Interactions between prebiotics and health-relevant commensal bacteria : impact on bacterial growth and metabolic activities

Cassandre Bedu-Ferrari

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Interactions entre prébiotiques et bactéries commensales d'intérêt santé : impact sur la croissance bactérienne et les activités métaboliques

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et la co-direction de **Claire CHERBUY**, Ingénieure de recherche (HDR)

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Cassandre BEDU-FERRARI

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Title : Interactions between prebiotics and health-relevant commensal bacteria: impact on bacterial growth and metabolic activities

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Abstract:

Complex microbial community inhabitants of the gastro-intestinal tract constitute the gut microbiota. This ecosystem contributes significantly to human health and wellbeing. The provision of dietary substrates to modify the composition and functions of the gut microbiota for health benefits is at the basis of the concept of prebiotics. The current challenge is to gain a better comprehension of the dynamic interplay between prebiotics and the human gut microbiota. In this study, several food ingredients were investigated as potential prebiotics. *In vitro* cultivation-based experiments were carried out using individual bacterial species and synthetic microbial assemblies. Our results revealed the metabolic capacities of health-relevant bacteria to utilise complex carbohydrates. In-depth metabolic profiles showed that bacterial growth and fermentation profiles appear to be significantly influenced by both carbon sources and taxonomic affiliation. Comparative genomic analyses highlighted the enzymatic potential of each bacterial species to metabolise the prebiotics of interest. These data revealed an adaptation of health-relevant bacteria to their nutritional environment. We expect that this work provides further rationales for the development of functional foods applied to prebiotics.

Titre : Interactions entre prébiotiques et bactéries commensales d'intérêt santé : impact sur la croissance bactérienne et les activités métaboliques

Mots clés : prébiotiques, microbiote intestinal, acides gras à chaine courte, métabolome, santé et nutrition humaine

Résumé :

Le tractus gastro-intestinal héberge une communauté microbienne riche et diversifiée appelée microbiote intestinal. Cet écosystème contribue de manière significative à la santé et au bien-être de l'homme. L'apport de substrats alimentaires permettant de modifier la composition et les fonctions du microbiote intestinal pour des bénéfices santé est à la base du concept de prébiotique. Aujourd'hui, il apparait déterminant d'acquérir une meilleure compréhension des interactions dynamiques entre les prébiotiques et le microbiote intestinal humain. Dans cette étude, plusieurs ingrédients alimentaires ont été étudiés comme prébiotiques potentiels. Des expériences de culture *in vitro* ont été réalisées sur des monocultures et des consortia bactériens d'intérêt. Nos résultats ont révélé les capacités métaboliques de bactéries d'intérêt santé à utiliser ces sucres complexes. Des profils métaboliques détaillés ont montré que la croissance bactérienne et les profils de fermentation semblent influencés de manière significative par les sources de carbone et l'affiliation taxonomique des bactéries. Des analyses génomiques comparatives ont mis en évidence les potentiels enzymatiques de chaque espèce bactérienne à métaboliser les prébiotiques d'intérêt. Ces données ont révélé une adaptation des bactéries d'intérêt santé à leur environnement nutritionnel. Nous proposons que ce travail apporte des arguments supplémentaires pour le développement d'aliments fonctionnels à base de prébiotiques.

Microbiote intestinal, santé humaine et nutrition

En référence aux communautés de micro-organismes qui peuplent l'environnement intestinal, le microbiote intestinal abrite des trillions de bactéries, champignons, archées et virus. En étroite interaction les unes avec les autres, ces communautés microbiennes contribuent à une multitude d'aspects de la biologie de l'hôte. Au cours des dernières décennies, l'expansion significative de la science liée au microbiote intestinal a ancré l'importance des micro-organismes pour la santé et le bien-être de l'Homme.

Aujourd'hui, la société mondiale est confrontée à un grand nombre de problèmes de santé, intimement liés à la nutrition humaine. Le syndrome métabolique (SM) désigne des symptômes métaboliques complexes qui reflètent une suralimentation, un mode de vie sédentaire et l'excès d'adiposité qui en résulte (Cornier et al., 2008). Le SM est fortement associé à l'obésité abdominale, à la résistance à l'insuline, à la dyslipidémie et à l'hypertension, qui contribuent collectivement au risque de maladies cardiovasculaires et de diabète de type II. La prévalence globale du SM a été estimée entre 20 et 25% de la population adulte mondiale (Saklayen, 2018). Par ailleurs, le trouble digestif le plus fréquemment observé dans les cliniques de gastroentérologie est le syndrome de l'intestin irritable (SII). Affectant environ 11 % de la population mondiale, le SII se caractérise par des douleurs abdominales, des ballonnements, des diarrhées, des constipations ou des périodes alternées de ces symptômes (Black et Ford, 2020). Les troubles gastro-intestinaux et métaboliques, tels que le SM et le SII, sont fréquemment associés à une altération du microbiote intestinal, à une dérégulation immunitaire et à un état pro-inflammatoire chronique. L'alimentation humaine, qui a l'un des impacts les plus importants connus sur le microbiote intestinal, est apparue comme la principale hypothèse de la prévalence accrue des troubles liés à l'alimentation, bien que la causalité puisse être difficile à établir (Sonnenburg et Sonnenburg, 2014).

En particulier, les faibles apports en fibres, typiques d'un mode de vie et d'une alimentation occidentalisés, ont été identifiés comme l'une des explications des perturbations du microbiote intestinal et du risque accru de maladies. Les fibres alimentaires englobent un large éventail de molécules complexes, dont la plupart se trouvent dans les légumes, les légumineuses, les céréales et les fruits. Parmi celles-ci, les molécules glucidiques, également appelées polysaccharides ou glycanes, sont résistantes à la digestion par les enzymes humaines. Par conséquent, la plupart des fibres ingérées atteignent le côlon et exercent des effets physiologiques importants sur la santé et le bien-être de l'Homme. En particulier, ces fibres peuvent être métabolisées par des microbes intestinaux qui présentent une capacité enzymatique extrême, ce qui entraîne une modulation favorable du microbiote (Kaoutari et al., 2013). **Grâce à une consommation plus élevée de fibres alimentaires, le maintien et/ou la restauration du microbiote intestinal est un objectif souhaitable pour la santé et le bien-être de l'Homme.**

Les fibres alimentaires sont appliquées, dans le cadre d'un régime alimentaire sain, aux actions de santé publique qui communiquent l'importance des aliments riches en fibres, pauvres en sucre et en matières grasses pour promouvoir notre santé. Des directives nationales et internationales ont été initialement établies pour éviter les carences en nutriments et les maladies associées. En particulier, l'organisation mondiale de la santé (OMS) a fixé la consommation quotidienne de fibres alimentaires à plus de 25 g/jour (Organisation mondiale de la santé, 2003). Néanmoins, de nombreuses personnes n'atteignent pas leur apport recommandé en fibres car elles ne consomment pas suffisamment de fruits, de légumes et de céréales (Stephen et al., 2017). **L'ajustement du régime alimentaire de l'Homme pour combler le "déficit en fibres" est une stratégie prometteuse dans la prévention et la gestion de nombreux troubles gastro-intestinaux et métaboliques.**

Le lien entre l'apport alimentaire, le microbiote intestinal et la santé humaine est un domaine de recherche actif à l'heure actuelle. Comprendre les mécanismes qui relient les régimes riches en fibres à une bonne santé est un défi.

Les prébiotiques, une stratégie nutritionnelle

L'étude du microbiote intestinal a révélé le rôle fondamental qu'il joue dans de nombreuses fonctions biologiques et métaboliques. Un déséquilibre dans les interactions au sein de cette vaste communauté de micro-organismes a des conséquences importantes pour la santé humaine. Ces observations ont conduit à l'émergence d'approches basées sur la nutrition pour soutenir le microbiote intestinal et ses fonctions métaboliques**. La manière dont l'alimentation pourrait être exploitée pour modifier facilement la composition des microbes intestinaux afin d'augmenter les niveaux des bactéries souhaitées n'est pas claire. Savoir comment les fibres alimentaires nourrissent les micro-organismes intestinaux pourrait suggérer des moyens de stimuler la croissance et les activités métaboliques des bactéries bénéfiques pour la santé.** Dans ce contexte, les prébiotiques font référence à l'utilisation de sources de fibres particulières qui fournissent de la nourriture aux microbes intestinaux souhaités.

Le terme "prébiotique" a été défini à l'origine en 1995 par Roberfroid et Gibson et récemment révisé par l'ISAPP (International Scientific Association for Probiotics and Prebiotics) comme "un substrat qui est utilisé de manière sélective par les micro-organismes de l'hôte conférant un avantage pour la santé" (Gibson et Roberfroid, 1995 ; Gibson et al., 2017). Les prébiotiques représentent une solution innovante pour l'avenir de la nutrition. L'utilisation d'une telle stratégie nutritionnelle nécessite une compréhension sophistiquée des interactions qui se produisent lorsque la communauté complexe des microbes intestinaux rencontre une source de fibres. En effet, bien que différentes fibres alimentaires puissent sembler physiologiquement équivalentes, les mécanismes d'action sous-jacents sont différents, et ce en ciblant des microbes intestinaux différents (Van Hul et al., 2020).

Par conséquent, les effets bénéfiques sur la santé de fibres alimentaires spécifiques doivent être documentés pour être considérés comme un prébiotique.

Le microbiote intestinal est complexe, dynamique et présente des variations intra- et inter-personnelles considérables dans sa composition et ses fonctions. Le grand nombre d'interactions potentielles entre les composants du microbiote rend difficile la définition des mécanismes sous-jacents par lesquels les ingrédients alimentaires affectent les propriétés de la communauté. **Un travail important reste à faire pour démêler les interactions entre les prébiotiques, le microbiote intestinal et la santé et le bien-être de l'hôte. Ce projet est consacré à une meilleure compréhension des mécanismes des fibres alimentaires ayant un potentiel prébiotique.**

La santé digestive dans l'industrie agroalimentaire

La science du microbiote intestinal a fait l'objet d'une attention croissante ces dernières années dans les domaines scientifique, médical et public. Conformément à ces avancées, la communication autour du microbiote intestinal a élargi la perception du public à la compréhension des rôles bénéfiques des micro-organismes dans la santé humaine (Cunningham et al., 2021). **Partout dans le monde, les gens commencent à envisager la santé à travers leurs intestins. Ils font de plus en plus le lien entre l'importance du microbiote intestinal et son impact sur la santé globale et le bien-être.** Ils recherchent des bénéfices nutritionnels qui pourraient réduire les risques de développer des maladies non transmissibles telles que les troubles cardiovasculaires, l'obésité, le diabète et les cancers (Mellentin, 2020).

Liée à un large éventail de préoccupations de santé, la culture de la santé intestinale est très majoritairement axée sur les probiotiques, les fibres alimentaires et les prébiotiques. Il existe une demande croissante pour des produits alimentaires plus sains, des aliments peu transformés avec la présence d'ingrédients fonctionnels (Nowosad et al., 2021). Attentifs aux demandes des consommateurs, l'industrie agroalimentaire cherche des solutions pour proposer des approches holistiques et proactives de la santé. Actuellement disponibles sur le marché, les probiotiques sous forme de compléments, d'aliments fermentés et de suppléments visent à optimiser la digestion, qui est de plus en plus reconnue comme la base de la santé et du bien-être. Les choix d'aliments et de boissons représentent une stratégie pour réduire l'inconfort ou les symptômes digestifs et pour gérer un état de santé général. Ces solutions nutritionnelles créent un paysage concurrentiel qui stimulent l'innovation en R&D. La recherche de nouveaux horizons en matière de santé intestinale peut soutenir les futures solutions nutritionnelles.

Après avoir évalué l'opportunité commerciale et défini les besoins des consommateurs, nous pensons que les prébiotiques représentent une solution émergente pour exploiter la santé et le bien-être digestifs grâce au développement d'aliments fonctionnels.

Comment réussir à influencer le microbiote intestinal ?

Dans ce projet, les prébiotiques sont proposés comme solution technologique pour nourrir le microbiote intestinal et maintenir la santé à travers l'intestin. La solution pour soutenir la santé et le bien-être digestifs peut être appliquée aux produits laitiers. Bien que les ventes de compléments alimentaires l'emportent sur celles des produits laitiers, cette matrice alimentaire semble mieux fonctionner que d'autres sur la base de l'histoire traditionnelle des produits laitiers. Les produits laitiers ont toujours été le véhicule le plus populaire pour offrir des avantages en matière de santé digestive à l'Homme en raison de la forte acceptation générale par les consommateurs (Champagne et al., 2018). Composé de cultures de bactéries vivantes, le yaourt est intuitivement perçu comme bénéfique pour la santé intestinale. Bien que les produits laitiers apparaissent comme la matrice la plus adaptée, nous pourrions imaginer des solutions pour délivrer avec succès des prébiotiques étendus à d'autres matrices alimentaires telles que les barres, les céréales, les repas préparés et les produits de boulangerie.

Les prébiotiques font partie d'une industrie alimentaire dont les ventes sur le marché augmentent. Actuellement, il existe une gamme étroite de substances prébiotiques confirmées, les galactanes et les fructanes dominant le marché. Explorer la culture de la santé intestinale peut garantir des avantages concurrentiels. Aujourd'hui, il existe une opportunité de développer un yaourt avec des fibres prébiotiques qui nourrissent le microbiote intestinal.

Un consortium d'experts

La faisabilité de ce projet nécessite un effort interdisciplinaire d'un consortium d'experts. Le travail présenté dans cette thèse s'inscrit dans un projet plus vaste « RestorBiome ». Ce projet collaboratif fédère des institutions académiques françaises, dont l'Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement (INRAE) et l'Université Clermont Auvergne (UCA), spécialistes de la science du microbiote intestinal. Le projet a également impliqué des acteurs clés de l'industrie agroalimentaire, dont GreenCell Biotechnologies et Yoplait France - General Mills, ce dernier étant le coordinateur du projet. Chaque acteur dispose d'expertises et de compétences complémentaires pour garantir le développement de produits alimentaires enrichis en prébiotiques pour maintenir et/ou restaurer le microbiote intestinal. Le projet associe également des pôles de compétitivité, dont Vitagora et Céréales Vallée, qui favorisent le développement de projets innovants en R&D et valorisent la mise sur le marché de nouveaux produits issus des projets. Ce projet est soutenu par le Programme d'Investissement d'Avenir opéré par BpiFrance.

Leader en écologie microbienne, Greencell Biotechnologies produit des ingrédients innovants favorisant une agriculture durable. Le premier axe d'innovation du projet RestorBiome est l'identification de nouvelles molécules issues de sources naturelles alternatives dans le respect de la planète et de ses ressources. Cet axe de recherche comprend le développement de nouveaux ingrédients alimentaires favorisant un microbiote intestinal équilibré. L'INRAE et l'UCA ont la responsabilité de l'évaluation scientifique de ces composés à effets prébiotiques potentiels. Cet axe inclut le développement d'une méthodologie standardisée pour évaluer la spécificité de ces substrats pour stimuler la croissance et/ou les activités fermentaires de bactéries favorables à la santé. Les perspectives du consortium RestorBiome sont de valider les effets prébiotiques des ingrédients alimentaires i) en maintenant et/ou en restaurant la composition et les activités fermentaires à l'échelle du microbiote intestinal à l'aide de systèmes de fermentation *in vitro* ii) et en préservant le dialogue entre le système intestinal microbiote et l'hôte à l'aide d'organoïdes et de systèmes gut-in-a-chip. Enfin, le projet comprend le développement de produits laitiers enrichis en prébiotiques.

Mon implication dans le projet RestorBiome a été de mettre en place une approche *in vitro* basée sur des cultures de bactéries commensales pour la sélection des ingrédients alimentaires les plus prometteurs à effets prébiotiques. Mon projet de thèse vise à caractériser les capacités des bactéries promotrices de santé en réponse aux prébiotiques pour une meilleure compréhension des mécanismes sous-jacents conférant des avantages pour la santé de l'hôte. Employée par Yoplait France – General Mills m'a permis de faire partie du processus d'innovation en R&D. Ma thèse vise à explorer les effets prébiotiques des ingrédients alimentaires fournis par l'industrie. Basée à l'INRAE, à Jouy en Josas, à l'Institut Micalis, l'équipe Probihôte m'a permis d'évoluer dans un environnement de travail adapté. Mon rôle dans le projet RestorBiome a été à l'interface de la recherche académique et privée pour transférer la recherche fondamentale dans le développement d'un produit alimentaire fonctionnel.

Plan de la thèse

Ce projet de thèse aborde les défis et les écueils de la recherche sur les prébiotiques et le microbiote. De nombreuses inconnues subsistent pour démêler les interactions dynamiques entre les prébiotiques, le microbiote intestinal et l'hôte. Dans ce contexte, les prébiotiques représentent une solution émergente pour améliorer la santé et le bien-être digestifs grâce au développement d'aliments fonctionnels. Cependant, la manière dont l'alimentation peut être exploitée pour manipuler facilement la composition des microbes intestinaux afin d'augmenter les niveaux des bactéries souhaitées n'est pas claire. Savoir comment les prébiotiques nourrissent les microorganismes intestinaux peut suggérer la stimulation sélective de la croissance et des activités métaboliques des bactéries bénéfiques pour la santé.

La ligne directrice de ce projet est de mieux comprendre les mécanismes sous-jacents des prébiotiques garantissant des effets physiologiques bénéfiques sur l'hôte. L'intérêt est de comprendre comment réussir à influencer avec succès le microbiote intestinal. Comment les interventions basées sur le microbiote peuvent-elles être utilisées pour prévenir les troubles gastro-intestinaux et métaboliques et promouvoir la santé et le bien-être humains ?

Le **chapitre 1** passe en revue la littérature scientifique de la recherche sur le microbiote intestinal désigné comme un médiateur clé de l'impact de l'alimentation sur la physiologie humaine. Les efforts se concentrent sur l'importance de la composition du microbiote intestinal et de ses capacités fonctionnelles vis-à-vis des prébiotiques. Bien qu'ils jouent un rôle dans l'écosystème intestinal, les champignons, les archées et les virus ne seront pas davantage mentionnés. Cette revue se concentre également sur les prébiotiques contenant des glucides non digestibles, qui représentent une grande partie de l'alimentation humaine et encouragent sélectivement la croissance des bactéries ciblées. La stimulation subséquente des activités de fermentation se traduit par la production de molécules bioactives conférant des bienfaits pour la santé. Les mécanismes sous-jacents aux effets prébiotiques englobent l'interaction complexe entre l'alimentation, le microbiote intestinal et le métabolisme énergétique de l'hôte. Pour souligner l'importance de la saccharification microbienne dans la santé humaine, ce chapitre résume les fondements moléculaires des processus de dégradation des polysaccharides à l'échelle bactérienne et à l'ensemble des communautés microbiennes. La modification intentionnelle de la composition et de la fonctionnalité du microbiote intestinal nécessite l'examen le plus complet de la recherche récente sur le microbiote prébiotique. Ce chapitre expose les connaissances actuelles sur la stratégie nutritionnelle prébiotique à des fins de santé et de bien-être. Ce chapitre est l'objet d'une communication scientifique : **Bedu-Ferrari, C.**, Biscarrat, P., Langella, P., & Cherbuy, C. (2022). **Prebiotics and the human gut microbiota: from breakdown mechanisms to the impact on metabolic health.** *Nutrients*, *14*(10), 2096. https://doi.org/10.3390/nu14102096.

Les principaux objectifs et stratégies expérimentales exposent les hypothèses de recherche avec un accent particulier sur les cinq ingrédients alimentaires fournis par Yoplait France - General Mills. Ces ingrédients répondent à la définition des fibres alimentaires non digestibles, ce qui signifie qu'ils sont résistants à l'acidité gastrique, à l'hydrolyse par les enzymes digestives de l'hôte et à l'absorption gastro-intestinale (FAO/WHO Codex Alimentarius Commission, 2009). Les avantages pour la santé médiés par les propriétés des fibres sont la régulation du temps de transit intestinal tout en augmentant le volume des selles, la réduction des réponses glycémiques et insulinémiques postprandiales et le maintien d'un taux de cholestérol normal dans le sang. Au-delà des actions physiologiques attendues des fibres alimentaires, ces cinq ingrédients alimentaires peuvent également agir comme prébiotiques (Gibson et Roberfroid, 1995 ; Gibson et al., 2017). La référence historique

pour mesurer l'efficacité des prébiotiques est la sélection des espèces de *Bifidobacterium* et *Lactobacillus*, les genres bactériens souvent utilisés comme probiotiques (Cockburn et Koropatkin, 2016). **L'hypothèse principale de ce travail est que ces cinq ingrédients alimentaires peuvent stimuler sélectivement la croissance et les activités métaboliques de bactéries intestinales clés, qui ne se limitent pas aux bifidobactéries et lactobacilles traditionnels.** En effet, les prébiotiques peuvent cibler d'autres bactéries bénéfiques pour la santé conférant des effets locaux ou systémiques sur la santé, d'où l'importance de décrire le métabolisme bactérien. Par conséquent, les cinq ingrédients alimentaires pourraient moduler favorablement le microbiote intestinal et entraîner des avantages physiologiques pertinents, notamment la promotion de la synthèse d'acides gras à chaines courtes (AGCC), le renforcement de l'intégrité du côlon et de la barrière intestinale, l'amélioration du métabolisme énergétique et de la satiété, et la régulation de la réponse immunitaire.

L'impact des prébiotiques sur la croissance bactérienne et les activités métaboliques a été étudié à l'aide d'une approche réductionniste du microbiote intestinal qui se concentre sur un panel de bactéries commensales importantes pour la santé. Cette sélection bactérienne couvre une diversité taxonomique répartie sur les quatre principaux phylums du microbiote intestinal humain. Le **chapitre 2** caractérise les effets prébiotiques des ingrédients alimentaires fournis par Yoplait France – General Mills sur les capacités métaboliques au niveau d'espèces bactériennes isolées favorables pour la santé humaine. Fait intéressant, la plupart de ces bactéries bénéfiques pour la santé ont été peu décrites pour leurs propriétés métaboliques. Cette étude fait progresser les connaissances actuelles sur le métabolisme des glucides des principales bactéries intestinales humaines possédant des propriétés pertinentes pour la santé. Des expériences *in vitro* ont révélé une variété de réponses aux prébiotiques d'une manière dépendante du phylum. Le déchiffrage des interactions entre les prébiotiques et les fonctions métaboliques cachées dans le microbiote intestinal au niveau de l'espèce bactérienne a fourni de nouvelles preuves scientifiques pour la modulation intentionnelle du microbiote intestinal à des fins de santé et de bien-être. Le contenu principal de ce chapitre est actuellement en préparation sous forme d'article de recherche : **Bedu-Ferrari C.**, Biscarrat P., Vati S., Pepke F., Castelli F., Chollet C., Almeida M., Meslier V., Langella P., Cherbuy C. **Functional characterisation of health-relevant intestinal bacteria revealed their metabolic capacities toward carbohydrate-containing prebiotics.**

Dans la continuité de ce travail de thèse, le **chapitre 3** étudie les effets prébiotiques sur le métabolisme bactérien au niveau de consortia simplifiés. En effet, étudier des bactéries isolées ne suffit pas pour comprendre l'environnement complexe de nos intestins. Si l'objectif des prébiotiques est de cibler précisément certains microorganismes bénéfiques, l'impact sur le microbiote s'avère généralement plus étendu. Il existe peu de glucides pour lesquels la capacité de dégradation est uniquement limitée à un petit sous-ensemble de bactéries bénéfiques, et l'alimentation croisée augmente encore le nombre d'espèces qui bénéficient de la présence du

prébiotique. En particulier, cette étude se concentre sur les substrats prébiotiques les plus prometteurs qui stimulent la croissance et les activités métaboliques du panel de bactéries bénéfiques pour la santé. Le contenu principal de ce chapitre est actuellement en préparation sous forme d'article de recherche : **Bedu-Ferrari C.**, Biscarrat P., Bruneau A., Pepke F., Langella P., Cherbuy C. **Impact of diverse carbohydrates on synthetic bacterial consortia shed light on cooperation and competition interactions.** Cette étude permet de mieux comprendre les interactions dynamiques entre les prébiotiques et les consortia bactériens. Les préférences nutritionnelles de certaines bactéries importantes pour la santé ont été mises en évidence et examinées plus en détail au chapitre 4.

Le lien entre les prébiotiques, le microbiote intestinal et la santé de l'hôte, bien que relativement bien caractérisé, est plutôt plus difficile à prédire et a souvent reposé sur des données de fermentation *in vitro* et des interventions humaines et/ou animales *in vivo*. En donnant un aperçu du potentiel fonctionnel des microorganismes, les techniques moléculaires permettent d'étudier en détail les capacités métaboliques bactériennes à utiliser des ingrédients glucidiques non digestibles et à produire des produits finaux de fermentation. En combinaison avec les expériences *in vitro*, nous avons avancé dans ce **chapitre 4** une approche *in silico* de génomique comparative basée sur la conservation évolutive des fonctions métaboliques chez les bactéries pour identifier. L'objectif est d'identifier les mécanismes moléculaires impliqués dans l'utilisation des glucides et des biomarqueurs génétiques potentiels pour la prédiction du potentiel génomique bactérien à fermenter les prébiotiques. Ces différentes approches fournissent des preuves scientifiques pour une meilleure compréhension des interactions dynamiques entre le microbiote intestinal humain et les prébiotique et pour la formulation rationnelle d'aliments fonctionnels contenant des prébiotiques. Le contenu de ce chapitre fait partie d'une collaboration en cours : **Bedu-Ferrari C.**, Lopez J., Lacroix T., Loux V., Almeida M., Meslier V., Langella P., Cherbuy C. **Identification of genetic biomarkers to monitor key functionalities of the gut microbiote in response to diet.**

A travers ces travaux de thèse, j'ai décrit l'évolution du concept scientifique des prébiotiques (Bedu-Ferrari *et al.,* 2022). En particulier, j'ai mis en évidence le fait qu'une définition consensuelle des prébiotiques conduit le secteur agroalimentaire dans la recherche translationnelle vers de nouveaux aliments fonctionnels. Elle a ancré l'importance de caractériser l'impact des prébiotiques sur le microbiote intestinal et leurs conséquences sur la santé et le bien-être humains. Ainsi, le projet RestorBiome a évalué le potentiel des ingrédients alimentaires pour leurs effets prébiotiques. Ces travaux ont apporté des connaissances liées aux ingrédients alimentaires les plus prometteurs, qui recèlent un formidable potentiel pour le processus d'innovation. Appliqué au développement d'un produit alimentaire fonctionnel, le transfert de technologie peut avoir un impact social et économique par le biais de la commercialisation. Pour anticiper les futurs produits prébiotiques et évaluer le concept d'innovation du projet RestorBiome, je me suis demandée comment le développement d'aliments fonctionnels pouvait

réellement être appliqué dans la pratique prébiotique. En effet, l'allégation d'effets prébiotiques sur la santé intestinale doit satisfaire les autorités réglementaires adéquates. Le **chapitre 5** donne une compréhension approfondie de la façon dont les réglementations en vigueur, les organismes de réglementation et les décideurs politiques aux États-Unis et en Europe, encadrent le développement des aliments fonctionnels appliqués aux prébiotiques. Cette analyse décrit comment les grands marchés économiques en matière d'aliments fonctionnels et de compléments alimentaires régissent les allégations de santé faites pour les prébiotiques. Le contenu principal de ce chapitre est en cours de préparation pour un article d'opinion : **Bedu-Ferrari C.**, Langella P., Cherbuy C. **Prebiotics in practice: how to develop functional foods in regards to regulatory frameworks?**

Enfin, **la discussion générale et la conclusion** examinent la contribution de ce travail aux connaissances actuelles à propos des prébiotiques en mettant l'accent sur les orientations futures de ce travail.

Conclusion

La modification intentionnelle du microbiote intestinal à des fins de santé et de bien-être nécessite l'étude du métabolisme des glucides des microbes intestinaux afin de définir leurs capacités à métaboliser les substrats prébiotiques. La dégradation des prébiotiques a été déterminée par l'affiliation phylogénétique des bactéries commensales. Une caractérisation approfondie des principales bactéries bénéfiques pour la santé a révélé que les bactéries réagissent de manière significative à la source de carbone au niveau des métabolites. Pour découvrir les mécanismes de l'interaction dynamique entre les prébiotiques et les bactéries commensales importantes pour la santé, des expériences *in vitro* en monoculture et dans des consortiums bactériens synthétiques ont révélé que les sources de carbone influençaient les comportements temporels de la croissance bactérienne et des activités métaboliques. La préférence nutritionnelle des conducteurs bactériens envers une source de carbone particulière suggère une adaptation nutritionnelle au sein d'interactions trophiques complexes allant de la coopération à la compétition. L'exploration du potentiel génomique des bactéries utiles à la santé promet de déchiffrer les mécanismes moléculaires détaillés du métabolisme des glucides pour utiliser les fructanes de type inuline et produire des produits finaux de fermentation. L'application de biomarqueurs indiquant des fonctions pertinentes pour la santé peut être utilisée pour surveiller l'écosystème intestinal et prédire la réactivité des microbiotes intestinaux individuels dans le cadre d'une intervention diététique. Une compréhension approfondie de la croissance bactérienne, des activités métaboliques et des mécanismes moléculaires qui sous-tendent la métabolisation des prébiotiques semble impérative pour la formulation d'aliments fonctionnels.

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"Life would not long remain possible in the absence of microbes."

–Louis Pasteur

"Let food be thy medicine and medicine be thy food."

–Hippocrates

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List of Communications

Scientific publications

Bedu-Ferrari, C., Biscarrat, P., Langella, P., & Cherbuy, C. (2022). **Prebiotics and the Human Gut Microbiota: From Breakdown Mechanisms to the Impact on Metabolic Health.** *Nutrients*, *14*(10), 2096. https://doi.org/10.3390/nu14102096

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Previous publication

Leclerc, M., **Bedu-Ferrari, C.**, Etienne-Mesmin, L., Mariadassou, M., Lebreuilly, L., Tran, S. L., Brazeau, L., Mayeur, C., Delmas, J., Rué, O., Denis, S., Blanquet-Diot, S., & Ramarao, N. (2021). **Nitric Oxide Impacts Human Gut Microbiota Diversity and Functionalities**. mSystems, 6(5), e0055821. https://doi.org/10.1128/mSystems.00558-21

Oral/Poster communications

Bedu-Ferrari, C., Vati, S., Chaudemanche, C., Benoit, V., Lacroix, T., Loux, V., Langella, P., Cherbuy, C. Impact of dietary fibres and prebiotics on the gut microbiota: consequences on the digestive health and wellness. ABIES Days, September 2020, Paris, France **(oral presentation)** – *best presentation award*.

Bedu-Ferrari, C., Biscarrat, P., Vati, S., Chaudemanche, C., Benoit, V., Lacroix, T., Loux, V., Langella, P., Cherbuy, C. Prebiotics for digestive health and wellness: how to successfully promote the gut microbiome? SFAM Early Career Scientist Research Symposium, 22-26 March 2021, virtual event **(live oral presentation)**.

Bedu-Ferrari, C., Biscarrat, P., Vati, S., Chaudemanche, C., Benoit, V., Lacroix, T., Loux, V., Langella, P., Cherbuy, C. Functional characterisation of keystone intestinal bacteria to metabolize prebiotics for the benefits of the digestive health and wellness. 12th International Symposium on Gut Microbiology, 13-15 October 2021, virtual event **(poster presentation)**.

Bedu-Ferrari, C., Biscarrat, P., Vati, S., Chaudemanche, C., Benoit, V., Lacroix, T., Loux, V., Langella, P., Cherbuy, C. Evidences for different abilities of human gut bacteria to metabolise prebiotics for the benefits of the digestive health and wellness. International Scientific Association for Probiotics and Prebiotics Annual Meeting, 1-3 June 2021, virtual event **(recorded oral presentation)**.

List of Abbreviations

AA auxiliary activity

ABC ATP-binding cassette

AXOS arabino-xylo-oligosaccharide

BCFA branched chain amino acid

CAZyme carbohydrate-active enzyme

CBM carbohydrate-binding module

CE carbohydrate esterase

CO_2 carbon dioxide

CUT carbohydrate utilisation locus-containing TBDT

DP degree of polymerization

ECF extra-cytoplasmic function

EFSA European food safety

FDA food and drug administration

FOS fructo-oligosaccharide

GC-MS gas chromatography mass spectrometry

GH glycoside hydrolase

GlOS gluco-oligosaccharide

GLP-1 glucagon-like peptide 1

GOS galacto-oligosaccharide

gpPUL Gram-positive PUL

GT glycosyltransferase

H_2 hydrogen

HGG human gastrointestinal bacteria genome collection

HMO human milk oligosaccharide

HMP human microbiome project

HTCS hybrid two-component transduction system

IBS irritable bowel syndrome

IMO isomalto-oligosaccharide

ISAPP international scientific association of probiotics and prebiotic

ITF inulin-type fructan

LPMO lytic polysaccharide monooxygenase

MAC microbiota-accessible carbohydrate

MetaHit metagenomics of the human intestinal tract

MetS metabolic syndrome

MFS major facilitator superfamily

MOS mannan-oligosaccharide

OMV outer membrane vesicle

OS oligosaccharide

PBP polysaccharide breakdown product

PDX polydextrose

PL polysaccharide lyase

PTS phosphoenolpyruvate-phosphotransferase system

PUL polysaccharides utilisation locus

PYY peptide YY

RD resistant dextrin

RFO raffinose family oligosaccharide

rRNA ribosomal ribonucleotide

RS resistant starch

SCF soluble corn fibre

SCFA short-chain fatty acid

SGBP surface glycan-binding protein

SO_4^{2-} sulphate

SUS starch utilisation system

SOS soybean oligosaccharide

TBDT TonB-dependent transporter

WHO World Health Organization

XOS xylo-oligosaccharide

Table of Contents

GENERAL INTRODUCTION

Gut microbiota, human health and nutrition

Referring to the communities of microorganisms inhabiting the intestinal environment, the gut microbiota harbours trillions of bacteria, fungi, archaea, viruses, and other microbial components. In close interactions with each other, these microbial communities contribute to myriad aspects of host biology. Over the past decades, the significant expansion of the science related to the gut microbiota has anchored the importance of microorganisms to the overall human health and wellbeing.

Today, the world society is facing a great burden of health concerns, intimately related to human nutrition. Metabolic syndrome (MetS) refers to complex metabolic symptoms that reflect overnutrition, sedentary lifestyles, and the resultant excess adiposity (Cornier et al., 2008). The MetS is strongly associated with abdominal obesity, insulin resistance, dyslipidaemia, hypertension, which collectively contribute to the risk of cardiovascular diseases and type II diabetes. The global prevalence of MetS has been estimated and be between 20 to 25% of the adult population worldwide (Saklayen, 2018). In addition, the most common digestive disorder seen in the gastroenterology clinic is irritable bowel syndrome (IBS). Affecting about 11% of the worldwide population, IBS is characterised by abdominal pain, bloating, diarrhoea, constipation or alternating periods of these symptoms (Black and Ford, 2020). Gastrointestinal and metabolic medical conditions, such as MetS and IBS, are frequently associated with altered gut microbiota, immune dysregulation and chronic pro-inflammatory state. Human nutrition, which has one of the largest known impacts on the gut microbiota, has emerged as the main hypothesis for the increased prevalence of diet-linked disorders, although causality can be difficult to establish (Sonnenburg and Sonnenburg, 2014).

In particular, the low fibre intakes, that are typical for a Westernized lifestyle and nutrition, has been pinpointed as one of the explanation for the disturbances of the gut microbiota and increased risk of diseases. Dietary fibre encompasses a wide range of complex molecules, most of which are found in vegetables, legumes, cereals and fruits. Among these, carbohydrate molecules, also referred to as polysaccharides or glycans, are resistant to digestion by human enzymes. Therefore, most ingested fibres reach the colon and exert important physiological effects on human health and wellbeing. In particular, these fibres can be metabolised by gut microbes that show an extreme enzymatic capacity, resulting in a favourable modulation of the microbiota (Kaoutari et al., 2013). **Through a higher consumption of dietary fibres, the maintenance and/or restoration of the gut microbiota is a desirable goal for human health and wellbeing.**

Dietary fibres are applied as part of a healthy diet to public health actions that communicate the importance of high-fibre, low-sugar, and low-fat foods to promote our global health. National and international guidelines have initially been established to avoid nutrient deficiencies and related diseases. In particular, the World Health Organization (WHO) fixed the daily intake of dietary fibres above 25 g/day (World Health Organization, 2003). Nevertheless, many people do not achieve their recommended fibre intake because they consume insufficient fruits, vegetables and cereals (Stephen et al., 2017). **The adjustment of the human diet to close the "fibre gap" is a promising strategy in the prevention and management of many gastrointestinal and metabolic disorders.**

The link between dietary intake, gut microbiota and human health is an active area of research at the present time. Understanding the mechanisms that link fibre-rich diets to good health is challenging.

Prebiotics as a nutritional strategy

The study of the gut microbiota has revealed the fundamental role it plays in many biological and metabolic functions. An imbalance in the interactions within this vast community of microorganisms has important consequences for human health. These observations have led to the emergence of nutrition-based approaches to support the gut microbiota and its metabolic functions. **It is unclear how diet could be harnessed to easily modify the composition of gut microbes to boost the levels of desired bacteria. Knowing how dietary fibre nourishes gut microorganisms might suggest ways to stimulate the growth and metabolic activities of health-promoting bacteria.** In this context, prebiotics refers to the use of particular fibre sources that provide food for the desired gut microbes (Delzenne & Bindels, 2019).

The term "prebiotics" has been originally defined in 1995 by Roberfroid and Gibson and recently endorsed by the ISAPP (International Scientific Association for Probiotics and Prebiotics) as "a substrate that is selectively utilised by host microorganisms conferring a health benefit" (Gibson and Roberfroid, 1995; Gibson et al., 2017). Prebiotics represent an innovative solution for the future of nutrition. The use of such nutritional strategy requires a sophisticated understanding of the interactions that occur when the complex community of intestinal microbes encounters a source of fibre. Indeed, although different dietary fibres may seem physiologically equivalent, the underlying mechanisms of action are different, and this by targeting different gut microbes (Van Hul et al., 2020). Therefore, the beneficial health effects of specific dietary fibres must be documented to be considered a prebiotic.

The gut microbiota is complex, dynamic and exhibits considerable intra- and inter-personal variation in its composition and functions. The large number of potential interactions between the components of the microbiome makes it challenging to define the underlying mechanisms by which food ingredients affect community properties. **Substantial work remains to disentangle the interactions between prebiotics, the gut microbiome and the host health and wellbeing. This project is devoted to gain insights into the mechanistic of dietary fibres with prebiotic potential.**

Culture of the gut health in the agrifood industry

The science of the gut microbiota has received escalating attention in recent years in the scientific, healthcare, and public arenas. Consistent with these advances, communication around the gut microbiota has broadened public perception to an understanding of the beneficial roles of microorganisms in human health (Cunningham et al., 2021). **Across the world, people start to envision health through their gut. They increasingly make the link between the importance of the gut microbiota and how it affects the overall health and wellbeing.** They look for nutritional benefits that could reduce the risks of developing non-communicable diseases such as cardiovascular disorders, obesity, diabetes and cancers (Mellentin, 2020).

Related to a wide range of health concerns, the gut health culture is overwhelmingly focused on probiotics, dietary fibres, and prebiotics. There is an increasing demand for healthier food products, minimally processed foods with the presence of functional ingredients (Nowosad et al., 2021). Attentive to consumer demands, manufacturers look for solutions to offer holistic proactive approaches to health. Currently available on the market, probiotics as add-on, fermented foods and supplements aim to optimise digestion, which is increasingly recognized as the root of health and wellbeing. The choices of foods and beverages represent one strategy to reduce digestive discomfort or symptoms and to manage a health condition. These nutrition solutions create a competitive landscape stimulating the innovation. Investigating new horizons in gut health can support future nutrition solutions.

After evaluating the business opportunity and the definition of consumer needs, we believe that prebiotics represent an emerging solution to harness digestive health and wellbeing through the development of functional foods.

How can we successfully influence the gut microbiota?

In this project, prebiotics are proposed as a technological solution to nourish the gut microbiota and maintain health through the gut. The solution to support digestive health and wellbeing can be applied

to dairy products. Although supplements sales outweigh dairy products, this food matrix appears to work better than others based on the traditional dairy back-story. Dairy foods have always been the most popular vehicle to deliver digestive health benefits to humans due to the general high acceptance by consumers (Champagne et al., 2018). Composed of live bacteria cultures, yogurt is intuitionally perceived as beneficial for gut health. Although dairy foods appear as the most suitable matrix, we could ideate solutions to successfully deliver prebiotics extended to other food matrices such as bars, cereals, meals, and bakery products.

Prebiotics are part of a food industry with increasing market sales. Currently, a narrow range of confirmed prebiotic substances exists, with galactans and fructans dominating the market. Exploring the gut health culture can secure competitive advantages. Today, there is an opportunity to develop a yogurt with prebiotic fibres that nurtures the gut microbiome.

The lack of dietary fibre consumption across the globe suggests people do particularly struggle to reach the daily recommendations. Incorporating the concept of prebiotics could constitute a strategy to renew the dietary fibre consumption. The development of a functional food product that incorporates dietary fibres with prebiotic properties rely on a preventive approach to improve health and wellbeing and/or reduce the risk of diseases through the gut microbiota.

A consortium of experts

The feasibility of this project requires an interdisciplinary effort from a consortium of experts. The work presented in this thesis was embedded in a larger project “RestorBiome”. This collaborative project federates academic French institutions, including the National Research Institute for Agriculture, Food, and Environment (INRAE) and the Université Clermont Auvergne (UCA), which are specialists in gut microbiome science. The project also involved key players from the food industry, including GreenCell Biotechnologies and Yoplait France - General Mills, the latter being the project coordinator. Each technical stakeholders have complementary expertise and skills to successfully develop food products enriched with prebiotics to maintain and/or restore the intestinal microbiota. The project brings together competitiveness clusters, including Vitagora and Céréales Vallée, which favour the development of innovative projects in R&D and valorises the marketing of new products resulting from the projects.

Leader in microbial ecology, Greencell Biotechnologies produces innovative ingredients promoting a sustainable agriculture. The first area of innovation in the RestorBiome project is the identification of new molecules from alternative natural sources with respect to the planet and its resources. This line

of research includes the development of novel food ingredients promoting a balanced intestinal microbiota. The INRAE and UCA have the responsibility of the scientific evaluation of these compounds with potential prebiotic effects. This axis includes the development of a standardized methodology to assess the specificity of these substrates to stimulate the growth and/or fermentative activities of key health-promoting bacteria. The perspectives of the RestorBiome consortium is to validate the prebiotic effects of food ingredients *i)* in restoring and/or maintaining the composition and fermentative activities at the scale of the gut microbiota using *in vitro* fermentation systems *ii)* and in preserving the dialogue between the intestinal microbiota and the host using organoids and gut-in-a-chip systems. Finally, the project includes the development of prebiotic-enriched dairy products.

My implication in the RestorBiome project has been to implement an *in vitro* approach based on single-carbohydrate cultures of commensal bacteria for the selection of the most promising food ingredients with prebiotic effects. My thesis project aimed to characterise key health-promoting bacteria capacities in response to prebiotics for a better understanding of the underlying mechanisms conferring health benefits to the host.

Employed by Yoplait France – General Mills have allowed being part of the innovation process in R&D. My thesis aims to explore the prebiotic effects of food ingredients provided by the industry. Based at the INRAE, in Jouy en Josas, at the Micalis Institute, the Probihote team has allowed me to evolve in an appropriate work environment. My role in the RestorBiome project has been at the interface of academic and private research to transfer the fundamental research into the development of a functional food product.

Thesis outline

The impact of prebiotics on the bacterial growth and metabolic activities were studied using a reductionist approach of the gut microbiota that focuses on a panel of health-relevant commensal bacteria. **Chapter 1** reviews the scientific literature and addresses the challenges and pitfalls of research on the human gut microbiota and prebiotics. Next, the **main objectives and experimental strategies** outline the research hypotheses with a particular focus on the five food ingredients supplied by Yoplait France - General Mills. **Chapter 2** characterises the prebiotic effects on metabolic capacities at the level of isolated bacterial species relevant to human health. This study advances current knowledge on the carbohydrate metabolism of key gut bacteria. *In vitro* single-carbohydrate experiments revealed a variety of responses to prebiotics in a phylum-dependent manner. In this continuity, **Chapter 3** investigates the prebiotic effects on the bacterial metabolism at the level of a simplified consortia.

Indeed, studying isolated bacteria is not enough to understand the complex environment of our guts. This study yields a better understanding of the dynamic interactions between prebiotics and synthetic bacterial assemblies. Nutritional preferences of certain health-relevant bacteria were highlighted and further scrutinized in **Chapter 4**. In combination with single-carbohydrate experiments, comparative genomic analyses allow the identification of molecular mechanisms involved in carbohydrate utilisation and biomarkers for the prediction of bacterial genomic potential to ferment prebiotics. These different approaches provide scientific evidence for the rational formulation of functional foods containing prebiotics. However, the claim of prebiotic effects on gut health must satisfy adequate regulatory authorities. **Chapter 5** exposes the current regulations that frame the development of functional foods applied to prebiotics. This analysis provides a description of how Europe and the United States of America, which govern the major economic markets in terms of functional foods and dietary supplements, do not approve health claims made for prebiotics. Finally, **general discussion and conclusion** consider the contribution of this work to the current knowledge of prebiotics with an emphasis on future directions of this work.

Chapter 1: Literature Review

Chapter 1 aims at providing a literature review of the current knowledge that point the gut microbiome as a key mediator of dietary impact on the human physiology. Efforts are focusing on the importance of the human gut microbiome composition and functional capacities towards prebiotics. In particular, the description of the bacterial composition highlights species that are further studied in this project. Noteworthy, most of the microbial biomass that resides in the gut is bacterial, which is of special attention in this review of the literature. Although they play a role in the intestinal ecosystem, fungi, archaea, and viruses will not be further mentioned. This review also focuses on non-digestible carbohydrate-containing prebiotics, which comprise a large fraction of the human diet and selectively encourage the growth of targeted bacteria. The subsequent stimulation of fermentation activities results in the production of bioactive molecules conferring health benefits. Mechanisms underlying prebiotic effects encompass the complex interplay between diet, gut microbiota, and host energy metabolism. To highlight the importance of microbial saccharification in human health, this chapter summarises the molecular underpinnings of polysaccharide degradation processes at the bacterial scale and at the entire microbial communities. The intentional modification of the gut microbiome composition and functionality requires the most complete consideration of the recent prebiotic microbiome research. This chapter exposes the current knowledge of the prebiotic nutritional strategy for health and wellbeing purposes.

The main content of this chapter constitutes a literature review (Annex 1).

Prebiotics and the human gut microbiota: from the breakdown mechanisms to the impact on metabolic health

Bedu-Ferrari Cassandre[1,2], Biscarrat Paul[1], Langella Philippe[1], Cherbuy Claire[1]

[1]Micalis Institute, INRAE, AgroParisTech, Université Paris-Saclay, F-78350 Jouy-en-Josas, France
[2]Yoplait France- General Mills, Vienne Technical Center, F-38200 Vienne, France

*Corresponding author: claire.cherbuy@inrae.fr

I. Human gut microbiome

The gut microbiome refers to the microorganisms encompassing bacteria, fungi, archaea, viruses and other microbial components, and their genomes. Inhabiting the human gastrointestinal tract, this dynamic and complex consortium of microbes plays critical physiological functions. Encompassing 150 times more genes and as much encoded functions than the human genome, the gut microbiome extends the biochemical and metabolic activities of the host essential for health (Qin et al., 2010). The gut microbiome supports the modulation of the immune system during health and disease throughout life (Gensollen et al., 2016; Zheng et al., 2020). It also provides colonisation resistance to invasive pathogenic infections through a multitude of mechanisms, such as competing for nutrients, producing antimicrobial peptides, and maintaining the integrity of the intestinal epithelial barrier (Leshem et al., 2020). Furthermore, the gut microbiome contributes to host metabolism: it provides essential nutrients (e.g. amino acids, vitamins, short-chain fatty acids (SCFAs)), facilitates the utilisation of nutrients (e.g. bile acids, xenobiotics) and allows energy harvesting from otherwise indigestible dietary polymers of carbohydrates (LeBlanc et al., 2013; Maurice et al., 2013; den Besten et al., 2013; Lynch and Pedersen, 2016). **To understand and exploit the gut microbiome for health and wellbeing purposes, it is necessary to characterise the composition and functions of the microbial gut communities.**

I.1. Microbial composition and diversity

The gut microbiota regroups approximately 10^{13} bacteria/mL residing in the distal gut. Methods based on 16S small subunit ribosomal gene sequences (rRNA) provide a phylogenetic framework for the structure and diversity of the gut microbiome (Eckburg et al., 2005; Bäckhed et al., 2005; Gill et al., 2006; Poretsky et al., 2014). Taxonomically, bacteria are classified according to phyla, classes, orders, families, genera, and species. Facilitated by the fast development of–omics technologies, international initiatives such as the Human Microbiome Project (HMP) (Turnbaugh et al., 2007, 2009; Arumugam et al., 2011; Human Microbiome Project Consortium, 2012) and the Metagenomics of the Human Intestinal Tract (MetaHit) project (Qin et al., 2010; Arumugam et al., 2011) revealed the composition of the human gut microbiome. **Gram-positive *Firmicutes* and Gram-negative *Bacteroidetes* are the most diverse and abundant bacterial phyla that colonise the human large intestine.** In healthy adults, more than 90% of the species belong to only these two phyla, and *Firmicutes* can account for 70% of known phylogenetic diversity. Further improvement in understanding bacterial composition and functions was investigated in the Human Gastrointestinal Bacteria Genome Collection (HGG) (Forster et al., 2019). The experimental and computational methods decipher the gene content of the human gut microbiome

and provide access to the uncultured microbial diversity. Thus, the analysis of 11850 human gut metagenome identified almost 2000 uncultured bacterial species with distinct functional capacity **(Figure 1)** (Almeida et al., 2019).

Members of the *Firmicutes* group are affiliated with four classes including *Bacilli*, *Clostridia, Erysipelotrichi* and *Negativicutes*. The predominant *Clostridia* class encompasses the most abundant families *Lachnospiraceae* and *Ruminococcaceae* that account, respectively, for 50% and 30% of the total intestinal microbiome, along with the families *Clostridiaceae*, *Christensenellaceae*, *Eubacteriaceae* and *Peptostreptococcaceae* (Biddle et al., 2013; Rajilić-Stojanović and de Vos, 2014). The *Lachnospiraceae* is the most abundant and diverse family clustering a number of species officially classified into the genera *Roseburia, Blautia, Eubacterium* and *Anaerostipes*. Several members of the *Lachnospiraceae* family are butyrate producers and exhibit health-promoting properties (Duncan et al., 2002a, 2002b; Liu et al., 2021). The *Ruminococcaceae* family groups the *Faecalibacterium*, *Subdoligranulum* and *Ruminococcus* genera, recognized as members of the *Clostridium leptum* cluster IV. Among the *Ruminococcaceae* family, *Faecalibacterium prausnitzii* is the most prevalent and abundant gastrointestinal microorganism to produce acetate, butyrate, formate and lactate (Duncan et al., 2002b; Zou et al., 2021). Based on phenotypic and phylogenetic considerations, *Subdoligranulum variabile* is the nearest relative to *F. prausnitzii* producing mostly butyrate and lactate as main end-products (Holmstrøm et al., 2004). The first human gastrointestinal *Ruminococcus* species reported is *Ruminococcus bromii* isolated in 1972 and producing acetate and formate as the main end-products (Moore et al., 1972; Ze et al., 2012).

The *Bacteroidetes* phylum clusters the major families *Bacteroidaceae*, *Prevotellaceae*, *Rikenellaceae* and *Porphyromonadaceae*. The *Bacteroides* genus is the most abundant but also the most variable in between individuals (Arumugam et al., 2011). Isolated in 1898, *Bacteroides fragilis* was the first species initially qualified as a human pathogen linked to appendicitis among other clinical cases. Although some *Bacteroides* species have initially been considered opportunistic microorganisms, they play an essential role in intestinal health by performing the metabolic conversions of proteins and complex carbohydrates to succinate, acetate and propionate as the main end-products (Rajilić-Stojanović and de Vos, 2014).

Less abundant *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia* represent subdominant bacterial phyla in humans. *Actinobacteria* are Gram-positive bacteria commonly present in human gastrointestinal tract. Members of the orders *Bifidobacteriales* (in particular *Bifidobacterium* species) and *Coriobacteriales* (mainly *Collinsella* species) are highly prevalent since early life. Particularly,

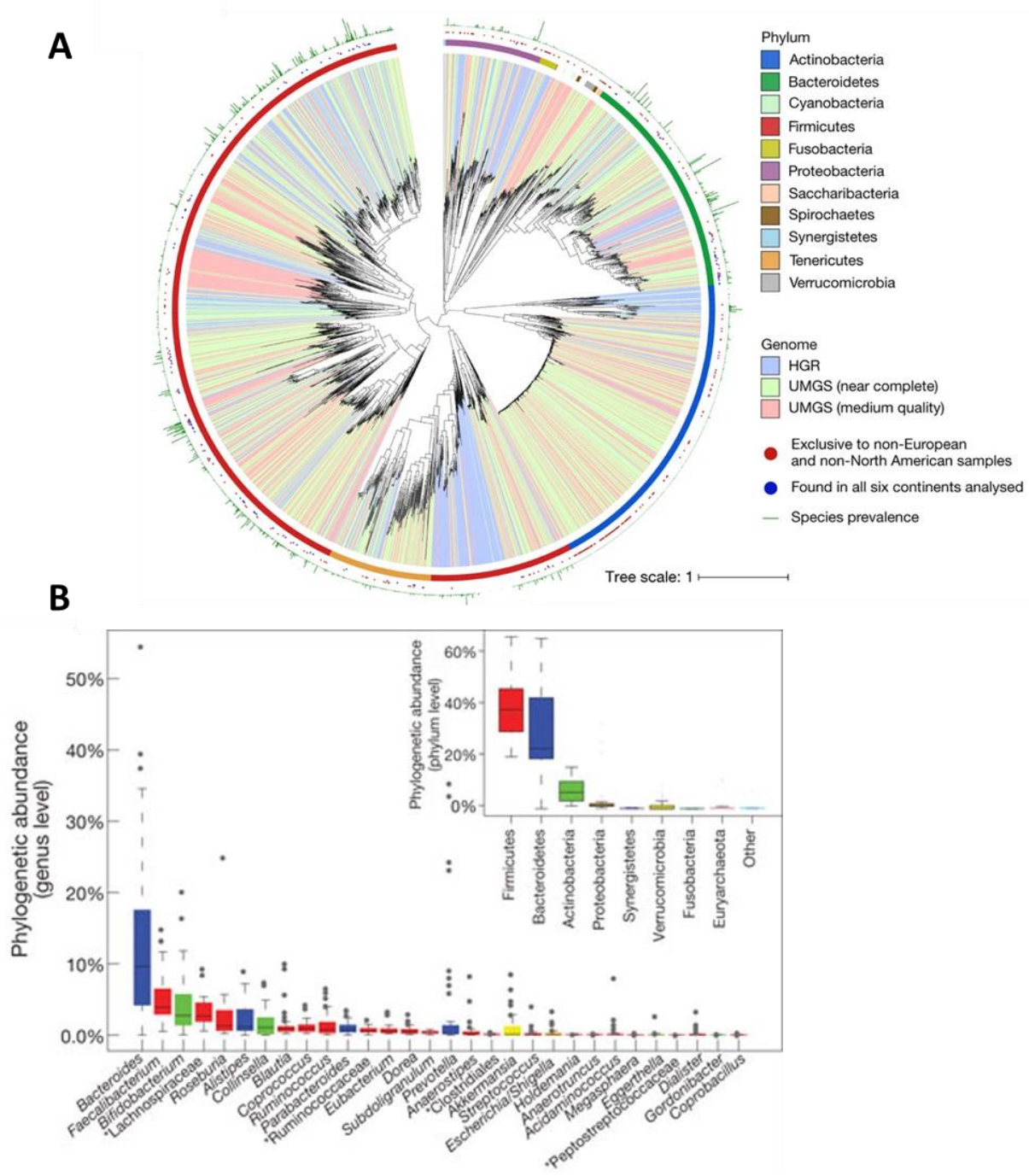

Figure 1: The abundance and the diversity of the human gut microbiome. A. Overview of the human gut microbiome phylogeny (from Almeida et al., 2019) A maximum-likelihood phylogeny was built on the basis of the 40 marker genes. Clades distinguish genome-types comprising 553 genomes belonging to the HGR and 1952 near-complete or medium-quality genomes to UMGS. The first outer layer depicts the phylum. The second layer denotes the geographical origin of genomes by blue and red dots. The last layer represents by a green bar the prevalence of the genome among 13133 metagenomic datasets. ***B. Phylogenetic profiles at genus level of the human gut microbiome (from Arumugam et al., 2011)*** The most abundant genera were determined by read abundance. Each genus is coloured respectively to their phylum affiliation displayed in the inset. The inset reports the most abundant phyla. Asterisks mark unclassified genera under a higher taxonomic rank.

Bifidobacterium species form a dominant fraction of the gut microbiome in infants, which constitutes the major lactose utilisers. Through a phosphoketolase pathway, also known as the bifid shunt, they produce acetate and lactate as the main end-products (O'Callaghan and van Sinderen, 2016). Moreover, they are assumed to have a beneficial effect on health and several members of the *Bifidobacterium* genus are commercially applied as probiotics. In fact, certain members of the *Lactobacillus* and *Bifidobacterium genera* make up the vast majority of functional ingredients present in currently commercialized food products. Members of the order *Actinobacteriales,* also known as *Actinomycetales,* are scarcely detected in the gastrointestinal tract (Rajilić-Stojanović and de Vos, 2014). *Proteobacteria* are particularly diverse Gram-negative bacteria, although not very abundant, representing about 1% of the gut microbiome. A unique keystone species within the *Verrucomicrobia* phylum has been detected in the human gastrointestinal tract. *Akkermansia muciniphila* was described in 2004 as a unique human gastrointestinal bacterium that is capable of growing in intestinal mucus as the sole carbon source (Derrien et al., 2004).

The democratization of high-throughput technologies strongly contributes to the current knowledge about the complex microbial communities inhabiting the intestinal environment (Clavel et al., 2022). Sequence-based assessment of human microbiomes revealed an extensive phylogenetic variability of the overall microbial communities unique to each individual (Arumugam et al., 2011; Human Microbiome Project Consortium, 2012; Lloyd-Price et al., 2017). This intervariability indicates a high degree of functional redundancy across gut microbes, which confers stability and resilience – that is, the capacity to return to an equilibrium state in response to chemical, physical or microbial perturbations – over time within the microbial communities **(Chapter 1, Section III.4)** (Moya and Ferrer, 2016). The myriad taxonomic compositions result in comparable functions of the intestinal ecosystem and ensure digestive functions of the host.

I.2. Digestive functions

Stably coexisting as a complex trophic network, the gut microbiome fulfils many digestive functions essential in human physiology. Indeed, anaerobic microorganisms in the gastrointestinal tract are involved in fermentation processes, regulation of food absorption through bile salts metabolisation, biosynthesis of bioactive compounds, such as vitamins and neurotransmitters, and elimination of exogenous toxins (A. Bernalier-Donadille, 2010; Lynch and Pedersen, 2016). **Forming a structural unit, the gut microbiome plays an important role in digestion ensuring the conversion of macronutrients into a variety of microbial-derived metabolites.** Actually, the degradation of

macronutrients, including carbohydrates, proteins, and to a lesser extend fats, is one of the core functions encoded in the gut microbiome (Lozupone et al., 2012; Oliphant and Allen-Vercoe, 2019) **(Figure 2)**.

Most digestive contributions rely on anaerobic microbes to digest the dietary macronutrients, which are distinctly available from the perspectives of the host and the microbes. In digestion, the reduced number human amylases in the oral sphere can hydrolyse very few macronutrients. These food components are further hydrolysed by pancreatic enzymes and are readily absorbed in the small intestine. However, high quantities of macronutrients can reach the colon upon either escaping the primary host-mediated digestion due to saturation of transporter systems or resisting due to the structural complexity of these polymeric biomolecules (Oliphant and Allen-Vercoe, 2019). Probably degraded to a certain degree, it was estimated that 2–10 g/day of sugars, 3–9 g/day of proteins and 20-40 g/day of carbohydrates are not completely assimilated in the small intestine of people on Western diets (Klurfeld et al., 2018). In countries with higher intakes of whole-grain cereals, legumes, fruits and vegetables, the amount of carbohydrates could reach 50 g/day (Elia and Cummings, 2007; Englyst et al., 2007). Due to day-to-day fluctuations in food intake and individual differences in gut function, uncertainty remains regarding the exact amounts and types of macronutrients that are available for bacterial fermentation (Mann et al., 2007). Nevertheless, the large intestine constitutes a favourable environment for commensal bacteria to hydrolyse and ferment non-digestible substrates.

In the forms of simple mono- and di- saccharides or the forms of complex oligo- and polysaccharides, as well as glycoconjugates, carbohydrates are one of the dominant nutrient sources in the human diet playing fundamental roles in all living systems. They constitute carbon sources essential for energy storage (starch in plants, glycogen in metazoans and fungi), structure (cellulose, hemicelluloses and pectins of plant cell walls, chitin of arthropods exoskeletons), host-pathogen interactions, signal transduction, inflammation, intracellular trafficking and diseases (Wali et al., 2021). **Depending on the dietary ratio presented to them, the gut microbiome preferentially consumes carbohydrates.** Found in cereals, legumes, fruits and vegetables, carbohydrates ranging from mono- and di-saccharides, such as glucose, fructose, sucrose, and lactose, to multitude of complex polysaccharides, typically oligosaccharides and resistant starch, account for 45-70% of total energy intake and expenditure (Terrapon et al., 2015). Noteworthy, mono- and di-saccharides escaping the host-mediated digestion can be rapidly consumed by the gut microbiome with often little

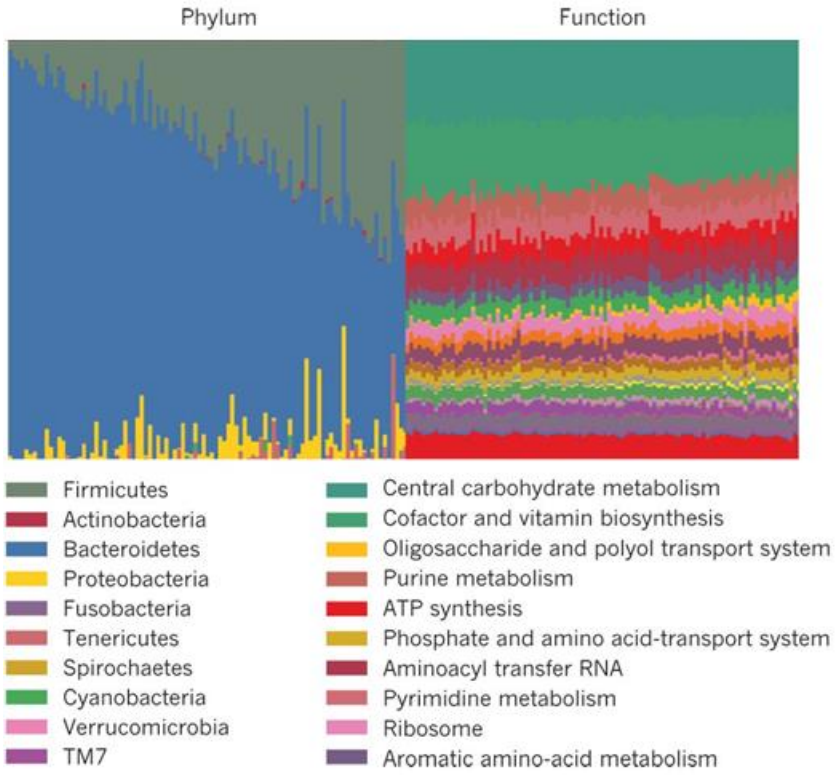

Figure 2: Functional diversity of the human gut microbiome (from Lozupone et al., 2012). From the Human Microbiota Project (HMP), the phylogenetic diversity of faecal communities was analysed using 16S rRNA (left panel). Microbial phyla show tremendous abundance diversity. These taxonomically disparate microbes often perform similar metabolic functions ensuring essential metabolic processes. The functional diversity (right panel) reported the redundancy of dominant pathways for central carbohydrate and amino acid metabolisms. Additional gut microbiome functions include cell signalling and membrane transport, cofactor and vitamin biosynthesis.

interconversion necessary for substrates to enter the Embden-Meyerhof-Parnas pathway, Entner-Doudoroff pathway, or Pentose phosphate pathway for pyruvate and subsequent ATP production (Wolfe et al., 2015). However, most of the complex carbohydrates require a metabolic dismantling from anaerobic microbes **(Chapter 1, Section II.1)**.

Anaerobic microbes are the major actors to degrade dietary substrates into microbial-derived metabolites. Hence, the gut microbiome has a profound effect on the host physiology in both positive and negative ways, exerting its influence both locally within the intestinal tract and systemically via the absorption of microbial-derived metabolites including SCFA and vitamins (Krautkramer et al., 2021). The relationships between bacteria and their hosts determine the microbial composition and functions. **In general, there is a great interest in the various contributors that influence the distribution of associated microorganisms and the host physiology. Diet is one of the most significant factor that shapes the structure of the gut microbiome, further influencing the human health.**

I.3. Shaping factors

Transiently passing through the lumen of gastrointestinal tract, the vast majority of gut bacteria are spatially distributed according to the properties of the local microenvironment that drives variations in both prevalence and abundance of taxa (Tropini et al., 2017) **(Figure 3A)**. Indeed, the gut microbiome is reflective of the physiological properties in a given region of the gastrointestinal tract. The density and composition of the microbiota vary substantially driven by chemical, immunological, and nutritional gradients along the gut, and thereby are stratified on both a transversal and longitudinal axis (Macpherson and McCoy, 2013). For example, the distribution of bile acids in the small and large intestine likely triggers significant changes in the dynamics of bacterial communities (Joyce and Gahan, 2016). Similarly, host immune mechanisms play an important role in controlling the intestinal microbiome and shaping the structure and function of the community (Hooper and Macpherson, 2010).

From infancy, the composition and function of the gut microbiome are tremendously personalised and strongly shaped by a plethora of selective pressures **(Figure 3B)**. The assembly of the human gut microbiota establishes through a succession of temporal colonisation and selection events of microorganisms from the environment. Each microbiome is shaped depending on infant-specific transitions, including the gestational date, the delivery pattern at birth, methods of milk feeding and weaning period (Koenig et al., 2011). Although early life events have strong effects on the gut microbiome, it retains some degree of flexibility. The gut microbiota can be subject to host intrinsic

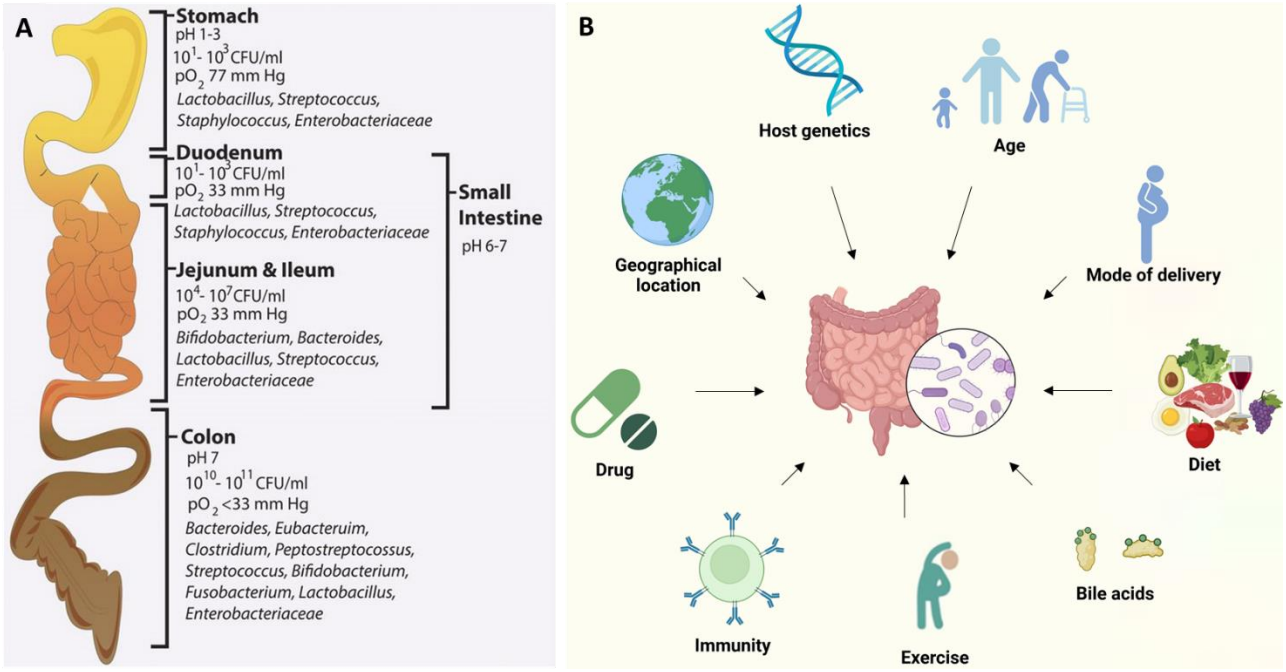

Figure 3: Selective pressures shaping the human gut microbiome (adapted from Clarke et al., 2019). A. Chemical and microbial gradients along the transverse axis of the gut. The composition and density of the intestinal microbes are not uniformly distributed along the gastrointestinal tract. Gradients of pH, bacterial concentration and oxygen partial pressures affect the spatial organisation of the gut microbiota. ***B. Overview of individual and environmental factors.*** The compositional characteristics of the gut microbiome are influenced by a number of factors including: mode of delivery (C-section vs. par vaginum); early feeding patterns (breast feeding vs. formula feeding); host genetics; geographical location; environmental exposures; exercise; gastric acid secretion; diet; ageing.

factors, including genetic forces (Benson et al., 2010) and physiological individual traits, such as age, sex, ethnicity, and body mass index (BMI) (Rothschild et al., 2018; Vujkovic-Cvijin et al., 2020). There is a complex interplay between dietary, gut microbiota and host physiology. In particular, circadian fluctuations and feeding rhythmicity induce microbial fluctuations and impact host physiology (Leone et al., 2015).

Nevertheless, environmental aspects dominate host intrinsic features in shaping the composition of the gut microbiome (Rothschild et al., 2018). Often associated with xenobiotics, including medications (antibiotic use, female hormones) and exposure to pollution (Clarke et al., 2019; Weersma et al., 2020), the host lifestyle, such as urbanization (De Filippo et al., 2010), is also involved in shaping the gut microbiota. In addition, fluctuations are closely related to geographical regions (Li et al., 2014), which reflect the cultural and dietary habits, and frequency of exercise (Lozupone et al., 2012). Evidence reported that seasonal dietary variations dynamically change the gut microbiome (Smits et al., 2017). The gut microbiota can be modulated through the exposure to a combination of environmental factors (Kolodziejczyk et al., 2019). Of these, diet influence is determinant.

There has been great attention to the role of diet components that exert a large effect on the gut environment, including gut transit time and pH of luminal contents (Oliphant and Allen-Vercoe, 2019). Previous studies have demonstrated that the gut microbiome is largely shaped by dietary macronutrients that exert a large influence on the intestinal environment (Turnbaugh et al., 2009; David et al., 2014). In particular, fat and protein intakes have been investigated to determine their influence on the gut microbiome composition (Kolodziejczyk et al., 2019). However, most studies have focused on carbohydrate intake, which can significantly affect the structure of the gut microbiome and ultimately influence the host's metabolic health. Complex dietary carbohydrates, including non-digestible dietary fibres (Walker et al., 2011; Tap et al., 2015), resistant starch (Martínez et al., 2010) and carbohydrate-containing prebiotics (Davis et al., 2011), appear to play a significant role in shaping the diversity of the human gut microbiome and readily link the microbiota to gut health. The site in the gastrointestinal tract, rate and extent of carbohydrate breakdown and the kinetics of absorption are key to understanding the many roles that carbohydrates play in the host metabolism and homoeostasis (Mann et al., 2007; Elia and Cummings, 2007).

How environmental and individual factors shape the human gut microbiota is still unclear, partly because these determinants are often confounded (Vujkovic-Cvijin et al., 2020). Thereby, the examination of the gut microbiome limits the capacity to identify causal relationships between host-associated microorganisms and human physiology. Although challenging, appreciation is growing for

how our gut microbes shape the physiological status in health and disease. It has been well established that a disruption in microbial communities, so called "dysbiosis", is a risk factor to the development of physio-pathological states (Wilmanski et al., 2021). Significant changes in diversity and composition within intestinal microbial communities have become hallmarks of many diseases, including irritable bowel syndrome (IBS) (Carroll et al., 2012; Tap et al., 2017) and complex metabolic disorders, denoted metabolic syndrome (MetS). The authors Ridaura *et al.* shed evidence on the remarkable role of the gut microbiome in lean and obese individuals, although the specific mechanisms being the cause or consequence of host metabolic responses were yet to be investigated (Ridaura et al., 2013). In general, substantial alterations in microbial community structure are predicted to be a major cause of non-communicable diseases, such as cardiovascular diseases, cancer, chronic respiratory diseases, diabetes, mental health and neurological disorders (Lloyd-Price et al., 2017). It is usually not known whether such associations are just correlative or a consequence of the health condition, or whether they might cause, or contribute to, the illness. Any disruption of the equilibrium between the gut microbiome and the host would be associated with modifications of the complex anaerobic trophic network and the functionality of the intestinal ecosystem, including bacterial fermentation activities.

I.4. Bacterial fermentation activities

Fermentation of dietary carbohydrates is one fundamental principle governed by gut microbial communities. The colon is the major site for complex carbohydrate fermentation due to favourable conditions for anaerobic bacterial metabolisms, such as its relatively high transit time and pH coupled with low redox potential. Fermentation activities vary along the gastrointestinal tract according to dietary residues. Not absorbed into the gastrointestinal tract, intestinal bacteria would adhere and degrade non-digestible carbohydrates into dietary residues. In the proximal colon, the bacterial growth and the fermentation are increased in accordance to the higher substrate quantity. In more distal zones of the colon, the availability of substrates to the gut microbiome progressively decreases (A. Bernalier-Donadille, 2010). Several factors influence fermentation activities, including the form and size of the food particles, the ratios of macronutrients, and transit time. Depending on the provision of adequate substrates, gut bacteria can generate metabolites that would be quickly absorbed by the intestinal epithelium (Krautkramer et al., 2021).

Fermentation by-products derived from dietary carbohydrates or host glycans primarily result in the formation of SCFAs, including acetate, propionate, and butyrate (Koh et al., 2016). These latter key biological molecules produced by anaerobic microorganisms are the main contributors to host health.

The proximal part of the large intestine is responsible for most of the absorption of the SCFAs. Intermediate fermentation metabolites include pyruvate, succinate, lactate, 1,2-propanediol, or acetyl-coA (Louis and Flint, 2017). These bacterial intermediates constitute precursors that can be further metabolised into end-products of energy metabolism. They usually do not accumulate to high levels in the human colon of healthy adults. Furthermore, small but significant amounts of alcohols, including ethanol, propanol, and 2,3-butanediol can be formed as end-products of carbohydrate fermentation (Louis and Flint, 2017). Furthermore, gaseous by- and end-products, such as hydrogen (H_2), carbon dioxide (CO_2), and sulphate (SO_4^{2-}) propel fermentation forward. Although energy needs are met primarily through fermentation, anaerobic respiration, through the membrane electron transport chain, confers the capacity to use H_2 and CO_2 by reductive acetogen and methanogen microorganisms, respectively, and SO_4^{2-} by sulphate-reducing microorganisms, as electron acceptors (Stams and Plugge, 2009). The utilisation of these gaseous substrates is mainly the result of cross-feeding between members of the gut microbiome, rather than host absorption (Rowland et al., 2018).

SCFAs are produced mainly through saccharolytic fermentation of carbohydrates, which have shown to exert multiple beneficial effects on host physiology. They are the main end-products of fermentation processes by the anaerobic intestinal microbiota that escape digestion and absorption in the small intestine. The role of SCFAs in human metabolism has been extensively reviewed together with the underlying molecular mechanisms. SCFAs improve intestinal health through a number of local effects. They constitute the main sources of energy for colonocytes, contributing approximately to 10% of the daily caloric content required by the human body for optimal functioning (McNeil, 1984). Actually, butyrate is the most preferred energy supply for colonocytes, although acetate and propionate are consumed to a much lesser degree than butyrate (Roediger, 1980). Depending on the fibre content in the diet, the composition of the microbiota and the transit time of the gut, SCFAs typically reach total concentrations of 50 to 200 mmol/kg of luminal content in the human large intestine (Macfarlane and Macfarlane, 2003). These relatively elevated concentrations of fermentation products tend to lower the pH of the colon, which directly contributes to protection against pathogens. Furthermore, SCFAs maintain intestinal barrier integrity by promoting cell proliferation and apoptosis, tight junctions, and mucus production (Morrison and Preston, 2016). SCFAs act as microbial signalling molecules that are recognized by specific host receptors. Thereby, they play a role in regulating the immune system and inflammatory response by influencing gene expression in colonocytes (Duscha et al., 2020). Relevant to host physiology, SCFAs participate in the short-term reduction of appetite through the secretion of glucagon-like peptide 1 (GLP-1) and peptide YY (PYY), and improvement of insulin response (Koh et

al., 2016). Other fermentation sources including proteins, peptides and amino acids constitute additional substrates for SCFA production. Nevertheless, amino acids fermentation also leads to the production of potential harmful metabolites such as phenolic and indolic compounds, amines and ammonia and branched chain amino acids (BCFA) (Flint & Louis, 2017).

SCFAs differ in relative production rates, concentrations and bacterial producers. The three predominant SCFAs acetate, propionate, butyrate are typically found in a proportion of 3:1:1 in the gastrointestinal tract (Cummings et al., 1987). Metagenomic approaches have facilitated the characterisation of bacteria responsible for SCFA production (Vital et al., 2013; Reichardt et al., 2014). Produced by most gut anaerobes, acetate is found in highest concentrations in the gut lumen. It plays an essential role in central metabolism. Distinctly, different subsets of gut bacteria are involved in the production of propionate and butyrate (Louis and Flint, 2017).

Propionate is distributed across a number of dominant phyla and relatively few bacterial genera (Reichardt et al., 2014). In particular, bacteria belonging to the *Bacteroidetes* phylum are involved in the synthesis of propionate through the main route of the succinate pathway **(Figure 4)**. Interestingly, some *Bacteroidetes* species produce succinate rather than propionate as their main and final fermentation products (Louis and Flint, 2017). Another pathway to synthesize propionate is through the propanediol pathway **(Figure 4)**. The latter is part of the metabolic activities of *A. muciniphila*, which is a dominant producer of propionate specialised in mucin degradation (Derrien et al., 2004). Mainly dependent of the nature of carbohydrates, some members of the *Lachnospiraceae* family could synthesize propionate, including *Roseburia inulinivorans* and *Blautia* species, while other *Firmicutes* produce 1,2 propanediol rather than propionate as their final product. For example, *Anaerobutyricum hallii* (formerly *Eubacterium hallii*) uses 1,2-propanediol in a cobalamin-dependent manner to form propionate (Engels et al., 2016). A minor route of propionate synthesis involves the acrylate pathway that consists in the conversion of lactate to propionate **(Figure 4)**.

Butyrate production is distributed across members of the *Firmicutes* phylum. For example, *Faecalibacterium prausnitzii, Eubacterium rectale* and *Roseburia* species are the most dominant butyrate-producing bacteria in the human gut microbiota in the colon both directly and through metabolic cross-feeding (Rivière et al., 2016; Louis and Flint, 2017). However, many dominant *Firmicutes* lack the ability to produce butyrate. In fact, members of the *Lachnospiraceae* family show disparate capacities to ferment diet-derived carbohydrates, which substantially varies between species and strains (Sheridan et al., 2016).

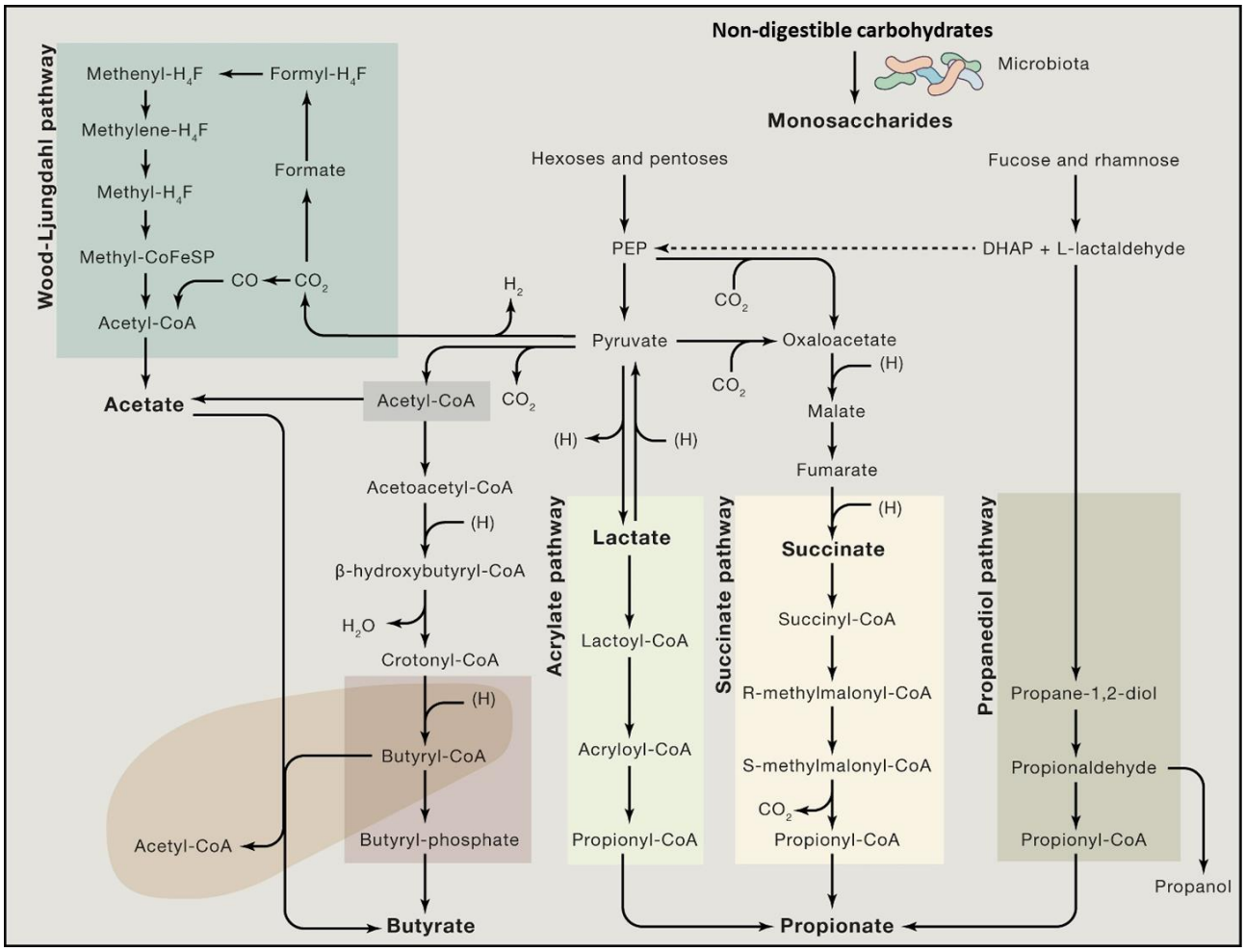

Figure 4: Overview of the pathways of bacterial fermentation resulting in the production of SCFA hydrolysis (adapted from Koh et al., 2016) Propionate is mainly produced by *Bacteroidetes* through two main pathways, the succinate or propanediol pathway, whereas the production of butyrate is dominated by *Firmicutes*.

The formation of SCFAs is the result of a complex interplay between dietary carbohydrates, the gut microbiome and the host metabolism. Fermentation of non-digestible carbohydrates by the gut microbiome is considered critical for mediating health benefits. A detailed understanding of SCFA metabolism by the gut microbiome is relevant to underpin dietary modulation strategies.

I.5. Nutritional strategies: toward a healthy gut microbiome

The evolution in human lifestyles, including the change in diets and the rapid development of modern society, disrupts the metabolic balance in humans, especially in the Western world (Sonnenburg and Sonnenburg, 2019). **Favouring processed starch and sucrose, the typical Western diet is believed to compromise the integrity of the gut microbiome. High caloric intake with a prolonged lack of dietary fibres is considered a health risk factor.** Le Chatelier *et al*. revealed the link between metabolic markers of obesity promoted by a Western diet and a reduced intestinal microbial diversity (Le Chatelier et al., 2013). Likewise, Sonnenburg *et al*. demonstrated in humanized mouse models that low-fibre diets had a negative effect characterised by the reduction of the diversity and abundance of fibre-degrading species in intestinal microbial communities. Some of these microbial strains were lost in offspring that were continually bred on low-fibre diets over multiple generations (Sonnenburg et al., 2016). Actually, several studies indicate that some dynamic lineages of microbes have decreased microbial diversity in modernized populations, with major shifts in composition and functions (Sonnenburg and Sonnenburg, 2019). Urbanized and industrialized lifestyles have been associated with a reduction of gut microbial diversity and loss of metabolic functionality in comparison to ancient and traditional rural populations (De Filippo et al., 2010; Smits et al., 2017; Wibowo et al., 2021). Furthermore, features of non-industrialized microbiomes are similar to the microbiomes of our human ancestors, and industrialized populations have diverged from this microbial signature (Olm and Sonnenburg, 2021).

Over the past century, the evolution of human diets has been designated as the main purported cause that explains the loss of microbial diversity in the populations of industrialized countries and the rises in cases of multiple non-communicable diseases, including obesity, asthma, diabetes, chronic inflammatory bowel diseases, among many others.

Diet is the major modifiable factor that can be targeted to counter the rising prevalence of metabolic diseases and intestinal disorders. The role of gut microbes has attracted intense research attention in mediating diet-induced effects on host physiology. The restoration of the gut microbiome stability seems a desirable goal for health and wellbeing purposes. Different nutritional strategies can offer considerable advantages in acquiring health, physical, and mental wellbeing (Vallianou et al., 2020). Diet-driven interventions that are rich in fibres promote changes in the microbial diversity often correlated to variations in faecal SCFA concentrations (Walker et al., 2011; Salonen et al., 2014; Johnstone et al., 2020). Thus, a high-fibre diet tends to increase concentrations of butyrate in the large intestine in correlation with an increase in *Firmicutes* bacterial members, conferring health benefits (De Filippis et al., 2016). In addition, dietary supplementation can be proposed to successfully modulate the human gut microbiome. Probiotics and prebiotics are the most studied functional components. Before the term "probiotic" was formally defined, they constituted the first strategy to modulate the gut microbiome for more than 100 years (Metchnikoff, 1908). Probiotics are defined as live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host (Hill et al., 2014). They are usually delivered through medicine/nutraceutical capsules as dietary supplements or foods and beverages. The probiotic approach would provide specific groups of saccharolytic bacteria known to produce beneficial metabolites. The *Lactobacillus* and *Bifidobacterium* groups are the most familiar genera used as probiotics for human consumption. The prebiotic approach would supplement the diet with fibres that favour the growth of beneficial resident gut bacteria **(Chapter 1, Section III)**. The combination of these two strategies constitutes a third approach that would directly exploit the mechanistic details provided by health-relevant microbial metabolites. Thereby, postbiotics were defined as preparing inanimate microorganisms and/or their components, including pili, cell wall compounds and other bacterial structures that confer a health benefit on the host (Swanson et al., 2020; Salminen et al., 2021). Recently, overlapping with, and adjacent to, the probiotic and prebiotic fields, new variants of microbiome-modulating solutions displaying nutritional and functional properties are developing, including fermented foods, postbiotics, microbial consortia, live biotherapeutic products, and genetically modified organisms, with renewed interest in polyphenols, fibres, and fermented foods (Cunningham et al., 2021).

Improving our health through the gut microbiome using different nutritional strategies is the key for achieving the restoration of the gut microbiome in its initial state considered healthy.

However, stating a "healthy" or "unhealthy" gut microbiome is difficult. There is no consensus definition of what is a "healthy" gut microbiome. In general, ecological principles of a "healthy" gut microbiome include microbial resilience- that is the ability to return to an original state (e.g. invasion by opportunistic pathogens) and resistance- that refers to the ability to resist to a perturbation (e.g. antibiotic treatment) (Bäckhed et al., 2012). Temporal stability is a key component to the inherent metabolic flexibility of the "healthy" gut microbiome that adapt to a variety of nutrient sources (Sholl et al., 2021). Therefore, a "healthy" gut microbial composition can be thought of as one that successfully maintains long-term stability, resists invasive pathogens, supplies key nutrients (including vitamins and fermentation by-products) to its host, and helps maintain host metabolic and immunological homeostasis (Koh et al., 2016).

A "healthy" gut microbiome is an elusive concept. The search for microbiome signatures of the gut microbiome is a key area in microbiome research. Unique taxonomic profiles and specific genera and species have been associated with health and disease (Gupta et al., 2020) as well as host biomarkers, dietary, and lifestyle characteristics (Mano et al., 2018). Taking into account the strongly individualised aspect, it is very challenging and complex to establish biomarkers and/or surrogate indicators of host function and pathogenic processes based on the gut microbiome (McBurney et al., 2019). Therefore, relevant authorities have not yet framed regulatory guidance for marketing and product claims pertaining to a "healthy human microbiome" across the world **(Chapter 5)**. Validation of a definition of scientific consensus could contribute to the framework that guides policymakers and regulators.

Reciprocally, there is no consensus definition of what is an "unhealthy" gut microbiome. Imbalances in the function and composition of intestinal microorganisms, known as dysbiosis, are associated with many health conditions. Certain gut bacterial species/groups and their metabolites have been related to diabetes, obesity, IBS and several other health conditions, but knowledge of individual microbial species is needed to decipher their biological roles in the human microbiome (Lloyd-Price et al., 2017). Hence, some of the key intestinal microbes, commonly considered crucial for gut health, are species belonging to the genera *Bifidobacterium*, *Lactobacillus,* and *Faecalibacterium*. The relative abundance of butyrate-producing bacteria belonging to the *Firmicutes* was reported reduced in pathological states whereas predominant in healthy individuals. However, the state of the gut microbiome should not be resumed to its idealized community composition. It is unknown whether dysbiosis is a cause, a consequence, or both.

Although the description of the gut microbiome composition has become routine, understanding the functionality of individual members in the context of their ecosystem remains challenging. To overcome this pitfall, the definition of a "healthy" microbiome has been proposed by the characterisation of its "core" functional potential uniformly present across a healthy population (Turnbaugh et al., 2009). Although controversial, a classification of individual microbiota arrangements in enterotypes has been proposed as a set of the same repertoire of microbial genes rather than a taxonomic profile of abundant organisms (Arumugam et al., 2011; Costea et al., 2018). The "core" functional capacities may be particularly useful in defining more universal markers of gut microbiome health. The multitude of factors that influence the human gut microbiome contributes substantially to the knowledge gap on what quantifiable characteristics constitute a "healthy" microbiome (Vujkovic-Cvijin et al., 2020).

The fundamental role that the microbiota plays in human health and disease has been accompanied by the challenge of trying to understand which features define a healthy gut community and how these may differ depending upon context. The influence of the diet is often indicated as a rapid change in the relative abundance of a single microbial taxon at the strain level (Patnode et al., 2021). However, the interpretation of such changes is controversial. These variations of bacterial species occupying specific niches within the gut environment may or may not affect the community function, depending on the composition of the rest of the community. The relative abundances of multiple interacting microbial taxa strongly shape the gut microbiome and ultimately host health. **Many microbial molecular mechanisms are still not fully understood. The contribution of gut microbiome modulation and the effects of diet-derived metabolites on human health in different scenarios is still uncertain. Establishing a causal link between bacterial shifts and host health is not straightforward.**

II. Carbohydrate metabolism by the gut microbiome

II.1. Digestibility of dietary carbohydrates

In forms of mono-, di-, oligo- and poly- saccharides, as well as glycoconjugates, carbohydrates have remarkable structural complexity, due to a rich diversity of monosaccharide building blocks, and the possibility of numerous glycosidic linkages giving rise to both simple linear and complex highly branched molecules. **Complex oligo- and polysaccharides, also designated as glycans, constitute the most heterogeneous and abundant polymer biomolecules in nature.** The primary classification of dietary carbohydrates is based on chemistry that is molecular weight, character of individual

monomers, degree of polymerization (DP) and type of linkage (α or β), as agreed at the Food and Agriculture Organization/World Health Organization Expert Consultation in 1997 (FAO/WHO Expert Consultation, 1998). Beyond the chemistry, carbohydrates are a diverse group of substances with a range of functional effects. A classification based solely on chemistry does not allow a simple translation into physicochemical and physiological properties since each of the major chemical classes of carbohydrates has a variety of overlapping effects (Cummings and Stephen, 2007). Thereby, their classification can also comprise nutritional dimensions of oligo- and polysaccharides reflecting physical effects in food matrix and food processing, specific health functionalities with regard to prebiotics, and biological origin with respect to dietary fibres.

In the human gastrointestinal tract, carbohydrate sources commonly include dietary plant and animal material. Mostly referred to as dietary fibres, a large variety of non-digestible carbohydrates supplies the gut microbiome (Hamaker and Tuncil, 2014; Martens et al., 2014). Carbohydrates can also be synthetized by other microbes that are food-borne or commensal to constitute a food source for some members of the gut microbiota. In addition, host-derived glycans of the dense layer of mucin forms a protective carbohydrate-rich barrier composed of O-linked glycans and glycosylated protein, providing a consistent nutrient source for bacteria when the host is between meals or when a suitable dietary glycan is not provided (Cockburn and Koropatkin, 2016). Each carbohydrate source displays different chemical structure characteristics, including the sugar moieties, the presence of substituents, the linkages and the side-chain branching, which affect carbohydrate physicochemical properties such as particle size and solubility. Subtle structural differences influence intestinal digestibility, carbon bioavailability and microbial accessibility. The carbon source accessibility incorporate the concept of microbiota-accessible carbohydrates (MACs), which refers to non-digestible carbohydrates, resistant to degradation and absorption by the host, and metabolically available to intestinal bacteria for fermentation (Sonnenburg and Sonnenburg, 2014). The amount of dietary MACs in food sources differs for each individuals upon their gut microbiomes. Human feeding trials estimated MACs about 75% to 90% for fruits and vegetables fibres and about 25% to 35% for whole grains fibres (Smith et al., 2020). This concept frameworks the investigation of metabolic abilities that a specific food or food nutrient can expect to exhibit within a given microbiome. **Variations in the accessibility of polysaccharides for fermentation are considered critical in shaping the microbial ecosystem to bringing about the health benefits of many carbohydrates in the diet.** Understanding the microbial digestibility of dietary carbohydrate by the gut microbiome is essential for application in dietary interventions, where MACs represent a promising tool for rationally modifying the gut microbial community.

II.2. Ubiquity of Carbohydrate-Active enZymes (CAZymes)

The enormous diversity of carbohydrates explains a large variety of carbohydrate-active enzymes (CAZymes) for the breakdown of complex material into the individual carbohydrate components. Analysis of the human genome revealed a paucity of genes encoding CAZymes for the breakdown of highly diverse structural carbohydrates. Indeed, the human genome has a reduced number of glycoside hydrolases (~17) and no polysaccharide lyases that are involved in carbohydrate digestion within the gut **(Figure 5A)**. Humans only produce a select set of enzymes capable of digesting non-resistant starch and simple oligosaccharides such as lactose, maltose, sucrose and trehalose (Kaoutari et al., 2013).

Although the human genome does not possess the enzymes necessary to digest dietary carbohydrates, the gut microbiome displays a plethora of CAZymes dedicated to the breakdown, biosynthesis or modification of complex carbohydrates. Microorganisms that feed on complex glycans have evolved large repertoires of CAZymes that expand digestive physiology. This functionality of the gut microbiome is reflected in an arsenal of prominent and highly diverse CAZyme-encoding genes, which comprise 1-5% of the predicted coding sequences in most bacterial genomes (Lombard et al., 2014). It is noteworthy that certain colonic bacteria are able to metabolise a remarkable variety of substrates, while other species carry out more specialised activities.

Members of the *Bacteroidetes* phylum, together with the *Firmicutes* phylum, dominate the human gut microbiome **(Chapter 1, Section I)**. Gram-negative *Bacteroidetes* bacteria exhibit a fascinating genetic repertoire encoding CAZymes, which supports the hypothesis of a better adaptation to the use of glycans and glycoproteins from food sources (Sonnenburg et al., 2010). *Bacteroidetes* species that utilise a large number of different carbohydrate structures often refer to themselves as "generalists" (Koropatkin et al., 2012). Contrarily, *Firmicutes* genomes tend to display low account of CAZyme-encoding genes mediating the breakdown of a narrower range of dietary polysaccharides. Utilizing relatively few carbohydrates, Gram-positive *Firmicutes* bacteria often refer to themselves as "specialists". Possessing more limited catabolism abilities, Flint and co-workers have suggested that *Firmicutes* may serve as "keystone" polysaccharide degraders (Ze et al., 2012). Important complex-fibre degraders from *Lachnospiraceae* and *Ruminococcaceae* families were associated with gut microbiomes presenting a high carbohydrate utilisation during *in vitro* batch fermentation (Smith et al., 2020). Harbouring a diverse microbial community results in a broader range of carbohydrate fermenters that are able to hydrolyse a wider range of glycosidic bonds and carbohydrate configurations.

CAZymes have been classified into families based upon their amino acid sequence similarities (http://www.cazy.org/) (Lombard et al., 2014). Diverse classes represent the numerous CAZymes families: glycoside hydrolases (GH) support the hydrolysis and/or rearrangement of glycosidic bonds; glycosyltransferases (GT) lead the formation of glycosidic bonds; carbohydrate esterases (CE) catalyze the hydrolysis of carbohydrate esters; and polysaccharide lyases (PL) operate non-hydrolytic cleavage of glycosidic bonds. In complement, enzymes for the auxiliary activities (AAs) including redox enzymes can supply CAZymes machinery. Carbohydrate-binding modules (CBM) are also involved in the adhesion of carbohydrates by displaying a modular structure with non-catalytic modules appended to the enzymes above (Cantarel et al., 2009). Functional prediction based on bacterial genomes revealed that GHs are the most representative CAZymes in the human gut microbiome (Kaoutari et al., 2013). These enzymes responsible for cleavage of glycosidic bonds in polysaccharides and oligosaccharides into di- and monosaccharide units are ubiquitous throughout all kingdoms of life **(Figure 5B)**. The exploration of GH diversity highlighted the number of families increasing steadily, and as of March 2022, 173 sequence-based families of GHs have been defined in the continuously updated CAZy database. Because there is a direct relationship between genetic sequence and folding similarities, the CAZy classification reflects the structural features of these enzymes better than their sole substrate specificity (Lombard et al., 2014; Grondin et al., 2017).

Based on amino acid sequences, the CAZy classification has proven to be particularly robust for the prediction and characterisation of CAZyme activities (Lombard et al., 2014). Key active-site residues, catalytic mechanism and the overall three-dimensional fold are strictly conserved allowing the investigation of substrate specificity, which is a key facet of microbial responses to dietary carbohydrate. Nevertheless, the broad sequence diversity illustrates the difficulty to differentiate between family membership with distinct activity profiles and substrate specificity (Hamaker and Tuncil, 2014). Indeed, CAZyme families are often "polyspecific" and include enzymatic activities with variations in glycosidic linkage specificity. Individual gut enzyme may be hypothetically associated with degradation of multiple polysaccharides leading to ambiguous functional predictions (Martens et al., 2014). For example, large families such as GH5 exemplify a wide variety of substrate specificities: this family currently contains close to 20 experimentally determined enzyme activities denoted with an EC number. Although the long and complex evolution of CAZymes families limits bioinformatic predictions, the classification helps to reveal evolutionary insights and provides a convenient tool to derive mechanistic information. Studies on CAZymes revealed new insights into the enzyme mechanisms and the dynamic evolution of microorganisms in carbohydrate degradation.

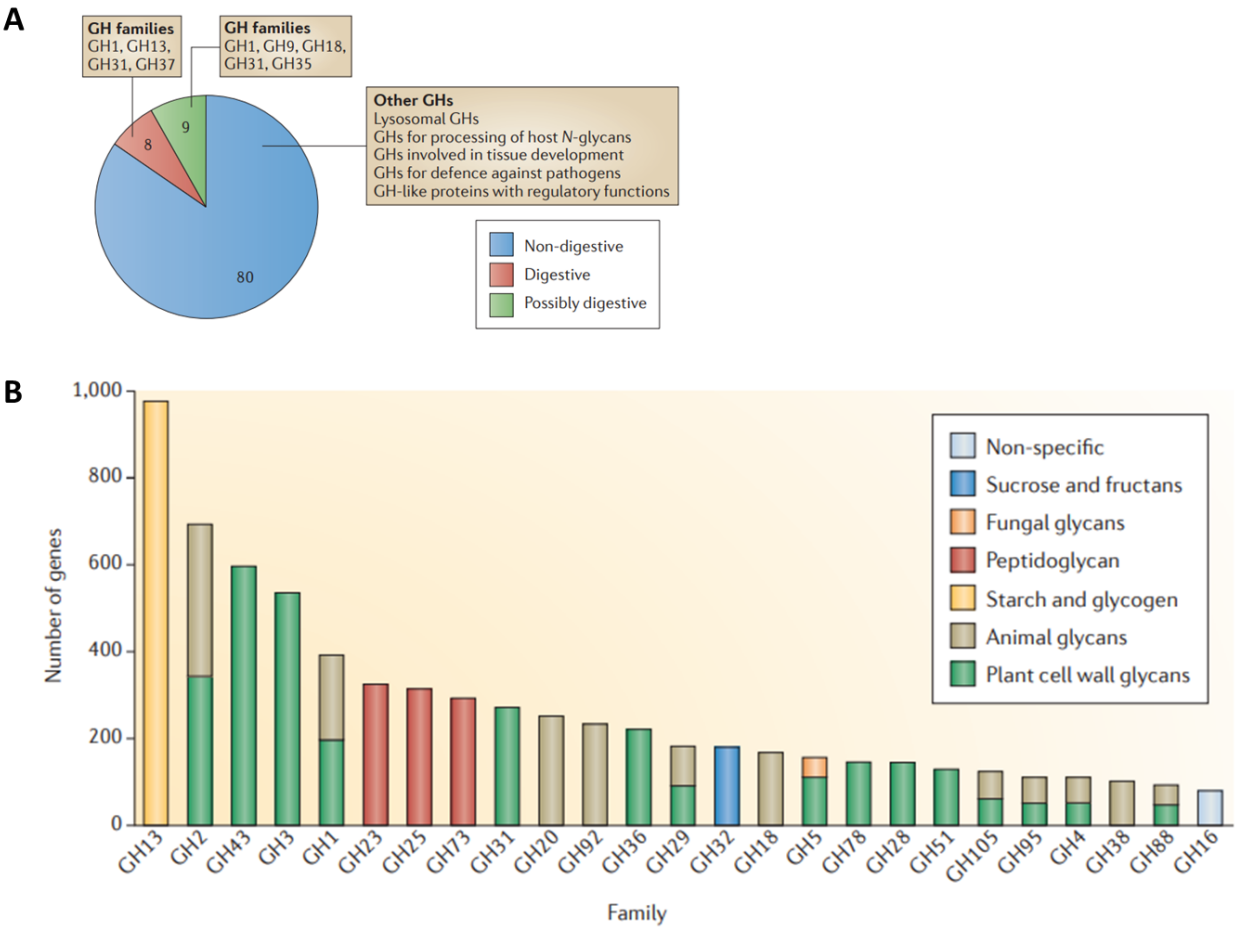

Figure 5: Distribution of CAZymes involved in the digestion of dietary carbohydrates in humans (adapted from Kaoutari, Armougom, Gordon, et al., 2013) ***A. Predictive enzyme cleavage of glycosidic bonds encoded by the human genome.*** The human genome encodes at most, only 17 enzymes for the digestion of food glycans, specially starch, sucrose and lactose. Among the 97 glycoside hydrolases (GHs), only 8 GHs are digestive and 9 are possibly digestive. The remaining enzymes have other functions than food digestion (e.g. GHs for processing host N-glycans, GHs involved in tissue development, GHs for the defence against pathogens, and GH-like proteins with regulatory functions). There are no polysaccharide lyases (PLs). ***B. The most abundant CAZymes based on a constructed model of a human gut mini-microbiome.*** Involved in starch breakdown, the glycoside hydrolase family 13 (GH13) is predictively the most represented family of CAZyme. GHs and PLs display different abilities to breakdown plant polysaccharides, animal glycans (e.g. hyaluronan, heparin and chondroitin) and other substrates.

II.3. Molecular insights of nutrient acquisition strategies

Polysaccharide Utilisation Locus (PUL) as the paradigm of bacterial foraging systems

Dictating the digestion of an array of complex dietary glycans, the large diversity and abundance of bacterial CAZymes revealed the *Bacteroidetes* phylum as a major actor in the breakdown of polysaccharides into a form that could otherwise not be absorbed and utilised by the host. **To varying extends among *Bacteroidetes* species, these Gram-negative bacterial genomes conserve a degrading and importing machinery that is encoded within clusters of contiguous and coregulated genes, known as polysaccharides utilisation loci (PULs).** First coined by Bjursell, Martens and Gordon in 2006, this elegant work provided the first mechanistic insights into how the symbiont *Bacteroides thetaiotaomicron* metabolises dietary starch and established a new paradigm of complex polysaccharide utilisation as a unique feature of *Bacteroidetes* genomes orchestrating the detection, sequestration, enzymatic digestion and transport of complex carbohydrates (Bjursell et al., 2006; Martens et al., 2011).

The archetypal PUL was initially defined for the starch utilisation system (SUS) described in *Bacteroides thetaiotaomicron* **(Figure 6A)**, which encodes all components necessary for starch metabolism, including carbohydrate binding, degradation, transport, and sensing proteins (Martens et al., 2008, 2009). Necessary to convert extracellular polysaccharides into intracellular monosaccharides, the SUS system-encoding genes are usually associated with three surface glycan-binding proteins (SGBPs), SusD, SusE and SusF, to recruit the starch to the cell surface, where it is cleaved into malto-oligosaccharides by the outer membrane localized CAZyme, SusG. Subsequently, these latter are transported into the periplasm via the TonB-dependent transporter (TBDT), SusC, where they are further degraded into glucose by the linkage-specific CAZymes, SusA and SusB. The key inner-membrane-bound regulatory protein, SusR, controls the expression of the susA-G genes in response to the presence of malto-oligosaccharides in the periplasm (Sonnenburg et al., 2010; Martens et al., 2011). As a signature of the canonical PUL machinery **(Figure 6B)**, the sequential susC/susD homologous genes constitute the TBDT and the cell surface glycan-binding proteins (SGBPs), respectively. In regards to the considerable structural diversity of SGBPs accounting for carbohydrate specificities, susD-like homologs refer to SGBP-A (Grondin et al., 2017).

The development of automatic bioinformatics by the CAZy team has led to the PUL database (http://www.cazy.org/PULDB), which catalogues experimentally characterised PULs from *Bacteroidetes*

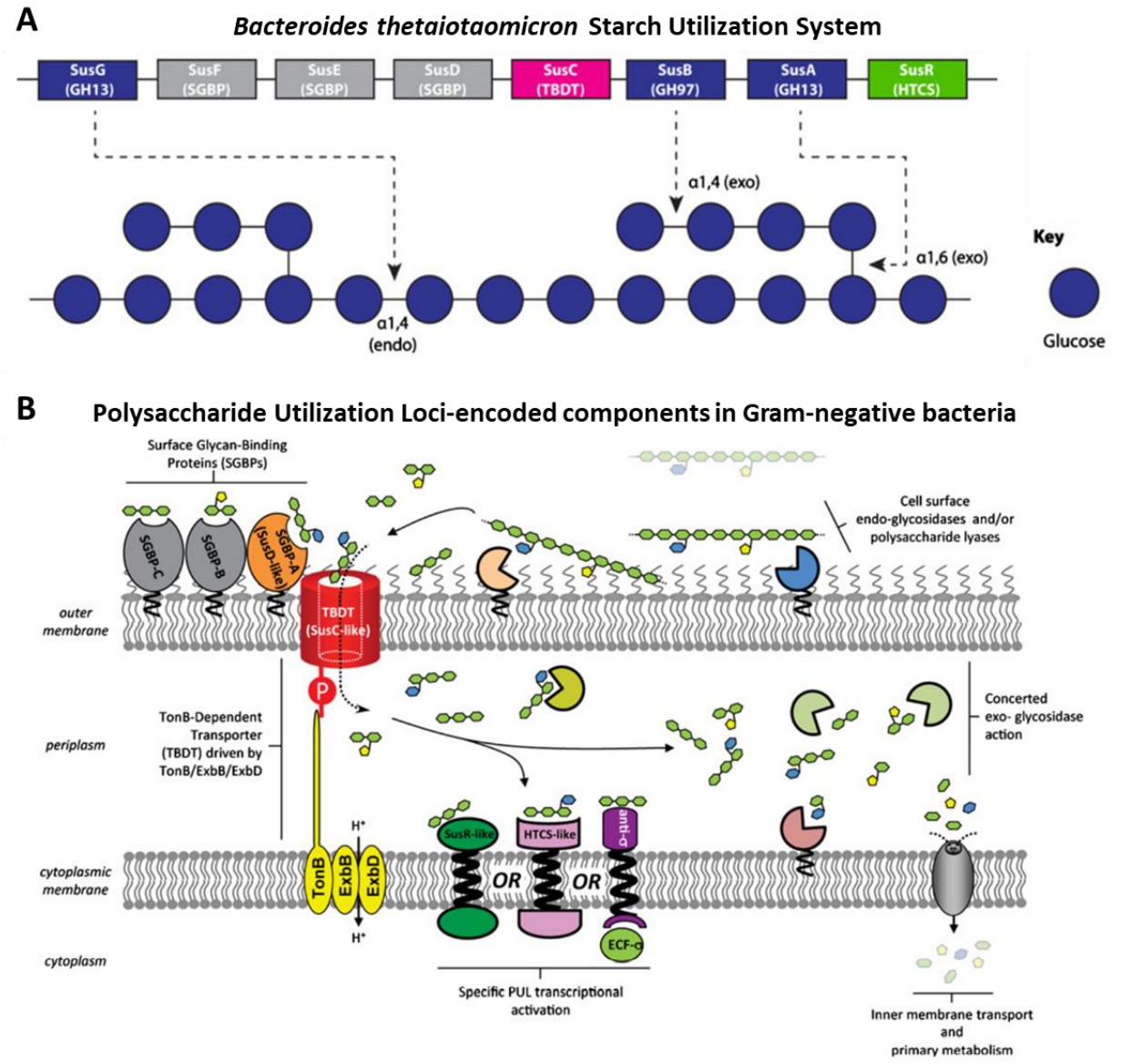

FIGURE 6: The paradigm of nutrient acquisition in the intestinal Bacteroidetes species (adapted from Hemsworth et al., 2016) ***A. The sus operon: a model system for starch uptake by the commensal Bacteroides thetaiotaomicron***. The SusD, SusE and SusF cell-surface glycan binding proteins (SGBPs) initially recruit the substrate from the outer membrane. The SusG endoglucanase hydrolyses starch into smaller maltooligosaccharides that are further imported into the periplasm by the SusC Ton-B dependent transporter (TBDT). Subsequently, oligosaccharides are catalysed into single sugars by the SusA and SusB exoglucosidases in the periplasmic space, before being imported into the cytoplasm for primary metabolism. In response to the presence of maltooligosaccharides, the prototypic PUL regulator SusR protein senses degradation products to control the PUL machinery. ***B. Polysaccharide utilisation loci (PULs) encoded components for carbohydrate metabolism, based on the archetypal SUS system in Bacteroidetes.*** Homologues of SusC and SusD are a hallmark of every Sus-like system. Intimately associated, these outer-membrane proteins adhere to polysaccharides and import digested products. However, carbohydrate-binding proteins such as SusE and SusF, as well as glycoside hydrolases, vary substantially between PULs. Likewise, different regulatory systems, responsible for the induction of PUL gene expression, include susR-like sensor and regulator, hybrid two-component transduction system (HTCS) and extracytoplasmic function (ECF) σ/anti-σ regulator factor for the transcriptional activation of the PUL machinery.

species (Terrapon et al., 2015, 2018). Along with structural biology and protein biochemistry studies, the range of complexity among individual PUL machineries has been revealed. **The gut microbiome exhibits extensive repertoires of PULs tailored to degrade a large variety of carbohydrate substrates.** Actually, the genetic variation of these PULs underlies divergent carbohydrate specificities and functional adaptation to polysaccharide diversity (Martens et al., 2008; Joglekar et al., 2018). Varying substantially between PULs, CAZymes most frequently release oligosaccharides as cell surface-anchored endoglycosidases and/or polysaccharide lyases. Additional linkage-specific CAZymes act in the periplasmic space to generate mono- and/or di-saccharides before being imported into the cytoplasm for primary metabolism. According to the substrate complexity, PULs can exhibit one or several CAZyme-encoding genes and ancillary enzymes such as esterases, proteases, sulfatases and phosphatases. The number of enzymes in a given system is directly correlated with the complexity of the target glycan (Koropatkin et al., 2012; Hemsworth et al., 2016).

Regulation of PUL-associated gene expression

In response to the transient changes in the nutrient environment, commensal bacteria of the gut microbiome strictly regulate the gene expression for adaptation to changes in resource availability. Broadly applicable to several prominent intestinal members, there are diverse local and global regulatory networks enabling the surveillance for available nutrients. Preferentially targeting plant cell wall carbohydrates, the hybrid two-component transduction system (HTCS) is the most prominent mode of PUL activated in the periplasm by oligosaccharides, coupling nutrient sensing and inducing an appropriate metabolic response (Sonnenburg et al., 2006). More particularly, the SusR-like sensor/regulator preferentially plays an important role in starch-derived oligosaccharides (but not glucose) (D'Elia and Salyers, 1996; Martens et al., 2011). Commonly found in PUL-associated induction mechanisms, the extra-cytoplasmic function (ECF) σ/anti-σ factor preferentially targets host-derived glycans rather than dietary plant polysaccharides. Additional regulators exist such as LacI, CRP, AraC (non-HTCS), GntR and SARP-OmpR (Grondin et al., 2017; Glowacki et al., 2021). Binding to free monosaccharides and simple disaccharides induces the transcription of the polysaccharide utilisation machinery (Sonnenburg et al., 2010). The ligand preference for an intermediate is thought to be primarily a result of breakdown of complex polysaccharides on the cell surface prior to their transport into the periplasm (D'Elia and Salyers, 1996; Martens et al., 2011). Recently, a global regulator has been determined to promote the transcription of genes involved in carbohydrate use, including one encoding a protein that promotes immunological tolerance in the gut environment (Townsend et al., 2020). Conserved in other *Bacteroides* species than *B. thetaiotaomicron*, this master regulator of

carbohydrate utilisation indirectly controls gut colonisation. Although the details of the promoters and potential signals mediate or modify basal expression of PULs have not been investigated deeply, regulatory strategy integrates sensory state of complex polysaccharides and its monomeric constituents in specific feedback inhibition of PUL expression (Schwalm et al., 2016; Townsend et al., 2020). These mechanisms of carbon catabolite repression integrate additional regulatory cues, which explain the global control mechanism underlying the overall hierarchy of preferred carbon source utilisation. Involved in the sensing of a broad spectrum of substrates, small RNAs have been identified as regulators for rapid down-regulation of PUL expression. Interestingly, substrate prioritization through small RNAs particularly concerns the model of sigma/anti-sigma factors, rather than the HTCS regulators (Cao et al., 2016; Ryan et al., 2020).

Extending the carbohydrate utilisation strategies

The PUL systems constitute the major strategy for harvesting carbohydrates deployed by *Bacteroidetes* bacteria **(Figure 7A)**. Nevertheless, analogs of *Bacteroidetes* PULs have been described beyond the tandem susC/susD-like pairs (Grondin et al., 2017). Inspection of bacterial genomes reveals that genes encoding CAZyme and susC-like TBDT may colocalize, although adjacent susD-like SGBP homologs and sensor/regulator systems are notably absent (Terrapon et al., 2015). Indeed, this advanced system of carbohydrate utilisation locus-containing TBDT (CUT) was described in a concerted action of a TBDT and periplasmic glycosidases, which coordinated complex carbohydrate metabolisation. Ubiquitous in *Bacteroidetes* PULs, the CUT system lacks genes encoding a SusD homologous protein and a sensor/regulatory system (Blanvillain et al., 2007). *Bacteroidetes* constitute only a fraction of the human gut microbiome. Gram-positive bacteria deploy alternative microbial strategies for carbohydrate harvesting **(Figures 7B and 7C)**. Although distinct in structure from Gram-negative systems, prominent *Firmicutes* and *Actinobacteria* bacteria elaborate additional cell surface-associated systems for the utilisation of polysaccharides, which are often built around enzymes with catalytic domains similar to those of *Bacteroidetes* (Glowacki et al., 2021). Substrate-specific gene clusters targeting a variety of plant- and host-based glycans were identified in the genomes of *Eubacterium rectale* and *Roseburia* species as Gram-positive PULs (gpPULs) (Cockburn et al., 2015; Sheridan et al., 2016; Tanno et al., 2021). The gpPULs contain carbohydrate transport systems that are mostly ATP-binding cassette (ABC), major facilitator superfamily (MFS) or phosphoenolpyruvate-phosphotransferase system (PTS) transporters. These genes are colocalized with associated LacI or AraC-like transcriptional regulators and a minimum of one CAZyme (Cockburn and Koropatkin, 2016; La Rosa et al., 2019). Some gut Gram-positive bacteria have adapted another extracellular multienzyme complex, known as a cellulosome, which targets

cellulose (originally), resistant starch, and possibly other substrates. This glycan acquisition paradigm of Gram-positive species have been well characterised in carbohydrate-degrading microorganisms from the bovine rumen and soil (Glowacki et al., 2021). Genomic, biochemical and molecular approaches of the human gut microbiome revealed the presence of protein components (dockerins and cohesins) that are signatures of cellulosomes in colonic *Ruminococcus* bacterial species (Ze et al., 2015). Another strategy constitutes the lytic polysaccharide monooxygenases (LPMOs), which rely on lignocellulose degradation. Classified as AA in the CAZy classification (Lombard et al., 2014), LPMOs have not been identified to date in any human microbiome data, presumably due to the anaerobic environment present in the distal human intestine (Hemsworth et al., 2016).

Unlike the multiple foraging strategies for utilising complex carbohydrates, the most common utilisation of gut nutrients predominantly confines to the utilisation of more simple mono- and disaccharides (Glowacki et al., 2021). Through the action of non-selfish bacterial catabolism, amounts of simple sugars or oligosaccharides may be transiently present in the colon. In competitive environment, the strategy of scavenging simple sugars liberated from larger polysaccharides by the action of commensal *Bacteroidetes*. These systems are generally less complex than PUL-encoded mechanisms. Many species broadly utilise simple sugars, which sometimes vary in their preference for metabolizing these sugars in complex mixtures, leading to alterations in the importance of certain sugars to different strains.

Nutrient acquisition strategies have increasingly been identified in species outside the *Bacteroides* genus, enhancing the metabolic repertoires of selected gut species. Identification of colocalized genes encoding CAZymes and transporters presents a valuable tool for bioinformatics analyses of complex carbohydrate utilisation. **Comprehensive molecular characterisation remains to be fully elucidated at the molecular level to understand the adaptive evolution of nutrient acquisition across intestinal microbial communities.**

II.4. Two common strategies at the level of the microbial community

Microbes inhabitant of the gastrointestinal tract must possess efficient strategies for harvesting carbohydrates in a dynamic nutrient environment. **Nutrient acquisition systems appear as a prerequisite for gut persistence to accommodate fluctuating nutrient levels.** Shifting bacterial metabolisms, regulatory systems mediate rapid and specific responses through coordinated gene expression. Connections exist between local PUL expression and the global nutrient status. However, the complete mechanisms for orchestrating carbohydrate utilisation remain to be determined.

A dietary carbohydrate could have disparate effects on the composition of the microbial communities depending on the degrading capacities of commensal bacteria. Community-driven metabolic changes could correspond to a complex hierarchical organization in response to nutritional changes on short timescales. The rapid changes likely reflect transient proliferation of the species that are the best equipped to metabolise specific substrates (Martens et al., 2014).

Coordination of nutrient acquisition strategies at molecular levels enables commensal bacteria to efficiently utilise dietary carbohydrates and thrives in a very competitive intestinal environment. Highly abundant *Bacteroidetes* are functionally diversified to assimilate a wide diversity of complex polysaccharides. Two common strategies of nutrient acquisition have been described for *Bacteroidetes* members at the level of the gut microbiome: one is selfish and the other is cooperative sharing. The degree to which carbohydrate degradation is mediated by syntrophic interactions between different members of the gut microbiome is unclear. It is possible that complex glycans and nutritionally adapted *Bacteroides* species share hydrolytic activities and/or partially degraded carbohydrates in a nutritional crisis context when competition for resources is intense within the gut microbiome. In some altruistic *Bacteroides* species, hydrolytic enzymes seem to act as public good resources in various networks of polysaccharide utilisers that benefit the whole microbial population (Elhenawy et al., 2014; Rakoff-Nahoum et al., 2014; Valguarnera et al., 2018). Indeed, harbouring signal sequences, hydrolases and proteases are preferentially packed in outer membrane vesicles (OMVs) and are further released into the extracellular environment. Most of these PUL-derived enzymes are OMV surface-exposed rescuing the growth of bacteria incapable of efficiently using complex polysaccharides. Ubiquitous among Gram-negative bacteria, including *B. ovatus*, *B. fragilis* and *B. thetaiotaomicron,* vesiculation appears to optimise the breakdown of nutrients by other bacteria with different metabolic capacities. Most of these OMV-enriched-enzymatic activities contributes to the maintenance of the gut microbiome.

Leaving the rise of metabolic cross-feeding, some primary *Bacteroides* degraders diffuse simple oligosaccharides produced at the cell surface into the extracellular environment. Releasing polysaccharide breakdown products (PBPs), this PUL-based distributive mechanism concomitantly benefits the bacterial community that lacks the enzymatic machinery to process the initial depolymerization step (Rogowski et al., 2015). Intermediate common resources, such as the secretion of hydrolytic enzymes and polymers, can increase the viability of primary degraders itself and benefit the growth of closely related neighbouring species (Rakoff-Nahoum et al., 2014). For instance, the large

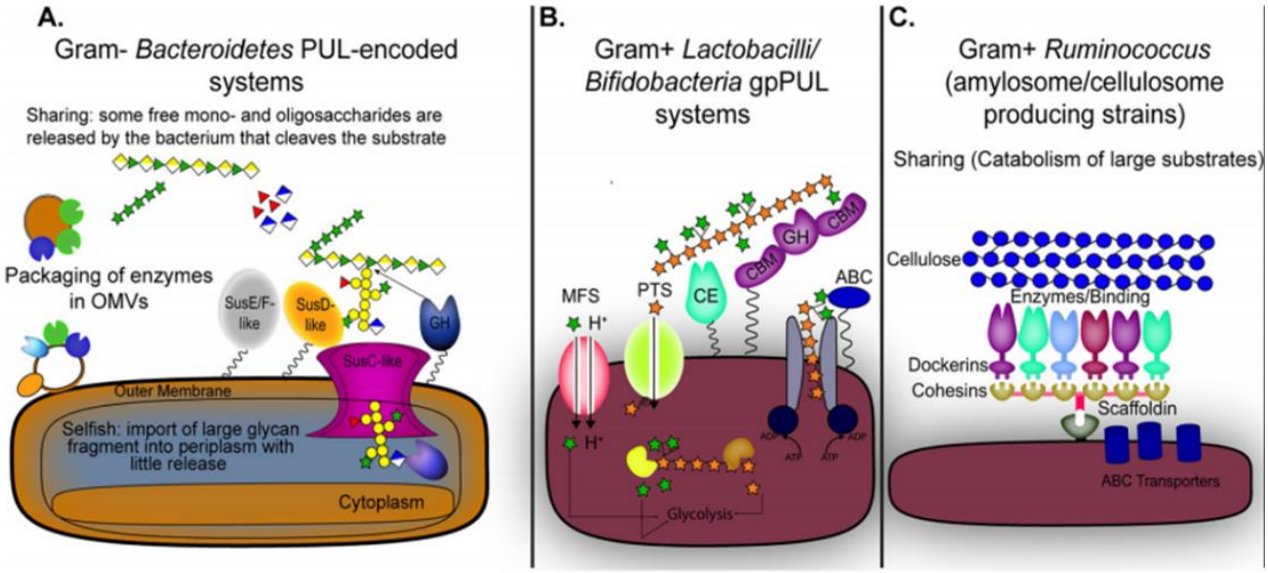

Figure 7: Mechanisms of nutrient utilisation for Gram-negative and Gram-positive intestinal bacteria (adapted from Glowacki et al., 2021). A. The archetypal polysaccharide utilisation loci (PULs) in the Gram-negative (G-) Bacteroidetes phylum. These multiprotein systems display glycoside hydrolases (GHs), SusC-, SusD- and SusE/F-like outer-membrane proteins. ***B. Prominent carbohydrate utilisation systems for some members of the Gram-positive (G+) Firmicutes and Actinobacteria phyla.*** These G+ PULs (gpPULs) often present cytoplasmic membrane importers such as ABC transporters, major facilitator superfamily (MFS) proteins, and (PTS) associated with degradative enzymes (GHs and carbohydrate esterases [CE]) and carbohydrate-binding modules (CBMs). ***C. Some Gram-positive Ruminococcus species exhibit multiprotein complexes termed cellulosomes or amylosomes to degraded cellulose and resistant starch, respectively.*** These strains and other related bacteria often rely on ABC transporters and display proteins including dockerins, cohesins and scaffoldins.

repertoire of GH/PL of *B. ovatus* appears more likely to degrade a wide range of polysaccharides with concomitant production of public goods, thriving the growth of other co-cultured *Bacteroidetes* members.

This cooperative evolution of mutualistic interactions in *Bacteroidetes* members results in positive effects on the bacterial fitness. Noteworthy, sharing common resources depends on the structural complexity of carbohydrates that influence their microbial accessibility (Rogowski et al., 2015). Based on carbohydrate structure, the symbiotic interaction network reflects the degradative hierarchies of carbohydrate utilisation, in which some simple glycans are prioritized above more complex polysaccharides (Rogers et al., 2013). In fact, individual bacteria exhibit multiple prioritization strategies in the presence of competing carbon sources. The simultaneous presence of dietary glycans, particularly in the form of monosaccharides, participates in consistent repression of the breakdown of host-derived glycans in *B. thetaiotaomicron* (Pudlo et al., 2015). In general, the presence of highly prioritized glycans repressed the transcription of genes involved in utilizing lower priority nutrients (Cao et al., 2016). Hierarchical orders promote the coexistence of stable microbial communities in a competitive environment (Tuncil et al., 2017). The selective metabolism of substrates supports the dynamics of microbial communities and promotes the metabolic plasticity of the entire gut microbiome, which is constantly faced with nutritional changes.

Interestingly, carbohydrate metabolisation does not always occur in the extracellular environment. Indeed, cell surface CAZymes can actually generate large oligosaccharides. Rapid transportation of PUL-produced oligosaccharides into the periplasm of Gram-negative *bacteria* may minimize substrate availability to other microbial residents. This "selfish" strategy prevents other bacterial species using partially degraded products (Cuskin et al., 2015). Exhibiting little collaboration during the digestion of complex carbohydrates, *B. thetaiotaomicron* rapidly imports some glycans into the periplasm for further breakdown, conferring no direct benefits on neighbouring species and enabling the microorganism to thrive in the competitive environment. Most PBPs would be for private competitive advantages and modest social benefits in the gut microbiome. These selfish ecological and evolutionary behaviours drive interdependent patterns for the bacterial fitness of the entire gut microbiome, which would stabilise in the context of reciprocal feedback benefits. The selfishness strategy is central to the eco-evolutionary stability of extracellular polysaccharide digestions between microbial species. Bacterial interactions tend to significantly benefit the fitness of the overall microbiome environment through the promotion of cooperation rather than competition (Rakoff-Nahoum et al., 2016; Wang et al., 2021).

The coordination of carbohydrate utilisation systems represent an impressive evolutionary solution for capturing valuable carbon sources. The rich interaction network between carbohydrates and bacteria is highly complex on the scale of an entire gut microbiome.

Strategies for carbohydrate metabolisation are often different at the level of individual microorganisms and microbial community (Grondin et al., 2017). Carbohydrate metabolisation varies according to the nutrient acquisition strategies, leading to different interdependent catalytic mechanisms that preferentially support the growth of certain microbes. There are clear of nutrients for the utilisation hierarchies driven by PULs-based systems targeting different polysaccharides. Preferential degradation of some glycans over others is likely to play a central role in the complex relationships of the gut microbiome (Schwalm and Groisman, 2017). Understanding the utilisation of intestinal bacterial glycan utilisation will be essential to induce predictable changes in the intestinal microbiota to improve health through diet. Predicting individual strategies of nutrient acquisition for each microbial type complete the characterisation of microbial adaptation to prebiotics to improve human metabolism (Klassen et al., 2021).

II.5. Exploration of the gut microbiome saccharification

Multidisplinary approaches provide a comprehensive understanding of the functional characterisation of carbohydrate metabolism. A combination of biochemical, enzymological, genetic and structural analyses revealed specific mechanistic insights in *Bacteroidetes* bacteria, which are important polysaccharide degraders in the gut environment (Cockburn and Koropatkin, 2016). Indeed, harbouring unique PULs exhibiting specialized CAZymes, the *Bacteroidetes* phylum gains attention for the dismantling of carbohydrate discrete structures. They have emerged as model organisms for functional microbiome research, especially the *Bacteroides thetaiotaomicron* species.

The abundance and diversity of CAZymes and PULs have enabled the comparative analysis of various *Bacteroidetes* bacteria, providing a fundamental basis to fully understand the roles of nutrient acquisition strategies directed toward complex polysaccharides in microbial communities. To decipher the nutrient acquisition strategies of specific *Bacteroidetes* members, different studies have explored the responses to a large variety of carbohydrate substrates, as done by Martens et al., 2008 for *B. thetaiotaomicron*, by Martens et al., 2011 for *B. ovatus,* and by McNulty et al., 2013 for *B. cellulosilyticus*. Showing few homologous and overlapping sets of PULs, individual *Bacteroidetes* species refer to nutrient adaptation that indicates their ability to respond dynamically to nutrient availability and participate in specific glycan niches within the gut microbiome. Prediction of metabolic abilities of

various *Bacteroidetes* bacteria at the molecular and cellular levels remains critical to understand nutrient niche colonisation and microbial ecological interactions (Terrapon et al., 2015, 2018). Reconstruction of metabolic and regulatory pathways and comparative genomics provide a comprehensive vision of carbohydrate utilisation to predict the functionality of the human gut microbiome.

Despite the -omic insights into the metabolic potential and the dynamics of the gut microbiome in response to dietary carbohydrates, the precise mechanisms of complex saccharification remain poorly elucidated. In the field of health and nutrition, exploring the interactions involved between carbohydrate metabolism and commensal bacteria is essential to better understand the plasticity of the human gut microbiome. **In addition, the investigation of regulatory mechanisms of nutrient acquisition strategies is not yet fully understood.** As potential functional biomarkers, utilisation of the clarification of CAZymes and PULs utilisation and their gene regulation can enable rational modification of human microbial communities through personalised nutrition. Diet can customize the structure of the gut microbiome to optimise function and interaction with the host and maximize human health. A prerequisite for incorporating microbial genomic data of an individual's microbiome into personalised is to achieve a mechanistic understanding of the most dominant aspects of microbiome function. It is essential, when manipulating the gut microbiome for human health and wellness, to apprehend at the best of our knowledge these seemingly distinct intestinal niches associated with targeted dietary substrates, which play a role in niche partitioning occupied by the different bacteria.

In these expectations, carbohydrate-containing prebiotics, through their bacterial metabolisation, are of particular interest for fuelling the host and the microbiome. They represent technological enablers to manipulate the structure and functions of our gut microbiome. However, rigorous evaluation of prebiotic outcomes are mandatory for engineering the intestinal microorganisms.

Currently, there is a lack of predictability in how the microbiome responds to dietary interventions, reflecting our limited understanding of nutrient sensing and utilisation by members of the intestinal microbiota. Deciphering the underlying mechanisms of carbohydrate metabolisation has a great potential to rationally modify the gut microbiome for future prebiotic interventions and food innovations.

III. Prebiotics: beyond the nutritional strategy

III.1. Evolution of the scientific concept of prebiotics

The emergence of the prebiotic concept occurred in the 1990s. Regrouping a panel of academic and industrial experts, the ISAPP regularly convened to state the definition and scope of prebiotics. New considerations were gradually included along with scientific research progress, consumer interest, and technological innovations from industrial scientists **(*Table I*)**. In 1995, Gibson and Roberfroid defined the term prebiotics as ***"non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health"*** (Gibson and Roberfroid, 1995). In 1995, non-digestible oligosaccharides (OS) in general and fructo-oligosaccharides (FOS) in particular were classified as prebiotics. A broad range of dietary ingredients classified as non-digestible carbohydrates could have been claimed with prebiotic status, whether the concept first would not have evolved.

To better demonstrate prebiotic health-enhancing attributes of food ingredients, the concept was revised in 2004 for candidates to fulfil three criteria: (a) resistance to gastric acidity, hydrolysis by mammalian enzymes and gastrointestinal absorption; (b) fermentation by intestinal microflora; (c) selective stimulation of the growth and/or activity of intestinal bacteria associated with health and wellbeing. This latter feature involves the description of the gut microbiome composition that confers health properties and a better characterisation of the gut microbiome functionality (Gibson et al., 2004). The inclusion of criteria expanded the concept highlighting the fact that prebiotics are not digested but rather fermented by the gut microbes within the digestive tract. Among dietary carbohydrates, inulin/FOS, galacto-oligosaccharides (GOS), lactulose were designated as prebiotic ingredients. Scientific evidence to assign prebiotic attributes was considered insufficient for many promising candidates, including isomalto-oligosaccharides (IMO), lactosucrose, xylo-oligosaccharides (XOS), soybean oligosaccharides (SOS), gluco-oligosaccharides (GlOS) and miscellaneous carbohydrates (Gibson et al., 2004).

Different aspects were further revisited in 2007 to validate and expand the prebiotic concept previously proposed (Roberfroid, 2007). Regarding the "*fermented ingredient*", the nature of the prebiotics should be specified as different types of molecules might unequally affect the bacterial populations. These food products with prebiotic properties involve "specific" microbial changes related to one or a limited

Table I: The prebiotic concept over the years developed by members of the International Scientific Association for Probiotics and Prebiotics (ISAPP)

Evolution of the prebiotic concept	Additional considerations for prebiotic classification	Ingredients incrementally considered as prebiotics
"non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health" (Gibson and Roberfroid, 1995)	*Not applicable*	FOS
"selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confer benefits upon host wellbeing and health" (Gibson et al., 2004)	(a) non-digestibility (b) fermentation by intestinal microflora (c) selective stimulation of the growth and/or activity of intestinal bacteria	Inulin tGOS Lactulose <u>Candidate* is listed:</u> IMO, lactosucrose, SOS, XOS, GlOS and other compounds
NB: there is no new definition of a prebiotic, but rather a validation and an expansion of the prebiotic concept (Roberfroid, 2007)	(a) nature of the prebiotics (b) dose-effect relation (c) animals and humans	*Not applicable*
NB: there is no new definition of a prebiotic, but rather a validation and an expansion of the prebiotic concept (Roberfroid et al., 2010)	(a) increase in the genus *Bifidobacterium* as a marker of intestinal health (b) selectivity of other genera or species than bifidobacteria (e.g. butyrate-producing bacteria) (c) beneficial effects in the colon and the whole body	*Not applicable*
"*a non-digestible compound that, through its metabolisation by microorganisms in the gut, modulates composition and/or activity of the gut microbiota, thus conferring a beneficial physiological effect on the host*" (Bindels et al., 2015)	(a) anatomical restriction to the gut; (b) requirement or not of fermentation; (c) restriction only to carbohydrates; and (d) requirement or not of microbiota modulation (possibility of having other direct positive effects)	HMO <u>Candidates* are listed:</u> RS, pectin, AX, whole grains, various dietary fibres and other non-carbohydrates such as polyphenols
"a substrate that is selectively utilised by host micro-organisms conferring a health benefit" (Gibson et al., 2017)	(a) microbes targeted by prebiotics should be health-promoting bacteria without specifying which one (b) effect is no longer limited to the microbial community of the gastrointestinal system associated with humans or animals (c) importance in describing selective bacterial metabolism and assessing microbial function and composition in reproducible randomized controlled studies that establish the direct link between prebiotics and health in the specific target host	<u>Candidates* are listed:</u> HMO, MOS, and other non-carbohydrates such as polyphenols, CLA and PUFA

*<u>Abbreviations:</u> FOS, fructo-oligosaccharides; tGOS, trans-galacto-oligosaccharides; IMO, isomalto-oligosaccharides; SOS, soya-oligosaccharides; XOS, xylo- oligosaccharides; GlOS, gluco-oligosaccharides; HMO, human milk oligosaccharides; RS, resistant starches; AX, arabinoxylans; MOS, mannan-oligosaccharides; PUFA, polyunsaturated fatty acids; CLA, conjugates linoleic acids *The prebiotic potential of candidate compounds has been investigated. However, scientific evidence is too sparse at the time to demonstrate any prebiotic effects.*

number of selectively microorganisms. The quantification of the bacterial increase, especially *Bifidobacterium* genus, and the description of the associated activities related to regular intake of prebiotics were considered to characterise a dose-effect relation. Originally considering microbial changes in humans only, the concept was extended to the colonic ecosystems of both animals and humans. Therefore, experimental demonstrations and human and/or animal intervention studies are required to evaluate prebiotic effects.

Other key qualifiers of the prebiotic concept were discussed in 2010 to precise the prebiotic concept (Roberfroid et al., 2010). Regarding the "activity in the gastrointestinal microbiota", scientific evidence requires demonstrations using the most up-to-date technology in the complex gut microbiome, including metabolic profile(s), molecular signalling, and prokaryote-eukaryote cell-cell interaction. Physiological effects should be established in controlled trials and correlated to selective changes in gut microbiota composition or activity. The specificity was considered "the key condition that needs to be demonstrated, *in vivo*, in the complex human (animal) gut microbiota by applying the most relevant and validated methodology(ies) to quantify a wide variety of genera/species composing the gut microbiota". About the "specific changes", a *Bifidobacterium* increase was considered as a marker of intestinal health that could be expanded towards other genera or species. The role of such changes remains to be definitively proven as beneficial for the host wellbeing and health. In reality, the beneficial physiological and pathophysiological effects of prebiotics are not only in the colon, but also within the entire body and/or contribute to reducing the risk of intestinal and systemic pathologies.

In 2015, Bindels et al. suggested a more comprehensive concept arguing the requirement that prebiotics have to be "specific" or "selective" for health-promoting taxonomic groups or beneficial metabolic activities (Bindels et al., 2015). The requirement for a specific effect towards health-promoting taxa conflicts the knowledge and comprehension of gut microbiome ecology and its relation to health. Prebiotic-induced changes cannot be as selective as previously assumed, probably due to functional redundancy among intestinal inhabitants and cross-feeding. No carbohydrates is likely to be fermented by only one or two bacterial groups in the gut and none is fermented by all. Thus, the definition was adapted to the current understanding of the gut microbiome as ***"a non-digestible compound that, through its metabolisation by microorganisms in the gut, modulates the composition and/or activity of the gut microbiota, thus, conferring a beneficial physiological effect on the host"***. Removing the requirement of selectivity or specificity from the prebiotic concept shifts the focus from the characterisation of the composition and functional features of gut microbiota more likely to be relevant for host physiology towards research on the mechanisms. This definition

emphasizes the causal link between the microbial metabolisation of the component and the resulting modulation of the gut microbiota and the beneficial physiological effects.

In 2017, the scientific community clarified the definition published by the ISAPP. Indeed, the expert panel proposed the most recent consensus definition that refines prebiotics as ***"a substrate that is selectively utilised by host microorganisms conferring a health benefit"*** (Gibson et al., 2017). The most accepted and candidate prebiotics are usually dietary carbohydrates. This definition expands the concept of prebiotics to possibly include non-carbohydrate substances, such as polyphenols and polyunsaturated fatty acids, applications to body sites other than the gastrointestinal tract, and diverse categories other than food. In particular, causal the link between the influence on the gut microbiome and the physiological effect must be demonstrated by reproducible health biomarkers in randomized controlled human or animal studies. The safety of prebiotics and their use in an appropriate dose are implicit in this definition. An appropriate dose must be sufficient to generate a prebiotic effect, but not too high to induce unwanted or adverse effects such as excessive gas formation or non-selective utilisation. Health effects must be documented for a substance to be considered a prebiotic. Further requirement for "selective utilisation" by a limited number of species or genera has been questioned. Scientific evidence showing the selective mechanisms mediated by the microbiota was retained. A prebiotic should not be broadly metabolised, but elicit a selective metabolism biased towards health-promoting microorganisms. Thus, prebiotic targets extend beyond stimulation of bifidobacteria and lactobacilli, and recognize that health benefits can derive from effects on other beneficial taxa.

The concept of prebiotics has evolved along with the advances in our understanding of diet–microbiome–host interactions. A consensus definition of prebiotics guides scientists in substantiating the physiological effects of a substance classified as prebiotic. It also leads the agrifood sector and stakeholders, allowing translational research into new therapies and functional foods. The definition is useful for regulatory agencies and policy makers in developing statutes and regulations, for suppliers or manufacturers to accurately characterise prebiotics and health benefits, and for end-product manufacturers to formulate and label prebiotic products. Thereby, marketed products do not overstate the strength of evidence for health benefits and avoid misleading messages. This statement also enables consumers to understand the terms used on the product labels, and the media and publishers of scientific papers to appropriately use the term "prebiotic".

III.2. Impact of prebiotics on the gut microbiome, health and wellbeing

Categorizing a prebiotic substance is complicated due to the overlap between the definitions of prebiotic and dietary fibre. Derived from the cell wall of vegetables, legumes, cereals, and fruits, most accepted and candidate prebiotics are non-digestible carbohydrates that satisfy the criteria of dietary fibres (FAO/WHO Codex Alimentarius Commission, 2009; Rezende et al., 2021). Indeed, the dietary fibres are classified into four distinct categories, including carbohydrate polymers, such as resistant oligosaccharides, non-starch polysaccharides and resistant starches, and non-carbohydrate associated phytochemicals, such as phenolic compounds, waxes, saponins, phytates, and phytosterols constitute (Stephen et al., 2017; Cronin et al., 2021). Similar to carbohydrates, certain of these phytochemicals have a low bioavailability, which suggests they may escape the absorption in the small intestine. For instance, it was estimated that 5-10% of the total intake plant polyphenols reach the colon. As a subset of dietary fibres, polyphenols may be metabolised by the gut microbiome to various degrees (Cardona et al., 2013). However, they may not lead to selective changes in the gut microbiota that may confer health benefits.

All fermentable fibres are candidate prebiotics provided that they function primarily through selective utilisation by the host microbiota and promote health and wellbeing **(Figure 8)**. Prebiotics are often equated with dietary fibres, but only a subset of dietary fibres actually qualify as prebiotics (Verspreet et al., 2016). Because the consensus definition includes the use of prebiotics for animals, a dietary fibre can be a prebiotic in one host but not another. For example, cellulose can be considered a prebiotic in ruminants but not in humans, as the human gut microbiome showed poor functional capacities to metabolise β (1→4) linkages of D-glucose polymers (Ben David et al., 2015).

As required to meet the definition, a prebiotic candidate must demonstrate physiological benefits through selective changes in the gut microbiome. Close relationships between prebiotics, microbiota composition, and health status must be established.

Characterisation of prebiotic-mediated physiological effects

Functional effects of prebiotics, equated dietary fibres, are dependent upon the natural or artificial source that defines the physicochemical structures of these carbohydrates (e.g. molecular weight, character of individual monomers, DP and type of linkage) **(Section II.1)**. These physicochemical properties drive physical effects in the food matrix and food processing such as particle size, viscosity, solubility, and fermentability (Gill et al., 2021). These determinant factors contribute to a variety of physiological effects on digestive health and wellbeing **(Figure 9)**.

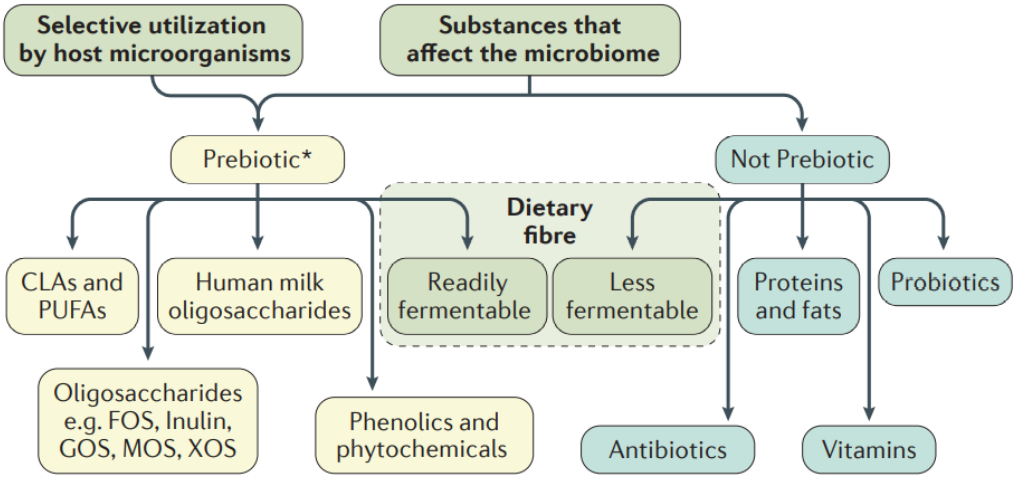

Figure 8: Distinction of what are prebiotics and dietary fibres (from Gibson et al., 2017). The definition clearly statutes the prebiotic function primarily through a selectively utilisation by the host microbiota conferring health benefits. Both candidate and accepted prebiotics are reported, and FOS and GOS are the most investigated prebiotics. CLA, conjugates linoleic acids; PUFA, polyunsaturated fatty acids; FOS, fructo-oligosaccharides; GOS, galacto-orligosacchairdes; MOS, mannan-oligosaccharides; XOS, xylo-oligosaccharides.

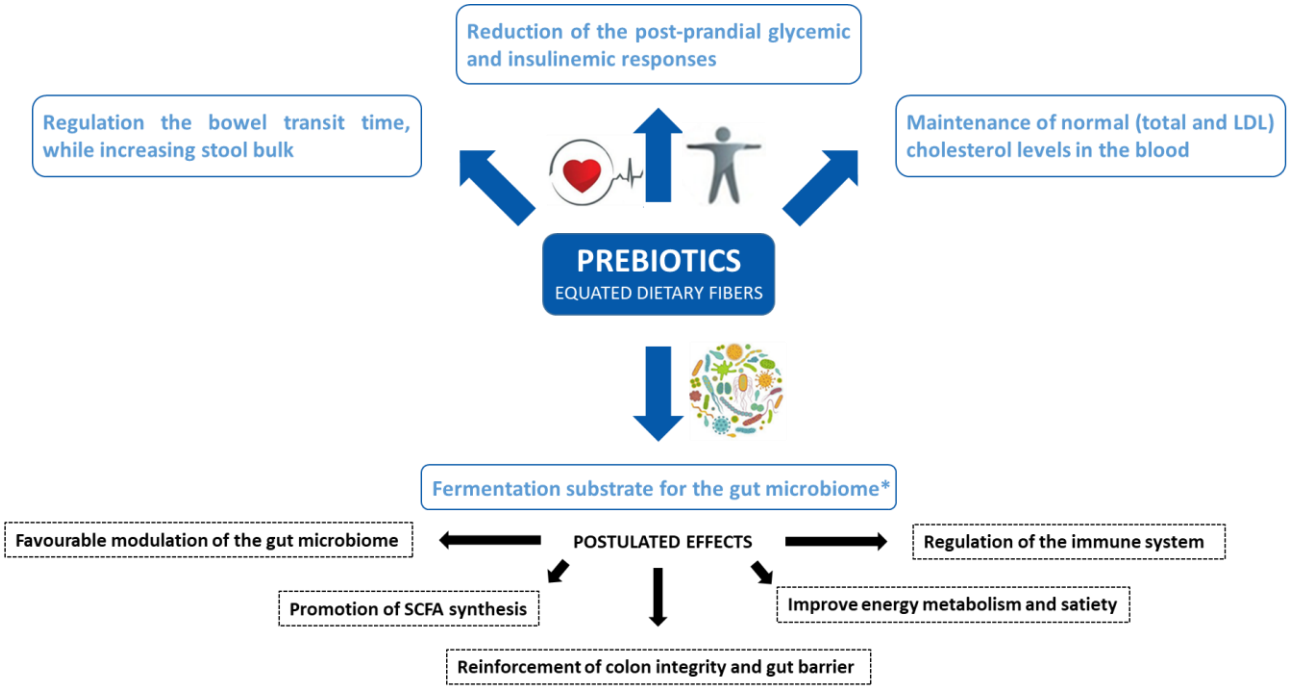

Figure 9: Summary of physiological effects mediated by prebiotics on host health and wellbeing

* Considering the role of colonic fermentation of prebiotics or dietary fibres as a source of health-promoting properties, there are some differences between countries. LDL, low-density lipoprotein; SCFA, short-chain fatty acid

Prebiotics, equated with dietary fibres, are associated with maintaining intestinal functions, including intestinal regularity and the reduction of occasional constipation and diarrhoea. Physiological effects also include the regulation of postprandial glycemia and insulinemia, the control of blood cholesterol levels, and fermentation by the intestinal microbiota (FAO/WHO Codex Alimentarius Commission, 2009; Zhang et al., 2018a; Delzenne et al., 2020). Noteworthy, the microbial fermentation of prebiotics, equated dietary fibres, is not recognised as a health benefit according to countries **(Chapter 5)**.

Through the favourable modulation of the gut microbiome, stimulation of fermentation activities provides bacterial-derived metabolites that can exert local and systemic physiological effects. In dietary interventions, the promotion of SCFA synthesis often appears as one of the main mechanism by which prebiotics mediate health and wellbeing benefits. Hence, SCFA-mediated effects contribute to colon integrity and gut barrier reinforcement through the upregulation of tight junctions (Baxter et al., 2019). Maintaining physiological gut permeability and anti-adhesive activity of intestinal cells, prebiotics can improve the competitive exclusion of pathogenic microorganisms and thus improve colonisation resistance and prevent a gastrointestinal infection (Koh et al., 2016). Nourishing gut bacteria, prebiotics can favour natural peptide excretion, such as PPY and GLP-1, involved in energy metabolism and satiety, and participate in improving the insulin response (Cani et al., 2009; Zhao et al., 2018). More evidence supports regulatory roles in the immune system, mineral metabolism, and bone health (Pujari and Banerjee, 2021; Whisner and Castillo, 2018).

Among the first group of dietary ingredients recognized for their ability to modulate the intestinal health and wellbeing were the human milk polysaccharides (HMOs) naturally present in breast milk. These mixtures of oligosaccharides, in combination with glycoproteins and glycolipids, result mainly in a dominant abundance of *Bifidobacterium* species in breastfed infants and, to a lesser extent, an abundance of some *Bacteroides* and *Lactobacillus* species (Walsh et al., 2020). As a result, different oligosaccharides have been utilised to mimic the function of HMOs, such as FOS and β-GOS. Evidence for the prebiotic status of HMOs is scarce relative to these two most investigated food ingredients.

Accepted prebiotics equated dietary fibres: fructans and galactans

Two chemical groups namely the inulin-type fructans (ITF) and the galactans fulfil the criteria for prebiotics, although many other classes are under investigation. There are two common types of galactans or GOS, both composed of galactose residues **(Figure 10A)**. Derived from lactose syrup, β-linked GOS, also known as trans-GOS (tGOS), are commercially synthesized through the trans-glycosylation activity of bacterial β–galactosidase or β-glycosidase enzymes (Tzortzis and Vulevic,

2009). This process commonly produces mixtures of short-chain tri to pentasaccharides, which present a terminal glucose monomer and a wide variety of β (1-6), β (1-2), β (1-3), and mostly β (1-4) linkages. The physiological effects of GOS have been described in many human intervention studies to improve bowel habits, insulin and lipid homeostasis, mineral absorption, and immune function through the gut health (Tzortzis and Vulevic, 2009; Vulevic et al., 2013). Further evidence reported beneficial properties the healthy subjects, in elderly and individuals with IBS. Indeed, β-GOS selectively stimulate the bacterial growth of the genera *Bifidobacterium*, *Lactobacillus,* and *Bacteroides* (Roberfroid, 2007; Davis et al., 2011). In particular, the administration of increasing doses of GOS showed the increasing relative abundances of certain species of *Bifidobacterium*, including *B. adolescentis* and *B. longum*, largely at the expense of *Bacteroides* species. Likewise, α-linked GOS is another type of non-digestible fibre commonly found in plants, consisting of α-linked galactose, α-linked glucose, and a terminal β-linked fructose. They are commonly called raffinose family oligosaccharides (RFO), including raffinose, stachyose, and verbascose. Interestingly, α-GOS demonstrated broad bacterial growth, suggesting that its prebiotic effects are less selective (Meyer, 2015). Metabolic capacities to metabolise GOS seem somewhat less widespread within the gut microbiome than ITF.

Belonging to the group of fructans, short-chain FOS or oligofructose and long-chain inulin constitute a mixture of linear fructan chains composed of both fructosyl-glucose linked by β (2-1) glycosidic bonds and fructosyl-fructose bound by β (2-1) linkages (Gibson et al., 2004) **(Figure 10B)**. From a chemical perspective, the DP distinguishes the FOS mixtures variying from 2 to 7 with an averaged DP of 4 sugar units, from the longer inulin molecules. For instance, the inulin from chicory is composed of a mixture of oligomers and polymers in which the DP ranges from 2 to 70 units with an averaged DP of 12. To note that about 10% of the fructan chains in native chicory inulin have a DP ranging between 2 and 5 units. ITF are often manufactured for use in the food industry. Inulin/FOS can be obtained by extraction from plants such as onions, Jerusalem artichoke, chicory root, and agave. Isolated inulin preparations often contain 5-10% monosaccharides. FOS can be produced by partial hydrolysis of long-chain inulin using endo-inulinase enzymes or derived from sucrose by trans-fructosylation using β-fructosidase enzymes. The large variety of inulin chemical structures and botanical origin result in a wide range of ITF mixtures with different functional effects. Several studies have already investigated the beneficial potency of prebiotics by interacting with the gut microbiota, mainly chicory inulin, on host metabolism and key intestinal functions. In healthy adults, the consumption of chicory inulin has reported the regulation of post-prandial glycemia and insulinemia (Parnell and Reimer, 2009; Parnell et al., 2017). In addition, inulin contributed to satietogenic effects through the suppression of ghrelin and increase of

PYY concentrations, and to the overall reduction in energy intake. These physiological effects were validated in adults and children with overweight and obesity (Hume et al., 2017). Furthermore, chicory inulin consumption has induced specific microbial modifications relative to the increase in the proportion of *Bifidobacterium* species and the increase in SCFA production (Vandeputte et al., 2017; Hiel et al., 2020). An inulin-enriched diet has been shown to improve intestinal discomfort, obesity, and diabetes-related complications (Vandeputte et al., 2017; Hiel et al., 2020). Promoting inulin intake enables to promote weight loss in obese patients and influenced microbial-derived metabolites and markers related to intestinal integrity and inflammation (Neyrinck, Rodriguez, Taminiau et al., 2021; Neyrinck, Rodriguez, Zhang et al., 2021). Noteworthy, the evaluation of the digestive tolerance reported a risk of flatulence episodes during the dietary intervention at doses higher than 15g/day (Hiel et al., 2019). Compared to chicory fructans, agave inulin constitutes another prebiotic source of complex mixtures with more diverse and highly branched structures that contain both β (2-1) and β (2-6) linkages, as well as internal and external glucose units. In vivo rodent models reported physiological effects, such as regulation of satiety, food intake, plasma glucose and lipids, and modulation of the gut microbiota, which together suggest that agave fructans may be interesting in the management of obesity (Márquez-Aguirre et al., 2016; González-Herrera et al., 2019). In two randomized, double-blind, placebo-controlled crossover trials undertaken in healthy adults, agave inulin changed the composition and activity of the intestinal microbiota to enrich *Bifidobacterium* species, which tended to be positively associated with an increase in SCFA production (Holscher et al., 2015). Likewise, the modulation of the gut microbiota was consistent with an increase of bifidobacteria and lactobacilli (Ramnani et al., 2015).

Two other well-established prebiotics are lactulose and lactilol. Strictly speaking, these disaccharides are not dietary fibres (Meyer, 2015). In addition to GOS and FOS, other oligosaccharides are not yet recognized as prebiotic fibres, such as IMO, SOS, XOS, and mannan-oligosaccharides (MOS).

Potential or emerging prebiotics: glucose-based dietary fibres

There is a wide range of glucose-based carbohydrates, including β-glucans, resistant starch (RS), polydextrose (PDX), and soluble corn fibre (SCF), which are considered prebiotic candidates but have not yet met the ISAPP consensus definition. Recommended as candidate prebiotics, β-glucans are well-recognised bioactive water-soluble non-starch carbohydrates commonly extracted from whole-grain cereals (Shoukat and Sorrentino, 2021). The β-glucan content can range from 1% in wheat (dry weight) to 3-7% in oat (dry weight) and from 5-11% in barley (dry weight) (Hughes et al., 2008; El Khoury et al., 2012). Cereal-derived β-glucans are linear and unbranched hemi-cellulosic polymers composed of D-glucose monomers joined by mixed-linkages β (1-4) and β (1-3) of glucose residues **(Figure 10C)**.

Promising functional, prophylactic and therapeutic potentials have been described in human studies to inhibit cardiovascular diseases and various metabolic disorders, such as diabetes and obesity, through improving the gut health (Wang et al., 2016; Gong et al., 2018; Velikonja et al., 2019). For example, a dietary intervention enriched in β-glucans reported cholesterol-lowering activity and increased SCFA production (Naumann et al., 2006; Cosola et al., 2017). Other effects on gut health were described, such as improving gut permeability flux, reducing intestinal inflammation, protecting against colon cancer, and modulating the immune system (Shoukat and Sorrentino, 2021). Furthermore, depending on intestinal microbial fermentation, β-glucans can stimulate the bacterial growth of lactobacilli and bifidobacteria and promote a saccharolytic shift towards the *Bacteroidetes* phylum. Since 2011, the European Food Safety Authority (EFSA) and the US Food and Drug Administration (FDA) recognize cereal β-glucans for their health claims in reducing postprandial glycemia and insulinemia responses and in cholesterol regulation in humans (EFSA Panel on Dietetic Products, 2011b). Barley and oat contain other dietary fibres, such as arabinoxylan and starch, insoluble fibres, proteins, and other bioactive phytochemicals. Noteworthy, the ratio of soluble/insoluble dietary fibres and phenolic compounds levels are the main factors that result in variation of compositional shifts in bacterial communities and bacterial-derived metabolites. The fermentation profile and ultimately the biological functionality of β-glucans depend on the primary structure, molecular weight, polymer charge, degree of branching, solubility, and viscosity (Hughes et al., 2008; Wang et al., 2016; Dong et al., 2017).

Starch is one of the most popular nutritional sources for humans and animals. The non-digestible starch fraction contains a mixture of two major components amylose and amylopectin and other polysaccharides including α-glucans, in a proportion of 80-90% of all polysaccharides. Amylose and amylopectin are glucans that differ in their structure and molecular weight **(Figure 10D)** (Cornejo-Ramírez et al., 2018). Amylopectin is a high molecular weight glucose polymer that consists of branched chains formed by α (1-6) glycosidic bonds and is susceptible to hydrolysis by amylases. Amylose is a more linear glucose polymer of lower molecular weight that consists of linear chains primarily bound by α (1-4) glycosidic linkages. The biochemical and nutritional properties determine the classification of starch resistant in four forms (Englyst et al., 1992). Starch with physical inaccessibility to digestion describes type 1 RS (RS1); the native granular structure defines type 2 RS (RS2); the starch obtained by retrograde processes of thermal heating and cooling treatments classifies type 3 RS (RS3); and the chemical or physical modified starch characterises type 4 RS (RS4). For example, resistant dextrins (RD) are produced by starch dextrinization, which replaces default α (1-4) and α (1-6) glycosidic bonds in starch with (1-2) and (1-3) α and β glycosidic bonds, resulting in a structure more resistant to amylolytic

enzymes (Śliżewska et al., 2012). The physicochemical structures of the high amylose and amylopectin content receive a considerable attention for their promising nutritional interventions in cardiovascular diseases and in a variety of metabolic disorders (Bindels et al., 2015; Yang et al., 2017). Dietary interventions enriched in RS in humans reported their digestive tolerance and their efficacy in improving a variety of metabolic characteristics, including the regulation of lipid metabolism and the post-prandial glycemia and insulinemia (Shen et al., 2017; Maier et al., 2017). Particularly, the enrichment in RS2 allowed the increase of SCFAs and the growth stimulation of *Bifidobacterium*, *Faecalibacterium*, *Ruminococcus* and *Eubacterium* genera (Venkataraman et al., 2016). Further evidence highlighted that RS4 allowed an increase in the relative abundance of *Bifidobacterium* and *Parabacteroides* genera (Upadhyaya et al., 2016). The functional differences between RS2 and RS4 highlighted the influence of the source on fermentation profiles, explained by the morphology of the starch granule, the amylose–amylopectin ratio and its association with other food component (Martínez et al., 2010). The impact of RS on the gut microbiome and microbial-derived metabolites, especially on the *Ruminococcus* genus, was consistent with other *in vivo* studies (Sun et al., 2016; Cherbuy et al., 2019). In fact, *Ruminococcus bromii* has been defined as a keystone species for the degradation of RS displaying a unique capacity to degrade RS2 and RS3 (Ze et al., 2012; Vital et al., 2018).

The lack of absorption of polydextrose (PDX) during gastrointestinal transit ensures that PDX remains available for fermentation by intestinal microbes and may therefore function as a prebiotic. Typically derived from corn glucose, PDX consists of a synthesized mixture of highly branched glucose polymers, which are randomly bonded by α and β (1-2), (1-3), (1-4) and (1-6) glycosidic linkages, but α (1-6) linkages predominate. The DP is highly variable from 2 to 100 units, with an averaged of 12. Commonly, over 90% of the molecules have a DP between 3 and 30, with DPs below 20 being the most common **(Figure 10E)**. *In vitro* fermentation models reported that branched PDX, in particular single-branched molecules displaying (1-6)-linked glucose moieties in a pyranose form, were the preferred substrates hydrolysed by the gut microbiota during gastrointestinal transit (Lahtinen et al., 2010). Further dietary interventions showed that polydextrose is well tolerated and stimulates the growth and/or activity of one or a limited number of intestinal bacteria associated with several physiological benefits on health (Bassaganya-Riera et al., 2011; Hooda et al., 2012; Konings et al., 2014). Although polydextrose is a promising prebiotic candidate, further consistent clinical studies are needed to confirm the prebiotic activity of this polysaccharide (do Carmo et al., 2016).

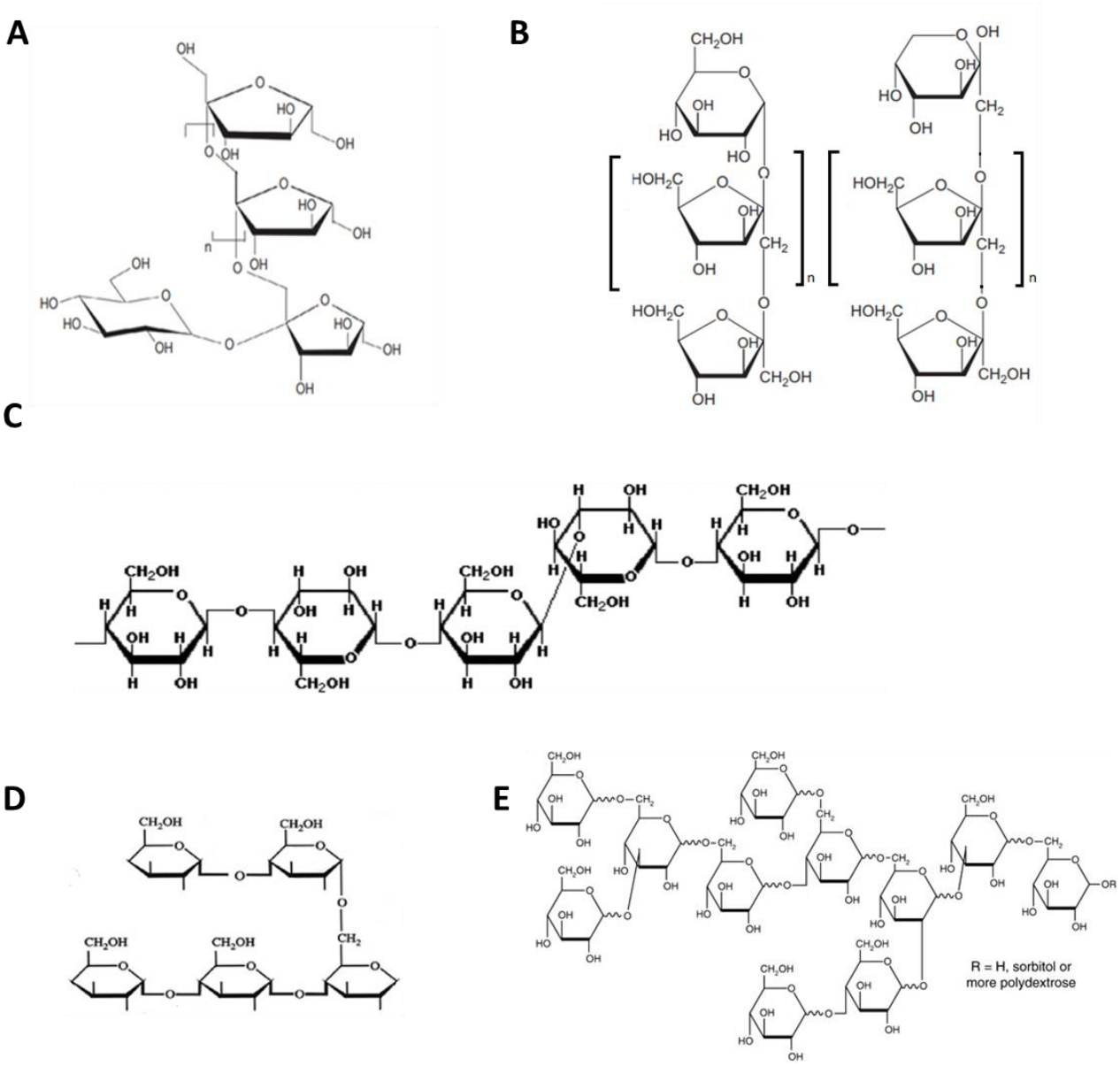

Figure 10: Structural diversity of prebiotic representatives (adapted from Meyer, 2015). (A) Basic structure of galactans. B-GOS are composed of β (1-6) linked galactose residues with a terminal monomer produced by enzymatic trans-glycosylation. ***(B) Chemical structure of linear inulin molecules with or without glucose residue.*** Inulin consists of β (1-2)-linked fructosyl backbone with often a terminal glucose residue or without a terminal glucose residue. ***(C) Basic structure of β-glucans in cereals from Shoukat and Sorrentino, 2021)*** combined with glycosidic linkage β-(1-3) and β-(1-4) ***(D) Chemical structure of resistant starches (from Cornejo-Ramírez et al., 2018)*** RS consists of glucose units linked by α-1,4 glycosides links of linear chains and α-1,6 links of the branches. ***(E) Typical structure of the polydextrose*** PDX consists of glucose units organised into a highly branched structure with a wide spectrum of glycosidic linkages represented, with 1–6 linkages predominate.

Soluble corn fibre is another potential glucose-based prebiotic produced from the partial hydrolysis of corn starch. Two parallel and short-term randomized interventions in healthy adult subjects investigated digestive tolerance after ingestion of food ingredients. Therefore, bowel habits determined by stool frequency, consistency, abdominal pain, intestinal bloating and flatulence were investigated (Stewart et al., 2010; Vester Boler et al., 2011; Konings et al., 2014; Costabile et al., 2016). These functional fibres appear well tolerated in doses as high as 21g/delivered/day while leading to no significant adverse effects and minimal gastrointestinal upset on bowel habits. Furthermore, the food ingredients elicit postprandial blood glucose and insulin responses. *In vitro* and *in vivo* studies highlighted the possible prebiotic effect of the increase in relative abundance of *Bifidobacterium* species in the overall gut microbiota. Microbial metabolite analysis showed an increase in short-chain fatty acids (Maathuis et al., 2009; Vester Boler et al., 2011; Hooda et al., 2012; Holscher et al., 2015; Costabile et al., 2016). *In vivo* rodent model also reported the potential for these dietary fibres to prevent gut inflammation (Bassaganya-Riera et al., 2011).

Significant efforts have been made identifying dietary fibres with prebiotic effects as well as providing deeper insights into their interactions with the gut microbiome and the host. Historically, the aim of prebiotic intervention was to precisely target *Bifidobacterium* and *Lactobacillus* species to confer beneficial effects on the host. These genera have been thoroughly studied as probiotic bacteria associated with human health benefits through a variety of mechanisms, including the biosynthesis of SCFAs, especially acetate and lactate. This production often refers to the "bifidogenic effect" that can lead to the further downstream stimulation of a number of bacterial groups, such as butyrate-producers (Fukuda et al., 2011; Rivière et al., 2016). Coculture experiments between *Faecalibacterium prausnitzii* and four different *Bifidobacterium* species showed that cross-feeding interactions could be competitive or mutually beneficial (Moens and De Vuyst, 2017). Thereby, the impact of prebiotics on the gut microbiome is more widespread within the intestinal community through cross-feeding interactions (Le Bastard et al., 2020). The consequences on the host health is likely to rely on intestinal community functions resulting from the complex ecological interactions of multiple microbial taxa. One prerequisite is a sophisticated understanding of the interactions that occur when the complex community of gut microbes encounters a source of fibre (Delzenne & Bindels, 2019).

Mechanistic research to clarify the impact of prebiotics on the gut microbiome is an important basis for the rational development of nutritional strategies. A comprehensive analysis of carbohydrate metabolisms and prebiotic mechanisms is necessary for the identification of health-promoting bacteria metabolically active when targeted by prebiotics.

III.3. Molecular mechanisms underpinning the prebiotic effects

To study the impact of prebiotics, many promising substrates from across the globe have been explored for their capacity to modulate the gut microbiome (McLaughlin et al., 2015). In particular, emphasis has been placed on the characterisation of changes in the composition of resident microbial communities and the analysis of microbial-derived metabolites (Spacova et al., 2020). Indeed, the compositional analysis of the gut microbiome is often correlated with the production of SCFA, which confers a range of health-relevant benefits, including regulation of gut epithelial and mucus barrier function, immunity, inflammation, glucose and lipid metabolism, energy expenditure, and satiety. These by-products from the bacterial metabolism are well-recognised for their ability to facilitate many prebiotic effects and interact with the host biological systems. However, it is clear that prebiotic research based on correlations does not provide all the evidence needed to identify prebiotic mechanisms defining health effects (Bindels et al., 2015).

Providing additional mechanisms of prebiotics is the step toward the hypothesis that intentional modulation of the gut microbiome is a valid strategy to improve human health and wellbeing. In this context, prebiotic research has considerable interest in understanding how commensal microorganisms selectively scavenge carbohydrate substrates and contribute to their degradation and consumption. Certain gut microorganisms can improve human health, but it is unclear how diet could be used to easily manipulate the composition of gut microbes, hence selectively stimulating the abundance of health-promoting gut microbes.

The mechanistic details of prebiotic selectivity are mediated through specific gene clusters encoded within saccharolytic bacteria controlled by signalling sensors for various substrates **(Chapter 1, Section II)**. Mostly investigated in *Bacteroides* species, carbohydrate utilisation pathways have provided new insight into how specific types of bacterium use and compete for prebiotic substrates. Unlikely, there are little evidence exploring the metabolic capacities of health-promoting bacteria, such as *Bifidobacterium*, *Lactobacillus* and butyrate-producing bacteria **(Table II)**. Specific gene clusters within the bacterial genome dictate the saccharolytic enzymes that the bacteria can activate and, therefore, whether they can metabolise the prebiotic substrates **(Chapter 1, Section II.3)**. First evidence was reported for the metabolisation of resistant starch in *Bacteroides thetaiotaomicron* (Bjursell et al., 2006; Martens et al., 2011). Actually, starch utilisation appeared widespread among gut bacteria, known as primary RS assimilators, including *Bacteroides, Bifidobacterium*, *Ruminococcus,* and *Eubacterium*. These highly abundant bacterial groups in the human microbiome are enzymatically equipped to degrade

glycosidic bonds and use glucose-based saccharides for energy and growth (Walker et al., 2011). In response to a nutritional environment enriched in RS, *-omics* approaches focused on *Eubacterium rectale* gave a better understanding of the nutrient acquisition strategies involved in the RS metabolisation (Cockburn et al., 2015, 2018). Indeed, the proteomic and transcriptomic profiles of *E. rectale* indicated the active expression of two GH13 enzymes, which were induced to target different starch molecules and the identification of ABC glycan-binding proteins, which were involved in the scavenge of malto-oligosaccharides and glucose. These findings allowed to propose a model of how gut microbes target starch in the intestinal tract (Cockburn et al., 2015). The ability to attach to starch particles in the intestinal tract is advantageous for *E. rectale* and may serve to place it in close proximity to resistant starch-degrading bacteria, such as *Ruminococcus bromii* (Ze et al., 2015). These mechanisms of starch degradation and importation systems described for *E. rectale* may constitute a conserved pathway in other *Clostridium* cluster XIVa microorganisms by which they take advantage of this abundant dietary carbohydrate. Furthermore, metagenomic evidence highlighted new mechanistic insights into RS degradation by the human gut microbiome, especially reporting the stimulation of active starch-degrading enzymes that belong to the GH13 and GH77 families in diverse bacterial responders (Vital et al., 2018).

Molecular mechanisms by which other glucose-based substrates, such as β-glucans, influence the gut microbiome, and ultimately human health, are largely unresolved and undoubtedly complex **(Table II)**. Recent efforts to elucidate the pathways by which diverse complex polysaccharides are utilised via PUL systems have provided structural insight into the CAZymes. Indeed, the authors Tamura *et al*. identified in *Bacteroides ovatus* a molecular mechanism that serves as a genetic marker dedicated to the metabolisation of β-glucans (Tamura et al., 2017). A particular utilisation locus encodes a GH16 endo-β-glucanase, which hydrolyses β (1-4) linkages that are preceded by a β (1-3) linked glucosyl residues, and a GH3 exo-β-glucosidase that digests the oligosaccharides to glucose. This PUL appears to be essential for the growth of *Bacteroides ovatus* on barley β-glucans. Nevertheless, homologous PULs of *Bacteroides ovatus* are present in the genomes of *Bacteroides xylanisolvens* and *Bacteroides uniformis*, which highlight the apparent prevalence of PULs dedicated to β-glucan metabolism among *Bacteroides* species. Probably, similar PULs in *Bacteroides uniformis* exhibiting GH30, GH158, and GH3 can involve the degradation of β-glucans and promote the growth of other bacteria (Déjean et al., 2020; Singh et al., 2020). However, very little is currently known about the molecular mechanisms of how Gram-positive bacteria, such as *Bifidobacterium* species and butyrate-producing bacteria, degrade different types of β-glucans.

Similarly, the molecular link between the gut microbiome and fructose-based prebiotics, such as inulin, has first been explored through the identification of inulin/FOS hydrolysing enzymes (Imamura et al., 1994). Belonging to the β-fructofuranosidase superfamily, these enzymes are present in *Bifidobacterium* species that confer their ability to breakdown the β-fructosidic bonds to release fructose monomers (Scott et al., 2011). These enzymes can display specific activities that vary according to the fructose, FOS, and inulin. An analysis of the global transcriptional response provided insight into the molecular mechanisms underlying prebiotics **(Table II)**. The identification of a fructan utilisation cluster in *R. inulinivorans* particularly identified the GH32 as the most strongly induced gene during growth on fructan substrates, together with co-located genes encoding carbohydrate transport function proteins, transcriptional regulators and carbohydrate kinases (Tanno et al., 2021). These results remind the carbohydrate utilisation strategy of gpPUL that illustrated the ability of this Gram-positive bacterial species to compete for inulin against a background of total gut microbiota (Sheridan et al., 2016). Other human butyrate-producers, including *Butyricicoccus pullicaecorum*, *Faecalibacterium prausnitzii*, *Eubacterium hallii,* and *Eubacterium rectale,* have shown different capacities to degrade ITF according to the DP of the inulin molecules (Moens and De Vuyst, 2017; Zhu et al., 2017). Interestingly, few studies have characterised the prevalence and activity of GH32 enzymes in taxonomically diverse health-relevant gut microbes.

The mechanistic utilisation systems that operate in the intestine during the digestion of prebiotic carbohydrates are complex, diverse, heterogeneous and often specific to a compound **(Chapter 1, Section II.4)**. The nutrient acquisition strategies may be conserved in bacterial groups, although the presence of functional genes encoding transporters and regulators, and arrangements, do not necessarily mean they are metabolically active and able to exhibit metabolic activities towards prebiotic substrates (Tanno et al., 2021). The mechanistic details of prebiotic selectivity may lie in the organization of CAZYmes in PULs and/or may rely in the ability of bacterial strains to adhere closely to the prebiotic substrate (Glowacki et al., 2021; Patnode et al., 2021). A recent study suggested that surface glycan-binding proteins, and more specifically their domain architectures, may explain the specificities and adaptability of bacterial strains in response to their nutritional environment (Tamura et al., 2019, 2021).

Table II: Molecular mechanisms of the key bacterial utilisers of prebiotic substrates, such as resistant starches, β-glucans and fructo-oligosaccharides. Other carbohydrate substrates have been investigated to decipher the mechanisms of action of prebiotics and their impact on human health and wellbeing, including β-GOS (Ambrogi et al., 2021) and pectic glycans (Ndeh et al., 2017; Luis et al., 2018).

Carbohydrate	Glycosidic linkage	CAZyme	Bacterial species	Reference
RS	α (1-6) glucose-glucose α (1-4) glucose-glucose	GH13 GH77	*Ruminococcus bromii* *Bifidobacterium adolescentis* *Eubacterium rectale* *Faecalibacterium prausnitzii* *Roseburia intestinalis* *Bacteroides thetaiotaomicron*	Ze et al., 2015 Cockburn et al., 2015 Vital et al., 2018
β-glucan	β (1-4) glucose-glucose β (1-3) glucose-glucose	GH16 GH3 GH30 GH158	*Bacteroides ovatus* *Bacteroides uniformis* *Bacteroides xylanisolvens*	Tamura et al., 2017 Déjean et al., 2020 Singh et al., 2020
FOS	β (2-1) fructose-glucose β (2-1) fructose-fructose	GH32	*Roseburia inulinivorans* and 13 other butyrate-producers	Scott et al., 2011 Tanno et al., 2021

Abbreviations: RS, resistance starch; FOS, fructooligosaccharide

To unravel the effects of nutrition on the microbiome and health, future research directions should consider multi-omic approaches for assessing interactions between gut microbes and the prebiotic that stimulates their existence. The impact of prebiotics on the composition and function of the gut microbiome can be captured through the combination of *-omics* approaches, including metagenomics, metaproteomics, and metabolomics (Maier et al., 2017). These cutting-edge technologies enabled to develop a more complete picture of the metabolic processes that occur in the gut during prebiotic digestion (Spacova et al., 2020). Novel investigations combining high-resolution proteomics, genetic screens, and artificial "food particles" consisting of glycan-coated magnetic beads allowed to investigate how bacterial species respond to different food sources at the strain level (Patnode et al., 2019, 2021).

The impact of prebiotics on the human gut microbiome is still insufficient to fully understand the link between the gut microbiome, prebiotics, and human health and wellbeing. Compositional shifts, SCFA production and health consequences remain speculative. Very few studies have undertaken a mechanistic approach by methods rather than correlations to establish the causative role of the gut microbiome in human health and wellbeing effects of bioactive substrates (Bindels et al., 2017). There still remain calls for greater understanding of the causal link of observed prebiotic effects and long-term influences (Cunningham et al., 2021).

The role of the prebiotics in the modulation of the gut microbiome have been hard to pin down in health and disease outcomes. The large number of potential interactions between the components of the microbiome makes it challenging to define the mechanisms by which prebiotics affect community properties. One of the main challenge in prebiotic research resides in the individualised variability of the gut microbiome.

III.4. Individualised responses of the gut microbiome to prebiotics

A substantial number of cohort studies have explored the impact of prebiotics on the gut microbial communities. However, the microbiome is a complex and dynamic ecosystem that exhibits considerable intra- and inter-personal variations in its composition and functions. Dietary intervention studies showed that the microbial response to prebiotics is highly individualised (Walker et al., 2011; Davis et al., 2011; Martínez et al., 2010). The gut microbiome responds in a variable manner between individuals, but dominant effects on the SFCA production were remarkably consistent (Cherbuy et al., 2019). This inter-individual heterogeneity may be explained by differences in the composition of the gut microbiome (Venkataraman et al., 2016). Indeed, the diversity and abundance

of microbial signatures vary extensively both within, specific to the body site (e.g. oral, gut, skin), and among individuals, regardless of their health status (Human Microbiome Project Consortium, 2012). Analyses of gut microbiomes of Chinese and Danish individuals revealed country-specific microbial signatures (Li et al., 2014). This individual variability across geography is a major obstacle in translating gut microbiome findings across populations, with dominant species in the gut showing a large degree of heterogeneity across cultures and continents (He et al., 2018). Evidence reported that over 20% of the inter-personal variability of the gut microbiome can be inferred from environmental factors associated with diet and lifestyle (Falony et al., 2016; Rothschild et al., 2018).

The tremendous inter-individual variability has been demonstrated in space and over time (Lloyd-Price et al., 2017). Owing to the individualised nature of their gut microbiota, a particular change in diet can have highly variable effects in different individuals only maintained so long as the substrate is consumed (Johnson et al., 2019; Falony et al., 2016). Indeed, human intervention studies have revealed that the gut microbiome responds rapidly to changes in our diet, resulting in temporal fluctuations on timescales from hours to days to a wide diversity of diet (Sonnenburg and Bäckhed, 2016). The availability of nutrients can restructure the microbial communities and the metabolic outputs on a time scale of as little as 24h (Ley et al., 2006; Johnson et al., 2019). However, after these initial shifts, the microbiota returned near its original baseline state for the remainder of the intervention, despite healthy subjects maintaining their dietary intervention (Fragiadakis et al., 2020). This microbial resilience results from long-term dietary trends that constitute a dominant force in determining the compositional features of an individual's gut microbiome. These dietary habits can even have intergenerational impacts on the gut microbiome (Arumugam et al., 2011; Wu et al., 2011).

The magnitude of individual shifts induced by non-digestible carbohydrates in human gut microbial composition can be substantial, with changes at the level of relative abundance restricted to specific species. The reasons for this individuality variations may be explained by the differences in the varying enzymatic abilities to utilise a specific prebiotic substrate (Zhao et al., 2018) and/or can reflect the absence of keystone species (Ze et al., 2012). The inconsistencies in response profiles of individuals in dietary intervention might be yielded by the absence of functional “guilds” able to access and utilise specific fibre sources. The differences in response to prebiotics do not imply the individual but rather the function of the host gut microbiome. A recent study reported that the variability in the gut microbiome composition at the strain level is expected to display diverse binding specificities for polysaccharides (Patnode et al., 2021). In particular, the physical-chemical and compositional properties of dietary glycans, including the source and linkage composition, appeared to determine the ability of

a microorganism to gain access to nutrients and to utilise the same substrate in a competitive environment (Patnode et al., 2019).

Inter-individual intestinal microbiome differences have been linked to differential clinical responses to prebiotics in healthy adults, which can be more effective in some individuals, identified as responders, than others, identified as non-responders. Responders are individuals with appropriate baseline commensal microbes for whom the prebiotic may confer a health benefit (Cunningham et al., 2021). The report of responders and non-responders in intervention studies supports the determination of the characteristics of the subjects enrolled in clinical cohort studies, including sex, age, ethnicity, diet, and the functional features of their gut microbiomes, which may be used for predicting individual responses to prebiotics (Spacova et al., 2020; Rodriguez et al., 2020). A previous human study characterised by high fibre intake and decreased energy intake has illustrated the gene richness of the gut microbiome as a key microbial feature to distinguish individuals who respond efficiently to short-term dietary intervention (Cotillard et al., 2013). This study has highlighted that the response in obese patients was person-specific and was less efficient in improving clinical phenotypes in individuals with lower microbial richness. The baseline of microbial diversity may be a predictor of the response to diet-induced improvements in clinical responses to prebiotics (Salonen et al., 2014). Individual variations in dietary interventions correlate with a range of confounding factors, including the use of medication (e.g. antibiotics, osmotic laxatives, female hormones, benzodiazepines, antidepressants, and antihistamines) and stool consistency, among others (Falony et al., 2016; Vujkovic-Cvijin et al., 2020).

The identification of specific characteristics of the gut microbiome provides the opportunity to develop personalised nutrition strategies (Kolodziejczyk et al., 2019). Indeed, there is a significant interest in targeted strategies to modulate microbial composition within hosts on a personalised approach to redirect microbial signatures towards health. The microbial signature of the gut can drive the efficacy of prebiotic intervention that can be useful to support the hypothesis that intentional modulation of the gut microbiome is a valid strategy to improve human health and wellbeing (Rodriguez et al., 2020). Recent personalised diet intervention has successfully identified personal and microbiome features to accurately predict personalised postprandial glycemic responses. Exposure to specific dietary components modulates the composition of the gut microbiome that influenced host metabolic responses to lower postprandial glucose (Zeevi et al., 2015). Therefore, the development of personalised diets that regulate blood sugar levels provides hope for further advancements in the control and treatment of disease. Future research could enhance the characterisation of biological

response to prebiotics in clinical trials to give a deeper understanding of these interventions and their potential for precision application.

The microbiome can influence the host physiology and respond to an array of environmental factors without being able to understand the link between causation and correlation. Related to individual differences, prebiotic interventions are still far from convincing in terms of outcome measures in metabolic health. The intra- and inter- variability of the gut microbiome limits the applications of prebiotics and complicates the translation of effects into real-life outcomes.

III.5. Future of prebiotics

Several considerations have been progressively included in the concept of prebiotics, along with the scientific research progress, consumer interest, and technological innovations of industrial scientists. Nutritional strategies modulate the gut microbiome to confer beneficial effects on the human health and wellbeing on a personalised or population subgroup level (Cunningham et al., 2021). Prebiotics were initially identified to stimulate *Bifidobacterium* and *Lactobacillus* bacterial groups, which are often used as probiotics (Gibson and Roberfroid, 1995). However, while the goal of prebiotics is to selectively target certain health-relevant microorganisms, there are few substrates for which metabolisation would be restricted to only a small subset of bacteria. The impact of prebiotics on the gut microbiota has generally been found to be more widespread through cross-feeding interactions. The main *Bifidobacterium* and *Lactobacillus* targets produce predominantly acetate and lactate, which can downstream stimulate a number of bacterial species benefiting from the presence of the prebiotic. Hence, a prebiotic intervention generally translates inconsistent changes in the gut microbiome composition across individuals, suggesting that the actual response to prebiotics likely implies the function of the gut microbiome rather than the taxonomic composition. Instead of nurturing over- or under-represented health-relevant bacteria for growth stimulation, next-generation prebiotics can assist metabolic microbiome pathways.

Target key functionalities rather than health-promoting bacteria

To overcome the individualised responses of the gut microbiome, prebiotics could target specific health-relevant functions of an intestinal ecosystem that stably coexists. Exploiting the high degree of functional redundancy could help to counteract the intervariability observed across individuals harbouring distinct microbial communities. The definition of groups of bacteria or keystone species could help personalise the prebiotic intervention. **What we propose is the search for microbial signatures to predict the functional response of the gut microbiome that would be associated**

with different combination of taxonomic profiles. Based on nutritional mechanisms at the level of the bacteria, the intestinal ecosystem and the epithelium, this strategy involves that the health-relevant functions of these bacterial groups are precisely elucidated for their metabolite-derived effects. It involves in-depth knowledge of highly diverse microbial communities in their dynamic interplay with the host. Nevertheless, beneficial mechanisms are not fully elucidated at the level of individual bacteria, intestinal ecosystem, and host. The focus of prebiotic research needs to be further shifted from individual microbes and their role in influencing health and disease toward ecology within gut microbiomes (Clavel et al., 2022). It is necessary to understand better the fundamental rules driving dynamic interactions between gut microbial communities and prebiotic polysaccharides. It is a prerequisite for next-generation prebiotics to know precisely the metabolic mechanisms of metabolisation and the fermentation characteristics of health-promoting bacteria. The ecological perspective will not be complete without identifying how changes in the relative abundances of one species impact the relative abundances of others and the overall community function. However, it seems necessary to overcome the benefits and underlying mechanisms beyond the SCFAs. More extensive studies are crucially warranted to fill the gap in knowledge about the beneficial mechanisms.

Design the microbial accessibility of biochemical structures

The specificity and the selectivity of prebiotic effects relate on the chemical structures of dietary substrates, which results in targeting different gut microbial populations (Van Hul et al., 2020). Thus, the prebiotic potential of different prebiotics must be investigated to better understand to which extent biochemical structures, including numerous possible linkage configurations among monomers, contribute to selectively modulate the gut microbiome. Indeed, subtle structural variations of dietary fibres impact the community structure and metabolic output of fermentation by colonic microbiota (Tuncil et al., 2020). A recent study suggested that physiological functions of prebiotics are highly structure dependent, thus the importance to characterize the complex structures of the carbohydrates employed (Deehan et al., 2020). Gas chromatography-mass spectrometry (GC-MS) can be used to analyse the composition of monosaccharides and glycosidic linkages of polysaccharides (Patnode et al., 2019).

In next-generation prebiotics, we propose to engineer the structure of polysaccharides by influencing monosaccharides, anomeric configurations, linkage types, backbone lengths, branching units, and terminal attachments. The utilisation of precise reactions operated by biosynthetic enzymes could generate rationally customised polysaccharides. Future prebiotic compounds may also be chemically or structurally modified by the application of sonication, high

pressure, acid, enzyme, and oxidation treatments, in order to modify functionality (Lam and Cheung, 2019). Many of the structural characteristics of prebiotics are known to influence the manner in which microbes can utilise the substrate, including monosaccharide structure, degree of polymerisation, branching, linkages, and addition of functional groups or other modifications. **Knowing precisely the physiological outcomes and the role of the physicochemical structure is a prerequisite to precisely modify the accessibility of prebiotics.** Machine learning could predict structural characteristics of a prebiotic required for the modulation of specific microbiome profiles, and lead to custom prebiotic production based on these physicochemical features.

These engineered prebiotic substrates could be further classify hierarchically according to their chemical and physical characteristics and their specificity to be metabolised by a narrow group of bacteria within the gut. Cantu-Jungles and Hamaker have first proposed this new vision. The use of such fibres as prebiotics targeted specific microbes could result in predictable shifts independent of the background microbial composition (Cantu-Jungles and Hamaker, 2020). Thus, carbohydrate structures potentially could align with the diversity of use of the colonic bacteria (Hamaker and Tuncil, 2014). Specific bacterial abilities would allow selective stimulation of the growth and/or activity of microbes associated with health and wellbeing **(Figure 11)**. Each carbohydrate may favour bacteria at the strain level because of competitive advantage encoded in bacterial genomes (Patnode et al., 2019, 2021). However, the conception of prebiotic fibres to sustain the growth of targeted bacteria in a predictable way in every individual hardly seems an achievable goal. **One of the main challenges would be to build knowledge for the intelligent use of fibre structures, grouping fibre structures that would favour colonic bacteria for health and wellbeing outcomes.**

Discover and explore new prebiotic molecules

In the future, novel and emerging prebiotic compounds may be able to be used in targeted ways to manipulate the microbiome and its metabolic output (Scott et al., 2020). The desire to stimulate a wider group of commensal organisms has allowed the search for novel prebiotic compounds. Other than carbohydrate-based substances derived from plants, yeast-based substances, and many non-carbohydrate substances, including polyphenolics, fatty acids, herbs, and other micronutrients, have been explored for their prebiotic potential. With new advancements of the extraction and identification techniques, many newly discovered polysaccharides are continuously discovered from various resources (Zhang et al., 2018a). These next-generation prebiotics can have a novel origin that may be peculiar to a geographical region or from aquatic sources (Scott et al., 2020). Sources largely

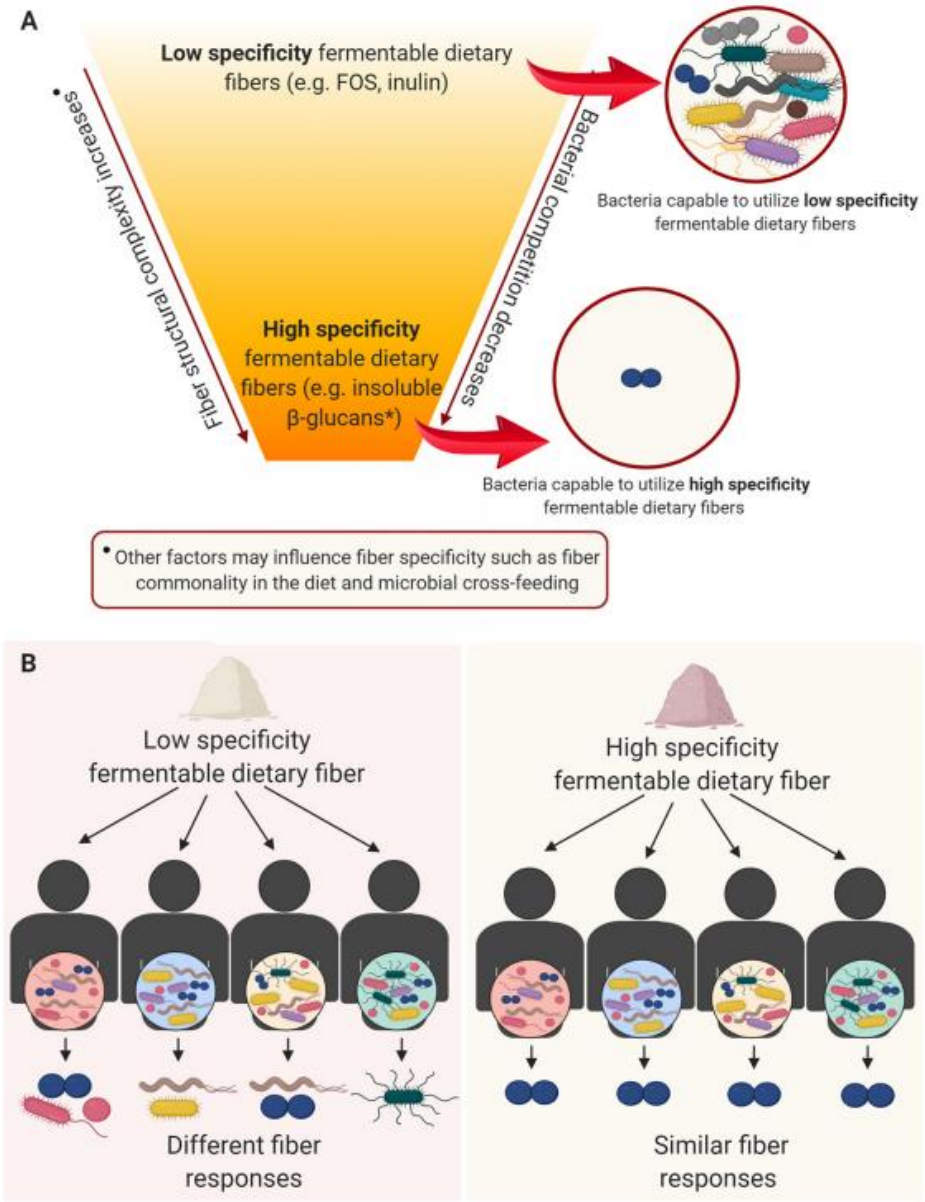

Figure 11: A hierarchical view of the specificity of dietary fibres toward intestinal microbes (from Cantu-Jungles and Hamaker, 2020). (A) Classification according to the specificity to gut microbes. (B) Variability in individual responses to dietary fibres related to the structure of intestinal communities. The top hierarchy includes dietary fibres with low microbial specificity. For example, FOS and inulin are highly accessible and metabolised by many bacterial taxa. Competitive interactions are based on the structure of intestinal communities that would use low-specificity dietary fibres generating divergent fibre responses across individuals. The bottom hierarchy includes dietary fibres with unique structural features resulting in high microbial specificity, which only few bacteria can access, degrade and utilise efficiently. Resulting in reduced competitiveness for high-specificity fibres would allow a more predictable and similar fibre response in a population, even in individuals harbour distinctly different microbial communities.

widespread across the world, such as legumes and algae, represent an opportunity for the valorisation of agriculture and the development of prebiotic components. From established origins, food waste generated annually in the food chain represents a rich and sustainable source of natural bioactive ingredients. Indeed, streams from fruit, vegetable, and grain processing contain potential prebiotics, such as pectin from citrus peel and raffinose family oligosaccharides from chickpeas. The revalorisation of certain wasted food sources is part of an intention to promote sustainable nutrition. In addition, next-generation prebiotics will likely be isolated from novel sources as the focus on sustainability, cost, and scale emerges (Cunningham et al., 2021). Therefore, the numerous studies to identify new nutritious targets suggests significant investment in the development of prebiotics as bioactive ingredients or supplements for a variety of potential applications **(Figure 12)** (Cunningham et al., 2021).

Future axes of research

High-throughput assays focused on analysis of DNA (metagenomics), proteins (proteomics), and small molecules (metabolomics) are providing key tools to interrogate the elaborate crosstalk among gut microbes and between microbes and their host. Likely, microbiome research will continue to accumulate in the future through the implementation of cutting-edge molecular technologies (Clavel et al., 2022), novel computational methods for the analysis of raw sequencing data and downstream interpretation (Quince et al., 2017), cultivation-free approaches (Almeida et al., 2019), and innovative workflows based on microfluidics (Tauzin et al., 2020). Ongoing developments in microbiome science will enable new frontiers of prebiotic research, addressing newly elucidated data-driven microbial niches and host targets. The scientific knowledge has the potential to expand the nutritional and healthcare applications of microbiome-modulating interventions.

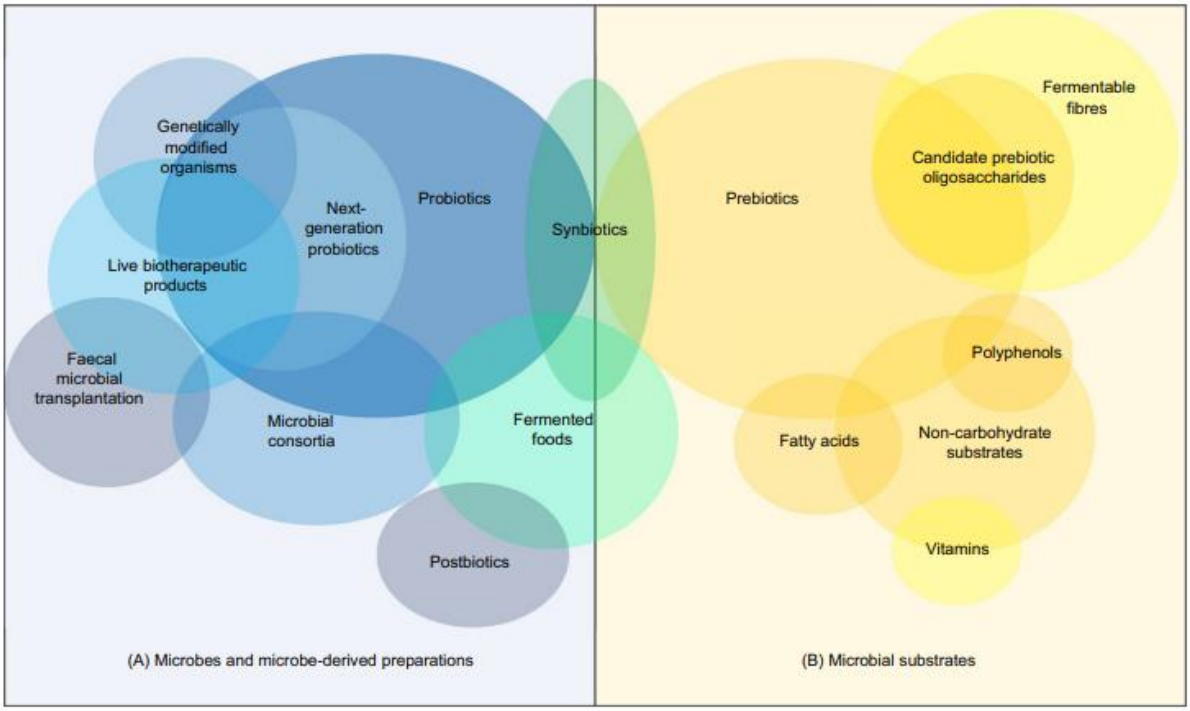

Figure 12: Adjacent fields of prebiotics and probiotics (from Cunningham et al., 2021) The figure depicts a conceptual map of established and emerging concepts, with (A) Microbes and microbe-derived preparations; and (B) Microbial substrates. Potential relationships between fields are depicted by the overlap of shapes.

MAIN OBJECTIVES & EXPERIMENTAL STRATEGIES

This PhD project addresses challenges and pitfalls of the prebiotic-microbiome research. Many unknowns remain in disentangling the dynamic interactions between prebiotics, the gut microbiome, and the host. Prebiotics represent an emerging solution to leverage digestive health and wellbeing through the development of functional foods. However, it is unclear how diet can be harnessed to easily manipulate the composition of gut microbes to boost the levels of desired bacteria. Knowing how prebiotics nourish gut microorganisms may suggest the selective stimulation of the growth and metabolic activities of health-promoting bacteria.

The guideline of this project is to gain insight into the underlying mechanisms of prebiotics warranting beneficial physiological impacts on the host. The interest is to understand how to successfully influence the gut microbiome. How can microbiome-based interventions be used to prevent gastrointestinal and metabolic disorders and promote human health and wellbeing?

In partnership with the giant agrifood Yoplait France – General Mills, five food ingredients were included in the project. These latter meet the definition of non-digestible dietary fibres, which means they are resistant to gastric acidity, hydrolysis by host digestive enzymes, and gastrointestinal absorption (FAO/WHO Codex Alimentarius Commission, 2009). Their health benefits mediated by fibre properties are regulation of the bowel transit time while increasing stool bulk, reduction of postprandial glycemic and insulinemic responses, and maintenance of normal cholesterol levels in the blood. Beyond the expected physiological actions of dietary fibres, these five food ingredients also may act as prebiotics (Gibson and Roberfroid, 1995; Gibson et al., 2017).

The main hypothesis of this work is that these five food ingredients can selectively stimulate the growth and metabolic activities of bacterial markers of intestinal health that are not limited to *Bifidobacterium* and *Lactobacillus* species. Indeed, prebiotics can target other health-promoting bacteria conferring local or systemic health effects, hence the importance of describing the bacterial metabolism. Therefore, the five food ingredients could favourably modulate the gut microbiome and result in relevant physiological benefits, including the promotion of SCFA synthesis, reinforcement of colon integrity and intestinal barrier, improvement of energy metabolism and satiety, and regulation of the immune response.

Previous studies have reported various physiological effects mediated by certain food ingredients among the five **(Table I)**.

Table I: Accumulated scientific evidence related to food ingredients provided by Yoplait France – General Mills. *Although the scientific evidence is unequal regarding the five food ingredients, their dietary fibre nature was assumed sufficient to designate these food ingredients as candidate prebiotics. Initially, psyllium husk was another food ingredient included in this study. Nevertheless, this compound proved to be poor substrate for the growth and the fermentation activities of most of health-promoting bacteria. We excluded this food ingredient from our analysis.*

Food ingredients	Health physiological effects	References
Agave Inulin Long-chain powdered **inulin** sourced from the Agave plant, containing **fructose polymers** and minor amounts (< 12% dry weight) of bound fructose, glucose and sucrose. Inulin is extracted and purified without the use of enzymes or processing aids (> 90% dry weight).	Among the selection of 32 human-derived bifidobacteria and lactobacilli strains, only *B. longum* subspecies *infantis* reported moderate growth stimulation	McLaughlin et al., 2015
Corn Fibre Maize-derived source of dietary fibres composed of a mixture of **glucose polymers** and non-digestible insoluble carbohydrates (>85% dry weight), which was obtained from a partial hydrolysed starch-made glucose syrup. This food ingredient may contain minor amounts of monosaccharides (< 15% dry weight)	High digestive tolerance after ingestion Low postprandial glycemia and insulinemia Changes in the composition of the gut microbiota: tendency to increase bifidobacteria and lactobacilli, increase of *Faecalibaterium* and *Roseburia* species Increase in SCFA Prevent gut inflammation	Maathuis et al., 2009 Stewart et al., 2010 Bassaganya-Riera et al., 2011 Vester Boler et al., 2011 Hooda et al., 2012 Konings et al., 2014 Holscher et al., 2015 Costabile et al., 2016
Polydextrose PDX is a mixture of highly branched and randomly bonded synthetic **polymers of glucose** (>90% dry weight), containing minor amounts (< 2% dry weight) of bound sorbitol and citric acid.	Low postprandial glycemia and insulinemia High feelings of fullness and satiety after consumption Prevent gut inflammation	Bassaganya-Riera et al., 2011 Konings et al., 2014
Citrus Pectin From citrus peel by-products (from the juice and citrus-oil processing industries), low-ester pectin standardized with sugars (dextrose)		Not applicable
Psyllium Husk		Not applicable

Abbreviations: PDX, polydextrose; DP, degree of polymerisation; SCFA, short-chain fatty acid

For instance, the corn fibre ingredient has been particularly investigated by short-term randomized and parallel interventions on healthy adult subjects to characterise the digestive tolerance after ingestion of the food ingredient. Hence, bowel habits determined by stool frequency, consistency, abdominal pain, intestinal bloating and flatulence were investigated (Stewart et al., 2010; Vester Boler et al., 2011; Costabile et al., 2016). This corn fibre appeared well tolerated in doses as high as 21g/fibre/delivered/day while leading to no significant adverse effects and minimal gastrointestinal upset on bowel habits. *In vitro* and *in vivo* studies highlighted the possible prebiotic effect of the increase in the relative abundance of *Bifidobacterium* species in the overall gut microbiota. The analysis of microbial metabolite showed an increase in short-chain fatty acids (Maathuis et al., 2009; Vester Boler et al., 2011; Hooda et al., 2012; Holscher et al., 2015; Costabile et al., 2016). Together with the polydextrose ingredient, these functional fibres also reported the potential for these dietary fibres to prevent gut inflammation using *in vivo* rodent model (Bassaganya-Riera et al., 2011).

Although the scientific evidence is unequal regarding the five food ingredients, their dietary fibre nature was assumed sufficient to designate these food ingredients as candidate prebiotics. The use of prebiotics as functional food ingredients is particularly interesting in the food industry because they combine health benefits and functional properties in food matrices **(Table II)**. Indeed, these five food ingredients show an excellent stability (both heat and acid) and compatibility with most food and beverage matrices. In addition, they are water-soluble carbohydrate-containing fibres that can be easily incorporated at high levels into food products, except psyllium husk. The latter has a physical nature that makes it less versatile in the food industry. In fact, this dietary fibre has been rapidly excluded from the experiments for their insolubility and their high viscosity.

The physicochemical properties and technological characteristics of the five food ingredients are interesting for use in different food applications (Lam and Cheung, 2019). They are ideal ingredients for applications that include dairy products, frozen desserts, baked goods, breakfast cereals, fruit spreads and fillings, processed meat, and baby food formulations. In addition to their nutritional advantages, the five food ingredients offer versatile functional properties when incorporated in foods as reducer of sugar and calorie content, fat replacer, textural modifier, organoleptic improver, and enhancer of fibre content and a prebiotic effects, where accepted (Lahtinen et al., 2010).

Table II: Prebiotic candidates as functional ingredients for food products. The property of nutritive sweetener is usually used for sugar reduction and/or replacement. The property of texture modifier is often used for providing viscosity and improving mouthfeel. The applications constitute a non-exhaustive list.

Food ingredients	Functional properties	Applications
Agave Inulin	white colour no off-odours nutritive sweetener texture modifier high solubility	in bakery, confectionery, frozen foods, formula-fed infants, dairy products, breakfast cereals, soft drinks in low-fat products
Corn Fibre	nutritive sweetener texture modifier	in a wide variety of foods and beverages in reduced calorie products for fibre fortification
Polydextrose	no off-colours no off-flavours no off-odours nutritive sweetener texture modifier	in bakery, confectionery, dairy foods and beverages in low-energy products
Citrus Pectin	no off-colours no off-flavours hydrocolloids gelation texture modifier slightly sweet and intense sweeteners	in a wide variety of foods and beverages in reduced energy products for fibre fortification

AXIS OF RESEARCH n°I: How to explore bacterial capacities to utilise prebiotics?

Our mission was to determine the prebiotic effects of the five food ingredients.

Based on the definition of prebiotics, dietary fibres can have prebiotic effects that rely upon microbial metabolism. To resume, a prebiotic is a "substrate" that implies bacterial growth through nourishment. A prebiotic should not be broadly metabolised, but elicit a selective metabolism biased towards health-relevant microorganisms. Thereby, prebiotic targets extend beyond stimulation of bifidobacteria and lactobacilli, and recognize that health benefits can derive from effects on other beneficial taxa. As selective utilisation of a prebiotic by host microorganisms is the key to its physiological effects, the metabolic results of this utilisation must, by deduction, be the main drivers of health and wellbeing (Gibson et al., 2017).

We have undertaken a comprehensive analysis of carbohydrate metabolism focusing on a panel of putative health-promoting bacteria. The investigation of prebiotic metabolisation at the scale of isolated species can provide evidence for further understanding of the interactions occurring within the overall communities of the gut microbiome. In fact, many microbial contributors that release and transform dietary carbohydrates are not well characterised (Wang et al., 2019).

In this project, we have the desire to target a wider group of commensal microorganisms, beyond *Bifidobacterium* and *Lactobacillus species*. The prebiotic metabolic properties of most human commensals from different taxonomic ranges have been poorly characterised. The functionality of the five food ingredients as prebiotic candidates were assessed at a low scale resolution of the gut microbiome to reduce the complexity of the intestinal ecosystem and to gain new insights into how the metabolism of intestinal health-promoting bacteria can be shaped through diet to improve human health and wellbeing.

Experimental strategy n°1: *in vitro* monocultures of health-relevant bacteria

To address this first question, the bacterial capacities to ferment candidate prebiotics were evaluated using single-carbohydrate monocultures. A similar approach has been already used to evaluate the functional ability of a substance to target bifidobacteria and lactobacilli probiotic strains (McLaughlin et al., 2015). Here, we developed a moderate-throughput cultivation approach to assess the prebiotic effects of the five food ingredients. Data collection allowed exploring the growth and

metabolic activities of health-promoting bacteria through the measurements of optical density, pH and concentrations of SCFAs.

SCFAs represent key molecular mediators in the dialogue between the gut microbiome and the host in response to nutrient residues. Deciphering the roles of prebiotics in the influence of host digestive health and wellbeing requires mechanistic studies. **Can we gain insights into other underlying prebiotic-mediated bacterial mechanisms, beyond SCFA stimulation?**

Recent studies have demonstrated the importance of the carbohydrate utilisation of the gut microbiota for host-microbial interaction and different aspects of host health, in particular energy metabolism (Krautkramer et al., 2021). **In-depth characterisation using *–omic* technologies expands the knowledge of functional capacity and activity of the microbiome.** Our untargeted metabolomic approach allowed the identification of a repertoire of bacterial-derived metabolites resulting from the prebiotic metabolisation that may be involved in interactions with the host. We provide detailed information on the overall carbohydrate utilisation capacity of key intestinal bacteria.

Experimental strategy n°2: *in vitro* cocultures of health-relevant commensal bacteria

Targeting the bacterial communities involved in the utilisation of a specific prebiotic is a though challenge. A better characterisation of trophic chains within the intestinal ecosystem is the basis for rational modification of the gut microbiome. Understanding prebiotic metabolisation within complex microbial communities can leverage dietary interventions.

The intentional modulation of the gut microbiome for health and wellbeing purposes involves deciphering the interactions between prebiotics and the health-relevant bacteria at higher resolution than individual microorganisms. It is not sufficient to collect data on individual organisms, and it is crucial to intensify research on microbial interactions within microbial communities (Clavel et al., 2022).

To uncover mechanistic insights into the interactions between the most promising food ingredients and health-relevant bacteria at a higher complexity level, we rationally designed synthetic bacterial assemblies. This experimental strategy supports the exploration of mutualistic symbiosis relationships and highlights the challenge of predicting the dynamics that occurs between prebiotics and the gut microbiome.

Importance of the project in the prebiotic-microbiome research

Several considerations have progressively been included in the concept of prebiotics, along with the scientific research progress, consumer interest, and technological innovations of industrial scientists. The goal of prebiotics is to select certain bacteria to confer beneficial effects on the host. The most reliable reference for the effectiveness of prebiotics is the selection of species of *Bifidobacterium* and *Lactobacillus*, the genera that are often used as probiotics (Cockburn and Koropatkin, 2016). An extrapolation to other genera or species is discussed, since the use of prebiotics may allow species-level changes in the microbiome (Gibson et al., 2004). **In this project, we explore the impact of food ingredients through the characterisation of selective stimulation of the growth and metabolic activities of key intestinal bacteria, beyond traditional *Bifidobacteria* and *Lactobacilli*. Most of these health-promoting bacteria have been poorly described for their metabolic properties.**

The main mediators of prebiotic beneficial effects are the SCFAs. However, the main prebiotic targets are typically not butyrate- and propionate-producing microorganisms. Instead, the main *Bifidobacterium* and *Lactobacillus* targets produce predominantly acetate and lactate, suggesting that cross-feeding is an important underlying mechanism of prebiotic effects or that we would have to better describe the underlying functional mechanisms of butyrate and propionate-producers.

While the goal of prebiotics is to precisely target certain beneficial organisms, the impact on the microbiota has generally been found to be more widespread. There are few carbohydrates for which the degradation capacity is uniquely restricted to only a small subset of beneficial bacteria, and cross-feeding further increases the number of species that benefit from the presence of the prebiotic.

AXIS OF RESEARCH n°II: How can we predict the genomic potential of the gut microbiome to utilise prebiotics?

Our mission was to better understand the molecular mechanisms involved in the utilisation of prebiotics and to propose gene biomarkers to monitor the microbial response to prebiotics.

There is limited understanding of how the human gut microbiome responds to a prebiotic-rich diet. The microbial signature of the gut can drive that often reports a heterogeneous efficacy in responses to prebiotic intervention. Identification of functional features would help to accurately predict individualised responses of the gut microbiome to prebiotics (Zeevi et al., 2015). The key challenge in prebiotic research is to predict the efficacy of prebiotics from the composition and functions of microbial communities.

We have undertaken a comprehensive analysis of prebiotic breakdown and fermentation mechanisms. Investigating bacterial genomes can provide new insights to support intentional modulation of the gut microbiome to improve human health and wellbeing. **Can we develop biomarkers of key functionalities of the gut microbiome?**

Experimental strategy n°3: *in silico* analysis of key metabolic pathways

To address this second question, bacterial capacities to degrade inulin-type fructans and to produce propionate and/or butyrate were investigated using an *in silico* approach. Although there are highly individualised responses, dominant metabolic outcomes of prebiotic intervention often report changes in SFCA production resulting from carbohydrate fermentation. They are described as one of the main mediators that elicit many physiological effects essential for health and wellbeing.

There is an interest to evaluate the selectivity of the carbohydrates and potential health properties hold through the metabolisation of gut microbes. This *in silico* approach aims to provide an in-depth characterisation of metabolic functions of the panel of health-promoting bacteria.

In this study, the characterisation of detailed mechanisms of carbohydrate utilisation allows to establish a list of potential biomarkers of the inulin-type fructan metabolisation and the propionate and butyrate syntheses. **Computational genomic analyses were performed to screen bacterial genomes to predict health-relevant microbial functionalities.**

AXIS OF RESEARCH n°III: How to develop functional foods applied to prebiotics

Our mission was to better understand the regulatory context of functional foods.

The outcome of the RestorBiome project is to incorporate the most promising food ingredients into dairy food matrices, in terms of prebiotic effects and their ability to target health-promoting bacteria. This functional product that contains prebiotics would intend to be consumed by the general population to maintain health and wellbeing through the gut microbiota. We can also envision restoring digestive health through the dietary supplementation of prebiotic ingredients that would be consumed by individuals suffering from metabolic syndrome (MetS) and irritable bowel syndrome (IBS). Hence, the consumption of this functional product would eventually intend to prevent gastrointestinal and metabolic symptoms. **How do the regulations in Europe and in the USA frame the application of prebiotic-containing functional foods?**

Experimental strategy n°4: detailed analysis of regulatory frameworks

This analysis provides an in-depth description of how regulatory agencies and policy markers in Europe and the United States of America, which govern the major economic markets in terms of functional foods and dietary supplements, do not approve health claims made for prebiotics.

RESULTS

Chapter 2: Functional characterisation of health-relevant intestinal bacteria revealed their metabolic capacities toward carbohydrate-containing prebiotics

In this **Chapter 2**, the prebiotic effects of the food ingredients provided by Yoplait France – General Mills were characterised for their ability to stimulate the growth and metabolic activities of key dominant intestinal bacteria. A panel of bacterial species covering a taxonomic diversity spread across the four main phyla of the human gut microbiome was investigated for their capacities to respond to diverse food sources. Interestingly, little is known about most of these intestinal anaerobes. This study yields a better understanding of the utilisation of carbohydrate-containing prebiotics by human gut bacteria holding health-relevant properties. Deciphering the interactions between prebiotics and the metabolic functions hidden within gut microbiome at the species level provided new evidence for intentional modulation of the gut microbiome to achieve health and wellbeing purposes.

The content of this chapter have been substantially prepared as a research article.

Impact of different complex carbohydrates on the growth and metabolic activities of health-relevant commensal bacteria

Bedu-Ferrari Cassandre[1,2], Biscarrat Paul[1], Vati Sarah[1], Pepke Frederic[1], Castelli Florence[3], Chollet Celine[3], Almeida Mathieu[4], Meslier Victoria[4], Langella Philippe[1], Cherbuy Claire[1,*]

[1]Micalis Institute, INRAE, AgroParisTech, Université Paris-Saclay, F-78350, Jouy-en-Josas, France
[2]Yoplait France - General Mills, Vienne Technical Center, F-38200, Vienne, France
[3]Service de Pharmacologie et Immuno-Analyse (SPI), Laboratoire d'Etude du Métabolisme des Médicaments, CEA, INRAE, Université Paris Saclay, MetaboHUB, F-91191 Gif-sur-Yvette, France
[4]Université Paris-Saclay, INRAE (Institut National de Recherche Pour L'agriculture, l'alimentation Et L'environnement), MGP (Metagenopolis), F-78350, Jouy en Josas, France.

*Corresponding author: claire.cherbuy@inrae.fr

ABSTRACT

Bacterial utilisation of complex carbohydrates is inextricably linked with the essential role of the gut microbiome, supporting the functional interdependence between the gut microbiome and its host. The role of intestinal microbes has attracted intense research attention in mediating diet-induced effects on host physiology. Inherent to the concept, a prebiotic substrate implies the stimulation of bacterial growth through nourishment, which relies upon microbial metabolism. In this study, we investigated the metabolic capacities of 17 health-relevant bacteria of the human gut microbiome towards potential prebiotics. *In vitro* experiments allowed the investigation of microbial profiling of growth and fermentation activities in response to different carbon sources. Each isolated key bacterial species showed different degrees of complex carbohydrate utilisation in terms of genomic repertoires dedicated to carbohydrate metabolism, growth, acidification level, and SCFA production at 24h culture. The results suggested that the metabolisation of prebiotics was driven by the phylogenetic affiliation of commensal bacteria rather than by the polysaccharide nature. In-depth characterisation of key health-promoting bacteria to deliver physiological benefits using an untargeted metabolomic approach revealed that bacteria respond significantly to the carbon source at the metabolite level. This study provides a holistic view of the underlying mechanisms of carbohydrate metabolism induced during the dynamic interplay between prebiotics and dominant health-promoting bacteria.

INTRODUCTION

Over the past decades, the evolution of human diet is thought to compromise the integrity of the gut microbiome resulting in the major changes in microbial diversity in the populations of industrialized countries. This phenomenon has been linked to the rises of multiple non-communicable diseases, including obesity, asthma, diabetes, chronic inflammatory bowel diseases, among many others (Sonnenburg and Sonnenburg, 2019). To improve health through the gut and reduce the risk of diseases, the development of functional foods has attracted intense research attention, with both scientific and commercial interest. There is an interest for the rational modulation of the composition and metabolic functions of the gut microbiome using nutritional strategies. Prebiotics define a substrate that is selectively utilised by host microorganisms that confer health benefits (Gibson et al., 2017). They can significantly influence the structure of the gut microbiome and ultimately the global health. In general, prebiotics refer to non-digestible dietary carbohydrates that can be fermented by health-promoting bacteria, resulting in the selective stimulation of growth and/or activity of commensal microorganisms (Bindels et al., 2015; Gibson et al., 2004). In this concept, *Bifidobacterium* and *Lactobacillus* strains are potential probiotic bacteria usually recognized as markers of intestinal health.

Prebiotic sources are mostly complex carbohydrates found in the human diet, such as non-starch polysaccharides, known as dietary fibres (Kolodziejczyk et al., 2019). These latter constitute a rich and diverse ubiquitous carbon source accessible by the gut microbiome. For example, inulin-type fructans, including short fructo-oligosaccharides (FOS) and longer inulin molecules, constitute one of the most accepted prebiotics that confer benefits on host health. Mediated physiological effects were investigated mainly for chicory inulin in dietary interventions intended first for adults and children with overweight and obesity (Hume et al., 2017; Neyrinck, Rodriguez, Zhang, et al., 2021). Indeed, chicory inulin has been associated with satietogenic effects (Vandeputte et al., 2017; Hiel et al., 2020), regulation of postprandial glycemia and insulinemia (Parnell and Reimer, 2009; Parnell et al., 2017), improvement of intestinal discomfort (Vandeputte et al., 2017; Neyrinck, Rodriguez, Taminiau, et al., 2021), and maintenance of normal cholesterol levels in the blood (Fromentin et al., 2022). In addition, this prebiotic supports favourable modulation of a gut microbiome through the consistent increase in relative abundance and metabolic activity of bifidobacteria and lactobacilli (Vandeputte et al., 2017; Hiel et al., 2020). This selective stimulation of bacteria, in particular *Bifidobacterium* species, can increase the acetate concentration in the gut environment which can lead to further downstream stimulation of a number of species, that indirectly benefit from the presence of the prebiotics (Glowacki et al., 2021).

Short Chain fatty Acid (SCFA) are the end-products of complex carbohydrate breakdown that often substantiate the health benefits of prebiotics (Bindels et al., 2017).

To gain new insights into the interactions between prebiotics, gut microbes and host health, efforts are made in the in-depth characterisation of the mechanistic underlying the prebiotic effects of dietary compounds. Saccharolytic fermentation of carbohydrate substrates involved a multitude of highly diverse carbohydrate-active enzymes (CAZymes) dedicated to the catalysis of complex material into individual carbohydrate components (Lombard et al., 2014). In particular, glycoside hydrolases (GH) support the hydrolysis and/or rearrangement of glycosidic bonds, which are essential for bacterial foraging systems such as the machinery encoded by polysaccharide utilisation loci (PUL) (Martens et al., 2011). According to the degradation capacities of commensal bacteria, dietary carbohydrates can have disparate effects on the composition of microbial communities.

In the field of health and nutrition, mechanistic research to clarify the impact of prebiotics on host health is an important basis for the rational development of nutritional strategies. In the current study, *in vitro* experiments of pure single-carbohydrate cultures were performed to characterise the functional capacities of key intestinal bacteria to metabolise dietary carbohydrates with prebiotic potential. To go further with the characterisation of prebiotic effects, the evaluation of five food ingredients was performed on a panel of 17 putative health-promoting microorganisms that encompass a broader range of taxonomic bacteria, beyond the most popular *Bifidobacterium* and *Lactobacillus* groups. This study advances new insights into the functional characterisation of dietary carbohydrates utilisation by health-promoting bacteria.

MATERIALS AND METHODS

Genome analysis

Selection of bacterial genomes Draft or complete genomic data were obtained for each selected bacteria from the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov). Assembly and annotation of the sequences, and quality check were carried out through computational analyses that run automatically the QUAST and PROKKA bioinformatic tools (Gurevich et al., 2013; Seemann, 2014). Selection of high-quality genomes met the following criteria: (1) only one genome per species was selected; (2) among multiple genomes for the same species, a genome with a minimal number of contigs was selected; (3) if more than one genome

with the maximum number of contigs exists, the genome with the maximal number of CDS was selected.

Phylogeny Phylogenetic analyses of the panel of health-promoting bacteria were estimated using MEGA X software (Kumar et al., 2018). 16S ribosomal DNA gene sequences were acquired from the NCBI Genbank database. Evolutionary relationships were inferred using the neighbour-joining method (Tamura et al., 2004) to estimate a tree from the aligned sequences performed using the ClustalW algorithm (Thompson et al., 1994). Evolutionary distances were calculated using the maximum composite likehood approach.

Prediction of enzymatic repertoires The prediction of all CAZymes of a genome was performed using the dbCAN2 v9 annotation tool (http://cys.bios.niu.edu/dbCAN2) (Zhang et al., 2018b). As recommended by the database, the combination of the HMMER search against the dbCAN hidden Markov model (HMM) database and the DIAMOND search against the CAZy database allowed to significantly identify the CAZyme-encoding genes. The results were filtered to exclude all matches with an e-value threshold of 1e-102 and an alignment coverage threshold of 0.35.

Bacterial strains and starter culture conditions

Most of the strains were provided by the *Deutsche Sammlung von Mikrooganismen und Zellkulturen* (DSMZ) and the *American Type Culture Collection* (ATCC) **(Table I)**. Each individual bacterial species was grown on BHI agar plates at 37°C in an anaerobic chamber containing 90% N_2, 5% CO_2, 5% H_2 atmosphere (Coy Lab Products, Grass Lake, MI, USA). A single isolated colony was subcultured at 37°C for 24h in BHI broth and used as an inoculum for the evaluation of prebiotic effects. The bacterial growth of the inoculum was assessed by the measurement of the optical density (OD) and the quantification of recoverable colony-forming units (CFU) per mL by plating on BHI supplemented agar. The BHI medium was supplemented with 10mg/L hemin, 5mg/mL vitamin K1, 0.5% L-cystein, 1% cellobiose and 1% maltose, which were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). A rich media was selected to support the growth of all monospecies.

The isolated strain identity was systematically verified by sequencing 16S rDNA amplicons. Colony PCRs were performed using oligonucleotide primers for the amplification of 16S rRNA-encoding gene of each bacteria. Eurofins synthesized the reverse primer [5'-ACGGCTACCTTGTTACGACTT-3'] and the forward primer [5'-AGAGTTTGATCCTGGCTCAG-3']. PCR products were amplified using DreamTaq enzyme (Thermo Scientific, Vilnius, Lithuania) following the manufacturer's instructions. PCR reactions

were performed using a T100™ Thermal Cycler (Biorad, Singapore). 16S rRNA gene-based amplicons were sequenced by Eurofins.

Single-carbohydrate experiments

A minimal medium (MM) was designed according to the culture conditions of the bacteria of interest described in the DSMZ German collection (https://www.dsmz.de). Adapted from YCFA medium (Duncan et al., 2009), this consensus medium has the particularity of containing a major source of carbon. The composition of the MM per 100 mL is as follows: 1.0g Bacto™ Tryptone (BD), 0.25g Bacto™ Yeast Extract (BD), 0.4g NaHCO3 (Prolabo), 0.045g K2HPO4 (Prolabo), 0.045g KH2PO4 (Merck), 0.09g NaCl (Merck), 0.009g MgSO4.7H2O (Prolabo), 0.009g CaCl2.2H2O (Fluka), 0.1mg resazurin (Sigma-Aldrich), 0.001g hemin (Sigma-Aldrich) and 0.1g cysteine (Sigma-Aldrich). The medium was supplemented with a filter-sterilised solution of vitamins (1 µg biotin, 1 µg cobalamin, 3 µg p-aminobenzoic acid, 5 µg folic acid, 15 µg pyridoxamine, 5 µg thiamine and 5 µg riboflavin). All vitamins were purchased from Sigma-Aldrich. Finally, the volume was adjusted to 100mL with deionized and sterilised H_2O. For the experiments carried out with *A. muciniphila*, the MM was enriched in 1% bovine mucin (Sigma-Aldrich). To study the metabolic properties of the panel of health-promoting bacteria, the MM was supplemented with diverse carbohydrate sources at 0.5% (w/v) and 0.1% (w/v) according to the viscosity properties of carbohydrate solutions. Five carbohydrates with potential prebiotic effects include agave inulin, corn fibre, polydextrose, citrus pectin, and psyllium husk **(Supplementary Table I)**. Carbon substrates were sterilised by 0.22µm filtration prior use. The MM supplemented with glucose and the MM supplemented with no carbohydrates were included as controls. The reconstituted culture media were allowed to be reduced for at least 48h in the anaerobic chamber.

Assessing growth and fermentation activities

Culture conditions in microtiter plates Each species preculture was diluted to an OD_{600} of 1 (Ultrapec 10 Cell Density Meter, Biochrom Ltd., Cambridge, UK). Because bacterial size and shape can influence OD600 measurements (Stevenson et al., 2016), counting of colony-forming units (CFU) estimated the bacterial inoculations from $1x10^7$ to $1x10^8$ CFU/mL. For each single carbohydrate experiment, 2% of the final volume of the inoculum culture was placed directly in 2mL 96-well v-bottom plates, in duplicate. We admit that nutrient carryover from pre-inoculations in rich media had only a marginal effect. Sample blanks containing no biological material were used as internal controls. The plates were covered and tightly sealed with a sterile semi-permeable membrane (Thermo Scientific, Rochester, USA) to prevent evaporation. Cultures were maintained in anaerobic conditions for 24h at 37°C.

Growth and fermentation parameters After sample homogenization, aliquots of each individual culture were transferred to 96-well microplates (96-well Costar® Assay Plate, Corning, Kennebunk, USA) to monitor growth using optical density (OD) at 600nm using a plate reader (Tecan Infinite 200 Pro Plate Reader, Grödig, Austria). The OD of inoculated cultures was measured before and after growth of bacteria. pH measurements were recorded for each of the bacterial cultures using a pH-meter 1140 (Mettler Toledo, Urdorf, Switzerland). The pH values of the inoculated cultures were measured before and after the growth of bacteria. From these values, we defined ΔOD and ΔpH for each individual bacterial culture to analyse the difference of OD and pH values. Variations of OD and pH estimate the growth and the strength of carbohydrate fermentation

Data analysis All measurements were recorded as 4 biologically independent replicates. Data were analysed using R Studio (Version 4.0.0) (R Development Core Team, 2008). The hierarchical clustering was based on the *dendextend* package using the *hclust* function with the agglomeration method of "ward.D2" and the *dist* function with the "Euclidean" method (Galili, 2015). The description of hierarchical categories was performed using the *FactoMinerR* package (Lê et al., 2008). The heatmap was generated using the *heatmap.2* function from the *gplots* package. Plots were generated using the *ggplot2* package (Wickham, 2016). Figures were generated using BioRender (https://biorender.com).

SCFA analysis by GC-FID

The determination and quantification of the SCFA content (acetic, propionic, i-butyric, butyric, i-valeric, valeric, i-caproic and caproic acids) was performed using gas chromatography equipped with a flame ionization detection (FID) (Agilent 7890 GC System, Courtaboeuf Les Ulis, France). Samples were acidified overnight with 1:11 (v/v) of a saturated phosphotungstic acid at 0.85M. Bacterial samples are centrifuged at 12,000 *g* for 15min at 4°C. Chromatographic separation was achieved using a Fused Silica capillary column (15m x 0.53mm x 0.5µm, film thickness) from Supelco (Sigma-Aldrich, Saint Quentin Fallavier, France). 0.3µL of samples were injected in splitless mode (splitless time 3min). The constant flow rate of the carrier gas (H_2) was 10mL/min and the inlet, column and FID detector temperatures were 200, 100 and 240°C, respectively. The oven temperature was initially set at 100°C for 10min, programmed at a rate of 20°C/min at 180 C and maintained for 2min. Calibration curves were obtained for each SCFA using a standard mixture at a final concentration of 4mM. The stock solution was stored at 4°C. The supernatants and calibration SCFA standard were spiked with 1:5 (v/v) internal standard solution of 2ethyl butyric acid at a final concentration 20mM in the samples. Phosphotungstic acid, 2ethyl butyric acid and volatile free acid mix were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). The water used in this study was deionized (resistivity greater than 18 MΩ cm) using

a Milli-Q SP Reagent Water System (Millipore, Molsheim, France). All samples were analysed in duplicate, without any previous derivatisation of the SCFAs. The data was collected and peaks integrated using OpenLab Chem station software (Agilent, Courtaboeuf Les Ulis, France). The ratio between each SCFA peak area and the internal standard peak areas was plotted against standard concentrations to calculate the quantities for each analyte. All data were expressed in mM as median.

Metabolomics

Culture conditions in Hungate tubes Single-carbohydrate batches of MM were used to examine the metabolic activities. Importantly, the MM includes non-defined protein sources, such as Bacto™ yeast extract and Bacto™ tryptone, which were reduced to 20% of the amounts. The pH was adjusted to 6.8. The quantitative determination of SCFAs produced by microorganisms can be measured in culture medium. Samples were centrifuged at 12,000 *g* for 15 min at 4°C and supernatants were immediately stored frozen at −80°C until metabolomic analyses were performed. Bacterial supernatants were immediately processed after thawing at room temperature.

Liquid chromatography coupled to high-resolution mass spectrometry (LC-MS) Ultra-high performance liquid chromatography separation was performed on a hypersil GOLD C18 1.9µm, 2.1mm × 150mm column (C18) at 30°C (Thermo Fisher Scientific, les Ulis, France) and on a Sequant ZICpHILIC 5µm, 2.1 x 150mm column (HILIC) at 15°C (Merck, Darmstadt, Germany). All chromatographic systems were equipped with an on-line prefilter (Thermo Fisher Scientific, Courtaboeuf, France). LC-MS analyses were performed using a U3000 liquid chromatography system coupled to an Exactive mass spectrometer from Thermo Fisher Scientific (Courtaboeuf, France) fitted with an electrospray source operated in the positive and negative ion modes. The software interface was Xcalibur (version 2.1) (Thermo Fisher Scientific, Courtaboeuf, France). The experimental settings for each LC/MS condition are described in Boudah et al., 2014.

Reagents, chemicals, and biological material Metabolite extraction was performed twice (for HILIC and C18 analyses) from 50µL of supernatants after precipitation of proteins assisted by methanol, as previously described in Boudah et al., 2014.

Data processing All raw data were manually inspected using the Qualbrowser module of Xcalibur version 2.1 (Thermo Fisher Scientific, Courtaboeuf, France). The raw files were first converted to mzXML format using MSConvert software. Automatic peak detection and integration were performed using the XCMS software package (Giacomoni et al., 2015). XCMS features were then filtered according to the following criteria: *(i)* the correlation between the dilution factors of the QC samples and the areas

of the chromatographic peaks (filtered variables should exhibit coefficients of correlation greater than 0.7 to account for metabolites occurring at low concentrations and which are not longer detected in the most diluted samples), *(ii)* the repeatability (the coefficient of variations obtained for the chromatographic peak areas of the QC samples should be less than 30%) and (iii) the ratio of the chromatographic peak area of the biological to blank samples above a value of 3. Optionally, if necessary, the chromatographic peak areas of each variable present in the XCMS peak lists were normalized using the LOESS algorithm in order to remove analytical drift induced by clogging of the ESI source observed in the course of analytical runs.

Annotation Features were annotated by matching their accurate measured masses ± 10ppm with theoretical masses contained in biochemical and metabolomic databases. Comparison of m/z and retention time from experimental data to referent molecules in intern (Boudah et al., 2014) and public (KEGG, METLIN and HMDB) databases (Kanehisa and Goto, 2000; Smith et al., 2005; Wishart et al., 2007). Note that the abbreviation m/z represents the quantity formed by dividing the ratio of the mass of an ion to the unified atomic mass unit, by its charge number (regardless of sign).

Statistical analysis LC-MS data matrices were statistically analysed using both multivariate and univariate non-parametric techniques in order to identify significant features that differ between groups of carbon sources and bacterial species in comparison with the initial non-inoculated minimum media. Data and statistical analyses have been performed in R Studio (Version 4.0.0) (R Development Core Team, 2008). A multivariate approach allows to highlight correlations and metabolite clustering generated by biological processes. The Sparse Partial Least Squares-Discriminant Analysis (sPLS-DA), implemented in the *mixOmics* R package and also in MetaboAnalyst, is an ubiquitous classification technique widely applied to metabolomics data (Rohart et al., 2017). To improve the interpretability of these data, sPLS-DA was particularly suited for large LC-MS data sets with numerous predictor variables (X = metabolites) to unravel the information contained in qualitative response variables (Y = carbon sources or bacteria). To estimate cluster validation, the optimal number of dimensions and variables was selected to minimize the misclassification error rate in the final sPLS-DA. The model was fitted with 10 components using repeated (× 10) 5-fold cross-validation procedure to evaluate the number of components sufficient to explain the covariance of the data set for any number of selected variables, and the classification performance, including the overall error rate (OER) and the balanced error rate (BER), for each type of prediction distance. Results were plotted using the scores of the first two components, where each point represents an individual sample. From the variables of each sPLS-DA that displayed individuals corresponding to either *Firmicutes* or *Bacteroidetes* bacteria, two lists of

discriminant metabolites were established for further statistical analyses. Separately, on each separation methods data sets, an univariate approach was performed using Wilcoxon tests to reveal pairwise differences of fold changes in metabolite levels between the initial non-inoculated MM vs. the inoculated MM after 24h culture. In addition, multiple test correction was performed on the p-values, which were adjusted with the Benjamini–Hochberg method. The significance threshold was established at 0.05. The summary statistics (mean, standard deviation and p-value) were computed on each separation methods data sets, separately. Normalized metabolite levels were analysed after log2-transformation to highlight the relative abundances of the discriminatory metabolites. It provided a way to operationally define the bacterial responses to the different carbon sources.

Gene expression

Culture conditions in Hungate tubes Single batches of MM adjusted to pH 6.8 were supplemented with or without carbon source and with a 20% reduction of the amounts of protein source. Single carbohydrate experiments of *B. thetaiotaomicron*, *B. xylanisolvens*, *R. intestinalis* and *S. variabile* were performed as previously described. Bacterial growth were stopped in the exponential growth phase 12h after the inoculation. The samples were centrifuged at 12,000 *g* for 15 min at 4°C and the pellets were collected and immediately stored frozen at −80°C until further downstream experiments.

RNA extraction Immediately processed after thawing at room temperature, total RNA were isolated with RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Total RNA quality was assessed on an Agilent Bioanalyzer 2100, using RNA 6000 pico kit (Agilent Technologies).

Libraries construction Directional RNA-Seq Libraries were constructed using the TruSeq Stranded Total RNA library prep kit, with bacteria Ribo-Zero reagents (Illumina), following the manufacturer's instructions. After the Ribo-Zero step, the samples were checked on the Agilent Bioanalyzer for proper rRNA depletion. Final libraries quality was assessed on an Agilent Bioanalyzer 2100, using an Agilent High Sensitivity DNA Kit.

RNA sequencing Libraries were pooled in equimolar proportions and sequenced on a single read 75pb run, on an Illumina NextSeq500 instrument. Demultiplexing was performed with bcl2fastq2 v2.18.12. Adapters were trimmed with Cutadapt v1.15, and only reads longer than 10pb were kept for further analysis. Reads have been mapped on high-quality genomes, and were counted using subread featureCounts v1.5.2. Differential analyses were performed in R using DESeq2.

RESULTS

Characterisation of putative health-promoting bacteria

Deciphering prebiotic effects of dietary carbohydrates with potential physiological benefits was based on a panel of key commensal bacteria of the human gut microbiome **(Table I)**. Indeed, we have chosen a total number of 17 cultivable bacterial species mostly isolated from human samples that illustrated the four major phyla including *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Verrucomicrobia* **(Figure 1A)**. At the scale of the overall diversity of the gut ecosystem, the selection encompassed bacterial members of the main taxa that displayed a wide range of phylogenetically representatives (Eckburg et al., 2005) **(Figure 1B)**.

The list of health-relevant bacteria was established through a bibliographic research strategy that focused on bacterial species in microbial signatures of health and gastrointestinal disorders. Indeed, clinical evidence reported that their relative abundances were modified in individuals suffering from a metabolic syndrome (MetS) (Hou et al., 2017; Jamar et al., 2018; Munukka et al., 2012; Org et al., 2017; Qin et al., 2012; Vrieze et al., 2012) and/or an irritable bowel syndrome (IBS) (Carroll et al., 2012; Jeffery et al., 2012; Palma et al., 2017; Rajilić–Stojanović et al., 2011; Saulnier et al., 2011) compared to healthy gut microbiomes. Therefore, these species were pinpointed as bacteria relevant to a healthy state of the intestinal ecosystem. Actually, most of the selected bacteria were considered for their roles to maintain the host digestive health as potential next-generation probiotics and/or live bacterial biotherapeutics (King et al., 2019; Ose et al., 2018; Rivière et al., 2016).

To better apprehend the enzymatic activities of health-promoting bacteria selected in this study, we performed functional prediction of CAZymes based on publicly available and high-quality annotated genomes **(Table I)**. Complete or draft genomes were used to search for CAZy proteins using dbCAN2 in the CAZy database (Zhang et al., 2018b). Based on CAZy annotation, the members of the *Bacteroidetes* phylum seem to harbour extensive enzymatic repertoires involved in carbohydrate degradation **(Figure 1C)**. As previously found, results showed that these *Bacteroidetes* species display broad utilisation capacities allowing them to grow and ferment a large variety of carbohydrate substrates (Terrapon et al., 2015; Martens et al., 2014). In comparison, the members of the *Actinobacteria* and *Firmicutes* phyla, such as *E. rectale* and other members of the Clostridium cluster XIVa, appear to possess smaller repertoires of CAZymes. Thus, enzymatic repertoires of the bacteria used in this study is in accordance with previous results revealing differential metabolic activities between the bacterial phyla (Kaoutari et al., 2013).

In addition, the occurrence and abundance of each health-relevant bacteria were estimated in metagenomic data prior a 8-week randomised controlled trial that explored the effects of a rich plant-based Mediterranean diet on metabolic health and gut microbiome in individuals with low fibre intake and sedentary lifestyle (Meslier et al., 2020). Each bacterial species appeared present and abundant to various degrees in the human gut microbiome, revealing that our selection contains members of the dominant and subdominant human gut microbiota **(Figure 1D)**.

Carbohydrate utilisation by health-promoting bacteria

In vitro single-carbohydrate experiments evaluated the bacterial growth and fermentation activities of 17 health-promoting bacteria in presence of prebiotic as the major carbohydrate source including agave inulin, corn fibre, polydextrose and citrus pectin **(Supplementary Table I)**. Their metabolic capacities to utilise various dietary polysaccharides were evaluated in a minimal media (MM) supplemented with the carbohydrates as the main fermentable carbon and energy source. In regards to appropriate controls, individual ΔOD profiles were used as indicators of the bacterial growth **(Figure 2A)**. The MM-glucose condition supported the growth of all bacterial species in monoculture enabling accurate assessment of their carbohydrate utilisation profiles, with the exception of *F. prausnitzii, B. pullicaecorum and R. bromii*. That is, the vast majority of the health-promoting bacteria showed appreciable growth on the positive MM-glucose control condition. In comparison, all bacterial species showed restricted growth on the MM without carbohydrate supplementation, serving as a negative control condition. Individual ΔpH measurements **(Figure 2B)** were also recorded as indicators of fermentation activities (Louis and Flint, 2017). As expected, the pH values for all species inoculated into MM without carbohydrate supplementation remained close to the initial pH of non-inoculated MM, with the exception of *Bacteroidetes* species. Indeed, pH values revealed a slight acidification of the MM without carbohydrate supplementation, especially for *B. thetaiotaomicron* and *B. xylanisolvens*, suggesting their capacities to utilise the yeast extract and tryptone content of the MM as energy source (Macfarlane et al., 1988). Carbohydrate utilisation activities resulted in the acidification of medium for most combination of bacterial species with one notable exception to the trend (*F. prausnitzii, B. pullicaecorum and R. bromii*). These results confirmed that MM is an appropriate medium for carbohydrate-dependent growth analysis in this study.

Each bacteria showed notable diversity in terms of carbohydrate metabolism, as they were able to utilise the variety of carbohydrates to different degrees. In regards to the appropriate control conditions, *Bacteroidetes* species were able to grow and ferment all complex carbohydrates appreciably. In particular, *B. xylanisolvens* exhibits the highest growth and fermentation activities in all carbon

sources. Other *Bacteroidetes* species displayed similar high growth and fermentation activities, which demonstrated a wide spectrum of carbohydrate utilisation. Compared to the *Bacteroidetes* species, growth and fermentation profiles revealed that *Actinobacteria* and *Firmicutes* species were less versatile in the utilisation of carbohydrates. It seems that *Firmicutes* species belonging to the *Lachnospiraceae* family have more metabolic capacities to degrade polysaccharides than *Ruminococcaceae* species, except for *S. variabile*.

Depending on each bacterial species, the carbon source influenced the ΔOD and ΔpH over 24h of single-carbohydrate monoculture. It appears that agave inulin and corn fibre were associated with higher growth and fermentation activities of health-promoting bacteria than polydextrose and pectin substrates. Actually, each food ingredients was able to stimulate growth and fermentation activities to various degrees according to the phylogenetic affiliation of each bacterial species **(Supplementary Figure 1)**. In particular, agave inulin and corn fibre reported highest growth and fermentation activities in *Bacteroidetes* and *Actinobacteria* species, compared to polydextrose and pectin sources.

Fermentation profiles based on SCFA production

As metabolic end-products of bacterial fermentation in the gut have been associated with human health, gas chromatography analysis was used to quantify SCFAs in single-carbohydrate experiments. Concentrations of the main metabolites, including acetate, propionate, and butyrate, were reported for each health-promoting bacteria **(Figure 3)**. SCFA concentrations allowed a deeper investigation of the utilisation of complex dietary carbohydrates by health-promoting bacteria. Most of the health-promoting bacteria appeared to ferment at least one carbon source into acetate. This predominant metabolite was measured at concentrations higher than the overall mean of the main SCFA (acetate, propionate and butyrate), with the exception of three species of *Ruminococcaceae*, including *F. prausnitzii, B. pullicaecorum and R. bromii*. Similarly, *A. muciniphila* species, representative of *Verrucomicrobia* phylum, displayed low concentrations of acetate and propionate, which confirmed their low capacities to grow and ferment carbon sources in these experimental culture conditions. In particular, the highest concentrations of acetate were revealed in cultures of *B. catenulatum* in MM-glucose and MM-inulin conditions. Similarly, cultures of *B. hansenii* reported elevated concentrations of acetate in MM-glucose and MM-inulin conditions. This latter species represents an exception related to the fermentation profiles of *Firmicutes* members in the panel of health-promoting bacteria. Indeed, most of *Firmicutes* species also produced butyrate. In particular, the most elevated concentrations were measured in the MM-glucose cultures of *A. caccae*, *A. hallii*, and *R. intestinalis*. Regarding the fermentation profiles of *Bacteroidetes* species, these bacteria were capable to ferment all carbon

sources into acetate and propionate. SCFAs were particularly high in MM-glucose cultures of *B. fragilis* and *B. xylanisolvens*, which produced acetate and propionate. Interestingly, the propionate concentrations were higher in the complex carbohydrates than in presence of glucose. Similar patterns were observed for all *Bacteroidetes* species, revealing the nutritional adaptation of these bacteria to grow and ferment complex dietary carbohydrates.

Each SFCA concentration varied greatly depending on both the species and the carbohydrate source. The highest growth and fermentation activities were reported for the MM-glucose condition, with the exception of *Bacteroidetes* species, *B. hansenii,* and *B. catenulatum,* which were more likely to grow on inulin. Agave inulin and corn fibre generated the highest concentrations of acetate, propionate and butyrate, compared to polydextrose and pectin. This data are consistent with the measurements of growth and acidification parameters **(Figure 2)**.

Diversity in carbohydrate utilisation driven by the bacterial phylum

Growth and fermentation variations revealed distinct metabolic capacities of health-promoting bacteria that seem dependent of the phylum-level **(Supplementary Figure 2)**. Indeed, *Bacteroidetes* species appeared to grow and ferment any given resistant carbohydrates, resulting in high production of acetate and propionate. On the contrary, most of the *Firmicutes* species displayed more restricted growth and fermentation activities on complex polysaccharides. Each monoculture was clustered according to its SCFA fermentation profiles **(Figure 4, Supplementary Table II)**. The application of a hierarchical classification method revealed the four functional groups of fermentation activities for the panel of health-promoting bacteria. Representing 64% of the single-carbohydrate experiments, the largest cluster N encompassed bacterial species that did not show growth and fermentation activities for none of the substrates. The fermentation profile revealed low SCFA contents and especially regrouped the pectin and negative conditions. The descriptive statistics indicated that *R. bromii*, *F. prausnitzii*, *B. pullicaecorum*, *B. adolescentis,* and *A. muciniphila* displayed low fermentation activities compared to the overall data set. In contrast, the other clusters encompassed intense fermentation activities with distinct SCFA contents. Representing 13% of experimental observations, the cluster G regrouped bacterial species that especially metabolise glucose. It was significantly distinguished by higher butyrate concentrations with a mean concentration of 8.9mM ± 5.47 compared to the overall mean of 1.6mM ± 3.46 (p-value = 7e-78). This cluster included only these butyrate-producers of the *Firmicutes* phylum and, in particular, members of the *Lachnospiraceae* family. These results suggested that *E. rectale*, *A. caccae*, *R. intestinalis,* and *S. variabile* can efficiently use glucose as their main carbon source. Representing 17% of the samples, the cluster A exclusively regrouped *Bacteroidetes* species that

were able to grow and ferment all carbon sources. It was significantly marked by a higher propionate with mean concentration of 10.3mM ± 5.20 compared to the overall mean of 2.1mM ± 4.48 (p-value = 4e-82) and isovalerate with mean concentration of 2.1mM ± 0.64 compared to the overall mean of 0.4mM ± 0.84 (p-value = 1e-104) concentrations. Interestingly, no monocultures based on glucose substrate were correlated to this latter group (p-value = 1e-06), which supported an evolutive adaptation of these *Bacteroidetes* species towards polysaccharide metabolisation. Representing 6% of the samples, the smallest cluster G/I regrouped bacterial species that metabolise in particular the glucose and inulin carbon sources. It was significantly characterised by higher acetate concentrations with mean concentration of 26.6mM ± 7.41 compared to the overall mean of 5.4mM ± 7.18 (p-value = 2e-70). Consistent with the fermentation profiles of health-promoting bacteria previously described, *B. catenulatum*, *B. hansenii*, *B. fragilis* and *B. xylanisolvens* displayed high fermentation activities in inulin and glucose conditions, resulting in high concentrations of acetate.

To resume, the hierarchical clustering of the SCFA production identified four functional groups of carbohydrate utilisation that partly reflected the phylogenetic affiliation of health-promoting bacteria. Indeed, butyrate and propionate were the metabolites that discriminated the functional clusters G and A strictly associated with *Firmicutes* and *Bacteroidetes*, respectively. Acetate was the predominant metabolite produced in most bacteria. Thus, acetate discriminated the function cluster G/I that was associated with three bacterial phyla. Furthermore, the statistical description of the categories indicated that the bacterial species appeared as a driving force of carbohydrate utilisation (p-value = 2e-103) compared to the conditions (p-value = 6e-33) **(Supplementary Table II)**.

In-depth metabolic activities of six carbohydrate-responding bacteria

The metabolic activities resulting from the interactions between the food ingredients and health-promoting bacteria allowed an in-depth characterisation of prebiotic effects beyond the investigation of fermentation SCFA products. The selection of six health-promoting bacteria was based on the effects of a rich plant-based Mediterranean diet that revealed the improvement of metabolic markers correlated with specific microbial taxa in individuals with low fibre intake and sedentary lifestyle (Meslier et al., 2020). Statistical comparison between the groups of control and treated individuals, the impact of rich plant-based diet seemed to be significantly correlate with the increase in relative abundances of *R. intestinalis*, *E. rectale*, *F. prausnitzii* and *B. adolescentis* **(Figure 5A)**. Further statistical comparison within the treated groups highlighted the tendency to the enrichment of B. *thetaiotaomicron*, *B. intestinalis* and *R. bromii* **(Figure 5B)**. These results suggested that these health-relevant bacteria were more prompted to respond to high intakes of complex carbohydrates. However, previous results

reported low growth and fermentation activities of *F. prausnitzii* and *R. bromii* in our experimental culture conditions. We have chosen to include three *Firmicutes* species, including *R. intestinalis*, *E. rectale* and the closest phylogenetic relative to *F. prausnitzii* that is *S. variabile* **(Figure 1)**. In addition, three *Bacteroidetes* species included *B. intestinalis*, *B. thetaiotaomicron* and *B. xylanisolvens* that referred to as bacterial models of the carbohydrate breakdown **(Review 1)**.

Using a LC-MS metabolomic approach, the experimental strategy explored the metabolic activities of six health-promoting bacteria in response to complex carbohydrates, including corn fibre, agave inulin and citrus pectin, in comparison to appropriate controls **(Figure 6A)**. This technology consisted of two types of chromatographic conditions (C18 and HILIC), and two ionization conditions (both positive and negative modes) that allowed cataloging of as many classes of small chemical compounds as possible (hydrophobic, hydrophilic, negatively charged and positively charged). This combination resulted in the detection of about 3085 features in the HILIC column and negative ionization mode, and 7862 features in the C18 columns and positive ionization mode. Approximately 28 metabolites were shared by the two LC systems. Applied to the global non-targeted metabolomic data, sparse partial least squares-discriminant analyses (sPLS-DA) were performed after their optimal minimisation **(Supplementary Figure 3)** for the identification of the metabolomic patterns at large-scale between the health-promoting bacteria and the carbon sources. Samples contributing to the first component allowed separation of health-promoting bacteria **(Figure 6B)**.

A clear distinction of *B. xylanisolvens* suggested a particular metabolic signature that differed from the *Firmicutes* species and the other *Bacteroidetes* species. *B. thetaiotaomicron* was also distinct from the *Firmicutes* species and displayed a metabolic profile similar to that of *B. intestinalis*. In general, the three *Firmicutes* species showed overlapping samples that suggested similar metabolic signatures, with the exception of *S. variabile* that appeared to have distinct samples from the *Bacteroidetes* and the other *Firmicutes* species. Furthermore, samples contributing to the first component allowed a separation of the carbon sources **(Figure 6C)**. The clusterization allowed a clear distinction of metabolic profiles obtained from the cultures in inulin and corn fibre, that from the cultures of pectin and glucose, those of the cultures without carbohydrates. The overlapping between metabolites between pectin and glucose conditions, and between inulin and corn fibre, suggested that these carbon sources can provide similar metabolomics information, respectively. Even if few overlapping samples were observed, metabolic signatures specifically associated with prebiotics were expected.

Consistent with the classification of SCFA fermentation profiles, the variance of metabolic profiles appeared more explained by bacteria (20%) than by carbon sources (12%), revealing that bacterial

species were a driving force of carbohydrate metabolism. Because the metabolic signatures seemed more pondered by bacteria, the metabolomics data sets were investigated for *Firmicutes* and *Bacteroidetes* profiles, distinctly, to examine the molecular determinants of carbohydrate metabolism at the phylum level. Thus, regarding the metabolic activities of *Firmicutes*, the profile of *S. variabile*, which belongs to the *Ruminococcaceae* family, was clearly distinct from the two other species **(Figure 6D)**. Being members of the *Lachnospiraceae* family, *R. intestinalis* and *E. rectale* shared some similarities in their metabolomic profiles. To some extent, the metabolic patterns can discriminate these three bacterial species, even if few overlapping samples were observed. For the metabolic activities of *Bacteroidetes*, although the three *Bacteroide*s species are closely related, the interpretation of their microbial signatures indicated metabolic diversity at species levels **(Figure 6E)**.

For both sPLS-DA on *Firmicutes* and *Bacteroidetes*, plotting the variables allowed the identification of the analytes that made the largest contribution to the group classification. After applying a 0.85 cut-off, approximately 399 and 468 features allowed to differentiate the metabolic signatures of each individual *Firmicutes* and *Bacteroidetes* species, separately. For each of these analytes, the differential analysis between conditions of non-inoculated versus cultivated medium revealed fold changes for each bacterium and each carbon source. Interestingly, the number of significant metabolites varies greatly between bacterial species, reflecting distinct activities in response to carbon sources **(Supplementary Figure 4)**. Hence, in accordance to previous results, *Bacteroidetes* species showed a high number of differential metabolites significantly produced in each medium. *B. xylanisolvens* seems particularly active in all conditions. Instead, the *Firmicutes* species activities resulted in lower number of differential metabolites significantly present in each medium. Compared to appropriate controls, *S. variabile* appeared to be particularly active in response to agave inulin and citrus pectin. A lower number of significant metabolites for *R. intestinalis* and *E. rectale* was reported, suggesting that these two *Firmicutes* species displayed more restricted metabolic activities compared to *S. variabile*. Interestingly, there were no differential metabolites for MM without carbohydrate supplementation in *Firmicutes* species, confirming this condition as a negative control for carbohydrate metabolism. Similarly, these bacterial species appeared to be unable to respond significantly to the levels of metabolites in at least one carbon source. *R. intestinalis* was unable to significantly metabolise citrus pectin; *E. rectale* did not show significant metabolites in agave inulin; and *S. variabile* did not report significant metabolism in corn fibre.

Log-2-transformed fold changes of metabolites discriminating health-promoting bacterial species were analysed for each phylum, separately, to better characterise their metabolic activities in presence of

prebiotic candidates **(Figure 7)**. In response to their nutritional environment, the bacterial activities involved a wide range of differential changes in metabolic profiles. Again, there were no differential metabolites significantly produced in *Firmicutes* species between the initial non-inoculated MM and the inoculated MM without carbohydrate supplementation. On the contrary, in the presence of pectin and inulin, *S. variabile* profiles revealed intense metabolic activities that resulted in significant changes in relative abundances of analytes **(Figure 7A)**. Interestingly, for that strain, the metabolic profile in presence of complex carbohydrates is close to that obtained in presence of glucose **(Figure 7B**). The metabolic productions of *E. rectale* and *R. intestinalis* in the presence of glucose and the three complex carbohydrates were lower than those of *S. variabile,* as shown by the weaker signals on the heatmap **(Figure 7A)**. Furthermore, the profiles of *E. rectale* and of *R. intestinalis* tend to cluster together in the presence of inulin, on the one hand, and corn fibre, on the other. This is in contrast to the profiles in presence of pectin that form a separate cluster, close to the non-supplemented condition **(Figure 7B)**. Results suggested similar metabolic signatures of *Lachnospiraceae* species that differed with the particular signature of *S. variabile*.

In contrast to *Firmicutes* bacteria, the differential analysis revealed that there was a significant production of metabolites in *Bacteroidetes* species between the initial non-inoculated MM and the inoculated MM without carbohydrate supplementation **(Figure 7C)**. This revealed the intense metabolic flexibility of this group, even in a restricted MM condition. With the exception of the similar metabolic patterns of the three *Bacteroidetes* species in presence of corn fibre **(Figure 7D)**, the profiles of *B. intestinalis* tend to be distinguished from those of *B. xylanisolvens* and *B. thetaiotaomicron*. Results reported that the metabolic patterns of *B. intestinalis,* in presence of glucose and inulin, in one hand, and in presence of pectin, in another hand, are distinct from the non-supplemented condition **(Figure 7D)**. Similarly, the profiles of *B. xylanisolvens* in presence of glucose and inulin cluster together and are distinct from the on in presence of non-supplemented condition. Interestingly, the metabolic patterns of *B. xylanisolvens* in the presence of pectin tend to be separated from the other conditions **(Figure 7D)**. Regarding *B. thetaiotaomicron,* the metabolic profiles in presence of pectin, inulin and corn fibre are distinct and scattered from the ones obtained in presence of glucose and non-supplemented condition (that tend to be clustered) **(Figure 7D)**.

Further investigations in the lists of analytes that largely contributed to the differences between carbon sources for each bacterial species could lead to the discovery of a specific health-relevant metabolite associated with a taxon **(Figure 8)**. Taking advantage of the hierarchical framework of metabolites for each bacterial phylum could highlight deeper features in the utilisation of carbohydrate-containing

prebiotics. Though the low degree of confidence in metabolite annotation, the metabolic activities of the *Firmicutes* species revealed the significant production of analytes with putative anti-microbial, anti-inflammatory, and anti-oxidant agents. This analysis highlighted metabolites with health-relevant functions. Common analytes were significantly produced by the two *Lachnospiraceae* species, suggesting similar patterns of metabolic activities. Interestingly, *S. variabile* produced few analytes in high fold changes that could hold promises of health-relevant functions. Similarly, the metabolic activities of the *Bacteroidetes* species revealed the significant production of different analytes in high abundance. The interpretation of the physiological effects of these significant metabolites is limited by the current knowledge of bacterial metabolites. Further investigations are required to identify non-annotated analytes.

Molecular mechanisms of carbohydrate-responding bacteria

Furthermore, metabolic activities was investigated at the molecular scale of four health-relevant bacteria to gain insights into the bacterial gene expression in response to their nutritional environment. Indeed, *R. intestinalis*, *S. variabile, B. thetaiotaomicron* and *B. xylanisolvens* were previously reported to respond significantly to carbon sources at the level of metabolites. Indeed, a transcriptomic approach allows characterising the molecular mechanisms underlying complex carbohydrate metabolism **(Supplementary Figure 5)**.

<u>Note to the jury members:</u> At the time of the first deposit of my thesis manuscript, the samples are still running at the transcriptomic platform. The results of gene expression are not included in this section of my thesis manuscript. However, we have chosen to integrate the transcriptomic experiments into the research article that will be submitted in a peer-reviewed journal.

DISCUSSION

The growth and metabolic activities of gut microbes to ferment a wide variety of dietary carbohydrates into health-relevant metabolites, such as SCFA, plays multiple beneficial effects on host health and wellbeing (Ríos-Covián et al., 2016). Nourishing the gut microbiota through carbohydrate-containing prebiotics constitutes an important basis to maintain and/or restore bacteria of the lower gastrointestinal tract that are essential in the host homeostasis (Cunningham et al., 2021). Their ability to metabolise a complex dietary carbon sources in a dynamic ecosystem defines the fitness and abundance of several bacterial taxa. Therefore, the functional characterisation of the dominant gut bacteria underlying prebiotic metabolisation is a very important basis for the rational development of

nutritional strategies (Scott et al., 2020). Different approaches have given insights into the impact of prebiotics on the gut microbiome composition and functions in terms of carbohydrate utilisation mechanisms and physiological effects (Spacova et al., 2020). In this study, the reduction of the gut microbiome to 17 health-relevant bacterial species offered a low-resolution scale of microbial complexity to elucidate metabolic capacities in terms of individual growth and fermentation profile to utilise five food ingredients. We expect our work will be a benchmark for the utilisation of carbohydrate-containing prebiotics to target health promoting bacteria beyond the *Bifidobacterium* and *Lactobacillus*.

The prediction CAZYme repertoires indicated enzymatic capacities.

Of particular interest, carbohydrate-active enzymes (CAZymes) are required to digest most of the extreme variety of dietary polysaccharides (Cantarel et al., 2009; Lombard et al., 2014). The prediction of enzymatic repertoires shed light on the high number of CAZymes harboured by *Bacteroidetes* species, suggesting flexible metabolic capacities to catabolize a broad range of carbohydrates (Kaoutari et al., 2013). In comparison, *Firmicutes, Actinobacteria and Verrucomicrobia* species exhibited narrower repertoires, suggesting more restricted metabolic capacities to degrade carbohydrates (Cockburn and Koropatkin, 2016). The distribution of CAZymes at the level of bacterial phyla confirmed the significant and important difference between *Bacteroidetes* and *Firmicutes* members, which contain mean numbers of GH and PL genes per genome of 137.1 and 39.6, respectively (Kaoutari et al., 2013). Devoted to carbohydrate metabolism, the abundance of CAZymes in *Actinobacteria* members was reported up to 8% of the Bifidobacterium genomes, which may indicate more versatile carbohydrate utilisation profile (Pokusaeva et al., 2011). Further analysis of the structural and functional diversity within CAZyme repertoires of the panel of health-promoting bacteria might reveal specific enzymatic capacities to metabolise certain carbohydrate-containing prebiotics. Interestingly, the variety of CAZymes per category of families reported that the arsenal of enzymes seemed representative of a simplified human gut microbiome. In particular, high abundances of GH13, GH23, GH3 and GH43 were observed **(data not shown)**. These most representative CAZymes have already been reported to be broadly distributed among the gut microbiome (Abot et al., 2016; Kaoutari et al., 2013). The capture of CAZyme diversity could support a better understand the metabolic roles of health-promoting bacteria. Proportions of CAZymes in bacterial genomes can reflect the enzymatic adaptation to metabolise non-digestible dietary carbohydrates. The different genetic backgrounds of health-promoting bacteria can be determinant factors in metabolic activities.

The metabolic activities revealed different degrees of carbohydrate utilisation.

In this study, the metabolic activities of isolated bacterial species were interpreted based on growth and fermentation parameters. The overall environmental pH of the monocultures partly reflected the SCFA production (Louis et al., 2017). Numerous by- and end-products of fermentation, such acetate, lactate and succinate, are prevalent metabolites that can also alter environmental pH, and thereby, influence microbial growth responses (Duncan et al., 2009; Ríos-Covián et al., 2016).

Related to large repertoires of CAZymes, *Bacteroidetes* species reported flexible nutritional capacities for the utilisation of any given resistant carbohydrates. In microbial ecology, these bacteria are commonly suggested as primary utilisers or "generalists" in the initiation of various and complex dietary carbohydrates degradation (Cockburn and Koropatkin, 2016). They were distinguishable by their fermentation activities, resulting in the production of acetate and propionate. Their carbohydrate breakdown products and metabolites, such as acetate and succinate, may facilitate the coexistence of many species that benefit these secondary utilisers in crossfeeding interactions, sustaining key members of the intestinal ecosystem (Wrzosek et al., 2013; Rivière et al., 2018). Nevertheless, according to their nutritional environment, a switch in metabolic outcomes could drive the bacterial dynamism towards competitive mechanisms for *Bacteroidetes* to access the energy present in dietary carbohydrates (Grondin et al., 2017).

Similar crossfeeding interactions have been reported for *Bifidobacterium* strains specializing in the degradation of different carbon substrates (Milani et al., 2015). In relatively low abundance, these bacteria can exert a strong influence on their communities (Banerjee et al., 2018). Described as keystone mutualistic microorganisms, *Bifidobacterium* species have generally been associated with the preferential utilisation of inulin-type fructans (ITF) carbohydrates. By releasing mono- or oligosaccharides or fermentation end-products, they are potentially involved in the cross-feed of other microorganisms (Moens et al., 2016).

Firmicutes species displayed more variable activities to grow and ferment complex polysaccharides. They may be more adapted to utilise a narrower range of complex glycans according to nutritional specialization (Cockburn and Koropatkin, 2016). Most of the *Firmicutes* species were distinguishable by their fermentation activities, resulting in the production of acetate and butyrate. In particular, most of the *Ruminococcaceae* members showed poor growth and fermentation activities on the positive MM-glucose control condition, with the exception of *Subdoligranulum variabile*. Indeed, the dominant colonic commensal microbes *F. prausnitzii*, *B. pullicaecorum,* and *R. bromii* differed in their ability to grow and ferment glucose. The MM composition might not be fully adapted for the culture of these

bacteria. Previous studies have investigated the metabolic activities of *F. prausnitzii* and *R. bromii* in richer medium cultures supplemented with various carbon sources (Tanno et al., 2021; Ze et al., 2012). As glucose is not a prominent carbon and energy source for bacteria in the colon, we suggest that these bacteria cannot grow on glucose due to poor adaptation to this carbon source in the colonic environment (McLaughlin et al., 2015). Although it makes difficult to interrogate the effects of carbohydrates, these results do not call into question the experimental strategy used to evaluate the prebiotic potential of food ingredients. The *Lachnospiraceae* members showed diverse capacities to utilise diet-derived carbohydrates. Consistent with a previous study, *E. rectale* was not able to utilise the agave inulin source (Tanno et al., 2021).

The absence of growth and fermentation activities in prebiotic conditions and the differences in carbohydrate utilisation capacities may be largely influenced by the nature of the available carbon source (Sonnenburg and Sonnenburg, 2014). The physicochemical properties of the components may also explain the inability of these bacteria to grow and ferment prebiotic candidates, such as agave inulin. The slight growth and fermentation activities recorded for some strains using these dietary carbohydrates could be due to the consumption of contaminants of lower molecular weight rather than dietary fibre itself (McLaughlin et al., 2015). Furthermore, bacterial metabolisms are expected to vary substantially between species and strain (Sheridan et al., 2016). Carbohydrate fermentation have been shown variable among strains tested of the same species for both genera (McLaughlin et al., 2015; Patnode et al., 2021).

Interestingly, not all carbohydrate substrates were equally capable of stimulating SCFA production in *Bacteroidetes* species. In particular, their fermentation activities appeared more related to acetate production on simple monosaccharides such as glucose. These latter appeared to be highly efficient for the degradation of complex dietary carbohydrates resulting in high concentrations of propionate. These observations are consistent with previous studies reporting that *Bacteroides species* show high flexibility to adapt to the nutritional conditions of the intestinal environment (Martens et al., 2011).

Significant responses to carbohydrate-containing prebiotics at the levels of metabolites.

The gut microbiome contributes to host physiology through the production of a myriad of metabolites. These metabolites exert their effects within the host as signalling molecules and substrates for metabolic reactions (Krautkramer et al., 2021). Beyond the production of SCFAs, little is known about the metabolites generated by the six key intestinal bacteria, certain of which may be beneficial for human physiology. The identification of specific microbial molecules may underlie physiological effects

on host, which can be interesting for further translation to clinical practice and/or interventional strategies (Clavel et al., 2022).

Untargeted LC-MS data analysis led to the statistical classification of metabolic features that successfully discriminated *Firmicutes* and *Bacteroidetes* species. Indeed, the advantage of PLS approaches is that components maximize phenotype covariance, which facilitates straightforward dimension reduction (Rohart et al., 2017). Interestingly, the phylogenetic proximity of *Bacteroidetes* species distinguishes different metabolomic profiles of prebiotic fermentation. Closely related species can utilise distinct resources and exhibit differences in metabolic profiles (Han et al., 2021). The metabolomic profiles of each bacteria reflect the adaptation to high concentrations of complex polysaccharides in the medium. Net environmental impact on each isolated species reported depleted and secreted metabolic features for specific carbon resources. Consistent with our previous results, the activities of the *Bacteroidetes* phylum seemed to be particularly stimulated compared to those of the *Firmicutes* phylum. It is likely in correlation with the expression of their respective CAZyme-encoding gene repertoires. In particular, the production of microbial metabolites is driven by a combination of dietary substrate availability (Gill et al., 2021).

Importantly, computational metabolite annotation remains a major bottleneck in untargeted LC-MS metabolomics (Han et al., 2021; Verdegem et al., 2016). Much work remains to identify unknown metabolic features and characterize the physiological effects of microbial metabolites that are important in human health. Recently, a construction of the MS pipeline aimed to accelerate the identification of microbiota-derived metabolites for the interrogation of mechanisms of the gut microbiome (Han et al., 2021). The annotated metabolites reported for each health-promoting bacteria provide information to further clarify the basic biological mechanisms underlying the prebiotic effects. Hierarchical clustering of the metabolites utilisation by each health-key intestinal bacteria can highlight the presence of complex correlation structures resulting from same underlying biological processes (Han et al., 2021). However, the clustering pattern may not recapitulate the metabolic pathways relationships in many cases, demonstrating the complexity of resource utilisation.

The diversity in carbohydrate utilisation capacities suggested varying adaptation of nutrient acquisition strategies differing at the species level. A previous study revealed that different prebiotic impacts were observed at the strain level for isolated bifidobacteria and lactobacilli species (McLaughlin et al., 2015; Patnode et al., 2021). Understanding these differences is essential for the application of nutritional strategies.

The metabolite changes of health-promoting bacteria can reveal their ability to adhere and degrade different forms of dietary ingredients. Other bacterial species might require cooperation for enzymatic hydrolysis to degrade and ferment prebiotic compounds (Rivière et al., 2016). An integrative approach of omics data at multiple levels, including genome, transcriptome, and metabolome, can yield better understanding and clearer picture of the complex interactions between prebiotics, the gut microbiome, and the host. Promising tools and methods have been developed for data integration and interpretation to highlight the relationships of the involved biomolecules and their functions (Subramanian et al., 2020). One perspective of this study is to integrate the genomic, transcriptomic, and metabolomic data sets available for the six health-promoting bacteria grown on the five food ingredients. This integrative analysis can depict a better comprehension of the underlying molecular processes of prebiotic functionality, which can mediate health effects on the host physiology.

The most promising food ingredients with prebiotic effects

Non-digestible food components that have the ability to selectively promote the growth and metabolism of commensal gut bacteria are defined as prebiotics. In regards to the growth and fermentation results, agave inulin and corn fibre are the most promising food ingredients to display prebiotic effects. Based on the metabolomics data, our statistical approach can allow further investigation of the metabolic signatures for the identification of biomarkers characterizing a prebiotic substrate. In prebiotic practice, the application of such biomarkers could be foreseen in dietary intervention and nutrition. These results provided metabolic biomarkers that help to understand the biological differences between prebiotic candidates. The application of metabolomics to identify biomarkers characterizing different prebiotics is an interesting first approach to understand prebiotic effects of food ingredients.

CONCLUSION

Health-promoting bacteria revealed different degrees of carbohydrate utilisation in response to diverse carbon sources. Carbohydrate-containing prebiotic metabolisation seemed driven by the phylogenetic affiliation of key intestinal microorganisms. In particular, generalist *Bacteroidetes* species were able to degrade a wide range of polysaccharides compared to more specialist *Firmicutes* species that were only able to utilise certain carbon sources. Metabolomics provided holistic insight in the bacterial responses to describe underlying mechanisms of carbohydrate metabolism induced at species levels. Identifying of specific microbial molecules can provide information on prebiotic effects that confer physiological benefits to the host. However, such bacterial-derived metabolites that influence host physiology with

local or additional systemic effects require further clinical translation. Indeed, the prebiotic effects of *in vitro* monoculture experiments may not translate into *in vivo* effects, as many factors can influence the ability of prebiotics to support beneficial changes. Understanding carbohydrate utilisation capacities in a reductionist approach is the first step in rational formulation of functional foods that include prebiotics.

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AUTHOR CONTRIBUTIONS

CBF, PB, SV and FP performed the experiments. MA and VM contributed to the metagenomic analyses. CC and PL conceived and supervised the project. CBF wrote the first draft of the manuscript. CC and PL corrected the manuscript.

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CONFLICT OF INTEREST

The authors declare that CBF has worked as an employee of Yoplait France – General Mills during the conduct of this study, as part of a CIFRE contract. The company had no role in the design, collection, analysis, or interpretation of data, the writing of this article, or the decision to submit it for publication. The other authors declare no competing interests.

FIGURES & TABLES

Phylum (Family)	*Bacterial Species*	International Collection	Strain Designation	RefSeq Assembly Accession	Assembly Level	Total Length (kb)	CDS
Actinobacteria (Bifidobacteriaceae)	*Bifidobacterium adolescentis*	DSM 20083T	E194an (variant a)	GCF_000010425.1	complete	2.1	1676
	Bifidobacterium catenulatum	DSM 16992T	B669	GCF_000173455.1	contig	2.1	1677
Bacteroidetes (Bacteroidaceae)	*Bacteroides fragilis*	DSM 2151T	EN-2	GCF_000025985.1	complete	5.2	4544
	Bacteroides intestinalis	DSM 17393T	341	GCF_000172175.1	contig	6.1	4655
	Bacteroides thetaiotaomicron	DSM 2079T	E50 (VPI-5482)	GCF_000011065.1	complete	6.3	4794
	Bacteroides xylanisolvens	DSM 18836T	XB1A	GCF_015668785.1	scaffold	6.1	4621
Firmicutes (Lachnospiraceae)	*Agathobacter rectalis* Syn. *Eubacterium rectale*	ATCC 33656T	VPI-0990	GCF_000020605.1	complete	3.4	3288
	Anaerobutyricum hallii Syn. *Eubacterium hallii*	DSM 17630	L2-7	GCF_900209925.1_EH1	complete	3.7	3076
	Anaerostipes caccae	DSM 14662T	L1-92	GCF_000154305.1	scaffold	3.6	3317
	Blautia hansenii	DSM 20583T	VPI-C7-24	GCF_002222595.2	complete	3.1	2974
	Roseburia intestinalis	DSM 14610T	L1-82	GCF_000156535.1	scaffold	4.4	4267
	Roseburia inulinivorans	DSM 16841T	A2-194	GCF_000174195.1	contig	4.0	3926
Firmicutes (Ruminococcaceae)	*Butyricicoccus pullicaecorum*	DSM 23266T	25-3	GCF_900167005.1	scaffold	3.3	3103
	Faecalibacterium prausnitzii	DSM 17677	A2-165	GCF_002734145.1	complete	3.1	2956
	Ruminococcus bromii	ATCC 27255	VPI 6883	GCF_002834225.1	contig	2.1	1998
	Subdoligranulum variabile	DSM 15176T	BI 114	GCF_000157955.1	scaffold	3.2	3040
Verrucomicrobia (Akkermansiaceae)	*Akkermansia muciniphila*	ATCC BAA-835T	Muc	GCF_000020225.1	complete	2.7	2257

T stands for "Type" bacterial strain.

Table I: Genomic features of bacterial strains used in the present study. *All strains are human isolates except Butyricoccus pullicaecorum.*

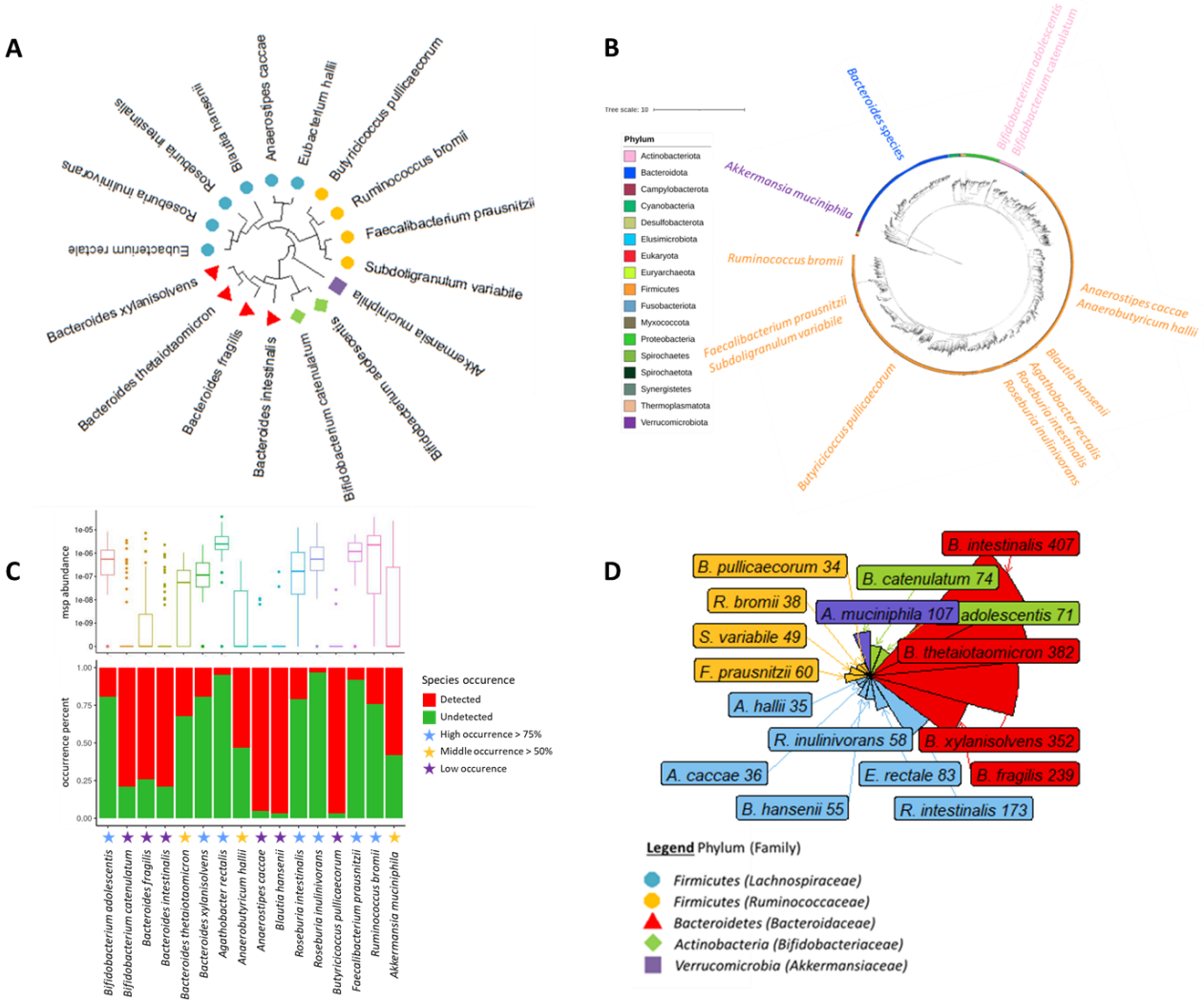


Figure 1: Selection of bacterial species for evaluating their metabolic capacities towards carbohydrate-containing prebiotics. (A) Phylogenetic relationships based on 16S rRNA genes. *The panel of bacterial species represents the four major phyla of the human gut microbiome, including Actinobacteria, Bacteroidetes, Firmicutes, and Verrucomicrobia. This unrooted phylogenetic tree was inferred using the neighbor-joining method computed with the maximum composite likehood approach. The bacterial phyla and families are indicated in the squared legend.* ***(B) Phylogenetic tree representing the diversity of the human intestinal microbiota.*** *Each bacterial species is placed in the context of the overall diversity of the gut ecosystem.* ***(C) Abundance and occurrence of each health-relevant bacteria in human metagenomic data.*** *The occurrence (in %) of the panel of health-relevant bacteria suggested these species are present in human gut microbiome and abundant to various degrees in metagenomic data prior a Mediterranean diet intervention. The abundance values were transformed in log10 for visualisation.* ***(D) Characterisation of enzymatic repertoires for carbohydrate metabolism.*** *The distribution of Carbohydrate-Active enZymes (CAZymes) was estimated in the genomes of corresponding health-promoting bacteria.*

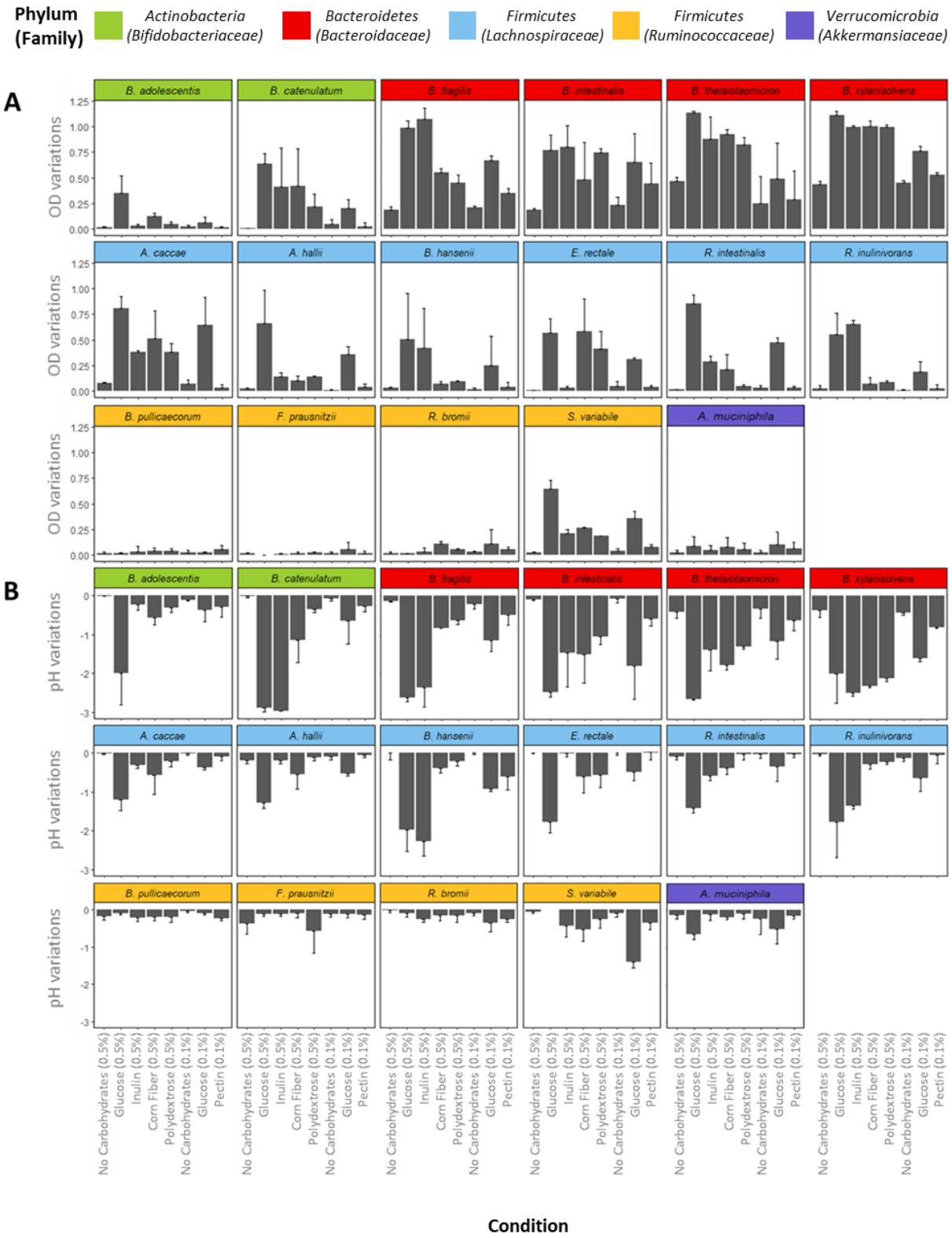


Figure 2: Metabolic capacities of putative health-promoting bacteria in the presence of various carbohydrate substrates. *Diverse carbohydrate sources were added to a minimal medium at 0.5% (w/v) or 0.1% (w/v) according to the solubility and the viscosity of food ingredients.* ***(A) Variations of optical density (ΔOD) reflect bacterial growth after 24h of culture. (B) Variations of pH (ΔpH) reveal the medium acidification after 24h of culture.*** *The acidification of the pH culture medium is a consequence of the production of SCFA, mainly acetate, propionate and butyrate.*

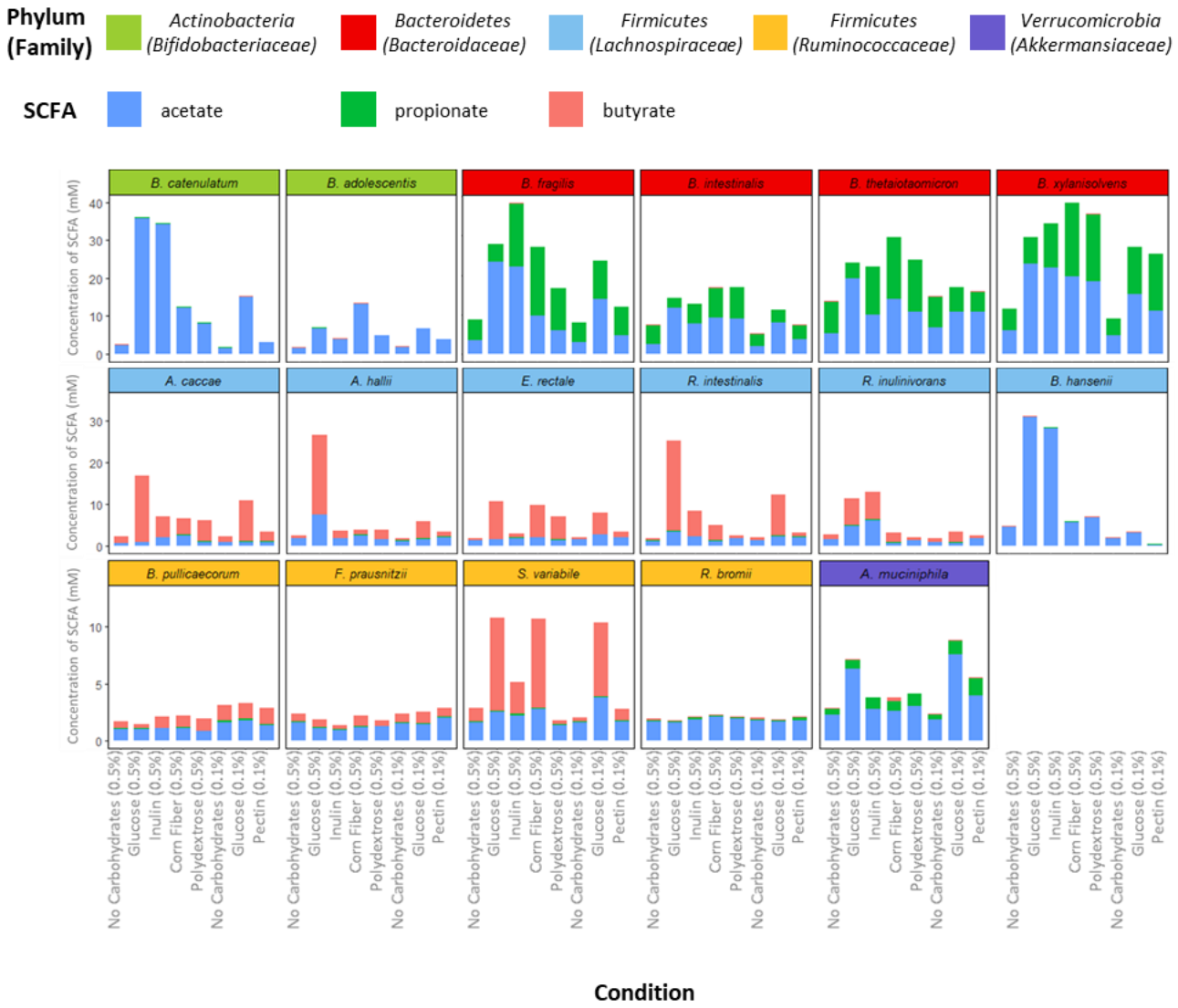


Figure 3: Fermentation profiles based on short-chain fatty acids produced by health-promoting bacteria. *The SCFA concentrations are expressed in mM. The bar plots represent the overall mean of acetate, propionate and butyrate.*

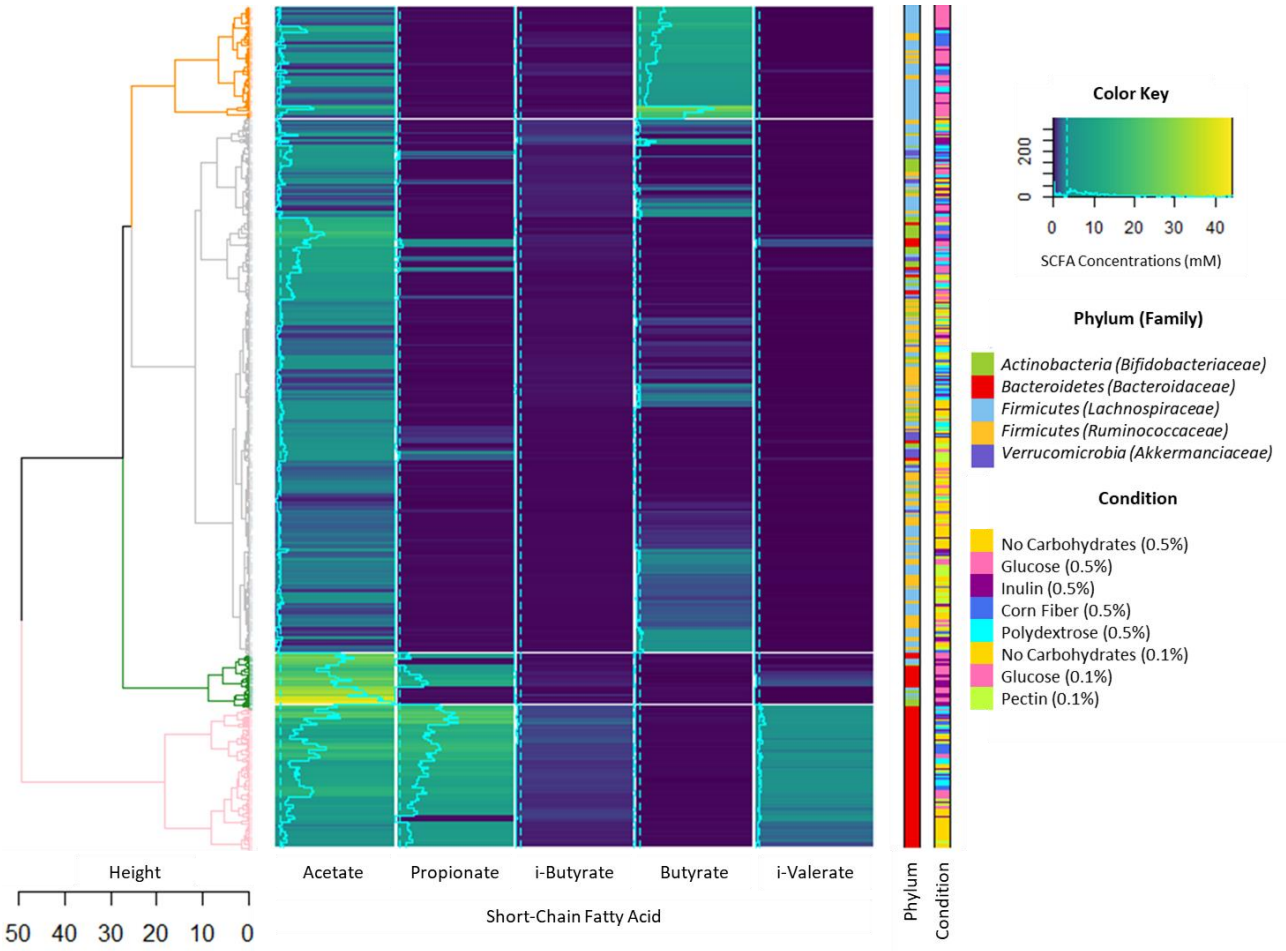


Figure 4: Classification of carbohydrate fermentation profiles of health-promoting bacteria. *The dendrogram contains all the bacterial monocultures classified according to their fermentation profile. Four groups highlight the functional capacities of health-promoting bacteria to ferment diverse carbohydrate substrates. The blue shade represents short-chain fatty acids (SCFAs) concentrations expressed in mM. The dotted lines represent the overall mean of total SCFAs (with the exception of valerate, i-caproate, and caproate for which the concentrations were null). The straight lines indicate the values of SCFAs for each bacterial culture.*

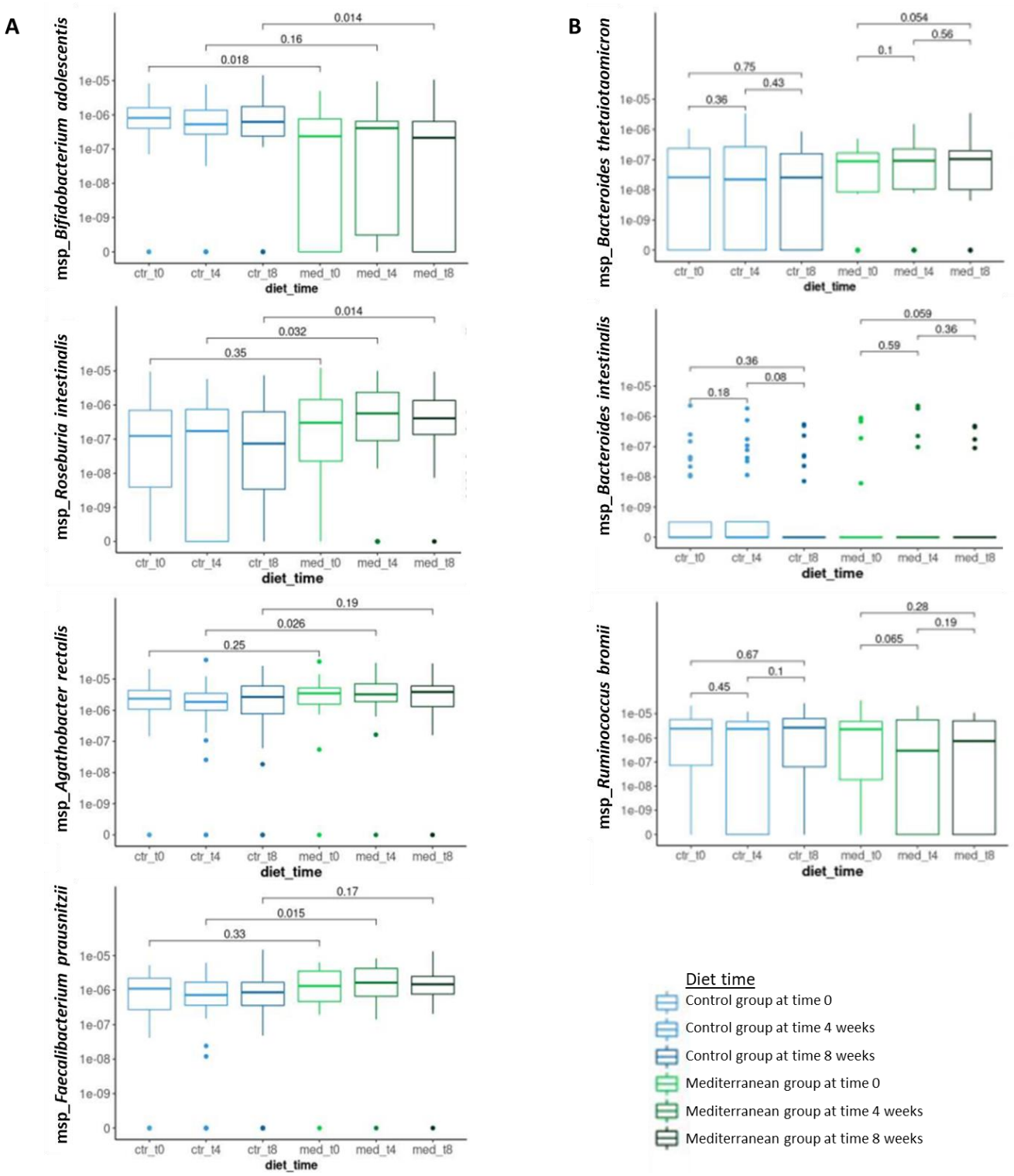


Figure 5: Bacterial responders to a Mediterranean diet in a human cohort. *Statistical analyses were performed using paired Wilcoxon tests with a significant threshold set at p-value ≤ 0.05.* ***(A) Health-relevant bacteria that were significantly more abundant in response to a rich plant-based diet.*** *R. intestinalis, E. rectale, F. prausnitzii and B. adolescentis were increased in response to high intakes of complex carbohydrates within gut microbiomes of control vs treated individuals.* ***(B) Health-relevant bacteria that showed a tendency to increase in response to a rich plant-based diet.*** *B. thetaiotaomicron, B. intestinalis and R. bromii reported a tendency to be more abundant in response to high intakes of complex carbohydrates within gut microbiome of treated individuals at different time points of the intervention.*

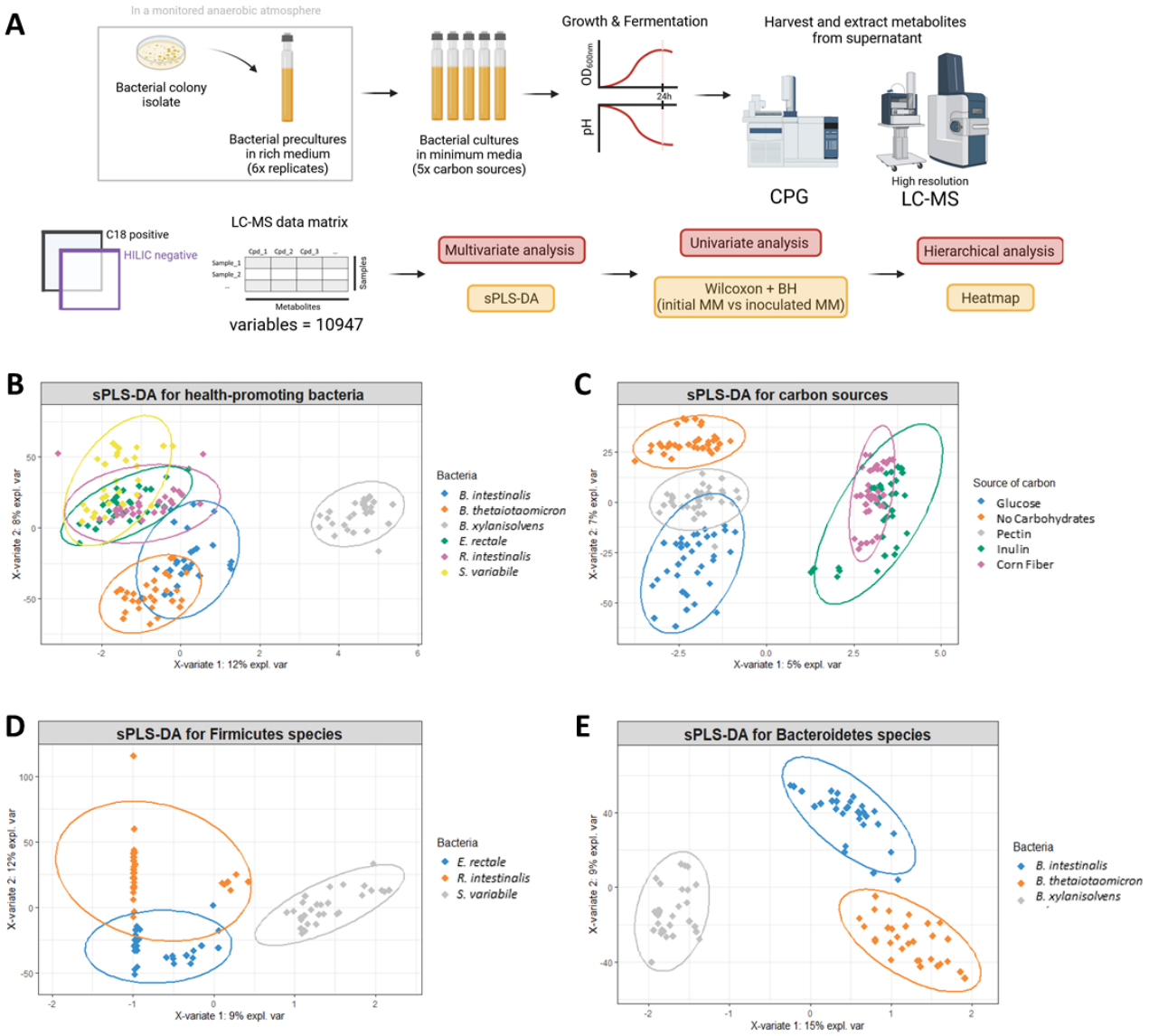


Figure 6: Metabolomic profiles highlighted the relationships between the carbon sources and the health-promoting bacteria. (A) Schematic of the experimental strategy and the untargeted metabolomic data analysis. *The metabolomic study was conducted on supernatants of six health-promoting bacteria, belonging to two different bacterial phyla including Bacteroidetes (B. intestinalis, B. thetaiotaomicron and B. xylanisolvens) and Firmicutes (E. rectale, R. intestinalis and S. variabile). These bacterial species were cultivated in five minimum media (MM), each supplemented or not with different carbon sources. Each condition was performed in 6 replicates, in addition to 5 replicates of the initial non-inoculated media.* ***(B) Scatter plot of the first two sPLS-DA components obtained for the panel of health-promoting bacteria.*** *Samples are projected into the space spanned by the first eight components. Samples are coloured by bacterial species.* ***(C) Scatter plot of the first two sPLS-DA components obtained for the five different carbon sources.*** *Samples are projected into the space spanned by the first eight components. Samples are coloured by carbon source.* ***D) Scatter plot of the first two sPLS-DA components obtained for Firmicutes species.*** *Samples are projected into the space spanned by the first eight components. Samples are coloured by bacterial species.* ***(E) Scatter plot of the first two sPLS-DA components obtained for Bacteroidetes species.*** *Samples are projected into the space spanned by the first eight components. Samples are coloured by bacterial species. All ellipses were drawn assuming a multivariate t-distribution with a confidence level of 0.95.*

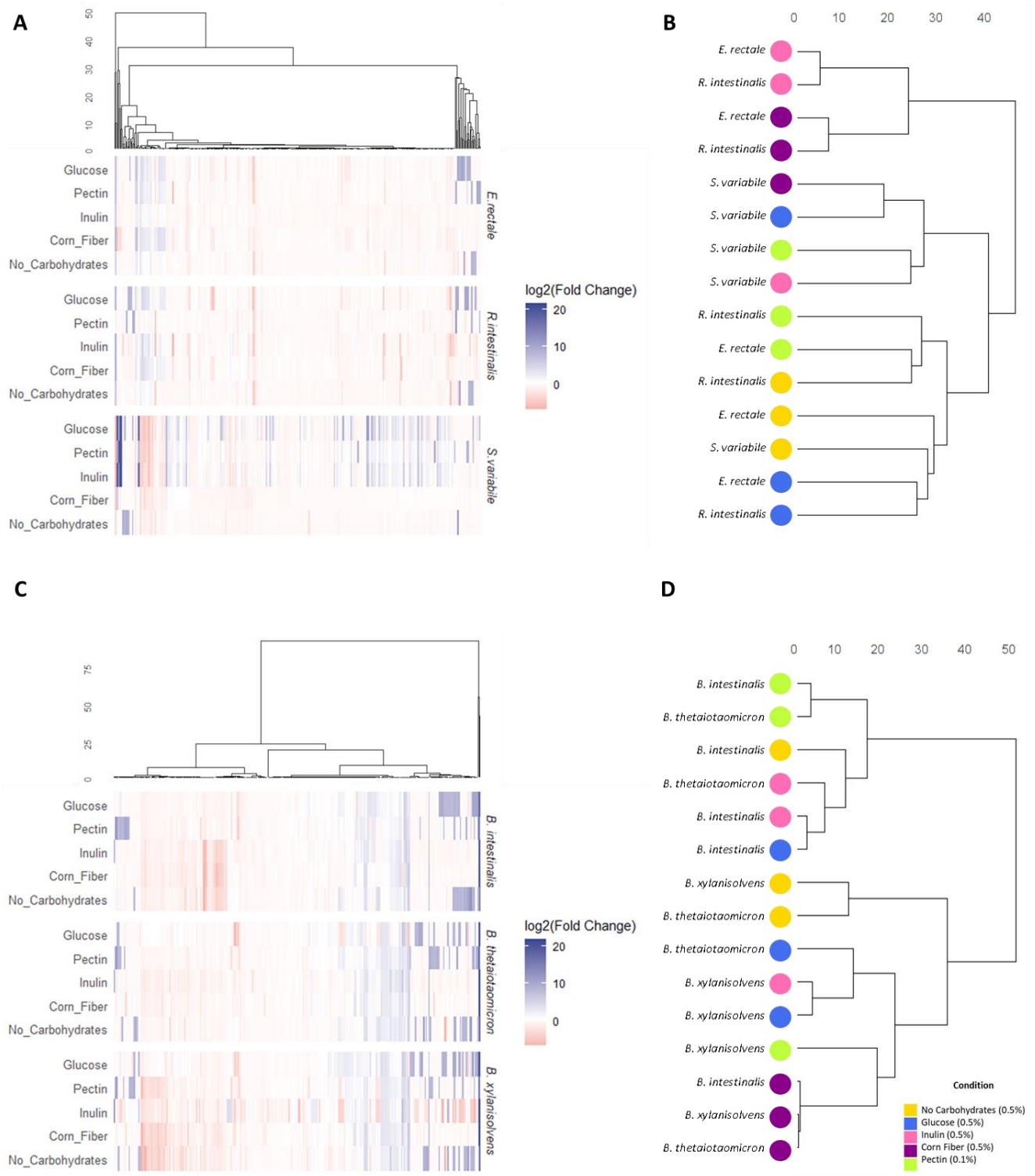


Figure 7: Metabolic profiles of Firmicutes and Bacteroidetes in response to dietary carbohydrates.

Differential analysis between conditions of non-inoculated versus cultivated medium revealed fold changes of analytes for each carbon source and each bacterium. Heatmaps depicted shows the log2-transformed fold changes of analytes that discriminate the metabolic patterns of each species of Firmicutes ***(A)*** *and each species of Bacteroidetes* ***(C)****, separately. Dendrograms organised each Firmicutes species* ***(B)*** *and each Bacteroidetes species* ***(D)*** *according to their metabolic profiles.*

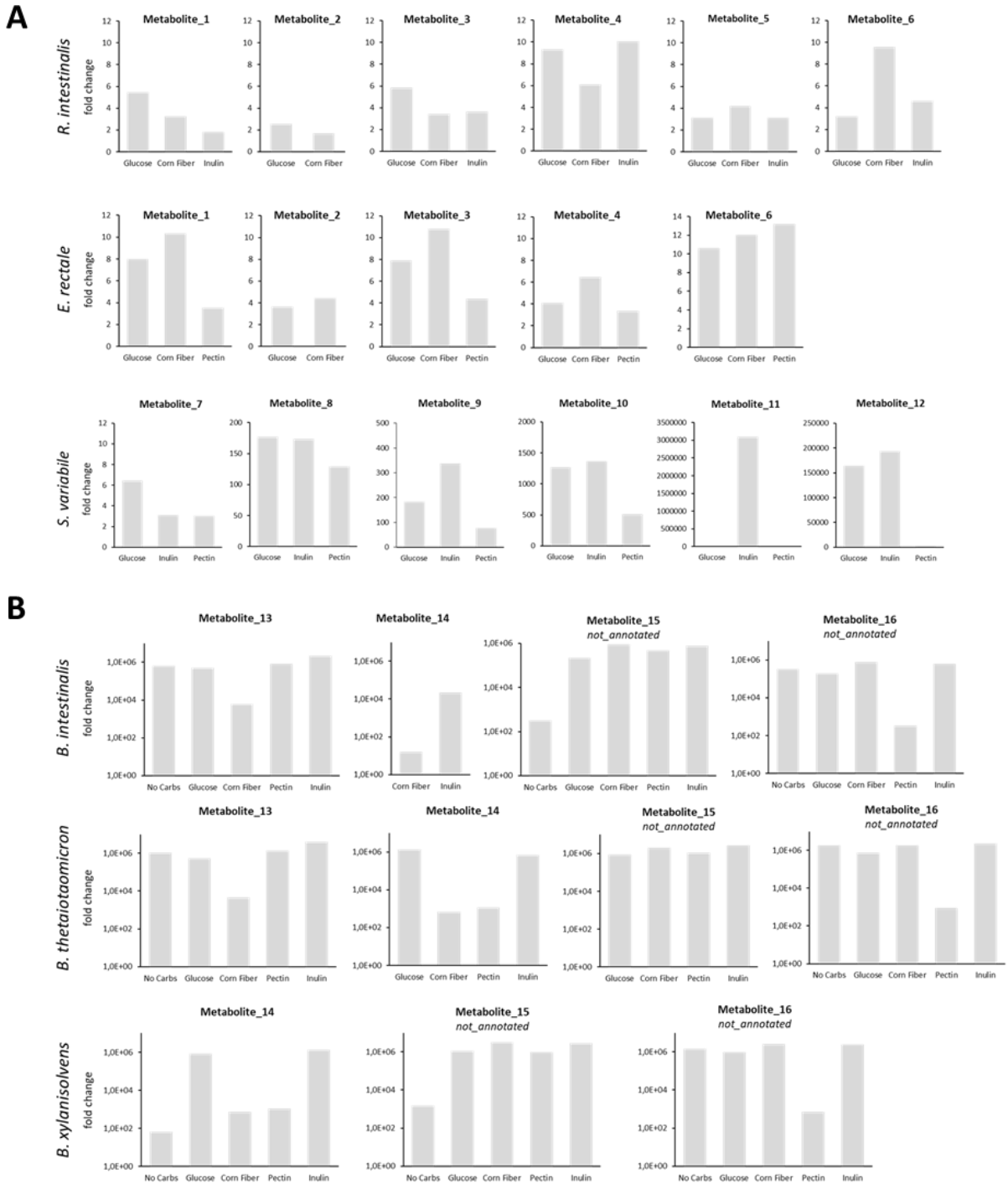
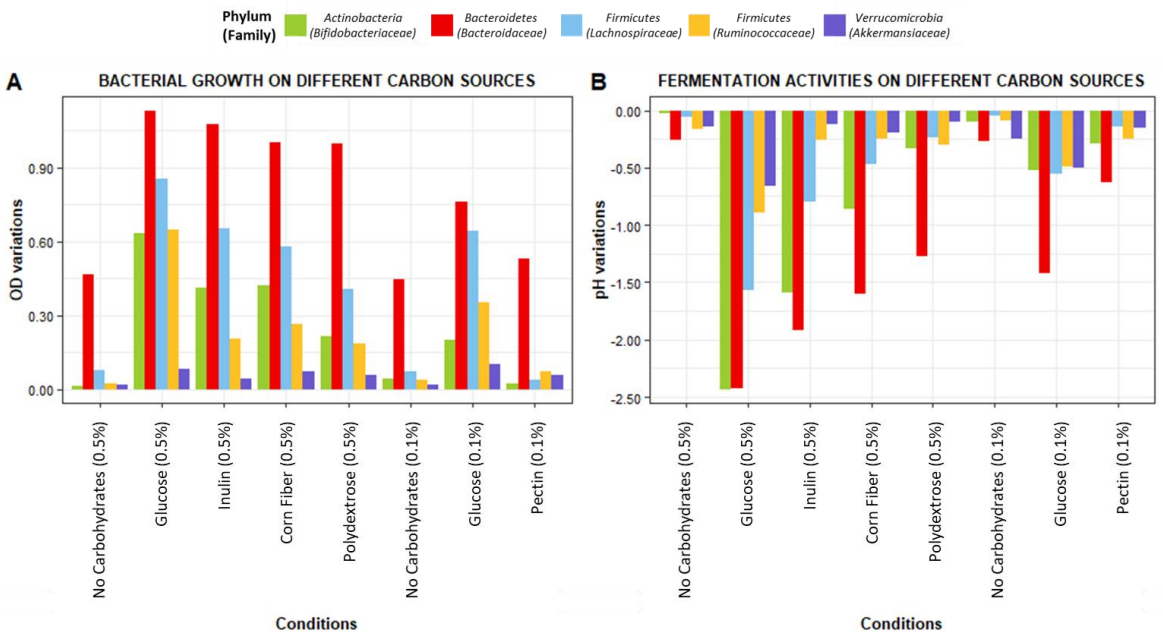


Figure 8: Significant production of analytes with putative effects by each commensal bacteria. *Differential analysis between conditions of non-inoculated versus cultivated medium revealed fold changes of analytes for each carbon source and each bacterium. This figure includes analytes that discriminate the metabolic patterns of each species of Firmicutes and each species of Bacteroidetes, separately. This figure includes conditions associated to analytes that have significant fold changes. Annotated metabolites were associated with putative effects recorded in the human metabolome database (HMDB) (https://hmdb.ca/).* ***(A) Discriminant metabolites produced by Firmicutes species.*** *Twelve metabolites would be linked to anti-microbial, anti-inflammatory and anti-oxidant properties.* ***(B) Discriminant metabolites produced by Bacteroidetes species*** *Two metabolites remained not annotated. Two metabolites would be linked to physiological effects. NB: For a better visualisation of the fold change values, the axis was expressed in log10.*

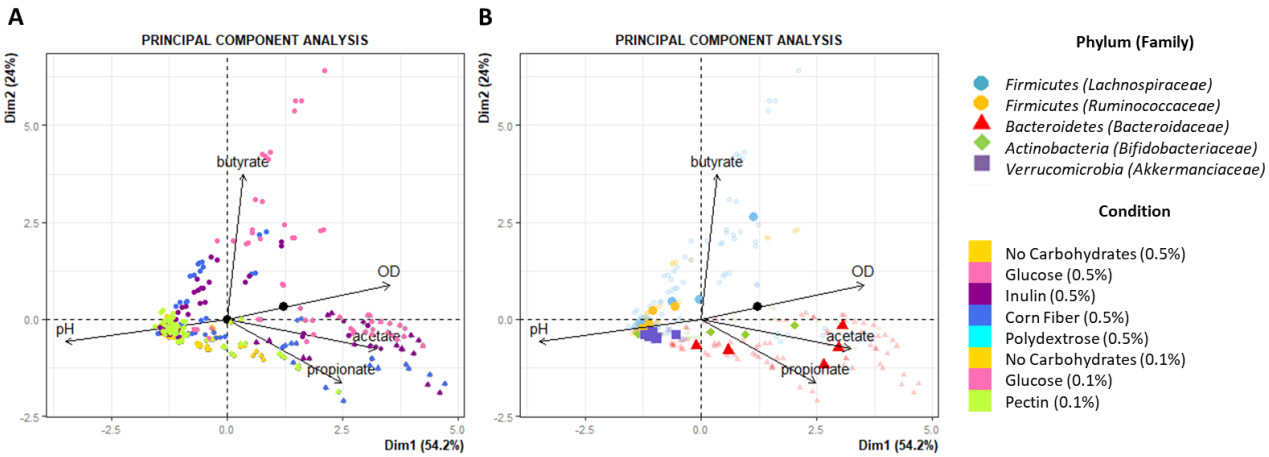
SUPPLEMENTARY DATA

Supplementary Table I: Physicochemical properties of carbohydrates with potential prebiotic effects used in this study. *Initially, psyllium husk was another food ingredient included in this study. Nevertheless, this compound proved to be poor substrate for the growth and the fermentation activities of most of health-promoting bacteria (results not shown). We excluded this food ingredient from our analysis.*

FOOD INGREDIENTS	PHYSICOCHEMICAL PROPERTIES
Agave Inulin	Powdered **long-chain inulin** sourced from the Agave plant, containing **fructose polymers** and minor amounts (< 12% dry weight) of bound fructose, glucose and sucrose. Inulin is extracted and purified without the use of enzymes or processing aids (> 90% dry weight).
Corn Fiber	Maize-derived source of dietary fibers composed of a mixture of **glucose polymers** and insoluble non-digestible carbohydrates (>85% dry weight), which was obtained from a partial hydrolysed starch made glucose syrup. This food ingredient may contain minor amounts of monosaccharides (< 15% dry weight)
Polydextrose	PDX is a mixture of synthetic highly branched and randomly bonded **polymers of glucose** (>90% dry weight), containing minor amounts (< 2% dry weight) of bound sorbitol and citric acid.
Citrus Pectin	From citrus peel by-products (from the juice and citrus-oil processing industries), low-ester pectin standardized with sugars (dextrose).



Supplementary Figure 1: Metabolic capacities of health-promoting bacteria at their phylum level in presence of diverse carbohydrate substrates. *Diverse carbohydrate sources were added to a minimal medium at 0.5% (w/v) or 0.1% (w/v) according to the solubility and the viscosity of food ingredients. Growth and medium acidification indicated high metabolic activity of bacteria.* ***(A) Variations of optical density (ΔOD) reflect the bacterial growth after 24h of culture. (B) Variations of pH (ΔpH) reveal the medium acidification after 24h of culture.*** *The acidification of the pH culture medium is a consequence of the production of SCFAs, mainly acetate, propionate and butyrate*



Supplementary Figure 2: Principal component analysis (PCA) of health-promoting bacteria based on growth and fermentation parameters, including OD, pH and SCFAs. *In both PCAs, the OD and pH variables are inversely correlated. It suggests that the bacterial growth is associated with the acidification of the pH culture medium, as a consequence of the production of short-chain fatty acids (SCFAs), mainly acetate, propionate and butyrate.* ***(A) PCA of health-promoting bacteria in response to different carbon sources.*** *Acetate, butyrate and propionate variables seem strongly correlated to the culture conditions of glucose, inulin and corn fibre. Propionate appears also correlated to the pectin condition.* ***(B) PCA of health-promoting bacteria in accordance with their phylogenetic affiliation.*** *Acetate and propionate variables seem particularly correlated to the Bacteroidetes and Actinobacteria phyla. Butyrate variable appears associated with Firmicutes phylum.*

Supplementary Table III: Statistical description of the four functional clusters. *These results suggested functional hierarchisation of the metabolic interactions between the dietary carbohydrates and the health-promoting bacteria.* ***(A) Statistical analysis of the clusters and the explicative variables that include bacteria, phyla and conditions.*** *Chi² tests are performed. The explicative variables are classified according to their decreasing statistical significance.* ***(B) Statistical analysis of the clusters and the quantitative variables that include acetate, propionate, isobutyrate, butyrate and isovalerate.*** *ANOVA tests are performed on each quantitative variable with each cluster as the explicative variable. The quantitative variables are classified according to their decreasing statistical significance.* ***(C) Description of each cluster based on the explicative variables.*** *Each bacteria, phyla and conditions are represented in different proportions according to each group.* ***(D) Description of each cluster based on the quantitative variables.*** *The mean and standard deviation of each quantitative variables are reported for each cluster in comparison with the overall data set. T-Student tests are performed to compare the mean of each cluster and the mean of the overall data set.*

A

	p-value	DF
Bacteria	1.86e-103	48
Phylum	1.89e-95	12
Condition	6.18e-33	21

B

	p-value
Isovalerate	3.54e-242
Propionate	8.45e-147
Acetate	1.64e-135
Butyrate	9.99e-122
Isobutyrate	4.35e-105

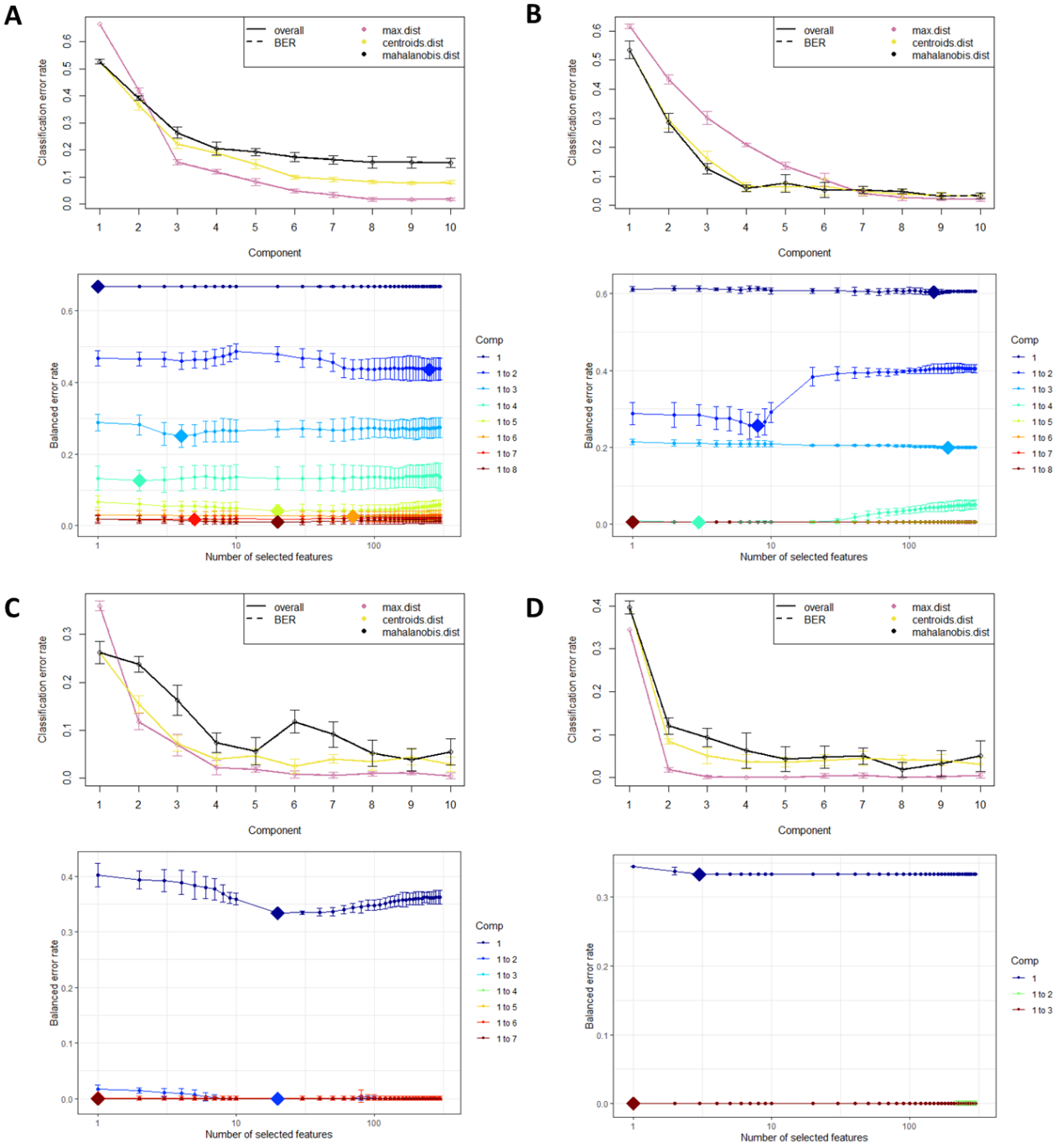
C

		Cla/Mod	p-value
Cluster N	Phylum=*Firmicutes_Ruminococcaceae*	91	6.89e-15
	Phylum=*Verrucomicrobia*	100	2.97e-07
	Bacteria=*R.bromii*	100	2.97e-07
	Bacteria=*F.prausnitzii*	100	2.97e-07
	Bacteria=*B.pullicaecorum*	100	2.97e-07
	Bacteria=*B.adolescentis*	100	2.97e-07
	Bacteria=*A.muciniphila*	100	2.97e-07
	Phylum=*Actinobacteria*	88	7.56e-06
	Condition=Pectin_0.1%	82	3.76e-04
	Condition=No_Carbohydrates_0.1%	82	3.76e-04
	Condition=No_Carbohydrates_0.5%	76	1.68e-02
	Bacteria=*A.caccae*	47	4.95e-02
	Condition=Glucose_0.1%	50	1.48e-02
	Bacteria=*B.intestinalis*	38	2.31e-03
	Condition=Glucose_0.5%	38	6.25e-06
	Bacteria=*B.thetaiotaomicron*	19	1.26e-07
	Bacteria=*B.xylanisolvens*	0	1.61e-15
	Bacteria=*B.fragilis*	0	1.61e-15
	Phylum=*Bacteroidetes*	14	1.15e-40
Cluster G	Phylum=*Firmicutes_Lachnospiraceae*	31	2.53e-19
	Bacteria=*A.caccae*	53	2.50e-08
	Bacteria=*E.rectale*	50	2.23e-07
	Condition=Glucose_0.5%	32	1.01e-05
	Condition=Glucose_0.1%	32	1.01e-05
	Bacteria=*S.variabile*	38	3.64e-04
	Bacteria=*R.intestinalis*	38	3.64e-04
	Phylum=*Verrucomicrobia*	0	9.20e-03
	Bacteria=*R.bromii*	0	9.20e-03
	Bacteria=*F.prausnitzii*	0	9.20e-03
	Bacteria=*B.xylanisolvens*	0	9.20e-03
	Bacteria=*B.thetaiotaomicron*	0	9.20e-03
	Bacteria=*B.pullicaecorum*	0	9.20e-03
	Bacteria=*B.intestinalis*	0	9.20e-03
	Bacteria=*B.hansenii*	0	9.20e-03
	Bacteria=*B.fragilis*	0	9.20e-03
	Bacteria=*B.catenulatum*	0	9.20e-03
	Bacteria=*B.adolescentis*	0	9.20e-03
	Bacteria=*A.muciniphila*	0	9.20e-03
	Phylum=*Actinobacteria*	0	6.11e-05
	Condition=Pectin_0.1%	0	3.18e-05
	Condition=No_Carbohydrates_0.5%	0	3.18e-05
	Condition=No_Carbohydrates_0.1%	0	3.18e-05
	Phylum=*Bacteroidetes*	0	8.17e-10

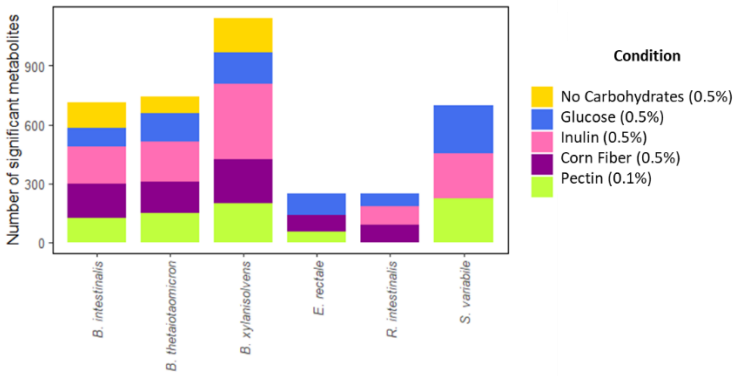
		Cla/Mod	p-value
Cluster A	Phylum=*Bacteroidetes*	72	7.71e-75
	Bacteria=*B.fragilis*	81	1.33e-16
	Bacteria=*B.xylanisolvens*	75	6.97e-14
	Bacteria=*B.thetaiotaomicron*	69	1.81e-11
	Bacteria=*D.intestinalis*	63	2.54e-09
	Phylum=*Verrucomicrobia*	0	2.19e-03
	Bacteria=*S.variabile*	0	2.19e-03
	Bacteria=*R.inulinivorans*	0	2.19e-03
	Bacteria=*R.intestinalis*	0	2.19e-03
	Bacteria=*R.bromii*	0	2.19e-03
	Bacteria=*F.prausnitzii*	0	2.19e-03
	Bacteria=*E.rectale*	0	2.19e-03
	Bacteria=*B.pullicaecorum*	0	2.19e-03
	Bacteria=*B.hansenii*	0	2.19e-03
	Bacteria=*B.catenulatum*	0	2.19e-03
	Bacteria=*B.adolescentis*	0	2.19e-03
	Bacteria=*A.muciniphila*	0	2.19e-03
	Bacteria=*A.hallii*	0	2.19e-03
	Bacteria=*A.caccae*	0	2.19e-03
	Phylum=*Actinobacteria*	0	3.10e-06
	Condition=Glucose_0.5%	0	1.32e-06
	Phylum=*Firmicutes_Ruminococcaceae*	0	1.25e-12
	Phylum=*Firmicutes_Lachnospiraceae*	0	2.97e-20
Cluster G/I	Condition=Glucose_0.5%	29	2.26e-11
	Condition=Inulin_0.5%	21	1.36e-05
	Phylum=*Bacteroidetes*	14	1.42e-04
	Bacteria=*B.xylanisolvens*	25	4.12e-04
	Bacteria=*B.hansenii*	25	4.12e-04
	Bacteria=*B.catenulatum*	25	4.12e-04
	Bacteria=*B.fragilis*	19	1.27e-02
	Phylum=*Actinobacteria*	13	4.68e-02
	Condition=Polydextrose_0.5%	0	9.15e-03
	Condition=Pectin_0.1%	0	9.15e-03
	Condition=Glucose_0.1%	0	9.15e-03
	Condition=Corn_Fiber_0.5%	0	9.15e-03
	Condition=No_Carbohydrates_0.5%	0	9.15e-03
	Condition=No_Carbohydrates_0.1%	0	9.15e-03
	Phylum=*Firmicutes_Ruminococcaceae*	0	7.84e-05

D

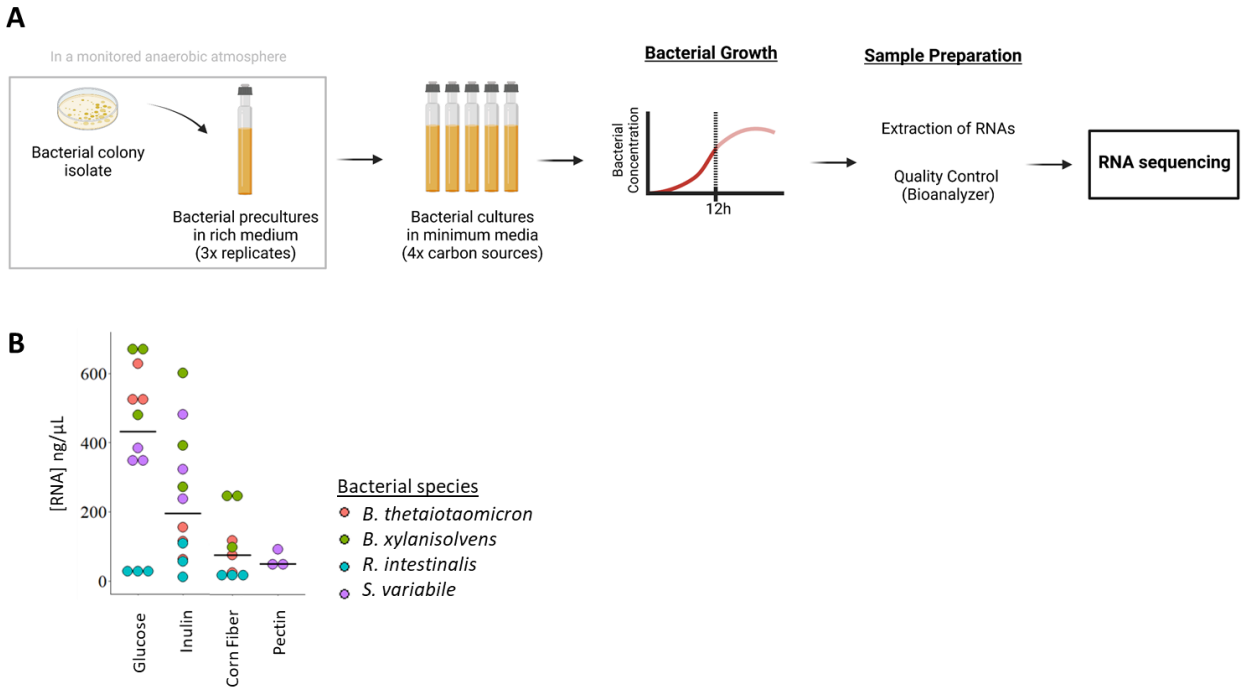
		Mean in Cluster	Overall Mean	SD in Cluster	Overall SD	p-value
Cluster N	Butyrate	0.65	1.61	0.89	3.56	6.71e-78
	Isobutyrate	0.16	0.24	0.14	0.23	8.75e-26
	Acetate	2.92	5.44	3.19	7.18	2.73e-27
	Propionate	0.24	2.15	0.45	4.48	2.89e-39
	Isovalerate	0.04	0.41	0.13	0.84	3.41e-41
Cluster G	Butyrate	8.91	1.61	5.47	3.56	6.71e-78
	Acetate	2.77	5.44	2.87	7.18	7.08e-04
	Propionate	0.09	2.15	0.04	4.48	3.03e-05
	Isobutyrate	0.13	0.24	0.10	0.23	2.40e-05
	Isovalerate	0.02	0.41	0.05	0.84	2.26e-05
Cluster A	Isovalerate	2.14	0.41	0.64	0.84	1.17e-104
	Propionate	10.33	2.15	5.20	4.48	3.69e-82
	Isobutyrate	0.64	0.24	0.22	0.23	1.51e-71
	Acetate	9.20	5.44	5.53	7.18	3.90e-08
	Butyrate	0.08	1.61	0.03	3.56	5.84e-06
Cluster G/I	Acetate	26.61	5.44	7.41	7.18	2.05e-70
	Propionate	3.76	2.15	4.03	4.48	3.04e-02
	Butyrate	0.09	1.61	0.04	3.56	9.93e-03



Supplementary Figure 3: Optimisation of sPLS-DA models for health-promoting bacteria (A), carbon sources (B), Firmicutes species (C) and Bacteroidetes species (D). *For each panel, the top graph represents the classification error rates obtained by the three prediction methods. Tuning the number of components in sPLS-DA on the LC-MS data sets. For each component, repeated cross-validation (10 × 5−fold CV) is used to evaluate the sPLS-DA classification performance (OER and BER), for each type of prediction distance metric. The estimated error rates obtained by the three prediction methods were not all stabilised after the same number of dimensions. For each panel, the bottom graph represents the balanced error rates of prediction distance metrics, including maximum, centroids and mahalanobis. Each coloured line represents the BER per component across all tested keepX values with the standard deviation based on the repeated cross-validation folds. The diamond indicates the optimal number of variables to keep for a given component to achieve a satisfying clustering of the samples with the lowest error rate. OER: overall error rate; BER: balanced error rate*



Supplementary Figure 4: Significant metabolites present in different carbon sources of Firmicutes and Bacteroidetes species. *Bacterial activities were investigated in response to different carbon sources, including glucose, agave inulin, corn fibre, citrus pectin, and in response to a minimal medium without supplementation of carbohydrates. Differential analysis between conditions of non-inoculated versus cultivated medium revealed fold changes of analytes for each carbon source and each bacterium. Statistical analysis allowed the identification of metabolites with significant fold changes.*



Supplementary Figure 5: Transcriptomic profiles highlighted the relationships between the carbon sources and the health-promoting bacteria. (A) Schematic of the experimental strategy. *The transcriptomic study was conducted on four health-promoting bacteria, belonging to two different bacterial phyla including Bacteroidetes (B. thetaiotaomicron and B. xylanisolvens) and Firmicutes (R. intestinalis and S. variabile). These bacterial species were cultivated in five minimum media (MM), each supplemented or not with different carbon sources. Each condition was performed in 3 replicates.* ***(B) Concentration of high-quality total RNA after extraction.*** *RNA integrity number (RIN) were all measured above 8. The analytic strategy of transcriptomic data will consist in the comparison of complex carbohydrate conditions versus the glucose condition. The gene expressions of Bacteroidetes species and R. intestinalis will be compared in inulin and corn fibre conditions versus the glucose condition. The gene expressions of S. variabile will be compared in inulin and pectin conditions versus the glucose condition.*

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Chapter 3: Impact of prebiotics on synthetic bacterial consortia shed light on cooperation and competition interactions

The investigation of carbohydrate metabolism of key intestinal bacteria revealed their metabolic capacities towards food ingredients with prebiotic effects. However, the intentional modulation of the gut microbiome for health and wellbeing purposes involves deciphering the interactions between prebiotics and the health-relevant bacteria at higher resolution than individual microorganisms. This **Chapter 3** is in the continuity of the previous results connecting the growth and fermentation profiles of isolated health-promoting bacteria *in vitro* single-carbohydrate consortia experiments. In particular, this study focuses on sources of agave inulin and corn fibre, which were the most promising prebiotic substrates that stimulate the growth and metabolic activities of the panel of health-promoting bacteria.

The main content of this chapter is currently being prepared as a research article.

Impact of diverse carbohydrates on the growth of health-relevant commensal bacteria in synthetic consortia

Bedu-Ferrari Cassandre[1,2], Biscarrat Paul[1], Bruneau Aurélia[1], Pepke Frédéric[1], Langella Philippe[1], Cherbuy Claire[1,*]

[1]Micalis Institute, INRAE, AgroParisTech, Université Paris-Saclay, F-78350, Jouy-en-Josas, France
[2]Yoplait France - General Mills, Vienne Technical Center, F-38200, Vienne, France

*Corresponding author: claire.cherbuy@inrae.fr

ABSTRACT

In the human gut, many environmental factors shape the ability of different microbes to coexist in microbial communities, one of which is diet. In particular, non-digestible dietary carbohydrates, also referred to as prebiotics, can influence metabolic activities of key intestinal bacteria that, ultimately, mediate physiological health effects through the production of bacterial-derived metabolites. Hence, food ingredients with prebiotic effects can modulate the gut microbiome to improve human health and wellbeing. However, the application of such a nutritional strategy involves deciphering the interactions between prebiotics, gut microbes, and the host. To uncover mechanistic insights into the interplay between prebiotics and health-relevant bacteria, we rationally designed synthetic bacterial assemblies. *In vitro* single-carbohydrate experiments allowed investigating the temporal behaviours of the bacterial composition in batch cultures. According to metabolic capacities of health-relevant species, the different carbon sources governed the relative abundance of microbes coexisting within synthetic bacterial assemblies. The results highlighted interspecies interactions and key drivers in carbohydrate metabolisation, including the nutritional preference of *Bifidobacterium* species in response to agave inulin. The particular adaptation of the bacterial drivers suggested complex trophic interactions ranging from cooperation to competition. This study advances a comprehensive understanding of the dynamics occurring between prebiotics and key health-relevant bacteria. Different methodological approaches were discussed to better understand how complex interactions between prebiotics, the gut microbiome, and the host could be investigated.

INTRODUCTION

Recent advances in microbiome science have anchored the importance of the metabolic activities of intestinal microbial communities in the host health and wellbeing. One of the core digestive functions of the gut microbiome is to transform complex dietary substrates into nutrients and fermentation by- and end-products that are determinant metabolites mediating host physiology (Lozupone et al., 2012; Krautkramer et al., 2021). The dynamic interplay of microbial communities is largely thrived by the interactions between the host and the carbohydrates that are non-digestible in the upper gastrointestinal tract. The delivery of dietary carbohydrates for intentional modulation of the gut microbiome incorporates the concept of prebiotics (Gibson et al., 2017). The mechanisms of carbohydrate-containing prebiotics are manifold, but are essentially direct or downstream, encouraging the proliferation of bacteria conferring health benefits to the host. A better understanding of how intestinal microbial communities are linked to health is crucial for the development of efficient nutritional strategies to preserve and/or restore the gut microbiome.

Although multiple variations in function and space occur, constitutive bacterial species of the gut microbiota appear to persist in an individual over extended time periods (Faith et al., 2013). Exhibiting stable coexistence of highly diverse microbial communities with high degree of functional redundancy contributes to the resilience of the intestinal ecosystem, that is, the capacity to return to an equilibrium state in response to chemical, physical, or microbial perturbations (Moya and Ferrer, 2016). Ensuring host digestive functions, the stability of the gut microbiome is driven by cooperative and competitive trophic interactions of diverse microbial species operating on multiple time and spatial scales at the community level, which could not be achieved by a single monospecies population (Gralka et al., 2020). While ecological competition is prevalent in natural microbial communities, it is assumed that cooperative networks maximise the functioning of stable microbiome communities (Coyte et al., 2015). Englobing the complexity of ecological networks, other types of interaction play a central role in microbiome stability, such as exploitation, commensalism, and amensalism (Coyte et al., 2015). Although bacteria fundamentally compete for nutrients, one by- and end-product of one strain can be the preferred energy source or a source of essential nutrients for another one. The nutritional preferences between bacteria often result in complex cross-feeding relationships (Smith et al., 2020). The population should converge to a single species with the greatest fitness to survive on that resource, resulting in competition mechanisms between microbes with overlapping metabolic capacities to utilise carbon sources (Goldford et al., 2018). Thus, in the intestinal environment, intense competition and

impressive cooperation led to a variety of strategies to process and scavenge dietary and host-produced glycans (Glowacki et al., 2021).

The complexity and temporality of interactions determine the dynamic behaviours and influence the functional activities of the gut microbiome. However, the prevalence of competition and cooperation in microbial communities of the human gut microbiome remains elusive. The ecological perspective of prebiotics would not be complete without identifying how changes in the relative abundances of one species impacts the relative abundances of others (Spacova et al., 2020). The manipulation of the metabolic activities of the gut microbiome for the benefits of host health and wellbeing benefits requires a comprehensive understanding of the dynamics of key health-promoting bacteria in the presence of prebiotics. Previous results reported that agave inulin and corn fibre stimulated the growth and fermentation activities of health-promoting bacteria to various degrees driven by their phylogenetic affiliation **(Article 1)**. In this study, the aim was to elucidate interspecies interactions and the nutritional preferences of the health-promoting bacteria within rationally designed bacterial assemblies. This reductionist approach to the complexity of the gut microbiome captures the dynamic behaviours of synthetic bacterial assemblies underlying the mechanistic of prebiotic metabolisation and the identification of key bacterial species driving the structure of the assembly. The examination of the microbial relative abundances with temporal resolution in single-carbohydrate batch cultures addressed the question of how microbes with distinct carbohydrate utilisation profiles coexist in synthetic bacterial assemblies.

MATERIAL AND METHODS

Bacterial species and starter culture conditions

The following bacterial strains were used in this study: *Bacteroides intestinalis* (DSM 17393), *Bacteroides xylanisolvens* (DSM 18836), *Bifidobacterium adolescentis* (DSM 20083), *Bifidobacterium catenulatum* (DSM 16992), *Blautia hansenii* (DSM 20583), *Agathobacter rectalis* (ATCC 33656) (formely Eubacterium rectale) (Rosero et al., 2016), *Roseburia intestinalis* (DSM 14610), *Faecalibacterium prausnitzii* (DSM 17677) and *Subdoligranulum variabile* (DSM 15176). A rich media was selected to support the growth of all monospecies: BHI medium was supplemented with 10mg/L hemin, 5mg/mL vitamin K1, 0.5% L-cystein, 1% cellobiose and 1% maltose, which were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). Each individual bacterial species were grown on BHI agar plates at 37°C in an anaerobic (90% N_2, 5% CO_2, 5% H_2 atmosphere) chamber (Coy Lab Products, Grass Lake, MI, USA). A single isolated colony was precultured at 37°C for 24h (to stationary phase) in supplemented BHI broth

and used as an inoculum for the evaluation of prebiotic effects in bacterial consortia. Bacterial growth of each starter culture was assessed by measuring optical density (OD) and quantifying recoverable colony-forming units (CFU) per mL by plating on BHI supplemented agar. The isolated strain identity was systematically verified by sequencing 16S rDNA amplicons, as previous described **(Article 1)**.

Growth culture conditions of synthetic bacterial assemblies

Single-carbohydrate cultures of synthetic bacterial assemblies were performed in minimum medium (MM) culture conditions previously described **(Article 1)**. To note, physicochemical modifications include the reduction to 20% of the initial amount of protein source and the adjustment of the pH to 6.8. The particularity of these culture conditions is to contain a unique predominant source of carbon that is the prebiotic. To study the temporal interactions and functionality of bacterial consortia, MM was supplemented with 0.5% (w/v) agave inulin or 0.5% (w/v) corn fibre. These two promising food ingredients with prebiotic effects were evaluated in comparison with appropriate controls that include the MM supplemented with 0.5% (w/v) glucose or supplemented with no carbohydrates. MM were inoculated with the following microorganisms: i) consortium n°1 contained *Bifidobacterium adolescentis*, *Bacteroides xylanisolvens*, *Subdoligranulum variabile* and *Roseburia intestinalis*; ii) consortium n°2 contained *Bifidobacterium catenulatum*, *Bacteroides intestinalis*, *Faecalibacterium prausnitzii*, *Eubacterium rectale* and *Blautia hansenii*. Under anaerobic conditions, all microorganism starter cultures were diluted to OD 600 0.1 for *Firmicutes* species and 0.01 for *Actinobacteria* and *Bacteroidetes* species (Ultraspec 10 Cell Density Meter, Biochrom Ltd. Cambridge, UK). The assembly inoculum with equivalent bacterial ratios was generated from these dilutions and inoculated with 2% (v/v) in a final volume of 45 mL glass bottles. We admit that nutrient carryover from starter cultures in rich media had only a marginal effect. Cultures were incubated anaerobically at 37°C for 24h. Samples were collected at times 0, 12, 14, 16, 18, 21 and 24 h and centrifuged at 12000 *g* for 15 minutes at 4°C. The supernatants were aliquoted to follow the acidification of the medium by pH measurement or to quantify the SCFA production. Cell pellets were used for genomic DNA extraction and subsequent quantification of bacterial concentrations by qPCR. All samples were stored at -20°C prior to use. Each synthetic bacterial assemblies were carried out in three independent biological replicates.

Medium acidification

Measures of pH were recorded for each the bacterial cultures using a pH-meter 1140 (Mettler Toledo, Urdorf, Switzerland).

Genomic DNA extractions

Total DNA was extracted from the cell pellets using the QIAamp® PowerFecal® DNA kit (Hilden, Germany) following the manufacturer's instructions. DNA was quantified using a Qubit™ 1X dsDNA HS Assay kit (Invitrogen, Thermo Fisher Scientific, Eugene, Oregon, USA).

Quantification of species abundance

Extracted DNA was diluted (100 times) to 10 ng/µL and used in PCR reactions using species-specific primers and probes **(Supplementary Table I)**. Reactions were run in StepOnePlus™ Real-time PCR system (Applied Biosystems®, Thermo Fisher Scientific, Singapore), using Takyon™ Rox SYBR® MasterMix or Takyon™ Probe MasterMix (Eurogentec, Seraing, Belgium) in MicroAmp® 96-well plates (Applied Biosystems®, life Technologies, Singapore). Reactions were carried out with an initial cycle for 2 min at 50°C (polymerase activation) and for 10 min at 95°C (DNA denaturation) followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. An additional step of curve fusion was included in SYBR systems and consisted in a cycle of 15 sec at 95°C, 1 min at 60°C and 15 sec at 95°C. Absolute quantification was expressed in the number of bacteria/mL of culture using standard curves of genomic DNA.

Standard of genomic DNA

For each individual bacteria, a single isolated colony was cultured at 37°C for 24h in supplemented BHI broth, as described above. Standard genomic DNA was obtained from the pellets of pure cultures, as described above. In addition, the absence of PCR inhibitors was validated using TaqMan™ exogenous IPC reagents (Applied Biosystems, Thermo Fisher Scientific, Vilnius, Lithuania). Internal positive control (IPC) reactions were proceeded following the instructions of the manufacturer. Quantitative PCR reactions were run using Ultra Clean Production (UCP) Probe PCR kit (QIAGEN, Hilden, Germany) and the *All bacteria* TaqMan system **(Supplementary Table I)**.

Data analysis and figures

For each bacterial consortium, data represent the averages of the three independent coculture experiments. Coculture outcomes were determined by calculating the individual abundance ratio for each species based on the quantitative bacterial measurements. Data were analysed using R Studio (Version 4.0.2) (R Development Core Team, 2008). Plots were generated using the R *ggplot2* package (Wickham, 2016). Figures were generated using BioRender (https://biorender.com).

RESULTS

Rational design of synthetic bacterial assemblies

As well-characterised and experimentally traceable, synthetic bacterial assemblies provide advantages over native communities in understanding the gut microbiome ecology, structure, and function (Clark et al., 2021). To decipher the temporal dynamics of key intestinal bacteria in carbohydrate metabolism, two simplified consortia were rationally designed.

Previous analyses revealed the metabolic capacities of individual health-promoting bacteria in single-carbohydrate cultures **(Article 1)**. The hierarchical clustering of the SCFA profiles reflected the phylogenetic relationships between bacterial species and their functional capacities to metabolise prebiotics. Indeed, the role of bacteria toward prebiotics may be organised at the phylum level. On one hand, *Actinobacteria* and *Bacteroidetes* species exhibited similar fermentation patterns in microbial interactions with prebiotics, resulting in the production of high propionate and acetate concentrations, respectively. In particular, *Bacteroidetes* species have demonstrated broad capacities to grow and ferment different carbon sources. On the other hand, *Firmicutes* species showed a varying range of capacities to grow and ferment different carbon sources. Few *Firmicutes* members of the *Lachnospiraceae* family reported high metabolic capacities for specific carbon sources, resulting in the production of butyrate. Hence, taxonomic level phylum may reflect metabolic functions that stabilise competition and promote coexistence (Goldford et al., 2018).

Metabolic exchanges mediate interspecies interactions. For engineering bacterial consortia, SCFAs were chosen as the most driving variables that reflect the bacterial metabolism of carbohydrates **(Article 1)**. Based on acetate that seems central in the bacterial fermentation and contributes to the production of propionate and butyrate end-products, we assumed a microbe-metabolite complementarity through cross-feeding interactions according to the production of SCFAs (Reichardt et al., 2014). Thereby, based on the fermentation profiles, the rationale to design each assembly was to associate one bacterial species belonging to *Actinobacteria* and *Bacteroidetes*, and two or three *Firmicutes* members of *Lachnospiraceae* and *Ruminococcaceae* families **(Supplementary Figure 1A)**.

This experimental strategy was supported by the fact that *Bacteroidetes* and *Firmicutes* have demonstrated unique and complementary metabolic specialisations in the gut microbiota (Fischbach and Sonnenburg, 2011). At the genus level, members of *Bacteroides* and *Bifidobacterium* have been reported as stable components of the human gut microbiome (Faith et al., 2013) and major drivers of configurational changes in gut microbiomes in response to dietary fibre supplementation (Delannoy-

Bruno et al., 2021). Additionally, the stability of a microbial ecosystem was proposed based on the metabolic capacities of generalist species rather than specialists (Goldford et al., 2018). Displaying broad metabolic capacities, *Bacteroides* species may play a key role in the growth of consistent community members opposed to production of specific metabolites. They may influence broad ecosystem functions, whereas *Firmicutes* species may contribute to specialised functions, such as the production of specific metabolites.

Bacterial growth in response to diverse carbon sources

Consortium experiments were performed to capture the temporal changes in the relative abundances of bacterial species in response to diverse single-carbohydrate sources. In batch cultures, growth and fermentation parameters of two rationally designed synthetic bacterial assemblies were followed over 24h in semi-defined minimal medium (MM) to highlight interspecies interactions and key drivers in carbohydrate metabolisation **(Supplementary Figure 1B)**. Experimental data consisted of relative abundances of bacterial species in single-carbohydrate cocultures for 24h at four different time points. The initial inoculum of bacterial assemblies were composed of distinct species proportions with the intention to lower, by a factor 10, *Bacteroidetes* and *Actinobacteria* inocula compared to *Firmicutes* inocula species. The description in bacterial proportions revealed the dynamic interactions between each bacterial species, which notably differed in response to the diverse carbon sources.

In MM supplemented without additional carbohydrates, the relative abundances of *Firmicutes* species in the consortium n°1 decreased over time to the benefit of the two other bacteria that together persisted well during the first 18h of the coculture. Then *B. xylanisolvens* appeared as the dominant single-species at 24h, outperforming *B. adolescentis* **(Figure 1A)**. Consistent with these observations, *S. variabile*, *R. intestinalis* and *B. adolescentis* showed a limited growth for the whole experiment compared to *B. xylanisolvens*, which reported a growth in a value of about 3-log increase **(Figure 1B)**. Noteworthy, *R. intestinalis* revealed a later behaviour after 18h coculture suggesting a fastidious growth in a value of about 1-log increase. Similar results were observed for the bacterial species in the consortium n°2, where the relative abundances of *B. intestinalis* rapidly take over *F. prausnitzii*, *E. rectale* and *B. catenulatum*. The *Blautia* species appeared to dominate at 12h of culture, before being left behind by *B. intestinalis,* which reported a growth in a value of about 3-log increase **(Figure 2)**. Therefore, in the absence of a dominant carbon source, the proteolytic capacities of the *Bacteroidetes* species were involved in competitive interactions with the other strains. These metabolic activities resulting from the degradation of the MM protein content are consistent with monoculture experiments **(Article 1)**. The extended period of coexistence between the two strains in cocultures

containing a particular carbon source might be due to their different hierarchical orders by which they avoid direct substrate competition. Thus, the preferences for carbon sources seem to facilitate their coexistence in microbial assemblies (Louis and Flint, 2017; Tuncil et al., 2017).

In the MM supplemented with glucose, the relative abundances of *Firmicutes* species in the consortium n°1 decreased over time to the benefit of the two other bacteria that together persisted for 24h of culture, with a particular advantage for *B. adolescentis* **(Figure 1A)**. Interestingly, the glucose carbon source supported the growth of all bacterial species in a value ranging from 1-log to 3-log increase. *Firmicutes* species showed rapid and early growth over 12h, with an increase of 2 logs, and persisted throughout the experiment. *B. xylanisolvens* and *B. adolescentis* grew extensively in values of about 3-log and 4-log increases, respectively **(Figure 1B)**. In the consortium n°2, the results reported a rapid growth for all bacterial species over 12h and persisted for the whole experiment, suggesting a stable coexistence **(Figure 2A)**. For the duration of the entire experiment, each species was maintained in stable proportions. Furthermore, different growth behaviours were reported for each species **(Figure 2B)**. *F. prausnitzii* showed a fastidious growth, in a value of about 1-log increase; *B. catenulatum*, *B. hansenii* and *B. intestinalis* showed a growth in a value of about 2-log increase; and *E. rectale* showed a high growth increase of about 3 logs. Interestingly, in presence of glucose as the dominant carbon source, the metabolic activities of all species in both bacteria consortia seemed in an environment where cooperative interactions may occur to maintain stable coexistence of all bacterial species. Indeed, *F. prausnitzii* reported a limited growth in the glucose carbon source, which was not previously reported in the monoculture experiments **(Article 1)**.

In the MM supplemented with corn fibre, the relative abundances of the consortium n°1 suggested similar growth behaviours than the ones observed in glucose carbon source, supporting the growth of all bacterial species from 2-log to 4-log increases **(Figure 1)**. In particular, *B. adolescentis* and *B. xylanisolvens* were the most dominant species, with bacterial growths that range from 3-log to 4-log increases, respectively. In the consortium n°2, the relative abundances reported that *E. rectale* dominated the bacterial assembly over the first 12h and persisted. Except for this single-dominant bacterium, each species appeared maintained in stable proportions during the whole experiment **(Figure 2A)**. In particular, the results reported a rapid growth of *E. rectale* and *B. hansenii* over 12h, in values of 2-log increase, and persisted for the whole experiment. *B. intestinalis* and *B. catenulatum* showed constant growth, in values of a 2-log increase, throughout the experiment. Interestingly, growth curves of *F. prausnitzii* revealed a later behaviour after 12h coculture **(Figure 2B)**. Therefore, in

presence of corn fibre as the dominant carbon source, the metabolic activities suggested an environment where cooperative interactions may occur to maintain stable coexistence of all bacterial species. Indeed, *F. prausnitzii* reported a limited growth after 12h coculture, which was not previously reported in the monoculture experiments **(Article 1)**. Although *E. rectale* dominated the bacterial assembly, the stable proportions of each bacterial species over time suggested a stable coexistence of all bacterial species.

In the MM supplemented with agave inulin, the relative abundances of *Firmicutes* and *Bacteroidetes* species in the consortium n°1 were rapidly outcompeted to the advantage of the dominant *B. adolescentis* **(Figure 1A)**. Bacterial growth curves reported bacterial growth of 2-log increase for the *Firmicutes* species, 3-log increase for *B. xylanisolvens*, and 5-log increase for *B. adolescentis* **(Figure 1B)**. In the consortium n°2, the agave inulin supported the stable coexistence of all bacterial species during 18h of culture, except *F. prausnitzii*, with a growth ranging from 1-log to 5-log increase **(Figure 2A)**. *F. prausnitzii* did not persist throughout the experiment. Rapid growth of *E. rectale* and *B. hansenii* over 12h, in values of 2-log increase, persisted for the whole experiment. *B. intestinalis* and *B. catenulatum* showed a constant growth of 2-log and 3-log increases, respectively **(Figure 2B)**. The high metabolic activities of *Bifidobacterium* species resulting from the degradation of the inulin carbon source are consistent with the monoculture experiments **(Article 1)**. These results suggested the nutritional preference of *Bifidobacteria* species that are likely enzymatically adapted for the metabolisation of inulin carbohydrate molecules, allowing competitive advantage over the other species. The dominance of these *Bifidobacterium* species is in accordance with previous studies that highlighted the genomic ability of these species to metabolise inulin-type prebiotics (Moens et al., 2014, 2016).

Bacterial fermentation in response to diverse carbon sources

The dynamics of the pH profiles were used as a measure of the extent of carbohydrate utilisation by the synthetic bacterial assemblages **(Figures 1C, Figure 2C)**. For both bacterial combinations, the pH values of the MM without additional carbohydrates remained close to the initial pH, whereas an important acidification of the medium was observed in the MM with glucose, partly indicating the bacterial fermentation capacities. Compared to the appropriate controls, the pH profiles revealed significant metabolisation for both synthetic bacterial assemblages in response to agave inulin and corn fibre. Interestingly, the fermentation patterns of these complex polysaccharides were similar to the one observed in glucose. In particular, the metabolic activities in agave inulin were very close to the glucose profile, suggesting high stimulation of growth and fermentation activities for both consortia experiments.

Further analyses of the metabolic profiles can provide a snapshot in time of the complex interactions within synthetic bacterial assemblies. Time-resolved measurements of SCFA production were performed for the two rationally designed communities to capture the dynamics of carbohydrate fermentation **(data not shown)**. Indeed, these metabolites, especially acetate, propionate and butyrate, are main by-products of complex carbohydrate fermentation processes carried out by the gut microbiome and play a major role in bacterial cross-feeding that, subsequently, impact host health (Ríos-Covián et al., 2016). In this study, further experimental adjustments would be required to validate the SCFA profiles.

DISCUSSION

A better understanding of how intestinal microbial communities are linked to health is crucial for the development of efficient nutritional strategies to preserve and/or restore the gut microbiome. Dietary interventions showed that the responses of microbial populations to prebiotics are highly individualised (Falony et al., 2016; Lloyd-Price et al., 2017). To gain a comprehensive understanding of the composition, stability, and functional activities of the gut microbiome ecosystem, there is an interest in interrogating anaerobic synthetic consortium models (Vrancken et al., 2019; Clark et al., 2021; Weiss et al., 2021). In this study, synthetic bacterial assemblies have characterised the dynamic behaviours of prevalent health-relevant species in responses to different single-carbohydrate sources.

Previous monoculture experiments reported that the isolated bacteria constituting the two synthetic bacterial assemblies were able to grow to different degrees under diverse single-carbohydrate conditions, except *F. prausnitzii* **(Article 1)**. In synthetic bacterial assemblies, *Firmicutes* species mostly reported early growth with a slowdown from 12h after inoculation. Interestingly, we observed a later growth behaviour of *R. intestinalis* in the MM without additional carbohydrates. These observations suggested that the metabolic activities of butyrate-producing bacteria could depend on cross-feeding interactions of smaller metabolites generated by *Bacteroidetes* species (Rivière et al., 2018; Fagundes et al., 2021). Exhibiting high abundance in the two synthetic bacterial assemblies, *Bacteroidetes* species may modulate the later growth of butyrate-producing species, and thus, community assembly, after the primary degradation of energy sources present in the MM without additional carbohydrates. For example, interactions between *Bacteroidetes* and butyrate producers may be important for acetate transformations, which is consistent with the conversion of acetate into butyrate (Clark et al., 2021).

Similarly, a later growth of *F. prausnitzii* was also observed in the glucose and corn fibre source. As opposed to the monoculture growth pattern, *F. prausnitzii* may grow to higher cell densities through indirect mechanisms operated by the other bacterial species showing active saccharolytic pathways. Actually, as generalists in the metabolisation of complex polysaccharides, *Bacteroidetes* may be involved in cross-feeding interactions in other single-carbohydrate conditions. Indeed, these species contain multiple polysaccharide utilisation loci (PULs), gene clusters comprising glycan binding proteins, sensors, transporters, enzymes for glycan degradation, and transcription factors that are coregulated in response to their specific glycan (Martens et al., 2011; Tuncil et al 2017; Grondin et al., 2017). This molecular adaptation can highlight the nutritional preferences of bacterial drivers within complex interactions that range from cooperation to competition depending on the bacterial species.

In our models of simplified microbial ecology, we were able to highlight the disproportionately large effect relative to their abundances of *Bacteroides* species in MM without additional carbohydrates. In cooperative interactions, they can be defined as keystone species that metabolise available substrates for other microorganisms (Berry and Widder, 2014). Nevertheless, cross-feeding interactions may also occur on other carbon sources among *Actinobacteria* strains. For instance, the predominance of *Bifidobacterium* species in the agave inulin condition supported the hypothesis of a favourable nutrient acquisition strategy adapted to the inulin source. Previous studies have demonstrated the cooperative interactions that stimulate the downstream growth of *Firmicutes* members and butyrate production resulting from the transformation of acetate and lactate produced by *Bifidobacterium* members (Moens et al., 2014, 2016). Hence, *Bifidobacterium* species are recognised as primary degraders of inulin-type fructans, which indicated the nutrient preferences and the bacterial adaptation conferring selective advantage in bacterial consortia (Claus, 2017).

The preferential degradation plays a central role in shaping the microbial relationships of the microbiota. Simplified experimental approaches can be used to identify key bacterial species and trophic interactions driving microbial communities. Once the underlying mechanisms of prebiotics are elucidated, the prebiotic could selectively target dynamic changes of specific metabolites produced by the gut microbiota and the relative abundances of health-relevant bacteria according to their metabolic capacities to ferment carbohydrates. In dietary intervention, there is significant and sustained interest in investigating the nutrient preferences of commensal bacteria to degrade one or more prebiotics. These biological insights could design prebiotic nutritional strategies to manipulate health-relevant functions at the community-level for prevention and therapeutic purposes.

Importantly, the metabolic activities of the two synthetic bacterial assemblies in response to carbon sources can be influenced by the environmental pH. On the one hand, fermentation by- and end-products, such as SCFA, can boost the growth of certain recipient species in cross-feeding exchanges (Venturelli et al., 2018). Depending on the environmental context, acetate can constitute a modest amount of carbon substrate, generated from carbohydrate metabolism, to produce propionate and butyrate (Louis and Flint, 2017; Clark et al., 2021). For instance, the bacterial consortia n°2 may provide acetate for the metabolic fermentation processes of *F. prausnitzii* to produce butyrate. Cross-feeding of metabolic by-products, such as acetate, is a prevalent mechanism that could alter environmental culture pH (Duncan et al., 2002; Wrzosek et al., 2013). On the other hand, levels of SCFAs can inhibit the growth of certain bacterial species (Tramontano et al., 2018). At low pH, SCFAs can be toxic to some species resulting in growth limitation (Ríos-Covián et al., 2016). The limit of this study is that we do not elucidate whether the relative abundance profiles are shaped by the carbon source or bacterial-derived metabolites. We cannot say whether the growth and fermentation activity of individual microbes changed in the presence of each other. Systematic mono- and pair-wise culture experiments lacked to identify the interaction types and the positive (primary degradation of carbon components into substrates or detoxification of the environment) or negative (depletion of key nutrients or production of toxic compounds) effects occurring within the synthetic bacterial assemblies (Venturelli et al., 2018). However, well-defined *in vitro* culture media and the selection of relevant bacterial strains supported the study of prebiotic effects. Additional *in vitro* experiments in batch or continuous cultures could be used to further describe the dynamic microbe-microbe and metabolite-microbe interactions.

This synthetic ecology approach has allowed exploring the growth and fermentation activities of health-relevant species in batch cultures, particularly, in response to carbohydrate-containing prebiotics. Developing methodologies to understand the dynamics of the microbial community is a first step toward elucidating the organisational principles of microbial communities and devising strategies for precisely manipulating ecological properties (De Roy et al., 2014). In this study, the rationally designed model systems supplied the acquisition of detailed knowledge not only of the carbohydrate utilisation capacities of individual health-promoting bacteria, but also of their behaviour within a web of complex interactions among them.

Predicting community dynamics is a key step to reveal ecological processes (Venturelli et al., 2018). Mathematical models of the ecosystem can support the discovery of significant microbial interactions within the gut microbiome and ecological driver species that have the most consistent and strong impacts on each metabolite and species abundance (Vrancken et al., 2019). For example, flux balance

analysis (FBA) used constraint-based modelling approach that allows to estimate how dietary carbon sources determine the taxonomic composition of microbial communities (Estrela et al., 2021). Such mathematical models can be robust coupling the biological information obtained in this study to overcome the lack of interpretability of machine learning (D'hoe et al., 2018). This integrative approach can generate networks that provide key insights into microbe-metabolite and microbe-microbe interactions that can further allow prediction of species relative abundances and overall metabolite production (Vrancken et al., 2019). Machine learning models represent a powerful tool for designing communities with a broad range of optimised health-relevant metabolites for therapeutic intention (Louis and Flint, 2017; Clark et al., 2021). Rather than tailoring the metabolic profiles of the synthetic bacterial assemblies, this study aimed to highlight nutritional preferences.

In the prebiotic research, ecological forces driving structural and functional dynamics within gut microbiomes have been neglected (Clavel et al., 2022). Translational approaches that estimate the rates at which species multiplied and consumed prebiotics could be useful to drive dietary interventions (D'hoe et al., 2018; Spacova et al., 2020). To go further, the use of synthetic communities, that is, stable consortia of well-defined strains, can be valuable for both experimental models and next-generation probiotics (Elzinga et al., 2019; Albright et al., 2022). In this study, we have highlighted the stable coexistence of bacterial consortia that strongly depend on the source or carbon. The sustainability of such bacterial consortia is a major challenge regarding the fact their maintenance is influenced by the dietary components. Sustained by selective dietary prebiotics, colonisation in animal models could support preclinical evidence for the application of synthetic bacterial communities in therapeutic interventions (Louis and Flint, 2017; Weiss et al., 2021).

The metabolisation of glycans is one of the main factors that thrives the human gut microbiome. Other interactions between microbial communities, including the production of antimicrobial compounds and quorum sensing networks, must also be considered in studying microbial co-existence (Louis, 2017). Leveraging multiple *-omics* levels of information can enable the modelisation of the gut microbiome at the level of entire microbial communities. Based on species composition, machine learning accurately predicting multiple functions of the gut microbiome constitutes a step forward in the intentional modulation of the trajectory of microbiome function.

CONCLUSION

Cultivation approach of simplified bacterial assemblies allowed the identification of compositional changes with temporal resolution, adaptability and ecological interactions of prebiotics within bacterial consortia. This synthetic microbial strategy can be used towards a more comprehensive understanding of the human gut ecosystem. Integrating *in vitro* culture experiments of isolated and combined bacteria with mathematical modelling can support the translation of mechanistic and ecological knowledge into novel and effective microbiome-based clinical tools for the maintenance and/or prevention of human health and wellbeing.

AUTHOR CONTRIBUTIONS

CBF, PB and CC elaborated the project and contributed to the design of the experimental strategy. CBF, PB, AB and FP performed the experiments. CBF and PB analysed the overall data. PL oversaw the project. CBF wrote the first draft of the manuscript.

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CONFLICT OF INTEREST

The authors declare that CBF has worked as an employee of Yoplait France – General Mills during the conduct of this study, as part of a CIFRE contract. The company had no role in the writing of this article or the decision to submit it for publication. The other authors declare no competing interests.

FIGURES & TABLES

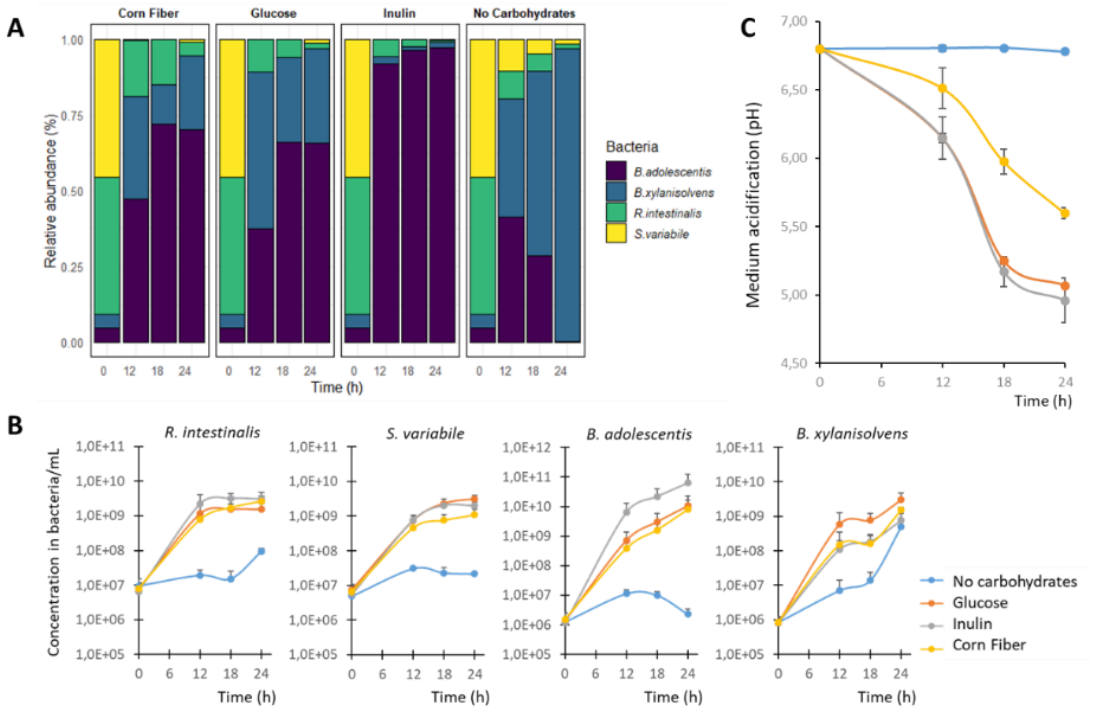


Figure 1: Bacterial synthetic assembly n°1. *The consortium n°1 is composed of four bacterial species that include Bacteroides xylanisolvens, Bifidobacterium adolescentis, Subdoligranulum variabile and Roseburia intestinalis. Data are the mean of three independent experiments in coculture. The initial inoculum of bacterial assemblies were composed of distinct species proportions with the intention to lower, by a factor 10, Bacteroidetes and Actinobacteria inocula compared to Firmicutes inocula species.* ***(A) Relative abundances of individual species as a function of time.*** ***(B) Growth curve of the individual species for 24h.*** *Data reported the concentration in bacteria per mL of culture over time.* ***(C) Acidification of the pH culture media reflects the fermentation activities.*** *The acidification of the pH culture medium is a consequence of the production of mixed acids, such as acetate, propionate and butyrate. Data reflect the fermentation activities of the overall synthetic bacterial communities.*

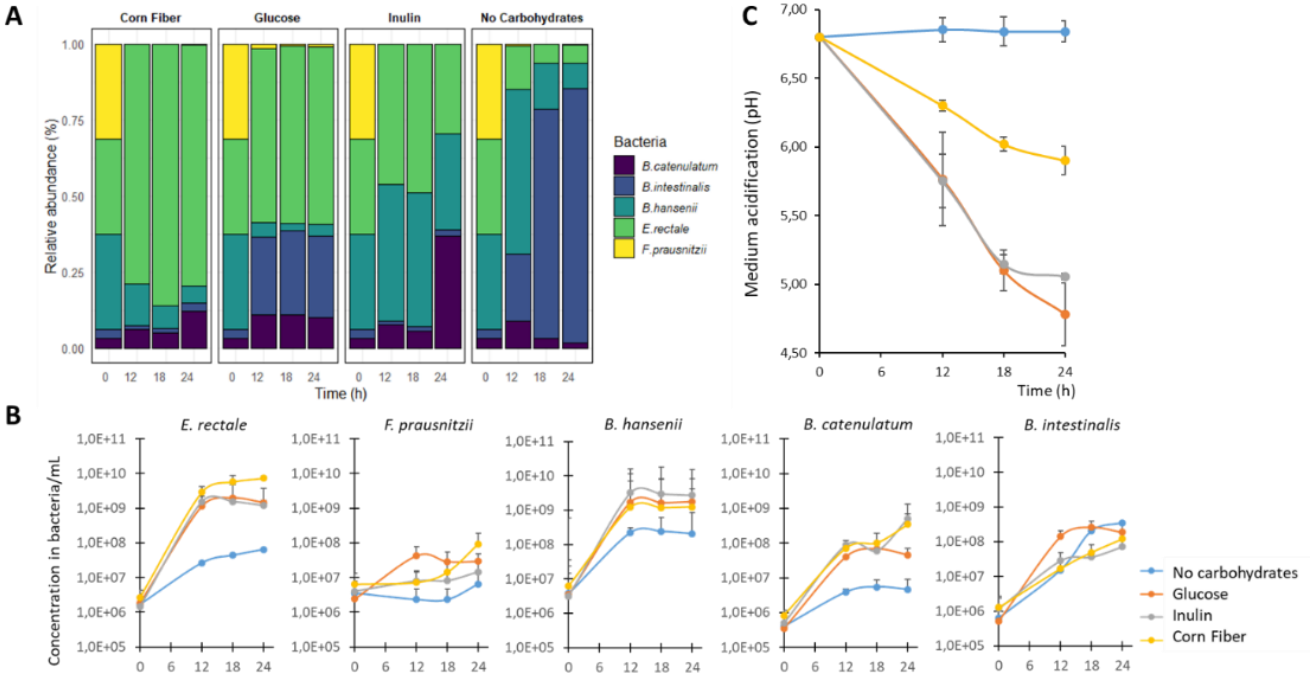
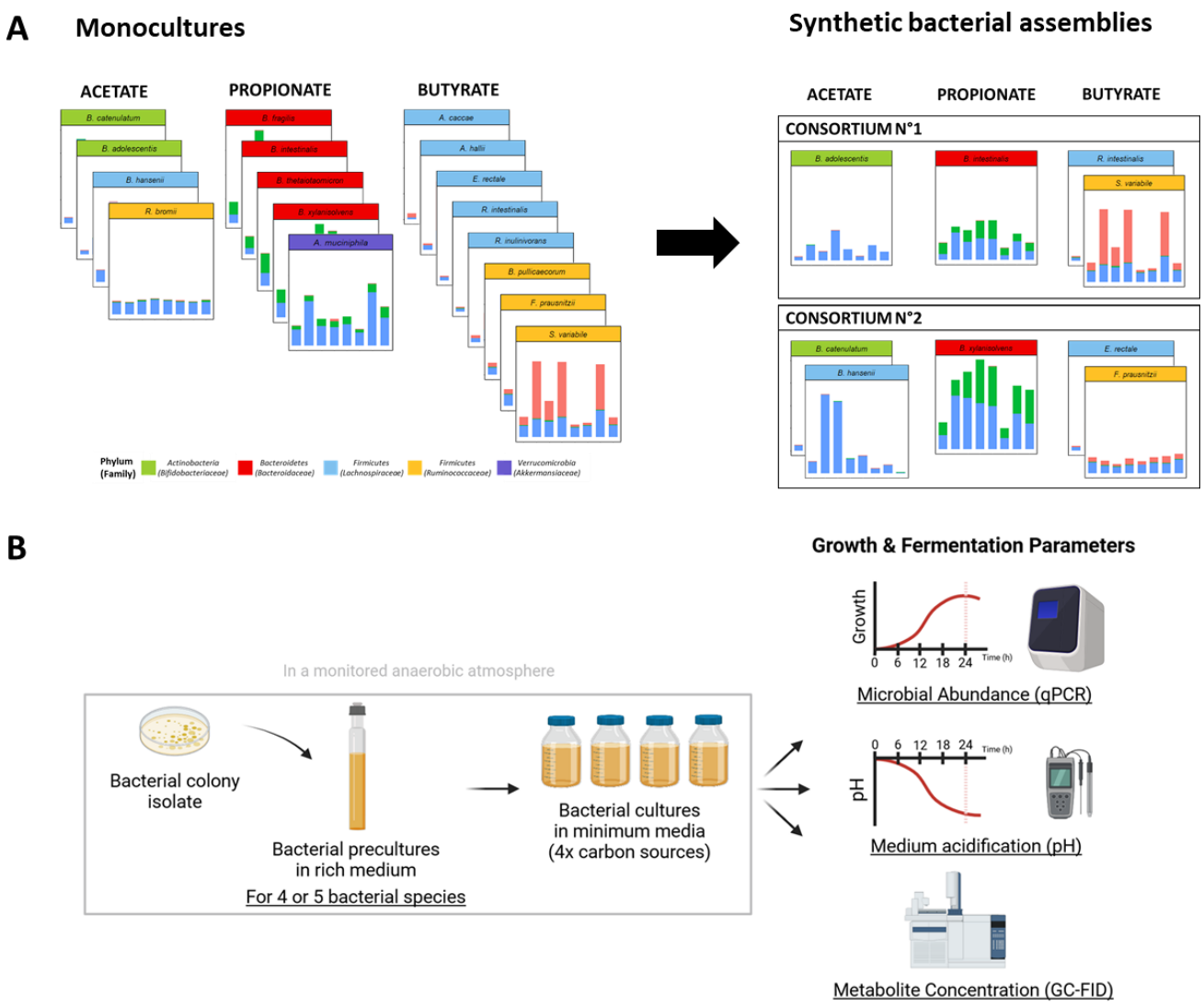


Figure 2: Bacterial synthetic assembly n°2. *The consortium n°2 comprises five bacterial species that include Bacteroides intestinalis, Bifidobacterium catenulatum, Faecalibacterium prausnitzii, Eubacterium rectale and Blautia hansenii. Data are the mean of three independent experiments in coculture. The initial inoculum of bacterial assemblies were composed of distinct species proportions with the intention to lower, by a factor 10, Bacteroidetes and Actinobacteria inocula compared to Firmicutes inocula species.* ***(A) Relative abundances of individual species as a function of time.*** *Colours represent different organisms in the community.* ***(B) Growth curve of the individual species for 24h.*** *Data reported the concentration in bacteria per mL of culture over time.* ***(C) Acidification of the pH culture media reflects the fermentation activities.*** *The acidification of the pH culture medium is a consequence of the production of mixed acids, such as acetate, propionate and butyrate. Data reflect the fermentation activities of the overall synthetic bacterial communities.*

SUPPLEMENTARY DATA

Supplementary Table I: qPCR systems to specifically quantify bacteria based on 16S rRNA gene-targeted primers and probes. Probes are annotated in bold.

	Target	Primer/Probe	Sequence 5'-3'	Reference
TaqMan system	All bacteria	F_Bact 1369 R_Prok 1492 **P_TEM1389F**	CGGTGAATACGTTCCCGG TACGGCTACCTTGTTACGACTT **CTTGTACACACCGCCCGTC**	Mayeur et al., 2013
	B. intestinalis *B. xylanisolvens*	F_Bacter11 R_Bacter08 **P_Bac303**	CCTWCGATGGATAGTGGTT CACGCTACTTGGCTGGTTCAG **AAGGTCCCCCACATTG**	Mayeur et al., 2013
	B. adolescentis *B. catenulatum*	F_Bifid09c R_Bifid06 **P_Bifid**	CGGGTGAGTAATGCGTGACC TGATAGGACGCGACCCCA **CTCCTGGAAACGGGTG**	Mayeur et al., 2013
	Blautia hansenii	F_Bhan R_Bhan **P_Bhan**	GACTAAGAAGCCCCGGCTAAC AACGCTTGCCCCCTACGT **TGCCAGCAGCCGC**	In this study
	S. variabile	F_Clept09 R_Clept08 **P_Clep01**	CCTTCCGTGCCGSAGTTA GAATTAAACCACATACTCCACTGCTT **CACATTAAGTAATCCACC**	Mayeur et al., 2013
SYBR system	*E. rectale*	s-Erec-F s-Erec-R	TTCTGACCGGTACTTAACCGTACC TTTGCTCGGCTTCACAGCTTT	Kurakawa et al., 2015
	F. prausnitzii	F_Fprau07 R_Fprau02	CCATGAATTGCCTTCAAAACTGTT GAGCCTCAGCGTCAGTTGGT	Mayeur et al., 2013
	R. intestinalis	RrecF Rrec630mR	GCGGTRCGGCAAGTCTGA CCTCCGACACTCTAGTMCGAC	Walker Alan W. et al., 2005 Ramirez-Farias et al., 2008



Supplementary Figure 1: Schematic representations (A) of the two synthetic bacterial assemblies and (B) of the experimental design of this study. *The rational design was based on the SCFA profiles identified in monoculture experiments. The phylogenetic affiliation of each bacterial species is indicated in the legend. In a monitored anaerobic atmosphere, the 4 or 5 bacterial members of each synthetic bacterial assemblies were prepared in distinct species proportions based on their fermentation profiles and cocultured in four different single-carbohydrate sources. Samples were collected for measurements microbial relative abundance in qPCR based on 16S rRNA gene. To capture the production of mixed fermentation acids, pH measurements of culture media were performed. In parallel, time-resolved SCFAs were measured in gas chromatography.*

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Chapter 4: Identification of genetic biomarkers to monitor key functionalities of the gut microbiome in response to diet

The link between prebiotics, gut microbiome and the host health, although relatively well characterised, is rather more difficult to predict and has often relied on *in vitro* fermentation data and *in vivo* human and/or animal interventions. Providing insights into the functional potential of organisms, molecular techniques enables detailed investigations into bacterial metabolic capacities to utilise non-digestible carbohydrate ingredients and to produce fermentation end-products.

In this chapter, we advanced an *in silico* approach based on the evolutionary conservation of metabolic functions in bacteria to identify potential genetic biomarkers that can enable a better comprehension of the dynamic interactions between the human gut microbiome and prebiotics.

The content of this chapter is part of a collaboration currently ongoing. The authors LJ, LT and LV contribute to the verification of the discriminant power of butyrate and propionate biomarkers and the evaluation of gene prevalence in metagenomic data. The authors MA and VM provide access to the metagenomic data.

BEDU-FERRARI Cassandre[1,2], LOPEZ Julien[2,3], LACROIX Thomas[3,4], LOUX Valentin[3,4], ALMEIDA Mathieu[5], MESLIER Victoria[5], LANGELLA Philippe[2], CHERBUY Claire[2,*]

[1]General Mills, Vienne Technical Center, Vienne, France
[2]Université Paris-Saclay, AgroParisTech, INRAE, MICALIS, Jouy-en-Josas, France
[3]Université Paris-Saclay, INRAE, MaIAGE, Jouy-en-Josas, France
[4]Université Paris-Saclay, INRAE, BioinfOmics, MIGALE bioinformatics facility, Jouy-en-Josas, France
[5]Université Paris-Saclay, INRAE (Institut National de Recherche Pour L'agriculture, l'alimentation Et L'environnement), MGP (Metagenopolis), F-78350, Jouy en Josas, France.

*Corresponding author: claire.cherbuy@inrae.fr

ABSTRACT

The health impact of prebiotic nutritional strategies emphasises the changes of composition and functions within the complex and diverse microbial community that constitutes the human gut microbiome. Non-digestible dietary carbohydrates are determinant to provide bacterial-derived metabolites, such as short-chain fatty acids (SCFAs), which play an important role in the dialogue between the gut microbiome and the host. *In silico* analysis holds promises enabling predictions of SCFA production from diet-gut microbiome interactions. A synteny approach based on the genomic environment analysis allowed the in-depth characterisation of molecular mechanisms that led to the discovery of biomarkers of key gut microbiome functionalities. The genomic potentials for the panel of health-promoting bacteria predicted metabolic capacities to utilise inulin-type fructans and produce fermentation end-products that vary substantially between species. Indicating health-relevant functions, the application of biomarkers can be used to monitor the gut ecosystem and to predict the responsiveness of individual gut microbiomes in dietary intervention. They provide paths toward rational engineering of functional outcomes of the gut ecosystem to promote human health in personalised nutritional approaches.

INTRODUCTION

The human gut microbiome is of considerable interest for health. Among the many functions attributed to the complex, diverse and dynamic microbial communities, the metabolism of non-digestible dietary carbohydrates is determinant to provide bacterial-derived metabolites, which play an important role in the dialogue between the gut microbiome and the host. The influence of dietary carbohydrates on functional aspects of the intestinal ecosystem can drive specific metabolic inputs through selectable pathways (Sonnenburg and Bäckhed, 2016). Inherent to the concept of prebiotics, the role of non-digestible carbohydrates relies on the modulation of the microbiome functions and their downstream physiological effects on human health (Gibson et al., 2017). The functions of the gut microbiome can shift in response to different dietary carbohydrates based on each species genomic potential to utilise these sources. For instance, inulin and/or fructo-oligosaccharides (FOS) are one of the most accepted prebiotics that are resistant to the action of human enzymes in the gastrointestinal tract (Gibson and Roberfroid, 1995). These food ingredients constitute a mixture of linear fructan chains composed of fructosyl-glucose and fructosyl-fructose linked by β (2-1) linkages. They provide substrates for the nutritional environment, which, through the metabolisation of intestinal microbes, can stimulate bacterial growth and metabolic activities (Gibson et al., 2017). Inulin/FOS are usually hydrolysed by glycoside hydrolase (GH) family 32 enzymes, and the resultant fructose and glucose are slotted into microbial glycolic pathways (Scott et al., 2011). To date, few studies have characterised the prevalence of inulin/FOS-degrading enzymes in health-relevant commensal bacteria.

The main mechanism of action by which prebiotics confer health benefits is through anaerobic fermentation processes, resulting in the production of short-chain fatty acids (SCFAs). Absorbed by the intestinal epithelium, the three main fermentation products are acetate, propionate and butyrate, which differ in their potential impacts upon host physiology (Louis and Flint, 2017). Indeed, they represent a source of energy and regulators of gene expression, immune cell development, and inflammation processes (Ríos-Covián et al., 2016). A detailed understanding of the SCFA biosynthesis pathways by the gut microbiome is necessary to support effective strategies supplying the host with health-relevant metabolites.

The development of high-throughput sequencing technologies has mostly described the gut microbiome at the taxonomic compositional level (Li et al., 2014). The annotation of high-quality bacterial genomes provides a robust source for a comprehensive significance of the gut microbiome functions (Ejigu and Jung, 2020). The challenge in microbiome research emphasizes the characterisation

of key metabolic functionalities (Reid et al., 2019). In this study, comparative analyses allowed the exploration of publicly available high-quality genomes referring to the panel of health-promoting bacteria previously described **(Article 1)**. We analysed genomic environments for the identification of biomarkers to utilise inulin/FOS prebiotics and to synthesise propionate and butyrate fermentation end-products. The perspective is the analysis of gene prevalence in metagenomic data to evaluate specific functionalities of the human gut microbiome in response to dietary changes.

MATERIALS & METHODS

Selection of bacterial genomes

Complete or draft genomic data for each bacteria was obtained from the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov). The assembly and annotation of the sequences and quality check were carried out as previously described **(Article 1)**.

In silico analysis of health-relevant bacteria

In bacterial genomes, metabolic functions are often encoded within conserved clusters of genes. The prediction of metabolic pathways was based on the exploration of these syntenic regions. The genetic context was investigated by exploring the functional annotations, which might represent a source of assignment errors and hypothetical proteins. Gaps in the identification of a gene function was manually filled. To avoid misannotations, the protein functions were checked for orthology in the UNIPROT database (https://www.uniprot.org/). The tBLASTn tool was used to query translated nucleotide sequences against amino acid sequences of functional genes of interest (Altschul et al., 1990). High-confidence matches were significantly homologous considering an *E* value threshold of 1e-6, amino acid identity ≥ 25% and bit score ≥ 100.

RESULTS

Characterisation of molecular mechanisms dedicated to inulin/FOS utilisation

Monomer moiety and glycosidic linkage type determine the bacterial utilisation of carbohydrates. Inulin/FOS ingredients are linear polymers made of fructose units linked by β-(2-1) glycosidic bonds with a typical terminal glucose unit **(Figure 1A)**. Among all CAZymes, the glycoside hydrolase family 32 (GH32) specifically supports the enzymatic hydrolysis of inulin β-(2-1) glycosidic bonds **(Figure 1B)** (Scott et al., 2011). Complete or draft genomes were screened to search for a functional reference GH32 originating from *Bacteroides intestinalis* **(Supplementary Table I)**. Translated nucleotide sequences of

each health-relevant bacteria were queried against amino acid sequences of this key gene reference responsible for fructan β-fructosidase activity. Focusing on *Bacteroidetes* species, the exploration of the genetic environment revealed clusters of genes encoding GH32 enzymes, carbohydrate transporters, and regulatory elements **(Figure 1C)**. These results were consistent with the description of the polysaccharide utilisation locus (PUL), which have previously been described to possess at minimum one polysaccharide-degrading enzyme and a carbohydrate transporter machinery characterised by a susC-like TonB-dependent transporter coupled to a susD-like nutrient importer (Martens et al., 2011; Sonnenburg et al., 2010). The exploration of bacterial genomes for the presence of the GH32 and adjacent genes suggested a carbohydrate utilisation locus supposedly dedicated to the inulin/FOS metabolism **(Figure 1D)**. Putative carbohydrate transport systems were adjacent to GH32, especially ATP-binding cassette (ABC) transporters or major facilitator superfamily (MFS) transporter and phosphotransferase system (PTS) transporters. Furthermore, the fructokinase and LacI transcription regulator were also located in the vicinity of the gene encoding GH32 proteins, and formed PULs as previously described (Tanno et al., 2021). Tough almost universally observed in gram-negative *Bacteroidetes* PULs, carbohydrate transporters encoded by susC/D-like homologues were not identified in the genomes of *Firmicutes* and *Actinobacteria* species. In addition, comparative genomic analyses have highlighted the prevalence of the PUL supposedly dedicated to the utilisation of inulin/FOS for each bacteria of interest **(Table II)**. In accordance with a previous study, the genomic potential within the panel of health-promoting bacteria to utilise fructans seemed to vary substantially between strains and species (Sheridan et al., 2016). Interestingly, the inulin/FOS utilisation cluster was particularly prevalent in *Blautia hansenii*, *Butyricicoccus pullicaecorum*, *Bacteroides xylanisolvens*, *Bifidobacterium adolescentis* and *Bifidobacterium catenulatum*.

Prediction of butyrate biosynthesis

Based on experimental data derived from the literature, the *bcd* gene encoding a butyryl-CoA dehydrogenase electron-transfer flavoprotein complex appeared central in all pathways of butyrate biosynthesis regardless of the initial substrate (Vital et al., 2013; Anand et al., 2016; Boynton et al., 1996). Exploring the genomic environment of this key gene allowed identifying the major route that leads to butyrate production though the acetyl-coenzyme A (CoA) and pyruvate substrates **(Figure 2A)**. Comparative genomic analyses allowed the reconstruction of the metabolic pathway through the identification of a conserved gene cluster **(Figure 2B)**, which has already been previously described (Boynton et al., 1996). According to previous studies, four initial substrates were suggested to lead to butyrate biosynthesis (Vital et al., 2013). Pyruvate constitutes the most common energy source for

commensal microbes and is directly supplied by carbohydrate metabolisation (Anand et al., 2016). The genomic potential of the panel of health-promoting bacteria to produce butyrate was based on the prevalence of gene copies involved in this pathway **(Figure 2C, Supplementary Table II)**. As expected, the absence of the butyrate gene cluster was predicted in *Bacteroidetes*, *Actinobacteria* and *Verrucomicrobia* phyla members (Vital et al., 2014). The results suggested that most *Firmicutes* species share a genomic potential to produce butyrate from the conversion of crotonyl-CoA to butyryl-CoA, with the exception of *R. bromii* and *B. hansenii*. Divergences in the organisation of butyrate genes were observed in the butyrate-producing species **(Figure 3)**. For example, *Roseburia* species lack the gene that encodes the crotonase enzyme. However, bacteria exhibiting all genes of a pathway (excluding terminal genes and allowing for one missing gene) were considered positive for that pathway. In addition, butyrate is synthesized by butyryl-CoA:acetate CoA-transferase (encoded by the but gene) or butyrate kinase (encoded by the buk gene) that ensure conversion from crotonyl-CoA to butyryl-CoA. It should be noted that *S. variabile* species utilise the butyrate kinase after phosphorylation of the butyryl-CoA rather than the CoA-transferase enzyme in the final step of butyrate formation. As a functional signature, the genomic context of the butyrate cluster was closely related to carbohydrate metabolism and bacterial fitness, including vitamin effectors, oxidative stress, and antibiotic resistance modules **(Figure 3)**.

Prediction of propionate biosynthesis

Previous studies revealed that propionate biosynthesis within the human gut microbiome involves three metabolic pathways whose functionality depends on the carbohydrate available for bacterial growth (Louis and Flint, 2017). The succinate pathway constitutes the major route for commensal microbes to produce propionate from carbohydrates and involves the conversion of succinate to propionate by the *mmdA* gene encoding a methylmalonyl-CoA transcarboxylase enzyme in presence of vitamin B12 (Reichardt et al., 2014). The propanediol pathway invokes the *pduP* gene encoding a key propionaldehyde dehydrogenase that can produce propionate and intermediate 1,2-propanediol as final products (Louis and Flint, 2017). From intermediary lactate fermentation product, the acrylate pathway involves *lcdA/B* genes encoding the lactoyl-CoA dehydratase key enzymes (Kandasamy et al., 2013). Based on experimental data derived from the literature, key genes were selected as potential discriminant biomarkers of propionate pathways **(Supplementary Table III)**. Comparative genomic analyses of health-promoting bacteria evaluated their genomic potential for propionate production **(Figure 4)**. The prevalence and abundance of propionate genes suggested that, among the panel of health-promoting bacteria, *Bacteroidetes* species synthesise propionate through the succinate pathway

encoding a propionyl-CoA:succinate CoA transferase. Similarly, *A. muciniphila* is a dominant propionate producer specialised in mucin degradation that synthesises propionate through the succinate pathway (Derrien et al., 2004). Based on the genes that were considered biomarkers of the three propionate pathways, we were unable to determine the genomic potential of *Ruminococcus bromii*. Interestingly, A. hallii displayed the genomic potential to produce propionate through the propanediol pathway. Previous study confirm the capacity of this dominant gut commensal bacteria belonging to *Lachnospiraceae* to produce propionate in response to its nutritional environment (Engels et al., 2016). To note that none of the health-relevant bacteria of interest seemed to utilise the acrylate pathway that convert lactate to propionate.

DISCUSSION

Inulin/FOS genomic potential of health-relevant bacteria

The degradation of complex polysaccharides has mostly scrutinized the presence of PULs in the *Bacteroidetes* members (Terrapon et al., 2015). In this study, the exploration of genomes of health-promoting bacteria allowed the identification of a cluster of genes possibly involved in the utilisation of inulin/FOS. Prevalent in *Actinobacteria* and Firmicutes, these putative PULs appeared widespread among the panel of health-promoting bacteria. There is an obvious need to better understand the utilisation of complex carbohydrates in Gram-positive bacteria. A previous study has described Gram-positive PULs (gpPULs) that englobe at minimum one polysaccharide-degrading enzyme, a carbohydrate kinase, a carbohydrate transporter system, and a regulatory factor. Compared to *Bacteroidetes*, the key difference in gpPULs is the absence of close homologues of susC/D pair genes (Sheridan et al., 2016). Analysing the prevalence of this cluster may indicate inulin/FOS bacterial responders especially adapted to this specific carbon source. Nevertheless, more evidence is required to evaluate the functionality of the putative inulin/FOS utilisation locus. Indeed, the prevalence of a gene does not reveal its expression and functionality. Previous results reported that *E. rectale* was not able to significantly utilise inulin source at the level of metabolites in culture condition enriched in agave inulin, though comparative genomic analysis revealed the presence of one GH32-containing PUL **(Article 1)**. According to the nutrient availability, the expression of this machinery could support divergent capacities of health-promoting bacteria to ferment inulin/FOS molecules. Transcriptional analyses may reveal the bacterial upregulation of a central gene encoding GH32 protein in single-carbohydrate experiments with inulin/FOS as the main source of carbon **(Article 1)**. Furthermore, this syntheny approach revealed variations in genetic arrangements that can be taxonomic-dependent

(data not shown). The organization of genes at a locus can distinguish the functional capacities and the specificity of polysaccharide utilisation related to a species/strain (Sonnenburg et al., 2010). It suggested that variations in gpPULs could underlie a wide range of fructan nutritional specificities and the fitness advantage given the appropriate environmental conditions.

Identification of genetic biomarkers for the production of propionate and butyrate

The analysis of the prevalence of genetic biomarkers associated to the production of butyrate was consistent with the literature. *Firmicutes* members of the *Ruminococcaceae* and *Lachnospiraceae* families were defined as butyrate-producers reporting the evolutionary conservation of a cluster of genes, except for *B. hansenii* and *R. bromii* (Louis and Flint, 2009; Vital et al., 2013). Interestingly, the genomic environment of the butyrate cluster was different from that of one species to that of another, suggesting that genetic expression may not be equivalent in every butyrate-producing bacteria. The co-transcription of other metabolites resulting from the cluster of butyrate seems to distinguish health-relevant bacteria. Interestingly, the dynamic of the bacterial genomes often leads to the functional clustering of several genes under the same regulation. A previous study analysing the GC content correlation and the evolutionary conservation of gene contexts has revealed the existence of fundamental 10-20 kb elementary structures transcriptionally coordinated in bacterial genomes (Junier et al., 2018). The prevalence of regulatory elements in the vicinity of the butyrate cluster strengthens the hypothesis of a transcriptional co-regulation of large genetic segments encoding functions involved in the carbohydrate metabolism and cofactor/vitamin biosynthesis. We raise the hypothesis that genetic expressions of butyrate clusters may not be equivalent among butyrate-producers, resulting in diverse fermentation coproducts potentially involved in the host-intestinal microbiota dialogue.

The phylogenetic distribution of putative propionate producers among the panel of health-promoting bacteria was less intuitive. Indeed, the identification of gene clusters for each pathway did not suggest the corresponding biochemical reactions. In particular, direct functional predictions based on protein homology can commonly result in misannotations if proteins with distinct functions share regions of high similarity (Vital et al., 2014). In this study, the homology of protein sequences excluded some biomarker genes belonging to the butyrate or propionate pathways and, therefore, were not considered as suitable indicators of SCFA production. Among the list of candidates, the *but* gene encoding a butyryl-CoA:acetate CoA-transferase and the *buk* gene encoding a butyrate kinase have already been used as biomarkers of the final step of butyrate formation for the identification of butyrate-producing communities (Vital et al., 2013). Importantly, we investigated the major route of

butyrate operated from the carbohydrate fermentation with the acetyl-CoA and pyruvate intermediates. However, there are other known butyrate-producing pathways, namely, the lysine, glutarate, and 4-aminobutyrate pathways (Louis and Flint, 2017). Similarly, propionate can also be produced from peptides and amino acids fermentation by gut microbes, with aspartate, alanine, threonine and methionine as major substrates (Reichardt et al., 2014). More evidence is required to confirm the discriminant power of these potential genetic biomarkers. Actually, targeting the entire pathway for functional predictions seems a robust way to circumvent difficulties associated with the analysis based on specific genes only.

Previous *in vitro* experiments confirmed the functional activity of the predicted butyrate and propionate producers, which suggested the validation of genetic biomarkers **(Article 1)**. Nevertheless, the metabolic potential of each bacterial species to produce propionate and/or butyrate depends on the nutritional environment. Among the panel of health-promoting bacteria, previous studies have reported two members of *Lachnospiraceae,* including *A. hallii* and *R. inulinivorans,* able to switch from butyrate to propionate and to sequentially synthesise these different SCFAs in the presence of different energy sources (Reichardt et al., 2014). In particular, *R. inulinivorans* was shown previously to utilise the propanediol pathway for propionate formation (Scott et al., 2006). In this study, we identified the genomic potential of *A. hallii* to synthesise propionate and butyrate. However, these metabolic capacities were not observed in single-carbohydrate experiments **(Article 1)**.

Identifying biomarker genes and/or clusters involved in specific metabolic pathways constitute powerful tools for predicting metabolic capacities. These genetic biomarkers could help to estimate in advance bacterial responders dedicated to the inulin/FOS utilisation and their fermentation end-products. Although some bacteria have the genomic potential to produce SCFA, it is not a guaranty of genetic expression and production of propionate and butyrate as fermentation end-products. For instance, some species of *Bacteroidetes* produce succinate rather than propionate as their main fermentation product. Bacterial fermentation processes can lead to the release of intermediates as final products.

Functional annotation can describe individual genomes of each bacterial species. However, they do not predict their contribution in the perspective of intestinal ecology or their effect on the host. The prevalence of a molecular mechanism does not indicate the resulting metabolic reaction at the level of the individual bacteria or the entire intestinal ecosystem. Indeed, the released fructose and glucose moieties can be utilised by the primary degrader itself or the bacteria have the ability to liberate monomers as public good resources for other bacteria, leaving the rise of metabolic cross-feeding

(Rakoff-Nahoum et al., 2014; Cuskin et al., 2015). A previous study suggested that *B. fragilis* can utilise inulin for its metabolism, nevertheless, the bacterium lacks the ability to liberate the fructose for other species (Chijiiwa et al., 2020). The catabolism of monomer units could be investigated to better understand the underlying interactions of the microbial communities in response to inulin/FOS in the gut environment.

The public availability of genomic and metagenomics sequences is a highly useful resource to foster our understanding of microbial metabolism in the gut, but care must be taken with assigning function to genes by sequence analysis, which should ideally be complemented by evidence from genetic or enzymatic studies (Wang et al., 2020). The availability and the designation of potential biomarkers are essential to investigate key microbial functionalities in complex microbial communities. Probing propionate and butyrate functionalities can provide insights into the effects of nutritional strategies on the gut microbiome (Vital et al., 2014).

Prediction of inulin/FOS metabolisation and other bacterial key capacities in metagenomic data of the human gut microbiome

The in-depth characterisation of molecular mechanisms led to the discovery of potential biomarkers of key gut microbiome functionalities. The perspective of this study is to screen metagenomic data to reveal the modulation of propionate and butyrate functionalities within the microbial communities in a dietary intervention. A previous study explored the effects of a rich plant-based Mediterranean diet on metabolic health and gut microbiome (Meslier et al., 2020). This 8-week randomised controlled trial includes overweighed and obese individuals who have risk factors for metabolic disease, with low fibre intake and sedentary lifestyle. The nutritional pattern of the Mediterranean diet revealed significant impacts on microbiome-mediated health outcomes, with improvement of metabolic markers correlated with the specific microbial taxa. In perspective, we want to evaluate the impact of a rich plant-based diet at the level of the entire intestinal ecosystem by targeting biomarker genes that potentially characterize key microbial functions. The estimation of the prevalence and abundance with a human metagenomic library can provide new insights into the gut microbiota that is most likely related to diet (Tauzin et al., 2020).

CONCLUSION

The shift from individual genome analysis to metagenomics is a pivotal milestone in the investigation of bacterial communities. Integrating metagenomics data from existing human cohorts can allow extending the research to uncultured microorganisms and gaining new insights into the mechanisms by which the gut microbiome contributes to health. Through their application, functional biomarkers have the potential to predict the responsiveness of individuals to prebiotic intervention or supplementation. They are determinant to better apprehend the heterogeneity of individual responses in the perspective of personalised plans to manage gut microbiomes for desirable functionality. Indeed, the composition of the human gut microbiome is unique to an individual, while its functionality is more likely to be universally shared across individuals (Visconti et al., 2019). A broader resolution of the gut microbiome functions would enable rational manipulation of the microbiome via dietary carbohydrates. The characterissation of functional adaptations to respective prebiotic substrates can provide a solution to predict the efficacy of a prebiotic intervention for which a responding individual is more likely to benefit.

FIGURES & TABLES

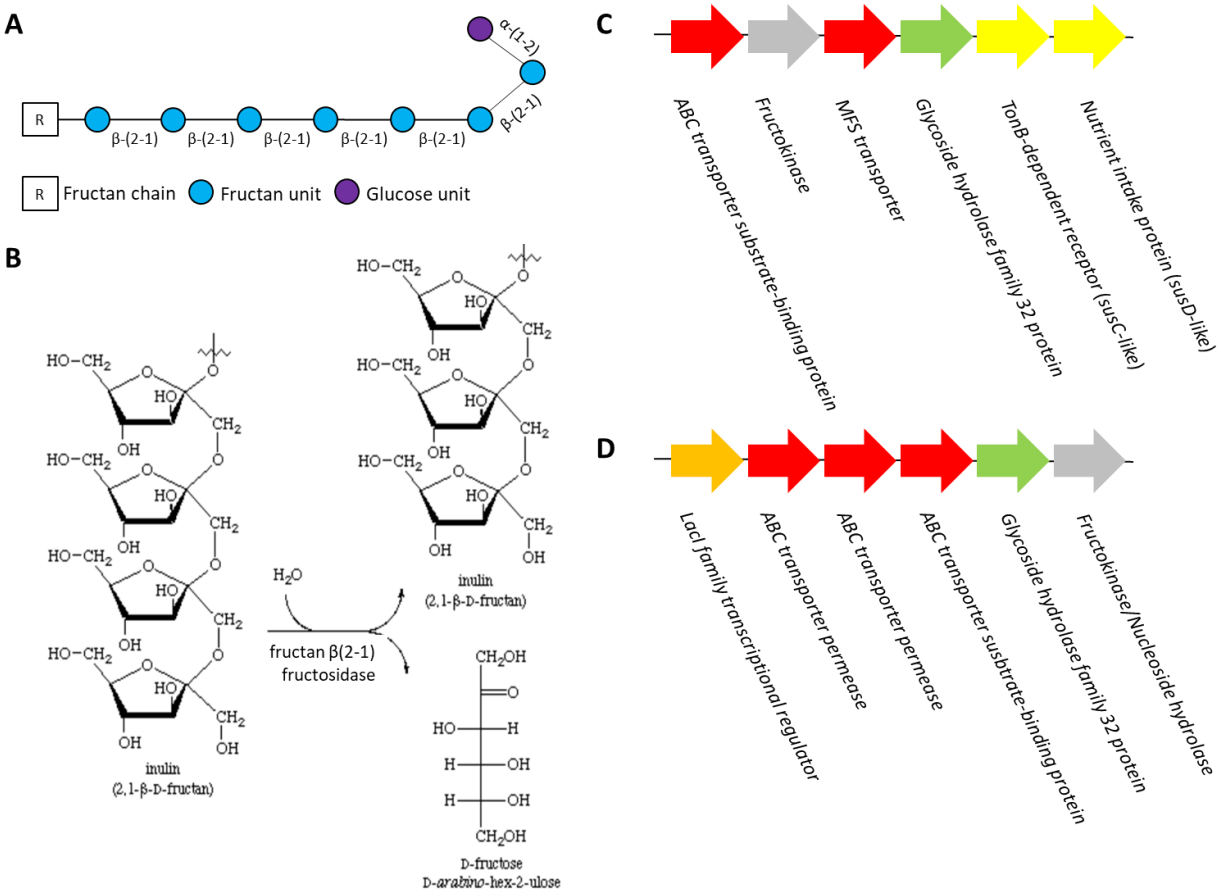
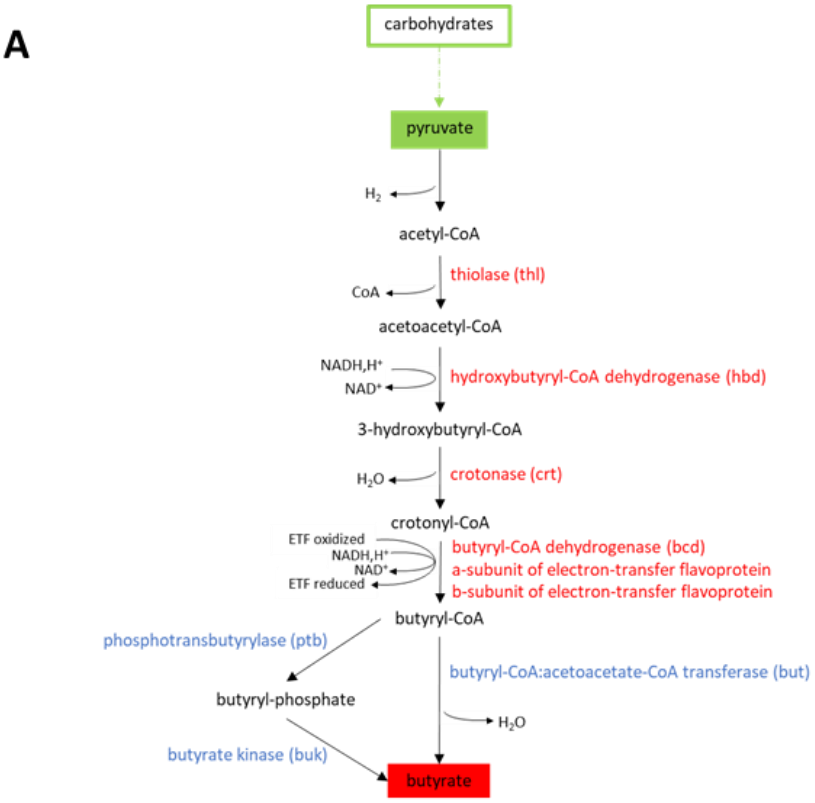


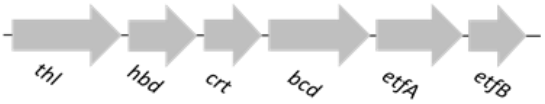
Figure 1: Putative molecular mechanisms involved in inulin/FOS utilisation among the panel of health-promoting bacteria. ***A)*** *Schematic representation of inulin/FOS reveals fructose units joined by β-(2-1) glycosidic bonds with a terminal glucose. Inulin/FOS molecules generally contain between 20 to several thousand units of fructose, indicated by R in the structure.* ***B)*** *The hydrolysis reaction of β-(2-1) glycosidic bonds is catalysed by a fructan β-(2-1) fructosidase classified as the glycoside hydrolase 32 family (GH32).* ***C)*** *Schematic representation of the putative inulin/FOS utilisation locus identified in Bacteroides spp. Glycoside hydrolase genes are coloured in green. ABC transporter system components are coloured in red. Fructokinase genes are coloured in grey. Outer membrane polysaccharide binding and import proteins are coloured in light yellow (susC/susD-like paired genes). The arrowhead indicates the direction of transcription. Differences in gene arrangement can occur between members of Bacteroidetes.* ***D)*** *Schematic representation of putative inulin/FOS utilisation cluster identified in the genomes of Firmicutes and Actinobacteria members. Glycoside hydrolase genes are coloured in green. ABC transporter system component are coloured in red. Transcriptional regulator genes are coloured orange. Fructokinase and nucleoside hydrolase genes are coloured in grey and are respectively found in Firmicutes and Actinobacteria members. The arrowhead indicates direction of transcription. Differences in gene arrangement can occur between bacterial species.*

Table II: Prevalence of a carbohydrate utilisation locus predicted for the inulin/FOS metabolisation in a panel of health-promoting bacteria. *For each bacteria, the absence and the presence of the gene cluster is respectively commented by an "absent" and a "1","2" or "3" whether all genes are present as a cluster. "Uncomplete" designates a cluster with one or two missing genes. Noteworthy, B. pullicaecorum* presented a distinct genetic arrangement compared to the other families of Firmicutes selected in this study.*

Phylum (Family)	*Bacterial Species*	International Collection	Strain Designation	RefSeq Assembly Accession	Assembly Level	CDS	Prevalence
Actinobacteria (Bifidobacteriaceae)	*Bifidobacterium adolescentis*	DSM 20083T	E194an (variant a)	GCF_000010425.1	complete	1676	2
	Bifidobacterium catenulatum	DSM 16992T	B669	GCF_000173455.1	contig	1677	2
Bacteroidetes (Bacteroidaceae)	*Bacteroides fragilis*	DSM 2151T	EN-2	GCF_000025985.1	complete	4544	1 + uncomplete
	Bacteroides intestinalis	DSM 17393T	341	GCF_000172175.1	contig	4655	uncomplete
	Bacteroides thetaiotaomicron	DSM 2079T	E50 (VPI-5482)	GCF_000011065.1	complete	4794	1 + uncomplete
	Bacteroides xylanisolvens	DSM 18836T	XB1A	GCF_015668785.1	scaffold	4621	2
Firmicutes (Lachnospiraceae)	*Agathobacter rectalis Syn. Eubacterium rectale*	ATCC 33656T	VPI-0990	GCF_000020605.1	complete	3288	1
	Anaerobutyricum hallii Syn. Eubacterium hallii	DSM 17630	L2-7	GCF_900209925.1_EH1	complete	3076	uncomplete
	Anaerostipes caccae	DSM 14662T	L1-92	GCF_000154305.1	scaffold	3317	uncomplete
	Blautia hansenii	DSM 20583T	VPI-C7-24	GCF_002222595.2	complete	2974	3
	Roseburia intestinalis	DSM 14610T	L1-82	GCF_000156535.1	scaffold	4267	1
	Roseburia inulinivorans	DSM 16841T	A2-194	GCF_000174195.1	contig	3926	1
Firmicutes (Ruminococcaceae)	*Butyricicoccus pullicaecorum*	DSM 23266T	25-3	GCF_900167005.1	scaffold	3103	2
	Faecalibacterium prausnitzii	DSM 17677	A2-165	GCF_002734145.1	complete	2956	1
	Ruminococcus bromii	ATCC 27255	VPI 6883	GCF_002834225.1	contig	1998	absent
	Subdoligranulum variabile	DSM 15176T	Bl 114	GCF_000157955.1	scaffold	3040	1 + uncomplete
Verrucomicrobia (Akkermansiaceae)	*Akkermansia muciniphila*	ATCC BAA-835T	Muc	GCF_000020225.1	complete	2257	absent

T stands for "Type" bacterial strain.





C

Present Absent

		Cluster of butyrate						Final step		
		etfB	*etfA*	*bcd*	*hbd*	*crt*	*thl*	*buk*	*ptb*	*but*
Actinobacteria	*Bifidobacterium adolescentis*	0	0	0	0	0	0	0	1	0
	Bifidobacterium catenulatum	0	0	0	0	0	0	0	1	0
Bacteroidetes	*Bacteroides fragilis*	1	1	1	0	1	0	2	2	1
	Bacteroides intestinalis	1	1	1	0	0	0	2	2	1
	Bacteroides thetaiotaomicron	1	1	1	0	1	0	1	2	1
	Bacteroides xylanisolvens	1	1	1	0	1	0	2	2	1
Firmicutes (Lachnospiraceae)	*Agathobacter rectalis*	1	1	1	1	1	1	1	1	1
	Anaerobutyricum hallii	3	3	4	1	1	1	1	1	2
	Anaerostipes caccae	6	6	6	1	3	1	1	1	2
	Blautia hansenii	0	0	0	0	0	0	0	0	0
	Roseburia intestinalis	1	1	1	1	0	1	1	1	1
	Roseburia inulinivorans	1	1	1	1	0	1	1	1	1
Firmicutes (Ruminococcaceae)	*Butyricicoccus pullicaecorum*	2	3	3	2	1	1	1	1	1
	Faecalibacterium prausnitzii	1	1	2	1	1	1	1	1	1
	Ruminococcus bromii	0	0	0	0	0	0	1	0	0
	Subdoligranulum variabile	1	1	1	1	1	1	2	2	0
Verrucomicrobia	*Akkermansia muciniphila*	0	0	0	1	1	0	0	0	0

Figure 2: Prediction of butyrate biosynthesis among the panel of health-promoting bacteria.
A) *Reconstruction of butyrate biosynthesis pathway from pyruvate initial substrate. Carbohydrate degradation into pyruvate via glycolysis is shown in green. The enzymes encoded by a cluster of genes are written in red. Terminal enzymes are written in blue.* ***B)*** *Cluster of genes involved in the butyrate biosynthesis. Differences in gene arrangement can occur among each bacterial strain. The arrowhead indicates direction of transcription. thl, thiolase; hbd, hydroxy-butyryl dehydrogenase; crt, crotonase; bcd, butyryl-CoA dehydrogenase; etfA/B electron transfer flavoprotein subunits A/B* ***C)*** *Estimation of the bacterial genomic potential for butyrate production. Numbers correspond to gene counts in this metabolic pathway. Bacteria exhibiting all genes of a pathway (excluding terminal genes and allowing for one missing gene) were considered positive for that pathway.*

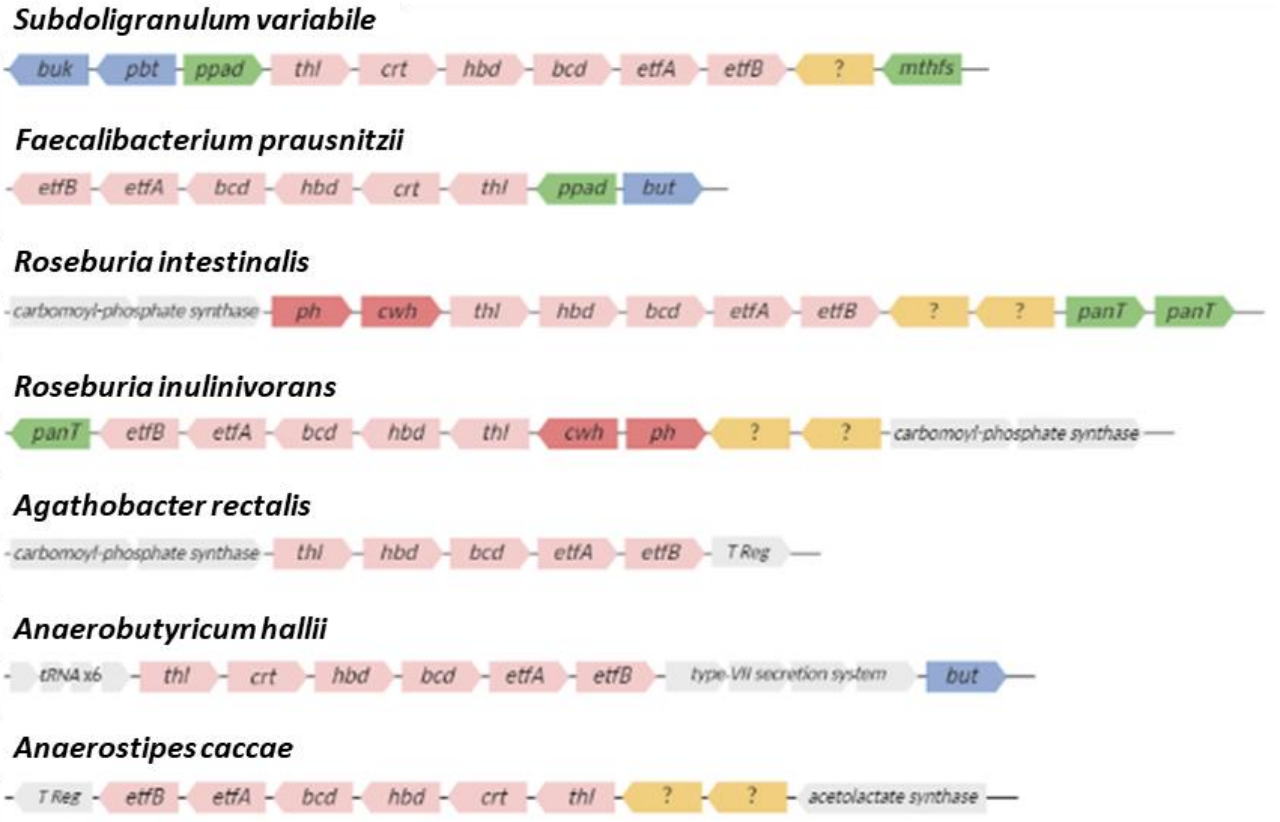


Figure 3: Exploration of the genetic context within 12 kb of the butyrate biosynthesis cluster. *Genes prevalent in the vicinity of the butyrate biosynthesis cluster are involved in vitamin B metabolism, gene regulation, sugar transport, oxidative stress and antibiotic resistance. Abbreviations:* ***but*** *butyryl-CoA dehydrogenase;* ***buk*** *butyrate kinase;* ***pbt*** *phosphotransbutyrylase;* ***ppad*** *pantetheine phosphate adenylyltransferase;* ***panT*** *pantothenic acid ECF transporter S component;* ***mthfs*** *5-formyltetrahydrofolate cycloligase;* ***ph*** *phosphohydrolase;* ***cwh*** *cell-wall hydrolase;* ***?*** *genes encoding hypothetical proteins*

Present Absent

		Succinate				Lactate		Propanediol			
		scpA	*scpC*	*mmdA*	*mce*	*lcdA*	*lcdB*	*pduC*	*pduD*	*pduE*	*pduP*
Actinobacteria	*Bifidobacterium adolescentis**										
	*Bifidobacterium catenulatum**										
Bacteroidetes	*Bacteroides fragilis*	1	1	3	1	0	0	0	0	0	0
	Bacteroides intestinalis	1	1	1	1	0	0	0	0	0	0
	Bacteroides thetaiotaomicron	1	1	3	1	0	0	0	0	0	0
	Bacteroides xylanisolvens	1	1	3	1	0	0	0	0	0	0
Firmicutes (Lachnospiraceae)	*Agathobacter rectalis**										
	Anaerobutyricum hallii	0	0	0	0	0	0	2	2	2	2
	*Anaerostipes caccae**										
	*Blautia hansenii**										
	*Roseburia intestinalis**										
	*Roseburia inulinivorans**										
Firmicutes (Ruminococcaceae)	*Butyricicoccus pullicaecorum**										
	*Faecalibacterium prausnitzii**										
	Ruminococcus bromii	0	0	1	0	0	0	0	0	0	1
	*Subdoligranulum variabile**										
Verrucomicrobia	*Akkermansia muciniphila*	1	1	1	1	0	0	0	0	0	0

Figure 4: Prediction of propionate biosynthesis among the panel of health-promoting bacteria.
Estimation of the bacterial genomic potential for propionate production. Numbers correspond to gene counts in each metabolic pathway. Bacteria exhibiting all genes of a pathway (allowing for one missing gene) were considered positive for that pathway. The asterisk indicates the bacterial genomes that were not analysed for their potential to produce propionate, on the basis of our previous in vitro results (Article 1).*

Table III: Prediction of butyrate and propionate biosynthesis among a panel of putative health-promoting bacteria. *The prevalence of the gene clusters described for both butyrate and propionate indicate the bacterial capacity to synthesize these two short-chain fatty acids. For each bacteria, the absence and the presence of the gene cluster is respectively commented by an "absent" and a "1" whether all genes are present. "Uncomplete" designates a cluster with one or two missing genes. To note, only bacteria further investigated by in vitro experiments were included in this table.*

Bacteria		Prevalence	
Phylum (family)	**Species**	**Butyrate**	**Propionate**
Firmicutes *(Lachnospiraceae)*	*A. caccae*	1	absent
	A. hallii	1	absent
	B. hansenii	absent	absent
	E. rectale	1	absent
	R. intestinalis	1	absent
	R. inulinivorans	1	absent
Firmicutes *(Ruminococcaceae)*	*B. pullicaecorum*	1	absent
	F. prausnitzii	1	absent
	R. bromii	absent	1
	S. variabile	1	absent
Bacteroidetes	*B. intestinalis*	uncomplete	1
	B. fragilis	uncomplete	1
	B. thetaiotaomicron	uncomplete	1
	B. xylanisolvens	uncomplete	1
Verrucomicrobia	*A. muciniphila*	absent	1
Actinobacteria	*B. adolescentis*	absent	absent
	B. catenulatum	absent	absent

SUPPLEMENTARY DATA

Supplementary Table I: Protein sequence of the key gene involved in the utilisation of inulin/FOS

GH32 **Glycoside Hydrolase Family 32** [Bacteroides intestinalis DSM 17393]	MNLKNNLLTVRWTLTFIIGTIWSVFSCQAINPPFVIKHLGDGQSIVQIENQKKFLLLPVEEASPEAKLYM IADNDVVRNMNVRLAINKVDYFVPVDLSGFDNKSLSFNFQLIPDTAICWDEMKLSDEFDTTNRENFR PLYHFTPQYGWMNDPNGMVYKDGVYHLFYQYNPYGSMWGNMHWGHATSTDLVSWEHQPVAI APDALGTIFSGSCVVDKDNTAGFGAGAIVAFYTSASDRQVQSMAYSLDNGRTFKKYARNPILTSTQR DFRDPKVFWHDATDKWIMILAVGQEMQIYSSANLKDWSYESSFGEGQGAHGGVWECPDLIELPIEG TELKKWILICNINPGGPFGGSATQYFVGTFDGKRFVNESPEATKWMDWGKDHYATVTWNNTPEGR HIALAWMSNWQYANNVPTTQYRSGNSVPRDLSLYTSAGETYLKSSPSRELLELRGKEEKKCSFKVDRT YNLDKLLSDNTGTYEIEMTIKNRNAEIVGFQLFNSKGEEVDIYYNLVEKKFAMDRSKSGIVSFSPDFPIV TFAPIEDNSEMTLRLFIDKSSIEAFGDDGRFAMTNLVFPSEPYNRISFYAKGGSYTVSSFKVYKLK

Supplementary Table II: Protein sequences of the putative genes involved in the microbial synthesis of butyrate.

BCD sp|P52042|1-379 **butyryl-CoA dehydrogenase** [Clostridium acetobutylicum ATCC 824]	MDFNLTREQELVRQMVREFAENEVKPIAAEIDETERFPMENVKKMGQYGMMGIPFSKEYGGAGGD VLSYIIAVEELSKVCGTTGVILSAHTSLCASLINEHGTEEQKQKYLVPLAKGEKIGAYGLTEPNAGTDSGA QQTVAVLEGDHYVINGSKIFITNGGVADTFVIFAMTDRTKGTKGISAFIIEKGFKGFSIGKVEQKLGIRAS STTELVFEDMIVPVENMIGKEGKGFPIAMKTLDGGRIGIAAQALGIAEGAFNEARAYMKERKQFGRSL DKFQGLAWMMADMDVAIESARYLVYKAAYLKQAGLPYTVDAARAKLHAANVAMDVTTKAVQLFG GYGYTKDYPVERMMRDAKITEIYEGTSEVQKLVISGKIFR
HBD sp|P52041|1-282 **hydroxybutyryl-CoA dehydrogenase** [Clostridium acetobutylicum ATCC 824]	MKKVCVIGAGTMGSGIAQAFAAKGFEVVLRDIKDEFVDRGLDFINKNLSKLVKKGKIEEATKVEILTRIS GTVDLNMAADCDLVIEAAVERMDIKKQIFADLDNICKPETILASNTSSLSITEVASATKRPDKVIGMHF FNPAPVMKLVEVIRGIATSQETFDAVKETSIAIGKDPVEVAEAPGFVVNRILIPMINEAVGILAEGIASVE DIDKAMKLGANHPMGPLELGDFIGLDICLAIMDVLYSETGDSKYRPHTLLKKYVRAGWLGRKSGKGF YDYSK
CRT sp|P52046|1-261 **crotonase** [Clostridium acetobutylicum ATCC 824]	MELNNVILEKEGKVAVVTINRPKALNALNSDTLKEMDYVIGEIENDSEVLAVILTGAGEKSFVAGADISE MKEMNTIEGRKFGILGNKVFRRLELLEKPVIAAVNGFALGGGCEIAMSCDIRIASSNARFGQPEVGLGI TPGFGGTQRLSRLVGMGMAKQLIFTAQNIKADEALRIGLVNKVVEPSELMNTAKEIANKIVSNAPVAV KLSKQAINRGMQCDIDTALAFESEAFGECFSTEDQKDAMTAFIEKRKIEGFKNR
THL sp|P45359|1-392 **acetyl-CoA acetyltransferase** [Clostridium acetobutylicum ATCC 824]	MKEVVIASAVRTAIGSYGKSLKDVPAVDLGATAIKEAVKKAGIKPEDVNEVILGNVLQAGLGQNPARQ ASFKAGLPVEIPAMTINKVCGSGLRTVSLAAQIIKAGDADVIIAGGMENMSRAPYLANNARWGYRM GNAKFVDEMITDGLWDAFNDYHMGITAENIAERWNISREEQDEFALASQKKAEEAIKSGQFKDEIVP VVIKGRKGETVVDTDEHPRFGSTIEGLAKLKPAFKKDGTVTAGNASGLNDCAAVLVIMSAEKAKELGV KPLAKIVSYGSAGVDPAIMGYGPFYATKAAIEKAGWTVDELDLIESNEAFAAQSLAVAKDLKFDMNKV NVNGGAIALGHPIGASGARILVTLVHAMQKRDAKKGLATLCIGGGQGTAILLEKC
ETFα **electron-transfer flavoprotein α-subunit** [Clostridium acetobutylicum ATCC 824]	MNKADYKGVWVFAEQRDGELQKVSLELLGKGKEMAEKLGVELTAVLLGHNTEKMSKDLLSHGADK VLAADNELLAHFSTDGYAKVICDLVNERKPEILFIGATFIGRDLGPRIAARLSTGLTADCTSLDIDVENRD LLATRPAFGGNLIATIVCSDHRPQMATVRPGVFFEKLPVNDANVSDDKIEKVAIKLTASDIRTKVSKVV KLAKDIADIGEAKVLVAGGRGVGSKENFEKLEELASLLGGTIAASRAAIEKEWVDKDLQVGQTGKTVRP TLYIACGISGAIQHLAGMQDSDYIIAINKDVEAPIMKVADLAIVGDVNKVVPELIAQVKAANN
ETFβ **electron-transfer flavoprotein β-subunit** [Clostridium acetobutylicum ATCC 824]	MNIVVCLKQVPDTAEVRIDPVKGTLIREGVPSIINPDDKNALEEALVLKDNYGAHVTVISMGPPQAKN ALVEALAMGADEAVLLTDRAFGGADTLATSHTIAAGIKKLKYDIVFAGRQAIDGDTAQVGPEIAEHLGI PQTYVEKVEVDGDTLKIRKAWEDGYEVVEVKTPVLLTAIKELNVPRYMSVEKIFGAFDKEVKMWTADD IDVDKANLGLKGSPTKVKKSSTKEVKGQGEVIDKPVKEAADMLSQN

The genes bcd, hbd, crt, thl, etfα and etfβ constitute a putative conserved cluster.

BUK sp|Q45829|1-355 **butyrate kinase** [Clostridium acetobutylicum ATCC 824]	MYRLLIINPGSTSTKIGIYDDEKEIFEKTLRHSAEEIEKYNTIFDQFQFRKNVILDALKEANIEVSSLNAVVG RGGLLKPIVSGTYAVNQKMLEDLKVGVQGQHASNLGGIIANEIAKEINVPAYIVDPVVVDELDEVSRIS GMADIPRKSIFHALNQKAVARRYAKEVGKKYEDLNLIVVHMGGGTSVGTHKDGRVIEVNNTLDGEG PFSPERSGGVPIGDLVRLCFSNKYTYEEVMKKINGKGGVVSYLNTIDFKAVVDKALEGDKKCALIYEAFT FQVAKEIGKCSTVLKGNVDAII
PTB sp|P58255|1-301 **phosphotransbutyrylase** [Clostridium acetobutylicum ATCC 824]	MIKSFNEIIMKVKSKEMKKVAVAVAQDEPVLEAVRDAKKNGIADAILVGDHDEIVSIALKIGMDVNDF EIVNEPNVKKAALKAVELVSTGKADMVMKGLVNTATFLRSVLNKEVGLRTGKTMSHVAVFETEKFDR LLFLTDVAFNTYPELKEKIDIVNNSVKVAHAIGIENPKVAPICAVEVINPKMPSTLDAAMLSKMSDRGQ IKGCVVDGPLALDIALSEEAAHHKGVTGEVAGKADIFLMPNIETGNVMYKTLTYTTDSKNGGILVGTS APVVLTSRADSHETKMNSIALAALVAGNK
BUT sp|P58255|1-301 **butyryl-CoA:acetoacetate-CoA transferase** [Faecalibacterium prausnitzii A2-165]	MDFTELYAQKKMTADQAAALVKSGDWVDYGWAVNTPVAVDAAIAKRLPELEDVNFRGGILMWVP AIFQIEDPAAHMTWNSWHMGGIERKAIAQGFSFYSPIRYSELPRYYRDSSDPVDVAVFQVTPMDEH GYFNFGPCASHLGAVCDKAKKIIVEVNRNMPKCLGGTENWVHISQVAGVVEGSNPPIGQMAAAGA ATEVDLKVANLIVPQIPDGACLQLGIGGMPNAIGNLIAQSDLKDLGVHTEMYVDAFVDIAKAGKITGR HKNLDKGRQVYAFGAGTQKMYDYLNDNPECMAAPVEYTNDIRSISAIDNFISINNAVDIDLFGQVN AESAGIKHISGAGGQLDFVLGAYLSNGGKSFICLSSTFMNKKTGKLESRIRPTLENGSIVTDTRANVHYL CTEYGCVNLKGLTSWEKAEALISVAHPDFRDELIAEAEKLHIWRRSNKR

The genes buk, ptb and but are involved in the terminal enzymatic steps of the butyrate synthesis.

Supplementary Table III: Protein sequences of the putative key genes involved in the metabolic pathways of propionate biosynthesis.

scpA sp|P27253 **methylmalonyl-CoA mutase** [Escherichia coli K12]	MSNVQEWQQLANKELSRREKTVDSLVHQTAEGIAIKPLYTEADLDNLEVTGTLPGLPPYVRGPRAT MYTAQPWTIRQYAGFSTAKESNAFYRRNLAAGQKGLSVAFDLATHRGYDSDNPRVAGDVGKAG VAIDTVEDMKVLFDQIPLDKMSVSMTMNGAVLPVLAFYIVAAEEQGVTPDKLTGTIQNDILKEYLC RNTYIYPPKPSMRIIADIIAWCSGNMPRFNTISISGYHMGEAGANCVQQVAFTLADGIEYIKAAISAG LKIDDFAPRLSFFFGIGMDLFMNVAMLRAARYLWSEAVSGFGAQDPKSLALRTHCQTSGWSLTEQ DPYNNVIRTTIEALAATLGGTQSLHTNAFDEALGLPTDFSARIARNTQIIIQEESELCRTVDPLAGSYYI ESLTDQIVKQARAIIQQIDEAGGMAKAIEAGLPKRMIEEASAREQSLIDQGKRVIVGVNKYKLDHED ETDVLEIDNVMVRNEQIASLERIRATRDDAAVTAALNALTHAAQHNENLLAAAVNAARVRATLGEI SDALEVAFDRYLVPSQCVTGVIAQSYHQSEKSASEFDAIVAQTEQFLADNGRRPRILIAKMGQDGH DRGAKVIASAYSDLGFDVDLSPMFSTPEEIARLAVENDVHVVGASSLAAGHKTLIPELVEALKKWGR EDICVVAGGVIPPQDYAFLQERGVAAIYGPGTPMLDSVRDVLNLISQHHD
scpC sp|P52043 **Propionyl-CoA:succinate CoA transferase** [Escherichia coli K12]	METQWTRMTANEAAEIIQHNDMVAFSGFTPAGSPKALPTAIARRANEQHEAKKPYQIRLLTGASIS AAADDVLSDADAVSWRAPYQTSSGLRKKINQGAVSFVDLHLSEVAQMVNYGFFGDIDVAVIEAS ALAPDGRVWLTSGIGNAPTWLLRAKKVIIELNHYHDPRVAELADIVIPGAPPRRNSVSIFHAMDRV GTRYVQIDPKKIVAVVETNLPDAGNMLDKQNPMCQQIADNVVTFLLQEMAHGRIPPEFLPLQSGV GNINNAVMARLGENPVIPPFMMYSEVLQESVVHLLETGKISGASASSLTISADSLRKIYDNMDYFAS RIVLRPQEISNNPEIIRRLGVIALNVGLEFDIYGHANSTHVAGVDLMNGIGGSGDFERNAYLSIFMAP SIAKEGKISTVVPMCSHVDHSEHSVKVIITEQGIADLRGLSPLQRARTIIDNCAHPMYRDYLHRYLEN APGGHIHHDLSHVFDLHRNLIATGSMLG
mmdA sp|P02904 **Methylmalonyl-CoA transcarboxylase** [Propionibacterium freudenreichii]	MAENNNLKLASTMEGRVEQLAEQRQVIEAGGGERRVEKQHSQGKQTARERLNNLLDPHSFDEVG AFRKHRTTLFGMDKAVVPADGVVTGRGTILGRPVHAASQDFTVMGGSAGETQSTKVVETMEQAL LTGTPFLFFYDSGGARIQEGIDSLSGYGKMFFANVKLSGVVPQIAIIAGPCAGGASYSPALTDFIIMTK KAHMFITGPQVIKSVTGEDVTADELGGAEAHMAISGNIHFVAEDDDAAELIAKKLLSFLPQNNTEEA SFVNPNNDVSPNTELRDIVPIDGKKGYDVRDVIAKIVDWGDYLEVKAGYATNLVTAFARVNGRSV GIVANQPSVMSGCLDINASDKAAEFVNFCDSFNIPLVQLVDVPGFLPGVQQEYGGIIRHGAKMLY AYSEATVPKITVVLRKAYGGSYLAMCNRDLGADAVYAWPSAEIAVMGAEGAANVIFRKEIKAADD PDAMRAEKIEEYQNAFNTPYVAAARGQVDDVIDPADTRRKIASALEMYATKRQTRPAKKHGNFPC
mce sp|O58010 **Methylmalonyl-CoA epimerase** [Pyrococcus horikoshii DSM 12428]	MIWMFKRIDHVGIAVKNLEEAIKIWEGLGFKVEEIEEVPDQKVKVAVIKVGENRIELLEATTEDSPIAK FIEKRGEGIHHLAIRVENIESKLEELKQKGYKLIDEKPRVGAGGAKIAFIHPKSVTGVLLELCERKE

The succinate pathway involves the decarboxylation of methylmalonyl-CoA into propionyl-CoA though the methylmalonyl-CoA transcarboxylase key enzyme.

lcdA sp|G3KIM4 **lactoyl-CoA dehydratase α-subunit** [Clostridium propionicum DSM 1682]	MSLTQGMKAKQLLAYFQGKADQDAREAKARGELVCWSASVAPPEFCVTMGIAMIYPETHAAGIG ARKGAMDMLEVADRKGYNVDCCSYGRVNMGYMECLKEAAITGVKPEVLVNSPAADVPLPDLVIT CNNICNTLLKWYENLAAELDIPCIVIDVPFNHTMPIPEYAKAYIADQFRNAISQLEVICGRPFDWKKF KEVKDQTQRSVYHWNRIAEMAKYKPSPLNGFDLFNYMALIVACRSLDYAEITFKAFADELEENLKA GIYAFKGAEKTRFQWEGIAVWPHLGHTFKSMKNLNSIMTGTAYPALWDLHYDANDESMHSMAE AYTRIYINTCLQNKVEVLLGIMEKGQVDGTVYHLNRSCKLMSFLNVETAEIIKEKNGLPYVSIDGDQT DPRVFSPAQFDTRVQALVEMMEANMAAAE
lcdB sp|G3KIM3 **lactoyl-CoA dehydratase β-subunit** [Clostridium propionicum DSM 1682]	MSRVEAILSQLKDVAANPKKAMDDYKAETGKGAVGIMPIYSPEEMVHAAGYLPMGIWGAQGKTI SKARTYLPAFACSVMQQVMELQCEGAYDDLSAVIFSVPCDTLKCLSQKWKGTSPVIVFTHPQNRG LEAANQFLVTEYELVKAQLESVLGVKISNAALENSIAIYNENRAVMREFVKVAADYPQVIDAVSRHA VFKARQFMLKEKHTALVKELIAEIKATPVQPWDGKKVVVTGILLEPNELLDIFNEFKIAIVDDDLAQES RQIRVDVLDGEGGPLYRMAKAWQQMYGCSLATDTKKGRGRMLINKTIQTGADAIVVAMMKFCD PEEWDYPVMYREFEEKGVKSLMIEVDQEVSSFEQIKTRLQSFVEML

The acrylate pathway reveals the lactoyl-CoA dehydratase sequence to be most suitable as a marker for this pathway.

pduC sp|P37450 **Propanediol dehydratase large subunit** [Salmonella typhimurium ATCC 700720]	MRSKRFEALAKRPVNQDGFVKEWIEEGFIAMESPNDPKPSIKIVNGAVTELDGKPVSEFDLIDHFIAR YGINLNRAEEVMAMDSVKLANMLCDPNVKRSEIVPLTTAMTPAKIVEVVSHMNVVEMMMAMQ KMRARRTPSQQAHVTNVKDNPVQIAADAAEGAWRGFDEQETTVAVARYAPFNAIALLVGSQVG RPGVLTQCSLEEATELKLGMLGHTCYAETISVYGTEPVFTDGDDTPWSKGFLASSYASRGLKMRFTS GSGSEVQMGYAEGKSMLYLEARCIYITKAAGVQGLQNGSVSCIGVPSAVPSGIRAVLAENLICSSLD LECASSNDQTFTHSDMRRTARLLMQFLPGTDFISSGYSAVPNYDNMFAGSNEDAEDFDDYNVIQ RDLKVDGGLRPVREEDVIAIRNKAARALQAVFAGMGLPPITDEEVEAATYAHGSKDMPERNIVEDI KFAQEIINKNRNGLEVVKALAQGGFTDVAQDMLNIQKAKLTGDYLHTSAIIVGDGQVLSAVNDVN DYAGPATGYRLQGERWEEIKNIPGALDPNEID
pduD sp|O31041 **Propanediol dehydratase medium subunit** [Salmonella typhimurium ATCC 700720]	MEINEKLLRQIIEDVLRDMKGSDKPVSFNAPAASTAPQTAAPAGDGFLTEVGEARQGTQQDEVIIA VGPAFGLAQTVNIVGLPHKSILREVIAGIEEEGIKARVIRCFKSSDVAFVAVEGNRLSGSGISIGIQSKG TTVIHQQGLPPLSNLELFPQAPLLTLETYRQIGKNAARYAKRESPQPVPTLNDQMARPKYQAKSAIL HIKETKYVVTGKNPQELRVAL
pduE sp|O31042 **Propanediol dehydratase small subunit** [Salmonella typhimurium ATCC 700720]	NTDAIESMVRDVLSRMNSLQGDAPAAAPAAGGTS
pduP sp|B1VB75 **Propanal dehydrogenase** [Citrobacter freundii ATCC 700720]	MNTSELETLIRNILSEQLAPAKAEVKGNGIFPSVSEAIDAAHQAFLRYQQCPLKTRSAIINALREELTP HLASLAAESAAETGMGNKEDKFLKNKAALDNTPGIEDLTTTALTGDGGMVLFEYSPFGVIGSVAPS TNPTETIINNSISMLAAGNSVVFSPHPGAKAVSLKLITMIEDIAFRCCGIRNLVVTVTEPTFEATQQM MAHPKIAVLAITGGPGIVAMGMKSGKKVIGAGAGNPPCIVDETADLVKAAEDIINGASFDFNLPCIA EKSLIVVDAVAERLVQQMQSFGAMRLNSEEIDKLRAVCLPEGIANKQLVGKSPATLLEAAGIPVPAK APRLLIGIVKADDPWVTSEQLMPMLPIVTVSDFDSALTLALKVEEGLHHTAIMHSQNVSRLNLAAR TLQTSIFVKNGPSYAGIGVGGEGFTTFTIATPTGEGTTSARTFARSRRCVLTNGFSIR

The propanediol pathway reveals different genetic sequences suitable as markers for this pathway.

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Chapter 5: Prebiotics in practice: How to develop functional foods in regards to regulatory frameworks?

Previously, I described the evolution of the scientific concept of prebiotics **(Review 1)**. In particular, I have highlighted the fact that a consensus definition of prebiotics leads the agrifood sector in translational research into new functional foods. It has anchored the importance of characterising the impact of prebiotics on the gut microbiome and their consequences on human health and wellbeing. Hence, the RestorBiome project has evaluated the potential of food ingredients for their prebiotic effects. This work provided knowledge related to the most promising food ingredients, which hold a tremendous potential for the innovation process. Applied in the development of a functional food product, the transfer of technology can have a social and economic impact through commercialization. To anticipate the future prebiotic products and to evaluate the innovation concept of the RestorBiome project, I wondered how the development of functional foods can actually be applied in prebiotic practice.

This chapter gives an in-depth understanding of how regulatory agencies and policy makers in the US and Europe frame prebiotic applications. Gaining insights into regulatory considerations of these two jurisdictions allowed apprehending the viability of the development of a functional food product containing prebiotics.

The main content of this chapter is currently being prepared as an opinion paper.

Satisfying the development of functional foods containing prebiotics in regards to the regulatory frameworks

Bedu-Ferrari Cassandre[1,2], Langella Philippe[1], Cherbuy Claire[1,*]

[1]Micalis Institute, INRAE, AgroParisTech, Université Paris-Saclay, F-78350, Jouy-en-Josas, France
[2]Yoplait France - General Mills, Vienne Technical Center, F-38200, Vienne, France

*Corresponding author: claire.cherbuy@inrae.fr

THE GUT MICROBIOME: BUSINESS OPPORTUNITIES AND REGULATORY CHALLENGES

Over the past decades, the significant expansion of cutting-edge technologies and original approaches have brought deeper insights into the microbiome science in recognizing the importance of food-based diets to the overall health. As a key trend in the global market, digestive wellness creates business opportunities to meet the population needs, who are eager to take advantage of new foods with an added value beyond the appropriate nutritional effects (Mellentin, 2020). In the agrifood sector and the pharmaceutic industry, targeting the gut microbiome represents an innovative strategy to deliver beneficial effects through functional products, in a way that is relevant to achieve an optimal state of health and wellbeing and/or reduce the risk of disease (Diplock et al., 2000).

Based on the definition of functional foods, which is "a food or dietary component that can exert health benefits and/or disease prevention, beyond nutritional needs", prebiotics can be considered as dietary ingredients that may have biologically active properties implying health benefits. Indeed, prebiotics are usually non-digestible carbohydrates that are "a substrate that is selectively utilised by host microorganisms conferring a health benefit" (Gibson et al., 2017). Thus, prebiotics and other gut microbiome enhancers represent enablers emphasizing the potential for food ingredients to promote nutrition and health effects, although public awareness regarding prebiotics are still growing appreciably in comparison to probiotics. The realization of the benefits and technological properties of prebiotic ingredients offers a new dimension for functional nutrition and new prophylactic and therapeutic strategies targeting the gut microbiome (Scott et al., 2020). Being increasingly incorporated into a wide range of foods, beverages and topical products, the uptake of prebiotics into functional products holds tremendous potential for innovation (Cunningham et al., 2021).

The coordination between scientists from food and agricultural academia and industry and regulatory agencies is essential for developing effective and safe functional products and substantiating health benefits. Emphasizing the characteristics of their products, manufacturers can promote their sale through the use of labelling or claims. Nutrition and health claims are one tool that can encourage competition in the marketplace leading consumers in their purchasing decisions. To guarantee a high level of consumer protection, governmental authorities protect consumer health, safety, and economic interests prohibiting misleading labelling and misleading advertising. This review examines the promises and limitations of the development of prebiotic applications in human nutrition exploring the requirements of applicable statutes and regulations established in Europe and USA to unlock the growing potential of the functional food industry.

OVERVIEW OF REGULATORY FRAMEWORKS FOR FUNCTIONAL FOODS IN A WORLDWIDE PROSPECT

Historically, Japan acted as the first global market leader in functional foods by conducting systematic research to highlight the nutritional and functional properties of dietary ingredients (Shimizu, 2003). To certify and label a health statement, the Ministry of Health, Labour and Welfare (MHLW) established in 1991 the regulatory system of Foods for Specified Health Use (FOSHU) under the Health Promotion Law. FOSHU defines foods with officially approved health claims based on scientific evaluation of the Council of Pharmaceutical Affairs and Food Hygiene in collaboration with many relevant bodies including consumers, food business operators and other stakeholders from various fields. According to the physiological effects on the human body, FOSHU also refers to foods for special dietary uses. For instance, most FOSHU-approved products promote the intestinal health. Other health claims involve blood sugar and blood pressure controls, cholesterol and triglyceride reductions, tooth care and mineral uptake (Iwatani and Yamamoto, 2019).

Together with Japan, the USA adopted three legislative acts in the 1990s regulating the concept of functional foods. The Food and Drug Administration (FDA) provides the legal authority for the Department of Health and Human Services to establish the Nutrition Labeling and Education Act (NLEA) introducing some regulatory requirements and procedures for labeling most foods. In addition, the Dietary Supplement Health and Education Act of 1994 (DSHEA) encouraged research on dietary supplements to support "reduction of disease risk or health-related condition" claims. Since 1997, functional foods have been regulated under the authority of the Federal Food, Drug, and Cosmetic Act (FD&C Act) and its subsequent amendments codified into Title 21 of the United States Code (USC). As biologically-based products, functional foods may not be subjected solely to this regulation. Not part of the FD&C Act, FDA develops other regulatory frameworks found in the Code of Federal Regulations (CFR) Title 21, which governs foods and drugs within the US. The statute of the regulatory product category is mainly based on the product intended uses, which include foods, dietary supplements or drugs, and on the claim associated with the products (Research, 2006).

The European Union (EU) strictly frames nutrition applications to guarantee free trade in the European market and the same level of protection to citizens throughout Europe. To ensure the safety and quality of food products, the European Commission (EC) provides labelling and standards regarding ingredients, additives, supplements and improvement agents, nutrition and health claims, and new food products. The member states are responsible for verifying the compliance of the food manufacturers with EU legislation and can adopt their own legislation to the extent that the EU has not

exercised its competence. Independently, the European Food Safety Authority (EFSA) is mandated since 2002 to assess and communicate all risks associated with the food chain in the EU. The EFSA influences the process of adoption or revision of European legislation and controls its respect at the request of European Commission and the European Parliament or on its own initiative. The EFSA provides administrative and scientific guidance for food manufacturers and verifies the scientific substantiation of health claims to authorize functional foods.

Interestingly, the statutes of a functional foods applied to prebiotics are not specifically mentioned in these regulatory frameworks. In Japan, components displaying prebiotic effects (oligosaccharides and dietary fibres) and probiotics (bifidobacteria and lactobacilli) are main representative of FOSHU-approved products classified into foods related to improving gastrointestinal conditions. Likewise, prebiotics are not specifically defined by law in the USA. Products displaying prebiotic effects are categorized into the biologically-based practices on complementary and alternative medicine products as dietary supplements, foods or drugs depending on the intended use (Scott et al., 2020). The same observation in Europe: prebiotics are neither covered nor mentioned by the regulatory frameworks. Although prebiotics do not have a statutory definition, they are generally deemed "*non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already established in the colon, and thus in effect improve host health*" according to the definition provided by Gibson and Roberfroid (1995).

INTERNATIONAL COORDINATION ON PREBIOTIC STATUTE

Before the international scientific community defined the prebiotic concept in the 1990s (Gibson and Roberfroid, 1995), dietary ingredients displaying physiological health benefits were subjected to regulatory requirements for functional foods. In 2007, the status of prebiotic was internationally recognized by the Food and Agriculture Organization (FAO) of the United Nations (Pineiro et al., 2008). The definition encompasses different criteria to put forward for any functional foods containing an ingredient associated with the modulation of the gut microbiome. These criteria include the characterisation of the prebiotic ingredient, the functionality of the prebiotic-containing food product, and the safety assessment of the prebiotic in the final product formulation. First, the characterisation of a prebiotic, mostly food-grade substances, should encompass the source, the origin, the purity, the chemical composition, the structure, and the stability of compounds into different food matrices. Impurities and/or contaminants should be declared out of concern for consumer safety. Any prebiotic-containing food product would specify the vehicle, the concentration, and the amount in which it is to be delivered to the host. Then, the functionality of prebiotic-containing food products should rely on

the measurable health benefit on the host without any adverse effects, and the effect should not be due to absorption of the component into the bloodstream or acting alone (i.e. drug). The prebiotic functionality is specifically based on the correlation between physiological outcomes in timeframe and the modulation of the gut microbiome in a specific intestinal compartment. Determining the events that occur within the compartments of the intestine is often difficult. The presence of a prebiotic component into a food formulation modulates the gut microbiome composition or activities through mechanisms including fermentation, receptor blockage or others. Targeted variables should be statistically significant, and the change should be biologically meaningful: scientific evidence should be feasible, valid, reproducible, sensitive, and specific for the substantiation of a possible health claim. To qualify the health claim, a prebiotic should affect the gut microbiome beyond bifidogenic effects, which are not sufficient to demonstrate physiological health benefits. Finally, the safety assessment of a prebiotic should cover the final product formulation. Scientific evidences should strengthen the safety assessment regarding the consumer, the user and the environment. Likely, safe consumption levels with minimal symptoms and side effects should be determined. Therefore, dose and duration for nutrition purposes should be better defined, and the effects related to gut microbiome modulations should not suggest long-term detrimental effects on the host. For instance, the optimal effect-response for inulin-type fructans (ITF) can be observed with dosage levels ranging from 3g/day to 8g/day although more may be consumed according to individual tolerance (Anadón *et al.*, 2010). Nevertheless, there are concerns over increased gas production especially detrimental for consumer showing fructose intolerance. Prebiotic ingredients with a history of safeness suggest that further animal and human toxicological studies may not be necessary. Noting prebiotics are often fibre-derived ingredients, they can be considered safe for the environment due to their natural origin, high biodegradability and low ecotoxicity. Actually, no additional scientific research is needed with regard to this point.

This broad FAO/WHO definition of prebiotics allows flexibility in terms of sources and health claims. Further criteria are required to evaluate the quality of food with health claims under legislation at national/international levels. Despites the expanded use of prebiotic ingredients across the world, there is an international regulation but not yet international guidelines for the development and the application of prebiotics. The regulatory framework protects consumers from potential health risks and misleading information, promotes innovation, and ensures fair competition in the food business. Standardization of prebiotic legislation seems difficult to operate considering the complexity of regulatory systems at national and international levels.

THE EUROPEAN UNION (EU) REGULATIONS AND DIRECTIVES

As a concept rather than specific food-derived categories, prebiotics do not have a recognized statute in the EU legislation and must meet general food laws. Depending on the product intended uses, prebiotics may align with Regulations and Directives for novel foods, nutrient sources as food supplements and fortified foods, nutrition and health claims and foods for specific categories **(Figure 1)**. The introduction of novel foods was first governed in 1997 by Regulation (EC) 258/1997 covering any food that was not used for human consumption to a significant degree within the Union before the amendment (European Parliament and Council of the European Union, 1997). Subsequently, the regulatory framework pursuant to Regulation (EU) 2015/2283 and amendments on novel foods and novel food ingredients currently supports the administrative and scientific guidance for EFSA authorization **(Table I)** (European Parliament and Council of the European Union, 2015). Displaying prebiotic effects, oligosaccharides from galactans (GOS) and fructans (FOS) are authorized as safe food ingredients under EU novel food regulations, since they have a safe history consumption. Prebiotic ingredients created after 1997 are considered novel, and thus require safety clearance in the EU within the novel food legislation. Mostly deriving from dietary fibres or phytochemicals, prebiotic ingredients can also be under the regulatory framework for nutrient sources **(Table II)**. Directive 2002/46/EC and subsequent amendments harmonize the supplementation of concentrated source of nutrients such as vitamins and minerals intended as food supplements (European Parliament and Council of the European Union, 2002). Having regard this Directive, Regulation (EC) 1925/2006 and amendments include more nutrients or ingredients, emphasising particular nutritional characteristics or physiological effects in order to "enrich" or "fortify" the food through the addition of vitamins, minerals including trace elements, amino acids, essential fatty acids, fibres, and various plants and herbal extracts (European Parliament and Council of the European Union, 2006b). In addition to food supplements and enriched/fortified foods, prebiotics can also aim to satisfy particular nutritional requirements of specific categories of the population, also called “dietetic foods” or “dietary foods”. Regulation (EC) 609/2013 and subsequent amendments set specific rules for infant formulae and follow-on formulae, foods for infants and young children (baby foods), foods for special medical purposes (medical foods) and foods for weight management (slimming foods) **(Table III)** (European Parliament and Council of the European Union, 2013). To complete the regulatory framework, the EFSA provides guidance for administrative submissions and scientific evaluation of substances and sources of nutrients added for specific purposes in the food products. In order to label nutritional and health allegations of foods, Regulation (EC) 1924/2006 and subsequent amendments rule out any claims displayed on the food labeling at the

European level **(Table IV)** and ensure that presentation or advertising is clear, accurate, and relied on a science-based approach (European Parliament and Council of the European Union, 2006a). Prebiotics as dietary fibres may be associated with authorized nutrition claims among which "source of fibre" or "high fibre" in compliance with the conditions established in the Annex (European Commission, 2016). An EU Register gathers permitted nutrition claims and authorized health claims made on labelled food for diverse applications (EU Register of nutrition and health claims made on foods).

In conjunction with Regulation (EC) 1924/2006, the EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) provides scientific and technical guidance for the authorization procedures of an application for nutrition and health claims (EFSA Panel on Dietetic Products, 2011; EFSA Panel on Dietetic Products et al., 2017). To deliver the scientific opinion of EFSA NDA Panel, the application for the health claim must contain information on the characteristics of the food/constituent such as composition, physical and chemical characteristics, manufacturing process, stability and bioavailability. The description must contain a proposal of the intended claim relevant for human health including the specific conditions of use, the target population, the quantity of the food/ingredient and pattern of consumption required to achieve the effect as part of a balanced diet, and any health risk or restriction of use. The application must report the totality of available scientific data to support the health claim, including the beneficial mechanism(s) of interaction between a functional component within a food matrix and one or more functions targeted in the body. The identification and the validation of markers of exposure and biological responses are required to assess the safety of the amount of food or its component(s) needed for functionality. In particular, evidence in human food trials should demonstrate the establishment of a cause-and-effect relationship (strength, consistency, specificity, dose-response, biological plausibility) related to the functional food/ingredient. These scientific requirements aim to substantiate the nutrition and health claims made on food with clear and accurate scientific evidence and/or include a request for the protection of proprietary data. The first digestive health claims attributed to fibres displaying prebiotic effects were authorized for their contribution to the normal bowel function due to their osmotic effect and their ability to reduce blood glucose rise after their consumption, as long as fibres are neither absorbed nor fermented into the gastrointestinal tract. In fact, fibres regulate bowel movement and regularity by increasing faecal bulk and reducing intestinal transit time. This property can be used to alleviate constipation. To date, the only health claim accepted within the EU for prebiotic application relates to chicory native inulin that contributes to the maintenance of normal bowel function by increasing the frequency of stool. The claim can be used only for food, which provides at least a daily intake of 12g of native chicory inulin. A second more general

health claim related to prebiotic states that 'non-digestible carbohydrates contribute to a reduction in post-prandial glycaemic response' (EFSA NDA Panel 2015).

Other EU health claim applications for prebiotics have been unsuccessful. The "healthy digestion" claim is currently not sufficiently defined and refers to aspects of promoting the growth of specific bacteria/groups and decreasing pathogenic intestinal microorganisms. The changes in the gut microbiome composition is not in itself considered as a beneficial physiological effect. Many prebiotic applications constitute non-authorized health claims, though scientifically evidence is valuable **(Supplementary Table I)**. The non-compliance with the current regulatory framework applicable is on the basis of scientific evidence. Often, a claimed effect for a food is insufficiently characterised for a scientific assessment, and therefore the claim cannot be substantiated. The EFSA has repeatedly expressed an insufficient characterisation and a weak correlation between their consumption and the claimed effect, which has not been recognized as a beneficial physiological effect. For instance, the prebiotic effect linked to an increasing number of intestinal microbes is not deemed beneficial physiological effect as defined and required by the Regulation (EC) No 1924/2006. The correlation between the number of intestinal microbes and health is not sufficiently characterised for a scientific assessment of the claimed effect to be completed (EFSA Panel on Dietetic Products, 2011).

UNITED STATES FOOD AND DRUG ADMINISTRATION (FDA)

Although prebiotics are not specifically recognized by US regulatory systems, ingredients that show physiological health benefits can be subjected to regulatory requirements for dietary fibres, if applicable. Indeed, the FDA has included since 2016 a definition of dietary fibres in Regulation 21 of the Code of Federal Regulation (CFR). Under the categories of conventional foods and dietary supplements, nutrients displaying prebiotic effects are mentioned into guidance to satisfy the requirements of applicable statutes and regulations (Nutrition, 2016). Naturally occurring fibres, often referred to as intact and intrinsic fibres, have been shown to be beneficial, and manufacturers do not need to demonstrate physiological benefits to human health provided by fibres from vegetables, whole grains, fruits, cereal, bran, flaked cereal and flours. In addition to intact and intrinsic fibres, FDA published a request for scientific data, information, and comments to determine whether certain isolated or synthetic non-digestible carbohydrates should be added to the definition of "dietary fibre". In 2018, a scientific review allowed to list accepted and candidate dietary fibres with health claims supported by scientific evidence (Nutrition, 2018) **(Table V)**. Accordingly, the definition of dietary fibres was updated in 2020 and now is defined as "*non-digestible soluble and insoluble carbohydrates (with 3 or more monomeric units), and lignin that are intrinsic and intact in plants; isolated or synthetic non-*

digestible carbohydrates (with 3 or more monomeric units) determined by the FDA to have physiological effects beneficial to human health" (Nutrition, 2020).

A final guidance for industry "Scientific Evaluation of the Evidence on the Beneficial Physiological Effects of Isolated or Synthetic Non-digestible Carbohydrates Submitted as a Citizen Petition in 21 CFR" was released about how FDA intends to evaluate scientific evidence (Nutrition, 2020). For instance, to determine whether a non-digestible carbohydrate meets the dietary fibre, FDA intends to consider, as part of the scientific evidence, clinical studies conducted on individuals diagnosed with a specific disease or at risk of developing a disease or who have an unrelated disease that would not affect the physiological endpoint being measured. Forasmuch as the mechanisms of action associated to a dietary fibre would not be different in the healthy or diseased populations, FDA intends to consider appropriate extrapolation of the scientific evidences to the healthy individuals.

In addition, the list of dietary fibres can be amended by submitting a citizen petition to request an amendment to the dietary fibre definition or by involving the petition process for the authorization of a health claim. As nutrients or dietary ingredients intended to affect the health and wellbeing of the human body, prebiotics can be based on health claims characterizing the relationship of any substance to a disease or health-related condition, and structure-function claims alleging the role of a particular substance in normal structure or functions in humans. Health claims must be preapproved by the FDA or must be issued as authoritative statements by an agency of the US government with responsibility for dietary guidance or public health. Likely, a health claim is allowed without FDA approval whether i) it is subjected to health claim petition requirements and filed review of regulation 21 CFR; ii) it is consistent with the weight of the scientific evidences; iii) the consumer health and safety are not threatened; iv) it meets the general requirements for health claims at 21 CFR. For instances, health claims alleviating a classical nutrient deficiency disease (prevalent in US) or related to general wellbeing from consumption of the food ingredient are not subjected to FDA approval. Other legal requirements can support health claims for foods in humans including long-standing traditional use, ethnomedical uses, case reports, and clinical or human volunteer trials. It should be noted that animal studies and in vitro experiments would not adequately support a health claim in humans. The qualification relies on the strength of the scientific evidence rated according to the quantity of studies, consistency, and relevance supporting a health claim. A health claim can be non-authorized by FDA whether evidence does not meet the significant scientific agreement requirements. A claim can be judged not conclusive or limited and not conclusive or with little scientific evidence supporting the claim. Interestingly, prebiotics are not considered by the regulatory framework to provide beneficial physiological effects

to human health. "*Fermentation and changes in the microbiota in the large intestine are considered to be processes, rather than physiological effects. Viscosity and fermentation of non-digestible carbohydrates are considered to be physicochemical properties, rather than physiological effects*". Prebiotic effects may be an outcome of viscosity, fermentation, or changes in the colonic microbiota. However, they are not measures of functions of the human body.

TOWARDS THE NEXT PREBIOTIC INNOVATIONS: HOW TO MODERNIZE THE LEGISLATION FRAMEWORKS?

Exploring regulatory frameworks for functional foods applied to prebiotics has highlighted the lack of legislative statutes. In the US and EU strict policies on nutrition applications, prebiotics are not specifically mentioned. Classified in different food categories across countries and regions, prebiotic-containing functional foods may vary according to different systems, as there is no legal definition for prebiotics. It implies that prebiotics in practice can refer to distinct appropriate regulations, guidelines and administrations according to their application scope. In particular, the intended usage can distinguish functional foods, nutraceuticals or pharmafoods and pharmaceutics **(Figure 2)**. The regulatory statutes of such products is still uncertain and participates to the lack of prebiotic applications in the market. Behind the lack of legal frameworks for prebiotic applications, we highlighted in this review similar regulations, but their legislative disparities create an interesting perspective for functional food products.

Relevant to prebiotic applications, there are different categories of health and nutrition claims. In both regulations, nutrient claims suggest that food has benefits by characterizing the level of a particular nutrient. Discrepancies appear in the definitions of health claims. In the EU, health claims establish the link between a nutrient and the health. They are submitted to the control of EFSA. Although many health claims are often considered as unsubstantiated, authorized claims are supported by science proven in healthy persons via clinical trials. On the contrary, in the US, health claims relate a substance to a disease or a related condition. The biggest concern with the FDA regulations is how it limits the claims to solely mention the correlation with the reduction of diseases. Therefore, health claims are distinguished from function/structure claims, which is important to highlight the greater capacity than the decrease in risk factor disease that the regulation of health claims allows (Martirosyan and Singharaj, 2016). US can have function/structure claims, which are not submitted to FDA authorization. These latter have different regulatory requirements that are easier to meet than health claims. Making a difference between function and health claims may pose a problem for food manufacturers. The distinction between function and health claims surely avoid abuses of misleading consumers. These

disparities highlight the lack of harmonized frameworks for health claims. The expanded consideration of regulatory standards related to functional foods and their internationalization enacted in harmony with the Codex Alimentarius are based on the growing public health concerns related to eating habits and lifestyle-related diseases and the advances in food science and technology that led to the development of new foods with more complex and diverse functions. In the future, the development of an international standardization that integrates prebiotic-containing functional foods could be used as a reference to resolve conflicts regarding food safety, consumer protection and the global market. To better frame their scope of application, regulations are essential to avoid the commercialisation of food products and supplements with unsubstantiated claims that can emphasize health and beauty enhancement (Scott et al., 2020).

Meeting the requirements for governmental approval, nutrition and health claims are protective of consumer rights. Strict policies provide legal certainty regarding the features, contents and usage of particular foods. Authorized labelling yields appropriate nutritional information to orientate increase the awareness and interests of consumers in making healthier food choices. Nutrition and health claims are one tool that can incentivize competition in the marketplace. However, in any regulatory framework, the interpretation of consumers is not considered. Consumers should remain critical of positive messages promoting healthy diets and food products. A modernizing nutrition and health claims to make marketing messages accessible to the consumers may discredit the substantiated nutrition and health claims reducing the transparency of the functional food. This observation supports the gap between scientific recognition and regulatory acceptance. An international regulatory framework could participate to modernize and control the use of claims in labelling. The aim of such update is to encourage the food industry to (re)formulate products to improve their healthy nutritional qualities and to empower people with information towards healthy food choices that are the most meaningful with respect to public health concerns to offer food groups for which American diets typically fall short of recommendations (Martirosyan and Singharaj, 2016).

The regulatory systems frame the strategies of innovation. The health promotion through of the gut microbiome represents a high potential for digestive health and wellness nutrition trend. Innovation strategies mining the gut microbiome through technological enablers such as probiotics, prebiotics, synbiotics, postbiotics, and fermented foods constitute a tremendous business opportunity. Nevertheless, the complexity of intestinal microbial populations constitute a challenge to achieve the prebiotic definition and criteria previously settled by the scientific community (Gibson et al., 2017). Many opportunities exist to allow researchers to elucidate the prebiotic functionality and their

underlying mechanisms (Spacova et al., 2020). Explosion of research in recent years into the links between the gut microbiome and host health have strengthened the evidences for their health benefits. A new evolution of the concept of prebiotics might be considered in the following years to focus more on the physiological benefits of the prebiotic and the bioactive substance. Although scientific evidences on the effects of isolated individual components are not sufficient, considering a pharmaco-ecological approach for prebiotic research might be a solution to encourage the R&D of prebiotic-containing functional foods and to modernize the regulatory systems.

CONCLUSION

The prebiotic concept evolved along with scientific research progress, consumer interest, and technological innovations from industrial scientists. The definition leads the agrifood and pharmaceutical sectors and stakeholders, allowing translational research into novel functional foods and new prophylactic and therapeutic interventions. The definition is also useful for regulatory agencies and policy makers in developing statutes and regulations, for suppliers or manufacturers to accurately characterise prebiotics and health benefits, and for end-product manufacturers to formulate and label prebiotic products. In prebiotic practice, the regulatory environment heavily influences the development of functional foods. To date, there is no international coordination on the statute of functional foods applied to prebiotics. In particular, the European and North American regulatory frameworks do not specifically mention prebiotic applications. The intended use of a product plays a central role in how it is regulated. Emanating from the public authorities, nutritional messages raise public awareness of prebiotics, emphasising the potential for nutritional and health claims. The major implementation in incorporation of prebiotics into nutrition and healthcare should encourage an international harmonization of regulatory approaches. The current disparities in the regulations in US and EU should converge toward frameworks adapted with future upcoming science and technologies. Regulatory frameworks should remain up-to-date for subsequent integration into policy, practice, and lifestyle for the protection of public health.

AUTHOR CONTRIBUTIONS

CBF wrote the first draft of the manuscript.

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CONFLICT OF INTEREST

The authors declare that CBF has worked as an employee of Yoplait France – General Mills during the conduct of this study, as part of a CIFRE contract. The company had no role in the writing of this article or the decision to submit it for publication. The other authors declare no competing interests.

FIGURES & TABLES

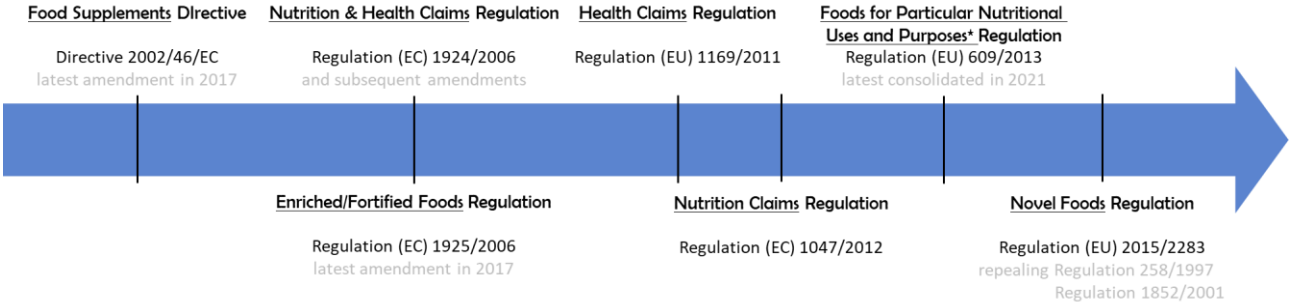

Figure 1: EU regulations frame nutrition applications of prebiotics. **Foods for specific categories are intended as infant formulae & follow-on formulae, specific medical purposes, and total diet replacement for weight management.*

Table I: Regulatory framework on novel foods and novel food ingredients in the European Union

Novel foods	
Regulation (EC) 258/1997	On novel foods and novel food ingredients
Regulation (EC) 1852/2001	On detailed rules for making certain information available to the public and for the protection of information
Regulation (EU) 2015/2283	On administrative requirements for the preparation and the submission of applications for authorization of a novel food
	On scientific requirements for the preparation and presentation of an application for authorization of a novel food and traditional foods from third countries
Regulation 2017/2468	On administrative and scientific requirements for traditional foods from third countries
Regulation 2017/2469	On administrative and scientific requirements for novel foods applications
Regulation 2017/2470	On establishing the Union list of novel foods

Table II: Regulations and Directives on nutrient sources in the European Union

Nutrient sources	
Directive 2002/46/EC	On the approximation of the laws of the Member States relating to food supplements
Directive 2006/37/EC	On the inclusion of certain substances
Regulation (EC) 1925/2006	On addition of vitamins and minerals and of certain substances to foods
Regulation (EC) 1170/2009	On the lists of vitamin and minerals and their forms that can be added to foods, including food supplements
Regulation (EC) 953/2009	On substances that may be added for specific nutritional purposes in foods for particular nutritional uses
Regulation (EU) 1161/2011	On the list of mineral substances that can be added to foods
Regulation (EU) 307/2012	On the addition of vitamins and minerals and of certain other substances to foods
Regulation (EU) 609/2013	On food intended for infants and young children, food for special medical purposes, and total diet replacement for weight control
Regulation (EU) 119/2014	On chromium-enriched yeast used for the manufacture of food supplements and chromium III lactate tri-hydrate added to foods
EC list	On authorised nutrient sources

Table III: Regulations and Directives on foods for specific categories (EFSA)

Foods for specific categories	
Directive 2006/141/EC	On infant formulae and follow-on formulae
Regulation (EU) 609/2013	On food intended for infants and young children, food for special medical purposes, and total diet replacement for weight control
Regulation 2016/127	As regards the specific compositional and information requirements for infant formulae and follow-on formula and as regards requirements on information relating to infant and young child feeding

Table IV: Regulations and Directives on nutrition and health claims in the European Union (EFSA)

Nutrition and health claims	
Regulation (EC) 1924/2006	On nutrition and health claims made on food on nutrition and health
Regulation 353/2008 Regulation (EC) 1169/2009	On applications for authorization of health claims
Regulation (EC) 1169/2011	On the provision of food information to consumers
Regulation 432/2012	List of permitted health claims made on foods other than reduction of disease risk and to children's development and health
Regulation (EC) 1047/2012	On the list of nutrition claims
Decision 2013/63/EU	Adopting guidelines for the implementation of specific conditions for health claims
EU register	Nutrition and health claims made on food

Table V: List of non-digestible carbohydrates listed as dietary fibres that can be declared on the Nutrition and Supplement Facts labels. The candidate fibres noted with an asterisk have recently been proposed by the FDA. *RS stands for resistant starch*

Dietary fibres with health benefits	Candidates
Beta-glucan soluble fibre	Mixed plant cell wall fibres (a broad category that includes fibres such as sugar beet fibre, sugar cane fibre and apple fibre, among many others)
Psyllium husk	Arabinoxylan
Cellulose	Alginate
Guar Gum	Inulin and inulin-type fructans
Pectin	High amylose corn/maize starch (RS type 2)
Locust bean gum	Retrograded corn starch (RS type 3)
Hydroxy-propyl-methyl cellulose	Resistant wheat and maize starch (RS type 4)
	Galacto-oligosaccharides (GOS)
	Polydextrose
	Resistant maltodextrin/dextrin
	Cereal fibres extracted from oat, wheat and corn
	Legume fibres extracted from pea and soy
	Xanthan Gum
	Xylo-oligosaccharides (XOS)
	Cross linked phosphorylated RS type 4*
	Glucomannan*

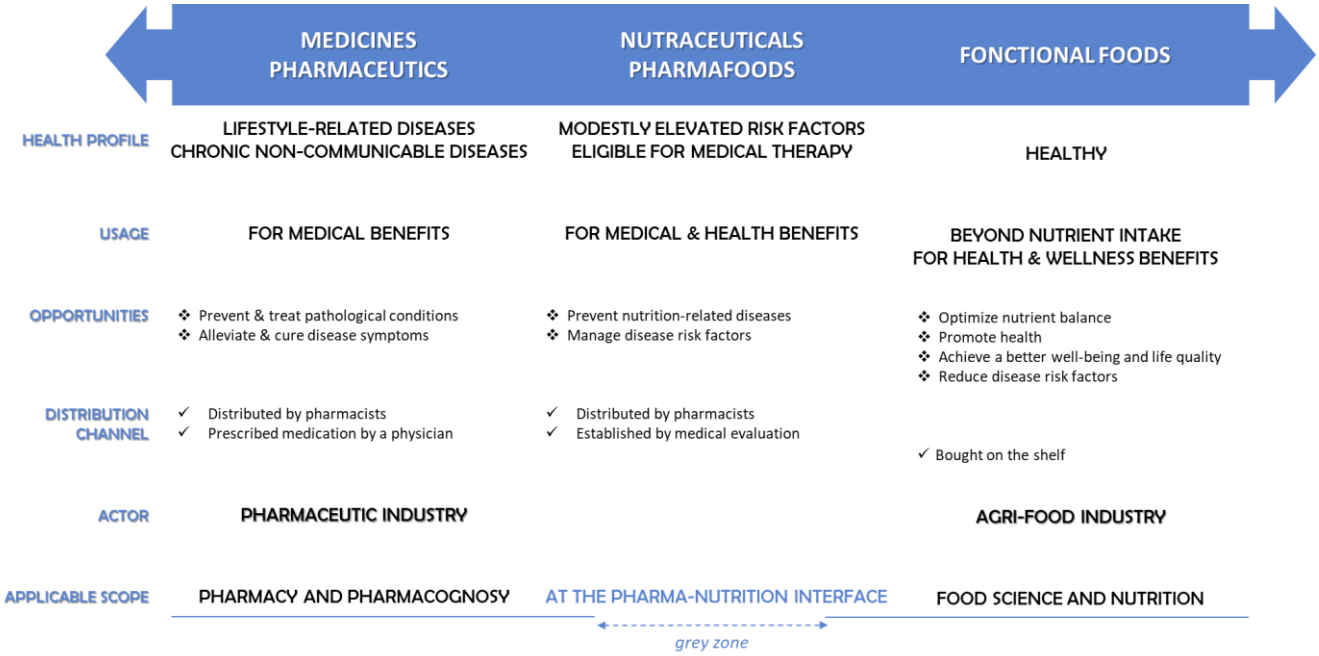

Figure 2: Applicable scopes of functional products. *Functional foods do not intend to diagnose, treat, cure, mitigate or prevent any disease, which is the definition of a medicine functional product.*

SUPPLEMENTARY DATA

Supplementary Table I: Non-exhaustive list of non-authorized claims and health relationship for prebiotics under the EU legislation (EU Register of nutrition and health claims made on foods)

Nutrient, substance, food or food category	Claim **Health relationship**	**EFSA opinion Commission regulation**
Prebiotics	Beneficially affects the intestinal flora Stimulates the growth of the good/beneficial gut flora Positively affects the intestinal health	2011;9(6):2244
GOS	Has a prebiotic effect Promotes growth of healthy organisms Stimulates the growth of bifidobacteria Helps maintain a healthy balance of beneficial bacteria Promotes the positive balance of the intestinal flora Maintains a healthy intestinal environment Maintains a healthy gut **Decreases potentially pathogenic gastrointestinal microorganisms** **Increasing numbers of gastrointestinal microorganisms**	2010;8(10):1809
Inulin/FOS	Has a prebiotic effect Promotes the growth of healthy gut bacteria Has bifidogenic effect Promotes good digestive health Promotes gastrointestinal/bowel/gut/colonic health	2011;9(6):2244
Inulin/FOS	Affects the intestinal flora Stimulates the growth of Bifidobacteria in the colon Promotes healthy gut bacteria Promotes a healthy bowel function Promotes a better intestinal flora supporting body's defence system Supports natural defence	2011;9(6):2044
FOS Oligofructose from sucrose	Has prebiotic effect Stimulates the growth of bifidobacteria in the colon Beneficially affects the intestinal flora Promotes healthy/good/balanced gut bacteria **Decreasing potentially pathogenic gastrointestinal microorganisms**	2011;9(4):2023
FOS Oligofructose from sucrose	Has prebiotic effect Stimulates the growth of Bifidobacteria in the colon Promotes growth of healthy organisms Stimulates the growth of good digestive bacteria Has bifidogenic effects Beneficially affect the intestinal flora Stimulates the growth of beneficial bacteria in the intestine Contributes to a better balance of the intestinal microflora Support the vitality of healthy gut flora **Decreasing potentially pathogenic gastrointestinal microorganisms** **Increasing numbers of gastro-intestinal microorganism**	2010;8(10):1809
Polydextrose	Has prebiotic effect Stimulates the growth of beneficial bacteria in the gut Stimulates the growth of Bifidobacteria in the colon Stimulates the growth of Lactobaccilli bacteria in the gut Promotes a healthy/well-balanced gut flora **Decreasing potentially pathogenic gastrointestinal microorganisms**	2011;9(6):2256
Glucomannan (Konjac)	Has a prebiotic effect Helps to restore the intestinal flora Helps to stimulate the growth of beneficial colon bacteria Helps to stimulate the growth of Bifidobacteria **Decreasing potentially pathogenic gastrointestinal microorganisms** Helps to control/ balance blood insulin/glucose level Can help to reduce the glycemic index of a meal Has beneficial effects on blood glucose and blood insulin level **Reduction of postprandial glycaemic responses**	2010;8(10):1798

Nutrient, substance, food or food category	Claim **Health relationship**	**EFSA opinion Commission regulation**
Partially Hydrolysed Guar Gum (PHGG)	Has a prebiotic effect Promotes the growth of beneficial intestinal bacteria (probiotics) that are important for the maintenance of the natural digestive balance Supports the natural, beneficial microflora Contributes to proper digestive function by regulating the microflora and short chain fatty acid production Helps promote healthy intestinal flora to support bowel function Helps maintain digestive balance by promoting "good bacteria" levels Helps to restore and maintain your natural digestive balance by stimulating the growth of beneficial intestinal flora Acts as a prebiotic to naturally nourish the beneficial bacteria found in your digestive system Stimulates the development of the intestinal flora Helps to maintain the natural balance of the intestinal flora Has a gentle, effective and progressive action for good development of the intestinal flora **Decreasing potentially pathogenic gastrointestinal microorganisms**	2011;9(6):2254
Xylo-oligosaccharides	Has prebiotic effect Stimulates a healthy intestinal flora Stimulates the growth of bifidobacteria **Increasing numbers of gastro-intestinal microorganisms**	2011;9(6):2228
Resistant starch- type 2 (RS) from high amylose maize	Delivers prebiotic activities Promotes a healthy digestive system. **Digestive health benefits**	2011;9(4):2024
Wheat Dextrin	Is fermented in the gut leading to the production of the beneficial SCFA Helps promote colon health Nourishes the digestive tract, where 70% of immune function occurs Stimulates the microflora to restore and maintain digestive system's healthy balance Stimulates the microflora (prebiotic effect) Stimulates the SCFA production in the bowel **Decreasing potentially pathogenic gastrointestinal microorganisms** Increases fibre intake helps maintain digestive health Promotes healthy functioning of your digestive system Helps your natural bowel regularity Helps to restore normal digestive health **Maintenance of normal bowel function** Helps to improve the absorption of calcium and magnesium; two minerals that are essential for healthy muscles and bones Improves magnesium and calcium absorption and retention Helps increase the absorption and retention of certain vital nutrients Promotes overall good health **Increasing in magnesium and/or calcium retention**	2010;8(10):1761
Lactulose	Has prebiotic effect Helps the development of the intestinal flora Has bifidogenic effects Stimulates the growth of Bifidobacteria in the colon Promotes healthy/balanced/good gut bacteria Supports healthy gastrointestinal flora Promotes healthy gut bacteria or microflora Promotes good digestive health Promotes gastrointestinal/bowel/gut/colonic health Promotes proper/healthy bowel function Supports healthy gastrointestinal flora Improves intestinal conditions Promotes intestinal health Promotes healthy conditions in the colon Improves digestive/bowel function Improves digestive/gut comfort **Decreasing potentially pathogenic gastrointestinal microorganisms**	2010;8(10):1806

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GENERAL DISCUSSION

It has become clear that diet influences the human gut microbiome. The incorporation of food ingredients with prebiotic potential into functional foods offers one avenue to successfully nourish the gut microbiome and maintain human health and wellbeing. A better description of the carbohydrate utilisation at the organismal and molecular levels of health-relevant commensal bacteria can help to disentangle their dynamic interactions with the gut microbiome. Thus, a better understanding of the carbohydrate breakdown in the gut can participate to tailor prebiotic interventions in order to reduce the incidence of metabolic-related diseases, including obesity, diabetes, irritable bowel syndrome, and potentially many others **(Review 1)**.

Experimental approaches to explore the metabolic interactions between prebiotics and the gut microbiome

Our relationship with the gut microbes is the product of close coevolution through thousands of successive generations (Foster et al., 2017). This study supported the hypothesis of an evolutional adaptation of the dominant phylum of *Bacteroidetes* for carbohydrate metabolisation. These commensal bacteria of the human gut microbiome are characterised by metabolic flexibility in adapting to changes in complex carbon sources. Likely, their large genomic repertoires encoding CAZymes allow them to switch readily between nutritional sources in the gut environment. *B. thetaiotaomicron*, *B. vulgatus*, *B. cellulosilyticus* and *B. ovatus* have constitute a bacterial paradigm for the investigation of carbohydrate metabolism (Lombard et al., 2014). However, to disentangle the complexity of the intestinal ecosystem, the characterisation of bacterial growth and metabolic activities of taxonomically diverse commensal bacteria of the human gut microbiome may enrich our general knowledge. This project was based on a panel of health-relevant bacteria established on a bibliographic research strategy of gastrointestinal disorders, including MetS and IBS. Beyond health and disease states, studying the biodiversity of the intestinal ecosystem with an emphasis on microbial conservation provides another research strategy to extend the panel of health-relevant bacteria (Olm and Sonnenburg, 2021). Placing the human microbiota in the context of our evolutionary history, a recent study suggested that the commensal *Butyrivibrio crossotus* was more prevalent in the ancient and non-industrial gut microbiomes (Wibowo et al., 2021). This bacterium can potentially contribute to the safeguarding of human health (Sonnenburg and Sonnenburg, 2019). Maintaining and/or restoring present-day gut microbiomes based on ancestral state can be an alternative research strategy for understanding the relationship between microbes and human health and nutrition.

In microbiome research, molecular approaches have provided instrumental breakthroughs. However, the limitations of bioinformatic tools have emphasised a renewed interest in cultivation-based approaches (Hitch et al., 2021). Innovative high-throughput approaches are developed and used broadly, although the large fraction of yet uncultured anaerobic gut microbes remain a laborious hurdle. In this context, the "culturomic" approach has helped recover novel taxa from the human gut (Lagier et al., 2016; Thomas and Segata, 2019; Afrizal et al., 2022). The major advantage of cultivating gut bacteria is to understand mechanisms underlying microbe–microbe and microbe–host interactions. For example, model organisms and/or minimal synthetic consortia that mimic major community functions can be used in batch/continuous cultures (Leclerc, Bedu-Ferrari et al., 2021) or in coculture systems to analyse interactions with host cells and community dynamics at the ecosystem scale. Furthermore, *in vivo* animal models allow researchers to have multiple biological replicates that represent the response of a single microbiota and to tightly control prebiotic intervention and. Experimental models that lack a gut microbiota, such as gnotobiotic mouse models, offer further power to dissect mechanisms underlying diet-microbe–host interactions (Clavel et al., 2016; Delannoy-Bruno et al., 2021). The combination of *in vitro* and *in vivo* models can be translated to human cohort studies for an application of prebiotics as nutritional strategies.

In prebiotic research, emphasis has been placed on characterisation of the microbial composition to assess the impact of prebiotic consumption on the resident gut microbiome (Scott et al., 2020). The combination of both cultivation and *–omic* technologies allows one to move beyond species catalogues and to gain a comprehensive understanding of the complex ecological and evolutionary relationships within microbes and their host (Spacova et al., 2020). Interestingly, functional traits of yet uncultured bacteria can be predicted from genomic information using synthetic biology and/or artificial intelligence (Borkowski et al., 2018; Dou and Bennett, 2018; Kumar et al., 2022). Future research of the gut microbiome and prebiotics is likely to rely on transdisciplinary approaches, which will require more collaboration between investigators in disparate fields, including evolution, ecology, microbiology, biomedicine, and computational biology.

Metabolomic approach gives holistic insights into the bacterial mediators of prebiotic health benefits

Designing a metabolic profiling study to assess prebiotic functionality requires careful considerations, such as the choice of the analytical platform, which are mainly hydrogen-nuclear magnetic resonance (H-NMR) spectroscopy and mass spectrometry (MS) eventually coupled with Liquid Chromatography (LC-MS) or Gas Chromatography (GC-MS) (Scott et al., 2020). In this study, we considered a "top down" approach where no prior knowledge was applied. The use of non-targeted LC-MS metabolomics has captured holistic information related to multiple mechanisms significantly associated with the breakdown of carbon sources. This high-throughput technique constituted a powerful strategy to decipher new mechanistic details of carbohydrate metabolism for a comprehensive understanding of the activities of six individual bacterial strains. Nevertheless, this exploratory approach came with its share of challenges. The generation of large data sets make the information difficult to analyse and summarize into a clear picture that unravels the mechanisms underlying the bacterial utilisation of prebiotics. Challenges associated with -*omics* data often lead to intensive exploration, visualization, and interpretation. The choice of analytical strategy has been critical to disentangle the information on metabolic analytes provided by the various samples (Scott et al., 2020). Today, gut microbiome research would substantially benefit from harmonized guidelines for processing standards and quality thresholds and a curated database of bacterial-derived metabolites (Han et al., 2021).

Based on the variable annotation against public databases, the analysis of bacterial profiles was carried out on metabolites with low confidence degree in comparison with an annotation method performed against an in-house curated database. The results identified biomolecules with potential physiological health benefits that participate in the prebiotic effects of particular food ingredients. Although assessing the flow of information from one -*omic* level to the other one is complex, the exploration of the bacterial metabolomic profiles yield useful insights into the interplay between prebiotics and key intestinal bacteria. In light of these complex potential influences of prebiotic metabolites on health, the choice of research methods becomes critical to draw comprehensive conclusions. The optimisation of -*omic* technologies and the development of various tools and methods participate to the provisioning data analysis, visualisation and interpretation. Combining–*omic* levels provides information for systematic and holistic understanding of microbiomics. Integrating multi-*omics* in the gut microbiome is a growing area of interest as researchers seek to reveal new understandings of the gut microbiome from functional and taxonomic perspectives.

From fundamental research to microbiome-based personalised nutrition

Translation of the fundamental research of the gut microbiome into clinical practice and/or interventional strategies constitutes a great challenge to improve public health. Inherent in the prebiotic concept, it is about time to deliver physiological benefits toward promises of microbiome-based applications. The field of gut microbiome needs to multiply efforts to reach practices using or targeting microbes themselves (Clavel et al., 2022). Clinical interventions accumulate evidence on the gut microbiome, health and wellbeing to characterise prebiotic-mediated physiological outcomes **(Review 1)**. The nutritional impact of a prebiotic on the gut microbiome composition and metabolic activities should be translated in intervention studies and in future public health recommendations (Delzenne et al., 2020).

The physiological effects of prebiotics depend on the nature of prebiotics that can unequally modify the composition and functions of the gut microbiome, and ultimately the human health and wellbeing (Gibson et al., 2017). Therefore, the biological source that defines their physicochemical properties, such as solubility, molecular weight, character of individual monomers, and complexity of chemical structures (Gill et al., 2021). The nutrient composition of natural plant sources creates complex food matrices for microbes. For example, barley and oat cereals contain beta-glucans, arabinoxylans, starches, insoluble fibres, proteins, and other bioactive phytochemicals (Hughes et al., 2008; Wang et al., 2016; Dong et al., 2017). Thus, the advantage of the natural mix of different substances with bioactive properties should be better considered to ensure that the utilisation by the gut microbiome functions prone to act on host physiology in intervention studies (Delzenne et al., 2020).

There is a nutritional importance to characterise the nature and source of prebiotics ensuring that their action on host physiology results from the utilisation by the gut microbiome **(Review 1)**. In this project, we have a characterissation of the food ingredients limited to a given fibre category and a given source (agave inulin, corn fibre, citrus pectin). Each food ingredient was analysed using nuclear magnetic resonance (NMR) for an accurate description of the complex carbohydrates. These analyses can also highlight biological ingredients present within the natural food matrix, including phenolic compounds, dietary fats and other fermentable carbohydrates, which may directly influence the composition of the gut microbiome and/or alter the prebiotic efficacy to confer health benefits (Jackson et al., 2022).

In addition, significant advances in the comprehensive interactions between the gut microbiome, nutrition and the host have not yet been formulated in the current public health recommendations. Public health efforts communicate about the importance of high-fibre, low-sugar, and low-fat foods to promote global health and outsize the spread of metabolic-related disorders, including obesity and diabetes. It would be nice for public health campaigns to consider the position of the gut microbiome to dynamically relaunch the consumption of dietary fibres. In fact, dietary fibre intake around the world refers to a certain amount of total dietary fibres for optimal health, typically 25 to 35 g/day of fibre in the adult (Stephen et al., 2017). Many people do not achieve their recommended fibre intake because they consume insufficient fruits, vegetables and cereals (Stephen et al., 2017). These dietary guidelines do not consider the ratio of soluble versus insoluble dietary fibres, the levels of phenolic compounds, the fermentation ability of the colonic microbiota (rapid, slow, completely fermentable or to a limited degree) and the digestive problems related to the consumption of dietary fibres. The balance between digestive tolerance and metabolic problems should be addressed in future dietary recommendations.

There is a need for rigorous randomised intervention studies in humans with strict dietary control to evaluate the consumption of the types and amounts of prebiotics and their efficacy in the management of metabolic-related health issues (Martinez et al., 2021). To date, a substantial number of human dietary interventions have revealed that prebiotics can have extreme individual, temporal, and variable effects on metabolic health outputs and the gut microbiome **(Review 1)**. Exhibiting considerable heterogeneity in taxonomic composition and functions regardless of individual health status, the signatures of the gut microbiome in dietary interventions vary substantially (Human Microbiome Project Consortium, 2012; Lloyd-Price et al., 2017). The inconsistent responsiveness of individuals to prebiotics highlighted that a dietary intervention can be more effective and confer health benefit in some individuals, identified as responders, than in others, identified as non-responders (Cunningham et al., 2021). The report of responders and non-responders encourages the determination of microbial signatures to better apprehend the efficacy of a prebiotic intervention, which can be useful to support the hypothesis that intentional modulation of the gut microbiome is a valid strategy to improve human health and wellbeing (Spacova et al., 2020; Rodriguez et al., 2020). Standardised microbial characteristics, including gene richness, microbial diversity, and CAZy-typing, could be used to predict individual responses with respect to various carbohydrate sources **(Review 1)**. Identifying specific characteristics of the gut microbiome provides the opportunity to develop individualised nutrition strategies (Gibson et al., 2017; Kolodziejczyk et al., 2019). Indeed, there is a significant interest in a personalised approach to redirect microbial signatures towards health. Advances in personalised

strategies will likely result from future refinements to the individual's microbiome features and its particular response to interventions (Bindels et al., 2015; Deehan et al., 2020). A recent personalised diet intervention has successfully identified personal and microbiome characteristics to accurately predict personalised postprandial glycemic responses (Zeevi et al., 2015). The development of personalised dietary components that reduce postprandial glucose provided hope for further advancements in the control and treatment of diabetes.

The microbiome can influence the host physiology and respond to an array of environmental factors without being able to understand the link between causation and correlation. Related to individual differences, prebiotic interventions are still far from convincing in terms of outcome measures in metabolic health. The variability of the gut microbiome limits the applications of prebiotics and complicates the translation of effects into real-life outcomes. Future research on short- and long-term dietary interventions could improve the characterisation of microbial responses to prebiotics to provide new information supporting the development of precision nutritional strategies (Leeming et al., 2019).

Incorporation of prebiotics into food-processed matrices

In this project, the interest was largely focused on better understanding the carbohydrate metabolism of health-relevant bacteria. *In vitro* experiments evaluated the prebiotic effects of food ingredients on bacterial growth and metabolic activities using classical microbiology and molecular methods. This objective highlights the dire need to set clear criteria to classify a food ingredient as prebiotic.

Delivering physiological benefits through microbiome-based applications can help promote health and wellbeing. In a favourable context for the development of novel functional food products to improve public health, prebiotics hold tremendous potential for innovation (Scott et al., 2020). The incorporation of isolated food ingredients within food-processed matrices may likely modify physicochemical properties of prebiotics influencing the dynamics among the gut microbes and the carbohydrate utilisation (Smith et al., 2020). For instance, breakdown of glycosidic linkages is commonly observed after conventional heating treatments, resulting in partial hydrolysis of complex carbohydrates (Misra et al., 2017). This food process can leading to different types of prebiotic molecules with modified physicochemical properties that might unequally affect the bacterial activities (Sonnenburg and Sonnenburg, 2014). A previous study advanced that complexity of the molecular structure might show less prebiotic effects due to the restricted accessibility to glycosidic bonds of certain inulin molecules (Sonnenburg and Sonnenburg, 2014). To avoid nutrient losses and maintain bioactive compounds during food processing, advanced technologies in food science can include non-thermal or optimised

thermal treatments such as high-pressure processing, high-intensity ultrasound, pulsed electric field and pulsed light (Zhang et al., 2019; Guimarães et al., 2019; Buchmann and Mathys, 2019). Beyond food-processing factors, such as milling, grinding, pH, pressure, and temperature, the nature of the food matrix delivering the prebiotic ingredient has the potential to influence to some extent the physicochemical properties of prebiotics, such as particle size, intactness, exposedness (Jackson et al., 2022). Indeed, the composition of the matrix can directly influence the bioavailability of nutrients due to its effect on the human digestion process. Recent efforts evaluated the microbiome-dorected metabolisation of dietary fibres (Patnode et al., 2021, 2019; Delannoy-Bruno et al., 2021).

One question to address is how food-containing prebiotics can be developed to target distinct features of the human microbiome. Most prebiotic investigations have been conducted with "purified" or isolated food ingredient at relatively high doses (Delzenne et al., 2020). Often, the consumption of isolated food ingredients is assumed to reach the distal intestine without any modifications to be metabolised by the gut microbiome. More investigations would be necessary to confirm this hypothesis. In this project, we have overlooked host-specific metabolism and largely underrepresenting the microbes that are more involved in host interactions. Effects on bacterial growth and metabolic activities of *in vitro* experiments may not translate to effects *in vivo* as other factors can influence the ability for prebiotics to support beneficial changes. To better understand the mechanisms of action and efficacy of prebiotics, the evaluation of microbiome-directed prebiotics in humans is essential (Delannoy-Bruno et al., 2021). Pilot controlled-diet study, in which participants consumed containing-prebiotic prototypes, can evaluate the host biological responses in terms of changes in the microbiome features and metabolic outputs, considering the degree of interpersonal variation in response to prebiotics. A consensus on the evaluation of food ingredients for their prebiotic potential could provide a rigorous scientific foundation for prebiotic claims in human nutrition.

Communicating the health benefits of prebiotics

As a whole, the RestorBiome project proposes a nutritional solution to promote health and wellbeing through the gut, in which the old concept of prebiotics represents an innovative solution. The translation of prebiotics into a microbiome-based functional food product remains a challenge in regards to the regulatory and policy requirements. Indeed, regulatory frameworks, clinical guidelines and industry trends influence the implementation of prebiotics into nutrition and healthcare. Thus, several regulatory, scientific, and industrial barriers limit the food application of prebiotics in viable practice.

People seek nutritional and health benefits to reduce the risks of developing metabolic-related diseases, including obesity, diabetes and cardiovascular diseases (Bohn et al., 2021). To date, public awareness and acceptance of prebiotics continues to expand, with a growth forecast of 12.7% over the next 8 years (Mano et al., 2018). Despite market opportunities, the lack of harmonised regulatory guidelines for the industry of functional foods and dietary supplements contributes to the difficulty to provide sufficient evidence to substantiate nutrition and health claims.

Valorisation and transfer of the fundamental research toward the development of nutritional solutions based on the microbiome appear to be challenging to implement in practice. Prebiotics constitute a controversial area in the field of public health relative to the potential of functional foods and dietary supplements in mitigating disease risks and improving metabolic health outcomes. This context highlights the difficulties obtaining a hard claim applied to a functional food product that would contain a prebiotic. For these reasons, food labelling represents an opportunity to communicate about the importance of the gut microbiota on the overall health and wellbeing. Although there are great disparities in regulations across the globe, the evaluation of functional food ingredients with prebiotic potentials participates to expand the prebiotic-microbiome research.

CONCLUSION

The intentional modification of the gut microbiome for health and wellbeing purposes requires the investigation of carbohydrate metabolism of gut microbes to define their capacities to metabolise prebiotic substrates. The breakdown of prebiotics was driven by the phylogenetic affiliation of commensal bacteria. In-depth characterisation of key health-promoting bacteria revealed that bacteria respond significantly to the carbon source at the metabolite level. To uncover mechanistic insights into the dynamic interplay between prebiotics and health-relevant commensal bacteria, *in vitro* single-carbohydrate experiments in monoculture and in synthetic bacterial consortia revealed that the carbon sources influenced the temporal behaviours of the bacterial growth and metabolic activities. The nutritional preference of the bacterial drivers towards a particular carbon source suggested a nutritional adaptation within complex trophic interactions ranging from cooperation to competition. Exploring the genomic potentials of health-relevant bacteria holds promises to decipher in-depth molecular mechanisms of carbohydrate metabolism to utilise inulin-type fructans and produce fermentation end-products. The application of biomarkers indicating health-relevant functions can be used to monitor the gut ecosystem and predict the responsiveness of individual gut microbiomes in dietary intervention. A comprehensive understanding of the bacterial growth, metabolic activities and molecular mechanisms underlying the metabolisation of prebiotics seems imperative for the formulation of functional foods.

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ANNEX 1: Prebiotics and the human gut microbiota: from breakdown mechanisms to the impact on metabolic health

Bedu-Ferrari Cassandre[1,2], Biscarrat Paul[1], Langella Philippe[1], Cherbuy Claire[1,*]

[1]Micalis Institute, Institut National de Recherche pour L'agriculture, L'alimentation et L'environnement (INRAE), AgroParisTech, Université Paris-Saclay, UMR1319, F-78350 Jouy-en-Josas, France

[2]Yoplait France - General Mills, Vienne Technical Center, F-38200 Vienne, France

*Corresponding author: claire.cherbuy@inrae.fr

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Review

Prebiotics and the Human Gut Microbiota: From Breakdown Mechanisms to the Impact on Metabolic Health

Cassandre Bedu-Ferrari [1,2], Paul Biscarrat [1], Philippe Langella [1] and Claire Cherbuy [1,*]

[1] Micalis Institute, Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement (INRAE), AgroParisTech, Université Paris-Saclay, 78350 Jouy-en-Josas, France; cassandre.bedu-ferrari@inrae.fr (C.B.-F.); paul.biscarrat@inrae.fr (P.B.); philippe.langella@inrae.fr (P.L.)
[2] Yoplait France—General Mills, Vienne Technical Center, 38200 Vienne, France
* Correspondence: claire.cherbuy@inrae.fr

Abstract: The colon harbours a dynamic and complex community of microorganisms, collectively known as the gut microbiota, which constitutes the densest microbial ecosystem in the human body. These commensal gut microbes play a key role in human health and diseases, revealing the strong potential of fine-tuning the gut microbiota to confer health benefits. In this context, dietary strategies targeting gut microbes to modulate the composition and metabolic function of microbial communities are of increasing interest. One such dietary strategy is the use of prebiotics, which are defined as substrates that are selectively utilised by host microorganisms to confer a health benefit. A better understanding of the metabolic pathways involved in the breakdown of prebiotics is essential to improve these nutritional strategies. In this review, we will present the concept of prebiotics, and focus on the main sources and nature of these components, which are mainly non-digestible polysaccharides. We will review the breakdown mechanisms of complex carbohydrates by the intestinal microbiota and present short-chain fatty acids (SCFAs) as key molecules mediating the dialogue between the intestinal microbiota and the host. Finally, we will review human studies exploring the potential of prebiotics in metabolic diseases, revealing the personalised responses to prebiotic ingestion. In conclusion, we hope that this review will be of interest to identify mechanistic factors for the optimization of prebiotic-based strategies.

Keywords: gut microbiota; prebiotics; carbohydrate metabolism; health and well-being; short-chain fatty acids; personalised nutrition

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1. Introduction

1.1. The Gut Microbiota as a Key Player in Human Health and Diseases

There is now a large body of evidence indicating that the gut microbiota plays a central role in human physiology and metabolism. Indeed, the gut microbiota supports the training and function of the host immune system [1,2], contributes to host metabolic homeostasis [3,4], influences neurocognitive function, and provides colonisation resistance to invasive pathogenic infections [5,6]. This gut microbial community is dominated by bacteria, which have been extensively studied in the past decades. The microbiota also includes commensal populations of fungi, viruses, and archaea that have not been as widely explored [7]. In healthy adults, over 90% of the gut bacterial diversity belongs to two phyla, Gram-positive *Firmicutes* and Gram-negative *Bacteroidetes* [8]. The *Bacteroidetes* phylum contains the major families *Bacteroidaceae*, *Prevotellaceae*, *Rikenellaceae*, and *Porphyromonadaceae*. Groups of *Firmicutes* are more diversified and, primarily affiliated with four classes, including, in decreasing order of relative abundance, *Clostridia*, *Bacilli*, *Erysipelotrichi*, and *Negativicutes*. Each is composed of several bacterial families, such as *Lachnospiraceae*, *Ruminococcaceae*, *Clostridiaceae*, *Christensenellaceae*, *Eubacteriaceae*, and *Peptostreptococcaceae* [9,10]. Less abundant *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia* represent subdominant bacterial phyla in humans.

A growing number of chronic disorders, with distinct clinical presentations, share underlying patterns of major compositional changes in the gut microbiota, often referred to as "dysbiosis". Thus, diabetes [11], cardiovascular disease [12,13], obesity [14], inflammatory bowel disease [15,16], and non-alcoholic fatty liver disease (NAFLD) are examples of diseases associated with a shift in microbial patterns compared to healthy individuals [17]. Such observations advocate for microbiota-targeted interventions to preserve human health and prevent or treat diseases. In this context, diet-based interventions are promising tools to modify the gut microbiota towards a favourable community structure [18].

1.2. Prebiotics: An Old Concept with Innovative Applications

The concept of prebiotics refers to the nutritional strategy that aims to fine-tune the composition and function of the gut microbiota to favour health and well-being. This concept was first introduced in 1995, and since then has evolved, along with advances in our understanding of the gut microbiota (Table 1 for the different definitions and their evolutions). The International Scientific Association for Probiotics and Prebiotics (ISAPP) proposed the most recent consensus view that redefines a prebiotic as "a substrate that is selectively utilised by host microorganisms conferring a health benefit" [19]. Prebiotics fulfil three criteria: (a) resistance to gastric acidity, hydrolysis by mammalian enzymes, and gastrointestinal absorption; (b) fermentation by intestinal microflora; and (c) selective stimulation of the growth and/or activity of intestinal bacteria associated with health and well-being [20]. A comprehensive understanding of the health properties of a substance classified as a prebiotic should require evaluating the physiological effect in randomized controlled human trials [21]. Thus, consistent clinical studies are needed to confirm the safety of prebiotics and their use in an appropriate dose. Historically, prebiotics were identified to stimulate *Bifidobacterium* and *Lactobacillus* bacterial groups, which are often referred to as probiotics [22]. This was based on the assumption that an increase in relative abundance of these bacteria could be used as a benchmark for a healthy gut microbiota, although the causal relationship between specific microbial changes and benefits for host health and well-being remains to be proven [23]. However, more recently, data based on high-throughput sequencing techniques, has revealed that the impact of prebiotic-induced changes on the gut microbiota is more widespread, probably due to functional redundancy and cross-feeding interactions [24]. Thus, for example, *Bifidobacterium* and *Lactobacillus* produce predominantly acetate and lactate, which can then stimulate a number of bacterial species benefiting from the presence of the prebiotic [25]. Therefore, the requirement that prebiotics must be "specific" or "selective" by a limited number of taxonomic groups/species or metabolic activities that promote health has been questioned, as it conflicts with the current understanding of gut microbiota ecology and its correlation with health [24]. The most recent ISAPP definition widened the concept of prebiotics beyond stimulation of bifidobacteria and lactobacilli and recognised that health benefits can derive from effects on other beneficial taxa [19]. Implicit in this definition is the idea that a prebiotic should not be broadly metabolised by the gut microbiota but should be selectively metabolised by health-promoting microorganisms.

Table 1. The prebiotic concept over the years. Consisting of a panel of academic and industrial experts, the ISAPP regularly convenes to state the definition and scope of prebiotics. New considerations are gradually included along with scientific research progress, consumer interest, and technological innovations from industrial scientists.

Evolution of the Prebiotic Concept	Additional Considerations from the Previous Definition	Ingredients Incrementally Considered as Prebiotics
"non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health" [22]	None	FOS
"selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confer benefits upon host well-being and health" [20]	(a) non-digestibility (b) fermentation by intestinal microflora (c) selective stimulation of the growth and/or activity of intestinal bacteria	Inulin tGOS Lactulose Candidates * are listed: IMO, lactosucrose, SOS, XOS, GlOS, and other compounds
NB: there is no new definition of a prebiotic, but rather a validation and an expansion of the prebiotic concept [26]	(a) nature of the prebiotics (b) dose-effect relation (c) animals and humans	None
NB: there is no new definition of a prebiotic, but rather a validation and an expansion of the prebiotic concept [23]	(a) increase in the genus *Bifidobacterium* as a marker of intestinal health (b) selectivity of other genera or species than bifidobacteria (e.g., butyrate-producing bacteria) (c) beneficial effects in the colon and the whole body	None
"a non-digestible compound that, through its metabolisation by microorganisms in the gut, modulates composition and/or activity of the gut microbiota, thus conferring a beneficial physiological effect on the host" [24]	(a) anatomical restriction to the gut (b) requirement or not of fermentation (c) restriction only to carbohydrates (d) requirement or not of microbiota modulation (possibility of having other direct positive effects)	HMO Candidates * are listed: RS, pectin, AX, whole grains, various dietary fibres and other non-carbohydrates such as polyphenols
"a substrate that is selectively utilised by host micro-organisms conferring a health benefit" [19]	(a) microbes targeted by prebiotics should be health-promoting bacteria without specifying which ones (b) effect is no longer limited to the microbial community of the gastrointestinal system associated with humans or animals (c) importance in describing selective bacterial metabolism and assessing microbial function and composition in reproducible randomized controlled studies that establish the direct link between prebiotics and health in the specific target host	Candidates * are listed: HMO, MOS, and other non-carbohydrates such as polyphenols, CLA, and PUFA

Abbreviations: ISAPP, international scientific association of probiotics and prebiotics; NB, nota bene; FOS, fructo-oligosaccharides; tGOS, trans-galacto-oligosaccharides; IMO, isomalto-oligosaccharides; SOS, soya-oligosaccharides; XOS, xylo- oligosaccharides; GlOS, gluco-oligosaccharides; HMO, human milk oligosaccharides; RS, resistant starches; AX, arabinoxylans; MOS, mannan-oligosaccharides; PUFA, polyunsaturated fatty acids; CLA, conjugates linoleic acids * The prebiotic potential of candidate compounds has been investigated. However, scientific evidence is too sparse at the time to demonstrate any prebiotic effects.

1.3. The Sources & Nature of Prebiotics

Derived from plants, non-digestible carbohydrate-based polymers are important sources of prebiotics, although non-carbohydrate substances have begun to emerge for their prebiotic potential, including polyunsaturated fatty acids and polyphenols [27–29]. Similar to carbohydrates, certain of these phytochemicals have a low bioavailability, which indicates that they may escape absorption in the small intestine. It was estimated that 5 to 10% of the total intake of plant polyphenols reach the colon, where they can be metabolised to various degrees by the gut microbiota [30]. As this has been recently reviewed, polyphenols will not be discussed further [31]. In the present article, we will focus only on complex carbohydrates that represent the best-studied class of prebiotic until now.

Non-digestible dietary carbohydrates, mainly dietary fibres and resistant starches, escape host digestion and reach the lower part of the gastro-intestinal tract, where they can supply the gut microbiota [32]. There is a body of evidence showing that diets, especially in Western countries, lack non-digestible substrates, which reduces the bacterial fermentation in the gut environment. The trend is toward a reduction in dietary fibre intake, with present consumption being the lowest recorded in human history [33]. The intake of dietary fibres was estimated at 15–20 g/day, which is below the daily recommendations of 25–35 g/day [34]. However, the amount of carbohydrates was shown to reach 50 g/day in countries where there is a high intake of whole-grain cereals, legumes, fruits, and vegetables [35,36]. Human nutrition trials show that dietary fibre fermentation represents about 75 to 90% in fruits and vegetables and 25 to 35% in whole grains [37]. Each dietary carbohydrate exhibits distinct structural characteristics, related to the length of the molecule, sugar moieties, the presence of substituents, the linkages, and the side-chain branching, which influence the microbial digestibility of dietary carbohydrates [38]. Variations in the accessibility of polysaccharides for fermentation are considered critical in shaping the gut microbial ecosystem.

Complex oligo- and polysaccharides, also designated as glycans, constitute the most heterogeneous and abundant polymer biomolecules in nature. The primary classification of carbohydrates is based on chemistry, which is molecular weight, character of individual monomers, degree of polymerization (DP), and type of linkage (α or β), as agreed at the Food and Agriculture Organization/World Health Organization Expert Consultation in 1997 [39]. Among the first group of dietary ingredients, human milk polysaccharides (HMOs), naturally present in breast milk, were recognised for their ability to modulate intestinal health and well-being. These mixtures of oligosaccharides, in combination with glycoproteins and glycolipids, result mainly in a dominant abundance of *Bifidobacterium* species in breastfed infants, and, to a lesser extent, in an abundance of some *Bacteroides* and *Lactobacillus* species [40]. As a result, different oligosaccharides have been used to mimic the function of HMOs, such as fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS).

Besides the fructan group, short-chain FOS or oligofructose and long-chain inulin constitute a mixture of linear fructan chains composed of both fructosyl-glucose and fructosyl-fructose linked by β (2-1) glycosidic bonds [20]. Inulin-type fructans (ITF) are often manufactured for use in the food industry by extraction from plants, such as onions, Jerusalem artichoke, chicory root, and agave. FOS can be produced by partial hydrolysis of long-chain inulin using endo-inulinase enzymes or derived from sucrose by transfructosylation using β-fructosidase enzymes [41]. The large variety of inulin chemical structures and botanical origins result in a wide range of ITF mixtures with different functional effects. From a chemical perspective, DP distinguishes FOS mixtures, in which DP varies from 2 to 7 with an average DP of 4 sugar units, from longer inulin molecules. For instance, the inulin from chicory is composed of a mixture of oligomers and polymers in which DP ranges from 2 to 70 units with an average DP of 12.

There are two common types of GOS, both composed of galactose residues. Derived from lactose syrup, β-linked GOS, also known as trans-GOS (tGOS), are commercially

synthesised by the transglycosylation activity of bacterial β-galactosidase or β-glycosidase enzymes [42]. This process commonly produces mixtures of short-chain tri- to penta-saccharides, which present a terminal glucose monomer and a wide variety of β (1-6), β (1-2), β (1-3), and mostly β (1-4) linkages. Likewise, α-linked GOS is another type of non-digestible fibre commonly found in plants, consisting of α-linked galactose, α-linked glucose, and a terminal β-linked fructose. They are commonly called raffinose family oligosaccharides (RFOs), including raffinose, stachyose, and verbascose.

ITF and GOS represent the main confirmed prebiotics [18]. However, many other classes of non-digestible carbohydrates derived from plants can be considered as potential prebiotics because they reach the large intestine and are broken down by the gut microbiota [20]. This is the case for starch, one of the most popular nutritional sources for humans, for which a fraction can be resistant to host enzymes. Indeed, besides the digestible starch, which is rapidly or slowly hydrolysed, a variable fraction called resistant starch (RS) resists digestion in the small intestine and is fermented in the large intestine, where it provides nutrients for the gut microbiota. The non-digestible starch fraction contains a mixture of two major components, amylose and amylopectin, and other polysaccharides such as α-glucans [43]. RS has been well documented for its promising nutritional interventions in cardiovascular disease and in a variety of metabolic disorders [44]. Many other sets of complex polysaccharides are also promising potential prebiotics. Among them, xylo-oligosaccharides (XOS)/arabino-xylo-oligosaccharides (AXOS) are abundant in cereals and are mainly composed of a chain of xylose units linked by β (1-4) bonds with branching sugar residues. Several clinical studies show that XOS/AXOS could exert protective effects on intestinal homeostasis and metabolic status [45]. Pectins are important cell-wall components of many fruits and vegetables that are composed of a chain of α (1-4)-linked D-galacturonic acid units with side chains containing arabinans, galactans, and arabinogalactans [18]. β-glucans are found in wheat, oat, and barley and are composed of D-glucose monomers joined by mixed-linkages β (1-4) and β (1-3) of glucose residues. Consumption of pectins and β-glucans has shown beneficial effects on metabolic homeostasis in humans [46]. In view of the richness of polysaccharides and the diversity of yet unexploited plant sources, the variety of compounds with prebiotic potential should grow in the coming years [18].

2. The Gut Microbiota: An Ecosystem Designed for Carbohydrate Breakdown

2.1. CAZymes, Abundant Enzymes in the Gut Microbiota

Several studies have highlighted the gut microbiota to be one of the most specialised and sophisticated ecosystems involved in the breakdown of complex polysaccharides [47]. Indeed, intestinal bacteria display efficient strategies for breaking down carbohydrates in this dynamic nutrient environment [48]. Providing mechanisms of prebiotic utilisation is a step toward an efficient intentional modulation of the gut microbiota to improve human health and wellbeing [24]. In this context, prebiotic research is focusing on understanding how commensal microorganisms selectively scavenge carbohydrate substrates and act on their degradation and consumption [21]. In particular, certain gut microorganisms have repeatedly been shown to improve human health, and it is important to know how diet could be used to fine-tune the composition of these gut microbes, selectively stimulating the abundance of the health-promoting faction.

The functionality of the gut microbiota to break down complex carbohydrates is reflected in an arsenal of prominent and highly diverse carbohydrate active enzymes (CAZymes)-encoding genes, which comprise 1 to 5% of the predicted coding sequences in most bacterial genomes [49]. Thus, the gut microbiome displays a plethora of CAZymes dedicated to the breakdown, biosynthesis, and modification of complex carbohydrates. In contrast, the CAZyme repertoire encoded by the human genome is minimal (approximately 17 enzymes) and is restricted to the ability to degrade digestible starch, sucrose, and lactose, revealing that gut microorganisms have evolved into large enzymatic repertoires that expand human digestive physiology [50].

It is noteworthy that certain colonic bacteria are able to metabolise a remarkable variety of substrates, whereas other species have more restricted metabolic capacities. In particular, as members of the Gram-negative *Bacteroidetes* phylum, the *Bacteroides* genus exhibits a wide diversity and a large abundance of CAZymes-encoding genes. This group of bacteria appears to be one of the major actors in the breakdown of polysaccharides, which are metabolised into a form that could otherwise not be absorbed and/or utilised by the host. Due to this vast genetic repertoire encoding CAZymes, *Bacteroidetes* utilise a large number of different carbohydrate structures and so are often known as "generalists" [51]. Devoted to carbohydrate metabolism, the abundance of CAZymes in *Actinobacteria* members represented up to 8% of the *Bifidobacterium* genomes [52]. The CAZyme genetic content of *Firmicutes* has received less attention, although this phylum accounts for about 70% of the microbial diversity within the colonic microbiota of adult individuals [8]. Typically, at the level of bacterial phyla, *Firmicutes* genomes show fewer genes (mean 39.6) involved in the polysaccharide hydrolysis than *Bacteroidetes* spp. (mean 137.1) [50]. This is likely to be due, partially, to their smaller genome sizes and reflects their greater nutritional specialisation. Therefore, *Firmicutes* members are known as "specialists". Some Gram-positive *Firmicutes* bacteria have been postulated to serve as "keystone" polysaccharide degraders, as their absence can limit the breakdown of a particular carbohydrate [53]. Interestingly, *Roseburia intestinalis* is unique among other *Firmicutes* studied, as strains of this species encode about 100 to 150 CAZymes [54].

CAZymes constitute an enzyme superfamily that has been classified into groups based on their amino acid sequence similarities and their biochemical reactions [49,55]. Diverse classes represent the numerous CAZyme families: glycoside hydrolases (GH) depolymerize carbohydrate substrates by hydrolysing glycosidic linkages; glycosyltransferases (GT) lead the formation of glycosidic bonds; carbohydrate esterases (CE) catalyze the hydrolysis of carbohydrate esters; and polysaccharide lyases (PL) operate the non-hydrolytic cleav- age of glycosidic bonds. In addition, enzymes with auxiliary activities, including redox enzymes and carbohydrate-binding modules (CBM), are also involved in carbohydrate metabolism [56]. Functional prediction based on bacterial genomes revealed that GHs are the most representative CAZymes in the human gut microbiome [50]. The exploration of GH diversity highlighted the number of families increasing steadily, and as of March 2022, 173 sequence-based families of GHs have been defined in the continuously updated CAZy database [55].

Based on amino acid sequences, the CAZy classification has proven to be particularly robust for the prediction and characterisation of CAZyme activities [49]. Key active-site residues, catalytic mechanism, and the overall three-dimensional fold are strictly preserved, allowing the investigation of substrate specificity, which is a key facet of microbial responses to dietary carbohydrates [57]. Nevertheless, the broad diversity of sequences illustrates the difficulty in differentiating between family membership with distinct activity profiles and substrate specificity [38]. Indeed, CAZyme families are often "polyspecific" and include enzymatic activities with variations in glycosidic linkage specificity. Individual gut enzymes may hypothetically be associated with the breakdown of multiple polysaccharides that leads to ambiguous functional predictions [58].

2.2. *Polysaccharide Utilisation Loci (PUL): The Paradigm of Bacterial Foraging Systems*

The complexity and diversity of the dietary carbohydrate structures that reach the lower digestive tract implies multi-step bacterial breakdown to efficiently utilise these macromolecules. Thus, nutrient acquisition strategies at the molecular level must be coordinated to enable commensal bacteria to thrive in this rich yet competitive intestinal environment. As primary degraders of polysaccharides in the gut, these orchestrated carbohydrate utilisation pathways were first investigated in *Bacteroidetes* species [59]. It has been shown that, to varying extents among *Bacteroidetes* species, bacterial genomes conserve a breakdown and importing machinery that is encoded within clusters of contiguous and coregulated genes, known as polysaccharides utilisation loci (PUL) [60]. This archetypal

PUL, known as the starch utilisation system (SUS), encodes all components necessary for starch metabolism, including carbohydrate binding, breakdown, transport, and sensing proteins (Figure 1) [61,62]. Necessary to convert extracellular polysaccharides into intracellular monosaccharides, the SUS system-encoding genes are usually associated with three surface glycan-binding proteins (SGBPs), SusD, SusE, and SusF, to recruit the starch to the cell surface, where it is cleaved into malto-oligosaccharides by the outer-membrane-localized CAZyme, SusG. Subsequently, these latter are transported into the periplasm via the TonB-dependent transporter (TBDT), SusC, where they are broken down further into glucose, by the linkage-specific CAZymes, SusA, and SusB. The key inner-membrane-bound regulatory protein, SusR, controls the expression of the susA-G genes in response to the presence of malto-oligosaccharides in the periplasm [47,60]. Sequential susC/susD homologous genes constitute the signature of the canonical PUL machinery. These hallmarks have been used to explore PUL components among the genomes of *Bacteroidetes* members, and this exploration revealed the effective nutrient adaptation of *Bacteroidetes* bacteria [60,63]. For instance, the genomes of *B. thetaiotaomicron* and *B. ovatus* contain about the same number of PUL (about 100), of which few are common to both species, suggesting that these two symbionts can have distinct glycan niches [60].

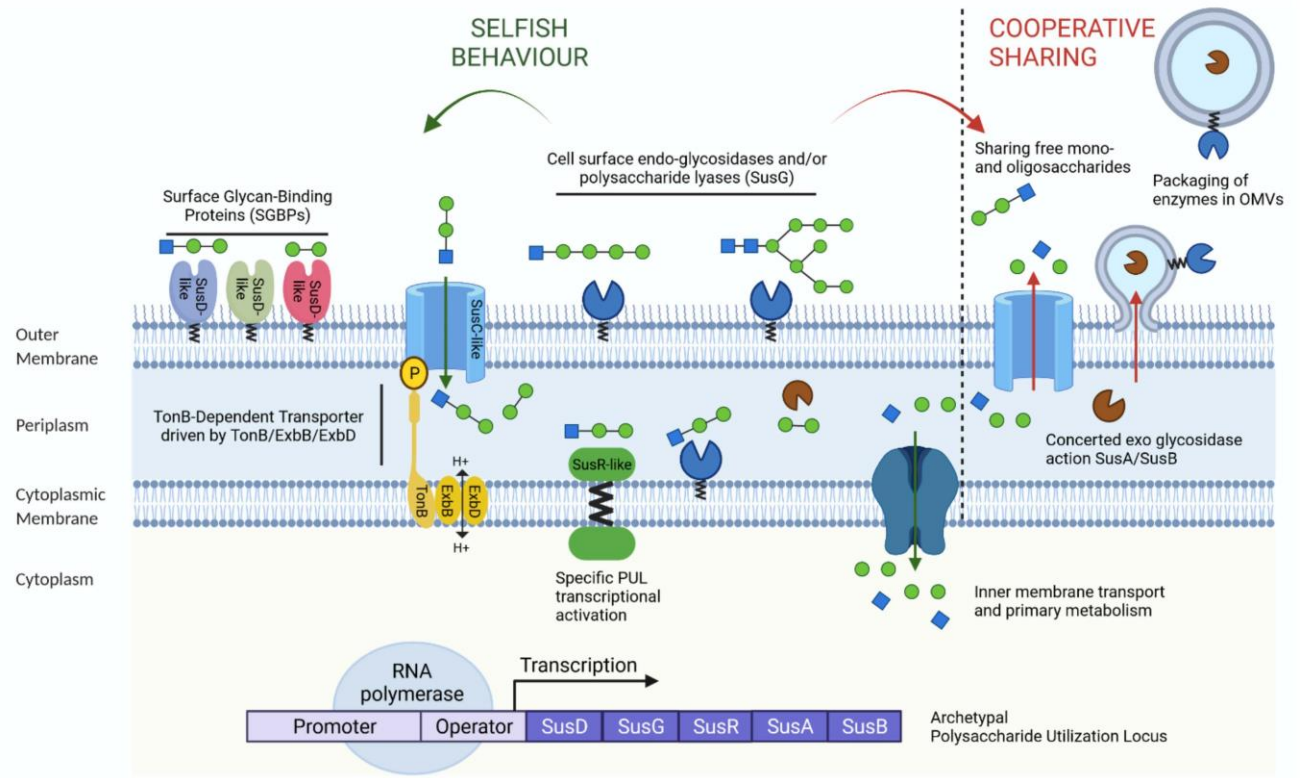

Figure 1. Nutrient acquisition strategies in two common trophic behaviours. The archetypal starch utilisation system (SUS) operon, a model system for starch uptake described in the commensal *Bacteroides thetaiotaomicron* at the origin of the polysaccharide utilisation locus (PUL). Intimately associated, the SusD cell-surface glycan binding proteins (SGBPs) initially adhere to and recruit the substrate from the outer membrane. The SusG endoglucanase proteins (GHs and PLs) hydrolyse starch into smaller malto-oligosaccharides that are further imported into the periplasm by the SusC Ton-B dependent transporter (TBDT). Carbohydrate-binding proteins and endoglucanase proteins vary substantially between PULs. Subsequently, oligosaccharides are catalysed into single sugars by the SusA and SusB exoglucosidases in the periplasmic space, before being imported into the cytoplasm for primary metabolism. In response to the presence of malto-oligosaccharides, the prototypic PUL regulator SusR protein senses degradation products to control the transcriptional

activation of the PUL machinery. In the intestinal environment, the products of carbohydrate breakdown can be either slotted into primary metabolic pathways, which could be called selfish behaviour, or act for the public good. These latter show cooperative behaviour, where polymers and hydrolytic enzymes can simply diffuse or can be shared in outer membrane vesicles (OMVs). P, phosphorus; H^+, hydrogen ions; SusA–SusD, SusG, SusR, SUS homologs; TonB, ExbB, ExbD, outer membrane receptors.

The development of automatic bioinformatics by the CAZy team has fuelled the PUL database, which catalogues all experimentally characterised PUL from *Bacteroidetes* species [57,64]. Along with genomic, transcriptomic, structural biology, and protein bio- chemistry studies, a tremendous diversity of PUL, targeting specific plant, algal, animal, and microbial glycans, has been identified and functionally characterised. Thus, the gut microbiome exhibits extensive repertoires of PUL that underlie divergent carbohydrate specificities and functional adaptation to break down a wide variety of carbohydrate substrates [65,66].

The PUL systems constitute the major strategy for harvesting carbohydrates deployed by *Bacteroidetes* bacteria. Nevertheless, analogs of *Bacteroidetes* PUL have been described beyond the archetypal tandem susC/susD-like pairs [67]. Although distinct in structure from Gram-negative systems, prominent *Firmicutes* and *Actinobacteria* members elaborate additional cell surface-associated systems for the utilisation of polysaccharides, which are often built around enzymes with catalytic domains, similar to those of *Bacteroidetes* [48]. Substrate-specific gene clusters targeting a variety of plant- and host-based glycans were identified in the genomes of *Eubacterium rectale* and *Roseburia* species as Gram-positive PUL (gpPUL) [54,68,69]. The gpPUL contain carbohydrate transport systems are mostly adenosine triphosphate (ATP)-binding cassette (ABC), major facilitator superfamily (MFS), and phosphoenolpyruvate-phosphotransferase system (PTS) transporters. These genes are colocalised with associated lactose repressor (LacI) or cytosine arabinoside (AraC)- like transcriptional regulators and a minimum of one CAZyme [70,71]. Some intestinal Gram-positive bacteria have adapted another extracellular multienzyme complex, known as a cellulosome, which targets cellulose, resistant starch, and possibly other substrates. This glycan acquisition paradigm of Gram-positive species has been well characterised in carbohydrate-degrading microorganisms from the bovine rumen and soil [48]. Genomic, biochemical, and molecular approaches of the human gut microbiome revealed the presence of protein components (dockerins and cohesins) that are signatures of cellulosomes in colonic *Ruminococcus* bacterial species [72]. Another strategy constitutes the lytic polysaccharide monooxygenases (LPMOs), which rely on lignocellulose degradation. Classified as enzymes for auxiliary activities in the CAZy classification [49], LPMOs have not been identified to date in any human microbiome data, presumably due to the anaerobic environment present in the distal human intestine [73].

2.3. Nutrient Acquisition Strategies by the Microbial Community

Members of the gut microbiota do not live in isolation but are part of a dynamic community where different types of interactions and nutrient resources exist. As specified below, *Bacteroidetes* are functionally diversified to assimilate a wide range of complex polysaccharides that reach the distal part of the intestine. Two common strategies for nutrient acquisition have been described in the complex gut microbial ecosystem: one is selfish and the other is cooperative sharing [66] (Figure 1). The degree to which carbohydrate degradation is mediated by syntrophic interactions between different members of the gut microbiota, as well as the ratio between selfish and cooperative strategies, are unclear. In some altruistic *Bacteroidetes* species, polymers and hydrolytic enzymes seem to act as purveyors of beneficial public resources in various networks of polysaccharide utilisers, which benefit the whole community [74]. Indeed, harbouring signal sequences, hydrolases, and proteases are preferentially packed in outer membrane vesicles (OMVs) and are further released into the extracellular environment. Most of these PUL-derived

enzymes are OMV surface-exposed, aiding bacteria incapable of efficiently using complex polysaccharides [75]. Hence, ubiquitous among Gram-negative bacteria, including *B. ovatus*, *B. fragilis*, and *B. thetaiotaomicron*, vesiculation appears to optimise the breakdown of nutrients by other bacteria with different metabolic capacities [76]. Most of these OMV-enriched-enzymatic activities contributes to the stability of the gut microbiota [77]. Leaving the rise of metabolic cross-feeding, some primary *Bacteroidetes* degraders diffuse simple oligosaccharides produced at the cell surface into the extracellular environment. Releasing polysaccharide breakdown products, this PUL-based distributive mechanism concomitantly benefits the bacterial community, which lacks the enzyme machinery to process the initial depolymerization step [78]. For instance, the large repertoire of GH/PL of *B. ovatus* appears more likely to break down a wide range of polysaccharides giving rise to the production of nutrients, which can help thrive other members of the community [77]. Similarly, it has been shown that *B. cellulosilyticus* initiates carbohydrate degradation through a surface endo-glycoside hydrolases, and releases oligosaccharides that are further metabolised by other members of the gut microbiota [79,80]. Thus, members of the *Bacteroidetes* act as primary polysaccharide degraders in orchestrating the initial polysaccharide breakdown that makes nutrients available not only for their own metabolic processes, but also to cross-feed the members of the gut microbiota depleted of hydrolytic enzymes.

This cooperative evolution of mutualistic interactions in *Bacteroidetes* members results in positive effects on bacterial fitness. It is noteworthy that sharing common resources depends on the structural complexity of carbohydrates that influences their microbial accessibility [78]. Based on carbohydrate structure, the symbiotic interaction network reflects the degradative hierarchies of carbohydrate utilisation, in which some simple glycans are prioritized above more complex polysaccharides [81]. In fact, individual bacteria exhibit multiple prioritization strategies in the presence of competing carbon sources. The simultaneous presence of dietary glycans, particularly in the form of monosaccharides, participates in consistent repression of the breakdown of host-derived glycans in *B. thetaiotaomicron* [82]. In general, the presence of highly prioritized glycans represses the transcription of genes involved in utilizing nutrients of lower priority [83]. Hierarchical orders promote the coexistence of stable microbial communities in a competitive environment [84]. The selective metabolism of substrates supports the dynamics of microbial communities and promotes the metabolic plasticity of the entire gut microbiota, which is constantly faced with nutritional changes.

Interestingly, carbohydrate metabolisation does not always occur in the extracellular environment. Indeed, CAZymes on the cell surface can actually generate large oligosaccharides. Rapid transportation of PUL-produced oligosaccharides into the periplasm of Gram-negative bacteria may minimize substrate availability to other microbial residents. This "selfish" strategy prevents other bacterial species using partially degraded prod- ucts [85]. Exhibiting little collaboration during the digestion of complex carbohydrates, *B. thetaiotaomicron* rapidly imports some glycans into the periplasm for further breakdown, conferring, at this step, no direct benefits on neighbouring species and enabling the microorganism to thrive in the competitive environment. Nevertheless, the metabolic activities of this bacterium can generate end-products that fuel other members of the gut commu- nity. These "partial" selfish ecological and evolutionary behaviours drive interdependent patterns for bacterial fitness and reciprocal feedback benefits of the entire gut microbiota. This selfishness strategy is central to the eco-evolutionary stability of extracellular polysaccharide digestions between microbial species. Bacterial interactions tend to significantly benefit the fitness of the overall microbial environment through the promotion of cooperation rather than competition [74,86]. The coordination of carbohydrate utilisation systems represents an impressive evolutionary solution for capturing valuable carbon sources. The rich interaction network between carbohydrates and bacteria is highly complex on the scale of an entire gut microbiota.

Strategies for carbohydrate metabolisation should be understood both at the level of individual microorganisms and at the level of the intermingled microbial community [67,87].

Carbohydrate metabolisation varies according to the nutrient acquisition strategies, leading to different interdependent catalytic mechanisms that preferentially support the growth of certain microbes. The preferential degradation of some glycans over others is likely to play a central role in the complex relationships of the gut microbiota [88]. Understanding the glycan utilisation of intestinal bacterial is essential in bringing about changes in the intestinal microbiota with the aim of improving health through diet. Predicting individual strategies of nutrient acquisition for each microbial type completes the characterisation of microbial adaptation to prebiotics to improve human metabolism [89].

2.4. Short-Chain Fatty Acids (SCFAs): Key Metabolites Underpinning the Prebiotic Impacts

Since the gut microbiota degrades complex dietary carbohydrates that cannot be broken down by human enzymes into digestible nutrients, it can be considered as "an energy harvesting" system. Conditions in the colon are highly favourable for complex carbohydrate fermentation due to the anaerobic environment, low transit time, and low pH coupled with low redox potential. Fermentation activities vary along the gastrointestinal tract according to dietary residues. In the proximal part of the colon, bacterial growth and fermentation are high, owing to the greater substrate availability. In the distal colon, the levels of substrates progressively decrease [90]. Several factors influence fermentation levels, including the shape and size of the food particles, the ratios of macronutrients, and transit time. Depending on the provision of adequate substrates, gut bacteria generate metabolites that can be quickly absorbed by the intestinal epithelium [91].

By-products derived from dietary carbohydrate fermentation are primarily SCFAs, mainly acetate, propionate, and butyrate [92]. Depending on the fibre content in the diet, the composition of the microbiota and the transit time of the gut, SCFAs typically reach total concentrations of 50 to 200 mmol/kg of luminal content in the human large intestine [93]. Acetate, propionate, and butyrate are typically found in a proportion of 3:1:1 in the gastrointestinal tract [94]. As detailed below, there are distinct pathways of bacterial fermentation that result in the production of short-chain fatty acids (SCFAs) involving different intermediate metabolites such as pyruvate, succinate, lactate, 1,2-propanediol, and acetyl-coenzyme A (coA) (Figure 2) [25]. In addition, small but significant amounts of alcohols, including ethanol, propanol and 2,3-butanediol can be formed as end-products of carbohydrate fermentation [25]. Furthermore, gaseous by- and end-products, such as hydrogen (H_2), carbon dioxide (CO_2), and sulphate (SO_4^{2-}) propel fermentation forward. Although energy needs are met primarily through fermentation, anaerobic respiration, through the membrane electron transport chain, confers the capacity to use H_2 and CO_2 by reductive acetogen and methanogen microorganisms, respectively, and SO_4^{2-} by sulphate-reducing microorganisms, as electron acceptors [95]. The utilisation of these gaseous substrates is mainly the result of cross-feeding between members of the gut microbiota, rather than host absorption [96].

Among the bacterial metabolites produced by anaerobic fermentation of carbohydrates, SCFAs have been shown to exert multiple beneficial effects on host physiology. They represent a major source of energy for the host, in general, and the colon, in particular [97,98]. In humans, these bacterial metabolites provide about 10% of the daily caloric requirements. Importantly, butyrate is the most preferred energy supply for colonocytes and is used in the Krebs cycle and in ketone bodies production pathway [99]. Observations in germfree condition have highlighted the important role of microbiota-derived butyrate in colonocyte feeding. Indeed, in this condition, where there is no butyrate production, a colonic mucosal atrophy is observed and is associated to metabolic starvation and a deficit in mitochondrial respiration [100–102]. Furthermore, the intestinal microbiota has been shown to contribute to the metabolic specialisation of the colonic epithelium with the use of butyrate, and in vivo data has shown that butyrate can modulate the expression of an enzyme involved in its own metabolism [103]. Butyrate also maintains the intestinal barrier integrity by promoting cell proliferation, apoptosis, tight junctions, and mucus production [104]. Once produced in the intestinal lumen, acetate and propionate are easily

absorbed by the colonic wall. They enter the portal blood compartment and are preferentially metabolised in the liver. They can fuel hepatic biosynthesis pathways, such as gluconeogenesis, cholesterol, and long-chain fatty acids synthesis, thus contributing to the whole host metabolism [105]. SCFAs can also act as microbial-signalling molecules that are recognised by specific host receptors, such as G-protein-coupled receptors, including GPR41 (also known as Free fatty acid receptor 2 (FFA2)), GPR43 (also known as Free fatty acid receptor 3 (FFA3)), GPR109A (also known as Hydroxycarboxylic acid receptor 2 (HCA2)), and Olfactory receptor 78 (Olfr78) [106,107]. In particular, GPR41 and GPR43 are expressed in entero-endocrine cells where they can activate a series of events conferring an array of metabolic effects, including the production of glucagon-like peptide-1 (GLP-1) and the appetite-regulating hormone peptide YY (PYY) [108]. Furthermore, the wide range of expression of these receptors, including in immune cells, suggests several other potential functions of SCFAs, especially those involving immune response and inflammation [109].

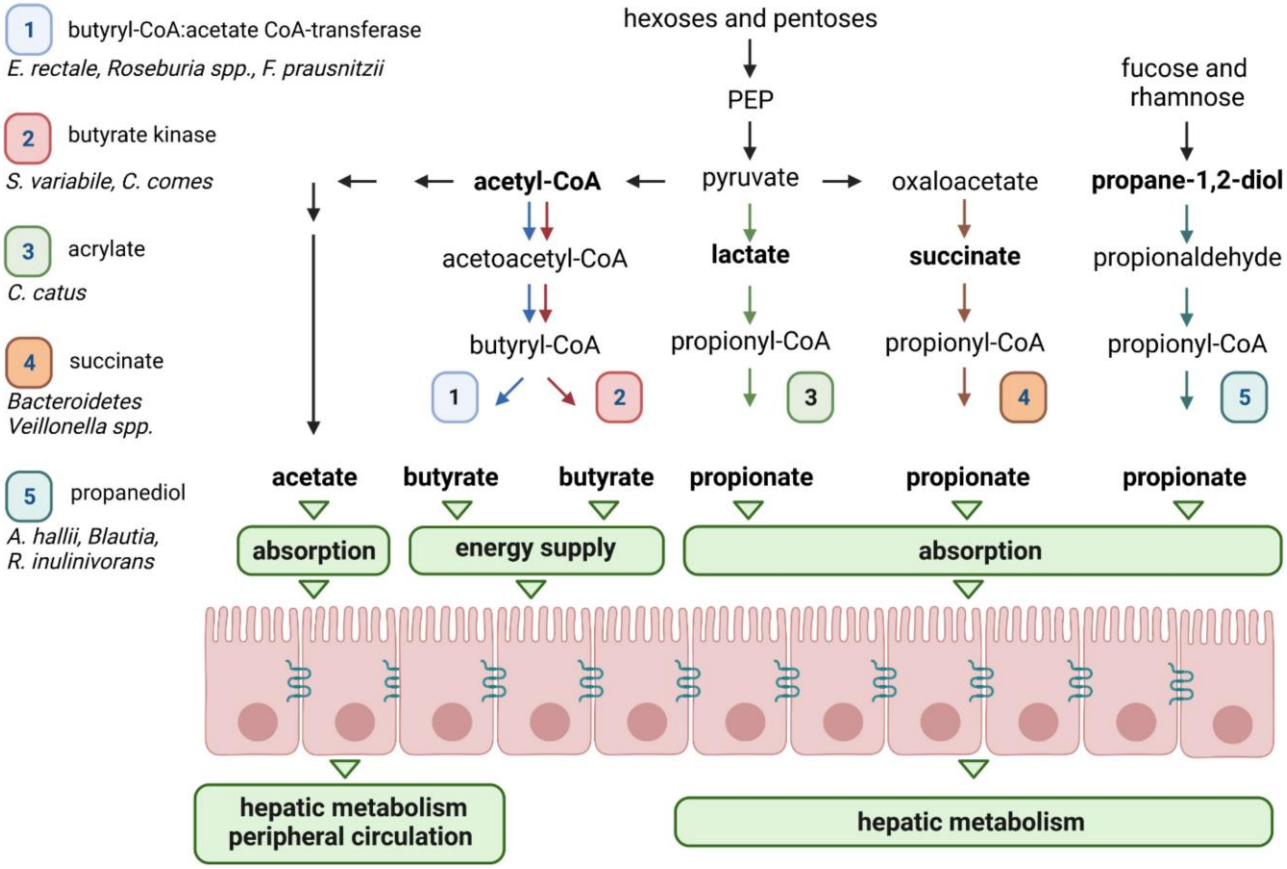

Figure 2. Short-chain fatty acid (SCFA) biosynthesis pathways by the gut microbiota. The different pathways involved in SCFA production are presented for acetate, butyrate, and propionate. SCFA-producing bacteria for each pathway are also shown. Acetate is mainly produced in the gut from pyruvate via acetyl-coenzyme A (CoA). Three pathways have been described for propionate synthesis, namely acrylate, succinate, and propanediol. The first two start from phosphoenolpyruvate (PEP) and the latter uses deoxysugars, such as fucose and rhamnose. Butyrate is produced via two pathways: the butyryl-CoA: acetate CoA-transferase and the butyrate kinase.

Given the role of these bacterial metabolites in human health, a detailed understanding of the metabolism of SCFAs by the gut microbiota is required to optimise efficient diet modulation strategies. SCFAs differ in relative production rates, concentrations, and bacterial producers. Metagenomic approaches have facilitated the characterisation of bacteria responsible for SCFA production (Figure 2) [110,111]. Found in the highest concentrations in the gut lumen, acetate is mainly produced by the decarboxylation of pyruvate. This

step is followed by the hydrolysis of acetyl-CoA to acetate by an acetyl-CoA hydrolase. Playing an essential role in the central metabolism, most acetate is synthesised through this pathway by gut commensal bacteria, including *Prevotella* spp., *Ruminococcus* spp., *Bifidobacterium* spp., *Bacteroides* spp., *Clostridium* spp., *Streptococcus* spp., *A. muciniphila*, and *B. hydrogenotrophica*. Different subsets of gut bacteria are involved in the production of propionate and butyrate [25]. Propionate biosynthesis involves three different biochemical pathways, namely succinate, acrylate, and propanediol routes that are distributed within different bacterial groups [111]. Propionate synthesis through the succinate pathway appears to be the main route in the gut and is found primarily in bacteria belonging to the *Bacteroidetes* phylum and in a few members of *Firmicutes* [25]. Interestingly, faecal propionate levels (as a percentage of all fermentation acids) have been reported to be significantly correlated to their relative abundance of *Bacteroidetes* [112]. The acrylate and propanediol pathways concern a reduced number of gut bacteria. Some members of the *Lachnospiraceae* family, including *Roseburia inulinivorans* [113], as well as *A. hallii*, *Blautia* species, and *A. muciniphila* can synthesise propionate through the propanediol pathway [114,115]. This involves the formation of 1,2-propanediol from deoxy sugars, such as rhamnose and fucose, and then its conversion into propionaldehyde and propionyl-CoA, leading to propionate. Consisting in the conversion of lactate to propionate, the acrylate pathway is the minor route of propionate synthesis and has been described in a small number of species such as *C. catus* [111]. Identification of butyrate-producing pathways revealed that they are mostly present in several different classes within the *Firmicutes* phylum. Indeed, butyrate producers are abundant in the gut and are primarily members of *Lachnospiraceae* and *Ruminococcaceae* [111]. For example, *Faecalibacterium prausnitzii* (which can represent more than 5% of the total bacterial population) [116], *Eubacterium rectale*, and *Roseburia* spp. (estimated to be 2 to 15% of the total bacteria within the *Lachnospiraceae*) are dominant butyrate-producing species in the human gut microbiota [117]. Butyrate production first involves the condensation of two molecules of acetyl-CoA to form butyryl-CoA through different steps, and then the liberation of butyrate from butyryl-CoA through two different types of reaction. In the first one, two enzymes are responsible for the conversion of butyryl-CoA into butyrate, i.e., phosphotransbutyrylase and butyrate kinase enzymes. In the second pathway, butyryl-CoA: acetate CoA-transferase converts butyryl-CoA into butyrate. Few bacteria in the gut use the butyrate kinase pathway, which seems to be limited to some *Coprococcus* species, whereas the butyryl-CoA:acetate CoA-transferase pathway is used by the majority of known butyrate-producing gut strains, such as *F. prausnitzii*, *E. rectale*, *E. hallii*, and *R. bromii* [118].

SCFA levels are frequently correlated to health. Indeed, metagenomic analyses, carried out on the butyrate production pathways, have shown that in diseases such as obesity and diabetes, the abundance of butyrate-producing species is lower than in healthy microbiota [119,120]. Interestingly, the amount of ingested dietary fibre has been shown to modulate the relative abundances of taxa and microbial genes associated with butyrate production. A low-fibre diet has been linked to reduced relative abundances of butyrate-producing microbes in humans [121], non-human primates [122], and mice, an effect which is more pronounced from generation to generation [123].

3. From Fundamental Research to Therapeutic Use

3.1. Prebiotics in Human Nutrition

Translating the fundamental research on the gut microbiota into clinical practice and/or interventional strategies constitutes a great challenge to improve public health. It is now time to revisit the prebiotic concept and deliver physiological benefits with rationally designed interventions based on gut microbiota engineering [124].

Recent human trials have shown the beneficial effects of prebiotics on the gut microbiota, health, and well-being. Given the nutritional impact of prebiotics on the gut microbiota composition and metabolic activities, this clinical data should be taken into account in future guidelines and public health recommendations [125]. Nevertheless, when

considering the prebiotic impact on the composition and functions of the gut microbiota, several factors contribute to the gap of knowledge for the translation of prebiotics [19]. For example, functional effects of prebiotics depend upon the biological source that defines their chemical structures (e.g., molecular weight, individual monomers, degree of polymerisation, and type of linkage). This influences their fermentation by the gut microbiota and can contribute to a variety of physiological effects on health and wellbeing. Also, the nutrient composition of natural plant sources creates complex food matrices for mi- crobes, which can impact the accessibility of polysaccharides to the gut microbiota. Finally, different types of polysaccharides co-exist in foods. For example, barley and oat cereals contain beta-glucans, arabinoxylans, starches, insoluble fibres, proteins, and other bioactive phytochemicals [126–128]. Thus, the advantage of the natural mix of different substances with bioactive properties should be better considered in intervention studies to ensure the utilisation by the gut microbiota functions prone to act on host physiology [125].

In addition, significant advances in the understanding of interactions between the gut microbiota, nutrition, and the host have not yet been considered in current public health recommendations. In particular, dietary fibre intake recommendations around the world refer to a certain amount of total dietary fibre for optimal health, typically 25 to 35 g/day [34]. However, these dietary guidelines do not consider the ratio of soluble versus insoluble dietary fibre, the levels of phenolic compounds, the fermentability by the colonic microbiota (rapid, slow, completely fermentable or to a limited degree), and the problems of digestive discomfort related to the consumption of dietary fibres. The balance between digestive tolerance and metabolic issues should be addressed in future dietary recommendations. There is a need for rigorous randomised intervention studies in humans with strict dietary control to evaluate the consumption of the types and amounts of prebiotics and their efficacy in the management of metabolic-related health issues [46].

3.2. Clinical Evidence of Prebiotic Effects

Several studies have already investigated the beneficial potency of ITF, including the two best characterized prebiotics inulin and FOS. In healthy adults, it has been shown that the consumption of chicory inulin regulates post-prandial glycemia and insulinemia. In a long-term dietary fibre supplementation, an acute dose of 24 g inulin in men with obesity decreased postprandial plasma glucose and insulin [129]. In overweight subjects, 20 g per day of inulin for 42 days decreased plasma insulin and improved insulin sensitivity [130]. Furthermore, ITF supplementation with a daily dose of 10 g for a duration of 6 weeks improved glycemic control for prediabetes and type 2 diabetes [131]. Similar effects were reported in healthy adults who consumed food products enriched in oligofructose [132].

In addition, inulin contributed to satietogenic effects through the suppression of ghrelin and increase of circulating GLP-1 and PYY concentrations, leading to the overall reduction in energy intake [133]. The improvement of appetite control was also reported in overweight and obese adults and children receiving a daily dose of 8 g for 16 weeks [134]. In overweight or obese adults, 21 g per day of oligofructose supplementation for 12 weeks attenuated body fat mass and weight gain. This was associated with a decrease in food intake and observed without changes in physical activity or lifestyle [133–135].

It has also been reported that a 30 day-inulin supplementation improved gastrointestinal disorders and inflammatory markers in middle-aged subjects [136]. The effect of inulin consumption on stool frequency was confirmed in healthy adults with mild constipation [137]. One potential mechanism for the beneficial effects of FOS could be through the improvement of obesity-associated intestinal permeability and inflammation. Indeed, a reduction of faecal markers related to intestinal integrity and inflammation was observed in obese patients after a prebiotic intervention consisting of a daily dose of 16 g of inulin for 3 months, in addition to dietary caloric restriction [138].

Inulin supplementation improved lipid metabolism in the management of dyslipidaemia associated with obesity and cardiometabolic risk. Physiological metrics identified

signature biomarkers related to vascular, inflammatory, and lipid dysmetabolic morbidities (obesity and type 2 diabetes) in middle-aged individuals [139].

Furthermore, oligofructose supplementation in human studies induced specific microbial modifications that were associated with an increase in *Bifidobacterium* and *Bacteroides* abundances [130,138]. Indeed, specific inulin-induced changes in relative abundances of *Anaerostipes*, *Bilophila*, and *Bifidobacterium* were identified in 4-week intervention periods of 12 g of inulin consumed on a daily basis [137]. These microbial changes might result in a rise in SCFA production, revealing an increase in carbohydrate fermentation [125]. Thus, oligofructose was reported to increase the concentrations of acetate, propionate, and butyrate in faecal samples of healthy adults [137,140]. However, in contrast, other studies did not report any effect of inulin supplementation on SCFA levels [141]. Given these contradictory results, more evidence is needed to further elucidate the effect of inulin on SCFA production. Indeed, these bacterial-derived metabolites are key players involved in the metabolic health and diseases [91]. These potential mechanisms driving the beneficial effects of oligofructose may be due to upstream alterations of the gut microbiota [132].

Overall, oligofructose is one of the most promising dietary fibres extensively studied in randomized, double-blind, placebo-controlled crossover trials, which have contributed to substantial improvement of metabolic parameters in humans.

3.3. The Inter-Individual Responses to Prebiotics Require Personalised Nutrition Strategies

A substantial number of human dietary interventions have revealed that prebiotics can have highly variable effects in the metabolic health outcomes and the gut microbiota [142]. Indeed, the gut microbiota responds rapidly to changes in our diet, on a time scale of as little as 24 h, resulting in temporal fluctuations on time scales from hours to days [143,144]. Despite maintaining the dietary intervention, the gut microbiota can nearly return to its original baseline state for the remainder of the intervention [145,146]. This microbial resilience phenomenon is the result of long-term dietary habits that constitute the determinant force of an individual's gut microbiota [147,148]. Hence, the gut microbiota exhibits considerable interpersonal variations in taxonomic composition and functions, regardless of individual health status [149,150]. Evidence reported that over 20% of the interpersonal variability of microbial signatures can be inferred from environmental factors associated with diet and lifestyle [145,151]. Geography is a major factor with dominant species in the gut showing a large degree of individual variation across cultures and continents [152]. The tremendous variability of microbial responses to dietary interventions has been correlated to a variety of confounding factors, including the use of medications (e.g., antibiotics, osmotic laxatives, female hormones, benzodiazepines, antidepressants, and antihistamines) and stool consistency, among others [153].

Differential clinical responses to prebiotics in adults can be more effective in some individuals, identified as responders, than in others, identified as non-responders (Figure 3). Responders are individuals with appropriate baseline commensal microbes for whom the prebiotic may confer a health benefit [21]. The reports of responders and non-responders in intervention studies encourage the determination of individual characteristics to better apprehend the efficacy of dietary intervention. The standardization of clinical protocols could provide details on the subjects enrolled in studies, including sex, age, ethnicity, diet, and the compositional and functional features of their gut microbiota. This gathering of in-depth clinical information would serve to predict individual responses to prebiotics [154,155]. For instance, a previous study characterised by high fibre and decreased energy intake illustrated the gene richness of the gut microbiome as a key microbial feature in pinpointing individuals who would respond efficiently to short-term dietary intervention [156]. This study highlighted that the positive response in obese patients was less effective in improving clinical phenotypes in individuals with lower microbial richness. Thus, the baseline of microbial diversity may be an important predictor of the response to diet-induced improvements in clinical responses to prebiotics [112]. Moreover, the magnitude of individual shifts induced by prebiotics can be restricted to selective responding bacterial species in the

composition of the gut microbiota. The reasons for this individual variability in response to dietary intervention can be explained by the differences in enzyme capacity to utilise a prebiotic substrate [157] and/or can be due to the absence of keystone species [53]. For instance, previous studies have proposed profiling the gut microbiome for CAZymes as a key microbial feature that can profoundly influence how the host interacts with respect to diverse carbohydrate sources [158]. Referred to as CAZy-typing, the analysis of the repertoires of gut microbiome CAZyme-encoding genes can be used as an indicator to predict the metabolisation of prebiotics on an individual scale [159]. Indeed, the inconsistent responsiveness of individuals in dietary interventions can be explained by the absence of functional "guilds" able to access and utilise a carbon source [160].

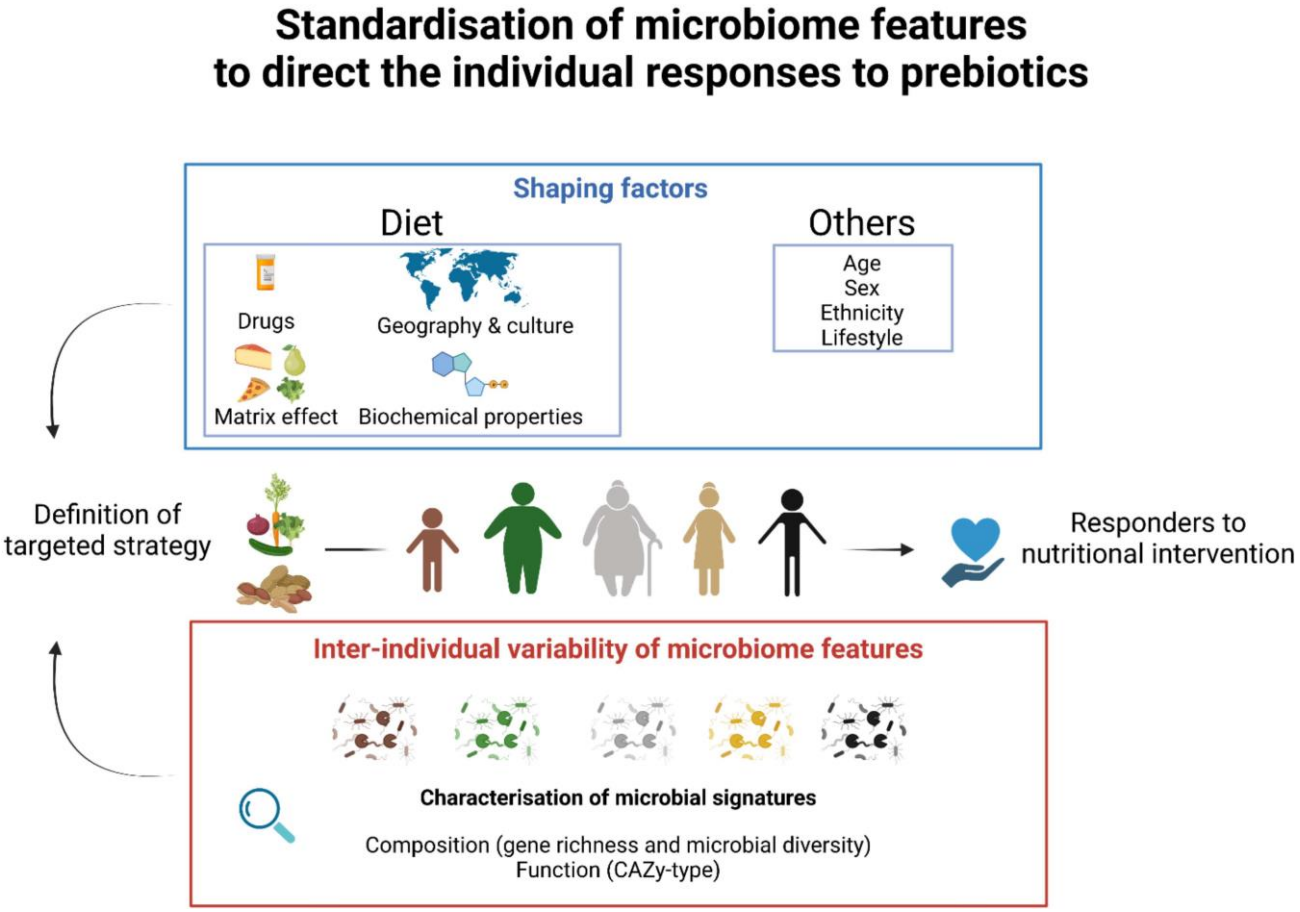

Figure 3. The determination of individual characteristics to direct an efficient prebiotic intervention. Individual and environmental shaping factors and interindividual variability of microbiomes modulate differential clinical responses to prebiotics. The description of microbiome signatures at the compositional and functional levels can provide insights to define a targeted nutritional strategy. A standardisation of multi-criteria aims to match the prebiotic intervention with individuals that would likely respond efficiently.

Identifying specific characteristics of the gut microbiome will enable the development of individualised nutrition strategies [161] (Figure 3). Indeed, there is significant interest in targeted strategies to modulate microbial composition within hosts in a personalised approach to redirect microbial signatures towards health [155]. A recent personalised diet intervention successfully identified personal and microbiome characteristics to accurately predict personalised postprandial glycemic responses. This study revealed that exposure to specific dietary components modulated the composition of the gut microbiota that influenced host metabolic responses to lower postprandial glucose [162]. Therefore, the

development of personalised diets that regulate blood sugar levels provides hope for further advancements in the control and treatment of disease.

Future research on short-term and long-term dietary interventions could improve the characterisation responses of the gut microbiota to prebiotics to give us a deeper understanding of these interventions and their potential for precision application [163]. Health and microbiome measurability is required before clinical recommendations and can be made for dietary modulation of the gut microbiota to improve health and well-being. Although general recommendations may lay the foundation for nutritional guidelines, the path to a personalised nutrition approach will be an increasingly desirable research area in the near future. The development of precision nutritional strategies may need to be unique to each individual and tailor-made to each individual's microbial composition.

4. Conclusions

Due to the variability of the responses of the gut microbiota, transforming theory into real-life host benefits is complicated. Future studies of the interactions between prebiotics and the gut microbiota is likely to rely on transdisciplinary fields, including evolution, ecology, microbiology, biomedicine, and computational biology. A better comprehension in the breakdown mechanisms of prebiotics increments requires fundamental research of the gut microbiota that supports the translation into clinical interventions, personalised nutrition approaches, and public recommendations. Collaborative efforts are still in their infancy and should be encouraged, taking into account public health issues.

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Abbreviations

SCFAs	short-chain fatty acids
ISAPP	international scientific association for probiotics and prebiotics
DP	degree of polymerization
HMOs	human milk polysaccharides
FOS	fructo-oligosaccharides
GOS	galacto-oligosaccharides
ITF	inulin-type fructans
XOS	xylo-oligosaccharides
AXOS	arabino-xylo-oligosaccharides
CAZymes	carbohydrate active enzymes
GH	glycoside hydrolase
GT	glycosyltransferase
CE	carbohydrate esterase
PL	polysaccharide lyase
CBM	carbohydrate-binding module

PUL	polysaccharide utilisation loci
SUS	starch utilisation system
SGBP	surface glycan-binding protein
TBDT	TonB-dependent transporter
gpPUL	Gram-positive PUL
ABC	ATP-binding cassette
MFS	major facilitator superfamily
PTS	phosphoenolpyruvate-phosphotransferase system
LPMOs	lytic polysaccharide monooxygenases
OMVs	outer membrane vesicles
H_2	hydrogen
CO_2	carbon dioxide
SO_4^{2-}	sulphate
GPL-1	glucagon-like peptide-1
PYY	peptide YY

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