3%) of the total sequences in puppies at 1WPW (**Figure 1C**).

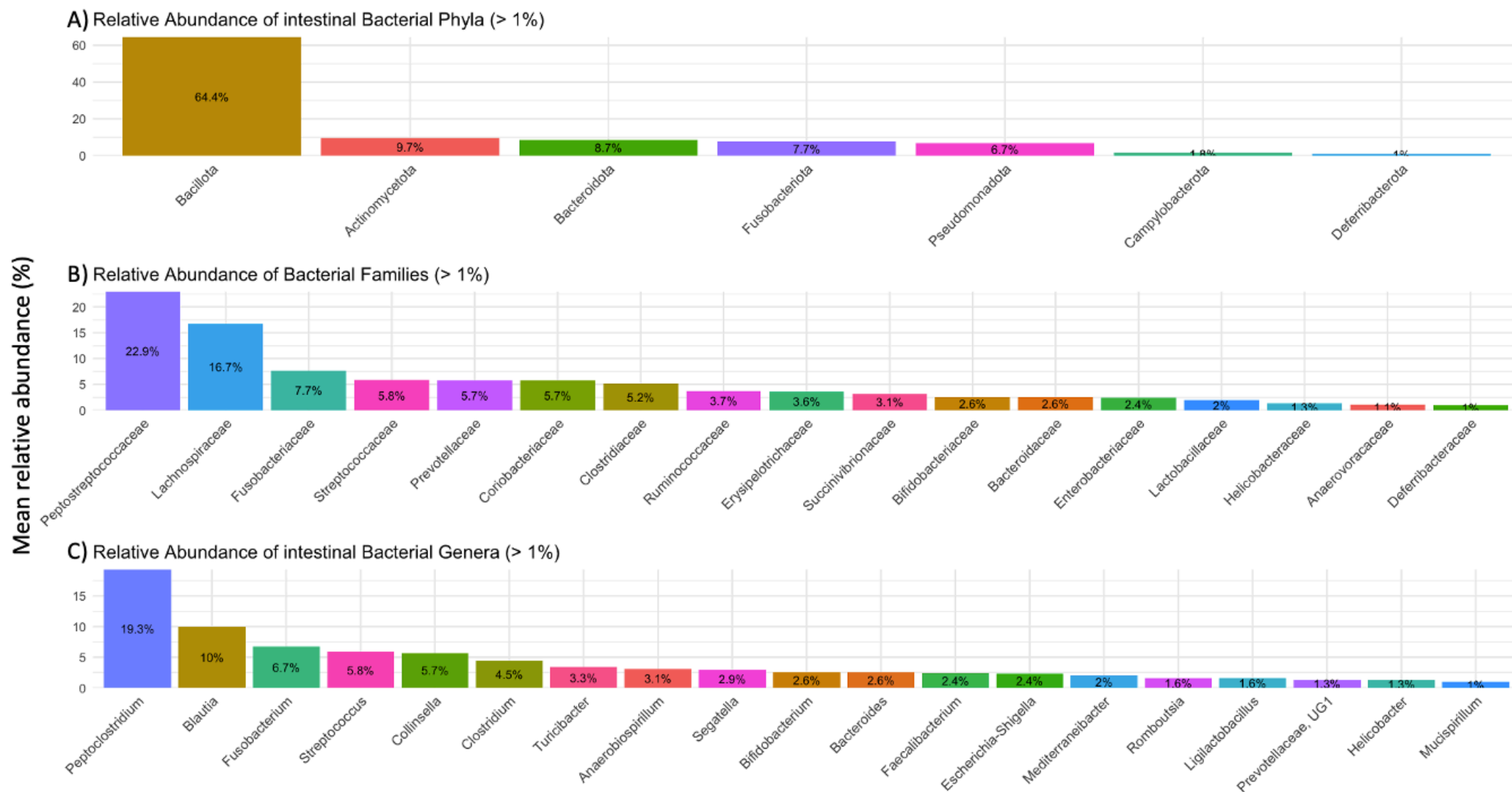


Figure 1. Relative abundance of taxa at the Phylum (A), Family (B), and Genus (C) levels. Only taxa with a relative abundance greater than 1% are shown.

Association between gut microbiota and the temperament at 1 week post weaning

At 1WPW, alpha and beta diversities of the GM, assessed at the ASV level using, respectively, Shannon Index and Bray-Curtis distance, were significantly associated with the puppy's temperament (**Table 2**). Bold puppies presented greater alpha diversity index than shy puppies ($p = 0.002$; **Figure 2A**). Although no clear separation of microbial communities was observed between the two temperaments in the NMDS space (reflecting beta diversity), the PERMANOVA analysis revealed a significant difference in microbiota composition of the two groups ($p = 0.001$, $R^2 = 6\%$) (**Figure 2B**).

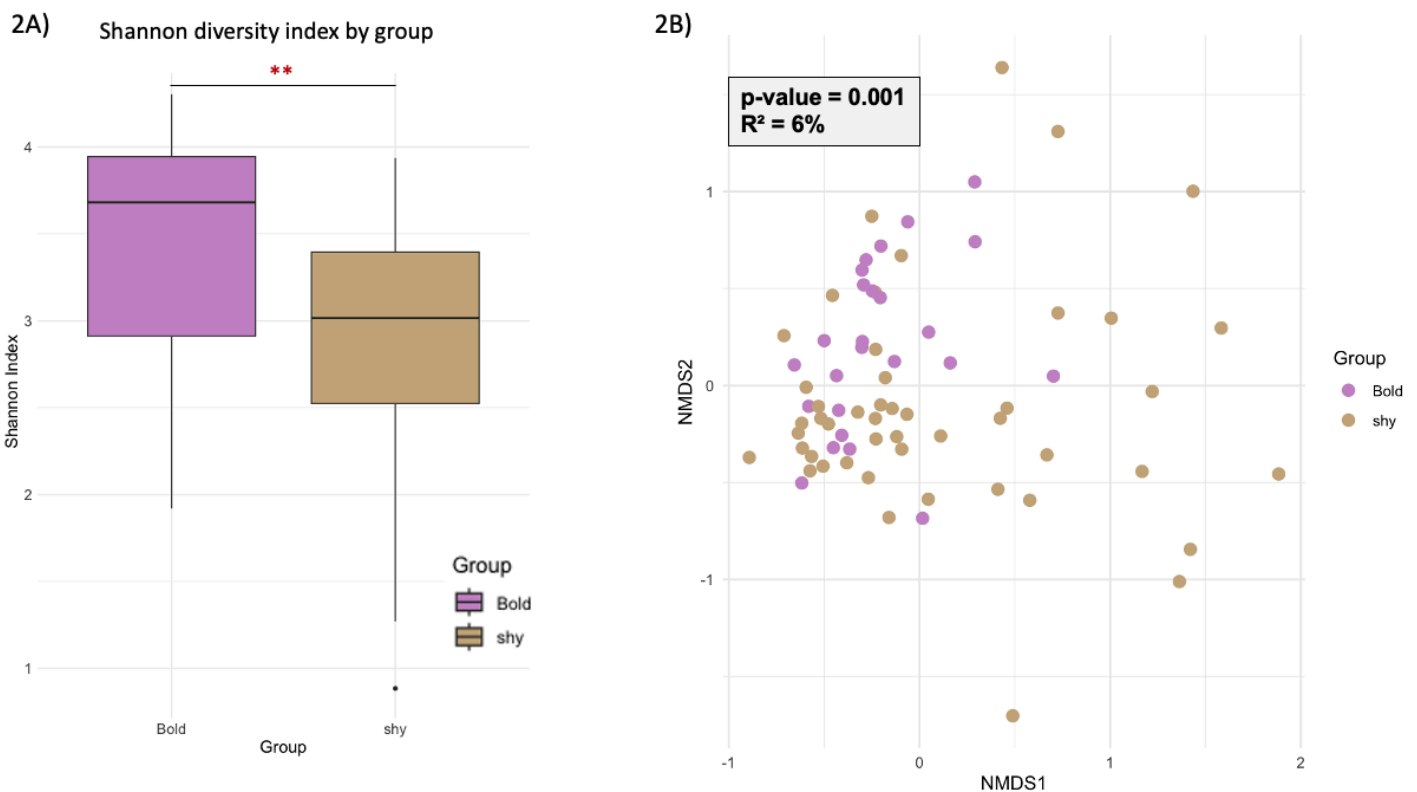


Figure 2. Bacterial diversity and composition in puppies' fecal microbiota across temperament groups. (A) Bacterial richness measured by the Shannon index. (B) Non-metric multidimensional scaling (NMDS) analysis of microbiota composition. Significant differences between groups were assessed using Tukey's HSD test, with significance levels indicated as ** for $0.001 < p\text{-value} \leq 0.01$.

Additionally, the batch showed a significant impact on beta diversity, although of lesser significance than that of the temperament (PERMANOVA; $p\text{-value} = 0.036$, $R^2 = 2\%$). In contrast, factors such as sex, weaning age, post-weaning weight and number of reads did not significantly influence either alpha or beta diversity (**Table 2**).

Table 2: Effect of cofactors on alpha diversity (Shannon Index) and beta diversity (Bray-Curtis) of the gut microbiota in puppies at one-week post-weaning at the ASV level.

Factors	P-value	
	Shannon Index	Bray curtis
Temperament group	0.001	0.001
Sex	0.768	0.805
Batch	0.538	0.036
Weaning age	0.694	0.567
Post weaning weight	0.193	0.253
Number of reads	0.929	0.162

To account for potential batch effects, subsequent differential abundance analyses were corrected for the batch variable.

Differential taxa in the gut microbiota according to the temperament

The ANCOM-BC differential abundance analysis allowed identification of several taxa associated with either boldness or shyness at both family and genus levels. At the family level, two families were significantly more abundant (**Figure 3A, Table 3**) (*Prevotellaceae* and *Campylobacteraceae*), while three families (*Enterococcaceae*, *Lactobacillaceae*, *Pasteurellaceae*) were less abundant in the bold group compared to the shy group.

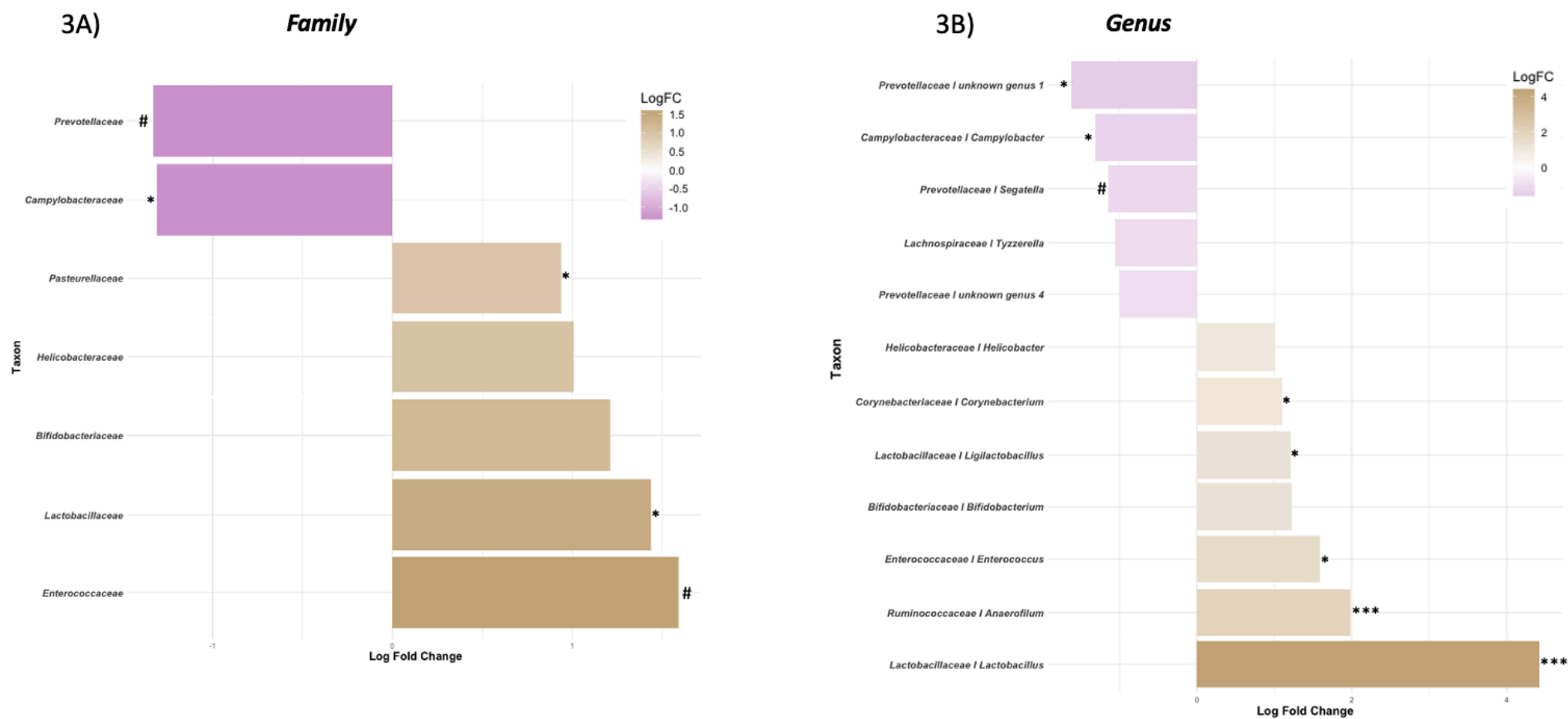


Table 3: Differential analysis of bacterial families' abundance between "shy" and "bold" groups using ANCOM-BC. Only bacterial families with a relative abundance greater than 0.2% of the total sequences were kept.

Taxon	Log fold change_GroupShy	P-value	Abundance_Relative (%)		Percent_Zero (%)	
			Bold	Shy	Bold	Shy
<i>Prevotellaceae</i>	-1,33	0,053	9,76±7,64	3,62±4,95	0	6
<i>Campylobacteraceae</i>	-1,31	0,027	0,58±1,00	0,48±2,66	32	44
<i>Pasteurellaceae</i>	0,94	0,048	0,23±0,89	0,20±0,62	44	40
<i>Helicobacteraceae</i>	1,01	0,175	2,00±3,26	0,87±2,06	20	19
<i>Bifidobacteriaceae</i>	1,21	0,180	0,67±2,46	3,57±10,38	48	31
<i>Lactobacillaceae</i>	1,44	0,024	0,13±0,35	2,94±8,39	60	33
<i>Enterococcaceae</i>	1,59	0,053	0,33±1,39	0,46±0,75	28	27

At the genus level, 7 out of 12 genera with relative abundance greater than 0.2% were identified using ANCOM-BC differential abundance analysis revealed a significant difference between the shy and bold

groups (**Figure 3B, Table 4**). Several were significantly more abundant in the bold group, including *Prevotellaceae*, *unknown genus 1*, *Campylobacter* and *Sagatella*. Conversely, genera such as *Lactobacillus*, *Anaerofilum*, *Enterococcus*, *Ligilactobacillus* and *Corynebacterium* were significantly more abundant in the shy group.

Table 4: Differential analysis of bacterial genera abundance between "shy" and "bold" groups using ANCOM-BC. Only bacterial genera with a relative abundance greater than 0.2% of the total sequences were kept.

Taxon	Log fold change_Groupshy	P-value	Abundance_Relative (%) Mean±SD		Percent_Zero (%)	
			Bold	Shy	Bold	Shy
<i>Prevotellaceae; unknown genus 1</i>	-1,62	0,022	1,89±1,62	1,01±1,54	8	21
<i>Campylobacteraceae; Campylobacter</i>	-1,31	0,023	0,58±1,00	0,48±2,66	32	44
<i>Prevotellaceae; Segatella</i>	-1,15	0,058	5,30±5,09	1,67±2,45	8	12
<i>Lachnospiraceae; Tyzzarella</i>	-1,06	0,105	0,47±0,58	0,49±1,66	16	17
<i>Prevotellaceae; unknown genus 4</i>	-1,00	0,101	0,73±0,73	0,23±0,47	24	37
<i>Helicobacteraceae; Helicobacter</i>	1,01	0,169	2,00±3,26	0,87±2,07	20	19
<i>Corynebacteriaceae; Corynebacterium</i>	1,10	0,033	0,24±0,42	0,30±0,54	32	37
<i>Lactobacillaceae; Ligilactobacillus</i>	1,21	0,026	0,06±0,23	2,38±8,07	76	41
<i>Bifidobacteriaceae; Bifidobacterium</i>	1,22	0,176	0,67±2,46	3,57±10,38	48	31
<i>Enterococcaceae; Enterococcus</i>	1,59	0,050	0,33±1,38	0,46±0,74	28	27

<i>Ruminococcaceae</i> ; <i>Anaerofilum</i>	1,98	<0,001	0,18±0,53	0,51±2,87	40	58
<i>Lactobacillaceae</i> ; <i>Lactobacillus</i>	4,42	<0,001	0,07±0,28	0,27±0,83	72	56

Correlation between fecal microbiota genera and behavioral parameters distinguishing temperaments.

Hierarchical clustering at both the family and genus levels revealed three coherent groups of bacteria, based on bacterial families and bacterial genera identified by ANCOM-BC as differentially abundant between bold and shy temperaments. The first group, associated with boldness-related behaviors such as exploration attitude, included the family *Prevotellaceae* and its representative genera *Unknown Genus 1* (UG1), *Unknown Genus 4* (UG4), and *Sagatella*. All the mentioned taxa showed positive correlations with boldness and negative correlations with shyness behaviors such as the "Glance" behavior in the Pearson correlation heatmap (**Figure 4**, **Figure 5**). The second group, which showed weak positive correlations with boldness-related behaviors, included the families *Campylobacteraceae* (*Campylobacter*), *Helicobacteraceae* (*Helicobacter*), and *Pasteurellaceae*, as well as genera from other families such as *Tyzerella* (*Lachnospiraceae*), *Anaerofilum* (*Ruminococcaceae*), and *Corynebacterium* (*Corynebacteriaceae*). In contrast, the third group exhibited negative correlations with boldness-related behaviors and consisted of the families *Lactobacillaceae*, *Bifidobacteriaceae*, and *Enterococcaceae*, along with their corresponding genera: *Lactobacillus*, *Ligilactobacillus*, *Bifidobacterium*, and *Enterococcus*.

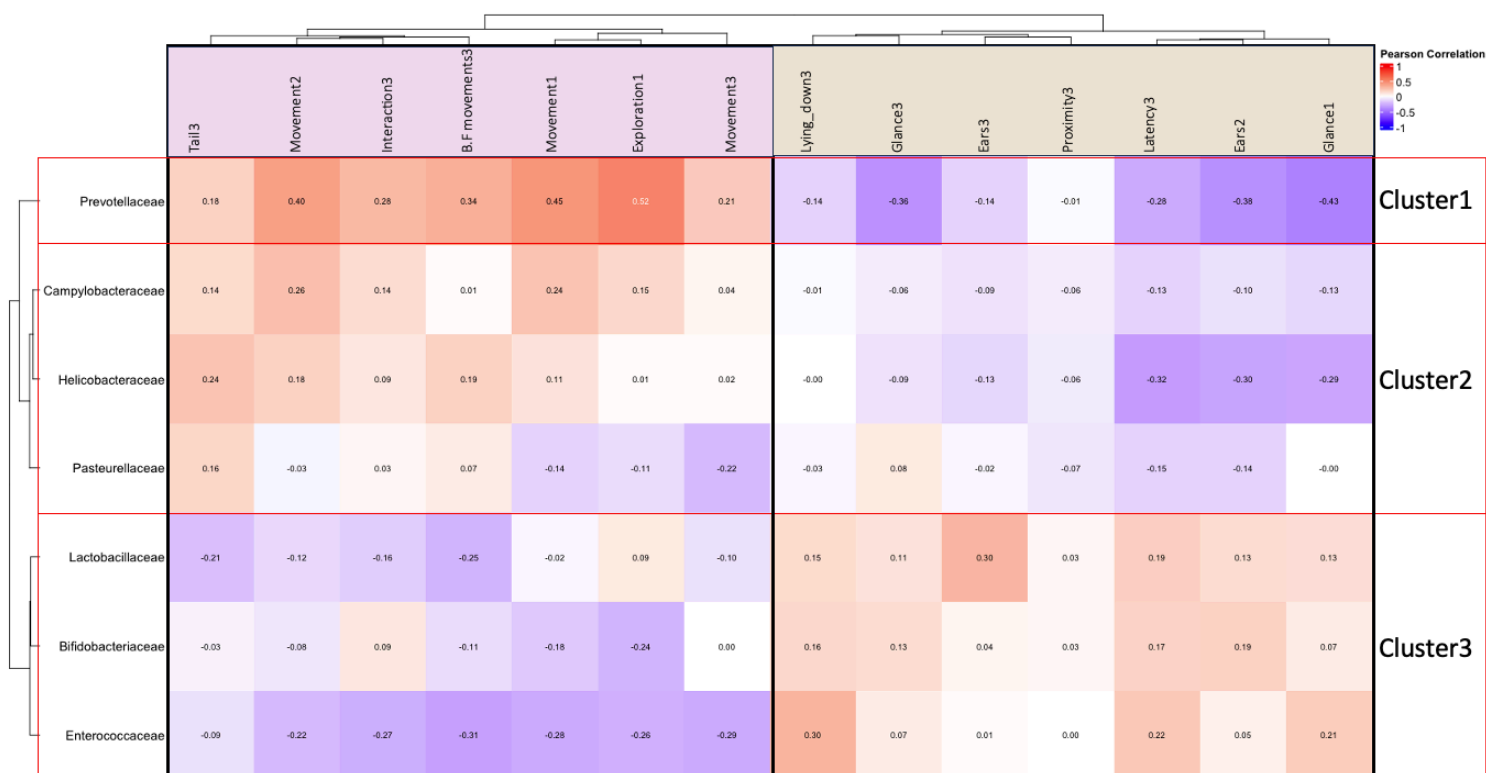


Figure 4. Correlation heatmap between the relative abundance of bacterial families (CLR-transformed and batch corrected data) and behavioral parameters associated with bold (purple squares) and shy (brown squares) temperament. This heatmap displays Pearson correlation coefficients, with the values explicitly shown within the figure. The color-coded squares highlight the strength and direction of correlations, with red representing positive correlation and blue representing negative correlation.

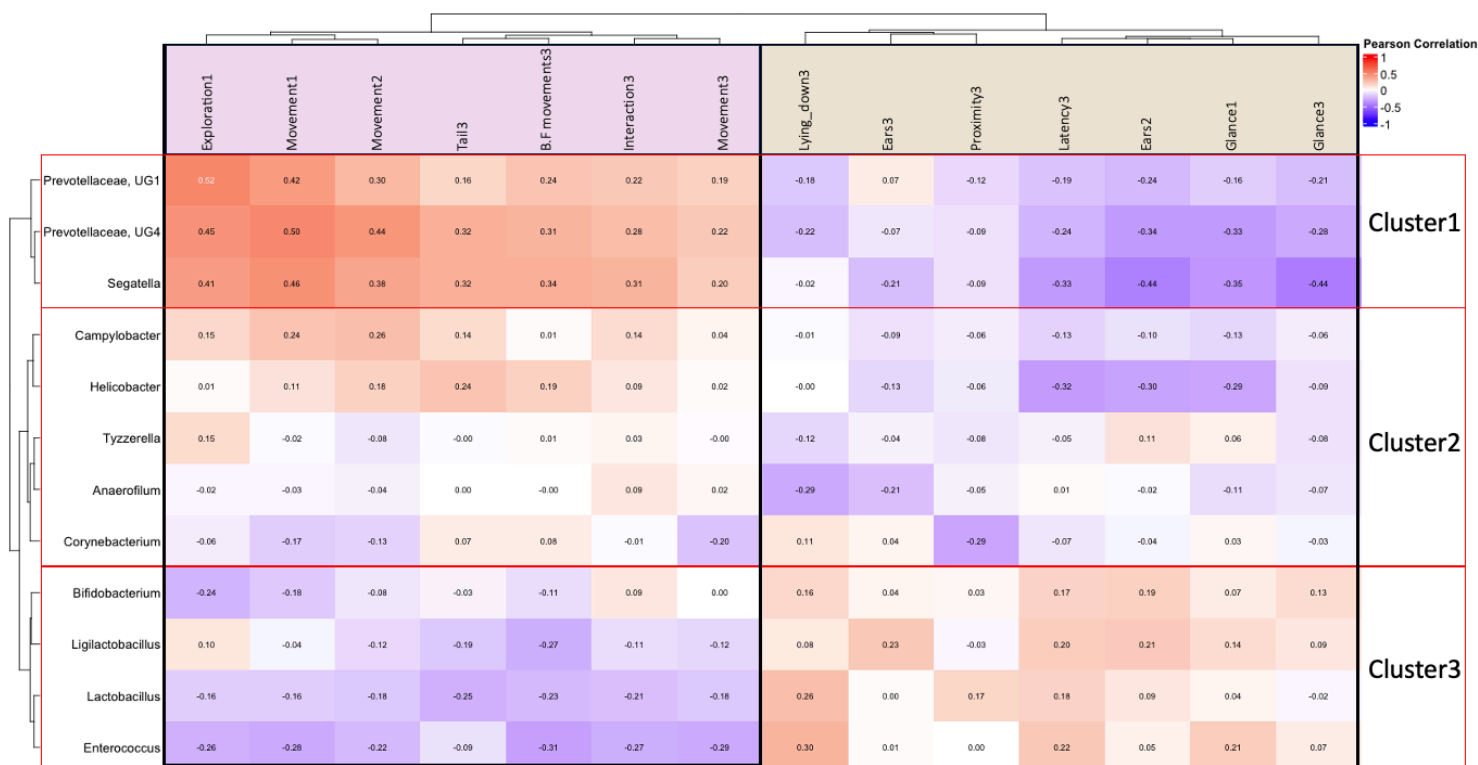


Figure 5. Correlation heatmap between the relative abundance of bacterial genera (CLR-transformed and batch corrected data) and behavioral parameters associated with bold (purple squares) and shy (brown squares) temperament. This heatmap displays Pearson correlation coefficients, with the values explicitly shown within the figure. The color-coded squares highlight the strength and direction of correlations, with red representing positive correlation and blue representing negative correlation.

Discussion

In this study, we explored the relationship between the GM and behavior in 73 Golden retriever puppies during the weaning period. Our findings reveal significant differences in alpha and beta diversity, as well as in specific bacterial taxa, among two distinct temperament groups one-week post-weaning. Although the link between the GM and behavior has been well-documented in humans (8) and other mammals (4, 5, 6), this study provides for the first time the evidence of such an association in puppies. Nonetheless, some limits must be taken into consideration. The study involved a single breed, a standardized diet, and a relatively limited sample size, which may reduce the generalizability of the results. Furthermore, approximately one-third of fecal samples were lost during preprocessing due to insufficient material collected via rectal swabbing. This issue reflects a common challenge when sampling puppies at the first week of weaning: at this stage, feces are often dry and compact, with less fecal matter in the rectal mucosa, which makes fecal recovery via swabbing particularly difficult and sometimes inadequate for downstream microbiota analysis. This technical limitation, although difficult to overcome, should be considered in future study designs aiming to sample weaning-age puppies.

The composition of the intestinal microbiota of puppies at one-week post-weaning (between 63 and 78 days of age) revealed a dominance of *Bacillota* ($64 \pm 21\%$) at the phylum level, followed by *Actinomycetota*, *Bacteroidota*, *Fusobacteriota*, and *Pseudomonadota*. (Figure 1A). These results are partially consistent with previous studies, although differences in relative proportions are observed (3, 25). Those differences could be due to differences in age, diet, or analytical methods. At the genus level, *Peptoclostridium*, *Blautia*, and *Fusobacterium* were identified as the most abundant (Figure 1C), which aligns with the results of Blake et al. and Garrigues et al (23, 25), where *Fusobacterium* and *Blautia* were also present as dominant genera.

In our study, we observed a different pattern of gut species richness (alpha diversity) at one-week post-weaning, with bold puppies exhibiting slightly higher alpha diversity than their shy counterparts. The relationship between temperament and alpha diversity has been previously explored. In children, extraversion and sociability have been associated with greater alpha diversity (Shannon index) (8). In contrast, Xia et al. found that bold macaques—characterized by high exploratory behavior and frequent interactions with novel objects—had lower alpha diversity (Shannon index) compared to their shy counterparts (22). This discrepancy highlights the need for further research to better understand the interplay between temperament and GM alpha diversity, in dogs as in other species.

One of the key differences identified in our study between bold and shy puppies concerns three families of lactic acid bacteria: *Lactobacillaceae* (particularly *Lactobacillus* and *Ligilactobacillus*), *Enterococcaceae* (*Enterococcus*), and *Bifidobacteriaceae* (*Bifidobacterium*). These groups are well-known not only for their probiotic properties (41, 42, 43) but also for their psychobiotic effects (44), involvement in metabolic regulation (45), prevention of allergic diseases (46), immune modulation (47), inhibition of pathogenic bacteria (48), and protection against antibiotic-associated diarrhea (49). Interestingly, we observed a significantly lower abundance of *Lactobacillus*, *Ligilactobacillus*, and *Enterococcus* in bold puppies compared to shy ones. Moreover, *Bifidobacterium* also appeared less abundant in bold individuals, although the difference was not statistically significant. The involvement of *Lactobacillus* in behavior and mood regulation has been documented across species. For example, supplementation with *Lactobacillus reuteri* and *Lactobacillus plantarum* reduced vigilance in piglets facing auditory stress (50), improved quality of life in children with ADHD (51), and alleviated depressive-like behaviors in mice exposed to chronic social stress (52). Similarly, *Lactobacillus* GM11 improved mood-related symptoms in depressed rats by enhancing serotonin levels and reducing cortisol both peripherally and centrally (53). More recently, research has pointed to *Enterococcus* as another candidate for behavioral modulation via the gut-brain axis. In murine models, *Enterococcus faecalis* 2001 (EF-2001) has demonstrated antidepressant-like effects by reducing intestinal inflammation and

restoring behavioral deficits in IBD-like pathology (54). It has also been linked to enhanced prefrontal cortex myelination, suggesting a neuroprotective mechanism in depression (55). Further, EF-2001 reduced anxiety-like behaviors and altered neurotransmitter receptor expression in the prefrontal cortex, while also promoting hippocampal neurogenesis and memory performance in mice with cognitive impairments (56). Together, these findings support the hypothesis that *Lactobacillus* and *Enterococcus* strains may influence behavior through both immune and neurochemical pathways. These results raise compelling questions about the role of lactic acid bacteria in shaping temperament—particularly traits related to boldness and shyness—in puppies. Further research is necessary to clarify the mechanisms by which these microbes may modulate behavior during early development.

In our study, *Prevotellaceae* was found to have a high relative abundance (6% of the detected bacterial microbiota) and high prevalence (present in 96% of individuals). In our study we demonstrated a tendency toward increased relative abundance of *Prevotellaceae* in bold puppies compared to shy ones. The potential association of *Prevotellaceae* with brain function and behavior has been explored in various studies. In humans, the proportion of *Prevotella* has been positively correlated with the Hamilton Depression Rating Scale, indicating a potential connection to depressive states (28). Similarly, in mice, treatment with *Prevotella copri* was shown to induce depression and impair motor function (29). In the context of comorbidity, such as irritable bowel syndrome (IBS) with anxiety/depression, the negative role of *Prevotella* and *Prevotellaceae* has been highlighted (30). Conversely, in rats, the administration of the antidepressant fluoxetine led to a significant reduction in *Prevotella* genera (31). However, the mechanism by which this bacterial family interacts with the CNS remains unclear. Some studies propose that *Prevotellaceae* may be implicated in modulating the immune system, contributing to intestinal inflammation (32), and influencing systemic inflammation (33). At the same time, inflammation is known as one of the major pathways modulating behavior (34).

A recent human study reported a greater abundance of *Prevotellaceae* (specifically *Prevotella copri* and *Prevotella buccae*) in patients with Attention-Deficit/Hyperactivity Disorder (ADHD) compared to controls (35). Interestingly, our previous study demonstrated that bold puppies had higher impulsivity and hyperactivity scores compared to shy ones (Bendahmane et al., in reviewing). Furthermore, the current study reveals a positive correlation between the *Prevotellaceae* family, including three genera (*Sagatella* and two *unknown genera*), and movement and exploratory behaviors (**Figure 4 – Figure 5**). Further research is needed to elucidate the mechanisms through which *Prevotellaceae* interacts with the brain in the context of boldness.

In addition, one of the most intriguing findings of our study is the increased relative abundance of *Campylobacter* in bold puppies. The positive association between fecal *Campylobacter* abundance and boldness in puppies may indicate its potential role in modulating behavior. For instance, in calves, boldness (measured by the time spent interacting with a novel object) was slightly associated with higher

fecal excretion of *Campylobacter* (37). One of the major pathways through which the GM may interact with the central nervous system is the vagal nerve (38). In mice, oral inoculation with *Campylobacter* has been shown to activate visceral sensory nuclei in the brainstem (39), as well as the basolateral and central nuclei of the amygdala (40), along with other brain regions involved in processing visceral and autonomic information. These findings suggest that *Campylobacter* is associated with behavior, although further research is needed to quantify *Campylobacter* levels via qPCR in the feces of bold versus shy puppies.

The potential link between gut *Pasteurellaceae* and behavior, to our knowledge, has not been previously documented in the literature. In our study, we observed that both *Pasteurellaceae* was more abundant in the shy group compared to the bold group. Further research is needed to clarify the potential association between this family and temperament.

In the previous study on the same cohort, the authors observed slightly lower fecal scores in bold puppies compared to shy ones (Bendahmane et al, in reviewing), raising the question about the role of gut microbiome in the interaction between brain-gut-health in puppies. This hypothesis aligns with the concept of the gut-brain axis, a bidirectional communication system between the central nervous system and the gastrointestinal tract, mediated by neurotransmitters, hormones, cytokines, and the autonomic nervous system (5). In light of the current findings showing reduced abundance of lactic acid bacteria and increased abundance of *Campylobacter* in bold individuals, this association becomes even more intriguing. *Campylobacter* is a well-known pathogenic bacterium associated with diarrhea in dogs (36). And lactic bacteria are known to contribute to gut health by producing lactic acid, which plays a protective role against diarrhea (57). Thus, future investigations should more directly examine the interplay between lactic acid bacteria, *Campylobacter*, behavioral traits such as boldness, and diarrhea by quantifying lactic acid and those bacteria in fecal samples at 1WPW and evaluating its associations with fecal consistency and behavioral patterns.

Conclusions

In conclusion, this study highlights significant associations between the GM and temperament in Golden retriever puppies during the weaning period. However, the mechanisms underlying these associations remain unclear and require further investigation. Future studies should focus on quantitative analyses of key taxa, as well as on exploring the role of blood and fecal metabolomes in mediating GM-brain communication. Such research could pave the way for targeted interventions, such as probiotics supplementation to support healthy behavioral development in puppies. Overall, this study contributes to the growing body of evidence linking the GM to temperament and underscores the need for further exploration of this complex relationship.

Comparative analysis of bacterial families associated with temperament

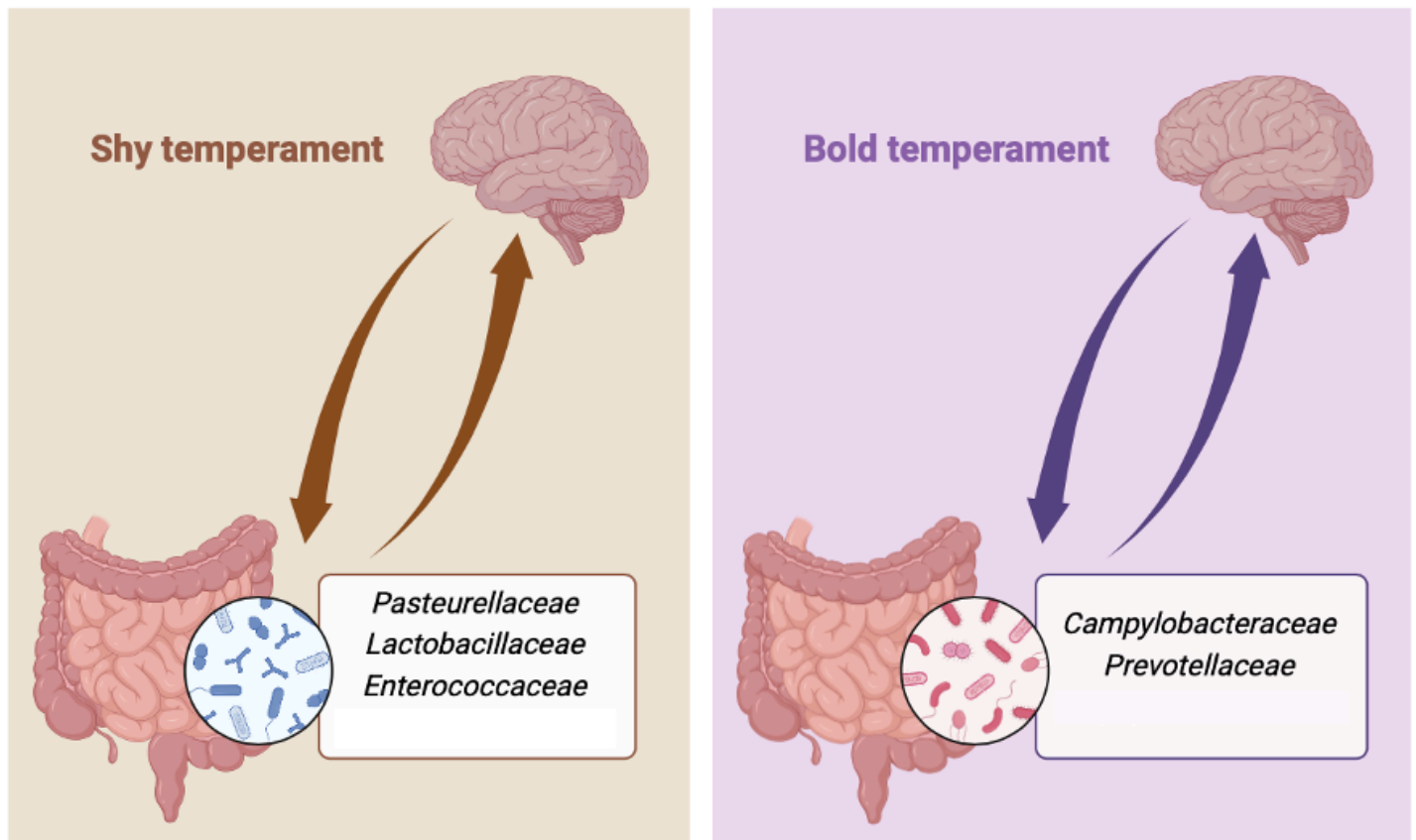


Figure 6. Overview of intestinal microbiota variations (family level) across bold and shy temperaments.

Author contributions

Conceptualization: I.B., H.M., A.M.; Data curation: I.B.; Formal analysis: I.B.; Funding acquisition: H.M.; Investigation: I.B.; Methodology: I.B., H.M., A.M.; Project administration: I.B., H.M., A.M.; Visualization: I.B.; Writing Original draft: I.B.; Writing Review & editing: I.B., H.M., A.M.

The manuscript was revised and approved by all co-authors.

Funding

This research was financially supported by the Occitanie Pyrénées-Méditerranée Region and the Visio Foundation (Bouchemaine, France). The authors acknowledge funding received for both the research and publication of this article.

Acknowledgments

The authors would like to thank the Visio Foundation for its financial support, which made this project possible. We are also grateful to the student volunteers from ENVT for their commitment and care provided to the dogs and their litters within the maternity unit. Special thanks go to the NeoCare support team—including animal caretakers, veterinary assistants, and clinicians—for their invaluable contribution to the daily management of the animals and the study. Finally, we thank all the puppy owners for their active participation and assistance in collecting follow-up data after adoption.

Conflict of interest

Authors declare no conflicts of interest.

References

1. Grellet A, Chastant-Maillard S, Robin C, Feugier A, Boogaerts C, Boucraut-Baralon C, Grandjean D, Polack B. Risk factors of weaning diarrhea in puppies housed in breeding kennels. *Preventive Veterinary Medicine* 2014;117:260–5.
2. Gaillard V, Chastant S, England G, Forman O, German AJ, Suchodolski JS, Villaverde C, Chavatte-Palmer P, Péron F. Environmental risk factors in puppies and kittens for developing chronic disorders in adulthood: A call for research on developmental programming. *Front Vet Sci* 2022;9:944821.
3. Guard BC, Mila H, Steiner JM, Mariani C, Suchodolski JS, Chastant-Maillard S. Characterization of the fecal microbiome during neonatal and early pediatric development in puppies. *PLoS One* 2017;12:e0175718.
4. Fung TC, Olson CA, Hsiao EY. Interactions between the microbiota, immune and nervous systems in health and disease. *Nat Neurosci* 2017;20:145–55.
5. Strandwitz P. Neurotransmitter modulation by the gut microbiota. *Brain Research* 2018;1693:128–33.
6. Cryan JF, O'Mahony SM. The microbiome-gut-brain axis: from bowel to behavior: From bowel to behavior. *Neurogastroenterology & Motility* 2011;23:187–92.
7. Gao W, Baumgartel KL, Alexander SA. The Gut Microbiome as a Component of the Gut–Brain Axis in Cognitive Health. *Biological Research For Nursing* 2020;22:485–94.
8. Christian LM, Galley JD, Hade EM, Schoppe-Sullivan S, Kamp Dush C, Bailey MT. Gut microbiome composition is associated with temperament during early childhood. *Brain, Behavior, and Immunity* 2015;45:118–27.

9. Dong Y, Han Y, Wang Z, Qin Z, Yang C, Cao J, Chen Y. Role of serotonin on the intestinal mucosal immune response to stress-induced diarrhea in weaning mice. *BMC Gastroenterol* 2017;17:82.
10. Riemer S, Müller C, Virányi Z, Huber L, Range F. Choice of conflict resolution strategy is linked to sociability in dog puppies. *Applied Animal Behaviour Science* 2013;149:36–44.
11. Lluch J, Servant F, Païssé S, Valle C, Valière S, Kuchly C, Vilchez G, Donnadiou C, Courtney M, Burcelin R, et al. The Characterization of Novel Tissue Microbiota Using an Optimized 16S Metagenomic Sequencing Pipeline. Heimesaat MM, editor. *PLoS ONE* 2015;10:e0142334.
12. Nadkarni MA, Martin FE, Jacques NA, Hunter N. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. *Microbiology* 2002;148:257–66.
13. GeT-PlaGe. GeT-PlaGe Genomics and Transcriptomics core facility of Toulouse [Internet]. INRAE; 2022 [cited 2025 May 18]. Available from: <https://get.genotoul.fr/la-plateforme/get-plage/>
14. Bernard M, Rué O, Mariadassou M, Pascal G. FROGS: a powerful tool to analyse the diversity of fungi with special management of internal transcribed spacers. *Briefings in Bioinformatics* 2021;22:bbab318.
15. Escudié F, Auer L, Bernard M, Mariadassou M, Cauquil L, Vidal K, Maman S, Hernandez-Raquet G, Combes S, Pascal G. FROGS: Find, Rapidly, OTUs with Galaxy Solution. Berger B, editor. *Bioinformatics* 2018;34:1287–94.
16. Rognes T, Flouri T, Nichols B, Quince C, Mahé F. VSEARCH: a versatile open source tool for metagenomics. *PeerJ* 2016;4:e2584.
17. Bokulich NA, Subramanian S, Faith JJ, Gevers D, Gordon JI, Knight R, Mills DA, Caporaso JG. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nat Methods* 2013;10:57–9.
18. Rstudio (version 2024.09.1+394) [Internet]. Available from: <https://posit.co/products/open-source/rstudio/>
19. Dixon P. VEGAN, a package of R functions for community ecology. *J Vegetation Science* 2003;14:927–30.
20. Lin H, Peddada SD. Analysis of compositions of microbiomes with bias correction. *Nat Commun* 2020;11:3514.
21. Gu Z. Complex heatmap visualization. *iMeta* 2022;1:e43.

22. Xia M, Xia Y, Sun Y, Wang J, Lu J, Wang X, Xia D, Xu X, Sun B. Gut microbiome is associated with personality traits of free-ranging Tibetan macaques (*Macaca thibetana*). *Front Microbiol* 2024;15:1381372.
23. Choudhury R, Middelkoop A, Bolhuis JE, Kleerebezem M. Exploring the association between microbiota and behaviour in suckling piglets. *Sci Rep* 2022;12:12322.
24. Rapp D, Schütz KE, Ross C, Sutherland MA, Hempstead MN, Hannaford R, Cave VM, Brightwell G. Fecal excretion of *Campylobacter jejuni* by young dairy calves and the relationship with neonatal immunity and personality traits. *Journal of Applied Microbiology* 2023;134:lxad094.
25. Garrigues Q, Apper E, Rodiles A, Rovere N, Chastant S, Mila H. Composition and evolution of the gut microbiota of growing puppies is impacted by their birth weight. *Sci Rep* 2023;13:14717.
26. Blake AB, Cigarroa A, Klein HL, Khattab MR, Keating T, Van De Coevering P, Lidbury JA, Steiner JM, Suchodolski JS. Developmental stages in microbiota, bile acids, and clostridial species in healthy puppies. *Veterinary Internal Medicine* 2020;34:2345–56.
27. Zhang S, Zeng L, Ma J, Xu W, Qu Y, Wang X, An X, Wang Q, Wu Y, Wang D, et al. Gut Prevotellaceae-GABAergic septohippocampal pathway mediates spatial memory impairment in high-fat diet-fed ovariectomized mice. *Neurobiology of Disease* 2023;177:105993.
28. Lin P, Ding B, Feng C, Yin S, Zhang T, Qi X, Lv H, Guo X, Dong K, Zhu Y, et al. Prevotella and Klebsiella proportions in fecal microbial communities are potential characteristic parameters for patients with major depressive disorder. *Journal of Affective Disorders* 2017;207:300–4.
29. Cai S, Lin J, Li Z, Liu S, Feng Z, Zhang Y, Zhang Y, Huang J, Chen Q. Alterations in intestinal microbiota and metabolites in individuals with Down syndrome and their correlation with inflammation and behavior disorders in mice. *Front Microbiol* 2023;14:1016872.
30. Simpson CA, Mu A, Haslam N, Schwartz OS, Simmons JG. Feeling down? A systematic review of the gut microbiota in anxiety/depression and irritable bowel syndrome. *Journal of Affective Disorders* 2020;266:429–46.
31. Cussotto S, Strain CR, Fouhy F, Strain RG, Peterson VL, Clarke G, Stanton C, Dinan TG, Cryan JF. Differential effects of psychotropic drugs on microbiome composition and gastrointestinal function. *Psychopharmacology* 2019;236:1671–85.

32. Chen L, Ye Z, Li J, Wang L, Chen Y, Yu M, Han J, Huang J, Li D, Lv Y, et al. Gut bacteria Prevotellaceae related lithocholic acid metabolism promotes colonic inflammation. *J Transl Med* 2025;23:55.
33. Cai S, Lin J, Li Z, Liu S, Feng Z, Zhang Y, Zhang Y, Huang J, Chen Q. Alterations in intestinal microbiota and metabolites in individuals with Down syndrome and their correlation with inflammation and behavior disorders in mice. *Front Microbiol* 2023;14:1016872.
34. Engler H, Doenlen R, Engler A, Riether C, Prager G, Niemi M-B, Pacheco-López G, Krügel U, Schedlowski M. Acute amygdaloid response to systemic inflammation. *Brain, Behavior, and Immunity* 2011;25:1384–92.
35. Li Y, Sun H, Huang Y, Yin A, Wang P, Han J, Qu D, Zhuo L, Tian M, Wang C, et al. Gut metagenomics discriminates unique microbial signatures in diverse symptomatic profiles with attention-deficit/hyperactivity disorder [Internet]. 2020 [cited 2025 Mar 23]. Available from: <https://www.researchsquare.com/article/rs-28862/v1>
36. A Review of *Campylobacter* Infections in Dogs. *Int J Vet Sci* 2021;10:259–66.
37. Rapp D, Schütz KE, Ross C, Sutherland MA, Hempstead MN, Hannaford R, Cave VM, Brightwell G. Fecal excretion of *Campylobacter jejuni* by young dairy calves and the relationship with neonatal immunity and personality traits. *Journal of Applied Microbiology* 2023;134:lxad094.
38. Breit S, Kupferberg A, Rogler G, Hasler G. Vagus Nerve as Modulator of the Brain–Gut Axis in Psychiatric and Inflammatory Disorders. *Front Psychiatry* 2018;9:44.
39. Gaykema RPA, Goehler LE, Lyte M. Brain response to cecal infection with *Campylobacter jejuni*: analysis with Fos immunohistochemistry. *Brain, Behavior, and Immunity* 2004;18:238–45.
40. Goehler LE, Park SM, Opitz N, Lyte M, Gaykema RPA. *Campylobacter jejuni* infection increases anxiety-like behavior in the holeboard: Possible anatomical substrates for viscerosensory modulation of exploratory behavior. *Brain, Behavior, and Immunity* 2008;22:354–66.
41. Pinchao YA, Serna-Cock L, Mora OO. Probiotic capacity of commensal lactic acid bacteria from the intestine of Guinea pigs (*Cavia porcellus*). *Heliyon* 2024;10:e29431.
42. Fijan S. Microorganisms with Claimed Probiotic Properties: An Overview of Recent Literature. *IJERPH* 2014;11:4745–67.
43. Yang Y, Song X, Wang G, Xia Y, Xiong Z, Ai L. Understanding *Ligilactobacillus salivarius* from Probiotic Properties to Omics Technology: A Review. *Foods* 2024;13:895.

44. Zhu R, Fang Y, Li H, Liu Y, Wei J, Zhang S, Wang L, Fan R, Wang L, Li S, et al. Psychobiotic *Lactobacillus plantarum* JYLP-326 relieves anxiety, depression, and insomnia symptoms in test anxious college via modulating the gut microbiota and its metabolism. *Front Immunol* 2023;14:1158137.
45. Mo S-J, Lee K, Hong H-J, Hong D-K, Jung S-H, Park S-D, Shim J-J, Lee J-L. Effects of *Lactobacillus curvatus* HY7601 and *Lactobacillus plantarum* KY1032 on Overweight and the Gut Microbiota in Humans: Randomized, Double-Blinded, Placebo-Controlled Clinical Trial. *Nutrients* 2022;14:2484.
46. Wang MF, Lin HC, Wang YY, Hsu CH. Treatment of perennial allergic rhinitis with lactic acid bacteria. *Pediatric Allergy Immunology* 2004;15:152–8.
47. Perrin Y, Nutten S, Audran R, Berger B, Bibiloni R, Wassenberg J, Barbier N, Aubert V, Moulin J, Singh A, et al. Comparison of two oral probiotic preparations in a randomized crossover trial highlights a potentially beneficial effect of *Lactobacillus paracasei* NCC2461 in patients with allergic rhinitis. *Clinical & Translational All* 2014;4:1.
48. Gao H, Li X, Chen X, Hai D, Wei C, Zhang L, Li P. The Functional Roles of *Lactobacillus acidophilus* in Different Physiological and Pathological Processes. *J Microbiol Biotechnol* 2022;32:1226–33.
49. Blaabjerg S, Artzi D, Aabenhus R. Probiotics for the Prevention of Antibiotic-Associated Diarrhea in Outpatients—A Systematic Review and Meta-Analysis. *Antibiotics* 2017;6:21.
50. Verbeek E, Dicksved J, Keeling L. Supplementation of *Lactobacillus* early in life alters attention bias to threat in piglets. *Sci Rep* 2021;11:10130.
51. Kumperscak HG, Gricar A, Ülen I, Micetic-Turk D. A Pilot Randomized Control Trial With the Probiotic Strain *Lactobacillus rhamnosus* GG (LGG) in ADHD: Children and Adolescents Report Better Health-Related Quality of Life. *Front Psychiatry* 2020;11:181.
52. Hashikawa-Hobara N, Otsuka A, Okujima C, Hashikawa N. *Lactobacillus paragasseri* OLL2809 Improves Depression-Like Behavior and Increases Beneficial Gut Microbes in Mice. *Front Neurosci* 2022;16:918953.
53. Ma J, Wang J, Wang G, Wan Y, Li N, Luo L, Gou H, Gu J. The potential beneficial effects of *Lactobacillus plantarum* GM11 on rats with chronic unpredictable mild stress- induced depression. *Nutritional Neuroscience* 2024;27:413–24.

54. Kambe J, Watcharin S, Makioka-Itaya Y, Inoue R, Watanabe G, Yamaguchi H, Nagaoka K. Heat-killed *Enterococcus faecalis* (EC-12) supplement alters the expression of neurotransmitter receptor genes in the prefrontal cortex and alleviates anxiety-like behavior in mice. *Neuroscience Letters* 2020;720:134753.
55. Takahashi K, Kurokawa K, Hong L, Miyagawa K, Mochida-Saito A, Iwasa M, Iwasa H, Nakagawasai O, Tadano T, Takeda H, et al. Antidepressant effects of *Enterococcus faecalis* 2001 through the regulation of prefrontal cortical myelination via the enhancement of CREB/BDNF and NF- κ B p65/LIF/STAT3 pathways in olfactory bulbectomized mice. *Journal of Psychiatric Research* 2022;148:137–48.
56. Takahashi K, Kurokawa K, Miyagawa K, Mochida-Saito A, Nemoto Y, Iwasa H, Nakagawasai O, Tadano T, Takeda H, Tsuji M. Antidementia effects of *Enterococcus faecalis* 2001 are associated with enhancement of hippocampal neurogenesis via the ERK-CREB-BDNF pathway in olfactory bulbectomized mice. *Physiology & Behavior* 2020;223:112997.
57. Heyman M. Effect of Lactic Acid Bacteria on Diarrheal Diseases. *Journal of the American College of Nutrition* 2000;19:137S-146S.

Article 4 : Early-life gut microbiota as a potential indicator of weaning-enterotypes associated with diarrhea susceptibility in puppies

I. Bendahmane^{1,2}, F. Lyazrhi³, A. Meynadier², and H. Mila¹

¹NeoCare, Université de Toulouse, ENVT, Toulouse, France

²GenPhySE, Université de Toulouse, INRAE, ENVT, Castanet Tolosan, France

³École Vétérinaire de Toulouse, Unité pédagogique Biostatistique, 23 chemin des Capelles, F-31076 Toulouse, France

Abstract

Weaning is a critical period for development of puppies, often associated with increased susceptibility to diarrhea. This study investigated the relationship between fecal microbiota composition and diarrhea risk in Golden Retriever puppies in the first week after weaning, and explored the potential for early-life microbial markers to predict post-weaning diarrhea. Seventy-seven puppies from 18 litters were followed from birth until one-week post-weaning (1WPW), with fecal samples collected at multiple timepoints and analyzed via 16S rRNA gene sequencing. K-means clustering analysis on fecal microbiota at 1WPW revealed two distinct enterotypes, one of which ("At risk") was associated with a significantly higher incidence of diarrhea. The "Healthy" enterotype showed enrichment in short-chain fatty acid (SCFA)-producing genera such as *Faecalibacterium*, *Collinsella*, and members of the *Lachnospiraceae* family, known for supporting gut barrier function and immune homeostasis. In contrast, "At risk" puppies harbored higher abundances of potentially pro-inflammatory taxa including *Campylobacter*, *Helicobacter*, *Mucispirillum*, and *Sutterella*. Importantly, microbial differences were already detectable in the pre-weaning period. Puppies that later developed the "At risk" enterotype had consistently higher levels of *Enterococcus* across all early-life timepoints—day 1, day 7, day 21, and just before weaning—suggesting early colonization may contribute to intestinal vulnerability. An unknown genus of *Prevotellaceae* family, enriched in "Healthy" enterotype just before weaning, also emerged as a potential protective marker. These findings highlight the potential of fecal microbiota as both a marker and modulator of post-weaning gut health. Early identification of at-risk profiles could inform targeted microbiota-based strategies—such as probiotic, prebiotic, or dietary interventions—to reduce diarrhea risk and promote resilience during this vulnerable life stage.

Keywords: Weaning, Diarrhea, Microbiota, Puppies, Dog

Introduction

The weaning is a critical event in early canine development, marked by social and nutritional changes. During this transition, puppies move from a combination of maternal milk and solid food to solid food exclusively, face separation from the dam and littermates, and experience new environmental exposures. These changes can place considerable stress on the immature digestive and immune systems (1, 2). One common consequence is the increased susceptibility to gastrointestinal disorders, particularly diarrhea which can affect up to 25% of puppies (3). This posing a significant concern for animal welfare and leading to economic consequences for breeders.

In recent years, the gut microbiota has emerged as a central player in early-life health. The intestinal microbial community undergoes dynamic development during the neonatal period and continues to evolve after weaning (4). This microbiota maturation is not merely a passive process; rather, it reflects a complex and reciprocal interaction between the host and its microbial inhabitants. It has become increasingly clear that perturbations in this process (whether due to diet, stress, antibiotic exposure, or other factors) can lead to dysbiosis, an imbalance in microbial composition that is often associated with disease (5, 6).

Evidence from studies in piglets, for example, has shown that post-weaning diarrhea is closely linked to alterations in the gut microbiota (7, 8, 9). Shifts in microbial composition and function during this vulnerable period was associated to compromised gut barrier integrity, reduced resistance to pathogens, and increased risk of gastrointestinal diseases (10). However, despite growing interest in the microbiome's role in gastrointestinal health, comparable studies in puppies are lacking. To date, to our knowledge, no study has examined the link between fecal microbiota and weaning diarrhea in dogs.

Moreover, recent studies (11, 12) have suggested that the composition of the gut microbiota prior to weaning may influence an individual's health trajectory. Early-life microbial signatures have been proposed as biomarkers capable of predicting disease risk, including susceptibility to diarrhea at weaning (13). This raises also the possibility of preventing the risk of diarrhea through early modulation of the gut microbiota, although data are still lacking to identify diarrhea-preventive microbes in the canine species.

The aims of this study were (I) to characterize the relationship between weaning-associated diarrhea enterotype and the fecal microbiota in puppies and (II) identify potential early-life microbial markers that may serve as predictors of enterotype associated with diarrheal outcomes.

Materials and methods

This study was conducted at the National Veterinary School of Toulouse (ENVT, Toulouse, France). All experimental procedures were reviewed and approved by the Ethics Committee of the University of Toulouse under approval number SSA_2023_007.

I. Animal enrolment

Seventy-seven Golden Retriever puppies from the Toulouse GOLD Cohort (www.toulousegoldcohort.envt.fr) born from 18 bitches across 6 batches and raised under standardized conditions were included in the study. All animals were housed in the same maternity unit at the National Veterinary School of Toulouse from birth until weaning (ENVT, France).

At birth (directly after expulsion), each puppy was individually identified using colored collars and its sex, body weight, and APGAR score (14)(a vitality score assessed at birth on a 10-point scale) were recorded. Throughout their stay in the maternity unit, puppies were weighed daily, and their general health was assessed weekly through clinical examination. Notably, weights were recorded at 2 days of life, and the neonate growth rate 0-2 days was calculated (**Formula 1**) in order to estimate the colostrum intake (15).

Formula 1: early growth rate (%) = [(weight Day 2 – weight Day 0) × 100]/weight Day 0.

In cases of weight loss or retarded growth during the first 4 weeks of life (define as daily growth rate <5%), puppies were supplemented with artificial milk (Lait Maternisé TVM Optima®, DÔMES PHARMA, Pont-du-Château, France) at the dose of 4ml/100g of body weight administrated via bottle feeding for a period ranging from 1 to 12 days.

Starting on day 28 and continuing until weaning (between 56 and 71 days of age), moistened kibbles (Hill's Science Plan – Puppy Large Breed – Chicken, Topeka, USA) were introduced as a complementary diet during the dietary diversification phase. During the entire stay in the maternity unit, puppies remained with their dams and were allowed to suckle their dam freely.

Once weaned at about two months of life, all puppies were adopted by pet owners and housed in individual households, separately from their littermates and dam. Since then, all puppies were fed the same dry kibble exclusively, with daily rations adjusted according to body weight: 270 g/day for puppies under 7 kg, 300 g/day for those between 7 and 9 kg, and 330 g/day for those over 9 kg. Body weight was recorded the day before weaning (BW) and again one-week post-weaning (1WPW). The weaning growth rate was then calculated using the following formula:

Formula 2: Weaning growth rate (%) = [(Weight 1WPW – Weight BW) × 100]/Weight 1WPW.

All puppies received antiparasitic treatment (at 4 weeks with Drontal® Chien HP, Vetoquinol S.A., Magny-Vernois, France, and at 8 weeks with Credelio Plus®, Elanco France, Sèvres, France) and were vaccinated against kennel cough, parvovirus, coronavirus, canine distemper, infectious hepatitis, and leptospirosis (at 4 weeks with Nobivac® KC, MSD, Kenilworth, USA; at 6 weeks with Primodog®, Boehringer Ingelheim, Lyon, France; and at 8 weeks with Nobivac® CHP and Nobivac® L4, MSD). All the puppies remained in good general health during the entire study period. Therefore, no medical treatment was administered to any puppy before 1WPW.

II. Assignment of Diarrhea Groups

Pet owners were asked to take pictures of feces of their puppy during the first week following weaning, specifically on post-weaning days 1, 5, and 7, and send it to the dedicated mail box in order to assess fecal consistency. Due to inconsistencies in the photo schedule, images were instead categorized into three time periods: the first taken between day 0 and day 2 post-weaning, the second between days 4 and 6, and the third between days 7 and 9 post-weaning. Each photography of feces was scored by trained personnel using a standardized fecal scoring scale (16) ranging from 1 (very liquid) to 13 (well-formed and firm), based on previously validated method (**Article 1 – Chapitre 4**).

To classify each puppy's diarrhea status, a composite score was determined over the three time points. As shown by the study of Grellet et al (3) puppies were assigned to the “Diarrhea” group if at least one of the three scores was ≤ 5 . Conversely, puppies were considered to have “No Diarrhea” if all three fecal scores were > 5 .

In addition, the mean fecal score across the three time points was calculated for each puppy and used to describe the characteristics of puppies' groups (**Table 1**).

III. Fecal microbiota sampling and Bioinformatics

Rectal swabs (ETM, COPAN, Brescia, Italy) were collected from all puppies at five key time points: at postnatal Day 1, Day 7, Day 21, the day before weaning (BW), and 1WPW. All samples were immediately stored at -80°C until further processing. DNA extraction, PCR amplification of the V3–V4 region of the 16S rRNA gene, and high-throughput sequencing using the Illumina MiSeq platform were performed as previously described by Bendahmane et al. (**Article 3 – Chapitre 5**). Briefly, DNA was extracted using the Quick-DNA™ Fecal/Soil Microbe Kit (ZymoResearch, Ville, USA), followed

by two rounds of PCR using universal primers 460F and 460R. Sequencing was carried out at the GeT-PlaGe (17) INRAE platform (Toulouse, France).

The sequenced amplicons were then processed using the FROGS pipeline (v3.2.3) (18), following the protocol detailed in our earlier study (**Article 3 – Chapitre 5**). Briefly, read pairs were assembled using VSEARCH, filtered by size (380–500 bp), presence of primer and aberrant base, and clustered using a distance of 1 with the fastidious option enabled. Chimeric sequences were removed, and low-abundance ASVs (present in <2 individuals or with an abundance <0.005% of total sequence) (19) were excluded. Taxonomic assignment was performed against the SILVA 138.1 database using a pintail score threshold of 100.

IV. Statistics

Statistical analyses were conducted using RStudio software (version 2024.09.1) (20).

a) Identification and characterization of puppy enterotypes at weaning

The overall methodological framework described in this section is summarized in **Figure 1**.

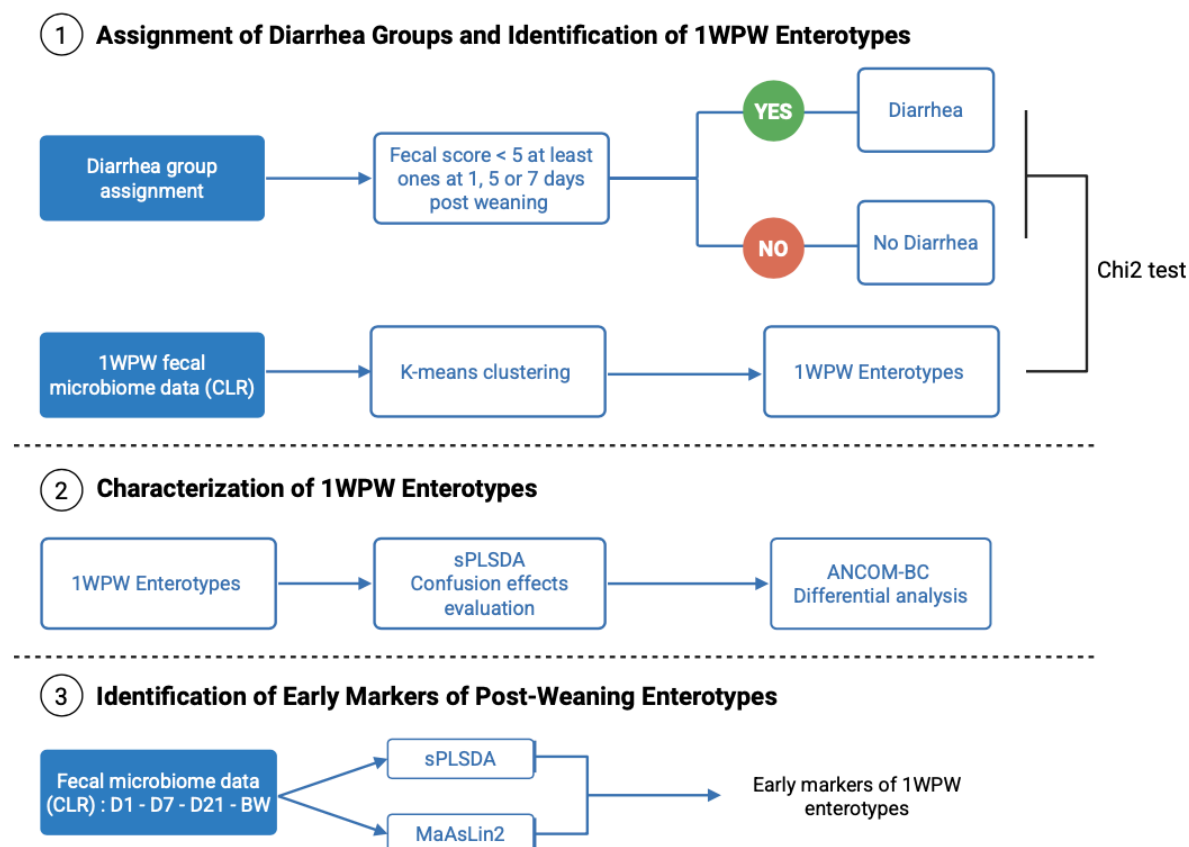


Figure 1. Integrative analytical workflow for post-weaning enterotype characterization, and early biomarker identification.

In order to identify the enterotype of puppies at 1WPW, a K-mean cluster analysis was performed on the fecal microbiome taxa at 1WPW. The optimal number of clusters was identified using the Average Silhouette Width method (21), which evaluates clustering performance across a range of k values (from 1 to 10 in this study). To visualize the resulting clusters, a two-dimensional cluster plot was generated. Subsequently, a chi-squared test was conducted to assess whether the prevalence of diarrhea differed significantly across the identified enterotype groups. Then, a supervised sPLS-DA analysis was performed to assess the level of discrimination between enterotypes based on microbial composition. To further investigate the contribution of individual explanatory variables to the microbial differences between enterotypes, an analysis of variance (ANOVA) was performed using the scores of the first component from the sPLS-DA model (**Formula 3**).

Formula 3 : $\text{sPLSDA.Comp} \sim \text{Enterotype} + \text{Sex} + \text{Batch} + \text{Temperament} + \text{Weaning_age} + \text{1WPW_weight}$

Among the explanatory variables included in this model, only Batch showed a significant confounding effect on the sPLS-DA first component scores (**Annexes Chapitre 5 ii - Supplementary Table 1**).

Finally, to identify microbial genera and families that differed significantly between enterotypes, differential abundance analysis was conducted using the Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) implemented via the “ANCOMBC” package (22). The model used is described by the following formula (**Formula 4**):

$$\text{Formula 4: } y = \beta_0 + \beta_1 \cdot \text{1WPW enterotype} + \beta_2 \cdot \text{Batch} + \epsilon$$

where y represents the centered log-ratio (CLR) abundance of each taxon, β_0 is the intercept, β_1 the effect size of the enterotype variable, β_2 the coefficient for the batch effect, and ϵ the residual error.

b) Identification of early markers of post-weaning enterotype

Since microbiome data are inherently compositional (23), for each time point (D1, D7, D21 and BW) zero values were first handled using the Geometric Bayesian-Multiplicative (GBM) method, which assumes that the probability of observing a taxon is not truly zero. This imputation was carried out using the `cmultRepl` function from the “zCompositions” package (24). The resulting abundance table was then transformed using the Centered Log-Ratio (CLR) method, implemented with “compositions” package.

Following data normalization, the optimal model parameters were determined using the “`tune.splsda`” function from the “mixOmics” package (25), which identified the best number of components (`ncomp`) and the number of variables to retain per component (`keepX`) for discriminating between enterotypes. Based on these parameters, a sparse Partial Least Squares Discriminant Analysis (sPLS-DA) was then

conducted using the “splsd” function. Model performance was evaluated using the balanced error rate (BER) and the area under the receiver operating characteristic curve (AUROC). To identify the most relevant discriminant features, the “vip” function was applied to the selected components. To assess the statistical significance of each selected genera, an analysis of variance (ANOVA) was performed on centered log-ratio (CLR)-transformed data using the model: CLR ~ Enterotype + Batch. And post-hoc pairwise comparisons were carried out using Tukey's Honest Significant Difference (HSD) test.

To identify potential bacterial biomarkers associated with the post-weaning enterotype, we used the MaAsLin2 package in R. The model included the 1WPW enterotype (classified as “Healthy” or “At risk”), sampling date, batch, and sex as fixed effects, and individual identity as a random effect to account for repeated measures across time (**Formula 3**). Taxa present in at least 10% of samples were retained, and data were normalized using Total Sum Scaling (TSS) followed by log transformation. Associations with an FDR-adjusted p-value below 0.05 were considered statistically significant.

Formula 3: Genus_abundance ~ 1WPW enterotype + date + batch + (1|Individuals)

Results

Description of the population

Among the 77 puppies included in the study originating from 18 litters and 6 batches, 34 were females and 41 were males (sex ration of 0.8). At birth, puppies’ mean Apgar score was 8 ± 1 , their mean birth weight was 428 ± 55 g and their mean weaning growth rate was $11 \pm 8\%$. The mean fecal score during the first week after weaning was 7 ± 1 . A total of 32% (25/77) of puppies presented diarrhea at least once during this period: 13/67 at one timepoint, 6/59 at two timepoints, and 7/48 at three timepoints.

Identification of enterotypes in puppies one week after weaning

In order to identify the enterotype of puppies at 1WPW, a K-mean cluster analysis was performed based on the 480 ASVs. According to the average silhouette score, 4 clusters were identified (**Figure 2A**). The cluster plot showed a clear separation between clusters along the first and second dimensions, explaining 26.6% of the overall variability (**Figure 2B**).

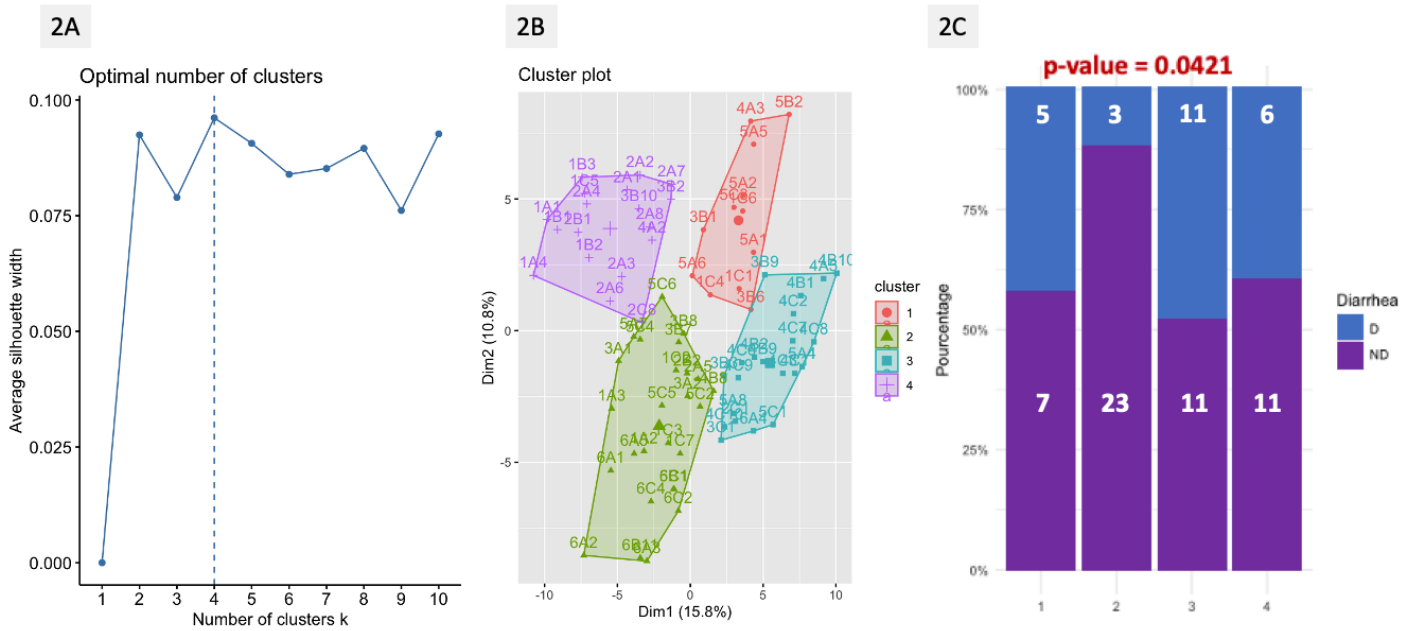


Figure 2 : K-means clustering analysis of 1 week post weaning fecal microbiome. (A) The optimal number of clustering is determined using the silhouette method. (B) The clusters are visualized on the first two principal components. (C) Chi-squared test showing the distribution of diarrhea cases across the identified clusters.

A Chi-squared test revealed a significant difference in diarrhea incidence across the four clusters ($p = 0.042$), with Cluster 2 containing the lowest number of individuals who experienced diarrhea, whereas Clusters 1, 3, and 4 showed an equal distribution between diarrheic and non-diarrheic puppies (**Figure 2C**). Based on this observation, Clusters 1, 3, and 4 were merged into a single group labeled “At risk” ($n = 51$) while Cluster 2 was classified as “Healthy” ($n = 26$).

Characteristics of the puppies according to their enterotype

No significant differences were observed between “Healthy” and “At risk” enterotype in terms of sex ratio, birth weight, weaning growth rate, or weaning age (**Table 1**). However, a significantly higher fecal score (indicating a better fecal consistency) at weaning was observed in the “Healthy” enterotype compared to the “At risk” enterotype ($p=0.004$).

Table 1: Characteristics of puppies according to their enterotype ($n=77$).

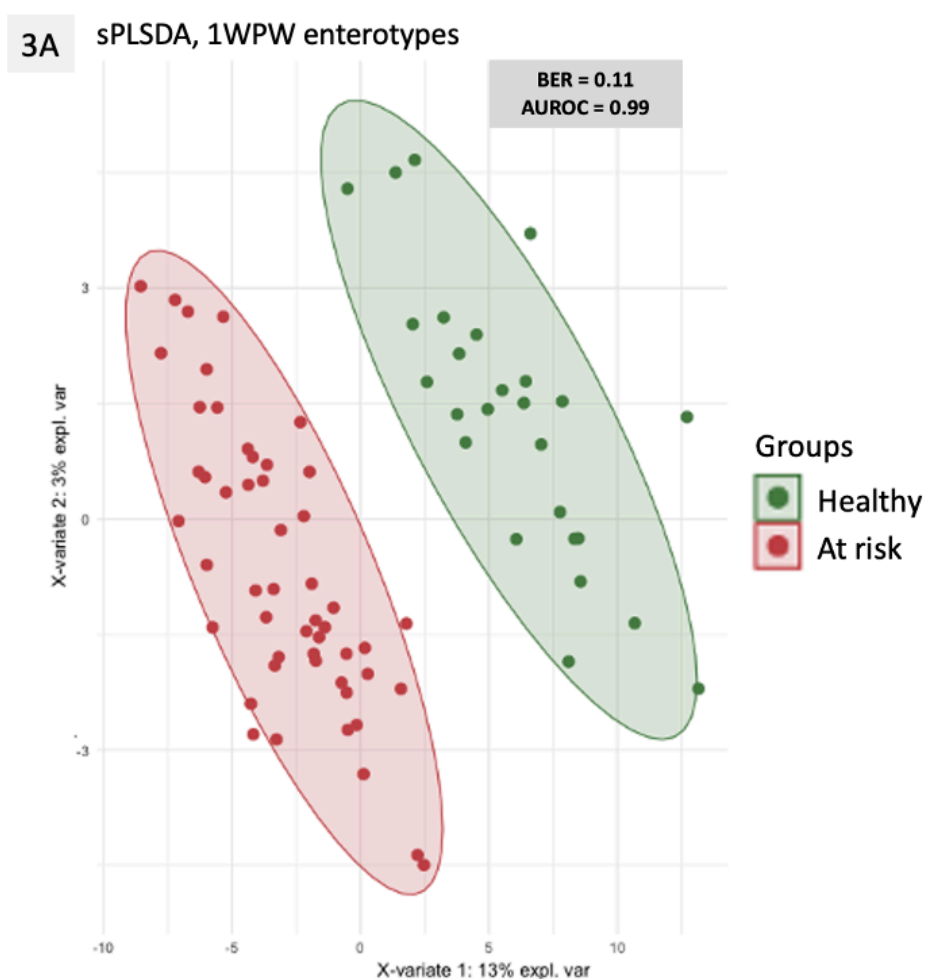
	Healthy (n=26)	At risk (n=51)	P-value
Sex ratio (F/M)	13/13	21/28	0.728
	Mean $\pm$ SD	Mean $\pm$ SD	

Weaning GR^a (%)	8.6 ± 9.7	12.4 ± 6.8	0.083
Weaning age (D)	61 ± 5	61 ± 4	0.688
Weaning FS^b	8.1 ± 1.6	6.8 ± 1.7	0.004

^aGR: Growth rate; ^bFS: Fecal score

Discriminating taxonomic units between the two enterotypes

To further investigate microbial differences between these two groups, a sPLS-DA was performed on the 1WPW gut microbiota composition. This analysis revealed a good discrimination (BER = 0.11 and AUROC = 0.99) between the two enterotypes called "Healthy" and "At risk" enterotype (**Figure 3A**).



Using ANCOM-BC, 27 bacterial genera were found to be differentially abundant between the two enterotypes. The “At risk” enterotype showed increased relative abundance of *Campylobacter*, *Helicobacter*, *Mucispirillum*, and *Anaerofilum*. In contrast, Genera such as *Collinsella*, *Peptoclostridium*, *Blautia*, and *Faecalibacterium* were significantly more abundant in the “Healthy” puppies (**Figure 3B**; **Table 2**)

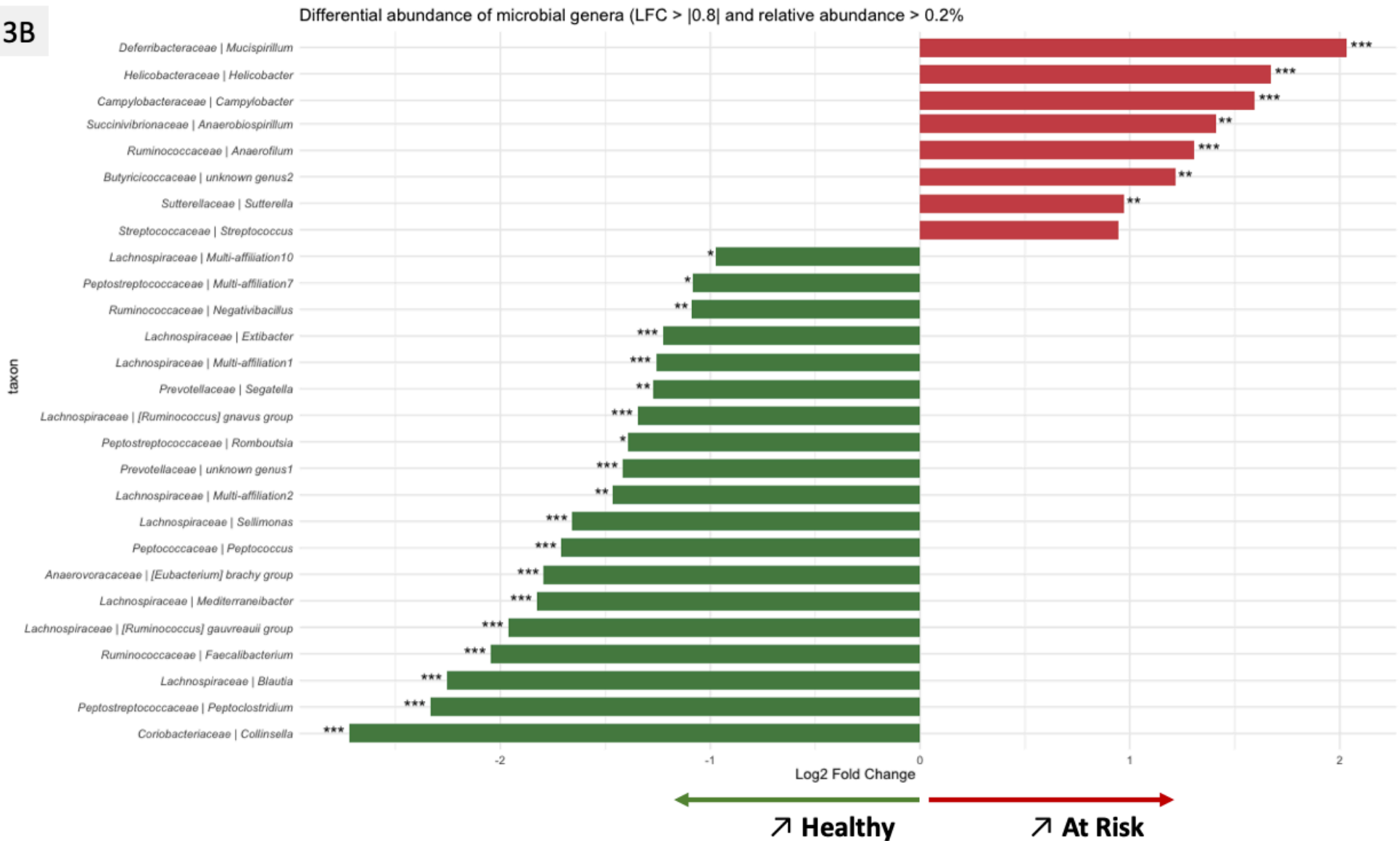


Figure 3. Sparse partial least-squares-discriminant analysis (sPLS-DA) of 1WPW enterotypes (A). Log-fold change (LFC) values of the of the fecal microbiome genera according the enterotype of puppies at one week post weaning (B). The bars represent the LFC of taxa between enterotype, with positive values indicating an increase and negative values indicating a decrease in taxa abundance in the "At risk" enterotype compared to the "Healthy" one. Significance levels: p -value ≤ 0.001 (***), $0.01 < p$ -value ≤ 0.05 (*), $0.05 < p$ -value ≤ 0.10 (#).

Table 2: Differential analysis of bacterial genera abundance between "Healthy" and "At risk" enterotype using ANCOM-BC. Only bacterial genera with a LFC > 10.81 and a relative abundance greater than 0.2% of the total sequences were kept.

Family	Genus	RelativeAbundance (%)		Percent_zeros	
		Mean±SD		Healthy	At risk
<i>Deferribacteraceae</i>	<i>Mucispirillum</i>	0,31±0,05	1,50±2,75	38	27
<i>Helicobacteraceae</i>	<i>Helicobacter</i>	0,10±0,23	2,33±4,53	12	14
<i>Campylobacteraceae</i>	<i>Campylobacter</i>	0.01±0.01	0,72±2,64	46	29
<i>Succinivibrionaceae</i>	<i>Anaerobiospirillum</i>	0,27±0,28	4,83±6,51	0	6
<i>Ruminococcaceae</i>	<i>Anaerofilum</i>	0,02±0,08	0,63±2,81	46	53
<i>Butyricicoccaceae</i>	<i>unknown genus2</i>	0,09±0,12	0,81±1,82	19	35
<i>Sutterellaceae</i>	<i>Sutterella</i>	0,20±0,26	0,94±1,16	8	35
<i>Streptococcaceae</i>	<i>Streptococcus</i>	3,30±7,84	7,03±11,32	4	2
<i>Lachnospiraceae</i>	<i>Multi-affiliation10</i>	0,27±0,35	0,26±0,56	4	41
<i>Peptostreptococcaceae</i>	<i>Multi-affiliation7</i>	0,72±1,42	0,80±1,40	12	20
<i>Ruminococcaceae</i>	<i>Negativibacillus</i>	0,58±0,58	0,43±0,55	4	33
<i>Lachnospiraceae</i>	<i>Extibacter</i>	0,95±2,10	0,53±0,77	0	16
<i>Lachnospiraceae</i>	<i>Multi-affiliation1</i>	0,87±0,90	0,55±0,62	0	8
<i>Prevotellaceae</i>	<i>Segatella</i>	1,97±2,74	2,97±4,23	0	14
<i>Lachnospiraceae</i>	<i>[Ruminococcus] gnavus group</i>	0,21±0,23	0,15±0,24	0	20

<i>Peptostreptococcaceae</i>	<i>Romboutsia</i>	0,67±1,07	0,38±0,65	8	31
<i>Prevotellaceae</i>	<i>unknown genus1</i>	1,39±1,59	1,12±1,61	0	22
<i>Lachnospiraceae</i>	<i>Multi-affiliation2</i>	0,51±0,63	0,26±0,76	0	33
<i>Lachnospiraceae</i>	<i>Sellimonas</i>	0,52±0,34	0,39±0,44	4	18
<i>Peptococcaceae</i>	<i>Peptococcus</i>	0,51±0,47	0,22±0,27	0	29
<i>Anaerovoracaceae</i>	<i>[Eubacterium] brachy group</i>	1,09±0,77	0,67±0,76	0	16
<i>Lachnospiraceae</i>	<i>Mediterraneibacter</i>	2,56±2,42	1,75±1,57	0	2
<i>Ruminococcaceae</i>	<i>Faecalibacterium</i>	2,77±1,91	1,89±2,07	0	6
<i>Lachnospiraceae</i>	<i>Blautia</i>	14,56±8,15	7,21±7,65	0	0
<i>Peptostreptococcaceae</i>	<i>Peptoclostridium</i>	30,03±12,42	14,91±15,11	0	0
<i>Coriobacteriaceae</i>	<i>Collinsella</i>	10,46±5,53	3,51±3,74	0	0

Identification of early-life microbial markers potentially associated with enterotype at weaning

We next investigated whether specific bacterial genera present early in life could serve as potential predictors of weaning-associated enterotypes. On Day 1, the sPLS-DA model (ncomp = 2, KeepX = 50) explained 23% of the variance and achieved a BER of 0.35 (**Figure 4A**). Among the selected features (**Annexes Chapitre 5 ii - Supplementary Table 2**), two genera were more abundant in the “At risk” enterotype: *Cellulosilyticum* (3.88% vs. 0.34% in “Healthy”, VIP = 1.55, $p = 0.014$) and *Enterococcus* (4.62% vs. 2.07%, VIP = 1.46, $p = 0.041$).

On Day 7, based on the sPLSDA with ncomp = 1 and KeepX = 30, six genera discriminated the two enterotypes (**Annexes Chapitre 5 ii - Supplementary Table 3**). Among the selected 4 genera were significantly different between the enterotype groups. *Peptoclostridium* (2.69% vs. 0.54% in “Healthy”, VIP = 3.35, $p = 0.006$) and *Roseburia* (1.34% vs. 0.57%, VIP = 3.19, $p = 0.003$) were more abundant, in “At risk” enterotype. *Streptococcus* (4.12% vs. 1.75% in “At risk”, VIP = 2.66, $p = 0.016$) and *Escherichia-Shigella* (33.6% vs. 28.6% 1.75%, VIP = 2.18, $p = 0.023$) were significantly more abundant in the “Healthy” enterotype group. Enterotype discrimination was maintained, although with moderate accuracy, as reflected by a BER of 0.35 and 22% of variance explained by the first two components (**Figure 4B**).

By Day 21, seven genera (**Annexes Chapitre 5 ii - Supplementary Table 4**) were retained by the sPLS-DA model (ncomp = 1, KeepX = 22), which explained 24% of the variance with a BER of 0.44 (**Figure 4C**). Among these, two genera showed significantly higher abundance in “At risk” enterotype: *Enterococcus* (2.53% vs. 0.75% in “Healthy”, VIP = 2.27, $p = 0.011$) and *Anaerobiospirillum* (5.93% vs. 4.10% in “Healthy”, VIP = 2.26, $p = 0.036$).

On the day BW, the sPLS-DA model (ncomp = 1, KeepX = 25) selected 14 genera, explaining 21% of the variance with a BER of 0.35 (**Figure 4D, Annexes Chapitre 5 ii - Supplementary Table 5**). Among these, 11 genera showed significant differences in abundance between enterotypes. Genera with higher abundance in the “At risk” group included *Turicibacter* (3.05% vs. 1.60%, VIP = 2.11, $p < 0.001$), *Escherichia-Shigella* (6.55% vs. 1.94%, VIP = 1.70, $p = 0.011$), *Enterococcus* (1.10% vs. 0.34%, VIP = 1.49, $p = 0.015$), *Cetobacterium* (1.02% vs. 0.03%, VIP = 1.29, $p = 0.047$) and *Clostridium* (7.58% vs. 3.75%, VIP = 1.28, $p = 0.048$). In contrast, several genera were more abundant in the “Healthy” group: *Fusobacterium* (7.99% vs. 3.90%, VIP = 2.33, $p = 0.001$), *Campylobacter* (1.22% vs. 0.26%, VIP = 1.88, $p = 0.008$), *Bacteroides* (2.99% vs. 1.25%, VIP = 1.77, $p = 0.017$), an unknown *Prevotellaceae* genus (1.30% vs. 0.28%, VIP = 1.77, $p = 0.011$), *Lachnispiraceae* Multi-

affiliation1 (2.13% vs. 0.79%, VIP = 1.66, $p = 0.018$) and an unknown *Butyricicoccaceae* genus (1.31% vs. 0.59%, VIP = 1.50, $p = 0.011$).

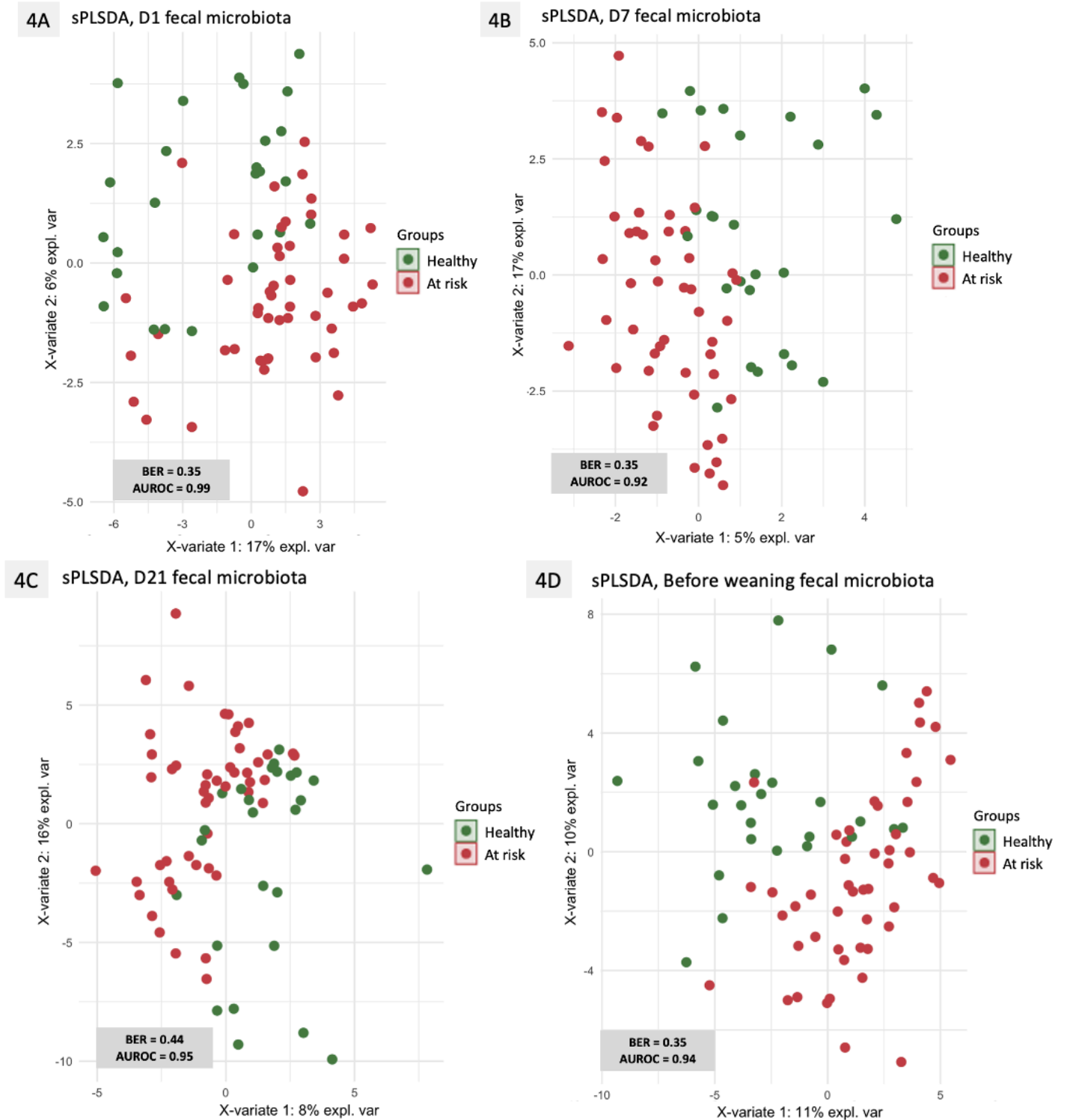


Figure 4 : Sparse partial least-squares-discriminant analysis (sPLS-DA) of fecal microbiota at Day 1 (A), Day 7 (B), Day 21 of life (C) and before weaning (D) in puppies according to their one-week post-weaning enterotype (“Healthy” in green and “At risk” of weaning diarrhea in red).

MaAsLin2 analysis identified *Enterococcus*, and *Prevotellaceae UG1* as significantly associated with 1WPW puppies' enterotypes. *Enterococcus* was consistently higher in the "At risk" group across time, with significant differences at day1 ($P=0.041$), day21 ($P=0.011$), BW ($P=0.015$) and tendency toward increased abundance at day7 ($P=0.097$) (Figure 5A). Conversely, the *Prevotellaceae UG1* genus increased progressively in both groups but reached significantly higher levels in the "Healthy" group at BW ($P=0.011$) (Figure 5B).

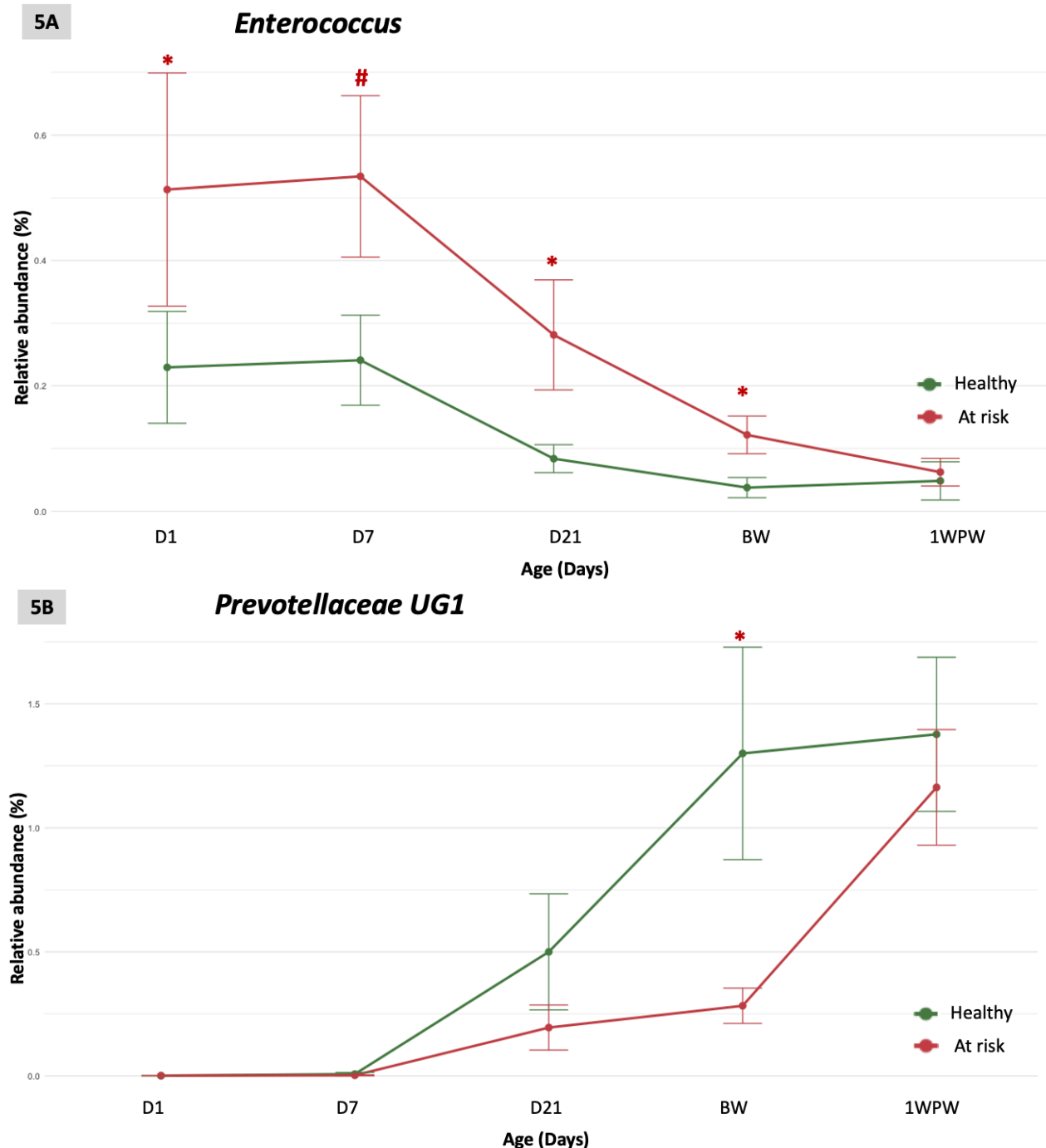


Figure 5 : Representation of genera selected from MaAslin2 analysis as associated with 1WPW enterotype. Evolution of relative abundance (%) of *Enterococcus* (C) and *Prevotellaceae* (D) over time in Healthy and At-risk puppies. Points represent the mean $\pm$ standard error of the mean (SEM) at each

age. Statistical comparisons at each time point were performed on CLR-transformed data using the ANOVA model (CLR ~ Enterotype + Batch). Significance levels: $0.01 < p\text{-value} \leq 0.05$ (*), $0.05 < p\text{-value} \leq 0.10$ (#).

Relationship between neonatal health parameters and enterotype at weaning

None of the studied neonatal health parameters, such as birth weight, APGAR score, early growth rate, and bottle-feeding, differ significantly between the “Healthy” and “At risk” enterotypes (**Table 3**).

Table 3: Early life characteristics of puppies according to their fecal microbiota type (n=77).

	Healthy (n=26)	At risk (n=51)	P-value
Birth weight (g)	442 ± 54	420 ± 55	0.101
APGAR score	8 ± 1	8 ± 1	0.778
Early GR ^a (%)	11 ± 7	13 ± 7	0.142
Bottle feeding (yes/no)	0.4 (8/18)	0.4 (15/36)	0.983

^aGR: Growth rate

Discussion

This study is the first to provides novel insight into the relationship between fecal microbiota composition and diarrhea risk in Golden Retriever puppies during the weaning transition. Our findings highlight the existence of two distinct enterotypes at 1WPW, one of which being “At risk” and associated with increased incidence of diarrhea. Notably, we also demonstrate that some early-life fecal microbes could be associated with the evolution to either enterotypes. These results suggest that the fecal microbiota could reflect or predict post-weaning gastrointestinal health.

In this study differential abundance analysis at 1WPW revealed that genera enriched in the “Healthy” enterotype predominantly belonged to beneficial taxa, including *Collinsella*, *Peptoclostridium*, *Eubacterium*, *Peptococcus*, *Faecalibacterium*, and several members of the *Lachnospiraceae* family (*Blautia*, *Mediterraneibacter*, *Sellimonas*, *[Ruminococcus] gnavus* group, *Extibacter*). These bacteria play a key role in maintaining gut homeostasis, strengthening the intestinal barrier, and providing protection against inflammation and diarrhea (26, 27, 28, 29). Consistent with our findings, Karasova et al. reported that *Prevotellaceae*, *Faecalibacterium*, and *Prevotella* were significantly more abundant in healthy piglets compared to those that developed weaning-associated diarrhea (30).

Conversely, the "At risk" enterotype showed increased abundances of genera commonly associated with gastrointestinal inflammation or dysbiosis, such as *Campylobacter*, *Helicobacter*, *Mucispirillum*, and *Sutterella* (31, 32, 33, 34). These taxa are frequently described as opportunistic or pro-inflammatory microbes, often enriched in diarrheic or dysbiotic gut profiles across species.

Our results are in line with other data, where *Campylobacter* is prevalent in pigs (35) and dogs (36) with enteric disorders. Zheng et al. (37) similarly reported increased *Campylobacter* abundance in piglets experiencing post-weaning diarrhea. Another study observed enrichment of *Sutterella* and *Campylobacter* in diarrheic versus healthy pre-weaning piglets (38). *Mucispirillum* has also been associated with intestinal inflammation in murine colitis models (39,40). *Sutterella* is of particular concern due to its ability to produce IgA proteases, which degrade secretory IgA, thereby impairing mucosal immunity and increasing gut vulnerability (41). Furthermore, its presence has been positively correlated with diarrhea in rodent models (42). To our knowledge, no prior studies have linked gut microbiota composition to weaning-associated diarrhea in puppies. Future research should aim to elucidate the underlying mechanisms (particularly the role of intestinal inflammation) and confirm these associations through targeted qPCR analyses of the implicated genera.

Beyond identifying distinct enterotypes at weaning, we next investigated early microbial markers that may predict post-weaning diarrhea risk. To this end, we applied a sPLS-DA to assess whether early-life microbiota composition could discriminate between puppies later classified into "Healthy" or "At risk" enterotypes. Our sPLS-DA results suggest a potential prediction value, although with moderate accuracy: the BER across pre-weaning timepoints was approximately 65%. Interestingly, when performing a bootstrap simulation using an expanded sample size ($n = 200$), the model's predictive accuracy markedly improved, reaching up to 80% for all early-life sampling days (**Annexes Chapitre 5 ii - Supplementary data1**). This finding highlights the potential of early microbiota profiles as predictive markers of post-weaning gut health but also underscores the importance of sample size in improving model robustness. Therefore, future studies involving cohorts of greater magnitude would be valuable to validate and refine these predictive microbial signatures.

Among the taxa identified, *Enterococcus* emerged as a notable taxon through both MaAsLin2 and sPLSDA analyses, showing significantly higher relative abundance in the fecal microbiota of puppies with the "At risk" enterotype. Importantly, this increased abundance was already evident during the pre-weaning period—on day 1, at day 21, and just BW—suggesting that early colonization by *Enterococcus* may contribute to shaping an intestinal environment that predisposes to gastrointestinal disorders.

We hypothesize that early *Enterococcus* dominance may lead to sustained luminal acidification, creating conditions less favorable for the establishment of beneficial microbial taxa. In neonates with

immature immune systems and limited intestinal buffering capacity (43), this low-pH environment could disrupt epithelial homeostasis and set the stage for dysbiosis. In turn, this imbalance may reduce intestinal resilience during the weaning transition, increasing the host's susceptibility to diarrhea.

Several lines of evidence support this hypothesis. *Enterococcus* species, as lactic acid bacteria, are known to produce lactate and reduce intestinal pH (44). A persistently low pH has been associated with lactate accumulation and shifts in microbial composition that promote intestinal inflammation (45,46). In calves, elevated luminal lactate concentrations have been positively associated with the occurrence of weaning-associated diarrhea (47). Similarly, in piglets, increased abundance of *Enterococcaceae* at 7 days of age has been shown to predict the development of post-weaning diarrhea (48). While *Enterococcus* is not typically classified as a primary enteric pathogen in dogs, its early-life dominance and metabolic activity may play a predisposing—rather than causative—role in disease development.

Further research should explore the functional metabolic output of early *Enterococcus*-dominated microbiota, including its influence on acid production. In addition, it would be valuable to quantify *Enterococcus* abundance across different developmental stages using qPCR in order to establish threshold levels that may predict susceptibility to weaning-associated diarrhea. Such insights could help identify microbial biomarkers of risk and inform targeted microbiota-based strategies (such as buffering agents, dietary fiber interventions, or precision probiotics) to promote healthy microbiota maturation and gastrointestinal resilience during the weaning period.

In addition, our study identified a *Prevotellaceae* unknown genus, which showed a significant increase just BW in puppies from the "Healthy" enterotype compared to the "At risk" enterotype. This suggests a potential protective role of this taxon against weaning-associated diarrhea. This aligns with findings in calves, where early colonization by *Prevotella* has been shown to reduce diarrhea risk during weaning (49). Likewise, in piglets, increased *Prevotellaceae* abundance was identified as a marker of gut health in the context of post-weaning diarrhea (30). In humans, reduced *Prevotella* abundance has also been linked to gastrointestinal disorders in children (50, 51). These findings suggest that certain *Prevotellaceae* may promote intestinal stability and merit further investigation as early-life indicators of gastrointestinal health.

Finally, no association was found in our study between neonatal parameters and enterotype after weaning predisposing to weaning diarrhea. However, studies in pigs have shown that piglets with insufficient colostrum intake are at greater risk of weaning diarrhea compared to those that received optimal quantity of colostrum (52). In our study, only one puppy out of 77 experienced colostrum intake deficiency, defined as a growth rate from Day 0 to Day 2 of less than -4% (15). It would therefore be necessary to re-evaluate the relationship between neonatal parameters and enterotypes in other

problematic breeding environments in order to define the factors determining the microbiota composition since the very beginning of the colonization process. In addition to environmental and physiological variables, other factors such as early feeding behavior, stress reactivity, or anxiety-related traits could also play a role and should be considered in future investigations to fully understand the early-life determinants of enterotype establishment.

Conclusion

In conclusion, our findings demonstrate a possible association between fecal microbiota and enterotype associated with weaning diarrhea in puppies and support the use of early-life microbial signatures as potential predictive markers of post weaning enterotypes (**Figure 6**). However, studies with larger sample sizes, providing greater statistical power, are needed to evaluate the potential predictive value of early microbiota on the risk of diarrhea later in life. Recent studies have shown that maternal probiotic supplementation can influence both the offspring's gut microbiota (53) and overall health from birth (54,55). Together, these insights open the door to preventive strategies, such as targeted probiotic or prebiotic supplementation, aiming to modulate the gut microbiota before weaning and reduce diarrhea risk during this critical transition.

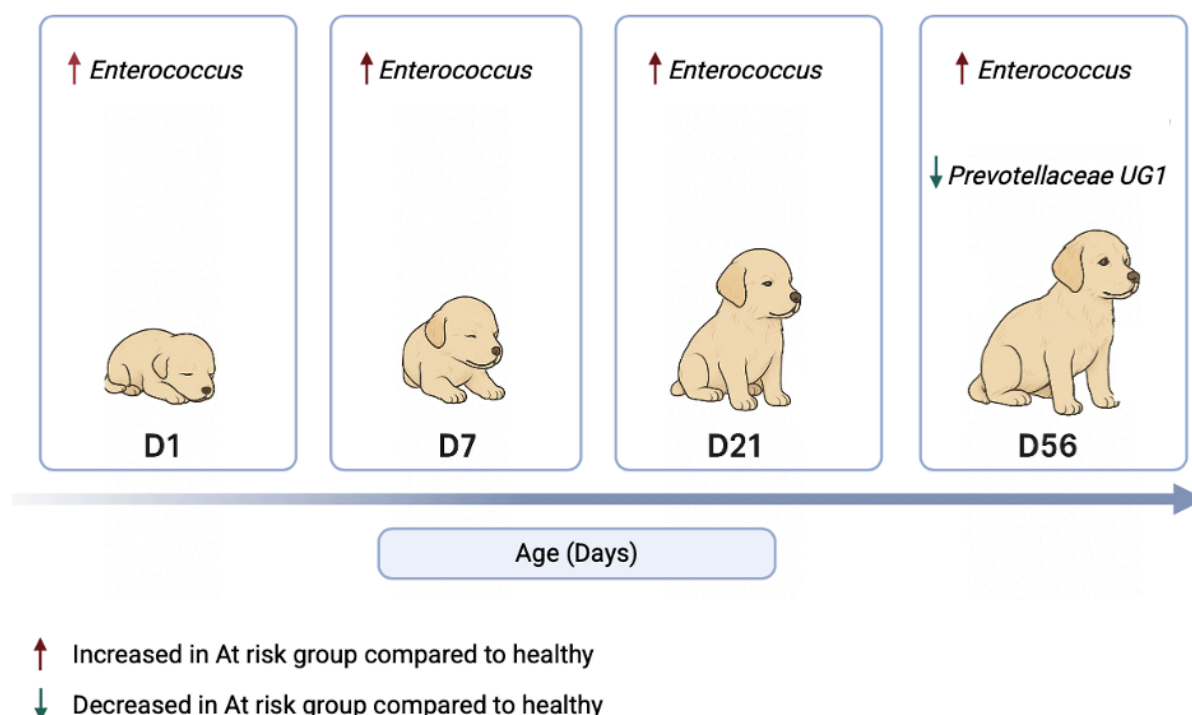


Figure 6. Summary of microbial signatures associated with enterotype At-risk of developing post weaning diarrhea in puppies. The figure illustrates the temporal pattern of two key genera (*Enterococcus* and *Prevotellaceae UG1*) from birth (D1) to weaning (D56). Red arrows indicate genera significantly more abundant in At-risk puppies compared to Healthy ones, while green arrows indicate genera less abundant in the At-risk enterotype.

References

1. Day MJ. Immune System Development in the Dog and Cat. *Journal of Comparative Pathology* 2007;137:S10–5.
2. Kick AR, Tompkins MB, Flowers WL, Whisnant CS, Almond GW. Effects of stress associated with weaning on the adaptive immune system in pigs¹. *Journal of Animal Science* 2012;90:649–56.
3. Grellet A, Chastant-Maillard S, Robin C, Feugier A, Boogaerts C, Boucraut-Baralon C, Grandjean D, Polack B. Risk factors of weaning diarrhea in puppies housed in breeding kennels. *Preventive Veterinary Medicine* 2014;117:260–5.
4. An J-U, Mun S-H, Kim W-H, Seong JK, Park K, Cho S. Dynamics of the canine gut microbiota of a military dog birth cohort. *Front Microbiol* 2025;16:1481567.
5. Hrnčir T. Gut Microbiota Dysbiosis: Triggers, Consequences, Diagnostic and Therapeutic Options. *Microorganisms* 2022;10:578.
6. Carding S, Verbeke K, Vipond DT, Corfe BM, Owen LJ. Dysbiosis of the gut microbiota in disease. *Microbial Ecology in Health & Disease* [Internet] 2015 [cited 2025 Jun 6];26. Available from: <http://www.microbecolhealthdis.net/index.php/mehd/article/view/26191>
7. Xie F, Zhou M, Li X, Li S, Ren M, Wang C. Macro-genomic and Metabolomic Analyses Reveal Mechanisms of Gut Microbiota and Microbial Metabolites in Diarrhea of Weaned Piglets. *Animals* 2024;14:2327.
8. Zheng X, Nie K, Xu Y, Zhang H, Xie F, Xu L, Zhang Z, Ding Y, Yin Z, Zhang X. Fecal Microbial Structure and Metabolic Profile in Post-Weaning Diarrheic Piglets. *Genes* 2023;14:1166.
9. Ren W, Yu B, Yu J, Zheng P, Huang Z, Luo J, Mao X, He J, Yan H, Wu J, et al. Lower abundance of Bacteroides and metabolic dysfunction are highly associated with the post-weaning diarrhea in piglets. *Sci China Life Sci* 2022;65:2062–75.
10. Tang X, Xiong K, Fang R, Li M. Weaning stress and intestinal health of piglets: A review. *Front Immunol* 2022;13:1042778.

11. Choudhury R, Middelkoop A, Boekhorst J, Gerrits WJJ, Kemp B, Bolhuis JE, Kleerebezem M. Early life feeding accelerates gut microbiome maturation and suppresses acute post-weaning stress in piglets. *Environmental Microbiology* 2021;23:7201–13.
12. Wang X, Tsai T, Deng F, Wei X, Chai J, Knapp J, Apple J, Maxwell CV, Lee JA, Li Y, et al. Longitudinal investigation of the swine gut microbiome from birth to market reveals stage and growth performance associated bacteria. *Microbiome* 2019;7:109.
13. Ma T, Villot C, Renaud D, Skidmore A, Chevaux E, Steele M, Guan LL. Linking perturbations to temporal changes in diversity, stability, and compositions of neonatal calf gut microbiota: prediction of diarrhea. *The ISME Journal* 2020;14:2223–35.
14. Veronesi MC, Panzani S, Faustini M, Rota A. An Apgar scoring system for routine assessment of newborn puppy viability and short-term survival prognosis. *Theriogenology* 2009;72:401–7.
15. Chastant S, Mila H. Passive immune transfer in puppies. *Animal Reproduction Science* 2019;207:162–70.
16. Grellet A, Feugier A, Chastant-Maillard S, Carrez B, Boucraut-Baralon C, Casseleux G, Grandjean D. Validation of a fecal scoring scale in puppies during the weaning period. *Preventive Veterinary Medicine* 2012;106:315–23.
17. GeT-PlaGe. GeT-PlaGe Genomics and Transcriptomics core facility of Toulouse [Internet]. INRAE; 2022 [cited 2025 May 18]. Available from: <https://get.genotoul.fr/la-plateforme/get-plage/>
18. Escudié F, Auer L, Bernard M, Mariadassou M, Cauquil L, Vidal K, Maman S, Hernandez-Raquet G, Combes S, Pascal G. FROGS: Find, Rapidly, OTUs with Galaxy Solution. Berger B, editor. *Bioinformatics* 2018;34:1287–94.
19. Bokulich NA, Subramanian S, Faith JJ, Gevers D, Gordon JI, Knight R, Mills DA, Caporaso JG. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nat Methods* 2013;10:57–9.
20. Rstudio (version 2024.09.1+394) [Internet]. Available from: <https://posit.co/products/open-source/rstudio/>

21. Batool F, Hennig C. Clustering with the Average Silhouette Width. *Computational Statistics & Data Analysis* 2021;158:107190.
22. Lin H, Peddada SD. Analysis of compositions of microbiomes with bias correction. *Nat Commun* 2020;11:3514.
23. Gloor GB, Macklaim JM, Pawlowsky-Glahn V, Egozcue JJ. Microbiome Datasets Are Compositional: And This Is Not Optional. *Front Microbiol* 2017;8:2224.
24. Palarea-Albaladejo J, Martín-Fernández JA. zCompositions — R package for multivariate imputation of left-censored data under a compositional approach. *Chemometrics and Intelligent Laboratory Systems* 2015;143:85–96.
25. Rohart F, Gautier B, Singh A, Lê Cao K-A. mixOmics: An R package for ‘omics feature selection and multiple data integration. Schneidman D, editor. *PLoS Comput Biol* 2017;13:e1005752.
26. Yin X-Q, An Y-X, Yu C-G, Ke J, Zhao D, Yu K. The Association Between Fecal Short-Chain Fatty Acids, Gut Microbiota, and Visceral Fat in Monozygotic Twin Pairs. *DMSO* 2022;Volume 15:359–68.
27. Liu J, Hao W, He Z, Kwek E, Zhu H, Ma N, Ma KY, Chen Z-Y. Blueberry and cranberry anthocyanin extracts reduce bodyweight and modulate gut microbiota in C57BL/6 J mice fed with a high-fat diet. *Eur J Nutr* 2021;60:2735–46.
28. Mukherjee A, Lordan C, Ross RP, Cotter PD. Gut microbes from the phylogenetically diverse genus *Eubacterium* and their various contributions to gut health. *Gut Microbes* 2020;12:1802866.
29. Fusco W, Lorenzo MB, Cintoni M, Porcari S, Rinninella E, Kaitsas F, Lener E, Mele MC, Gasbarrini A, Collado MC, et al. Short-Chain Fatty-Acid-Producing Bacteria: Key Components of the Human Gut Microbiota. *Nutrients* 2023;15:2211.
30. Karasova D, Crhanova M, Babak V, Jerabek M, Brzobohaty L, Matesova Z, Rychlik I. Development of piglet gut microbiota at the time of weaning influences development of postweaning diarrhea – A field study. *Research in Veterinary Science* 2021;135:59–65.

31. Imbrea A-M, Balta I, Dumitrescu G, McCleery D, Pet I, Iancu T, Stef L, Corcionivoschi N, Liliana P-C. Exploring the Contribution of *Campylobacter jejuni* to Post-Infectious Irritable Bowel Syndrome: A Literature Review. *Applied Sciences* 2024;14:3373.
32. Peng W, Zhao X, Li X. *Helicobacter bilis* Contributes to the Occurrence of Inflammatory Bowel Disease by Inducing Host Immune Disorders. Garg H, editor. *BioMed Research International* 2022;2022:1837850.
33. Herp S, Durai Raj AC, Salvado Silva M, Woelfel S, Stecher B. The human symbiont *Mucispirillum schaedleri*: causality in health and disease. *Med Microbiol Immunol* 2021;210:173–9.
34. Hiippala K, Kainulainen V, Kalliomäki M, Arkkila P, Satokari R. Mucosal Prevalence and Interactions with the Epithelium Indicate Commensalism of *Sutterella* spp. *Front Microbiol* [Internet] 2016 [cited 2025 Jun 2];7. Available from: <http://journal.frontiersin.org/article/10.3389/fmicb.2016.01706/full>
35. Burrough E, Terhorst S, Sahin O, Zhang Q. Prevalence of *Campylobacter* spp. relative to other enteric pathogens in grow-finish pigs with diarrhea. *Anaerobe* 2013;22:111–4.
36. Candellone A, Badino P, Girolami F, Cerquetella M, Nebbia P, Aresu L, Zoppi S, Bergero D, Odore R. Concomitant *Campylobacteriosis* in a Puppy and in Its Caregiver: A One Health Perspective Paradigm in Human-Pet Relationship. *Veterinary Sciences* 2023;10:244.
37. Zheng X, Nie K, Xu Y, Zhang H, Xie F, Xu L, Zhang Z, Ding Y, Yin Z, Zhang X. Fecal Microbial Structure and Metabolic Profile in Post-Weaning Diarrheic Piglets. *Genes* 2023;14:1166.
38. Yang Q, Huang X, Zhao S, Sun W, Yan Z, Wang P, Li S, Huang W, Zhang S, Liu L, et al. Structure and Function of the Fecal Microbiota in Diarrheic Neonatal Piglets. *Front Microbiol* [Internet] 2017 [cited 2025 Jun 2];8. Available from: <http://journal.frontiersin.org/article/10.3389/fmicb.2017.00502/full>
39. Herp S, Durai Raj AC, Salvado Silva M, Woelfel S, Stecher B. The human symbiont *Mucispirillum schaedleri*: causality in health and disease. *Med Microbiol Immunol* 2021;210:173–9.

40. Yoon K-N, Lee S-J, Keum GB, Song K-Y, Park J-H, Song B-S, Yu SY, Cho JH, Kim ES, Doo H, et al. Characteristics of *Lactococcus petauri* GB97 lysate isolated from porcine feces and its *in vitro* and *in vivo* effects on inflammation, intestinal barrier function, and gut microbiota composition in mice. Chen W-H, editor. *Microbiol Spectr* 2024;12:e01334-23.
41. Kaakoush NO. Sutterella Species, IgA-degrading Bacteria in Ulcerative Colitis. *Trends in Microbiology* 2020;28:519–22.
42. Lv W, Liu C, Ye C, Sun J, Tan X, Zhang C, Qu Q, Shi D, Guo S. Structural modulation of gut microbiota during alleviation of antibiotic-associated diarrhea with herbal formula. *International Journal of Biological Macromolecules* 2017;105:1622–9.
43. Evermann JF, Wills TB. Immunologic Development and Immunization. *Small Animal Pediatrics*. [Internet] Elsevier; 2011 [cited 2025 Jun 3]. p. 104–12. Available from: <https://linkinghub.elsevier.com/retrieve/pii/B9781416048893000140>
44. Matsunaga K, Komatsu Y. Ratio of l-(+)- and d-(-)-Lactic Acids Produced by *Enterococcus faecalis* Changes Depending on the Culture pH. *Microbiology Research* 2024;15:2703–12.
45. Wang SP, Rubio LA, Duncan SH, Donachie GE, Holtrop G, Lo G, Farquharson FM, Wagner J, Parkhill J, Louis P, et al. Pivotal Roles for pH, Lactate, and Lactate-Utilizing Bacteria in the Stability of a Human Colonic Microbial Ecosystem. Cotter PD, editor. *mSystems* 2020;5:e00645-20.
46. Firman J, Liu L, Mahalak K, Tanes C, Bittinger K, Tu V, Bobokalonov J, Mattei L, Zhang H, Van den Abbeele P. The impact of environmental pH on the gut microbiota community structure and short chain fatty acid production. *FEMS Microbiology Ecology* 2022;98:fiac038.
47. He Z, Ma Y, Chen X, Yang S, Zhang S, Liu S, Xiao J, Wang Y, Wang W, Yang H, et al. Temporal Changes in Fecal Unabsorbed Carbohydrates Relative to Perturbations in Gut Microbiome of Neonatal Calves: Emerging of Diarrhea Induced by Extended-Spectrum β -lactamase-Producing Enterotoxigenic *Escherichia coli*. *Front Microbiol* 2022;13:883090.
48. Dou S, Gadonna-Widehem P, Rome V, Hamoudi D, Rhazi L, Lakhal L, Larcher T, Bahi-Jaber N, Pinon-Quintana A, Guyonvarch A, et al. Characterisation of Early-Life Fecal Microbiota in

- Susceptible and Healthy Pigs to Post-Weaning Diarrhoea. Loh G, editor. PLoS ONE 2017;12:e0169851.
49. Chen H, Liu Y, Huang K, Yang B, Zhang Y, Yu Z, Wang J. Fecal microbiota dynamics and its relationship to diarrhea and health in dairy calves. *J Animal Sci Biotechnol* 2022;13:132.
 50. Kang D-W, Park JG, Ilhan ZE, Wallstrom G, LaBaer J, Adams JB, Krajmalnik-Brown R. Reduced Incidence of *Prevotella* and Other Fermenters in Intestinal Microflora of Autistic Children. Gilbert JA, editor. PLoS ONE 2013;8:e68322.
 51. Pop M, Walker AW, Paulson J, Lindsay B, Antonio M, Hossain MA, Oundo J, Tamboura B, Mai V, Astrovskaya I, et al. Diarrhea in young children from low-income countries leads to large-scale alterations in intestinal microbiota composition. *Genome Biol* 2014;15:R76.
 52. Blavi L, Solà-Oriol D, Llonch P, López-Vergé S, Martín-Orúe SM, Pérez JF. Management and Feeding Strategies in Early Life to Increase Piglet Performance and Welfare around Weaning: A Review. *Animals* 2021;11:302.
 53. Benahmane I, Apper E, Garrigues Q, Chastant S, Meynadier A, Mila H. ESAR, Nantes 2023. Oral communications. *Reprod Domestic Animals* 2023;58:184–98.
 54. Bendahmane I, Garrigues Q, Apper E, Mugnier A, Svilar L, Martin J, Chastant S, Meynadier A, Mila H. Maternal oral supplementation with *Saccharomyces boulardii* I-1079 during gestation and early lactation impacts the early growth rate and metabolic profile of newborn puppies. *Front Nutr* 2025;12:1500600.
 55. Garrigues Q, Mugnier A, Chastant S, Sicard F, Martin J-C, Svilar L, Castex M, Ramis-Vidal MG, Rovere N, Michaud L, et al. The supplementation of female dogs with live yeast *Saccharomyces cerevisiae* var. *boulardii* CNCM I-1079 acts as gut stabilizer at whelping and modulates immunometabolic phenotype of the puppies. *Front Nutr* 2024;11:1366256.

Annexes Chapitre 5

i. Supplementary data Article 2

Supplementary data 1: The socialization program employed in this study. The table provide details of the stimuli that were applied to the puppies.

Socialisation activity	Age in weeks							
	1	2	3	4	5	6	7	8
Manipulations by different operators daily, clinical examinations, samples, vacuum cleaner noises, collar; radio	X	X	X	X	X	X	X	X
Encouraged to interact with the rug, toys with unknown noise, soft toys and balls			X					
Discover of the outside area: false grass, different floor textures, playing with bottle, balls, stuffed animals, agility material (high platforms)				X	X	X	X	X
Plastic bags shaking next to them, phone noises, training (sit, leash...)					X	X	X	X
Walking in arms to discover horses, dogs, cars, urban noises						X	X	X
Walking on a leash in urban area (traffic), car travel							X	X

Supplementary data 2 : Details of behavioral testing performed.

Period	Behavioral test	Details
Before weaning	Free exploration	The puppy is placed in the center of the arena, while the evaluator stands in a corner without any physical or visual interaction with the puppy.
	Plush toy interaction test	The interactive plush toy is placed in the center of the arena by the evaluator, then activated once. The evaluator moves to a corner, and after one minute, the plush toy is reactivated.

	Rope interaction	The evaluator stimulates the puppy with a rope for about ten seconds, then stands up and leaves the rope hanging at the puppy's level.
	Walking on plastic bag test	The evaluator places a plastic bag on the ground and encourages the puppy to walk on it by placing a rope in the middle.
	Noise exposure test	The evaluator plays a video with machine gun sounds.
1 week post-weaning	Free exploration	The puppy is placed in the center of the arena, while the evaluator stands in a corner without any physical or visual interaction with the puppy.
	Greeting test	The evaluator kneels down, claps hands, and calls the puppy by its name. Then, the evaluator moves to another spot and repeats the same action.
	Rope interaction	The evaluator stimulates the puppy with a rope for about ten seconds, then throws the rope to the other side of the arena and asks the puppy to bring it back, repeating the process.
	Leader-following test	The evaluator walks around the arena while calling the puppy and clapping hands.

	Problem solving	The evaluator places a transparent, open, and overturned glass jar containing kibble in the center of the arena.
	Vet check test	The evaluator sits on the ground, holds the puppy, and examines its teeth, eyes, ears, and belly.
	Noise exposure test	The evaluator plays a video with the sound of balloons popping.
	Plush toy interaction test	The interactive plush toy is placed in the center of the arena by the evaluator, then activated once. The evaluator moves to a corner, and after one minute, the plush toy is reactivated.

Supplementary data 3 :

Questionnaire 1:

What is your age?

.....

How many people live in the house?

.....

Do you have other animals? What species?

.....

Do you have a garden?

Yes No

Did the puppy travel in:

A cage

In your arms

Other (specify):

During the journey, did the puppy:

Vocalize (cry)

Pant

Urinate

Defecate

Salivate

Upon arrival at the house, did the puppy:

Explore the house right away?

Stay still?

If yes, for how long?

A few minutes

15 minutes

More than 15 minutes

Follow you? If yes, for:

A few minutes

15 minutes

More than 15 minutes

Play/explore with other family members?

Yes

No

Not applicable

Play/explore/interact with other animals?

Yes

No

Not applicable

Where did the puppy sleep the first night (multiple answers possible)?

In your room?

In a separate room?

In a cage?

On its bed?

In your bed?

Other (specify):

Does your puppy urinate or defecate at night?

If yes, how often?

Every day

Several times a day

Rarely

Does your puppy urinate or defecate in the house?

If yes, how often?

Every day

Several times a day

Rarely

Supplementary data 4 :

Questionnaire 2:

Puppy Behavior Questionnaire

FEAR / SHYNESS

The following questions address your puppy's potential fear/shyness reactions towards strangers, unfamiliar dogs, places, or new/strange situations. Please circle the correct answer. If the situation has not occurred, leave the question unanswered. If your puppy's behavior has changed at any point, please describe this change in the "Modified Behavior" section.

1. TOWARDS STRANGERS

Think about how your puppy usually reacts when meeting a stranger. If your puppy shows shyness towards strangers, please check YES, my puppy exhibits fearful behavior, and then check the behavior(s) that best describe your puppy's reaction. If none of the listed behaviors accurately describe your puppy's

reaction, you can add an appropriate behavior to the list. If your puppy does not behave fearfully, check NO and note how your puppy behaves when meeting a stranger.

Does your puppy show shyness or fear when meeting an unknown person?

- a. Yes
- b. No
- c. I don't know

If you answered YES, please mark one or more behaviors that describe your puppy's reaction:

- a. Withdraws
- b. Barks but does not approach the person
- c. Growls but does not approach the person
- d. Tail low/between the legs
- e. Does not want to make contact
- f. Stays close to the owner
- g. Barks/growls and approaches a stranger
- h. Other (specify):

If you answered yes, how often does the puppy react with fear? My puppy shows shyness or fearful behavior towards a stranger:

- a. Always (100% of the time)
- b. Almost always (60 to 100% of the time)
- c. Often (40 to 60% of the time)
- d. Sometimes (20 to 40% of the time)
- e. Rarely (0 to 20% of the time)

If you answered NO, please specify how your puppy behaves when meeting a stranger:

- a. If allowed, always goes to greet the person
- b. Jumps, licks, is very excited
- c. Sniffs, wags tail, and is relaxed
- d. Makes contact but loses interest quickly
- e. Is not interested in people but does not withdraw or mind if a person wants to pet them
- f. Not interested, quite indifferent
- g. Barks, growls
- h. Other (specify):

Behavior Change:

Former behavior:

Current behavior:

2. TOWARDS UNFAMILIAR PUPPIES

Think about how your puppy usually reacts when meeting unfamiliar puppies. If your puppy is shy when meeting unfamiliar puppies, please check YES, my puppy shows a fearful reaction, and then mark the appropriate behavior(s) that best describe your puppy's reaction. If none of the listed behaviors accurately describe your puppy's reaction, you can add an appropriate behavior to the list. If your puppy does not behave fearfully, check NO and note how your puppy behaves.

Does your puppy show shyness or fear when meeting an unfamiliar puppy?

- a. Yes
- b. No
- c. I don't know

If you answered YES, please mark one or more behaviors that describe your puppy's reaction:

- a. Withdraws
- b. Barks (does not approach the puppy)
- c. Tail low/between the legs
- d. Does not want to make contact
- e. Stays close to the owner (even when not commanded)
- f. Barks/growls and approaches an unfamiliar puppy
- g. Other (specify):

If you answered YES, how often does the puppy react with fear? My puppy shows shyness or fearful behavior towards an unfamiliar puppy:

- a. Always (100% of the time)
- b. Almost always (60 to 100% of the time)
- c. Often (40 to 60% of the time)
- d. Sometimes (20 to 40% of the time)
- e. Rarely (0 to 20% of the time)

If you answered NO, please specify how your puppy behaves when meeting an unfamiliar puppy:

- a. Eager to meet, always friendly
- b. Eager to meet, but quickly loses interest

- c. Generally friendly, but reacts aggressively if another puppy shows aggressive behavior
- d. Generally friendly, but sometimes starts a fight
- e. Generally growls
- f. Generally barks
- g. Barks/growls AND approaches a puppy
- h. Indifferent, not interested in other puppies
- i. Other (specify):

Behavior Change:

Former behavior:

Current behavior:

AGGRESSIVENESS

The following questions address your puppy's aggressive behavior towards strangers, the owner/family members, and other puppies. Please mark the frequency of the behavior.

1. TOWARDS STRANGERS

The puppy barks when the doorbell rings or someone knocks on the door

- a. Never
- b. Rarely
- c. Sometimes
- d. Often
- e. Always or almost always
- f. I don't know

The puppy barks when strangers enter and does not want to greet them at first

- a. Never
- b. Rarely
- c. Sometimes
- d. Often
- e. Always or almost always
- f. I don't know

The puppy barks when a stranger tries to touch or pet it in its home

- a. Never
- b. Rarely
- c. Sometimes
- d. Often
- e. Always or almost always
- f. I don't know

The puppy tries to snap or bite when a stranger tries to touch or pet it in its home

- a. Never
- b. Rarely
- c. Sometimes
- d. Often
- e. Always or almost always
- f. I don't know

The puppy is happy and excited when strangers come to its home

- a. Never
- b. Rarely
- c. Sometimes
- d. Often
- e. Always or almost always
- f. I don't know

When a stranger wants to pet the puppy outside the home and the puppy is on a leash, my puppy barks at the stranger

- a. Never
- b. Rarely
- c. Sometimes
- d. Often
- e. Always or almost always
- f. I don't know

When a stranger wants to pet the puppy outside the home and the puppy is on a leash, my puppy growls at the stranger

- a. Never
- b. Rarely
- c. Sometimes

- d. Often
- e. Always or almost always
- f. I don't know

When a stranger wants to pet the puppy outside the home and the puppy is on a leash, my puppy tries to snap or bite the stranger

- a. Never
- b. Rarely
- c. Sometimes
- d. Often
- e. Always or almost always
- f. I don't know

The puppy barks or growls at passersby when in the car

- a. Never
- b. Rarely
- c. Sometimes
- d. Often
- e. Always or almost always
- f. I don't know

2. TOWARDS OWNER/FAMILY MEMBER

When the owner/family member handles (washes, grooms, clips nails, etc.) the puppy, it growls

- a. Never
- b. Rarely
- c. Sometimes
- d. Often
- e. Always or almost always
- f. I don't know

When the owner/family member handles (washes, grooms, clips nails, etc.) the puppy, it tries to snap or bite

- a. Never
- b. Rarely
- c. Sometimes
- d. Often

- e. Always or almost always
- f. I don't know

When the owner/family member takes a bone/food/toy from the puppy, it growls

- a. Never
- b. Rarely
- c. Sometimes
- d. Often
- e. Always or almost always
- f. I don't know

When the owner/family member takes a bone/food/toy from the puppy, it tries to snap or bite

- a. Never
- b. Rarely
- c. Sometimes
- d. Often
- e. Always or almost always
- f. I don't know

Are there any other situations where your puppy behaves aggressively? Please specify:

.....

Behavior Change:

Former behavior:
 Current behavior:

SURFACES AND HEIGHTS

The following questions focus on your puppy's behavior when walking on various surfaces and in elevated places.

Does your puppy have difficulty walking on a metal grid?

- a. Yes, always
- b. Often
- c. Sometimes, depends on the place

- d. Rarely
- e. Never
- f. I don't know

Does your puppy have difficulty walking on shiny floors (such as in shopping malls, veterinary clinics, etc.)?

- a. Yes, always
- b. Often
- c. Sometimes, depends on the place
- d. Rarely
- e. Never
- f. I don't know

Does your puppy have difficulty climbing stairs where you can see "through" the steps?

- a. Yes, always
- b. Often
- c. Sometimes, depends on the place
- d. Rarely
- e. Never
- f. I don't know

Does your puppy have difficulty climbing stairs where you cannot see "through" the steps?

- a. Yes, always
- b. Often
- c. Sometimes, depends on the place
- d. Rarely
- e. Never
- f. I don't know

Does your puppy have difficulty walking next to glass railings, for example on the second floor of a shopping mall?

- a. Yes, always
- b. Often
- c. Sometimes, depends on the place
- d. Rarely
- e. Never
- f. I don't know

Does your puppy have difficulty climbing metal stairs where you can see "through" the steps?

- a. Yes, always
- b. Often
- c. Sometimes, depends on the place
- d. Rarely
- e. Never
- f. I don't know

Does your puppy have difficulty crossing narrow bridges?

- a. Yes, always
- b. Often
- c. Sometimes, depends on the place
- d. Rarely
- e. Never
- f. I don't know

Does your puppy have difficulty moving from one surface to another (e.g., from outside to inside in a new place)?

- a. Yes, always
- b. Often
- c. Sometimes, depends on the place
- d. Rarely
- e. Never
- f. I don't know

Does your puppy have problems on other surfaces or at heights? If your puppy has other problems with surfaces or heights not included in the questionnaire, please describe them below in your own words:

.....
.....

SEPARATION ANXIETY

Does your puppy exhibit separation anxiety when left alone or with other puppies?

- a. Yes

- b. No
- c. I don't know

How does your puppy react when the owner is not home and the puppy is alone or with other puppies?

The puppy destroys/chews things when alone at home

- a. Never
- b. Rarely
- c. Sometimes
- d. Often
- e. Very often
- f. I don't know

The puppy urinates or defecates when alone at home

- a. Never
- b. Rarely
- c. Sometimes
- d. Often
- e. Very often
- f. I don't know

The puppy vocalizes (e.g., howls, barks) when alone at home

- a. Never
- b. Rarely
- c. Sometimes
- d. Often
- e. Very often
- f. I don't know

The puppy drools when alone at home

- a. Never
- b. Rarely
- c. Sometimes
- d. Often
- e. Very often
- f. I don't know

The puppy pants when alone at home

- a. Never
- b. Rarely
- c. Sometimes
- d. Often
- e. Very often
- f. I don't know

HYPERACTIVITY/IMPULSIVITY

Please indicate how FREQUENTLY the statement is true for your puppy.

My puppy is hyperactive

- a. Never
- b. Rarely
- c. Sometimes
- d. Often
- e. Always or almost always
- f. I don't know

My puppy's hyperactivity/impulsivity disrupts daily routines and/or training

- a. Never
- b. Rarely
- c. Sometimes
- d. Often
- e. Always or almost always
- f. I don't know

If your puppy is hyperactive, when did the hyperactive or impulsive behavior begin?

- a. From the first day of adoption
- b. A few days after adoption

Please indicate how often the statement is true for your puppy.

My puppy has difficulty learning because he is inattentive or other things easily capture his attention

- a. Never
- b. Sometimes
- c. Often
- d. Very often

It's easy to get his attention, but he quickly loses interest

- a. Never
- b. Sometimes
- c. Often
- d. Very often

He has difficulty concentrating on a task or game

- a. Never
- b. Sometimes
- c. Often
- d. Very often

He leaves his place when he should stay

- a. Never
- b. Sometimes
- c. Often
- d. Very often

He cannot be calm, he cannot be easily calmed

- a. Never
- b. Sometimes
- c. Often
- d. Very often

He is restless all the time

- a. Never
- b. Sometimes
- c. Often
- d. Very often

He seems not to listen even when he knows someone is talking to him

- a. Never
- b. Sometimes
- c. Often
- d. Very often

He is excessive, hard to control, and if he rushes, he is difficult to hold back

- a. Never
- b. Sometimes
- c. Often
- d. Very often

He plays and/or runs all the time

- a. Never
- b. Sometimes
- c. Often
- d. Very often

He solves simple tasks easily but often has difficulty with complex tasks even if he knows them and has practiced them often

- a. Never
- b. Sometimes
- c. Often
- d. Very often

He is likely to react quickly, which is why he fails in his tasks

- a. Never
- b. Sometimes
- c. Often
- d. Very often

His attention can be easily distracted

- a. Never
- b. Sometimes
- c. Often
- d. Very often

He cannot wait because he has no self-control

- a. Never
- b. Sometimes
- c. Often
- d. Very often

Please indicate to what extent the statement describes your puppy.

My puppy shows extreme physical signs when excited (e.g., drooling, panting, raising his hackles, urinating, licking his lips, wide-eyed)

- a. Strongly disagree
- b. Generally disagree
- c. Partly agree, partly disagree
- d. Generally agree
- e. Strongly agree

When my puppy is very excited, it can lead to repetitive fixed behavior (i.e., an action that repeats in the same way over and over), like chasing his tail or circling

- a. Strongly disagree
- b. Generally disagree
- c. Partly agree, partly disagree
- d. Generally agree
- e. Strongly agree

I would consider my puppy very impulsive (i.e., he has sudden and strong urges to act; acts without foresight; acts without considering the effects of his actions)

- a. Strongly disagree
- b. Generally disagree
- c. Partly agree, partly disagree
- d. Generally agree
- e. Strongly agree

My puppy does not like to be approached or hugged

- a. Strongly disagree
- b. Generally disagree
- c. Partly agree, partly disagree
- d. Generally agree

- e. Strongly agree

My puppy becomes aggressive (e.g., growls, snaps, bites) when excited

- a. Strongly disagree
- b. Generally disagree
- c. Partly agree, partly disagree
- d. Generally agree
- e. Strongly agree

My puppy seems "sorry" after doing something wrong

- a. Strongly disagree
- b. Generally disagree
- c. Partly agree, partly disagree
- d. Generally agree
- e. Strongly agree

My puppy does not think before acting

- a. Strongly disagree
- b. Generally disagree
- c. Partly agree, partly disagree
- d. Generally agree
- e. Strongly agree

My puppy can be very persistent (e.g., he will continue to do something even if he knows he will be punished or reprimanded)

- a. Strongly disagree
- b. Generally disagree
- c. Partly agree, partly disagree
- d. Generally agree
- e. Strongly agree

My puppy can become aggressive (e.g., growls, snaps, bites) if frustrated by something

- a. Strongly disagree
- b. Generally disagree
- c. Partly agree, partly disagree
- d. Generally agree
- e. Strongly agree

My puppy is easy to train

- a. Strongly disagree
- b. Generally disagree
- c. Partly agree, partly disagree
- d. Generally agree
- e. Strongly agree

My puppy is eager to approach new situations

- a. Strongly disagree
- b. Generally disagree
- c. Partly agree, partly disagree
- d. Generally agree
- e. Strongly agree

My puppy takes a long time to lose interest in new things

- a. Strongly disagree
- b. Generally disagree
- c. Partly agree, partly disagree
- d. Generally agree
- e. Strongly agree

My puppy calms down very quickly after being excited

- a. Strongly disagree
- b. Generally disagree
- c. Partly agree, partly disagree
- d. Generally agree
- e. Strongly agree

My puppy seems to have a lot of control over how he reacts

- a. Strongly disagree
- b. Generally disagree
- c. Partly agree, partly disagree
- d. Generally agree
- e. Strongly agree

My puppy is very interested in new things and new places

- a. Strongly disagree
- b. Generally disagree
- c. Partly agree, partly disagree
- d. Generally agree
- e. Strongly agree

My puppy is not very patient (e.g., he fidgets while waiting for his food or waiting to go for a walk)

- a. Strongly disagree
- b. Generally disagree
- c. Partly agree, partly disagree
- d. Generally agree
- e. Strongly agree

My puppy seems to get excited for no reason

- a. Strongly disagree
- b. Generally disagree
- c. Partly agree, partly disagree
- d. Generally agree
- e. Strongly agree

Supplementary data 5 : Behavioral parameters Before and one week post-weaning: (Mean $\pm$ SD)

Paramètre	BW (Moyenne $\pm$ SD)	1WPW (Moyenne $\pm$ SD)
Exploration1 (sec)	54 $\pm$ 30	43 $\pm$ 32
Movement1 (sec)	25 $\pm$ 13	37 $\pm$ 28
Sitting1 (sec)	22 $\pm$ 24	21 $\pm$ 22
lying_down1 (sec)	4 $\pm$ 11	8 $\pm$ 13
Crying_Time (sec)	27 $\pm$ 38	7 $\pm$ 13
Barking (count)	2 $\pm$ 10	14 $\pm$ 23
Observation1 (sec)	6 $\pm$ 8	5 $\pm$ 7
Glance1 (count)	3 $\pm$ 2	3 $\pm$ 2
Proximity1 (sec)	81 $\pm$ 27	51 $\pm$ 34
Ears2 (count)	3 $\pm$ 2	3 $\pm$ 2

Proximity2 (sec)	14 ± 12	18 ± 12
Origin2 (count)	2 ± 2	3 ± 2
Sitting2 (sec)	12 ± 9	11 ± 10
lying_down2 (sec)	3 ± 8	7 ± 11
Movement2 (sec)	6 ± 5	8 ± 8
Observation2 (sec)	5 ± 5	5 ± 6
Glance2 (count)	1 ± 1	1 ± 1
Latency3 (sec)	79 ± 55	51 ± 54
Star_movments3 (count)	0 ± 1	3 ± 4
Growling (count)	1 ± 2	3 ± 5
Proximity3 (sec)	71 ± 37	48 ± 41
Sitting3 (sec)	49 ± 30	25 ± 27
lying_down3 (sec)	7 ± 21	40 ± 32
Tail3 (count)	7 ± 7	15 ± 10
Observation3 (sec)	11 ± 12	4 ± 4
Glance3 (count)	5 ± 3	4 ± 3
Ears3 (count)	3 ± 2	4 ± 2
Interaction3 (sec)	4 ± 11	14 ± 22
Movement3 (sec)	17 ± 14	23 ± 27
Environment1 (sec)	6 ± 10	2 ± 5
Movement_Latency1 (sec)	4 ± 5	3 ± 3

Supplementary data 6: PERMANOVA results (adonis2) for the first and second principal component scores using explanatory factors: K.mean (group), Mother, Sex, Batch, and Litter Size

PC scores ~ K.mean + Mother + Sexe + Batch + LitterSize				
	PC1		PC2	
	R2	F Pr(>F)	R2	F Pr(>F)
K.mean_After (group)	0.72875	0.001	0.00221	0.631
Mother	0.01450	0.005	0.00109	0.757
Sexe	0.00278	0.180	0.00201	0.642
Batch	0.08506	0.001	0.13508	0.013
LitterSize	0.01129	0.019	0.01603	0.196
Residual	0.15762		0.84358	
Total	1.00000		1.00000	

ii. Supplementary data Article 4

Supplementary Table 1: ANOVA results for the first principal component scores (Comp1) using explanatory factors including diarrhea status, sex, batch, temperament, age at departure, and post-adoption environment.

Variables	Df	Sum Sq	Mean Sq	F value	Pr(>F)	Signif.
Enterotype	1	314.8	314.81	28.634	1.44e-06	***
Sex	1	4.3	4.34	0.395	0.532040	
Batch	5	291.1	58.21	5.295	0.000429	***
Temperament	1	0.9	0.88	0.080	0.778233	
Weaning_age	1	14.8	14.83	1.349	0.250902	
1WPW_weight	1	1.4	1.37	0.124	0.725503	
Residuals	60	659.7	10.99			

Supplementary Table 2: Bacterial genera identified by sPLS-DA as distinctive between the 'Healthy' and 'At risk' enterotypes on Day 1 of life, with a Variable Importance in Projection (VIP) score > 1 and a mean relative abundance > 1% of the total sequences.

Family	Genus	p-val	VIP_comp1.2	Relative Abundance (%)		Percent_zeros	
				Mean±SD		Healthy	At risk
<i>Lachnospiraceae</i>	<i>Cellulosilyticum</i>	0,014	1,55	0,34± 0,91	3,88± 9,44	58	39
<i>Enterococcaceae</i>	<i>Enterococcus</i>	0,041	1,46	2,07± 5,56	4,62±11,22	23	8
<i>Lachnospiraceae</i>	<i>Multi.affiliation2</i>	0,099	1,41	0,10± 0,28	0,93±3,89	62	39
<i>Pasteurellaceae</i>	<i>Pasteurella</i>	0,204	1,02	1,29±3,93	0,15±0,42	46	41

Supplementary Table 3: Bacterial genera identified by sPLS-DA as distinctive between the 'Healthy' and 'At risk' enterotypes on Day 7 of life, with a Variable Importance in Projection (VIP) score > 1 and a mean relative abundance > 1% of the total sequences.

Family	Genus	p-val	VIP_comp1	Relative Abundance (%)		Percent_zeros	
				Mean±SD		Healthy	At risk
<i>Peptostreptococcaceae</i>	<i>Peptoclostridium</i>	0,006	3,35	0,54±1,35	2,69±5,74	35	10
<i>Lachnospiraceae</i>	<i>Roseburia</i>	0,003	3,19	0,57±2,02	1,34±3,50	81	41
<i>Streptococcaceae</i>	<i>Streptococcus</i>	0,016	2,66	4,12±6,71	1,75±4,86	12	10
<i>Enterobacteriaceae</i>	<i>Escherichia-Shigella</i>	0,023	2,18	33,57±23,66	28,55±23,75	0	2
<i>Coriobacteriaceae</i>	<i>Collinsella</i>	0,056	1,68	1,51±2,81	4,19±7,94	23	8
<i>Enterococcaceae</i>	<i>Enterococcus</i>	0,097	1,03	2,17±3,12	4,81±9,65	19	10

Supplementary Table 4: Bacterial genera identified by sPLS-DA as distinctive between the 'Healthy' and 'At risk' enterotypes on Day 21 of life, with a Variable Importance in Projection (VIP) score > 1 and a mean relative abundance > 1% of the total sequences.

Family	Genus	p-val	VIP_comp1	Relative Abundance (%)		Percent_zeros	
				Mean±SD		Healthy	At risk
<i>Enterococcaceae</i>	<i>Enterococcus</i>	0,011	2,27	0,75±1,21	2,53±5,08	50	22
<i>Succinivibrionaceae</i>	<i>Anaerobiospirillum</i>	0,036	2,26	4,10±6,12	5,93±7,14	31	14
<i>Helicobacteraceae</i>	<i>Helicobacter</i>	0,071	1,83	0,33±0,93	1,41±3,53	65	51
<i>Lachnospiraceae</i>	<i>Blautia</i>	0,134	1,56	3,41±6,29	0,91±2,39	38	37
<i>Peptostreptococcaceae</i>	<i>Clostridioides</i>	0,272	1,54	0,79±1,52	1,59±3,26	62	43
<i>Lachnospiraceae</i>	<i>Multi-affiliation4</i>	0,296	1,13	1,30±6,53	0,01±0,03	81	84
<i>Clostridiaceae</i>	<i>Clostridium</i>	0,168	1,06	2,64±5,02	2,29±2,85	27	10

Supplementary Table 5: Bacterial genera identified by sPLS-DA as distinctive between the 'Healthy' and 'At risk' enterotypes at the day before weaning, with a Variable Importance in Projection (VIP) score > 1 and a mean relative abundance > 1% of the total sequences.

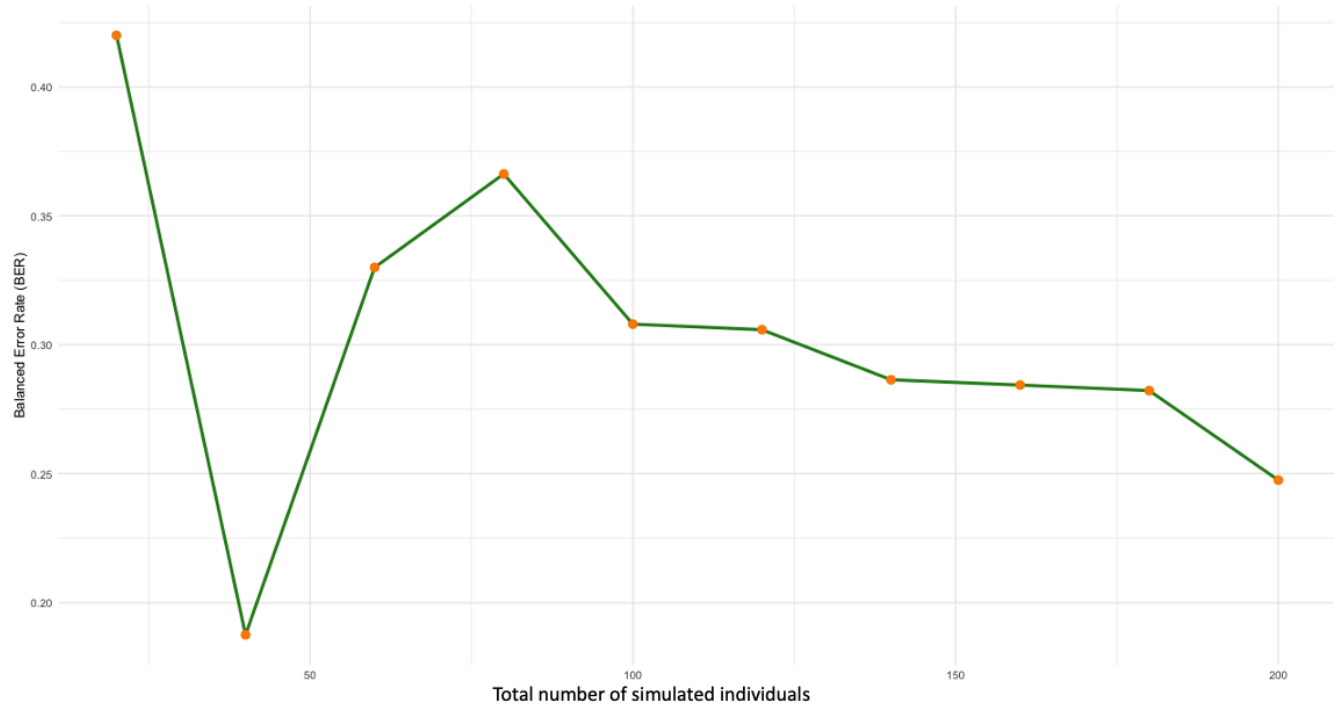
Family	Genus	p-val	VIP_comp1	Relative Abundance (%)		Percent_zeros	
				Mean±SD		Healthy	At risk
<i>Fusobacteriaceae</i>	<i>Fusobacterium</i>	0,001	2,33	7,99±6,91	3,90±5,42	0	4
<i>Erysipelotrichaceae</i>	<i>Turicibacter</i>	0,000	2,11	1,60±5,41	3,05±5,47	23	10
<i>Campylobacteraceae</i>	<i>Campylobacter</i>	0,008	1,88	1,22±2,39	0,26±0,77	19	47
<i>Bacteroidaceae</i>	<i>Bacteroides</i>	0,017	1,77	2,99±3,85	1,25±2,11	4	12
<i>Prevotellaceae</i>	<i>unknown genus1</i>	0,011	1,77	1,30±2,18	0,28±0,50	15	16
<i>Enterobacteriaceae</i>	<i>Escherichia-Shigella</i>	0,011	1,70	1,94±9,28	6,55±16,16	15	2
<i>Lachnospiraceae</i>	<i>Multi-affiliation1</i>	0,018	1,66	2,13±5,75	0,79±2,12	4	4
<i>Butyricicoccaceae</i>	<i>unknown genus2</i>	0,037	1,50	1,31±2,28	0,59±1,24	12	31
<i>Enterococcaceae</i>	<i>Enterococcus</i>	0,015	1,49	0,34±1,01	1,10±2,27	31	24
<i>Fusobacteriaceae</i>	<i>Cetobacterium</i>	0,047	1,29	0,03±0,15	1,02±4,04	92	78
<i>Clostridiaceae</i>	<i>Clostridium</i>	0,048	1,28	3,75±5,21	7,58±10,31	0	2

<i>Lachnospiraceae</i>	<i>Blautia</i>	0,071	1,27	5,91±6,05	4,34±5,80	0	4
<i>Lachnospiraceae</i>	<i>Multi-affiliation4</i>	0,065	1,17	0,78±1,55	1,28±2,51	19	14
<i>Helicobacteraceae</i>	<i>Helicobacter</i>	0,177	1,03	5,45±10,44	2,22±8,52	12	18

Supplementary data1

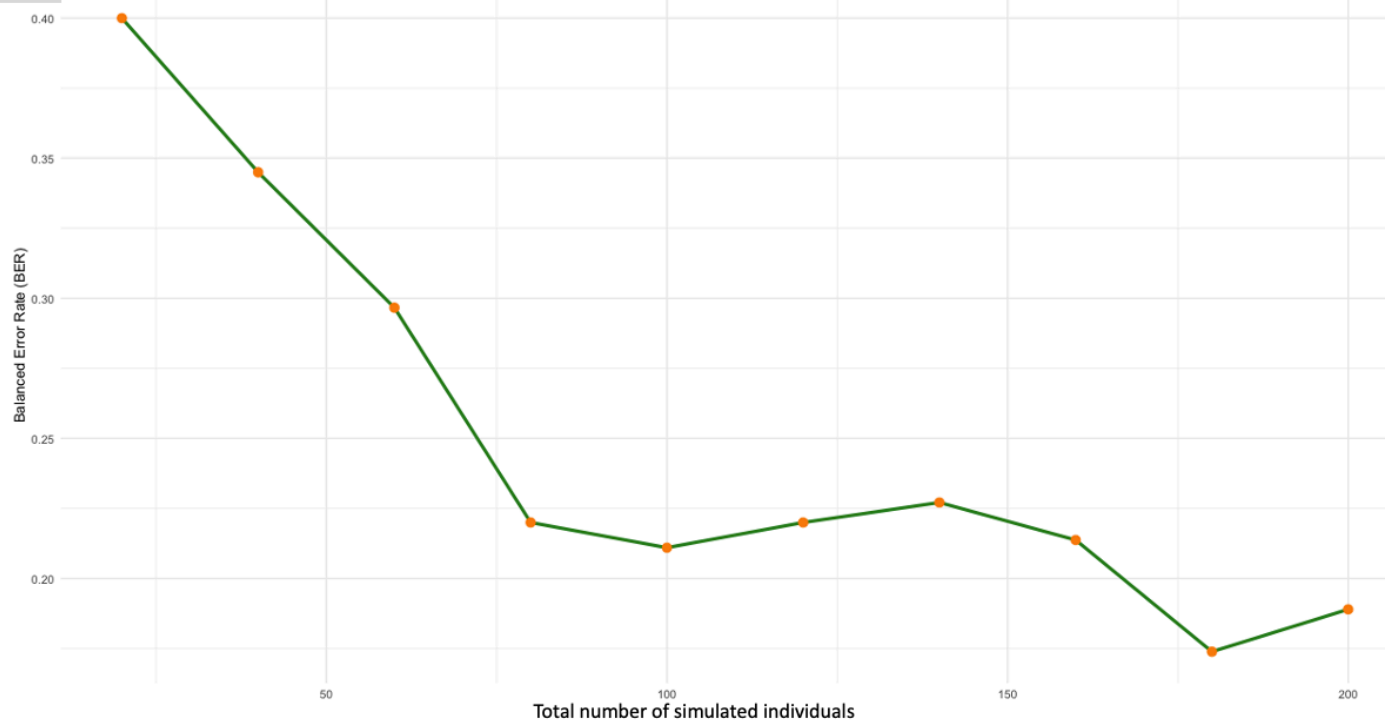
S1A

Effect of simulated sample size (bootstrap) on sPLS-DA classification performance (BER) at Day 1 based on fecal microbiota data



S1B

Effect of simulated sample size (bootstrap) on sPLS-DA classification performance (BER) at Day 7 based on fecal microbiota data



S1C Effect of simulated sample size (bootstrap) on sPLS-DA classification performance (BER) at Day 21 based on fecal microbiota data



S1D Effect of simulated sample size (bootstrap) on sPLS-DA classification performance (BER) before weaning based on fecal microbiota data

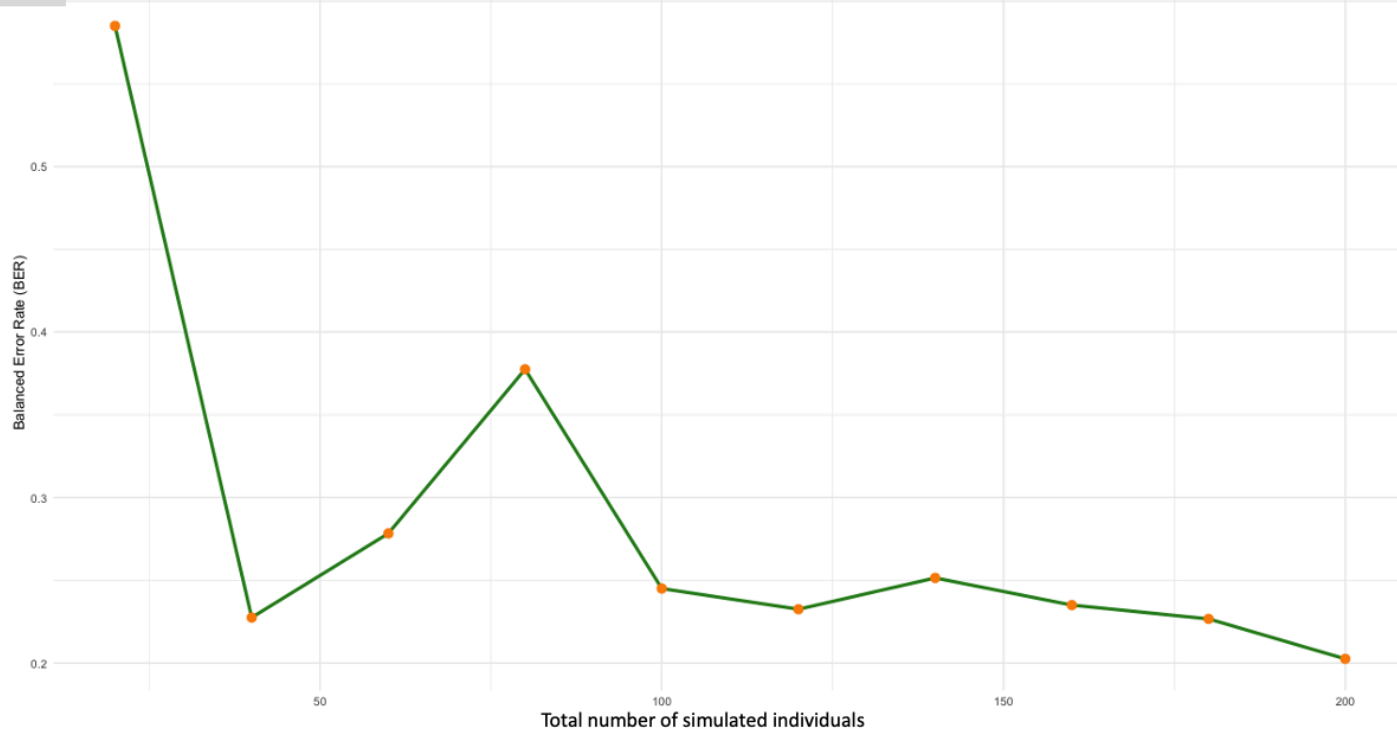


Figure S1 : Figure 6. Effect of simulated sample size on sPLS-DA balanced error rate (BER) based on fecal microbiota data. The plot shows the evolution of BER with increasing numbers of individuals used in bootstrap simulations at four time points: (A) Day 1, (B) Day 7, (C) Day 21, and (D) just before weaning. Each curve represents the mean BER obtained from multiple bootstrap iterations.

Chapitre 6

Modulation de la santé des chiots à travers la programmation nutritionnelle

6.1 Introduction

Dans le chapitre précédent, nous avons mis en évidence l'existence de deux entérotypes distincts chez le chiot au moment du sevrage, dont l'un, qualifié « à risque », était associé à une prévalence élevée de diarrhée. Nous avons également démontré que ce profil microbien pouvait être partiellement prédit dès la période pré-sevrage, notamment par la détection d'un marqueur bactérien précoce : le genre *Enterococcus*. Ces résultats suggèrent que le microbiote précoce joue un rôle clé dans la susceptibilité digestive et ouvrent la voie à des stratégies de prévention ciblées, centrées sur la modulation de ce microbiote, comme discuté dans le **Chapitre 2**.

Dans cette perspective, ce chapitre s'intéresse à la possibilité d'intervenir sur le microbiote intestinal du chiot de manière indirecte, en modulant celui de la mère au cours de la gestation et de la lactation. Plus précisément, nous avons évalué l'impact d'une supplémentation maternelle en probiotiques, ici la levure *Saccharomyces boulardii* I-1079, sur le microbiote précoce des chiots. L'objectif de cette étude est ainsi de répondre à la troisième hypothèse de ce travail : La supplémentation maternelle en probiotiques pendant la gestation et la lactation constitue un levier de modulation du microbiote intestinal du jeune chiot.

Les résultats obtenus confirment l'effet de cette approche : la supplémentation maternelle en *S. boulardii* a significativement diminué l'abondance des ASVs appartenant au genre *Enterococcus* chez les chiots à 21 et 56 jours de vie, ces derniers ayant été identifiés comme des marqueurs bactériens précoce associés à un risque accru de diarrhée au sevrage. Cette observation suggère un effet protecteur potentiel de la modulation maternelle, en réduisant précocement l'implantation d'un microbiote à risque chez les jeunes.

Dans un second temps, nous avons exploré les mécanismes sous-jacents à cette modulation, en nous intéressant au métabolome urinaire et du sérum des chiots. L'objectif était de déterminer si l'effet de la supplémentation pourrait être médié, au moins en partie, par des altérations du profil métabolique intestinal en lien avec le microbiote. Les résultats ont mis en évidence plusieurs modifications métaboliques associées à la supplémentation. Toutefois, en raison d'un effectif plus restreint dans la cohorte analysée pour le microbiote ($n = 54$) par rapport à celle du métabolome ($n = 81$), le lien entre ces deux dimensions – microbiote et métabolome – n'a pas pu être établi dans cette étude.

Ces résultats soulèvent une question essentielle : la modulation du microbiote précoce via la mère peut-elle induire un effet protecteur/préventif contre la diarrhée au sevrage chez le chiot ? Bien que cette hypothèse nécessite encore d'être validée par des études cliniques longitudinales, nos observations fournissent des arguments en faveur d'une approche préventive, ciblant la période périnatale comme fenêtre d'intervention stratégique.

Article 5 : Effect of maternal oral supplementation with *Saccharomyces boulardii* I-1079 on the microbiota and fecal score of puppies during the first 8 weeks of life

I. Bendahmane ^{1,3*}, Q. Garrigues ¹, A. Rodiles ², E. Apper ², A. Mugnier ², S. Chastant ^{1,4},
A. Meynadier ³ and H. Mila ¹

¹NeoCare, Université de Toulouse, ENVT, Toulouse, France

²Lallemand SAS, Blagnac, France

³GenPhySE, Université de Toulouse, INRAE, ENVT, F-31326, Castanet Tolosan, France

⁴Present address: Ecole Nationale Vétérinaire d'Alfort, BREED, F-94700 Maison Alfort, France

*Correspondence: ilyas.bendahmane@envt.fr

Abstract

Gut microbiota (GM) is known to play a critical role in the health of neonates. Maternal diet can influence the gut microbiota of their offspring, consequently driving developmental programming. Thus, one of the ways to promote healthy microbiota could be by adding live yeast to the diet of the mother. Therefore, this study aimed to explore the effect of maternal yeast supplementation on the GM of their puppies during the first two months of life. *Saccharomyces boulardii* I-1079 or placebo was administered orally from day 28 of gestation until day 56 postpartum to 10 bitches (5 per group). Feces of their 54 puppies were collected via rectal swabs on postnatal day 0 (D0), day 2 (D2), day 21 (D21) and 56 (D56) to extract and sequence the bacterial DNA via next generation sequencing method (16S rRNA). According to the sparse Partial Least-Squares Discriminant Analysis (sPLS-DA), the dam's supplementation in yeast led to a significant modification of the GM of their puppies at D0, D2, D21 and D56. Moreover, puppies from yeast supplemented mothers, presented higher fecal score (more hard feces) at D21 and D28 compared with placebo puppies. This study showed that maternal supplementation during gestation and lactation with yeast is a possible tool to promote a healthy gut microbiome in their puppies. Further studies are needed to evaluate the effect of maternal supplementation with *Saccharomyces boulardii* I-1079 on the risk of weaning diarrhea in puppies.

Keywords: Nutritional programming, Yeast, Microbiota, Puppies, Fecal score

Introduction

The gut microbiota, constituted mainly of bacteria, was demonstrated to be essential for the host functioning and homeostasis. Indeed, its role is multiple, from nutrient digestion and assimilation, like synthesis of the essential vitamins, to immune stimulation (1).

The main colonization process in dogs begins at birth, starting with exposure to the mother's vaginal and fecal microbiota during passage through the birth canal (2). Additional colonization occurs through contact with the mother's oral microbiota during nursing, as well as with her udder skin and milk microbiota during suckling (3). At birth, the gut microbiota is highly heterogeneous, with environmental aerobes (e.g., the *Moraxellaceae* family) and facultative anaerobes (e.g., *Aerococcaceae* and *Enterobacteriaceae* families, including *Escherichia coli*) being the initial colonizers. Within two days, bacterial diversity decreases as facultative and strict anaerobes (e.g., *Bacteroidaceae* and *Clostridiaceae* families) begin to dominate, becoming the prevalent bacteria in the gut by around three weeks of age. When solid food is introduced at approximately one month of age, the gut microbiota undergoes another shift, becoming dominated by members of the *Bacteroidetes* and *Fusobacteria* phyla (4).

While the rapid evolution of the gut microbiota during the first weeks of life reflects the necessary physiological adaptation to early development, any abnormal condition may disrupt this colonization process, potentially leading to dysbiosis (an imbalance in the microbial community that can negatively affect health) (5). One of the consequences of the gut dysbiosis in young animals is the weaning diarrhea. Indeed, weaning diarrhea is a frequent problem in breeding kennels, with approximately 25% of puppies presenting liquid feces between 6 and 8 weeks of age (6). In addition to the welfare concerns for the puppies, this condition also creates financial and emotional strain for breeders and owners.

Probiotics, defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (7) have been extensively studied over the past two decades as effective health modulators in several species, including humans, pigs, calves, and poultry. Among them, *Saccharomyces boulardii* (SB), a yeast originally isolated from lychee and mangosteen fruit, has demonstrated beneficial effects on gastrointestinal health notably during the weaning period.

Administration of SB to pre- or post-weaning piglets (8–10) and calves (11) has been shown to improve average daily weight gain and reduce the incidence of diarrhea and mortality. Similar benefits have been observed in piglets born to sows supplemented with *S. boulardii* during pregnancy and lactation (12–14). One of the mechanisms of action of *S. boulardii* in the host consists on modulation of the gut microbiota composition.

Although weaning diarrhea is a common issue in puppies, strategies to mitigate it remain limited. To date, the potential of maternal yeast supplementation to improve digestive health in dogs has not been investigated. Thus, the aim of this study was to evaluate the effects of *Saccharomyces boulardii*

administered to pregnant and lactating female dogs on the gut microbiota composition and fecal scores of their offspring during the first two months of life.

Materials and methods

1. Ethics, animals and study design

The present study received approval from the local Ethics Committee for Animal Experimentation (Animal Science and Health No. 115; reference number: SSA_2020-004, Toulouse, France).

1.1 Dams' enrollment and monitoring

For this study, 11 medium- to large-sized female dogs were selected from a cohort of 17 animals previously included in an earlier study (15), all housed within the same breeding kennel. The bitches were kept under identical housing conditions, each housed individually. Ovulation was identified based on serum progesterone concentrations between 4 and 10 ng/mL. Following confirmation of ovulation, two natural matings were scheduled on the 1st and 3rd day post-ovulation. Pregnancy was confirmed via abdominal ultrasonography performed between days 25 and 28 post-ovulation.

1.2 Maternal diet and supplementation

According to the nutritional recommendations for pregnant and lactating dogs, two distinct diets were provided to the included bitches:

Diet 1: Starting from the day of ovulation, all bitches received the same diet, manufactured by CRUSTY FOOD SAS (Montardit, Verteuil d'Agenais, France), in accordance with the nutritional recommendations of the NRC 2006 (16). The diet used was administered from ovulation until Day 27 of gestation. It provided a metabolizable energy content of 5.7 kcal per 100 g of raw matter. The nutritional composition included 30.8% crude protein, 15.1% ether extract (fat), and 2.7% crude fiber. Carbohydrates, calculated by difference on a dry matter basis, amounted to 37.0%.

Diet 2: At Day 28 of gestation, the pregnant bitches were assigned to one of two groups (Placebo or SB) based on breed size, age, parity, body condition score, and fecal score. All bitches were fed a second diet, also manufactured by CRUSTY FOOD SAS and compliant with NRC 2006 nutritional guidelines. This diet provided a metabolizable energy content of 6.3 kcal per 100 g of raw matter. Its composition included 28.4% crude protein, 16.8% ether extract (fat), and less than 2% crude fiber. Carbohydrates, calculated by difference on a dry matter basis, accounted for 37.7%.

In addition to the second diet, the bitches received a supplementation with *Saccharomyces cerevisiae* var. *boulardii* CNCM I-1079 (Levucell SB®, Lallemand SAS, Blagnac, France) since D28

until post-partum D56. The additive was administered in 400-mg plant-based capsules (hydroxypropylmethylcellulose (HPMC), size 1 clear; Suheung Co., South Korea) containing 6.25% of the active yeast (corresponding to 6.4×10^8 CFU per capsule), 92.75% potato starch, and 1% E551a (precipitated and dried silicic acid). Capsules were given twice daily, in the morning and evening, embedded in a small amount of wet pet food to ensure full ingestion. Each dog therefore received a daily dose of 1.3×10^9 CFU of the probiotic. The control group received identical capsules lacking the active yeast (placebo).

1.3 Puppies' enrollment and diet

From these dams, only 54 healthy puppies born via natural delivery and with rectal swabs available at all timepoints were enrolled. Within the first 8h after birth, each puppy was individually identified using color-coded collars and weighed, and its breed, sex and litter size were recorded. All puppies stayed with their dams and had continuous access to maternal milk until the end of the study. From birth to the 3rd week of life, the puppies were exclusively fed with maternal milk. Then, they were introduced to the same diet as their mothers (**Diet 2**), as a complement to maternal milk until the end of the study.

2. Microbiome sampling and analysis

In order to analyze fecal microbiota of puppies, rectal sample using a sterile swab (ETM, COPAN, Brescia, Italy) were taken at birth (D0), 2 days (D2), 21 day (D21) and 56 days after birth (D56) (**Figure1**). The swab was stored at -80°C until analysis.

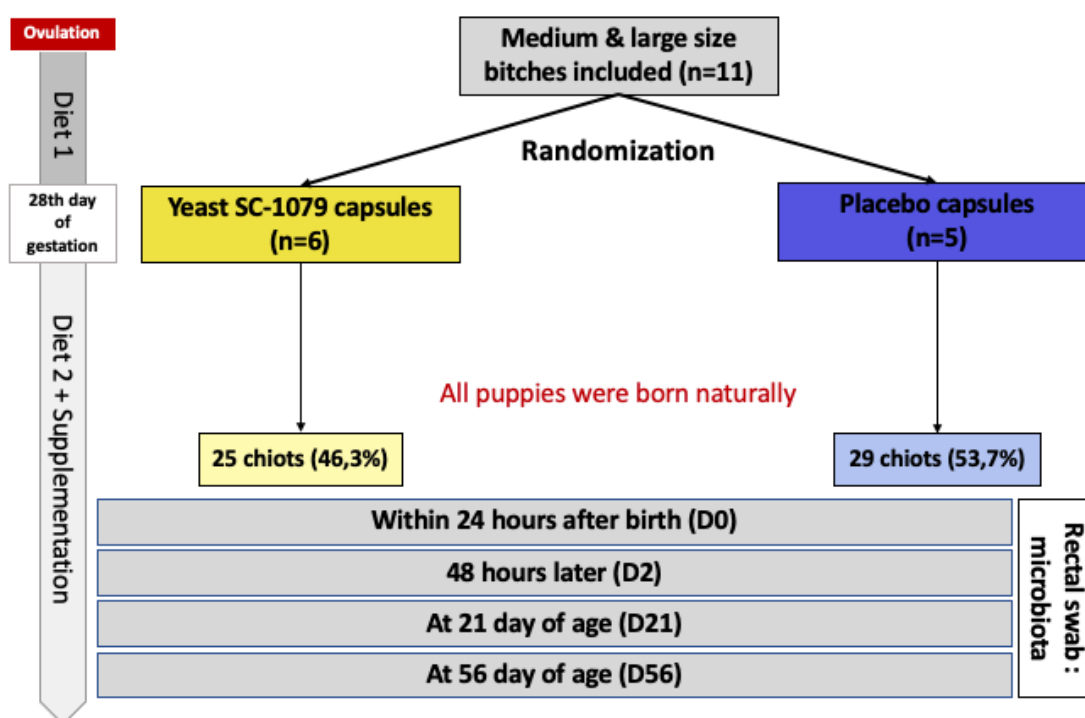


Figure 1: Experimental design of the study

2.1 DNA extraction and 16S rRNA gene sequencing

In order to extract genomic DNA from the swabs, the Quick-DNA Fecal/Soil Microbe KIT (ZymoResearch, Irvine, CA, USA) was used according to the manufacturer's protocol. Then the V3-V4 region of the 16s ribosomal RNA gene was amplified by PCR using universal primers 341F (CCTACGGGAGGCAGCAG) (17) and 806R (GGACTACNVGGGTWTCTAAT) (18). At the end of PCR, amplicons underwent purification using HighPrep PCR system from Magbio Genomics (Gaithersburg, MD, USA) and were then used in library construction employing the Illumina NEXTflex PCR-Free DNA sequencing kit from Bioo Scientific Corp. (Austin, TX, USA). These amplicon libraries were subsequently sequenced on the Illumina MiSeq 2500 platform (Illumina, San Diego, CA, USA) at the GeT-PlaGe INRAE Platform in Toulouse (19), France, generating paired-end fragments of 250 base pairs.

2.2 Analysis of sequence Data

Fastq files were processed, involving importation, demultiplexing, quality filtering, and dereplication, utilizing DADA2 in QIIME 2 (version 2020.2). This process allowed the identification of amplicon sequence variants (ASVs), with default parameters excluding primer length. Subsequently, ASVs were aligned and a phylogenetic tree was constructed using MAFFT and FastTree2, respectively. Taxonomic classification was performed for the resulting 16S rRNA marker genes, employing the sklearn classifier method described by Bokulich et al., against Greengenes (gg-13-8-99-nb-classifier) and SILVA (v138).

Raw data underwent rarefaction by removing ASVs found in fewer than five samples or representing less than 0.005% of the total sequences.

3. Fecal score evaluation

Fecal consistency in puppies was assessed once on freshly collected stools at 21, 28, 35, and 49 days of age, based on a fecal scoring scale ranging from 1 (Feces completely liquid) to 13 (Formed, dry and hard feces) specifically developed for puppies (20).

4. Statistical analysis

All statistical analysis was performed using RStudio version (2023.06.0).

4.1 Microbiota data

Bacterial richness.

Alpha diversity was assessed using the Shannon index, calculated with the vegan package (version 2.6-2) (21). The effects of treatment and age were evaluated by ANOVA, followed by Bonferroni-adjusted post hoc tests.

Abundance data.

Microbiome are compositional data (22). Firstly, the ASVs were filtered by removing those that were present in less than 15% of individuals. Subsequently, we applied the Geometric Bayesian-multiplicative method (GBM) to handle the zero values, assuming that the probability of detecting the ASV in the samples is not zero. The `cmultRepl` function from the `zCompos` package was employed to perform this method (23). Then, using the abundance table generated from the GBM output, the `compos` package was utilized to transform the data into the Centred Log Ratio (CLR). Subsequently, after obtaining the CLR output, a linear model was applied to identify the impact of each fixed effect on each ASV, using the following **Formula 1**:

Formula 1: ANOVA (`lm (formula=abundance ~ mother + breed + litter size)`)

P-values were obtained and used to calculate the percentage of Amplicon Sequence Variants (ASVs) affected by each confusion effect. The results revealed that the breed effect impacts approximately 50% of the ASVs, while the maternal effect and litter size affect approximately 12%. The breed effect was thus corrected and the residual data retrieved from table for subsequent multivariate analyses (**Formula 2**):

Formula 2: `resid(lmer(y~ (1| breed))`

Notably, the correction for the breed effect using the linear mixed-effect model (explained above) eliminated the influence of the maternal and litter size effects.

Multivariate analysis.

The residuals derived from the final model were employed for multivariate analyses. Utilizing the corrected data matrix obtained from **Formula2**, a sparse Partial Least Squares Discriminant Analysis (sPLS-DA) was performed using the "splsgda" function from the “**mixOmics**” package (24), with the parameters (ncomp and KeepX) recommended by "tuneo.plsgda" for achieving the most effective discrimination between the two groups (Placebo and SB). Subsequently, we employed Plotloadings and variable importance in projection (VIP) functions on the principal components, in order to extract the relevant variables that discriminate the two studied groups.

4.2 Fecal score data

The effects of maternal supplementation with SB, breed sex and litter size on fecal score were analyzed using an ANOVA model separately for each timepoint.

Results

Characteristics of the included puppies

A total of 54 puppies from 11 bitches were included in the study, with 29 puppies (54.7%) born from 5 mothers supplemented with a placebo and 25 puppies (46.3%) born from 6 mothers supplemented with SB during gestation and lactation. Puppies belonged to three different breeds in each group: 5 Golden Retriever, 12 Australian Shepherd and 12 Labrador Retriever in the placebo group; 11 Golden Retriever, 9 Australian Shepherd and 5 White Swiss Shepherd in the SB group. The two groups were comparable in terms of litter size (t.test $P=0.305$), sex ratio (Chi2 $P = 0.424$), number of medium and large breeds, and birth weight (t.test $P=0.423$) (**Table 1**).

Table 1. Characteristics of puppies whose mothers are treated with *Saccharomyces boulardii* or Placebo from mid-gestation until two months of lactation.

	Placebo group (n=29)	SB group (n=25)
Mothers (n)	5	6
Puppies (n)	29 (54,7%)	25 (46,3%)
Sex (M/F)	1.4 (17/12)	0.8 (11/14)
Litters size (n)	6±2	4±2
Breeds (n)	5 Golden Retriever 12 Australian Shepherd 12 Labrador Retriever	11 Golden Retriever 9 Australian Shepherd 5 White Swiss Shepherd
Birth weight (g)	377±84	390±61

Impact of the maternal supplementation with SB on the fecal microbiota of puppies

To assess the effect of maternal supplementation with SB on the early fecal microbial diversity in puppies, alpha diversity was calculated using the Shannon index at four time points: D0, D2, D21 and D56 (**Figure 2**). A significant interaction between age and treatment was observed ($P < 0.001$). Although the treatment effect alone was not statistically significant over time, alpha diversity changed significantly between time points ($P < 0.001$). At D0, the SB group exhibited a significantly lower Shannon index compared to the placebo group ($P < 0.001$). However, this difference disappeared at later time points (D2, D21, and D56).

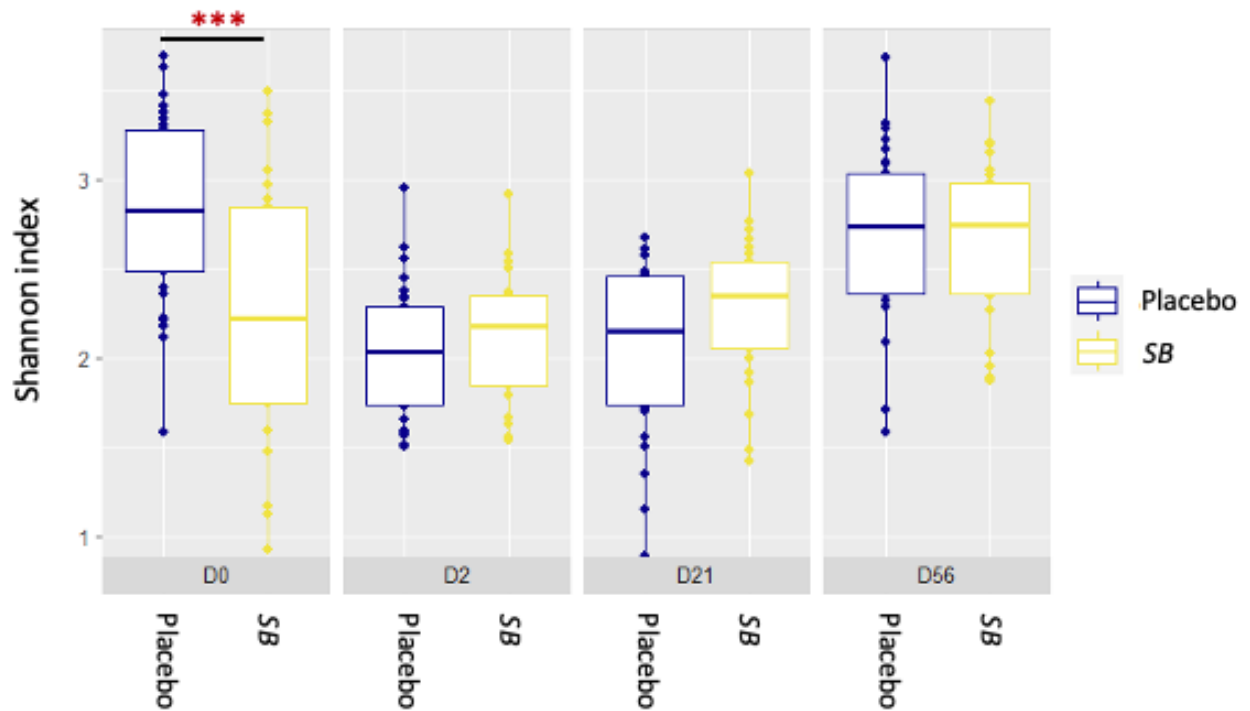


Figure 2. Shannon diversity index of puppies' fecal microbiota from birth (D0) to day 56 (D56) according to maternal supplementation. Statistical analysis was performed using two-way ANOVA with Bonferroni correction. Significance level: *** $P < 0.001$.

To further assess the effect of maternal supplementation on the fecal microbiota of puppies, sPLS-DA was performed at each timepoint (**Figure 3**).

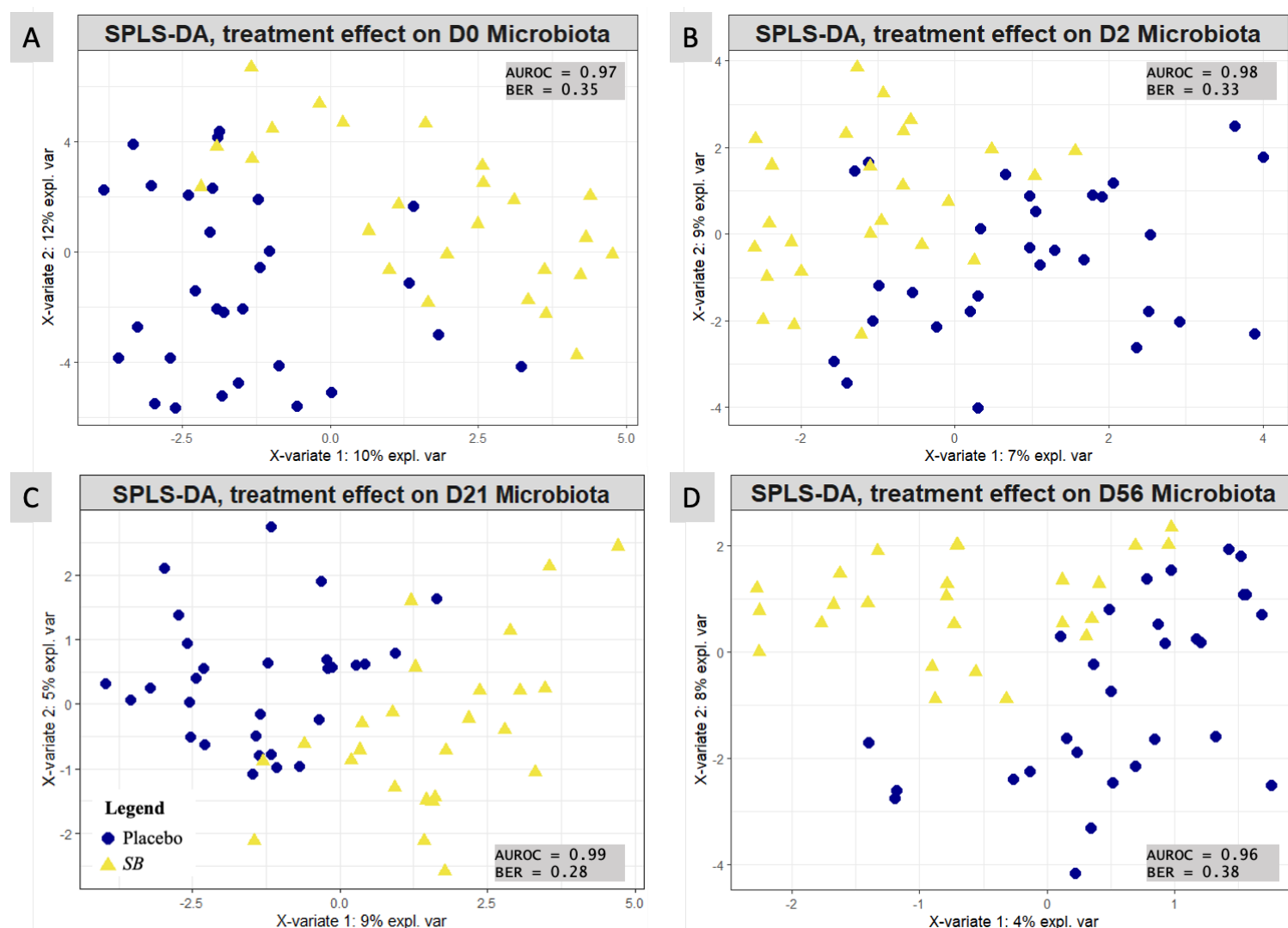


Figure 3. Sparse partial least-squares-discriminant analysis (sPLS-DA) of maternal supplementation effect on offspring fecal microbiota at birth (A), day 2 (B), day 21 (C) and day 56 (D). AUROC and BER values are shown in each sPLS-DA. Yellow triangles represent puppies from *Saccharomyces boulardii* supplemented mothers (n=25). Blue dots represent puppies from Placebo supplemented mothers (n=29). The sPLS-DA models were built with the following parameters: at birth (D0), one component (ncomp = 1) with 70 variables kept (keepX = 70); at day 2 (D2), one component with keepX = 60; at day 21 (D21), one component with keepX = 40; and at day 56 (D56), two components (ncomp = 2) with 20 and 5 variables kept for components 1 and 2, respectively.

At D0, the first two components of the sPLS-DA explained 22% of the variance, with an AUROC of 0.97 and a BER of 0.35 (**Figure 3A**). Puppies born to SB-supplemented mothers showed a higher relative abundance of *Staphylococcus* (unknown species 1: 2.00% vs. 0.90%; unknown species 2: 0.36% vs. 0.05%), *Providencia* (1.12% vs. 0.34%), and an unknown species of the *Enterobacteriaceae* family (6.98% vs. 4.61%) compared to the placebo group. In contrast, the relative abundance of an unknown species of *Dorea* (0.41% vs 1.06%), unknown genera/species of the *Pasteurellaceae* family (0.04% vs.

0.44%) and an unknown species of *Peptostreptococcus* (0.51% vs. 1.57%) was significantly reduced in the SB group (**Annexes Chapitre 6 - Supplementary Table 2**).

At D2, components 1 and 2 explained 7% and 9% of the variance, respectively, with an AUROC of 0.98 and a BER of 0.33 (**Figure 3B**). Only one taxon, *Streptococcus luteciae*, was significantly more abundant in the SB group compared to the placebo group (12.82% vs. 9.80%) (**Annexes Chapitre 6 - Supplementary Table 3**).

On D21, the first two sPLS-DA components explained 14% of the variance (AUROC: 0.99; BER: 0.28) (**Figure 3C**). Puppies from SB-supplemented dams had an increased abundance of *Peptostreptococcus* (0.34% vs. 0.02%), *Lactobacillus reuteri* (1.55% vs. 0.11%), *Lactobacillus* (unknown species: 3.41% vs. 0.58%), and *Streptococcus luteciae* (7.42% vs. 0.62%). Conversely, lower relative abundance was observed in the SB group for unknown species of *Klebsiella* (0.006% vs. 0.25%), *Helicobacter* (3.35% vs. 5.25%), and *Enterococcus* (0.05% vs. 2.16%), as well as for unknown genera/species of the *Anaeroplasmataceae* family (0.02% vs. 0.36%) (**Annexes Chapitre 6 - Supplementary Table 4**).

Finally, at D56, the first two sPLS-DA components explained 12% of the variance, with an AUROC of 0.96 and a BER of 0.38 (**Figure 3D**). Puppies from the SB group showed higher relative abundance of *Bacteroides coprophilus* (0.25% vs. 0.03%) and *Cetobacterium somerae* (0.68% vs. 0.39%). In contrast, *Corynebacterium* (unknown species: 0.009% vs. 0.92%) and *Enterococcus cecorum* (0.73% vs. 1.68%) were significantly decreased compared to the placebo group (**Annexes Chapitre 6 - Supplementary Table 5**).

Effect of the maternal supplementation with *Saccharomyces boulardii* on the fecal score of puppies

In order to evaluate the effect of maternal SB supplementation on digestive health in puppies, fecal score was assessed at different timepoints between D21 and D49. The mean fecal score of puppies between D21 and D56 was 5.5 ± 1.8 . In total, 72% (39/54) puppies presented fecal score <5 at least once during the study period. No effect of breed, sex or litter size was observed on the fecal score. Conversely, a significant difference was observed at D21, with improved fecal consistency in the SB group compared to the Placebo group ($P=0.014$) and tendency toward increased fecal score at D28 ($P=0.094$). No significant differences were detected at later timepoints (**Table 6**).

Table 6: Mean fecal scores ($\pm$ standard deviation) in puppies from mothers supplemented with either placebo or *Saccharomyces boulardii* on days 21, 28, 35, 42, and 49.

	Placebo (mean $\pm$ SD)	SB (mean $\pm$ SD)	Anova P-value

FS D21	5.2±2.0 (n=18)	7.0±1.7 (n=16)	0.014
FS D28	4.2±1.7 (n=21)	5.2±1.7 (n=16)	0.094
FS D35	5.5±2.1 (n=21)	5.6±1.1 (n=19)	0.737
FS D42	5.9±1.7 (n=26)	5.6±1.3 (n=23)	0.497
FS D49	5.6±2.0 (n=27)	5.0±1.8 (n=23)	0.201

FS = Fecal score; D = Days post birth

Discussion

Diarrhea is a major health concern in puppies, frequently occurring during the early stages of life. Indeed, our data seem to corroborate with this statement as 72% of puppies presented fecal score <5 - considered as diarrhea (6) - at least once during the study period.

In the present study we investigated whether maternal supplementation with the probiotic - yeast SB - during gestation and lactation could modulate the fecal score and microbiota at pre-weaning period in puppies. First, we observed a significant reduction in alpha diversity in the SB group compared to the placebo group but only at birth day. However, our results also showed a high inter-individual variability in microbiota diversity at this early time point. The implications of reduced alpha diversity at such an early age remain unclear in the current literature. While higher microbial diversity is often associated with a mature and resilient microbiome in adults (25), this may not apply to the neonatal period, which is characterized by dynamic colonization and microbial succession (26). In fact, some human studies have suggested that an increased alpha diversity during early life may not be always beneficial. For instance, a prospective cohort study reported that infants with higher gut microbial diversity at 3 months of age had an increased risk of obesity by 12 years of age (27). In line with this, Differding et al. (28) suggested that early increases in diversity might reflect a more random and unregulated microbial development, potentially predisposing individuals to chronic disease later in life. This hypothesis needs further investigation in longitudinal studies to understand the direct consequences of reduced alpha diversity on the evolution of the gut microbiota.

Our results on the microbiota composition show that at birth, maternal SB supplementation led to an increased abundance of two taxa from the *Staphylococcaceae* family and two from the *Enterobacteriaceae* family. These bacterial families are primarily composed of facultative

anaerobes, which play a potentially important role during the neonatal period. At birth, the neonatal intestine contains oxygen (26), and the colonization by facultative anaerobes contributes to consuming this oxygen, thereby creating a more suitable anaerobic environment for the establishment of strict anaerobes.

Inversely, at the same time point, SB supplementation resulted in a decreased abundance of one unknown species of *Dorea* (family *Lachnospiraceae*), one species of *Peptostreptococcus* (family *Peptostreptococcaceae*), and an unknown genus and species from the *Pasteurellaceae* family. These taxa have been previously associated with neonatal gut dysbiosis or pathogenic outcomes in various species. For example, *Dorea* has been implicated in neonatal diarrhea in calves (29). Furthermore, members of the *Pasteurellaceae* family have been identified as potential contributors to neonatal illness in dogs; notably, Tikhonov et al. reported increased relative abundance of *Pasteurellaceae* one day after birth in puppies affected by the fading puppy syndrome (30).

Moreover, on day 2, maternal supplementation with SB was associated with an increased relative abundance of *Streptococcus luteciae*. Interestingly, *S. luteciae* has recently been highlighted as a potentially beneficial commensal in dogs. In a study by Carapeto et al., the administration of lyophilized fecal capsules containing approximately 8% relative abundance of *S. luteciae* over a 30-day period led to improved fecal consistency in dogs (31).

At the end of neonatal period (21 day of age), we observed that maternal supplementation with SB led to a significant increase in the relative abundance of several taxa considered beneficial, including *Lactobacillus reuteri*, an unclassified *Lactobacillus* species, *S. luteciae*, and an unclassified species of *Peptostreptococcus*. Conversely, maternal SB supplementation was associated with a reduced abundance of unknown species from genera *Klebsiella*, *Helicobacter*, and *Enterococcus*.

Lactobacillus, *Streptococcus*, and *Enterococcus* are classified as lactic acid bacteria (LAB), known for their ability to ferment carbohydrates into lactic acid and for their potential pH modulation and antimicrobial activity (32) (33). These features make them promising probiotic candidates, contributing to intestinal homeostasis and protection against enteric pathogens.

However, the role of *Enterococcus* in intestinal health remains controversial. While some studies in puppies (34) highlight the beneficial effects of *Enterococcus* strains, others have reported its role as potential early markers of neonatal diarrhea in pigs (35) or puppies (**Article 4 – Chapitre 5**).

The pathogenic potential of *Klebsiella* and *Helicobacter* has been previously reported in puppies. *Klebsiella* have been isolated from internal organs of septicemic puppies (36), supporting their role as systemic pathogens. Moreover, *Helicobacter* species are known to thrive in acidic environments and

have been associated with gastrointestinal disorders. As discussed by Bendahmane et al. (**Article 4 – Chapitre 5**), one hypothesis is that *Enterococcus* may promote an acidic intestinal environment, which in turn selects for acid-tolerant opportunistic pathogens like *Helicobacter* (37).

Importantly, both *Enterococcus* and *Helicobacter* were found to be more abundant at 21 days of age in puppies identified as being at higher risk of post-weaning diarrhea (**Article 4 – Chapitre 5**). Interestingly, our current findings showed that maternal SB supplementation reduced their abundance and simultaneously improved fecal consistency.

Therefore, the observed decrease in *Enterococcus* and *Helicobacter* abundance following maternal SB supplementation may reflect a beneficial ecological shift that supports gut health. Further studies are needed to evaluate the effect of maternal SB supplementation on the risk of diarrhea during the weaning.

Interestingly, the maternal SB supplementation was also associated with a significant reduction in the relative abundance of *Enterococcus cecorum* at 56 days of age. Interestingly, a previous study (Bendahmane et al., **Article 4 – Chapitre 5**) reported that puppies presenting a higher relative abundance of *Enterococcus* just before weaning (between 56 and 71 days) were more likely to develop post-weaning diarrhea. These observations raise important questions regarding the role of *Enterococcus* in digestive vulnerability during the early life stages.

While *Enterococcus* are often considered beneficial and are included in some commercial probiotic formulations for dogs (38), our findings suggest that a higher abundance of *Enterococcus* at the genus level may be associated with digestive problems in young puppies. However, given the substantial variability in function and pathogenic potential across *Enterococcus* species, further studies focusing on species-level or even strain-level characterization are needed to better assess the safety and potential role of *Enterococcus*-based probiotics in neonatal and pre-weaning puppies.

Conclusions

Altogether, our findings highlight the potential positive effect of maternal SB supplementation on digestive health in young puppies, possibly through early modulation of the gut microbiota. However, the mechanisms by which SB influences gut health and microbial composition in offspring remain to be elucidated. Several hypotheses can be proposed to explain how maternal SB intake may influence the microbiota of offspring. First, SB may alter the composition of the maternal microbiota, which can be vertically transmitted to the puppies through direct contact, grooming, and environmental exposure. Second, SB supplementation might influence the composition of maternal milk, thereby shaping the microbial colonization in the puppies' gut. Future studies involving larger cohorts are warranted to

confirm these results and to explore the long-term impact of maternal probiotic supplementation on digestive vulnerability from weaning to adulthood.

References

1. Landman C, Quévrain E. Le microbiote intestinal : description, rôle et implication physiopathologique. *La Revue de Médecine Interne* 2016;37:418–23.
2. Barko PC, McMichael MA, Swanson KS, Williams DA. The Gastrointestinal Microbiome: A Review. *Veterinary Internal Medicine* 2018;32:9–25.
3. Ge Y, Zhu W, Chen L, Li D, Li Q, Jie H. The Maternal Milk Microbiome in Mammals of Different Types and Its Potential Role in the Neonatal Gut Microbiota Composition. *Animals* 2021;11:3349.
4. Garrigues Q, Apper E, Rodiles A, Rovere N, Chastant S, Mila H. Composition and evolution of the gut microbiota of growing puppies is impacted by their birth weight. *Sci Rep* 2023;13:14717.
5. Gresse R, Chaucheyras-Durand F, Fleury MA, Van De Wiele T, Forano E, Blanquet-Diot S. Gut Microbiota Dysbiosis in Postweaning Piglets: Understanding the Keys to Health. *Trends in Microbiology* 2017;25:851–73.
6. Grellet A, Chastant-Maillard S, Robin C, Feugier A, Boogaerts C, Boucraut-Baralon C, Grandjean D, Polack B. Risk factors of weaning diarrhea in puppies housed in breeding kennels. *Preventive Veterinary Medicine* 2014;117:260–5.
7. Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, Morelli L, Canani RB, Flint HJ, Salminen S, et al. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat Rev Gastroenterol Hepatol* 2014;11:506–14.
8. Zhu W, Mo X, Zeng W, Ren W, Liu M. Effects of *Saccharomyces boulardii* cell wall polysaccharide on growth performance, immunity, antioxidant ability and ileal mucosal morphology of Jinhua weaned piglets in Northern Xinjiang region. *Front Anim Sci* 2025;6:1554880.

9. Parada J, Magnoli A, Isgro MC, Poloni V, Fochesato A, Martínez MP, Carranza A, Cavaglieri L. In-feed nutritional additive probiotic *Saccharomyces boulardii* RC009 can substitute for prophylactic antibiotics and improve the production and health of weaning pigs. *Vet World* 2023;1035–42.
10. Zhang W, Bao C, Wang J, Zang J, Cao Y. Administration of *Saccharomyces boulardii* mafic-1701 improves feed conversion ratio, promotes antioxidant capacity, alleviates intestinal inflammation and modulates gut microbiota in weaned piglets. *J Animal Sci Biotechnol* 2020;11:112.
11. Lee J-S, Kacem N, Kim W-S, Peng DQ, Kim Y-J, Joung Y-G, Lee C, Lee H-G. Effect of *Saccharomyces boulardii* Supplementation on Performance and Physiological Traits of Holstein Calves under Heat Stress Conditions. *Animals* 2019;9:510.
12. Rocha VP, Araújo LRS, De Mendonça IB, Martins LP, De Alcântara Araújo GG, Watanabe PH, Andrade TS, Evangelista JNB. Effects of *Saccharomyces cerevisiae* var. *boulardii* CNCM I-1079 on performance, colostrum and milk composition, and litter performance of mixed-parity sows in a tropical humid climate. *Trop Anim Health Prod* 2022;54:41.
13. Sun H, Laguna FBD, Wang S, Liu F, Shi L, Jiang H, Hu X, Qin P, Tan J. Effect of *Saccharomyces cerevisiae* *boulardii* on sows' farrowing duration and reproductive performance, and weanling piglets' performance and IgG concentration. *J Anim Sci Technol* 2022;64:10–22.
14. Tan CQ, Wei HK, Sun HQ, Long G, Ao JT, Jiang SW, Peng J. Effects of supplementing sow diets during two gestations with konjac flour and *Saccharomyces boulardii* on constipation in periparturient period, lactation feed intake and piglet performance. *Animal Feed Science and Technology* 2015;210:254–62.
15. Bendahmane I, Garrigues Q, Apper E, Mugnier A, Svilar L, Martin J, Chastant S, Meynadier A, Mila H. Maternal oral supplementation with *Saccharomyces boulardii* I-1079 during gestation and early lactation impacts the early growth rate and metabolic profile of newborn puppies. *Front Nutr* 2025;12:1500600.

16. Nutrient Requirements of Dogs and Cats [Internet]. Washington, D.C.: National Academies Press; 2006 [cited 2024 Dec 9]. Available from: <http://www.nap.edu/catalog/10668>
17. Muyzer G, De Waal EC, Uitterlinden AG. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* 1993;59:695–700.
18. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, Fierer N, Knight R. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc Natl Acad Sci USA* 2011;108:4516–22.
19. GeT-PlaGe. GeT-PlaGe Genomics and Transcriptomics core facility of Toulouse [Internet]. INRAE; 2022 [cited 2025 May 18]. Available from: <https://get.genotoul.fr/la-plateforme/get-plage/>
20. Grellet A, Feugier A, Chastant-Maillard S, Carrez B, Boucraut-Baralon C, Casseleux G, Grandjean D. Validation of a fecal scoring scale in puppies during the weaning period. *Preventive Veterinary Medicine* 2012;106:315–23.
21. Dixon P. VEGAN, a package of R functions for community ecology. *J Vegetation Science* 2003;14:927–30.
22. Gloor GB, Macklaim JM, Pawlowsky-Glahn V, Egozcue JJ. Microbiome Datasets Are Compositional: And This Is Not Optional. *Front Microbiol* 2017;8:2224.
23. Palarea-Albaladejo J, Martín-Fernández JA. zCompositions — R package for multivariate imputation of left-censored data under a compositional approach. *Chemometrics and Intelligent Laboratory Systems* 2015;143:85–96.
24. Rohart F, Gautier B, Singh A, Lê Cao K-A. mixOmics: An R package for ‘omics feature selection and multiple data integration. Schneidman D, editor. *PLoS Comput Biol* 2017;13:e1005752.
25. Relman DA. The human microbiome: ecosystem resilience and health. *Nutrition Reviews* 2012;70:S2–9.

26. Garrigues Q, Apper E, Rodiles A, Rovere N, Chastant S, Mila H. Composition and evolution of the gut microbiota of growing puppies is impacted by their birth weight. *Sci Rep* 2023;13:14717.
27. Stanislawski MA, Dabelea D, Wagner BD, Iszatt N, Dahl C, Sontag MK, Knight R, Lozupone CA, Eggesbø M. Gut Microbiota in the First 2 Years of Life and the Association with Body Mass Index at Age 12 in a Norwegian Birth Cohort. Pettigrew MM, editor. *mBio* 2018;9:e01751-18.
28. Differding MK, Benjamin-Neelon SE, Hoyo C, Østbye T, Mueller NT. Timing of complementary feeding is associated with gut microbiota diversity and composition and short chain fatty acid concentrations over the first year of life. *BMC Microbiol* 2020;20:56.
29. Ku J-Y, Lee M-J, Jung Y, Choi H-J, Park J. Changes in the gut microbiome due to diarrhea in neonatal Korean indigenous calves. *Front Microbiol* 2025;16:1511430.
30. Tal S, Tikhonov E, Aroch I, Hefetz L, Turjeman S, Koren O, Kuzi S. Developmental intestinal microbiome alterations in canine fading puppy syndrome: a prospective observational study. *npj Biofilms Microbiomes* 2021;7:52.
31. Carapeto S, Cunha E, Serrano I, Pascoal P, Pereira M, Abreu R, Neto S, Antunes B, Dias R, Tavares L, et al. Effect of the Administration of a Lyophilised Faecal Capsules on the Intestinal Microbiome of Dogs: A Pilot Study. *Genes* 2023;14:1676.
32. Pancheniak E de FR, Maziero MT, Rodriguez-León JA, Parada JL, Spier MR, Soccol CR. Molecular characterisation and biomass and metabolite production of *Lactobacillus reuteri* LPB P01-001: A potential probiotic. *Braz J Microbiol* 2012;43:135–47.
33. Cui J, Xiao M, Liu M, Wang Z, Liu F, Guo L, Meng H, Zhang H, Yang J, Deng D, et al. Coupling metagenomics with cultivation to select host-specific probiotic micro-organisms for subtropical aquaculture. *J Appl Microbiol* 2017;123:1274–85.
34. Benyacoub J, Cavadini C, Sauthier T, Schiffrin EJ, Von Der Weid T, Czarnecki-Maulden GL, Anderson RE. Supplementation of Food with *Enterococcus faecium* (SF68) Stimulates Immune Functions in Young Dogs. *The Journal of Nutrition* 2003;133:1158–62.

35. Dou S, Gadonna-Widehem P, Rome V, Hamoudi D, Rhazi L, Lakhal L, Larcher T, Bahi-Jaber N, Pinon-Quintana A, Guyonvarch A, et al. Characterisation of Early-Life Fecal Microbiota in Susceptible and Healthy Pigs to Post-Weaning Diarrhoea. Loh G, editor. PLoS ONE 2017;12:e0169851.
36. Schäfer-Somi S, Spargser J, Breitenfellner J, Aurich JE. Bacteriological Status of Canine Milk and Septicaemia in Neonatal Puppies – a Retrospective Study. Journal of Veterinary Medicine, Series B 2003;50:343–6.
37. Husnik R, Klimes J, Kovarikova S, Kolorz M. Helicobacter Species and Their Association with Gastric Pathology in a Cohort of Dogs with Chronic Gastrointestinal Signs. Animals 2022;12:1254.
38. Schmitz S, Suchodolski J. Understanding the canine intestinal microbiota and its modification by pro-, pre- and synbiotics – what is the evidence? Veterinary Medicine & Sci 2016;2:71–94.

Article 6 : Maternal oral supplementation with *Saccharomyces boulardii* I-1079 during gestation and early lactation impacts the early growth rate and metabolic profile of newborn puppies

I. Bendahmane ^{1,3*}, Q. Garrigues ³, E. Apper ², A. Mugnier ², L. Svilar ⁴, JC. Martin ⁴, S.

Chastant ^{1,5}, A. Meynadier ³ and H. Mila ¹

¹NeoCare, Université de Toulouse, ENVT, Toulouse, France

²Lallemand SAS, Blagnac, France

³GenPhySE, Université de Toulouse, INRAE, ENVT, F-31326, Castanet Tolosan, France

⁴CriBioM, Aix Marseille Université, 13005 Marseille, France

⁵Present address: Ecole Nationale Vétérinaire d'Alfort, BREED, F-94700 Maisons-Alfort, France

*Correspondence: ilyas.bendahmane@envt.fr

Keywords: Yeast; Nutritional Programming; Early growth; Proline; Metabolome; Newborn; Dog



OPEN ACCESS

EDITED BY

Yalin Zhou,
Peking University, China

REVIEWED BY

Zhicheng Peng,
University of Pennsylvania, United States
Achraf Adib Lesaux,
Phileo Lesaffre Animal Care, France

*CORRESPONDENCE

Ilyas Bendahmane
✉ ilyas.bendahmane@envt.fr

†PRESENT ADDRESS

Sylvie Chastant,
Ecole Nationale Vétérinaire d'Alfort, INRAE,
BREED, Maisons-Alfort, France

RECEIVED 23 September 2024

ACCEPTED 11 February 2025

PUBLISHED 27 February 2025

CITATION

Bendahmane I, Garrigues Q, Apper E,
Mugnier A, Svilar L, Martin J-c, Chastant S,
Meynadier A and Mila H (2025) Maternal oral
supplementation with *Saccharomyces*
boulardii I-1079 during gestation and early
lactation impacts the early growth rate and
metabolic profile of newborn puppies.
Front. Nutr. 12:1500600.
doi: 10.3389/fnut.2025.1500600

COPYRIGHT

© 2025 Bendahmane, Garrigues, Apper,
Mugnier, Svilar, Martin, Chastant, Meynadier
and Mila. This is an open-access article
distributed under the terms of the [Creative
Commons Attribution License \(CC BY\)](#). The
use, distribution or reproduction in other
forums is permitted, provided the original
author(s) and the copyright owner(s) are
credited and that the original publication in
this journal is cited, in accordance with
accepted academic practice. No use,
distribution or reproduction is permitted
which does not comply with these terms.

Maternal oral supplementation with *Saccharomyces boulardii* I-1079 during gestation and early lactation impacts the early growth rate and metabolic profile of newborn puppies

Ilyas Bendahmane^{1,2*}, Quentin Garrigues¹, Emmanuelle Apper³,
Amélie Mugnier³, Ljubica Svilar⁴, Jean-charles Martin⁴,
Sylvie Chastant^{1†}, Annabelle Meynadier² and Hanna Mila¹

¹NeoCare, Université de Toulouse, ENVT, Toulouse, France, ²GenPhySE, Université de Toulouse, Institut national de recherche pour l'agriculture, l'alimentation et l'environnement (INRAE), École nationale vétérinaire de Toulouse (ENVT), Castanet Tolosan, France, ³Lallemand SAS, Blagnac, France, ⁴CriBioM, Aix Marseille Université, Marseille, France

Nutritional programming is a manipulation of fetal and neonatal development through maternal feeding. In humans and pigs, maternal yeast supplementation was demonstrated as a promising approach to positively modulate newborns' health. This study aimed to investigate the effects of *Saccharomyces cerevisiae* var. *boulardii* CNCM I-1079 (SB) supplementation in pregnant and lactating bitches on the newborns' early growth rate (EGR, between birth and 2 days of life), metabolic profiles, and the association between both of them. A total of 17 female dogs and their 81 puppies were included. From day 28 of gestation until the end of the study, bitches were divided into two groups, one of which received orally 1.3×10^9 colony forming units of live yeast per day. Puppies from mothers receiving the live yeast were defined as the SB group ($n = 40$) and the others were defined as the placebo group ($n = 41$). For each puppy, EGR was calculated, and blood and urine samples were collected at D2 for metabolome analysis using liquid chromatography-mass spectrometry (LCMS). Puppies from the SB group presented higher EGR compared with the placebo group (12% vs. 7%; $p = 0.049$). According to the Sparse Partial Least Squares Discriminant Analysis (sPLS-DA), both urine and serum metabolome profiles were significantly different between the two groups with a total of 29 discriminating metabolites in urine and serum. Fourteen of them were implicated in the nitrogen metabolism pathway including, gamma-aminobutyrate, 3-methyl-L-histidine and xanthosine (less abundant in SB compared with placebo group, all $p < 0.05$), adenine, aspartate and proline (more abundant in SB compared with placebo group, all $p < 0.05$). Metabolic pathways pointed to proline synthesis, a crucial component in collagen synthesis and osteoarticular system development. Urinary proline abundance was positively correlated with EGR ($r = 0.45$; $p < 0.001$). These findings highlight the potential benefits of maternal supplementation with SB promoting early neonatal growth, essential for the neonatal survival, through nitrogen metabolism orientation.

KEYWORDS

nutritional programming, early growth, proline, metabolome, newborn dog, dog, yeast

1 Introduction

The maternal-fetal relationship plays a pivotal role in shaping the development and health of offspring. According to the fetal programming theory initially proposed by Barker (1), factors and experiences during pregnancy may induce lasting modifications in the fetal anatomy, physiology, and metabolism, potentially contributing to the development of diseases in childhood and even in adulthood.

In the early stages of life, dogs encounter a spectrum of health challenges. One of the major concerns that puppies face during the 1st months of life is growth problems. For instance, the early growth rate (EGR) is strongly associated with neonatal mortality (2). Indeed, the first 2 days of life are crucial for the newborn dog, as they reflect the adaptation to the extrauterine life but also the colostrum ingestion.

As all of the mentioned phenomena are strongly related to the dam, one could hypothesize that acting on the maternal organism could improve the newborn's health. Indeed, studies in humans and pigs demonstrated the possibility of modulating offspring growth through maternal nutrition during gestation and lactation (3, 4). One of the strategies of maternal programming via nutrition aiming to improve neonatal outcomes is maternal probiotic supplementation during gestation.

Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (5–8). Among probiotics, *Saccharomyces cerevisiae* var. *bouardii* (SB) is a yeast extracted from lychee and mangosteen fruit that has been shown to have beneficial effects on digestive health, general immunity in humans (9) and on mental health in rats (10). In addition to effects observed on the host, its potential role as a tool for maternal nutritional programming on the offspring's health has been demonstrated recently. In pigs, supplementation of sows with SB during gestation and lactation modifies the intestinal microbiota of piglets, specifically increasing *Lactobacillus* species abundances, known to be beneficial for the host's health (11). The positive influence of SB supplementation in pregnant sows has been highlighted on the immunological status and response to stress of the newborn at birth, via a decreased blood cortisol concentration and increased white blood cell counts (12). To date, the effect of the maternal nutritional programming by yeast has never been investigated in the canine species.

The objectives of this study were thus (1) to evaluate the effect of live yeast SB supplementation of the pregnant and lactating bitches on EGR, urine and serum metabolome of their puppies at 2 days of life and (2) to study the relationship between these two elements.

2 Materials and methods

2.1 Ethics statement

This study was reviewed and approved by the local ethical committee (Ethics Committee for Animal Experimentation, Animal Science and Health No. 115; reference number: SSA_2020-004, Toulouse, France).

TABLE 1 Characteristics of the 17 dams included in the study at the 28th days of gestation.

	Placebo (<i>n</i> = 7)	SB (<i>n</i> = 10)
	<i>n</i>	<i>n</i>
Breed		
Medium	3	4
Large	4	6
	Mean ± SD	Mean ± SD
Body weight (kg)	26.3 ± 4.5	25.4 ± 5.0
Age (years)	3.2 ± 1.2	3.3 ± 1.4
Parity	2 ± 2	3 ± 2
Body condition score*	4 ± 1	4 ± 1
Fecal score [#]	3 ± 1	3 ± 1

SB, Females supplemented with *Saccharomyces bouardii*. *Body condition score evaluated on 9-point scale according to Laflamme (41). [#]Fecal score was evaluated on 5-point scale (<https://my.royalcanin.com/UserFiles/Docs/Digital-Toolkit/gi-fecal-scoring-chart-dog.pdf>) on the basis of 3 consecutive days.

2.2 Animals, diet, and experimental design

2.2.1 Dams

Of the thirty-six bitches included in the previous study by Guarigues et al. (13), 17 were selected for our study, to balance the cohort, ensure homogeneous groups, and healthy puppies (Table 1). These female dogs of medium-sized breed (Australian Shepherd, body weight between 15 kg and 25 kg) and large-sized breeds (Golden Retriever, Labrador Retriever, White Swiss Shepherd; body weight between 25 kg and 45 kg) were included within one breeding kennel and housed individually in the same conditions. No dam presented any health trouble at the time of inclusion.

Ovulation was identified using a progesterone assay, with progesterone levels ranging between 4 ng/mL and 10 ng/mL. Then two natural matings were conducted on days 1 and 3 post-ovulation. Pregnancy diagnosis was carried out between days 25 and 30 post-ovulation using abdominal ultrasonography.

At the 28th day of gestation (G28), bitches were randomized according to their breed size, breed, age, parity, body condition score and fecal score and subsequently, they were allocated into two groups: placebo (*n* = 7) or supplemented group (*n* = 10; SB group) (Details in Table 1).

2.2.2 Diet and probiotic supplementation in dams

Two extruded foods manufactured by CRUSTY FOOD SAS (Montardit, Vertheuil d'Agenais, France) and formulated to meet NRC recommendations (14) were provided during the study: The first diet (Diet 1; Table 2) was given from the date of ovulation until the G28 and the 2nd diet from G28 until the end of the study (56 days *post-partum*; Diet 2). Vitamin A, vitamin D3 and vitamin E were added as nutritional feed additives (Table 2) (15). All dogs had *ad libitum* access to water for drinking during the whole experiment.

The additive tested was *Saccharomyces cerevisiae* var. *bouardii* CNCM I-1079 (Levucell SB[®], Lallemand SAS, Blagnac, France) given in 400-mg vegetal capsules (hydroxypropylmethylcellulose (HPMC) caps size 1 clear, Suheung Co, South Korea) containing 6.25% additive (YEAST, measured at 6.4×10^8 CFU of additive/capsule), 92.75% potato starch and 1% E551a (silicic acid, precipitated and dried). Capsules were fed twice per day, in the morning and the evening, in a bullet of wet pet food to ensure full consumption. Each dog received 1.3×10^9 CFU of additive/day. The control group was fed the same capsules but without the additive (Placebo).

TABLE 2 Nutrient composition of diets used in the study.

		Feed	
		Diet 1	Diet 2
Nutrient content (on raw mater basis)	Moisture, %	5.7	6.3
	Crude ash, %	8.7	9.3
	Crude protein, %	30.8	28.4
	Ether fat, %	15.1	16.8
	Crude fiber, %	2.7	<2
	Carbohydrates ^a , %	37.0	37.7
Feed additives	Vitamin E, mg/kg	124	114
	Vitamin A, IU/kg	7,670	7,330
	Vitamin D, IU/kg	1,080	1,000
Metabolizable energy ^b , kcal/100 g		385.8	408.6

^aCarbohydrate was calculated from equation: %Dry Matter – (%Ether extract + %Crude Protein + %Crude Ash + %Crude fiber); ^bMetabolizable energy was calculated from equation (14, 15). Diet 1: from ovulation until 27 day of gestation. Diet 2: from the 28 day of gestation until the end of the study.

2.2.3 Puppies

Among a total of 113 puppies born, 105 were born alive and only 81 puppies having all samples needed (urine and serum) were selected for this study. At birth, puppies were identified using a different colored collar, and their weight and sex were recorded. All included puppies were born naturally and remained healthy at the time of sampling (2 days *post-partum*). Puppies remained with their dams during the study period with unlimited access to maternal milk.

Included puppies underwent weight measurements at the minimum 0 and maximum 12 h following birth (D0). About 24 h later (D1) all puppies were examined and their rectal temperature and glycemia were measured to evaluate their health status. Puppies were weighed again 24 h later (D2), and at the same time urine and serum samples were taken as follows (Figure 1): about 1 mL of urine was obtained by gentle swabbing of perineal area (to stimulate spontaneous miction) and blood was collected from the jugular vein (1 mL), both on Eppendorf tubes. The blood was centrifugated (16,000 RCF for 10 min) to isolate the serum, and aliquot was stored at –20°C for 2 weeks and then transferred to –80°C until analysis.

2.3 Serum and urine metabolome analyses

Urine and serum metabolome analyses and data pre-processing were conducted at the BIOMET platform attached to the cardiovascular and nutrition research center (C2VN) located in Marseille, France.

2.4 Samples preparation

2.4.1 Urine

The tubes were thawed on ice, then vortexed for 1 min. The samples were then centrifuged at 4°C for 15 min at 16,000 RCF.

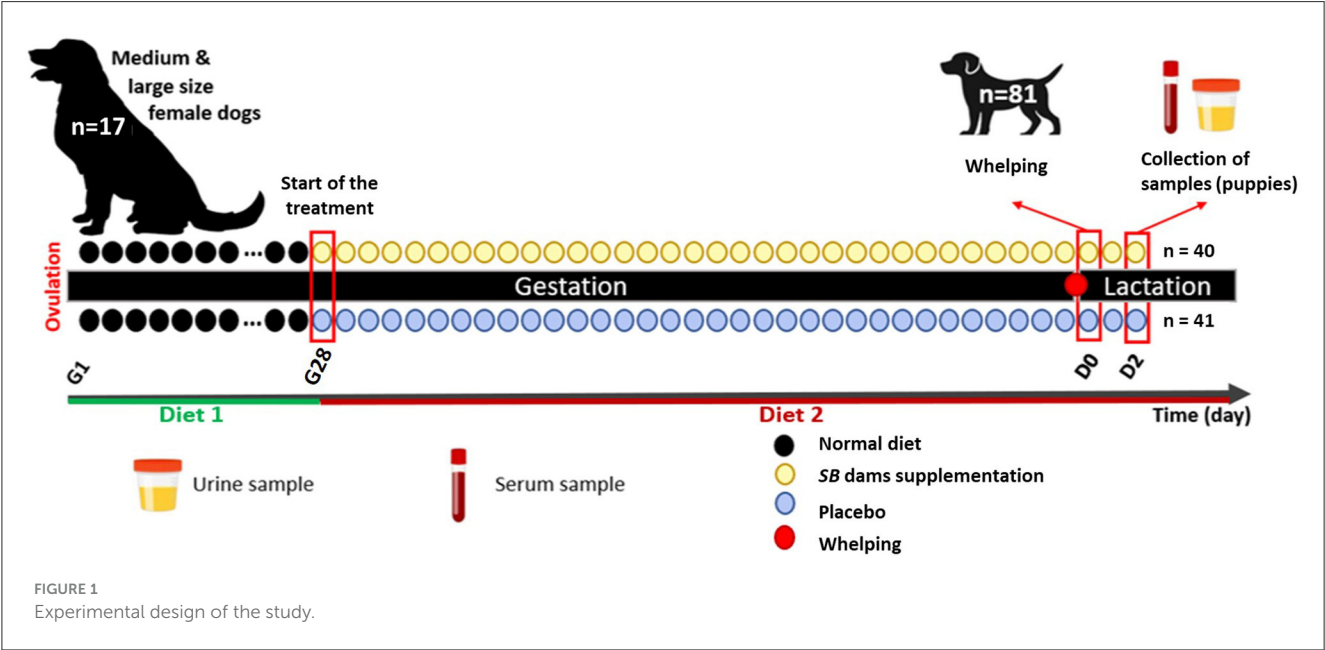


TABLE 3 Characteristics of puppies selected and included for metabolome analysis.

		Placebo group	SB group
Puppies (n)		41 (51%)	40 (49%)
Sex	Males (n)	27 (33%)	22 (27%)
	Females (n)	14 (18%)	18 (22%)
Litters (n)		7	10
Puppies fed by each lactating bitch (mean $\pm$ SD)		7 $\pm$ 2	8 $\pm$ 2
Puppies included per litter (mean $\pm$ SD)		6 $\pm$ 2	4 $\pm$ 2
Breeds (n)		4	4
Birth weight (mean $\pm$ SD)		395 $\pm$ 75 g	379 $\pm$ 68 g

Placebo group includes the following breeds: Golden Retriever (30% of puppies), Australian shepherd (46% of puppies), Labrador (10% of puppies), and Bernese Mountain dog (14% of puppies). The *Saccharomyces boulardii* group includes the following breeds: Golden Retriever (52% of puppies), Australian shepherd (35% of puppies), Labrador (5% of puppies), and Swiss White Shepherd (7% of puppies).

Following this, 50 μ L of urine was drawn into 2 mL Eppendorf tubes and 400 μ L of deionized water was added. After vortexing the mixture for 30 seconds, another round of centrifugation was performed. A total of 350 μ L of the supernatant was collected in a 0.45 μ m filter, then recentrifuged (4°C, 15 min 16,000 RCF). Then, 50 μ L of the supernatant was collected in a glass vial with the insert then stored at -80°C before analysis by liquid chromatography–mass spectrometry (LC-MS).

2.4.2 Serum

The tubes were thawed on ice then vortexed for 1 min. Then 50 μ L of the sample was taken and 200 μ L of cold methanol (MeOH) (-20°C) was added. The mixture was slowly agitated for 1 min then incubated in the freezer (-20°C) for 1 h, vortex mixed for 30 seconds then centrifuged (4°C, 15 min, 11,000 RPM). A total of 200 μ L of the supernatant was collected in a 10 kDa filter then recentrifuged (4°C, 45 min, 16,000 RCF). Following this, the tubes were evaporated under a stream of nitrogen then the dry extract was taken up in 120 μ L Water/acetonitrile (90/10, v/v +0.1% formic acid) then vortexed for 1 min. The mixture was then transferred to a 0.45 μ m filter tube and centrifuged for 15 min (4°C, 16,000 RCF). Then, 50 μ L of the supernatant was collected in a glass vial with the insert then stored at -80°C before analysis by LC-MS.

For both sample types (urine and serum), two controls for each were carried out: the pool (quality control samples) as a mixture of 20 μ L of each serum sample, urine sample and a blank samples, prepared the same way as the samples by taking 50 μ L of deionized water.

2.5 LC-MS analysis

The LC-MS analysis and data treatment were performed as described by Garrigues et al. (13). Briefly, the metabolic profile

of the samples was acquired using a high-performance liquid chromatography (HPLC) system, a Thermo Fisher Scientific Ultimate 3,000 Series, coupled with a Q-Extractive Plus, a high-resolution mass spectrometer (Thermo Fisher Scientific). Chromatographic separation was achieved using hydrophilic and reverse phase chromatography in order to largely cover the metabolome identification. A mass spectrometry data were achieved in positive and negative ionization modes with a resolving power set to 35,000 full width at half maximum (FWHM). A tandem mass spectrometry spectra (MS/MS) were acquired using a Data Dependent Analysis experiment, at 17,500 FWHM resolving power.

Samples were analyzed randomly in an analytical batch and in the LC-MS system applying the quality control procedure. MS/MS spectra were acquired on a pooled sample for the metabolite annotation and identification.

2.6 Data pre-processing

Full scan spectra were converted to a mzXML format using Proteo Wizard and pre-processed using XCMS library script under R language. The following parameters were applied: peak picking: CentWave, peak grouping: density, retention time correction: obiwrap. Analytical batch was corrected using Van-der-Kloet algorithm and further filtering was applied on the extracted signals, such as removal of signals with coefficient of variation higher than 30% in quality control samples, and removal of noise signal.

Signal annotation was achieved using an “in-house” database with information about m/z and retention times in reverse and hydrophilic phase of nearly 800 metabolites. A match between the experimental and database information was done on the Galaxy Workflow 4 metabolomics platform using following conditions: m/z accuracy limit was set to 5 ppm, and retention time drift up to 0.7 min. After an automatic annotation, each annotated feature was visually verified and the annotation confirmed by fragmentation spectra comparison.

From this annotation, a table is obtained containing 205 identified/annotated metabolites with their intensities detected in the urine samples and 228 metabolites for serum. Subsequently, probabilistic quotient normalization (PQN) was carried out on each of the matrices.

2.7 Statistical analysis

All statistical analysis was performed using R software, version 4.2.1. (16).

2.7.1 EGR

The early growth rate was calculated for each individual as follows: $(\text{weight D2} - \text{weight D0}) \times 100 / \text{weight D0}$. An analysis of variance (ANOVA) was then performed to evaluate

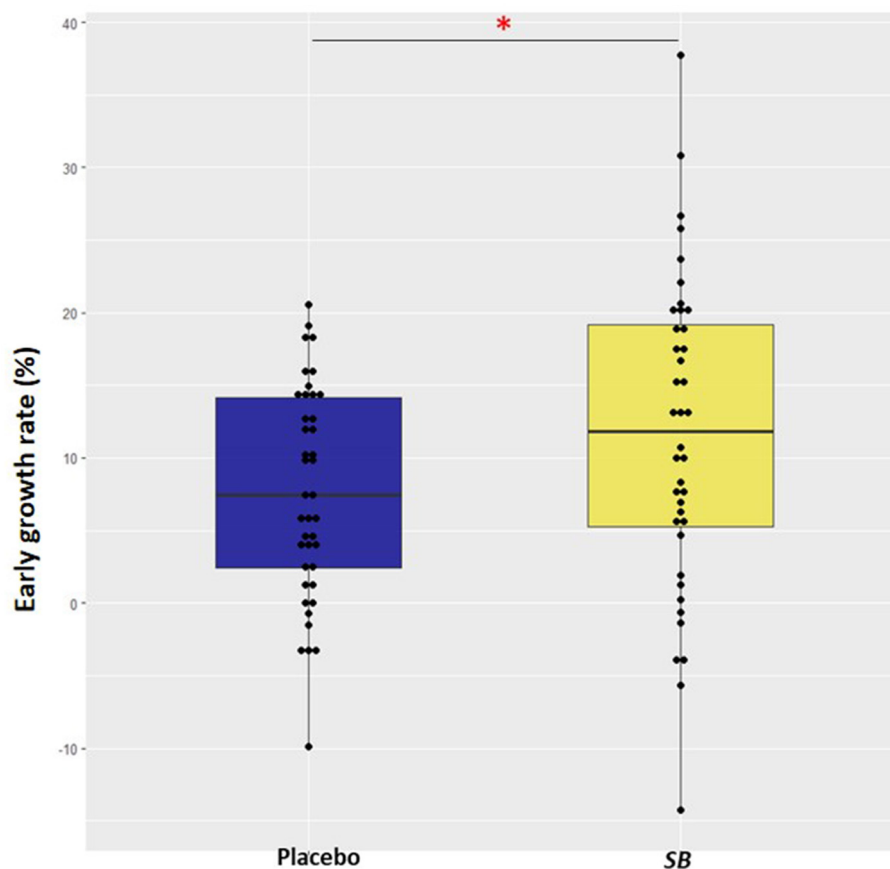


FIGURE 2

Box and whisker plot of early growth rate in puppies from mothers supplemented with *Saccharomyces boulardii* ($n = 40$; in yellow) during gestation and lactation and puppies from mothers supplemented with placebo ($n = 41$; in blue). Early growth rate (%) = (weight at 2 days - weight at birth)/weight at birth * 100. An ANOVA was carried out. One asterisk (*) indicates p value < 0.05.

the effect of treatment, breed and litter size on growth rate, using the model:

$$\text{EGR} = \mu + \text{treatment} + \text{breed} + \text{litter size} + \text{sex} \varepsilon$$

2.7.2 Metabolome

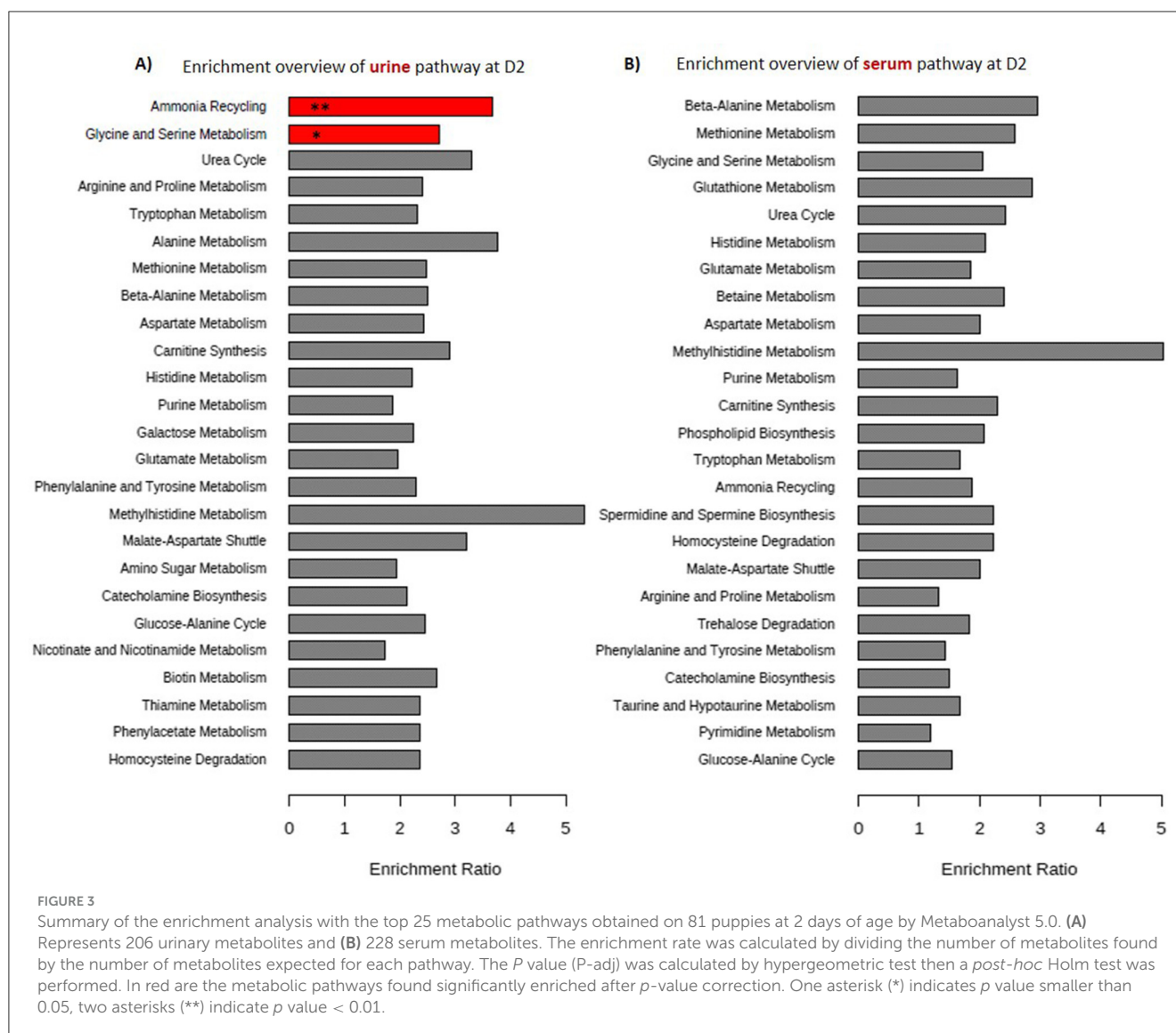
As metabolomes are compositional data (17), we firstly applied the Geometric Bayesian-multiplicative method (GBM) to handle the zero values, assuming that the probability of detecting the metabolite in the samples is not zero. The `cmultRepl` function from the `zComposition` package (18) was employed to perform this method. Then, using the abundance table generated from the GBM output, the composition package was utilized to transform the data into the Centered Log Ratio (CLR). Subsequently, after obtaining the CLR output, the effects of sex, mother, breed, and litter size were visually assessed using PLS-DA. No significant differences were observed for sex. A linear model was applied to identify the impact of the remaining fixed effect on each metabolite, using the following formula:

$$\text{Formula 1: } y_1 = \mu_1 + \text{mother} + \text{breed} + \text{litter size} + \varepsilon_1$$

P -values were obtained and used to calculate the percentage of metabolites affected by each confusion effect. The results revealed that the breed effect impacts 50% of the metabolites, while the maternal effect and litter size affect approximately 20%. Notably, the correction for the breed effect eliminates the influence of the maternal and litter size effects. Consequently, we opted to correct for the breed effect and proceeded to retrieve the residual data table for subsequent multivariate analyses:

$$\text{Formula 2: } y_2 \sim \mu_2 + (1 | \text{breed}) + \varepsilon_2$$

The residuals derived from the final model were employed for the following analyses. Utilizing the corrected data matrix obtained from Formula 2, we employed the `tune.splsda` function to determine the optimal number of principal components (`ncomp`) and the number of variables (`KeepX`) per component for achieving the most effective discrimination between the two groups (Placebo and SB). Subsequently, a sparse Partial Least Squares Discriminant Analysis (PLS-DA) was performed using the `splsda` function from the `MixOmics` package (19), with the parameters (`ncomp` and `KeepX`) recommended by `tune.splsda`. Evaluation of error rate was conducted through the utilization of balanced error rate (BER) analysis and area under the receiver operating characteristic



curve (AUROC) was performed to assess the classification performance. Subsequently, PlotLoadings and variable importance in projection (vip) functions were employed on the principal components selected (as determined by “tune.splsda” function), in order to extract the relevant variables that discriminate our groups.

Metabolite set enrichment analysis was performed using Metaboanalyst 5.0 (20) website in order to identify significant urine and serum metabolic pathways. Correlations between the variables of different matrices were calculated based on multiblock sPLS-DA using the “network” function of the MixOmics package. A cut-off score of 0.50 was set in order to only represent variables whose scores are higher than the latter.

2.7.3 Link between metabolome and EGR

Pearson’s correlation with a threshold of 0.4 was used to highlight the links between individual metabolites and growth rate. Then, scatterPlot was produced using the ggplot2 package (21). The

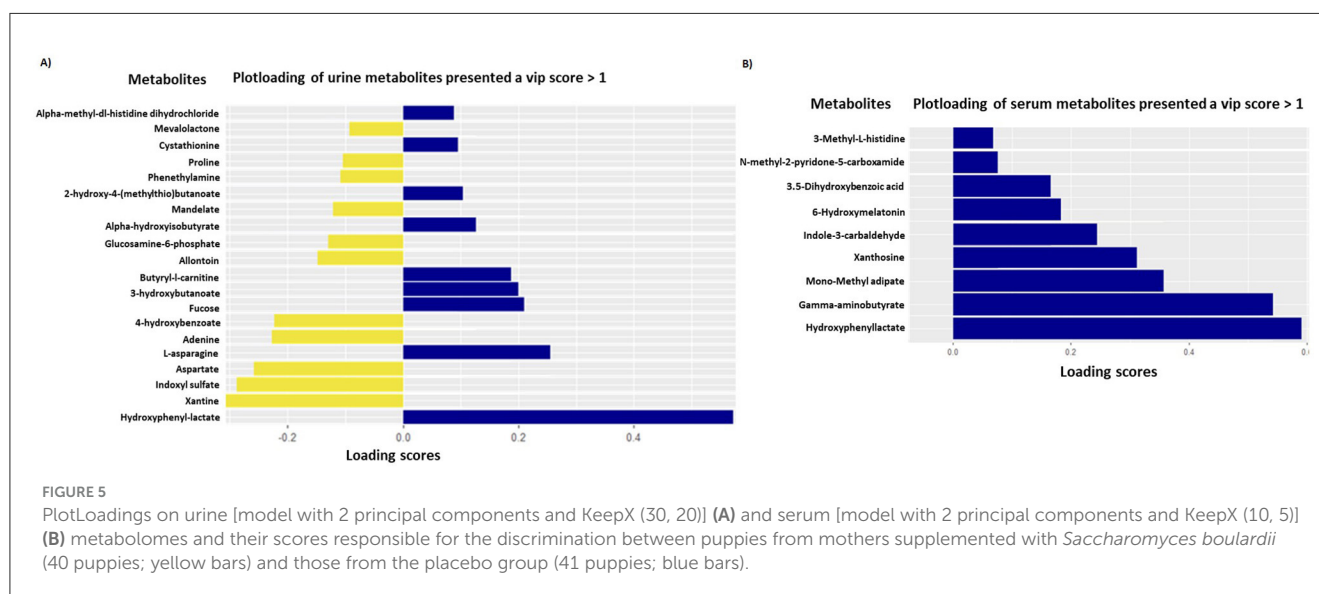
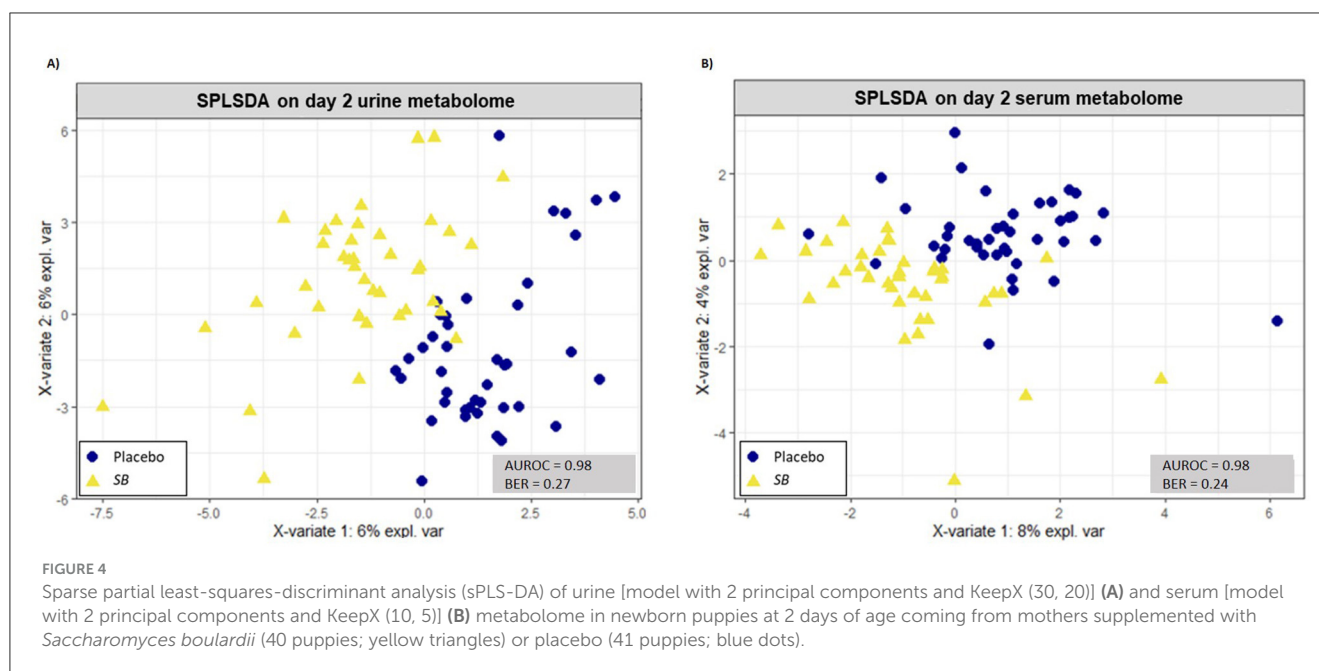
final diagram was produced using the BioRender website. Data are presented as mean ± standard deviation (SD).

3 Results

3.1 Characteristics of the included puppies

A total of 81 puppies alive at D2 (49 males and 32 females, 7 per litter on average) from 17 selected bitches were included in the study. Birth weights ranged from 214 g to 576 g with an average of 387 g. At D1, the mean rectal temperature was $36.4 \pm 0.8^\circ\text{C}$ (min: 33.8°C – max: 38.3°C) and the glycemia was 120 ± 30.6 mg/dl (min: 64 mg/dl – max: 207 mg/dl) with no sign of diseases.

Among the included puppies, 49% (40 puppies) were born from 10 mothers supplemented with SB during gestation and lactation, while 51% (41 puppies) were born from 7 mothers supplemented with a placebo. Both groups of puppies were balanced according to the sex ratio, litter size, breeds, and birth weight (Table 3).



Out of all puppies, EGR varied between -14.2% and 37.7% . According to the ANOVA test, the EGR was significantly higher in puppies from the SB group compared to those from the placebo group ($+57\%$; $p\text{-value} = 0.049$) despite a high variability in the SB group (Figure 2).

3.2 Urinary and serum metabolome in newborn puppies

A total of 228 serum and 206 urine metabolites were detected in puppies at D2 according to the LC-MS analysis (Supplementary Table 1). The metabolites found in the urine and serum of neonates were for the vast majority part of the nitrogen metabolism pathway (Figure 3).

After the enrichment analysis and p -value correction, only the ammonia recycling and glycine-serine metabolism pathways were significantly overrepresented in urine samples (respectively, $p\text{-adj} < 0.01$, $p\text{-adj} = 0.016$; Figure 3), while no pathway was significantly overrepresented in serum samples.

3.3 Impact of maternal SB supplementation on the urinary metabolic profiles of newborn puppies

The sPLSDA analyses performed on urinary metabolites allowed us to discriminate puppies of the SB group from those in the placebo group with a high accuracy of the model (AUROC

TABLE 4 Uniquely distinctive urinary metabolites between *Saccharomyces boulardii* and placebo groups, based on Variable Importance in Projection (VIP).

Urine metabolites	VIP	Values in SB compared to placebo	P-adj
Hydroxyphenyllactate	7.12	↓	$p = 0.000$
Xantine	4.15	↑	$p = 0.001$
Indoxyl sulfate	3.92	↑	$p = 0.001$
Aspartate	3.59	↑	$p = 0.002$
L-Asparagine	3.54	↓	$p = 0.002$
Adenine	3.22	↑	$p = 0.004$
4-Hydroxybenzoate	3.19	↑	$p = 0.004$
Fucose	3.03	↓	$p = 0.005$
3-Hydroxybutanoate	2.93	↓	$p = 0.005$
Butyryl-L-carnitine	2.78	↓	$p = 0.006$
Allantoin	2.34	↓	$p = 0.010$
Glucosamine 6-Phosphate	2.12	↑	$p = 0.013$
Alpha-Hydroxyisobutyrate	2.09	↓	$p = 0.014$
Mandelate	2.03	↑	$p = 0.014$
Phenethylamine	1.89	↑	$p = 0.017$
Proline	1.85	↑	$p = 0.017$
2-Hydroxy-4-(Methylthio)butanoate	1.84	↓	$p = 0.018$
Cystathionine	1.75	↓	$p = 0.019$
Mevalolactone	1.71	↑	$p = 0.020$
Alpha-methyl-DL-histidine dihydrochloride	1.68	↓	$p = 0.021$

Arrow (↓) means a lower abundance of metabolites and the arrow (↑) a higher relative abundance in *Saccharomyces boulardii* compared to the placebo group. P -adj was obtained using Anova and *post-hoc* Bonferroni test.

≥ 0.98 for urine metabolome). The two principal components explained 12% of the variance for urine metabolome and discriminated groups with an error rate (BER) of 0.27 (Figure 4A). On these principal components, VIP analyses allowed the selection of 20 out of the initial 206 urinary metabolites (Loading score comprised between −0.31 and 0.57), discriminating puppies of the SB from the placebo group (Figure 5A; Table 4). All these metabolites presented a VIP score >1.0.

Significant differences were noted between the SB and placebo groups in the abundance of all discriminating urine metabolites. Specifically, within the purine metabolism pathway, the SB group exhibited a significant increase in the abundance of urine adenine and xanthine, accompanied by a significant decrease in urine allantoin compared to the placebo group. Regarding amino acid metabolism, the SB group showed a significant increase in urinary abundance of phenethylamine, aspartate, and proline. In contrast, urinary abundances of cystathionine and butyryl-L-carnitine and L-asparagine were decreased compared to the placebo group.

TABLE 5 Uniquely distinctive serum metabolites between *Saccharomyces boulardii* and placebo groups, based on Variable Importance in Projection (VIP).

Serum metabolites	VIP	Values in SB compared to placebo	P-adj
Hydroxyphenyllactate	8.91	↓	$p = 0.000$
Gamma-aminobutyrate	8.19	↓	$p = 0.000$
Mono-Methyl adipate	5.38	↓	$p = 0.001$
Xanthosine	4.70	↓	$p = 0.001$
Indole-3-carbaldehyde	3.68	↓	$p = 0.002$
6-Hydroxymelatonin	2.75	↓	$p = 0.004$
3,5-Dihydroxybenzoic acid	2.49	↓	$p = 0.004$
N-methyl-2-pyridone-5-carboxamide	1.14	↓	$p = 0.007$
3-Methyl-L-histidine	1.02	↓	$p = 0.008$

Arrow (↓) means a lower relative abundance of metabolites in *Saccharomyces boulardii* compared to the placebo group. p -adj was obtained using Anova and *post-hoc* Bonferroni test.

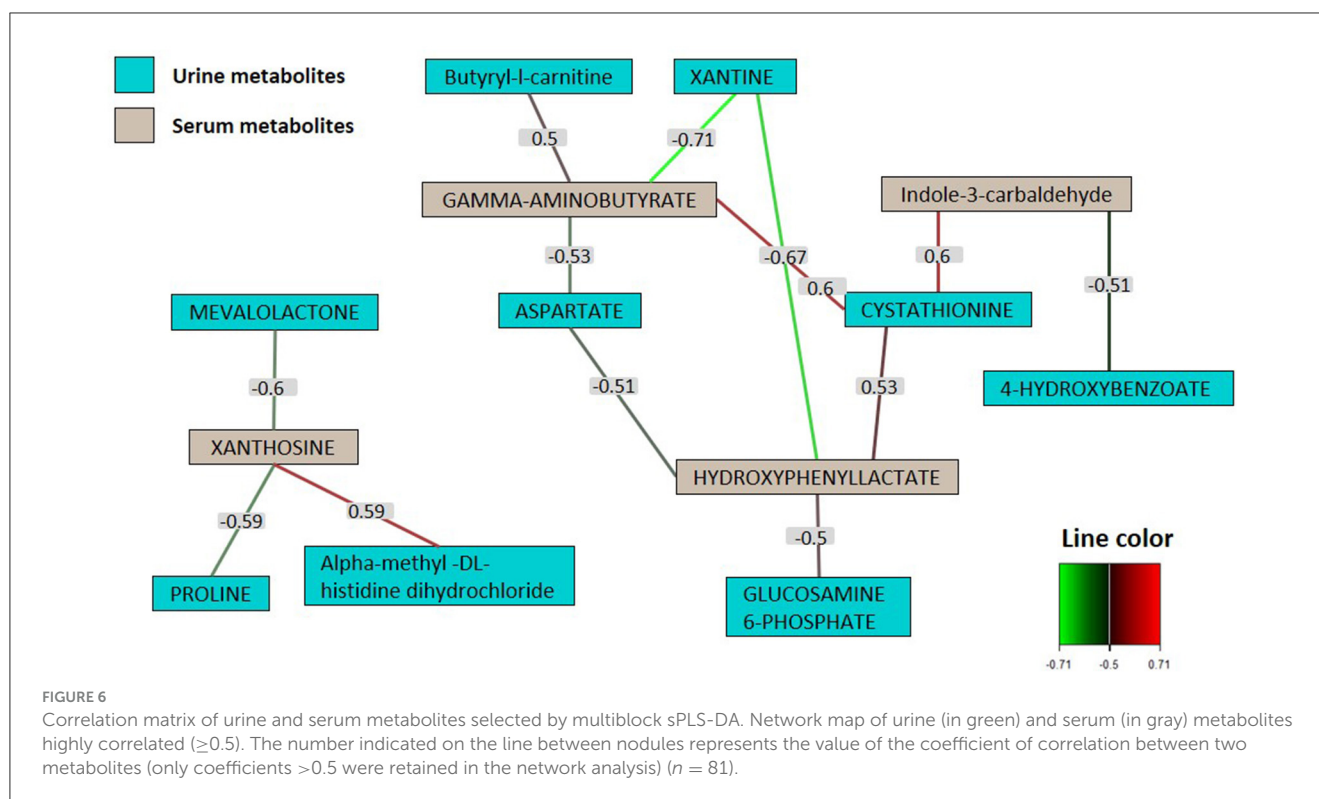
3.4 Impact of maternal SB supplementation on the serum metabolic profiles of newborn puppies

The sPLSDA analyses performed on serum metabolites also achieved high accuracy in discriminating puppies of the SB group from those in the placebo group (AUROC ≥ 0.98 for serum metabolome). Similarly, the two principal components explained 12% of the variance of serum metabolome and distinguished the groups with an error rate (BER) of 0.24 (Figure 4B). From these components, VIP analyses identified 9 serum metabolites out of the initial 228 (Loading score between 0.07 and 0.59), which differentiated the SB group from the placebo group (Figure 5B; Table 5). Each of these metabolites had a VIP score >1.0.

Significant differences were observed between the SB and placebo groups in the abundance of these discriminating serum metabolites. Notably, within the purine metabolism pathway, the SB group showed a significant decrease in serum xanthosine levels compared to the placebo group. Additionally, the SB group had decreased levels of serum gamma-aminobutyrate and 3-methyl-L-histidine compared to the placebo group.

3.5 Correlations between urinary and serum metabolites and early growth of puppies

A network based on multiblock sPLS was used to investigate the correlation between urinary and serum metabolites (Figure 6). Correlation analysis was restricted to metabolites that exhibited significant differences between SB and placebo groups. The network exhibited a considerable degree of connectivity with each node being connected to several nodes. In line with the correlation score, two distinct correlation network groups were



identified. Serum metabolites, particularly gamma-aminobutyrate and hydroxyphenyl lactate (both with a connectivity of 4), along with xanthosine (with a connectivity of 3), emerged as the most central, while urinary metabolites were peripheral. Xanthine presented the strongest (negative) correlations, in particular with hydroxyphenyl lactate ($r = -0.67$) and gamma-aminobutyrate ($r = -0.71$), followed by cystathionine positively correlated with indole-3-carbaldehyde ($r = 0.60$) gamma-aminobutyrate ($r = 0.60$) and hydroxyphenyl lactate ($r = 0.53$).

According to Pearson's correlation analysis, the early growth rate was positively correlated with proline ($r = 0.45$) and mevalolactone ($r = 0.56$) in urine (Figures 7A, B), while it was negatively correlated with alpha-methyl-DL-histidine dihydrochloride ($r = -0.64$) in urine and 3-methyl-L-histidine ($r = -0.46$) in serum (Figures 7C, D).

4 Discussion

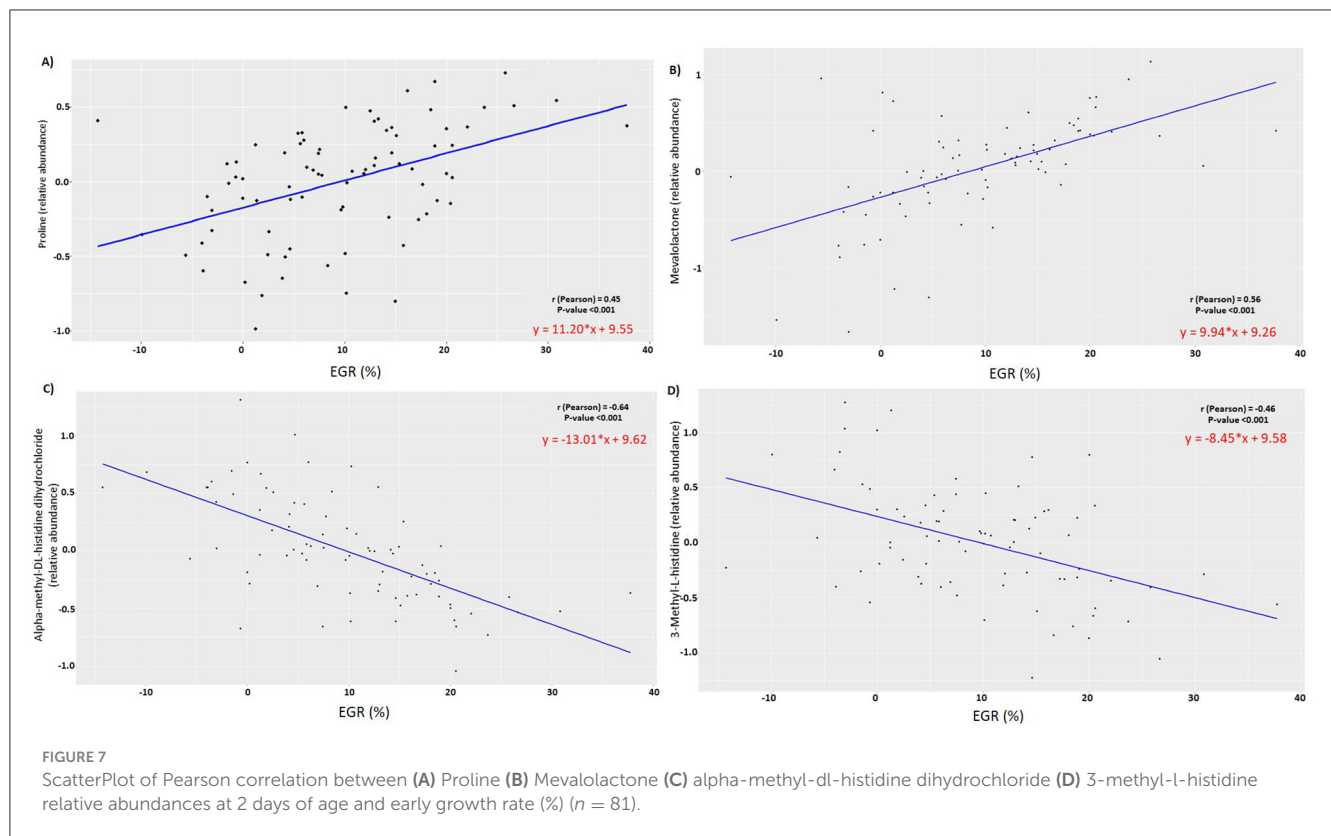
Although the interest in research on the neonatal period (i.e., the first 3 weeks of life) in the dog is continuously increasing, the canine neonatal morbidity and mortality rates remain relatively high (11%) (22). Several strategies are proposed to canine breeders and their veterinarians (23) to improve the newborn's health, mostly focused on whelping and suckling periods management. The modulation of offspring vitality and vulnerability through the mother during the gestation and lactation period is increasingly documented in humans and pigs (8), but very few studies focused on canine developmental programming through maternal nutrition in dogs. Our study demonstrated for the first time the interest of maternal supplementation with yeast during gestation

and early lactation on the neonatal growth and metabolic profile, proposing a new strategy to breeders and veterinary practitioners.

The median growth rate during the first 2 days of life in the dog was estimated at about 3% in the study by Mila et al. (2). In our study, the growth rate in the placebo group was found to be twice as high at 7%. Despite this notable result, the SB group presented even a 1.5 higher growth rate over the first 2 days of life than puppies from the placebo group; however, there was a large variability among individuals (Figure 2).

In order to understand the mechanism explaining the increased growth in puppies born from SB mothers in our study, we decided to explore their urinary and serum metabolomes. Firstly, we investigated the different metabolic pathways found in the urine and serum of newborn puppies at 2 days of age. Our results showed that whether in urine or serum, the identified metabolites are mostly involved in nitrogen metabolism (amino acids and purines metabolic pathways, urea cycle, or even ammonia recycling cycle). Nitrogen metabolism is essential for cell division (24), which is important for the development of tissues and organs in puppies at this age. The need for nitrogen is the highest right after birth due to the intensive growth process and then decreases with age. In humans during the first 6 months, the daily protein requirement of infants is 2.2 g/kg, then this requirement gradually decreases to reach 0.75 g/kg/day in adulthood (25). The same pattern could be observed in puppies, supporting the overrepresentation of the nitrogen metabolism in puppies at 2 days of life in our study.

Secondly, we aimed to explore how maternal SB supplementation during gestation and lactation affect the metabolome of puppies. We have demonstrated that maternal yeast supplementation modifies the metabolic profile of offspring as observed in piglets (12). A total of 29 metabolites from serum



or urine were impacted by the supplementation, 14 were amino acids, precursors, or products of their degradation, nucleotides, and energy metabolites. The metabolic pathways in which these metabolites are involved as well as their relationship were compiled in Figure 8. This figure shows that two major nitrogen metabolic pathways are modified thanks to the addition of SB: amino acids and purine metabolic pathways. The main changes are observed in reactions involving amination and transamination, in particular those using the glutamine/glutamate system. The glutamine/glutamate system is essential for protein anabolism and nucleotide synthesis, because of its ability to donate or take up an amine group (26, 27). The transformation of glutamine by deamination and the conversion of aspartate, histidine, and tyrosine by transamination are crucial processes for the biosynthesis of glutamate, an essential precursor of gamma-aminobutyrate and proline (28) (Figure 9). One of the most significant findings in our study is the increased urinary levels of proline and reduced levels of gamma-aminobutyrate observed in the SB group.

Proline is a crucial component in collagen synthesis and osteoarticular system development (29). This finding is particularly significant in light of the observed increased growth rate of puppies from mothers supplemented by SB. Interestingly, we also found a significant correlation between urinary proline abundance and growth rate in puppies. A similar positive correlation between blood proline concentration at 12 months and growth between 6 and 12 months was observed in babies, growth being evaluated using weight-for-height ratio and length velocity (30).

In parallel to this change in amino acid metabolism after SB maternal supplementation, we hypothesized a modification of the purine metabolism pathway in puppies at 2 days of age (Figure 8). In the same way as proline, we have noted an increase in urine adenine abundance. This could reflect an increase of purine metabolism (31) aligning with the needs of rapid growth.

During the 1st days of life it is known that the tissues of the body grow at different rates, with bone tissue development being prioritized over other tissues, including the nervous system (32). Overall, according to here presented results, we hypothesize that yeast promotes glutamate utilization for proline synthesis, possibly at the expense of gamma-aminobutyrate production, which could be probably associated with accelerated osteoarticular growth in the supplemented newborns presenting increased overall growth rate values. Imaging studies must be conducted to confirm if these changes in nitrogen metabolism induced by maternal SB supplementation are linked to the bone development of puppies at this age.

This effect of maternal SB supplementation on changes in metabolites involved in the nitrogen metabolism pathway in the offspring can be elucidated through various mechanisms. First, maternal SB supplementation could modify the fetal environment during gestation with its effect observed postnatally. Indeed, maternal amino acids have the potential to modify fetal epigenetics, promoting growth and development (33). In a parallel study, we noticed that SB supplementation in late gestation led to a change in maternal metabolism, thus reinforcing the idea of fetal epigenetic modulation through the transplacental pathway (12). In the previous study mothers supplemented with SB had a higher

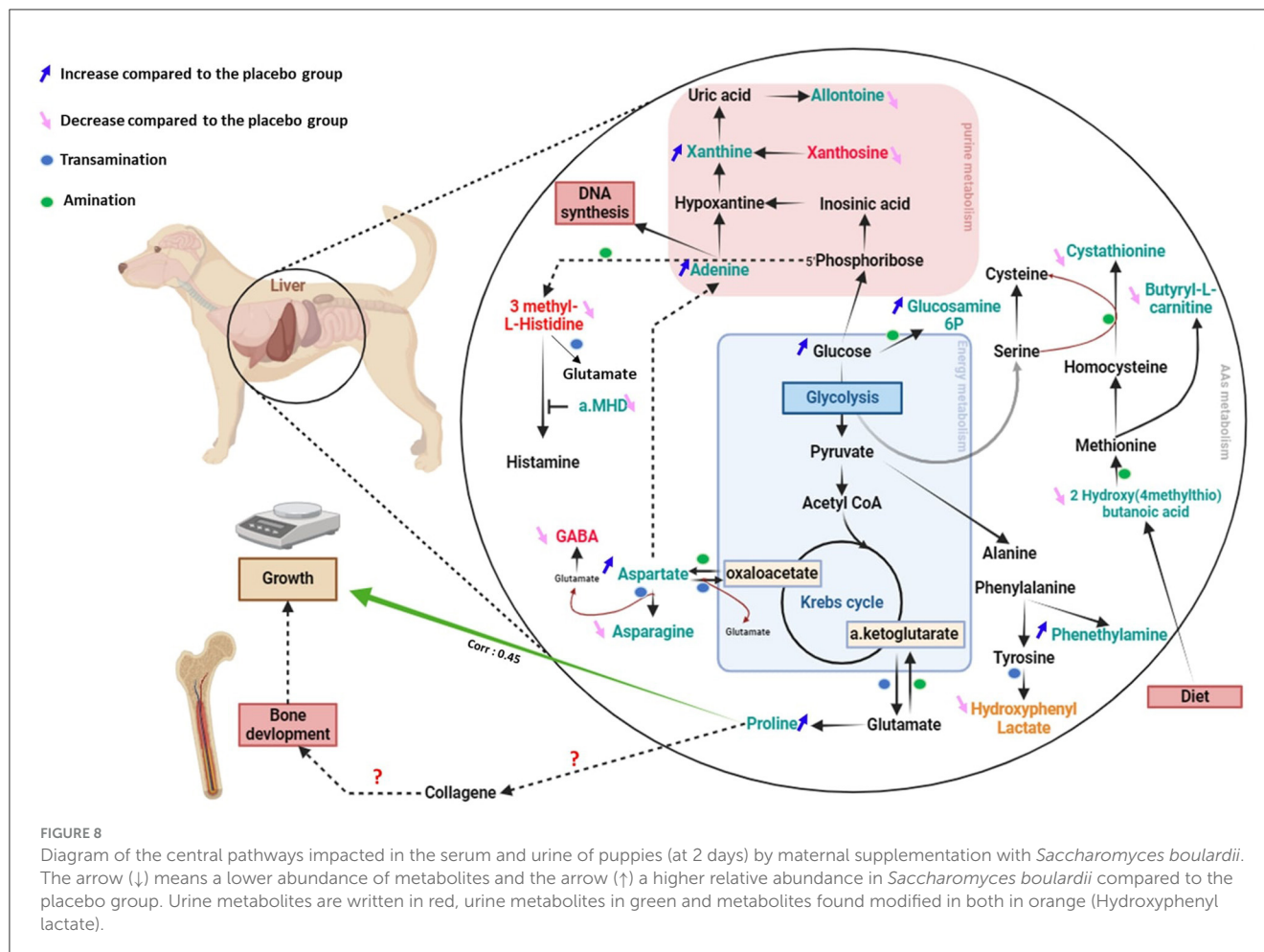


FIGURE 8

Diagram of the central pathways impacted in the serum and urine of puppies (at 2 days) by maternal supplementation with *Saccharomyces boulardii*. The arrow (↓) means a lower abundance of metabolites and the arrow (↑) a higher relative abundance in *Saccharomyces boulardii* compared to the placebo group. Urine metabolites are written in red, urine metabolites in green and metabolites found modified in both in orange (Hydroxyphenyl lactate).

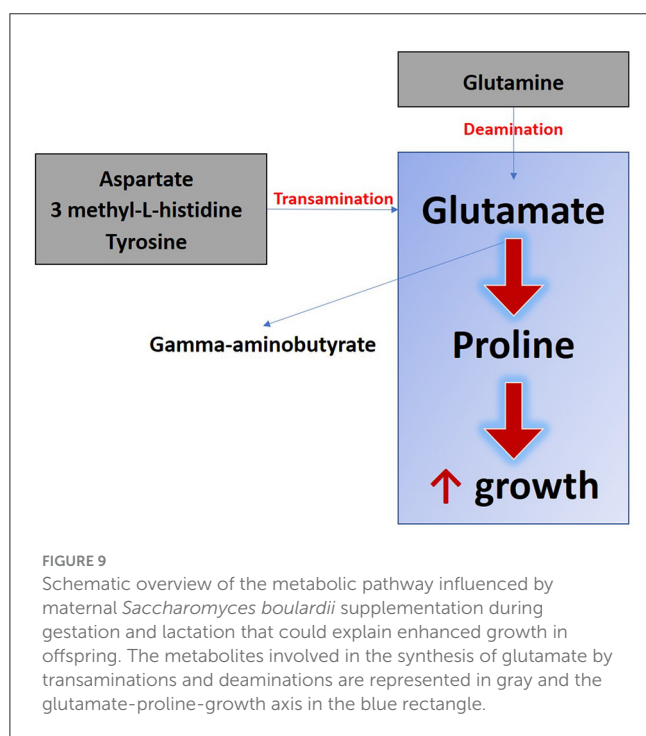


FIGURE 9

Schematic overview of the metabolic pathway influenced by maternal *Saccharomyces boulardii* supplementation during gestation and lactation that could explain enhanced growth in offspring. The metabolites involved in the synthesis of glutamate by transaminations and deaminations are represented in gray and the glutamate-proline-growth axis in the blue rectangle.

blood level of metabolites involved in the metabolism of amino acids such as proline, tryptophan, beta-alanine, glycine, serine, and threonine during gestation compared to placebo group. We cannot conclude at this stage whether the changes in the nitrogen metabolism of the mothers have an influence on the nitrogen metabolism of the young, or whether these two changes are the result of the same cause.

In addition, changes in nitrogen metabolism could be influenced by maternal milk quality. In the study of Garrigues et al. (13), no significant protein modification was observed in the colostrum after SB supplementation of the bitches. However, breast milk contains also a fraction of non-protein nitrogen in the form of free amino acids, peptides, which could be ingested and used by newborns, playing an important role in their growth and development (34). A detailed analysis of the colostrum should be carried out to reach a conclusion.

Yeasts can influence the body in various ways, either indirectly by affecting the intestinal microbiota or directly by providing nutrients. Of all these possibilities, the one that could explain at least part of our observations is its richness in vitamins B, including B6 (35, 36). Vitamin B6 acts as an essential cofactor for more than 100 enzymes playing a crucial role in catalytic reactions, including decarboxylations and transaminations (37). Vitamin B6

can be transmitted transplacentally to the fetus. Colostrum is rich in vitamins, including vitamin B6 (38). Intake of vitamin B6 transplacentally or via colostrum could play an important role in modulating nitrogen metabolism in newborns from SB supplemented dams (39). In future studies, it would be interesting to evaluate the effect of maternal SB supplementation on the abundance of vitamin B6 in the plasma and colostrum of female dogs and its link with offspring metabolome and growth changes.

Moreover, the study by Garrigues et al. (13) demonstrated in dogs that maternal supplementation with SB altered the composition of the maternal gut microbiota, increasing the abundance of fiber-degrading bacteria while reducing undesirable bacteria like *Campylobacter*. Maternal intestinal microbiota during gestation and lactation plays a crucial role in shaping the gut microbiota of newborns (40). Modulating the maternal microbiota through SB during gestation could positively influence the microbial colonization of the newborn, potentially impacting their metabolome and growth. This hypothesis remains to be demonstrated in further studies.

These findings highlight the potential benefits of maternal supplementation with SB in promoting offspring growth potentially through metabolic nitrogen metabolism regulation. However, this pathway requires further investigation, in particular taking into account the effect of the age and time of sampling non-studied in this work. Additionally, more research is needed to investigate whether there is a dose-dependent effect of SB and explore the long-term implications of these modifications on the overall health and development of the offspring.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Ethics statement

The animal study was approved by the Local Ethical Committee (Comité d'Éthique en Expérimentation Animale, Science et Santé Animale no. 115; reference number: SSA_2020-004, Toulouse, France). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

IB: Data curation, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. QG: Conceptualization, Data curation, Writing – review & editing. EA: Conceptualization, Project administration, Writing – review & editing, Formal analysis, Validation. AMu: Supervision, Writing – review & editing. LS: Methodology, Writing – original draft,

Writing – review & editing. J-cM: Methodology, Writing – review & editing. SC: Conceptualization, Methodology, Project administration, Writing – review & editing, Funding acquisition. AMe: Methodology, Supervision, Writing – original draft, Writing – review & editing. HM: Conceptualization, Data curation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing, Formal analysis, Funding acquisition, Validation.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This research project was financially supported by the Occitania Pyrenees-Mediterranean Region and Lallemand SAS, Blagnac, France (R3301-ope-2021-0002).

Acknowledgments

The authors would like to thank the breeder for enabling the feasibility of this project, and likewise, all veterinary students participating in the project (Pauline David, Elodie Mansalier, Fanny Mercier, and Nicolette Rover) for their aid in conducting the samplings.

Conflict of interest

EA and AM were employees of Lallemand SAS, Blagnac, France.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fnut.2025.1500600/full#supplementary-material>

SUPPLEMENTARY TABLE 1

Raw data of the urinary and serum metabolome of puppies, categorized by maternal supplementation group.

References

- Barker DJP. The origins of the developmental origins theory. *J Intern Med.* (2007) 261:412–7. doi: 10.1111/j.1365-2796.2007.01809.x
- Mila H, Grellet A, Feugier A, Chastant-Maillard S. Differential impact of birth weight and early growth on neonatal mortality in puppies 1,2. *J Anim Sci.* (2015) 93:4436–42. doi: 10.2527/jas.2015-8971
- Quyen PN, Nga HT, Chaffee B, Ngu T, King JC. Effect of maternal prenatal food supplementation, gestational weight gain, and breast-feeding on infant growth during the first 24 months of life in rural Vietnam. *PLoS One.* (2020) 15:e0233671. doi: 10.1371/journal.pone.0233671
- Liu Y, Jia X, Chang J, Jiang X, Che L, Lin Y, et al. Effect of yeast culture supplementation in sows during late gestation and lactation on growth performance, antioxidant properties, and intestinal microorganisms of offspring weaned piglets. *Front Microbiol.* (2023) 13:1105888. doi: 10.3389/fmicb.2022.1105888
- Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, et al. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat Rev Gastroenterol Hepatol.* (2014) 11:506–14. doi: 10.1038/nrgastro.2014.66
- Bernaola Aponte G, Bada Mancilla CA, Carreazo NY, Rojas Galarza RA. Probiotics for treating persistent diarrhoea in children. *Cochr Datab System Rev.* (2013) 2013:CD007401. doi: 10.1002/14651858.CD007401.pub3
- Ritchie ML, Romanuk TN. A meta-analysis of probiotic efficacy for gastrointestinal diseases. *PLoS ONE.* (2012) 7:e34938. doi: 10.1371/journal.pone.0034938
- Cuinat C, Stinson SE, Ward WE, Comelli EM. Maternal intake of probiotics to program offspring health. *Curr Nutr Rep.* (2022) 11:537–62. doi: 10.1007/s13668-022-00429-w
- Kazmierczak-Siedlecka K, Ruskowski J, Fic M, Folwarski M, Makarewicz W. *Saccharomyces boulardii* CNCM I-745: a non-bacterial microorganism used as probiotic agent in supporting treatment of selected diseases. *Curr Microbiol.* (2020) 77:1987–96. doi: 10.1007/s00284-020-02053-9
- Babaei F, Mirzababaei M, Mohammadi G, Dargahi L, Nassiri-Asl M. *Saccharomyces boulardii* attenuates lipopolysaccharide-induced anxiety-like behaviors in rats. *Neurosci Lett.* (2022) 778:136600. doi: 10.1016/j.neulet.2022.136600
- Le Floch N, Achard CS, Eugenio FA, Apper E, Combes S, Quesnel H. Effect of live yeast supplementation in sow diet during gestation and lactation on sow and piglet fecal microbiota, health, and performance. *J Animal Sci.* (2022) 100:skac209. doi: 10.1093/jas/skac209
- Salak-Johnson JL, Reddout C, Hernandez L, Visconti A. Maternal supplementation of *Saccharomyces cerevisiae boulardii* during late-gestation through lactation differentially modulated immune status and stress responsiveness of the progeny to farrowing and weaning stressors. *Animals.* (2022) 12:164. doi: 10.3390/ani12020164
- Garrigues Q, Mugnier A, Chastant S, Sicard F, Martin J-C, Svalir L, et al. The supplementation of female dogs with live yeast *Saccharomyces cerevisiae* var. *boulardii* CNCM I-1079 acts as gut stabilizer at whelping and modulates immunometabolic phenotype of the puppies. *Front Nutr.* (2024) 11:1366256. doi: 10.3389/fnut.2024.1366256
- National Research Council. *Nutrient Requirements of Dogs and Cats.* Washington, DC: National Academies Press (2006).
- Fédération Européenne de l'Industrie des Aliments pour Animaux Familiers. *Nutritional Guidelines for Complete and Complementary Pet Food for Cats and Dogs.* (2019).
- R Core Team. *R: a language and environment for statistical computing.* Vienna, Austria: R found stat Comput (2022).
- Kalivodová A, Hron K, Filzmoser P, Najdekr L, Janečková H, Adam T. PLS-DA for compositional data with application to metabolomics. *J Chemom.* (2015) 29:21–8. doi: 10.1002/cem.2657
- Palarea-Albaladejo J, Martín-Fernández JA. zCompositions—R package for multivariate imputation of left-censored data under a compositional approach. *Chemom Intell Lab Syst.* (2015) 143:85–96. doi: 10.1016/j.chemolab.2015.02.019
- Rohart F, Gautier B, Singh A, Lê Cao K-A. mixOmics: an R package for omics feature selection and multiple data integration. *PLoS Comput Biol.* (2017) 13:e1005752. doi: 10.1371/journal.pcbi.1005752
- Xia J, Psychogios N, Young N, Wishart DS. MetaboAnalyst: a web server for metabolomic data analysis and interpretation. *Nucleic Acids Res.* (2009) 37:W652–60. doi: 10.1093/nar/gkp356
- Wickham H. *ggplot2 Elegant Graphics for Data Analysis.* Second Edition. Cham: Springer (2009). doi: 10.1007/978-0-387-98141-3
- Nielsen ALJ, van der Gaag I, Knol BW, Schukken YH. Investigation of mortality and pathological changes in a 14 month birth cohort of boxer puppies. *Veter Record.* (1998) 142:602–6. doi: 10.1136/vr.142.22.602
- Reid A, Donnelly E. Improving early neonatal survival in puppies and kittens: resuscitation and beyond. *In Pract.* (2024) 46:108–17. doi: 10.1002/inpr.415
- Eagle H. Amino acid metabolism in mammalian cell cultures. *Science.* (1959) 130:432–7. doi: 10.1126/science.130.3373.432
- Webb G. Modern nutrition in health and disease, 9th edition. M. E. Shils, J. A. Olsen, M. Shike and A. C. Ross (editors). 1999. Baltimore: Williams & Wilkins. \$105.00. ISBN 0-683-30769-X. *Br J Nutr.* (1999) 82:331–2. doi: 10.1017/S0007114599001555
- Newsholme P, Procopio J, Lima MMR, Pithon-Curi TC, Curi R. Glutamine and glutamate—their central role in cell metabolism and function. *Cell Biochem Funct.* (2003) 21:1–9. doi: 10.1002/cbf.1003
- Newsholme P, Lima MMR, Procopio J, Pithon-Curi TC, Doi SQ, Bazotte RB, et al. Glutamine and glutamate as vital metabolites. *Braz J Med Biol Res.* (2003) 36:153–63. doi: 10.1590/S0100-879X2003000200002
- Tapiero H, Mathé G, Couvreur P, Tew KDII. Glutamine and glutamate. *Biomed Pharmacother.* (2002) 56:446–57. doi: 10.1016/S0753-3322(02)00285-8
- Danelatos-Athanassiadis CT, Constantas NS, Agathopoulos AS, Matsaniotis NS. Urinary free proline and collagen metabolites in newborns, healthy children, and in collagen diseases. *Clinica Chimica Acta.* (1969) 23:153–64. doi: 10.1016/0009-8981(69)90022-9
- Ordiz MI, Semba RD, Moaddel R, Rolle-Kampczyk U, von Bergen M, Herberth G, et al. Serum amino acid concentrations in infants from malawi are associated with linear growth. *Curr Dev Nutr.* (2019) 3:nzz100. doi: 10.1093/cdn/nzz100
- Mills GC, Schmalstieg FC, Koolkin RJ, Goldblum RM. Urinary excretion of purines, purine nucleosides, and pseudouridine in immunodeficient children. *Biochem Med.* (1982) 27:37–45. doi: 10.1016/0006-2944(82)90006-0
- The dog encyclopedia. *A dog's life: from start of life to ageing.* 2020th ed. Royal Canin (2020). p. 156.
- Lin G, Wang X, Wu G, Feng C, Zhou H, Li D, et al. Improving amino acid nutrition to prevent intrauterine growth restriction in mammals. *Amino Acids.* (2014) 46:1605–23. doi: 10.1007/s00726-014-1725-z
- Atkinson S, Lonnerdal B. *Protein and Non-Protein Nitrogen in Human Milk.* 1st ed. New York: CRC Press (2019). doi: 10.1201/9780367812805
- Jach ME, Sajnaga E, Kozak E, Malm A. Use of yeasts for prevention and therapy. *Curr Issues Pharm Med Sci.* (2013) 26:198–202. doi: 10.12923/j.2084-980X/26.2/a.17
- Datta S, Timson DJ, Annapure US. Antioxidant properties and global metabolite screening of the probiotic yeast *Saccharomyces cerevisiae* var. *boulardii*. *J Sci Food Agric.* (2017) 97:3039–49. doi: 10.1002/jsfa.8147
- Percudani R, Peracchi A. A genomic overview of pyridoxal-phosphate-dependent enzymes. *EMBO Rep.* (2003) 4:850–4. doi: 10.1038/sj.embor.embor914
- Hurley WL. Composition of sow colostrum and milk. In: Farmer C, editor. *The gestating and lactating sow.* Brill: Wageningen Academic (2015). p. 193–229. doi: 10.3920/9789086868032_010
- Hampel D, Allen LH. Analyzing B-vitamins in human milk: methodological approaches. *Crit Rev Food Sci Nutr.* (2016) 56:494–511. doi: 10.1080/10408398.2013.783550
- Nyangahu DD, Lennard KS, Brown BP, Darby MG, Wendoh JM, Havyarimana E, et al. Disruption of maternal gut microbiota during gestation alters offspring microbiota and immunity. *Microbiome.* (2018) 6:124. doi: 10.1186/s40168-018-0511-7
- Laflamme D. Development and validation of a body condition score system for dogs. In: *Agricultural and food sciences, Medicine* (1997).

Annexes Chapitre 6

i. Supplementary data Article 5

Supplementary Table 2: Birthday distinctive taxa between *Saccharomyces boulardii* and placebo groups based on VIP. Only taxa with VIP>1 and relative abundance >0,2% of the total sequences of sample were showed.

D0 TAXA	SB effect	VIP score	Relative abundance (%) Mean±SD		P-val	Percentage of zeros	
			Placebo	SB		Placebo	SB
<i>Lachnospiraceae</i> ; <i>Dorea</i> ; US	↓	2.98	1.06±1,15	0.41±0.98	P<0.01	44.4%	20.4%
<i>Pasteurellaceae</i> ; UG, US	↓	2.63	0.44±1.08	0.04±0.11	P<0.05	33.3%	12.9%
<i>Staphylococcaceae</i> ; <i>Staphylococcus</i> ; US1	↑	2.48	0.90±1.50	2.00±5.00	P<0.05	42.6%	42.6%
<i>Staphylococcaceae</i> ; <i>Staphylococcus</i> ; US2	↑	2.23	0.05±0.16	0.36±0.85	P<0.05	7.4%	14.8%
<i>Peptostreptococcaceae</i> ; <i>Peptostreptococcus</i> ; US	↓	2.25	1.57±1.89	0.51±0.81	P<0.05	50.0%	31.5%
<i>Enterobacteriaceae</i> ; <i>Providencia</i> ; US	↑	2.16	0.34±0.97	1.12±2.79	P<0.05	18.5%	29.6%
<i>Enterobacteriaceae</i> ; UG; US	↑	1.89	4.61±8.78	6.98±9.69	P<0.05	50.0%	46.3%

US = unknown species; UG = unknown genus. Arrow (↓) means a lower relative abundance of bacteria and the arrow (↑) a higher relative abundance in *Saccharomyces boulardii* puppies compared to the placebo group.

Supplementary Table 3: Day 2 distinctive taxa between *Saccharomyces boulardii* and placebo groups based on VIP. Only taxa with VIP>1 and relative abundance >0,2% of the total sequences of sample were showed.

D2 TAXA	SB effect	VIP score	Relative abundance (%)		P-val	Percentage of zeros	
			Placebo	SB		Placebo	SB
<i>Streptococcus ; luteciae</i>	↑	1.94	9.80±11.41	12.82±13.03	P<0.05	50.0%	46.3%

US = unknown species; UG = unknown genus. Arrow (↓) means a lower abundance of bacteria and the arrow (↑) a higher relative abundance in *Saccharomyces boulardii* puppies compared to the placebo group.

Table 4: Day 21 distinctive taxa between *Saccharomyces boulardii* and placebo groups based on VIP. Only taxa with VIP>1 and relative abundance >0,2% of the total sequences of sample were showed.

D21 TAXA	SB effect	VIP score	Relative abundance (%)		P-val	Percentage of zeros	
			Placebo	SB		Placebo	SB
<i>Enterococcaceae ; enterococcus ; US</i>	↓	2.91	2.16±7.73	0.05±0.15	P<0.001	48.1%	18.5%
<i>Streptococcus ; luteciae</i>	↑	2.35	0.62±1.51	7.42±11.47	P<0.01	29.6%	
<i>Lactobacillus ; reuteri</i>	↑	2.25	0.11±0.29	1.55±2.49	P<0.01	18.5%	29.6%
<i>Enterobacteriaceae ; klebsiella ; US</i>	↓	2.16	0.25±0.72	0.006±0.02	P<0.001	18.5%	3.7%
<i>Lactobacillaceae ; lactobacillus ; US</i>	↑	2.07	0,58±1,44	3,41±5,18	P<0,01	35,1%	38,9%
<i>Anaeroplasmataceae ; gut ; metagenome</i>	↓	1.74	0.36±1.34	0.02±0.09	P<0.05	14.8%	3.7%

<i>Peptostreptococcaceae</i> ; <i>peptostreptococcus</i> ; US	↑	1.57	0.02±0.09	0.34±0.76	P<0.05	7.4%	22.2%
<i>Helicobacteraceae</i> ; <i>helicobacter</i> ; US	↓	1.41	5,25±6,31	3,35±8,17	P<0,05	48,1%	35,1%

US = unknown species; UG = unknown genus. Arrow (↓) means a lower abundance of bacteria and the arrow (↑) a higher relative abundance in *Saccharomyces boulardii* puppies compared to the placebo group.

Table 5: Day 56 distinctive taxa between *Saccharomyces boulardii* and placebo groups based on VIP. Only taxa with VIP>1 and relative abundance >0,2% of the total sequences of sample were showed.

D56 TAXA	SB effect	VIP score	Relative abundance (%) Mean±SD		P-val	Percentage of zeros	
			Placebo	SB		Placebo	SB
<i>Corynebacteriaceae</i> ; <i>corynebacterium</i> ; US	↓	3.58	0.92±3.00	0.009±0.02	P<0.001	12.9%	7.4%
<i>Bacteroides</i> ; <i>coprophilus</i>	↑	3.35	0.03±0.11	0.25±0.73	P<0.001	5.0%	27.7%
<i>Cetobacterium</i> ; <i>somerae</i>	↑	2.25	0.39±1.22	0.68±0.93	P<0.01	22.2%	37.0%
<i>Enterococcus</i> ; <i>cecorum</i>	↓	1.21	1.68±2.07	0.73±0.57	P<0.001	53.7%	44.4%

US = unknown species; UG = unknown genus. Arrow (↓) means a lower abundance of bacteria and the arrow (↑) a higher relative abundance in *Saccharomyces boulardii* puppies compared to the placebo group.

Chapitre 7

Discussion générale

L'axe intestin-cerveau

La diarrhée du sevrage constitue un enjeu majeur en élevage canin. Fréquente chez le chiot, elle représente un double défi : éthique, en compromettant le bien-être animal, et économique, en augmentant la charge de soins et les pertes pour les éleveurs et nouveaux adoptants. Malgré son importance, les mécanismes sous-jacents à ce trouble digestif restent encore mal compris. Dans ce contexte, notre travail vise à mieux comprendre ce trouble multifactoriel en adoptant une approche intégrative, centrée sur les associations potentielles entre cette diarrhée du sevrage, la composition du microbiote fécal et le comportement des chiots. Cette orientation est basée sur la synthèse de littérature réalisée en amont, qui a souligné le manque de connaissances spécifiques à l'espèce canine. Les travaux existants tendent à examiner séparément les dimensions digestives, microbiennes et comportementales sans en considérer les interrelations. Or, les troubles digestifs, et plus largement les pathologies complexes, impliquent l'interaction de multiples systèmes physiologiques (digestif, nerveux, immunitaire, etc.), et leur compréhension nécessite de croiser plusieurs disciplines. C'est ce que recouvre une approche pluridisciplinaire en biologie ou en médecine. Ce type d'approche est aujourd'hui reconnu comme essentiel dans l'étude des maladies multifactorielle (Schiopu et al., 2023; Chaudhuri et al., 2021), car il permet de dépasser les visions fragmentées des processus biologiques. Chez d'autres espèces, telles que le porcelet ou la souris, cette démarche a permis de mettre en lumière des liens étroits entre santé digestive, microbiote intestinal et comportement (**Figure 4 – Chapitre 2**). Par ailleurs, des stratégies préventives ont été testées dans ces modèles, notamment la modulation du microbiote par supplémentation maternelle en probiotiques durant la gestation et la lactation, avec des résultats prometteurs sur la santé des jeunes (**Table 2 – Chapitre 2**).

Dans cette perspective, notre étude visait à combler en partie le manque de connaissances chez le chiot, en examinant (I) les relations entre le comportement, le microbiote digestif et le score fécal au sevrage chez le chiot, (II) l'effet préventif de la supplémentation maternelle par probiotique sur la fonction digestive. Au-delà de l'analyse séparée de chaque dimension, l'objectif était de croiser ces niveaux d'analyse pour explorer leurs interrelations. C'est dans cette logique que s'inscrit l'objectif transversal de ce travail : comprendre les liens entre microbiote, santé digestive et comportement et déterminer pourquoi certaines associations apparaissent robustes, tandis que d'autres restent partiels ou absents.

Nos travaux ont d'abord mis en évidence deux profils comportementaux distincts chez les chiots au moment du sevrage : audacieux et timides. Ces profils, caractérisés par des différences d'exploration,

de proximité humaine et de réactivité physiologique, s'accompagnent de différences significatives dans la composition du microbiote fécal. Les chiots audacieux présentaient une abondance réduite de bactéries lactiques à potentiel psychobiotique et une abondance plus élevée de bactéries pro-inflammatoires, notamment *Campylobacter* et certains genres de la famille *Prevotellaceae*. Cette association suggère une interaction précoce entre tempérament et microbiote, en cohérence avec le concept d'axe intestin-cerveau fonctionnel dès le jeune âge.

En parallèle, nos analyses ont également révélé une association claire entre certains profils microbiens (entérotypes) et le risque de diarrhée post-sevrage. Ces entérotypes à risque se caractérisaient par une abondance plus élevée de genres bactériens connus pour leur pouvoir pathogène ou inflammatoire (*Helicobacter*, *Campylobacter*, *Mucispirillum*...). De plus, l'abondance précoce du genre *Enterococcus*, détectée dès la naissance, apparaît comme un potentiel biomarqueur précoce de la survenue de troubles digestifs.

Toutefois, et de manière surprenante, le lien direct entre lentempérament et le risque de diarrhée n'a pas été mis en évidence dans notre étude. Malgré des différences de croissance et de réactivité au stress entre chiots audacieux et timides, les scores fécaux n'étaient pas significativement différents entre les deux groupes. Cette absence de lien direct contraste avec des résultats observés chez d'autres espèces (notamment le macaque), où des profils comportementaux audacieux sont associés à une incidence accrue de troubles digestifs (Gottlieb et al., 2018). De manière intéressante, dans notre cohorte, les taxons microbiens associés au tempérament diffèrent de ceux associés à la diarrhée. Seul le genre *Campylobacter* est impliqué dans les deux dimensions. Ce pathogène est d'ailleurs bien connu pour son double effet : il peut d'une part moduler l'activité de structures cérébrales impliquées dans la régulation émotionnelle, telles que l'amygdale, via l'activation du nerf vague (Breit et al., 2018; Gaykema et al., 2004; Goehler et al., 2008), et d'autre part altérer l'intégrité de l'épithélium intestinal en sécrétant des toxines, induisant une perméabilité accrue et une réponse inflammatoire locale (Fischer et al., 2025; Lobo De Sá et al., 2021). Ces effets suggèrent que *Campylobacter* pourrait représenter un point de convergence entre le comportement et la santé digestive. De futures recherches devraient s'attacher à mieux caractériser l'implication de ce genre bactérien dans l'axe intestin-cerveau, en particulier dans le contexte des troubles digestifs comme la diarrhée.

En synthèse, nos travaux montrent que le microbiote intestinal du chiot est associé au tempérament d'une part et au risque de diarrhée post-sevrage d'autre part. Toutefois, aucun lien clair n'a pu être démontré entre le tempérament et l'apparition des troubles digestifs, suggérant que ces deux phénomènes — bien que tous deux liés au microbiote — relèvent probablement de mécanismes distincts. L'ensemble de ces résultats sont présentés dans le schéma **Figure 1.7C**.

Cette dissociation observée dans notre étude entre le comportement et le risque de troubles digestifs, mérite d'être discutée à la lumière des données disponibles chez d'autres espèces. En effet, chez la souris ou le macaque, une interconnexion plus nette entre tempérament, microbiote et troubles digestifs a été rapportée, notamment dans des contextes de stress (Dong et al., 2017; Elfenbein et al., 2016; Gottlieb et al., 2018). Cela suggère que des conditions expérimentales spécifiques introduisant un stress important, absentes dans notre protocole, pourraient être nécessaires pour révéler pleinement ces interactions chez le chiot. Toutefois, une telle démarche n'est pas envisageable chez le chiot pour des raisons éthiques liées au bien-être animal.

Plusieurs hypothèses peuvent être avancées pour expliquer l'absence d'association directe entre tempérament et diarrhée dans notre cohorte. D'abord, la taille relativement restreinte de l'échantillon pourrait limiter la capacité à détecter des effets d'interaction modérés. Une faible puissance statistique, notamment dans des modèles multivariés complexes intégrant microbiote, santé digestive et comportement, peut masquer des liens réels, mais de faible amplitude.

Une autre piste d'explication plausible réside dans le rôle potentiel des troubles d'origine alimentaire, tels que l'intolérance et l'hypersensibilité alimentaires, qui pourraient favoriser l'émergence de troubles digestifs de manière indépendante du tempérament. L'intolérance alimentaire, qui toucherait entre 5 à 20 % de la population générale, repose sur des mécanismes non immunologiques impliquant par exemple des effets pharmacologiques de certains composants alimentaires ou encore des déficiences enzymatiques ou de transport (Lomer, 2015). À l'inverse, l'hypersensibilité alimentaire constitue un terme plus large, incluant à la fois ces intolérances et les véritables allergies alimentaires, ces dernières étant médiées par des mécanismes immunologiques, IgE-dépendants ou non. Selon certaines études, 17% des chiens (Chesney, 2001) et jusqu'à 28 % des jeunes enfants peuvent présenter une forme d'hypersensibilité alimentaire dès les premiers mois de vie (Azad et al., 2015), ce qui souligne la fréquence de ces phénomènes au cours du développement.

Ces réactions peuvent perturber la fonction digestive et provoquer des troubles comme la diarrhée, sans lien évident avec le comportement. Ce type de manifestations pourrait s'expliquer par la complexité du système immunitaire intestinal synthétisée dans la revue de Verlinden et al., (2006). Celui-ci repose notamment sur le tissu lymphoïde associé à l'intestin (GALT), chargé de différencier les nutriments des agents potentiellement pathogènes. Le GALT, bien caractérisé chez le chien, comprend les plaques de Peyer, les cellules immunitaires de la lamina propria et les ganglions mésentériques. Il assure la régulation de la réponse immune via un équilibre délicat entre tolérance et élimination des antigènes. L'ensemble de ces mécanismes, souligne que toute altération — barrière muqueuse, régulation immunitaire ou reconnaissance antigénique — peut favoriser l'émergence de troubles digestifs liés à l'hypersensibilité alimentaire. Ainsi, une sensibilité alimentaire latente, non détectée dans le cadre de notre protocole, pourrait contribuer à la survenue de diarrhées chez certains chiots, indépendamment de

leur tempérament. L'association entre la composante immunitaire intestinale, la diarrhée du sevrage et une éventuelle sensibilité alimentaire mérite d'être évaluée dans de futures études.

Enfin, la présence éventuelle de facteurs infectieux (viraux, ou parasitaires), bien documentés pour leur capacité à induire des troubles digestifs indépendamment du tempérament (**Figure 4 – Chapitre 2**), pourrait également avoir contribué à masquer une relation sous-jacente entre comportement et le risque des troubles digestifs. Ces agents pathogènes, en interagissant directement avec le système immunitaire intestinal ou en perturbant l'équilibre du microbiote, peuvent entraîner des diarrhées aiguës ou chroniques sans médiation comportementale (Li et al., 2021). Par ailleurs, certains pathogènes peuvent également moduler la composition microbienne de manière transitoire ou durable (Grondin et al., 2024), ce qui complique l'interprétation des liens entre microbiote, comportement et troubles digestifs. Ainsi, l'absence de relation démontrée entre tempérament et diarrhée pourrait résulter d'une combinaison de ces facteurs non contrôlés cités au-dessus.

Des études futures, reposant sur des cohortes plus larges, un suivi longitudinal avec un contrôle systématique des variables environnementales, alimentaires et sanitaires (y compris les statuts infectieux), seront nécessaires pour clarifier les associations dynamiques entre comportement, microbiote intestinal et santé digestive chez le chiot. L'intégration de mesures immunologiques et métagénomiques permettrait également de mieux appréhender les mécanismes sous-jacents à ces interactions complexes.

Modulation du microbiote

Enfin, notre étude s'est attachée à explorer le potentiel d'une intervention maternelle préventive, via la supplémentation probiotique pendant la gestation et la lactation. Nos résultats montrent que cette stratégie permet de moduler significativement la croissance précoce des chiots, leur santé digestive pendant la période néonatale, ainsi que l'évolution de leur microbiote fécal. De manière particulièrement intéressante, nous avons observé une réduction marquée de l'abondance de deux ASV appartenant au genre *Enterococcus* chez les chiots issus de mères supplémentées en SB, à la fois à J21 et à J56, ce dernier correspondant à l'âge du sevrage. Cette observation suggère une action indirecte de la supplémentation maternelle en probiotique sur la colonisation intestinale du chiot, probablement via des modifications du microbiote du lait maternel et/ou de sa composition en vitamines, oligosaccharides et autres composés susceptibles d'influencer le développement du microbiote intestinal.

Ce résultat peut être rapproché de notre étude précédente (**Article 4**), dans laquelle nous avons observé un lien potentiel entre l'évolution de l'abondance relative du genre *Enterococcus* et l'enterotype microbien après sevrage. En effet, leur abondance relative est supérieure dès les premiers jours de vie chez les chiots présentant l'entérotipe à risque élevé de diarrhée, soulignant leur rôle potentiel dans la

prédisposition aux troubles digestifs post-sevrage. Ainsi, la réduction significative de ces bactéries par la voie maternelle représente une piste prometteuse pour prévenir la diarrhée au sevrage, en agissant en amont de son apparition, dès la phase de développement du microbiote intestinal. Des études similaires chez le porcelet, a montré que la supplémentation maternelle par *Saccharomyces cerevisiae* pendant la gestation et lactation a induit une modulation du microbiote intestinal de la descendance plus tard au sevrage (Kantas et al., 2015; Law et al., 2024).

Ces résultats soutiennent l'hypothèse selon laquelle la nutrition maternelle constitue un levier d'intervention précoce sur la santé du chiot, agissant dès les premiers jours de vie. Miser sur une approche préventive centrée sur la mère permettrait d'anticiper les troubles digestifs au lieu d'y remédier une fois apparus. Toutefois, plusieurs questions restent ouvertes quant à la persistance de l'effet de la programmation nutritionnelle. Il conviendra notamment de déterminer si l'impact observé sur le microbiote persiste au-delà du sevrage, et s'il est associé à des bénéfices cliniques mesurables à moyen et long terme.

Dans ce contexte, la question des mécanismes par lesquels la supplémentation maternelle en SB agit sur la descendance mérite d'être approfondie. En effet, si les bénéfices observés sur la croissance, la santé digestive et la composition du microbiote fécal des chiots sont désormais documentés, les voies biologiques impliquées dans ces effets restent encore partiellement élucidées. Plusieurs hypothèses peuvent être avancées pour expliquer ceci.

Ces effets de la supplémentation maternelle par SB sur la santé des chiots semblent reposer sur plusieurs mécanismes complémentaires. D'une part, la qualité nutritionnelle du colostrum et du lait maternel peuvent jouer un rôle essentiel sur la santé et l'évolution du microbiote de la progéniture (Chu et al., 2016). Chez le chien, il a été démontré que la supplémentation maternelle par SB a amélioré l'énergie brute du colostrum ainsi que la concentration en protéines du lait au 7^{ème} jour après la mise bas (Garrigues et al., 2024).

D'autre part, la modification du microbiote fécal de la mère au moment de la mise bas constitue un autre mécanisme de modulation de la santé et du microbiote des nouveaux nés (Suárez-Martínez et al., 2023). Garrigues et al. (2024) ont également montré que la supplémentation par SB augmente l'abondance de bactéries digestives bénéfiques (dégradant les fibres) tout en réduisant des pathogènes comme *Campylobacter* au moment de la mise-bas chez la chienne. Ces altérations du microbiote maternel au péri-partum pourraient moduler directement celui des chiots, comme le suggèrent plusieurs travaux soulignant l'influence du microbiote maternel sur celui de la descendance (Nyangahu et al., 2018; Russell et al., 2023; Tochitani et al., 2024).

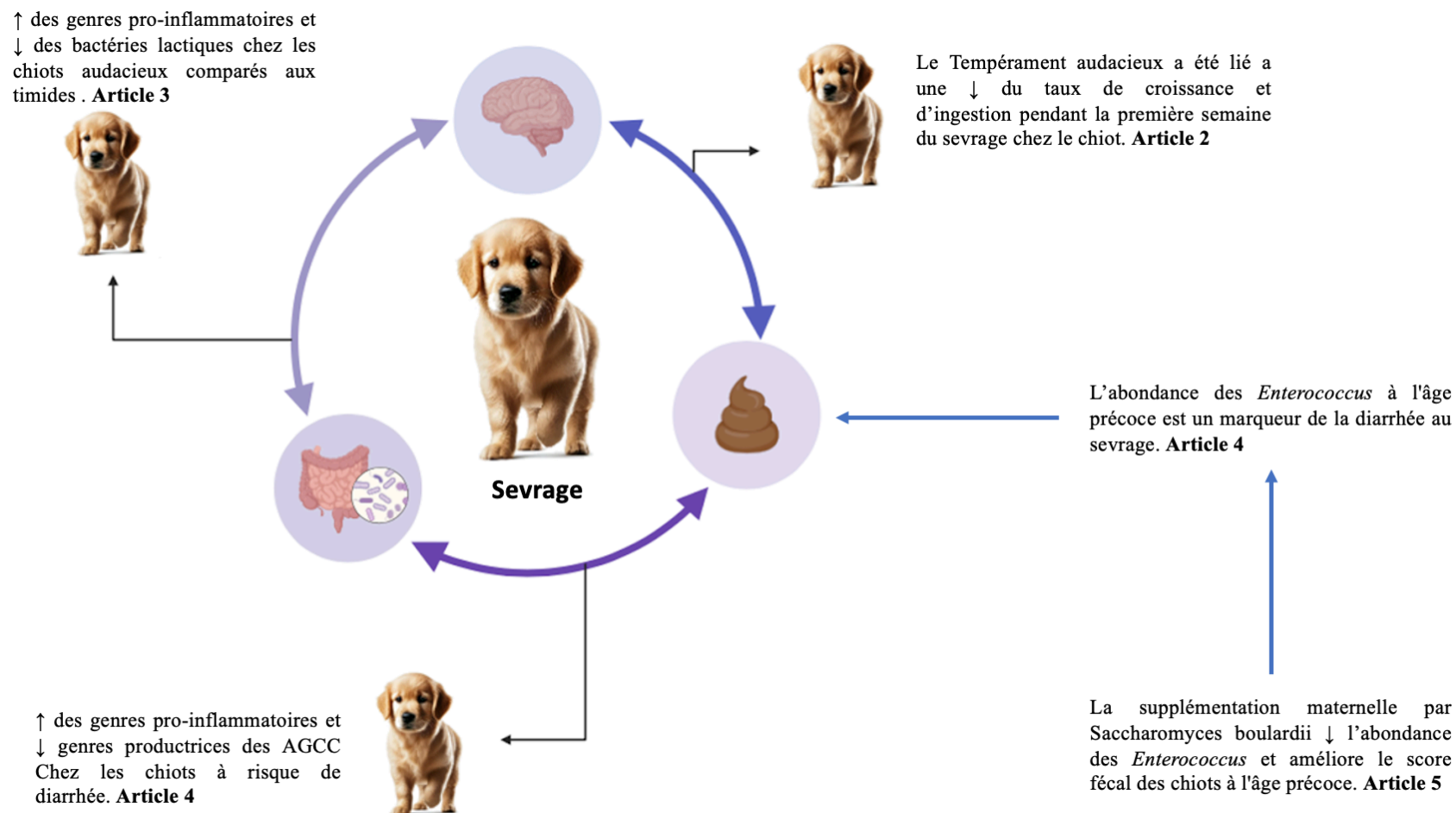
Enfin, une transmission directe du probiotique de la mère aux chiots est également envisageable. Certaines études menées chez la souris, le rat et le porc ont détecté la présence du probiotique dans les fèces de la portée après avoir supplémenté la mère. Par exemple, (Buddington et al., 2010) ont administré des souches de *Lactobacillus acidophilus* et *Bifidobacterium lactis* à des femelles gestantes de souris, rats et truies ; ils ont ensuite retrouvé ces souches dans les fèces de leurs petits à 14 jours après la naissance, respectivement chez 22 %, 33 % et 75 % des cas pour *L. acidophilus*, et chez 30 % (souris) et 80 % (porcelets) pour *B. lactis*. Ces résultats confirment un transfert vertical du probiotique, probablement facilité par le contact avec le microbiote vaginal et fécal maternel. Une hypothèse similaire mériterait d'être explorée chez le chien, notamment en évaluant la présence de SB dans les selles néonatales par PCR après supplémentation maternelle.

Par ailleurs, une méta-analyse récente a mis en évidence les bénéfices de la supplémentation directe en SB chez les nourrissons, avec une réduction significative du risque de gastroentérites pédiatriques (McFarland & Li, 2025). Dans ce contexte, il serait également pertinent de s'interroger sur l'impact potentiel d'une supplémentation directe des chiots nouveau-nés. Notre étude a en effet montré que les toutes premières heures de vie sont marquées par une évolution rapide du microbiote fécal, avec des changements significatifs détectables dès 8 heures après la naissance. Une augmentation notable de l'abondance des anaérobies facultatifs tels que les *Pasteurellaceae* (*Haemophilus* et *Frederiksenia*), ainsi qu'une apparition progressive des bactéries anaérobies strictes telles que *Clostridium sensu stricto* I et certaines *Peptostreptococcaceae* (*Terrisporobacter*, *Romboutsia*) ont été observées (**Annexes thèse ; Figure 1, Poster IPC2025**). Ces premières phases de colonisation intestinale pourraient donc constituer une fenêtre d'intervention intéressante pour moduler le développement initial du microbiote par l'administration directe de probiotiques aux chiots. Une telle stratégie pourrait ouvrir la voie à des actions ciblées précoces en matière de prévention des troubles digestifs au sevrage.

De plus, nos résultats montrent que la supplémentation maternelle en SB modifie le métabolome sérique et urinaire des chiots dès le deuxième jour de vie, notamment en améliorant le métabolisme azoté. Cette modulation semble orienter les voies métaboliques vers une production de proline associée à une augmentation de taux de croissance précoce des chiots. Or, le métabolome est reconnu pour être associé avec la composition du microbiote intestinal (Visconti et al., 2019). Cette hypothèse mériterait d'être confirmée sur une cohorte plus large, afin de mieux comprendre le lien entre le métabolome néonatal et le développement du microbiote du chiot.

En se basant sur l'ensemble de ces résultats (**Figure 1.C7**), nous formulons l'hypothèse que la supplémentation maternelle en SB réduisant à l'âge précoce l'abondance des *Enterococcus*, améliorera l'environnement du microbiote intestinal (notamment le pH) favorisant un entérotipe bactérien avec un risque de survenue de diarrhée après le sevrage plus faible (**Figure 2.C7**).

Figure 1.7C : Schéma synthétique des principaux résultats issus des différents chapitres de la thèse, illustrant les interactions entre le tempérament, le microbiote intestinal et la survenue de la diarrhée au moment du sevrage chez le chiot, ainsi que l'impact de la supplémentation maternelle en *Saccharomyces boulardii* sur le microbiote intestinal et la santé digestive précoce des chiots. AGCC : Acides gras à chaîne courte



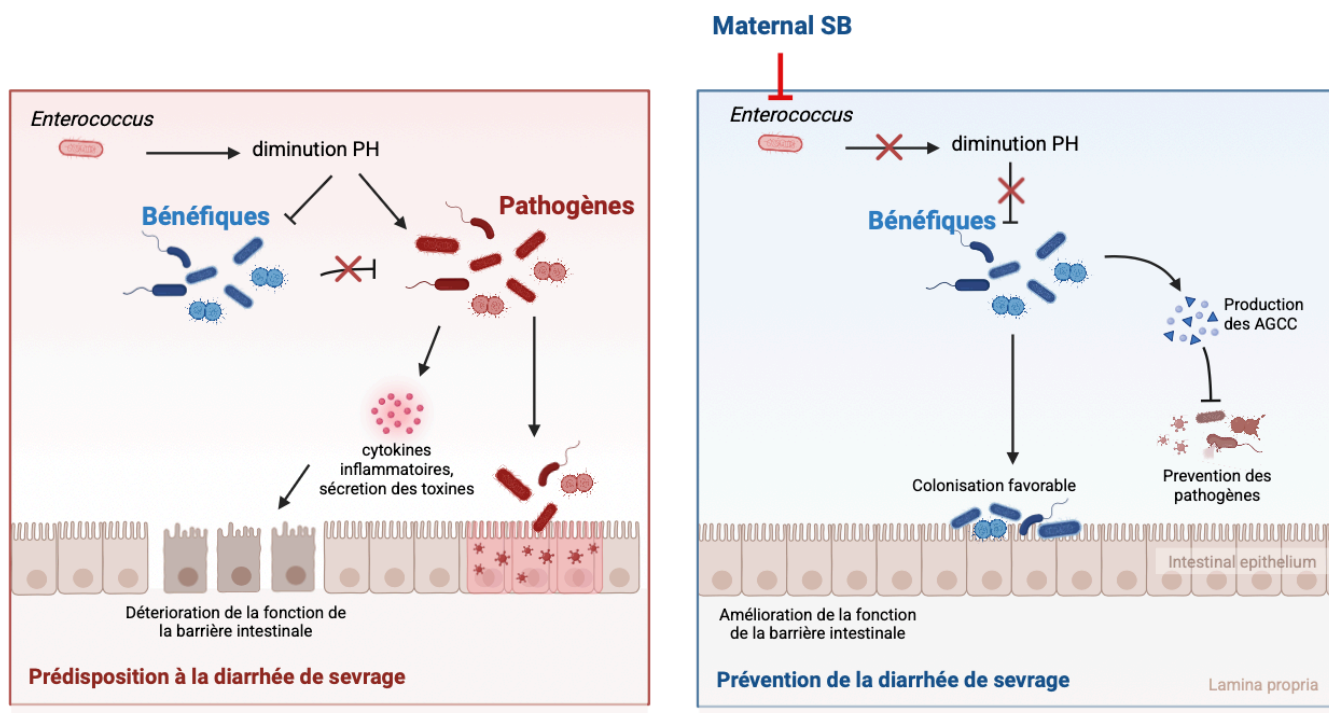


Figure 2.7C : Schéma hypothétique illustrant le rôle prédisposant d'*Enterococcus* dans la diarrhée du sevrage chez le chiot, ainsi que l'effet préventif potentiel de la supplémentation maternelle en *Saccharomyces boulardii* (SB). À gauche : une abondance plus élevée d'*Enterococcus* en période néonatale conduit à une baisse du pH intestinal, défavorable à l'implantation de bactéries bénéfiques et favorisant la prolifération de bactéries pathogènes. Cela entraîne une inflammation intestinale, une altération de la barrière épithéliale et une susceptibilité accrue à la diarrhée au sevrage. À droite : la supplémentation maternelle en SB limite la colonisation par *Enterococcus*, favorise l'implantation de bactéries bénéfiques productrices d'acides gras à chaînes courtes (AGCC), et améliore la fonction barrière intestinale, contribuant ainsi à la prévention de la diarrhée du sevrage.

Pour répondre à l'hypothèse selon laquelle les *Enterococcus* modifient le pH intestinal et influencent l'évolution du microbiote, ainsi que pour évaluer l'effet potentiel de la supplémentation maternelle en SB sur ce processus, il serait intéressant de mesurer précisément le pH intestinal, notamment à l'aide de capsules spécifiques (Warrit et al., 2017). Parallèlement, il conviendrait de quantifier les espèces d'*Enterococcus*, par qPCR, afin de tenter d'établir une corrélation entre leur abondance et les variations de pH observées. Une analyse approfondie de la composition du microbiote par séquençage 16S rRNA permettrait également d'examiner comment ces fluctuations de pH et la présence d'*Enterococcus* pourraient influencer la dynamique bactérienne globale et le risque de la diarrhée au sevrage. Au vu de l'effet observé de la supplémentation sur l'abondance des *Enterococcus*, il serait essentiel d'investiguer le mécanisme d'action des levures. Il est possible que la supplémentation maternelle en SB modifie la composition du lait, en altérant par exemple la composition en vitamines, oligosaccharides, acides gras

ou peptides bioactifs, ce qui pourrait ensuite influencer la colonisation microbienne néonatale ainsi que l'environnement digestif du chiot. Dans cette perspective, il serait essentiel d'investiguer le mécanisme d'action des levures, notamment par réalisation d'analyse métabolique et lipidomique du lait maternel et évaluation de son lien avec le microbiote des nouveau-nés. Cette approche permettrait de mieux comprendre les interactions microbiennes impliquées, et de proposer une formulation optimisée à base de levures, accompagnée de recommandations adaptées pour leur usage pratique sur le terrain comme stratégie de prévention des troubles digestifs

Limites de l'étude

Malgré les résultats obtenus, notre étude présente quelques limites qu'il convient de considérer. Tout d'abord, la première phase de notre étude a été menée dans un contexte expérimental standardisé, au sein de la Toulouse GOLD Cohort, composée uniquement de chiots Golden Retriever. Cette cohorte présente de nombreux atouts, notamment une homogénéité génétique, un contrôle rigoureux de l'alimentation, de la socialisation et des conditions sanitaires, permettant de limiter les biais environnementaux. Toutefois, cette approche limite la représentativité des résultats, qui ne peuvent être généralisés à l'ensemble de l'espèce canine en raison des variations comportementales et microbiennes connues entre races. De plus, l'organisation logistique de l'étude nous a contraints à répartir les chiennes en plusieurs lots successifs, introduisant un effet lot qui a été démontré comme influençant la composition du microbiote fécal des chiots (**Article 3 et 4 – Chapitre 5**). Pour élargir les conditions d'observation, une seconde cohorte a été constituée dans un élevage commercial, plus proche des pratiques de terrain, incluant quatre races et une plus grande diversité sanitaire. Malgré cette hétérogénéité, l'alimentation et la prise en charge des animaux étaient standardisées, assurant une certaine cohérence dans l'analyse des effets de la supplémentation probiotique. En termes d'effectifs, notre étude mobilise un nombre de chiots relativement important au regard des standards de la littérature en recherche vétérinaire, souvent limités à 50 à 77 individus par groupe (Berry et al., 2019; Bertero et al., 2024; Blake et al., 2020; Kajdič et al., 2021). Toutefois, malgré ces effectifs, certaines comparaisons statistiques manquent de puissance, notamment pour établir des modèles prédictifs robustes. Nos calculs initiaux, réalisés via BiostatTGV, ont estimé qu'un effectif d'environ 100 chiots serait nécessaire pour détecter une différence de 10 % de prévalence de diarrhée avec une puissance de 80 %. Or, nos analyses ont révélé une grande variabilité interindividuelle du microbiote, et notamment un pouvoir discriminant non négligeable vis-à-vis des entérotypes, suggérant que les effectifs nécessaires doivent être réévalués en se basant sur des performances des modèles de classification. D'après nos simulations basées sur la capacité du microbiote précoce à prédire l'enterotype au sevrage (**Article 4 Annexe ii– Chapitre 5**), un effectif d'environ 200 chiots serait requis pour atteindre un taux de réussite de classification d'environ 80 %. Dans cette perspective, la TGC constitue une ressource unique pour les études futures. Par ailleurs, la méthode de séquençage haut débit du gène 16S rRNA, bien qu'efficace pour explorer la

composition globale du microbiote et identifier des taxa associés à la diarrhée du sevrage ainsi que des marqueurs microbiens précoces, ne permet ni la quantification absolue des micro-organismes ni une identification à l'échelle des espèces. Cette limite technique restreint notre capacité à définir des seuils d'abondance critiques ou à cibler les espèces spécifiques impliquées dans le risque digestif. Des approches complémentaires comme la qPCR ciblée ou le séquençage métagénomique shotgun devront être mobilisées dans de futures études pour confirmer et approfondir ces résultats.

Chapitre 8

Conclusion générale

Le sevrage chez le chiot est un événement critique sur le plan de la santé digestive. Cette phase, marquée par d'importants changements nutritionnels et sociaux, coïncide souvent avec l'apparition des troubles digestifs, notamment la diarrhée. Fréquente en élevage, cette dernière peut compromettre la croissance, le bien-être et la socialisation des chiots. Comprendre les facteurs associés à ces troubles digestifs est donc essentiel pour améliorer la santé des jeunes chiens et prévenir les complications à court et long terme. Nos résultats suggèrent que la diarrhée du sevrage est associée au microbiote fécal, mais pas au tempérament des chiots. Certaines signatures microbiennes présentes dès les premières semaines de vie pourraient permettre d'anticiper le risque de troubles digestifs au sevrage. Enfin, la supplémentation maternelle en probiotiques pendant la gestation et la lactation constitue une stratégie prometteuse pour moduler positivement le microbiote et améliorer la santé digestive chez les chiots. Cette approche pourrait ainsi représenter un levier prometteur pour des applications pratiques en élevage, visant à améliorer la santé des chiots dès les premiers stades de vie.

L'impact du microbiote précoce sur la santé constitue un champ de recherche vaste et en pleine expansion. Si cette étude a permis d'apporter de premières données sur l'association entre le microbiote précoce et la survenue de la diarrhée du sevrage chez le chiot, les mécanismes sous-jacents restent à préciser. En particulier, le rôle des *Enterococcus* dans la modulation du pH intestinal et l'évolution du microbiote associée à la diarrhée au sevrage mérite une attention approfondie. Par ailleurs, l'effet de la supplémentation maternelle en probiotiques sur la prévention de ces troubles digestifs requiert une exploration détaillée, notamment pour comprendre comment elle agit sur le microbiote des chiots. Les résultats obtenus dans cette étude apportent ainsi des éléments de réponse à des questions clés, et pourraient contribuer à améliorer la santé digestive et le bien-être des chiots durant cette période critique.

Poster 1: Early dynamics of the gut microbiota in Golden Retriever puppies during the first 12 hours of life: A preliminary study

I. Bendahmane^{1,2}, M. Rotter¹, A. Meynadier², and H. Mila¹

¹NeoCare, Université de Toulouse, ENVT, Toulouse, France

²GenPhySE, Université de Toulouse, INRAE, ENVT, Castanet Tolosan, France

INTRODUCTION AND AIM. The early hours of life represent a critical period for the dynamic development of the gut microbiota (GM). At birth, the newborn's intestinal tract contains oxygen, creating an environment that favors the initial colonization by aerobic bacteria. This early colonization is essential, as these bacteria consume the available oxygen, facilitating the establishment of facultative and strict anaerobic bacteria later on (Garrigues et al., 2023). Despite the fundamental importance of this process, its detailed progression remains poorly understood and understudied in dogs and other mammals.

MATERIALS AND METHODS. In this preliminary study, we aimed to assess the evolution of the gut microbiota in Golden Retriever puppies during the first 12 hours of life. The study included 14 healthy puppies born vaginally from 3 dams. Rectal swabs were collected at birth (H0) and at 4 (H4), 8 (H8), and 12 hours (H12). Bacterial DNA was extracted, amplified (V3-V4 regions of 16S rRNA), and sequenced (Illumina MiSeq). Taxonomic affiliation used the 16S SILVA database (version 138.1 PINTAIL 100). Statistical analysis (ANCOM) evaluated changes in taxa at H4, H8, and H12 relative to H0, with p-values adjusted using the Holm method.

RESULTS. Four phyla were identified in the 56 collected fecal samples: *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Actinobacteria*. Altogether, 33 taxa were present at the family level, and 45 at the genus level. Between H0 and H12, a significant increase was observed in the abundances in 6 of those taxa ($p < 0.05$ for all comparisons; Fig.1). Among them, *Pasteurellaceae* (0% at H0, $16 \pm 29\%$ at H12), and specifically *Haemophilus* (1% at H0, $2 \pm 3\%$ at H12) and *Frederiksenia* (0% at H0, $3.31 \pm 8\%$ at H12) were facultative anaerobes. Whereas, *Clostridiaceae* (0% at H0; $16 \pm 29\%$ at H12) and mainly *Clostridium sensu stricto 1* (0% at H0, $13 \pm 29\%$ at H12), and *Peptostreptococcaceae* ($1 \pm 2\%$ at H0, $12 \pm 25\%$ at H12) and mainly *Terrisporobacter* (0% at H0, $3 \pm 7\%$ at H12) and *Romboutsia*, (0% at H0, $8 \pm 18\%$ at H12) were strict anaerobes (**Figure Annexes1**).

DISCUSSION. The results confirm the rapid shift in GM during the first hours after birth, from an oxygen-rich to oxygen-poor environment, favoring colonization by strict anaerobes and facultative anaerobes. These findings emphasize the importance of these early dynamics in facilitating the transition to a more mature microbiota. Further studies with larger sample sizes and longer follow-up periods are needed to fully understand the functional implications of these microbial changes in the development of gastrointestinal dysbiosis, being most probably responsible for numerous chronic conditions in adulthood.

Figure Annexes 1: Poster représentant l'évolution du microbiote fécal pendant les 12 premières heures de vie chez le chiot Golden Retriever. Les résultats ont été présentés au congrès IPC2025 à Athènes, Grèce.

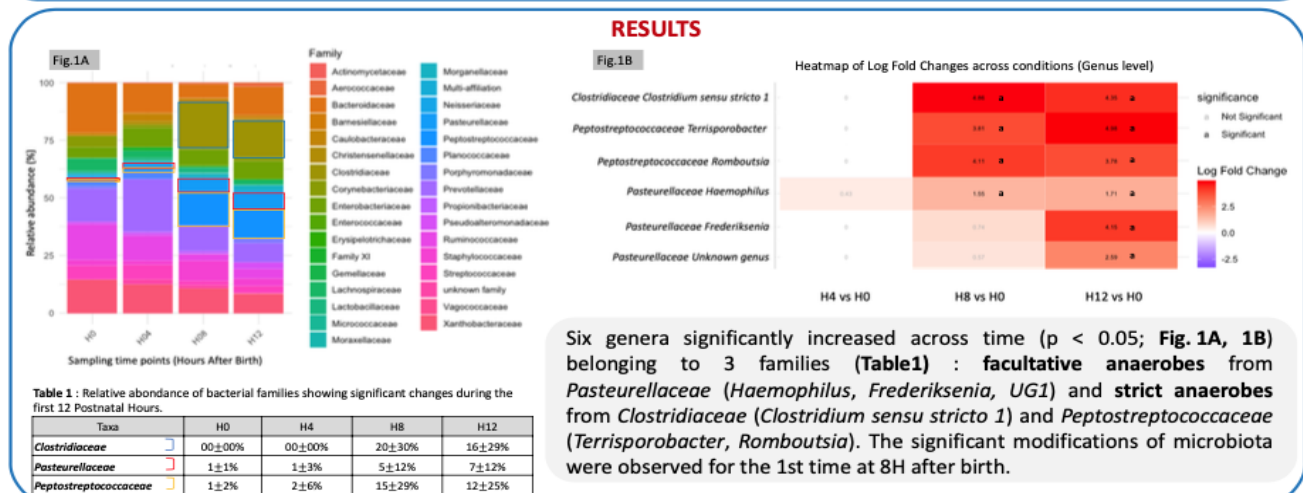
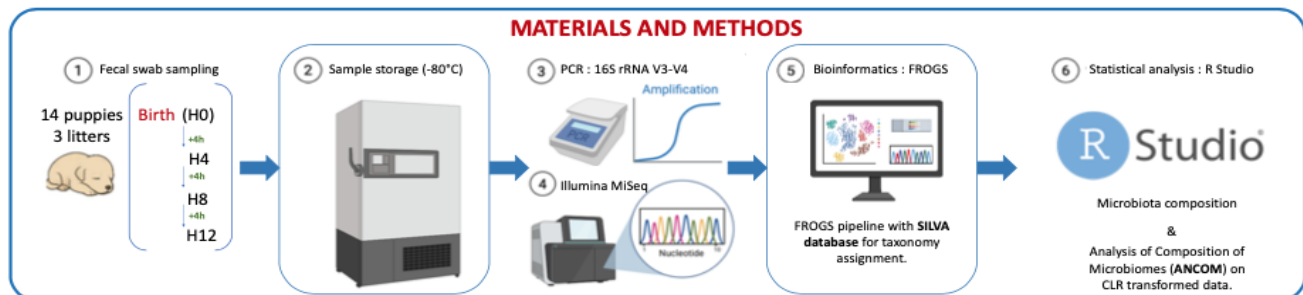


Early Dynamics of the Gut Microbiota in Golden Retriever Puppies During the First 12 Hours of Life: A Preliminary Study

I.BENDAHDANE^{1,2}, M.ROTTER¹, A.MEYNADIER², H.MILA¹

¹NeoCare, ENVT, Université de Toulouse, France - ²GenPhySE, Université de Toulouse, INRAE, ENVT, F-31326, Castanet Tolosan, France.

- **CONTEXT :** At birth, the neonatal gut contains oxygen, creating an environment favorable to the initial colonization by aerobic and facultative anaerobic microbes. These bacteria as well as enterocytes consume progressively the oxygen, allowing a progressive shift toward anaerobic conditions and the colonization by facultatif and strict anaerobes^[1]. While this ecological succession is considered to influence long-term health^[2], its early dynamics in puppies but also other species remain poorly described.
- **AIM :** To characterize the evolution of the gut microbiota in Golden Retriever puppies during the first 12 hours of life



DISCUSSION

This study, although conducted on a limited population, confirms a rapid and structured shift in gut microbiota composition during the first 12 postnatal hours. The progressive transition from an oxygen-rich environment at birth to an anaerobic one favors the early colonization by facultative and strict anaerobes, such as *Clostridiaceae*, *Pasteurellaceae* and *Peptostreptococcaceae*. However, the most significant changes were observed only at 8h after birth suggesting the potential role of colostrum in the modification of the gut environment and microbiota.

The identification of significantly evolving taxa within just a few hours post-birth underscores the critical window of microbial colonization immediately after delivery, offering a potential opportunity for modulation in unfavorable conditions.

[1] Composition and evolution of the gut microbiota of growing puppies is impacted by their birth weight. Scientific Reports 2023. Q. Garrigues et al.

[2] The long-term health effects of neonatal microbial flora. Curr Opin Allergy Clin Immunol 2009. E. Conroy et al.



Corresponding author
Ilyas BENDAHDANE
3rd year Ph.D student
Ilyasbendahmane@hotmail.com



Références

- Alexopoulos, C., Georgoulakis, I. E., Tzivara, A., Kyriakis, C. S., Govaris, A., & Kyriakis, S. C. (2004). Field Evaluation of the Effect of a Probiotic-containing *Bacillus licheniformis* and *Bacillus subtilis* Spores on the Health Status, Performance, and Carcass Quality of Grower and Finisher Pigs. *Journal of Veterinary Medicine Series A*, 51(6), 306–312.
<https://doi.org/10.1111/j.1439-0442.2004.00637.x>
- American Gastroenterological Association medical position statement: Guidelines for the evaluation and management of chronic diarrhea☆☆. (1999). *Gastroenterology*, 116(6), 1461–1463.
[https://doi.org/10.1016/S0016-5085\(99\)70512-3](https://doi.org/10.1016/S0016-5085(99)70512-3)
- Appleby, D. L., Bradshaw, J. W. S., & Casey, R. A. (2002). Relationship between aggressive and avoidance behaviour by dogs and their experience in the first six months of life. *Veterinary Record*, 150(14), 434–438. <https://doi.org/10.1136/vr.150.14.434>
- Azad, M. B., Konya, T., Guttman, D. S., Field, C. J., Sears, M. R., HayGlass, K. T., Mandhane, P. J., Turvey, S. E., Subbarao, P., Becker, A. B., Scott, J. A., Kozyrskyj, A. L., & the CHILd Study Investigators. (2015). Infant gut microbiota and food sensitization: Associations in the first year of life. *Clinical & Experimental Allergy*, 45(3), 632–643.
<https://doi.org/10.1111/cea.12487>
- Benyacoub, J., Cavadini, C., Sauthier, T., Schiffrin, E. J., von der Weid, T., Czarnecki-Maulden, G. L., & Anderson, R. E. (2003). Supplementation of Food with *Enterococcus faecium* (SF68) Stimulates Immune Functions in Young Dogs. *The Journal of Nutrition*, 133(4), 1158–1162.
<https://doi.org/10.1093/jn/133.4.1158>
- Berry, A. S. F., Kelly, B. J., Barnhart, D., Kelly, D. J., Beiting, D. P., Baldassano, R. N., & Redding, L. E. (2019). Gut microbiota features associated with *Clostridioides difficile* colonization in puppies. *PLOS ONE*, 14(8), e0215497. <https://doi.org/10.1371/journal.pone.0215497>

- Bertero, A., Banchi, P., Del Carro, A., Corrà, M., Colitti, B., Van Soom, A., Bertolotti, L., & Rota, A. (2024). Meconium microbiota in naturally delivered canine puppies. *BMC Veterinary Research*, 20(1). <https://doi.org/10.1186/s12917-024-04225-2>
- Blake, A. B., Cigarroa, A., Klein, H. L., Khattab, M. R., Keating, T., Van De Coevering, P., Lidbury, J. A., Steiner, J. M., & Suchodolski, J. S. (2020a). Developmental stages in microbiota, bile acids, and clostridial species in healthy puppies. *Journal of Veterinary Internal Medicine*, 34(6), 2345–2356. <https://doi.org/10.1111/jvim.15928>
- Blake, A. B., Cigarroa, A., Klein, H. L., Khattab, M. R., Keating, T., Van De Coevering, P., Lidbury, J. A., Steiner, J. M., & Suchodolski, J. S. (2020b). Developmental stages in microbiota, bile acids, and clostridial species in healthy puppies. *Journal of Veterinary Internal Medicine*, 34(6), 2345–2356. <https://doi.org/10.1111/jvim.15928>
- Bray, E. E., Sammel, M. D., Cheney, D. L., Serpell, J. A., & Seyfarth, R. M. (2017). Effects of maternal investment, temperament, and cognition on guide dog success. *Proceedings of the National Academy of Sciences*, 114(34), 9128–9133. <https://doi.org/10.1073/pnas.1704303114>
- Breit, S., Kupferberg, A., Rogler, G., & Hasler, G. (2018). Vagus Nerve as Modulator of the Brain–Gut Axis in Psychiatric and Inflammatory Disorders. *Frontiers in Psychiatry*, 9, 44. <https://doi.org/10.3389/fpsy.2018.00044>
- Buchi, É., & Schweickard, W. (Eds.). (2014). *Dictionnaire Étymologique Roman (DÉRom): Genèse, méthodes et résultats*. DE GRUYTER. <https://doi.org/10.1515/9783110313482>
- Buddington, R. K., Elnif, J., Malo, C., & Donahoo, J. B. (2003). Activities of gastric, pancreatic, and intestinal brush-border membrane enzymes during postnatal development of dogs. *American Journal of Veterinary Research*, 64(5), 627–634. <https://doi.org/10.2460/ajvr.2003.64.627>
- Buddington, R. K., Williams, C. H., Kostek, B. M., Buddington, K. K., & Kullen, M. J. (2010). Maternal-to-Infant Transmission of Probiotics: Concept Validation in Mice, Rats, and Pigs. *Neonatology*, 97(3), 250–256. <https://doi.org/10.1159/000253756>
- Caldji, C., Tannenbaum, B., Sharma, S., Francis, D., Plotsky, P. M., & Meaney, M. J. (1998). Maternal care during infancy regulates the development of neural systems mediating the expression of

- fearfulness in the rat. *Proceedings of the National Academy of Sciences*, 95(9), 5335–5340.
<https://doi.org/10.1073/pnas.95.9.5335>
- Carabotti, M., Scirocco, A., Maselli, M. A., & Severi, C. (2015). The gut-brain axis: Interactions between enteric microbiota, central and enteric nervous systems. *Annals of Gastroenterology*, 28(2), 203–209.
- Caron-Lormier, G., England, G. C. W., Green, M. J., & Asher, L. (2016). Using the incidence and impact of health conditions in guide dogs to investigate healthy ageing in working dogs. *The Veterinary Journal*, 207, 124–130. <https://doi.org/10.1016/j.tvjl.2015.10.046>
- Chapillon, P., Patin, V., Roy, V., Vincent, A., & Caston, J. (2002). Effects of pre- and postnatal stimulation on developmental, emotional, and cognitive aspects in rodents: A review. *Developmental Psychobiology*, 41(4), 373–387. <https://doi.org/10.1002/dev.10066>
- Chaudhuri, A., Goddard, E. A., Green, M., & Ardura, M. I. (2021). Diarrhea in the pediatric solid organ transplantation recipient: A multidisciplinary approach to diagnosis and management. *Pediatric Transplantation*, 25(2), e13886. <https://doi.org/10.1111/petr.13886>
- Che, J., Ye, S., Liu, B., Deng, Y., Chen, Q., Ge, C., Liu, G., & Wang, J. (2016). Effects of *Brevibacillus brevis* FJAT-1501-BPA on growth performance, faecal microflora, faecal enzyme activities and blood parameters of weaned piglets. *Antonie van Leeuwenhoek*, 109(12), 1545–1553. <https://doi.org/10.1007/s10482-016-0756-8>
- Cheng, Z., Zhou, S.-T., Zhang, X.-H., Fu, Q., Yang, Y., Ji, W.-B., & Liu, H.-G. (2023). Effects of early intermittent maternal separation on behavior, physiological, and growth performance in piglets. *Journal of Animal Science*, 101, skad122. <https://doi.org/10.1093/jas/skad122>
- Chesney, C. J. (2001). Systematic review of evidence for the prevalence of food sensitivity in dogs. *Veterinary Record*, 148(14), 445–448. <https://doi.org/10.1136/vr.148.14.445>
- Chorell, E., Karlsson Videhult, F., Hernell, O., Antti, H., & West, C. E. (2013). Impact of probiotic feeding during weaning on the serum lipid profile and plasma metabolome in infants. *British Journal of Nutrition*, 110(1), 116–126. <https://doi.org/10.1017/S0007114512004618>

- Choudhury, R., Middelkoop, A., Bolhuis, J. E., & Kleerebezem, M. (2022). Exploring the association between microbiota and behaviour in suckling piglets. *Scientific Reports*, 12(1), 12322. <https://doi.org/10.1038/s41598-022-16259-3>
- Chu, D. M., Meyer, K. M., Prince, A. L., & Aagaard, K. M. (2016). Impact of maternal nutrition in pregnancy and lactation on offspring gut microbial composition and function. *Gut Microbes*, 7(6), 459–470. <https://doi.org/10.1080/19490976.2016.1241357>
- Collier, C. T., Carroll, J. A., Ballou, M. A., Starkey, J. D., & Sparks, J. C. (2011). Oral administration of *Saccharomyces cerevisiae* boulardii reduces mortality associated with immune and cortisol responses to *Escherichia coli* endotoxin in pigs1. *Journal of Animal Science*, 89(1), 52–58. <https://doi.org/10.2527/jas.2010-2944>
- Crespo-Piazuelo, D., Gardiner, G. E., Ranjitkar, S., Bouwhuis, M. A., Ham, R., Phelan, J. P., Marsh, A., & Lawlor, P. G. (2022). Maternal supplementation with *Bacillus altitudinis* spores improves porcine offspring growth performance and carcass weight. *British Journal of Nutrition*, 127(3), 403–420. <https://doi.org/10.1017/S0007114521001203>
- Cryan, J. F., & Dinan, T. G. (2012). Mind-altering microorganisms: The impact of the gut microbiota on brain and behaviour. *Nature Reviews Neuroscience*, 13(10), 701–712. <https://doi.org/10.1038/nrn3346>
- Dinler Ay, C. (2022). Neutrophil to lymphocyte ratio as a prognostic biomarker in puppies with acute diarrhea. *Journal of Veterinary Emergency and Critical Care*, 32(1), 83–89. <https://doi.org/10.1111/vec.13159>
- Dong, Y., Han, Y., Wang, Z., Qin, Z., Yang, C., Cao, J., & Chen, Y. (2017a). Role of serotonin on the intestinal mucosal immune response to stress-induced diarrhea in weaning mice. *BMC Gastroenterology*, 17(1), 82. <https://doi.org/10.1186/s12876-017-0634-5>
- Dong, Y., Han, Y., Wang, Z., Qin, Z., Yang, C., Cao, J., & Chen, Y. (2017b). Role of serotonin on the intestinal mucosal immune response to stress-induced diarrhea in weaning mice. *BMC Gastroenterology*, 17(1), 82. <https://doi.org/10.1186/s12876-017-0634-5>
- Downes, J., Dewhirst, F. E., Tanner, A. C. R., & Wade, W. G. (2013). Description of *Alloprevotella* rava gen. Nov., sp. Nov., isolated from the human oral cavity, and reclassification of

- Prevotella tanneriae Moore et al. 1994 as Alloprevotella tanneriae gen. Nov., comb. Nov. *International Journal of Systematic and Evolutionary Microbiology*, 63(Pt_4), 1214–1218.
<https://doi.org/10.1099/ijs.0.041376-0>
- Elfenbein, H. A., Rosso, L. D., McCowan, B., & Capitanio, J. P. (2016). Effect of Indoor Compared with Outdoor Location during Gestation on the Incidence of Diarrhea in Indoor-Reared Rhesus Macaques (*Macaca mulatta*). *Journal of the American Association for Laboratory Animal Science: JAALAS*, 55(3), 277–290.
- Elliot, O., & Scott, J. P. (1961a). The Development of Emotional Distress Reactions to Separation, in Puppies. *The Journal of Genetic Psychology*, 99(1), 3–22.
<https://doi.org/10.1080/00221325.1961.10534386>
- Elliot, O., & Scott, J. P. (1961b). The Development of Emotional Distress Reactions to Separation, in Puppies. *The Journal of Genetic Psychology*, 99(1), 3–22.
<https://doi.org/10.1080/00221325.1961.10534386>
- Enquête NeoCare “Sevrage.”* (n.d.). <https://neocare.pro/wp-content/uploads/2023/05/Enquete-NeoCare-4-vente.pdf>
- Ephraim, E., Jackson, M. I., Cochrane, C.-Y., Brockel, C. E., & Jewell, D. E. (2020). Developmental Changes in the Canine Gut Microbiome during Breastfeeding, Weaning, Socialization and Maturation to Adulthood. *The FASEB Journal*, 34(S1), 1–1.
<https://doi.org/10.1096/fasebj.2020.34.s1.02259>
- Farshim, P., Walton, G., Chakrabarti, B., Givens, I., Saddy, D., Kitchen, I., R. Swann, J., & Bailey, A. (2016). Maternal Weaning Modulates Emotional Behavior and Regulates the Gut-Brain Axis. *Scientific Reports*, 6(1), 21958. <https://doi.org/10.1038/srep21958>
- Fava, G. A., & Pavan, L. (1976). Large Bowel Disorders. *Psychotherapy and Psychosomatics*, 27(2), 100–105. <https://doi.org/10.1159/000287003>
- Fischer, G. H., Hashmi, M. F., & Paterek, E. (2025). *Campylobacter Infection*. In *StatPearls*. StatPearls Publishing. <http://www.ncbi.nlm.nih.gov/books/NBK537033/>
- Foster, J. A., Rinaman, L., & Cryan, J. F. (2017). Stress & the gut-brain axis: Regulation by the microbiome. *Neurobiology of Stress*, 7, 124–136. <https://doi.org/10.1016/j.ynstr.2017.03.001>

- Foyer, P., Wilsson, E., Wright, D., & Jensen, P. (2013). Early experiences modulate stress coping in a population of German shepherd dogs. *Applied Animal Behaviour Science*, 146(1–4), 79–87.
<https://doi.org/10.1016/j.applanim.2013.03.013>
- Garrigues, Q., Apper, E., Rodiles, A., Rovere, N., Chastant, S., & Mila, H. (2023). Composition and evolution of the gut microbiota of growing puppies is impacted by their birth weight. *Scientific Reports*, 13(1), 14717. <https://doi.org/10.1038/s41598-023-41422-9>
- Garrigues, Q., Mugnier, A., Chastant, S., Sicard, F., Martin, J.-C., Svilar, L., Castex, M., Ramis-Vidal, M. G., Rovere, N., Michaud, L., David, P., Mansalier, E., Rodiles, A., Mila, H., & Apper, E. (2024a). The supplementation of female dogs with live yeast *Saccharomyces cerevisiae* var. Boulardii CNCM I-1079 acts as gut stabilizer at whelping and modulates immunometabolic phenotype of the puppies. *Frontiers in Nutrition*, 11, 1366256.
<https://doi.org/10.3389/fnut.2024.1366256>
- Garrigues, Q., Mugnier, A., Chastant, S., Sicard, F., Martin, J.-C., Svilar, L., Castex, M., Ramis-Vidal, M. G., Rovere, N., Michaud, L., David, P., Mansalier, E., Rodiles, A., Mila, H., & Apper, E. (2024b). The supplementation of female dogs with live yeast *Saccharomyces cerevisiae* var. Boulardii CNCM I-1079 acts as gut stabilizer at whelping and modulates immunometabolic phenotype of the puppies. *Frontiers in Nutrition*, 11, 1366256.
<https://doi.org/10.3389/fnut.2024.1366256>
- Gaultier, E., Bonnafous, L., Vienet-Legu  , D., Falewee, C., Bougrat, L., Lafont-Lecuelle, C., & Pageat, P. (2008). Efficacy of dog-appeasing pheromone in reducing stress associated with social isolation in newly adopted puppies. *Veterinary Record*, 163(3), 73–80.
<https://doi.org/10.1136/vr.163.3.73>
- Gaykema, R. P. A., Goehler, L. E., & Lyte, M. (2004). Brain response to cecal infection with *Campylobacter jejuni*: Analysis with Fos immunohistochemistry. *Brain, Behavior, and Immunity*, 18(3), 238–245. <https://doi.org/10.1016/j.bbi.2003.08.002>
- Gerbaux, A., Luce. (n.d.). *Pathologie de groupe en   levage canin fran  ais: Dominantes et impacts*. Ecole nationale v  t  rinaire d’Alfort.

- Gibson, G. R., Hutkins, R., Sanders, M. E., Prescott, S. L., Reimer, R. A., Salminen, S. J., Scott, K., Stanton, C., Swanson, K. S., Cani, P. D., Verbeke, K., & Reid, G. (2017). Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nature Reviews Gastroenterology & Hepatology*, 14(8), 491–502. <https://doi.org/10.1038/nrgastro.2017.75>
- Godsall, S. A., Clegg, S. R., Stavisky, J. H., Radford, A. D., & Pinchbeck, G. (2010). Epidemiology of canine parvovirus and coronavirus in dogs presented with severe diarrhoea to PDSA PetAid hospitals. *Veterinary Record*, 167(6), 196–201. <https://doi.org/10.1136/vr.c3095>
- Goehler, L. E., Park, S. M., Opitz, N., Lyte, M., & Gaykema, R. P. A. (2008). *Campylobacter jejuni* infection increases anxiety-like behavior in the holeboard: Possible anatomical substrates for viscerosensory modulation of exploratory behavior. *Brain, Behavior, and Immunity*, 22(3), 354–366. <https://doi.org/10.1016/j.bbi.2007.08.009>
- Gophna, U., Konikoff, T., & Nielsen, H. B. (2017). *Oscillospira* and related bacteria – From metagenomic species to metabolic features. *Environmental Microbiology*, 19(3), 835–841. <https://doi.org/10.1111/1462-2920.13658>
- Gottlieb, D. H., Del Rosso, L., Sheikhi, F., Gottlieb, A., McCowan, B., & Capitanio, J. P. (2018). Personality, environmental stressors, and diarrhea in *Rhesus macaques*: An interactionist perspective. *American Journal of Primatology*, 80(12), e22908. <https://doi.org/10.1002/ajp.22908>
- Grellet, A. (n.d.). *Weaning diarrhea in puppies*. <https://academy.royalcanin.com/en/veterinary/weaning-diarrhea-in-puppies>
- Grellet, A. (2016). Weaning diarrhea in puppies. *Veterinary Focus*.
- Grellet, A., Chastant-Maillard, S., Robin, C., Feugier, A., Boogaerts, C., Boucraut-Baralon, C., Grandjean, D., & Polack, B. (2014). Risk factors of weaning diarrhea in puppies housed in breeding kennels. *Preventive Veterinary Medicine*, 117(1), 260–265. <https://doi.org/10.1016/j.prevetmed.2014.07.016>
- Grellet, A., Feugier, A., Chastant-Maillard, S., Carrez, B., Boucraut-Baralon, C., Casseleux, G., & Grandjean, D. (2012). Validation of a fecal scoring scale in puppies during the weaning

- period. *Preventive Veterinary Medicine*, 106(3–4), 315–323.
<https://doi.org/10.1016/j.prevetmed.2012.03.012>
- Grellet, A., Mila, H., & Chastant, S. (2017). *Parvovirus canin de type 2 chez le chiot*.
https://neocare.pro/wp-content/uploads/2023/06/ID_10_2017_Grellet_Parvovirus-canin-de-type-2-chez-le-chiot-De-nouvelles-donnees-sur-un-virus-classique-La-depeche-technique_compressed.pdf
- Grondin, J. A., Jamal, A., Mowna, S., Seto, T., & Khan, W. I. (2024). Interaction between Intestinal Parasites and the Gut Microbiota: Implications for the Intestinal Immune Response and Host Defence. *Pathogens*, 13(8), 608. <https://doi.org/10.3390/pathogens13080608>
- Gross, B., Garcia-Tapia, D., Riedesel, E., Ellinwood, N. M., & Jens, J. K. (2010). NORMAL CANINE BRAIN MATURATION AT MAGNETIC RESONANCE IMAGING: MRI Appearance of Normal Postnatal Canine Brain Maturation with Histology Correlation. *Veterinary Radiology & Ultrasound*, 51(4), 361–373. <https://doi.org/10.1111/j.1740-8261.2010.01681.x>
- Grzeškowiak, Ł., Grönlund, M.-M., Beckmann, C., Salminen, S., von Berg, A., & Isolauri, E. (2012). The impact of perinatal probiotic intervention on gut microbiota: Double-blind placebo-controlled trials in Finland and Germany. *Anaerobe*, 18(1), 7–13.
<https://doi.org/10.1016/j.anaerobe.2011.09.006>
- Guard, B. C., Barr, J. W., Reddivari, L., Klemashevich, C., Jayaraman, A., Steiner, J. M., Vanamala, J., & Suchodolski, J. S. (2015). Characterization of Microbial Dysbiosis and Metabolomic Changes in Dogs with Acute Diarrhea. *PLOS ONE*, 10(5), e0127259.
<https://doi.org/10.1371/journal.pone.0127259>
- Guard, B. C., Mila, H., Steiner, J. M., Mariani, C., Suchodolski, J. S., & Chastant-Maillard, S. (2017). Characterization of the fecal microbiome during neonatal and early pediatric development in puppies. *PLOS ONE*, 12(4), e0175718. <https://doi.org/10.1371/journal.pone.0175718>
- Guardini, G., Mariti, C., Bowen, J., Fatjó, J., Ruzzante, S., Martorell, A., Sighieri, C., & Gazzano, A. (2016). Influence of morning maternal care on the behavioural responses of 8-week-old

- Beagle puppies to new environmental and social stimuli. *Applied Animal Behaviour Science*, 181, 137–144. <https://doi.org/10.1016/j.applanim.2016.05.006>
- Henning, S. J. (1986). Development of the gastrointestinal tract. *Proceedings of the Nutrition Society*, 45(1), 39–44. <https://doi.org/10.1079/PNS19860033>
- Howell, T., King, T., & Bennett, P. (2015). Puppy parties and beyond: The role of early age socialization practices on adult dog behavior. *Veterinary Medicine: Research and Reports*, 143. <https://doi.org/10.2147/VMRR.S62081>
- Hsiao, E. Y., McBride, S. W., Hsien, S., Sharon, G., Hyde, E. R., McCue, T., Codelli, J. A., Chow, J., Reisman, S. E., Petrosino, J. F., Patterson, P. H., & Mazmanian, S. K. (2013). Microbiota Modulate Behavioral and Physiological Abnormalities Associated with Neurodevelopmental Disorders. *Cell*, 155(7), 1451–1463. <https://doi.org/10.1016/j.cell.2013.11.024>
- Hu, Y., Dun, Y., Li, S., Zhang, D., Peng, N., Zhao, S., & Liang, Y. (2015). Dietary *Enterococcus faecalis* LAB31 Improves Growth Performance, Reduces Diarrhea, and Increases Fecal *Lactobacillus* Number of Weaned Piglets. *PLOS ONE*, 10(1), e0116635. <https://doi.org/10.1371/journal.pone.0116635>
- Isolauri, E., Arvola, T., S  tas, Y., Moilanen, E., & Salminen, S. (2000). Probiotics in the management of atopic eczema. *Clinical & Experimental Allergy*, 30(11), 1605–1610. <https://doi.org/10.1046/j.1365-2222.2000.00943.x>
- Kajdi  , L., Plavec, T., Zdovc, I., Kalin, A., & Zako  sek Pipan, M. (2021). Impact of Type of Parturition on Colostrum Microbiota Composition and Puppy Survival. *Animals*, 11(7), 1897. <https://doi.org/10.3390/ani11071897>
- Kang, P., Toms, D., Yin, Y., Cheung, Q., Gong, J., De Lange, K., & Li, J. (2010). Epidermal Growth Factor-Expressing *Lactococcus lactis* Enhances Intestinal Development of Early-Weaned Pigs. *The Journal of Nutrition*, 140(4), 806–811. <https://doi.org/10.3945/jn.109.114173>
- Kantas, D., Papatsiros, V. G., Tassis, P. D., Giavasis, I., Bouki, P., & Tzika, E. D. (2015a). A feed additive containing *Bacillus toyonensis* (Toyocerin^{  }) protects against enteric pathogens in postweaning piglets. *Journal of Applied Microbiology*, 118(3), 727–738. <https://doi.org/10.1111/jam.12729>

- Kantas, D., Papatsiros, V. G., Tassis, P. D., Giavasis, I., Bouki, P., & Tzika, E. D. (2015b). A feed additive containing *Bacillus toyonensis* (Toyocerin[®]) protects against enteric pathogens in postweaning piglets. *Journal of Applied Microbiology*, 118(3), 727–738.
<https://doi.org/10.1111/jam.12729>
- Kim, H.-N., Yun, Y., Ryu, S., Chang, Y., Kwon, M.-J., Cho, J., Shin, H., & Kim, H.-L. (2018). Correlation between gut microbiota and personality in adults: A cross-sectional study. *Brain, Behavior, and Immunity*, 69, 374–385. <https://doi.org/10.1016/j.bbi.2017.12.012>
- Kirjavainen, P. V., Arvola, T., Salminen, S. J., & Isolauri, E. (2002). Aberrant composition of gut microbiota of allergic infants: A target of bifidobacterial therapy at weaning? *Gut*, 51(1), 51–55. <https://doi.org/10.1136/gut.51.1.51>
- Kyriakis, S. C., Tsiloyiannis, V. K., Vlemmas, J., Sarris, K., Tsinas, A. C., Alexopoulos, C., & Jansegers, L. (1999). The effect of probiotic LSP 122 on the control of post-weaning diarrhoea syndrome of piglets. *Research in Veterinary Science*, 67(3), 223–228.
<https://doi.org/10.1053/rvsc.1999.0308>
- Langley-Evans, S. C. (2009). Nutritional programming of disease: Unravelling the mechanism. *Journal of Anatomy*, 215(1), 36–51. <https://doi.org/10.1111/j.1469-7580.2008.00977.x>
- Law, K., Johnston, L. J., Urriola, P. E., & Gomez, A. (2024). Maternal Programming of Nursery Pig Performance and Gut Microbiome through Live Yeast Supplementation. *Animals*, 14(6), 910. <https://doi.org/10.3390/ani14060910>
- Li, Y., Guo, Y., Wen, Z., Jiang, X., Ma, X., & Han, X. (2018). Weaning Stress Perturbs Gut Microbiome and Its Metabolic Profile in Piglets. *Scientific Reports*, 8(1), 18068.
<https://doi.org/10.1038/s41598-018-33649-8>
- Li, Y., Xia, S., Jiang, X., Feng, C., Gong, S., Ma, J., Fang, Z., Yin, J., & Yin, Y. (2021). Gut Microbiota and Diarrhea: An Updated Review. *Frontiers in Cellular and Infection Microbiology*, 11. <https://doi.org/10.3389/fcimb.2021.625210>
- Liao, P., Yang, K., Huang, H., Xin, Z., Jian, S., Wen, C., He, S., Zhang, L., & Deng, B. (2023). Abrupt Dietary Change and Gradual Dietary Transition Impact Diarrheal Symptoms, Fecal

- Fermentation Characteristics, Microbiota, and Metabolic Profile in Healthy Puppies. *Animals*, 13(8), 1300. <https://doi.org/10.3390/ani13081300>
- Lindsay, S. R. (Ed.). (2001). *Handbook of Applied Dog Behavior and Training, Volume Two*. Iowa State University Press. <https://doi.org/10.1002/9780470376997>
- Lobo De Sá, F. D., Schulzke, J.-D., & Bücker, R. (2021). Diarrheal Mechanisms and the Role of Intestinal Barrier Dysfunction in *Campylobacter* Infections. In S. Backert (Ed.), *Fighting Campylobacter Infections* (Vol. 431, pp. 203–231). Springer International Publishing. https://doi.org/10.1007/978-3-030-65481-8_8
- Lomer, M. C. E. (2015). Review article: The aetiology, diagnosis, mechanisms and clinical evidence for food intolerance. *Alimentary Pharmacology & Therapeutics*, 41(3), 262–275. <https://doi.org/10.1111/apt.13041>
- López-Arias, Á., Villar, D., López-Osorio, S., Calle-Vélez, D., & Chaparro-Gutiérrez, J. J. (2019). Giardia is the most prevalent parasitic infection in dogs and cats with diarrhea in the city of Medellín, Colombia. *Veterinary Parasitology: Regional Studies and Reports*, 18, 100335. <https://doi.org/10.1016/j.vprsr.2019.100335>
- Lu, H., Wilcock, P., Adeola, O., & Ajuwon, K. M. (2019). Effect of live yeast supplementation to gestating sows and nursery piglets on postweaning growth performance and nutrient digestibility. *Journal of Animal Science*, 97(6), 2534–2540. <https://doi.org/10.1093/jas/skz150>
- Lund, J. D., & Vestergaard, K. S. (1998). Development of Social Behaviour in Four Litters of Dogs (*Canis familiaris*). *Acta Veterinaria Scandinavica*, 39(2), 183–193. <https://doi.org/10.1186/BF03547791>
- Mair, C., Plitzner, C., Domig, K. J., Schedle, K., & Windisch, W. (2010). ORIGINAL ARTICLE: Impact of inulin and a multispecies probiotic formulation on performance, microbial ecology and concomitant fermentation patterns in newly weaned piglets: Dietary modulation of microbial ecology in piglets. *Journal of Animal Physiology and Animal Nutrition*, 94(5), e164–e177. <https://doi.org/10.1111/j.1439-0396.2010.01000.x>
- Marks, S. L. (2013a). Diarrhea. In *Canine and Feline Gastroenterology* (pp. 99–108). Elsevier. <https://doi.org/10.1016/B978-1-4160-3661-6.00011-0>

- Marks, S. L. (2013b). Diarrhea. In *Canine and Feline Gastroenterology* (pp. 99–108). Elsevier.
<https://doi.org/10.1016/B978-1-4160-3661-6.00011-0>
- Martín, R., Olivares, M., Pérez, M., Xaus, J., Torre, C., Fernández, L., & Rodríguez, J. M. (2010). Identification and evaluation of the probiotic potential of lactobacilli isolated from canine milk. *The Veterinary Journal*, 185(2), 193–198. <https://doi.org/10.1016/j.tvjl.2009.04.014>
- Masson, S., & Gaultier, E. (2018). Retrospective study on hypersensitivity-hyperactivity syndrome in dogs: Long-term outcome of high dose fluoxetine treatment and proposal of a clinical score. *DOG BEHAVIOR*, 4(2). <https://doi.org/10.4454/db.v4i2.79>
- Mayer, E. A., Tillisch, K., & Gupta, A. (2015). Gut/brain axis and the microbiota. *Journal of Clinical Investigation*, 125(3), 926–938. <https://doi.org/10.1172/JCI76304>
- McFarland, L. V., & Li, T. (2025). Efficacy and safety of *Saccharomyces boulardii* CNCM I-745 for the treatment of pediatric acute diarrhea in China: A systematic review and meta-analysis. *Frontiers in Cellular and Infection Microbiology*, 15, 1587792.
<https://doi.org/10.3389/fcimb.2025.1587792>
- Meineri, G., Cocolin, L., Morelli, G., Schievano, C., Atuahene, D., & Ferrocino, I. (2023). Effect of an Enteroprotective Complementary Feed on Faecal Markers of Inflammation and Intestinal Microbiota Composition in Weaning Puppies. *Veterinary Sciences*, 10(7), 434.
<https://doi.org/10.3390/vetsci10070434>
- Melandri, M., Aiudi, G. G., Caira, M., & Alonge, S. (2020). A Biotic Support During Pregnancy to Strengthen the Gastrointestinal Performance in Puppies. *Frontiers in Veterinary Science*, 7, 417. <https://doi.org/10.3389/fvets.2020.00417>
- Moeser, A. J., Pohl, C. S., & Rajput, M. (2017). Weaning stress and gastrointestinal barrier development: Implications for lifelong gut health in pigs. *Animal Nutrition*, 3(4), 313–321.
<https://doi.org/10.1016/j.aninu.2017.06.003>
- Morrow, M., Ottobre, J., Ottobre, A., Neville, P., St-Pierre, N., Dreschel, N., & Pate, J. L. (2015). Breed-dependent differences in the onset of fear-related avoidance behavior in puppies. *Journal of Veterinary Behavior*, 10(4), 286–294. <https://doi.org/10.1016/j.jveb.2015.03.002>

- Münnich, A., & Küchenmeister, U. (2014). Causes, Diagnosis and Therapy of Common Diseases in Neonatal Puppies in the First Days of Life: Cornerstones of Practical Approach. *Reproduction in Domestic Animals*, 49(s2), 64–74. <https://doi.org/10.1111/rda.12329>
- Nabuurs, M. J. A., Hoogendoorn, A., Van Der Molen, E. J., & Van Osta, A. L. M. (1993). Villus height and crypt depth in weaned and unweaned pigs, reared under various circumstances in the Netherlands. *Research in Veterinary Science*, 55(1), 78–84. [https://doi.org/10.1016/0034-5288\(93\)90038-H](https://doi.org/10.1016/0034-5288(93)90038-H)
- Nielsen, S. S., Alvarez, J., Bicout, D. J., Calistri, P., Canali, E., Drewe, J. A., Garin-Bastuji, B., Gonzales Rojas, J. L., Gortazar Schmidt, C., Herskin, M., Michel, V., Miranda Chueca, M. A., Padalino, B., Pasquali, P., Roberts, H. C., Spooler, H., Stahl, K., Velarde, A., Viltrop, A., ... Winckler, C. (2023). Welfare of calves. *EFSA Journal*, 21(3). <https://doi.org/10.2903/j.efsa.2023.7896>
- Nocerino, R., Di Costanzo, M., Bedogni, G., Cosenza, L., Maddalena, Y., Di Scala, C., Della Gatta, G., Carucci, L., Voto, L., Coppola, S., Iannicelli, A. M., & Berni Canani, R. (2019). Dietary Treatment with Extensively Hydrolyzed Casein Formula Containing the Probiotic *Lactobacillus rhamnosus* GG Prevents the Occurrence of Functional Gastrointestinal Disorders in Children with Cow's Milk Allergy. *The Journal of Pediatrics*, 213, 137-142.e2. <https://doi.org/10.1016/j.jpeds.2019.06.004>
- Nyangahu, D. D., Lennard, K. S., Brown, B. P., Darby, M. G., Wendoh, J. M., Havyarimana, E., Smith, P., Butcher, J., Stintzi, A., Mulder, N., Horsnell, W., & Jaspan, H. B. (2018). Disruption of maternal gut microbiota during gestation alters offspring microbiota and immunity. *Microbiome*, 6(1), 124. <https://doi.org/10.1186/s40168-018-0511-7>
- Pan, C., Xu, S., Zhang, W., Zhao, Y., Song, M., & Zhao, J. (2025). Investigating hair cortisol dynamics in German Shepherd Dogs throughout pregnancy, lactation, and weaning phases, and its potential impact on the hair cortisol of offspring. *Domestic Animal Endocrinology*, 92, 106921. <https://doi.org/10.1016/j.domaniend.2025.106921>

- Paulsen, D. B., Buddington, K. K., & Buddington, R. K. (2003). Dimensions and histologic characteristics of the small intestine of dogs during postnatal development. *American Journal of Veterinary Research*, 64(5), 618–626. <https://doi.org/10.2460/ajvr.2003.64.618>
- Pirrone, F., Pierantoni, L., Mazzola, S. M., Vigo, D., & Albertini, M. (2015). Owner and animal factors predict the incidence of, and owner reaction toward, problematic behaviors in companion dogs. *Journal of Veterinary Behavior*, 10(4), 295–301. <https://doi.org/10.1016/j.jveb.2015.03.004>
- Pluske, J. R., Thompson, M. J., Atwood, C. S., Bird, P. H., Williams, I. H., & Hartmann, P. E. (1996). Maintenance of villus height and crypt depth, and enhancement of disaccharide digestion and monosaccharide absorption, in piglets fed on cows' whole milk after weaning. *British Journal of Nutrition*, 76(3), 409–422. <https://doi.org/10.1079/BJN19960046>
- Pohl, C. S., Medland, J. E., & Moeser, A. J. (2015). Early-life stress origins of gastrointestinal disease: Animal models, intestinal pathophysiology, and translational implications. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 309(12), G927–G941. <https://doi.org/10.1152/ajpgi.00206.2015>
- Rapp, D., Schütz, K. E., Ross, C., Sutherland, M. A., Hempstead, M. N., Hannaford, R., Cave, V. M., & Brightwell, G. (2023). Fecal excretion of *Campylobacter jejuni* by young dairy calves and the relationship with neonatal immunity and personality traits. *Journal of Applied Microbiology*, 134(5), lxad094. <https://doi.org/10.1093/jambio/lxad094>
- Ren, W., Yu, B., Yu, J., Zheng, P., Huang, Z., Luo, J., Mao, X., He, J., Yan, H., Wu, J., Chen, D., & Luo, Y. (2022a). Lower abundance of Bacteroides and metabolic dysfunction are highly associated with the post-weaning diarrhea in piglets. *Science China Life Sciences*, 65(10), 2062–2075. <https://doi.org/10.1007/s11427-021-2068-6>
- Ren, W., Yu, B., Yu, J., Zheng, P., Huang, Z., Luo, J., Mao, X., He, J., Yan, H., Wu, J., Chen, D., & Luo, Y. (2022b). Lower abundance of Bacteroides and metabolic dysfunction are highly associated with the post-weaning diarrhea in piglets. *Science China Life Sciences*, 65(10), 2062–2075. <https://doi.org/10.1007/s11427-021-2068-6>

- Rieger, J., Janczyk, P., Hünigen, H., Neumann, K., & Plendl, J. (2015). Intraepithelial lymphocyte numbers and histomorphological parameters in the porcine gut after *Enterococcus faecium* NCIMB 10415 feeding in a *Salmonella* Typhimurium challenge. *Veterinary Immunology and Immunopathology*, 164(1–2), 40–50. <https://doi.org/10.1016/j.vetimm.2014.12.013>
- Ríos-Covián, D., Ruas-Madiedo, P., Margolles, A., Gueimonde, M., de los Reyes-Gavilán, C. G., & Salazar, N. (2016). Intestinal Short Chain Fatty Acids and their Link with Diet and Human Health. *Frontiers in Microbiology*, 7. <https://doi.org/10.3389/fmicb.2016.00185>
- Romaniuk, A. C., Barnard, S., Shreyer, T., & Croney, C. (2025). Effects of dam fear and stress on metrics of puppy welfare in commercial breeding kennels. *Scientific Reports*, 15(1), 2820. <https://doi.org/10.1038/s41598-025-85936-w>
- Russell, A. L., McAdams, Z. L., Donovan, E., Seilhamer, N., Siegrist, M., Franklin, C. L., & Ericsson, A. C. (2023). The contribution of maternal oral, vaginal, and gut microbiota to the developing offspring gut. *Scientific Reports*, 13(1), 13660. <https://doi.org/10.1038/s41598-023-40703-7>
- Salak-Johnson, J. L., Reddout, C., Hernandez, L., & Visconti, A. (2022). Maternal Supplementation of *Saccharomyces cerevisiae boulardii* during Late-Gestation through Lactation Differentially Modulated Immune Status and Stress Responsiveness of the Progeny to Farrowing and Weaning Stressors. *Animals*, 12(2), 164. <https://doi.org/10.3390/ani12020164>
- Scharek, L., Altherr, B. J., Tölke, C., & Schmidt, M. F. G. (2007). Influence of the probiotic *Bacillus cereus* var. *Toyoi* on the intestinal immunity of piglets. *Veterinary Immunology and Immunopathology*, 120(3–4), 136–147. <https://doi.org/10.1016/j.vetimm.2007.07.015>
- Schiopu, C. G., Gavril, R. S., Ștefănescu, C., Eșanu, I. M., & Mitu, F. (2023). The Gut Microbiome, a Possible Key to Multidisciplinary Clinical Practice—Literature Review. *Internal Medicine*, 20(2), 35–54. <https://doi.org/10.2478/inmed-2023-0246>
- Schoeman, J. P., Goddard, A., & Herrtage, M. E. (2007). Serum cortisol and thyroxine concentrations as predictors of death in critically ill puppies with parvoviral diarrhea. *Journal of the American Veterinary Medical Association*, 231(10), 1534–1539. <https://doi.org/10.2460/javma.231.10.1534>

- Serpell, J., Duffy, D. L., & Jagoe, J. A. (2016). Becoming a dog: Early experience and the development of behavior. In J. Serpell (Ed.), *The Domestic Dog* (2nd ed., pp. 93–117). Cambridge University Press. <https://doi.org/10.1017/9781139161800.006>
- Suárez-Martínez, C., Santaella-Pascual, M., Yagüe-Guirao, G., & Martínez-Graciá, C. (2023). Infant gut microbiota colonization: Influence of prenatal and postnatal factors, focusing on diet. *Frontiers in Microbiology*, *14*, 1236254. <https://doi.org/10.3389/fmicb.2023.1236254>
- Suchodolski, J. S., Markel, M. E., Garcia-Mazcorro, J. F., Unterer, S., Heilmann, R. M., Dowd, S. E., Kachroo, P., Ivanov, I., Minamoto, Y., Dillman, E. M., Steiner, J. M., Cook, A. K., & Toresson, L. (2012). The Fecal Microbiome in Dogs with Acute Diarrhea and Idiopathic Inflammatory Bowel Disease. *PLoS ONE*, *7*(12), e51907. <https://doi.org/10.1371/journal.pone.0051907>
- Taras, D., Vahjen, W., Macha, M., & Simon, O. (2005). Response of performance characteristics and fecal consistency to long-lasting dietary supplementation with the probiotic strain *Bacillus cereus* var. Toyoi to sows and piglets. *Archives of Animal Nutrition*, *59*(6), 405–417. <https://doi.org/10.1080/17450390500353168>
- Tochitani, S., Tsukahara, T., & Inoue, R. (2024). Perturbed maternal microbiota shapes offspring microbiota during early colonization period in mice. *Proceedings of the Japan Academy, Series B*, *100*(6), 335–352. <https://doi.org/10.2183/pjab.100.020>
- Trevisi, P., Latorre, R., Priori, D., Luise, D., Archetti, I., Mazzoni, M., D’Inca, R., & Bosi, P. (2017). Effect of feed supplementation with live yeast on the intestinal transcriptome profile of weaning pigs orally challenged with *Escherichia coli* F4. *Animal*, *11*(1), 33–44. <https://doi.org/10.1017/S1751731116001178>
- Trybocka, R. (2010). Character assessment testing to test suitability for guide dogs. *Veterinary Nursing Journal*, *25*(11), 32–33. <https://doi.org/10.1111/j.2045-0648.2010.tb00101.x>
- Verlinden, A., Hesta, M., Millet, S., & Janssens, G. P. J. (2006). Food Allergy in Dogs and Cats: A Review. *Critical Reviews in Food Science and Nutrition*, *46*(3), 259–273. <https://doi.org/10.1080/10408390591001117>

- Vilson, Å., Ramadan, Z., Li, Q., Hedhammar, Å., Reynolds, A., Spears, J., Labuda, J., Pelker, R., Björkstén, B., Dicksved, J., & Hansson-Hamlin, H. (2018a). Disentangling factors that shape the gut microbiota in German Shepherd dogs. *PLOS ONE*, *13*(3), e0193507. <https://doi.org/10.1371/journal.pone.0193507>
- Vilson, Å., Ramadan, Z., Li, Q., Hedhammar, Å., Reynolds, A., Spears, J., Labuda, J., Pelker, R., Björkstén, B., Dicksved, J., & Hansson-Hamlin, H. (2018b). Disentangling factors that shape the gut microbiota in German Shepherd dogs. *PLOS ONE*, *13*(3), e0193507. <https://doi.org/10.1371/journal.pone.0193507>
- Visconti, A., Le Roy, C. I., Rosa, F., Rossi, N., Martin, T. C., Mohn, R. P., Li, W., De Rinaldis, E., Bell, J. T., Venter, J. C., Nelson, K. E., Spector, T. D., & Falchi, M. (2019). Interplay between the human gut microbiome and host metabolism. *Nature Communications*, *10*(1), 4505. <https://doi.org/10.1038/s41467-019-12476-z>
- Vital, M., Howe, A. C., & Tiedje, J. M. (2014). Revealing the Bacterial Butyrate Synthesis Pathways by Analyzing (Meta)genomic Data. *mBio*, *5*(2), e00889-14. <https://doi.org/10.1128/mBio.00889-14>
- Wang, K., Kong, X., Azad, Md. A. K., Zhu, Q., Xiong, L., Zheng, Y., Hu, Z., Yin, Y., & He, Q. (2021). Maternal Probiotic or Synbiotic Supplementation Modulates Jejunal and Colonic Antioxidant Capacity, Mitochondrial Function, and Microbial Abundance in Bama Mini-piglets. *Oxidative Medicine and Cellular Longevity*, *2021*(1), 6618874. <https://doi.org/10.1155/2021/6618874>
- Wang, Y. B., Du, W., Fu, A. K., Zhang, X. P., Huang, Y., Lee, K. H., Yu, K., Li, W. F., & Li, Y. L. (2016). Intestinal microbiota and oral administration of *Enterococcus faecium* associated with the growth performance of new-born piglets. *Beneficial Microbes*, *7*(4), 529–538. <https://doi.org/10.3920/BM2015.0099>
- Warrit, K., Boscan, P., Ferguson, L. E., Bradley, A. M., Dowers, K. L., Rao, S., & Twedt, D. C. (2017). Minimally invasive wireless motility capsule to study canine gastrointestinal motility and pH. *The Veterinary Journal*, *227*, 36–41. <https://doi.org/10.1016/j.tvjl.2017.08.005>
- West, C. E., Gotheffors, L., Granström, M., Käyhty, H., Hammarström, M. K. C., & Hernell, O. (2008). Effects of feeding probiotics during weaning on infections and antibody responses to

- diphtheria, tetanus and Hib vaccines. *Pediatric Allergy and Immunology*, 19(1), 53–60.
<https://doi.org/10.1111/j.1399-3038.2007.00583.x>
- West, C. E., Hammarström, M., & Hernell, O. (2009). Probiotics during weaning reduce the incidence of eczema. *Pediatric Allergy and Immunology*, 20(5), 430–437.
<https://doi.org/10.1111/j.1399-3038.2009.00745.x>
- Xia, T., Yin, C., Comi, M., Agazzi, A., Perricone, V., Li, X., & Jiang, X. (2022). Live Yeast Supplementation in Gestating and Lactating Primiparous Sows Improves Immune Response in Dams and Their Progeny. *Animals*, 12(10), 1315. <https://doi.org/10.3390/ani12101315>
- Xie, F., Zhou, M., Li, X., Li, S., Ren, M., & Wang, C. (2024a). Macrogenomic and Metabolomic Analyses Reveal Mechanisms of Gut Microbiota and Microbial Metabolites in Diarrhea of Weaned Piglets. *Animals*, 14(16), 2327. <https://doi.org/10.3390/ani14162327>
- Xie, F., Zhou, M., Li, X., Li, S., Ren, M., & Wang, C. (2024b). Macrogenomic and Metabolomic Analyses Reveal Mechanisms of Gut Microbiota and Microbial Metabolites in Diarrhea of Weaned Piglets. *Animals*, 14(16), 2327. <https://doi.org/10.3390/ani14162327>
- Xie, Y., He, Y., Irwin, P. L., Jin, T., & Shi, X. (2011). Antibacterial Activity and Mechanism of Action of Zinc Oxide Nanoparticles against *Campylobacter jejuni*. *Applied and Environmental Microbiology*, 77(7), 2325–2331. <https://doi.org/10.1128/AEM.02149-10>
- Xu, S., Jia, X., Liu, Y., Pan, X., Chang, J., Wei, W., Lu, P., Petry, D., Che, L., Jiang, X., Wang, J., & Wu, D. (2023). Effects of yeast-derived postbiotic supplementation in late gestation and lactation diets on performance, milk quality, and immune function in lactating sows. *Journal of Animal Science*, 101, skad201. <https://doi.org/10.1093/jas/skad201>
- Yu, Z., Ruan, Y., Zhou, M., Chen, S., Zhang, Y., Wang, L., Zhu, G., & Yu, Y. (2018). Prevalence of intestinal parasites in companion dogs with diarrhea in Beijing, China, and genetic characteristics of Giardia and Cryptosporidium species. *Parasitology Research*, 117(1), 35–43. <https://doi.org/10.1007/s00436-017-5631-7>
- Zeyner, A., & Boldt, E. (2006). Effects of a probiotic *Enterococcus faecium* strain supplemented from birth to weaning on diarrhoea patterns and performance of piglets*. *Journal of Animal*

Physiology and Animal Nutrition, 90(1–2), 25–31. <https://doi.org/10.1111/j.1439-0396.2005.00615.x>

Zheng, X., Nie, K., Xu, Y., Zhang, H., Xie, F., Xu, L., Zhang, Z., Ding, Y., Yin, Z., & Zhang, X.

(2023a). Fecal Microbial Structure and Metabolic Profile in Post-Weaning Diarrheic Piglets.

Genes, 14(6), 1166. <https://doi.org/10.3390/genes14061166>

Zheng, X., Nie, K., Xu, Y., Zhang, H., Xie, F., Xu, L., Zhang, Z., Ding, Y., Yin, Z., & Zhang, X.

(2023b). Fecal Microbial Structure and Metabolic Profile in Post-Weaning Diarrheic Piglets.

Genes, 14(6), 1166. <https://doi.org/10.3390/genes14061166>