Understanding the role of raffinose family oligosaccharides in pea on plant fitness and gut health

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Abstract

Raffinose family oligosaccharides (RFOs), including raffinose, stachyose and verbascose, are abundant in legumes, contributing to plant stress tolerance. Although once considered anti-nutritional due to their indigestibility and flatulence effects, RFOs are now being regarded as emerging prebiotics.

This study investigated the role of RFOs in pea on plant performance and their effects on human gut microbiota composition and function. Mutations in *Raffinose synthase* (*Rfs*) and *Rfs*-like genes resulted in pea seeds with decreased RFO content and accumulation of the RFO precursors, sucrose and galactinol. The BCFN 1551 mutant exhibited reduced tolerance to drought stress in the glasshouse and poor productivity in the field. The performance of the BCRfsTILL W521* mutant was similar to that of the commercial cultivar Caméor in the field.

Batch fermentations with human faecal samples revealed that Caméor flour led to higher *Bifidobacterium longum* levels compared to the BCFN 1551 mutant, along with alterations in bile acid and tryptophan metabolism. Fermentation with sucrose led to increased *Bifidobacterium* spp., whereas galactinol fermentation was characterised by increased *Citrobacter freundii*. Individual RFO fermentation assays revealed that raffinose and verbascose increased *Bifidobacterium* spp., with verbascose increasing butyrate production. Dynamic colon model fermentation of Caméor and BCRfsTILL W521* showed no significant differences in microbiota composition attributable to RFO reduction.

Gas production did not differ significantly among fermentations of BCFN 1551, BCRfsTILL W521*, and Caméor flours, suggesting that other fermentable carbohydrates may overshadow RFO effects. Preliminary studies with human intestinal organoids showed that fermented Caméor flour increased interleukin-8 production under healthy conditions but not following inflammatory challenge, but intestinal integrity remained unaffected.

Together, we showed that RFOs are important for pea drought tolerance and positively influence human gut microbiota. This work paves the way to the development of stress-resilient pea varieties with improved nutritional traits.

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Table of contents

Abbreviations 7

List of Figures 11

List of Tables 14

1 Chapter One: Introduction 15

1.1 *Pisum sativum* 16

1.1.1 Global distribution and adaptation of pea and other legumes to the environment 16

1.1.2 Advancements and key milestones in legume and pea breeding 22

1.1.3 Nutritional value of pea and other legumes 24

1.2 The human gastrointestinal (GI) tract 27

1.2.1 Structure and function of the GI tract 27

1.2.2 The intestinal epithelium 28

1.3 The gut microbiota 34

1.3.1 Establishment and composition of the microbiota in the GI tract 34

1.3.2 Biogeography of the gut microbiota in the gut 35

1.3.3 Role of the gut microbiota in human health 39

1.3.4 Modulation of the gut microbiota by the diet 45

1.4 Raffinose Family Oligosaccharides (RFOs) 53

1.4.1 Biosynthesis and accumulation of RFOs in plants 53

1.4.2 Role of RFOs in plants 55

1.4.3 Effect of RFOs on animal and human health 57

1.5 Aim and objectives 58

2 Chapter Two: Materials and methods 59

2.1 Plant-based assays 60

2.1.1 Pea seeds used in this study 60

2.1.2 Field trial 66

2.1.3 Controlled water deficit in the glasshouse 66

2.1.4 Pre-emergence herbicide trials 67

2.1.5 *Rhizobium* nodulation 67

2.1.6 Pea flour and extract preparation 68

2.1.7 Analytical measurements 68

2.2 Microbiology 70

2.2.1 *Bifidobacteria* growth assays 70

2.2.2 Fermentation assays 71

2.2.3 Metagenomics analyses 73
2.2.4 Analytical measurements 76
2.3 Intestinal organoid cultures 78
2.3.1 Organoid culture expansion 78
2.3.2 Culture of organoids on transwells 79
2.3.3 Cytokine-induced inflammatory challenge on organoid monolayers 79
2.3.4 Fermented pea flour treatment on organoid monolayers 79
2.3.5 Transepithelial electrical resistance (TEER) measurement assays 80
2.4 Immunological assays 80
2.4.1 LEGENDplex™ flow cytometry-based multiplex 80
2.5 *In silico* analyses 81
2.6 Statistical analyses 82
3 Chapter Three: Characterisation and agronomical traits of *P. sativum* raffinose synthase mutants 83
3.1 Introduction 84
3.2 Results 85
3.2.1 Identification of *rfs* pea mutants 85
3.2.2 Characterisation of RFOs in pea *rfs* mutants 90
3.2.3 Effect of water deficit on agronomical traits of *rfs* pea mutants in the glasshouse 92
3.2.4 Evaluation of agronomical traits of pea RFO mutants in the field 95
3.2.5 The effects of pre-emergence herbicides on FN *rfs* mutant and control pea plants 101
3.2.6 Nodulation of *R. leguminosarum* A3841 on *rfs* mutants and control pea plants 104
3.3 Discussion 106
4 Chapter Four: Effects of RFOs and RFOs in pea on the human gut microbiota and barrier function 111
4.1 Introduction 112
4.2 Results 113
4.2.1 Effect of RFOs on gut microbiota structure and function 113
4.2.2. Effects of RFO fermentation from pea on gut barrier function using *in vitro* human-derived colonic organoid model 134
4.3 Discussion 140
5 Chapter Five: Effects of BCTILLRfs W521* pea mutant on gut microbiota structure, function and gas production 149
5.1 Introduction 150
5.2 Results 151
5.2.1. The effect of BCTILLRfs W521* and Caméor pea flour on the human gut microbiota structure and function using a dynamic fermentation system 151

5.2.1.2 Effect of BCTILLRfs W521* and Caméor pea flour on the human gut microbiota composition..... 152

5.2.2. Effect of BCTILLRfs W521*, BCFN 1551 and Caméor pea flour on cumulative gas pressure using the ANKOM^{RF} fermentation system 166

5.3 Discussion 169

6 Chapter Six: Conclusions, limitations and future perspectives 173

6.1 The FN 1551/11 mutant showed altered plant resistance to stress and poor performance in the field compared to Caméor, while the BCRfsTILL W521* mutant performed well in field conditions 174

6.2 RFOs and precursors impact the gut microbiota composition and function *in vitro* 176

6.3 *In vitro* fermentation of Caméor and FN 1551/11 and BCRfsTILL W521 pea flours led to differences in microbial composition and metabolism but cannot be attributed to RFO content

..... 177

6.4 Concluding remarks and perspectives 178

Supplementary figures 182

Abbreviations

2’-FL	2`-fucosyllactose
AA	Amino acid
AC	Ascending colon
ACE	Angiotensin I- converting enzyme
AhR	Aryl hydrocarbon receptor
ALDH	Aldehyde dehydrogenase superfamily
AMG	Amyloglucosidase
ASVs	Amplicon sequence variants
AUC	Area under the curve
AXOS	Arabinoxylooligosaccharides
BC	Backcross
BCAAs	Branched amino acids
BCFAs	Branched-chain fatty acids
BLAST	Basic Local Alignment Search Tool
BNF	Biological nitrogen fixation
BP	Boiling point
BSA	Bovine serum albumin
bsh	Bile salt hydrolase
CAzymes	Carbohydrate-active enzymes
CBCs	Crypt base columnar cells
CD	Crohn`s disease
CEP	C-terminally encoded peptide
CID	Compound identifier
CLR	Centred log ratio transformation
CRISPR-Cas9	Cas clustered regularly interspaced short palindromic repeats associated 9
cscA	Sucrose-6-phosphatehydrolase
DC	Descending colon
DCA	Deoxycholic acid
DMEM/F-12	Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12
DNA	Deoxynucleic acid
EC	Enzyme commission
EGF	Human epidermal growth factor
EMS	Ethylmethane sulphonate
FAO	Food and Agriculture Organisation of the United Nations
FN	Fast neutron
FOS	Fructo-oligosaccharides
FSC	Forward scatter
GALT	Gut-associated lymphoid tissue
GAP	Goblet cell associated passage

GGT	Galactan-galactan galactosyl transferase
GH	Glycoside hydrolase
GI	Gastrointestinal
GLP-1, GLP-2	Glucagon-peptide like 1 and 2
GolS	Galactinol synthase
GOS	Galacto-oligosaccharides
GSMMs	Genome-scale metabolic models
H2S	Hydrogen sulphide
HCl	Hydrochloric acid
HIF	Hypoxia-inducible factors
HILIC	Hydrophilic interaction liquid chromatography
HMOs	Human milk oligosaccharides
HMOs	Human milk oligosaccharides
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
ICNP	International Code of Nomenclature for Prokaryotes
IFN-γ	Interferon- γ
IgA	Immunoglobulin A
IL	Interleukin
INRAE	National Research Institute for Agriculture, Food and Environment in France
IP-10 I	nterferon γ-induced protein 10 kDa
IRLC	Inverted Repeat-Lacking Clade
ISAPP	International Scientific Association for Probiotics and Prebiotics
ISCs	Intestinal stem cells
ITF	Inulin-type fructans
JIC	John Innes Centre
LCA	Lithocholic acid
LC-MS	Liquid chromatography-mass spectrometry
ldh	Lactate dehydrogenase
LEfSe	Linear Discriminant Analysis Effect Size
LNnT	Lacto-N-neotetraose
MALT	Mucosa-associated lymphoid tissue
MCP-1	Monocyte chemoattractant protein-1
mMRS	Modified Man-Rogosa-Sharpe
MRM	Multiple reaction monitoring
MRS	Man-Rogosa-Sharpe
MUC2	Type-2 mucin
N	Nitrogen
NCR	Nodule-specific cysteine-rich peptides
NO	Nitric oxide
NodD	Nodulation protein D
OC	Outcross

OD	Optical density
ODM	Organoid differentiation medium
OGM	Organoid growth medium
OTUs	Operational Taxonomic Units
PBS	Phosphate buffer solution
PCA	Principal component analysis
PCoA	Principal coordinate analysis
PICRUSt2	Phylogenetic Investigation of Communities by Reconstruction of Unobserved Stats 2
PSA	Polysaccharide A
PUL	Polysaccharide utilisation loci
PYY	Peptide YY
QIB	Quadram Institute Bioscience
qPCR	Quantitative polymerase chain reaction
QQ	Quantile-quantile
QTL	Quantitative trait loci
RFOs	Raffinose Family Oligosaccharides
Rfs	Raffinose synthase
Rfs-like	Raffinose synthase-like
RNA	Ribonucleic acid
ROCKi	ROCK-inhibitor Y-27632 dihydrochloride
RS2, RS3	Soybean Rfs isoforms 2 and 3
RT	Room temperature
SCFAs	Short-chain fatty acids
SCR	Stop codon readthrough
SHIME®	Simulator of the Human Intestinal Microbial System
SIM	Selected ion monitoring
SIMGI®	SIMulator of the GastroIntestinal tract
SNPs	Single nucleotide polymorphisms
SSC	Side scatter
TC	Transverse colon
TEER	Transepithelial electrical resistance
TGF-β	Transforming growth factor β
TILLING	Targeted Induced Local Lesions in Genomes
TMAO	Trimethylamine N-oxide
TNF- α	Tumour necrosis factor-α
TY	Tryptone-yeast extract
UC	Ulcerative colitis
UDP	Uridine diphosphate
URGV	Plant Genomics Research Unit
USDA	United States Department of Agriculture
UTILLdb	URGV TILLING database
WD	Water deficit
Wnt3	Wnt family member 3

wps	Weeks post sowing
XOS	Xylo-oligosaccharides
ZO	Zonula occludens

Bacteria phylum nomenclature

International Code of Nomenclature for Prokaryotes (ICNP) (Oren et al., 2023)	Former nomenclature
Bacillota	Firmicutes
Bacteroidota	Bacteroidetes
Methanobacteriota	Euryarchaeota
Actinomycetota	Actinobacteria
Pseudomonadota	Proteobacteria

List of Figures

Figure 1. Fabaceae phylogeny tree…… 17
Figure 2. Stages of legume-rhizobia symbiosis…… 19
Figure 3. Morphology of a mature pea seed…… 22
Figure 4. TILLING pipeline for UTILLdb pea population…… 24
Figure 5. Gut layer organisation…… 27
Figure 6. Cross-sectional structure of the small and large intestines and the major cell constituents of the epithelium…… 30
Figure 7. Flux across the epithelial barrier and depiction of intercellular junction…… 32
Figure 8. Dominant bacteria families in the small and large intestine…… 36
Figure 9. Chemical structures of acetate, propionate, butyrate and valerate…… 40
Figure 10. Effect of bacterial cross-feeding from prebiotic dietary sources on SCFA production…… 50
Figure 11. Chemical structures of RFO precursors (sucrose and galactinol) and RFOs (raffinose, stachyose and verbascose), with detail of the galactinol-dependent biosynthesis pathway…… 54
Figure 12. Schematic showing introgression of a donor locus (*rfs/rfs*) into the commercial cultivar Caméor using backcrossing, and pea plant compound leaf morphology…… 65
Figure 13. Experimental design used to study plant responses to water deficit (WD)…… 67
Figure 14. Transepithelial/endothelial electrical resistance (TEER) measurement using EVOM ® instrument…… 80
Figure 15. Structural model of Rfs predicted by AlphaFold…… 89
Figure 16. Alignment of the catalytic region of α-galactosidase AtAkαGal3 and raffinose synthases Rfs and Rfs-like from pea…… 89
Figure 17. LC-MS analysis of RFOs and their precursors in pea mutants and control lines…… 90
Figure 18. Starch concentrations (% w/w) in *rfs* pea mutants and Caméor cultivar…… 91
Figure 19. Development of Caméor and BCFN 1551 pea plants under water deficit (WD) conditions in the glasshouse…… 92
Figure 20. Seed yield traits in BCFN 1551 mutant and Caméor cultivar pea plants grown under controlled and WD conditions in the glasshouse…… 93
Figure 21. Flowering production and pod formation onset during the development of BCFN 1551 and Caméor cultivar under controlled watering and WD conditions…… 94
Figure 22. LC-MS analysis of RFOs and their precursors in mature seeds from pea mutants under controlled watering or WD conditions in the glasshouse…… 95
Figure 23. Germination percentage of *rfs* pea mutants and Caméor cultivar in the field…… 96
Figure 24. Close-up of plant leaflets of *rfs* pea mutants and Caméor cultivar in the field…… 97
Figure 25. Development of *rfs* pea mutants and Caméor cultivar pea plants in the field…… 98
Figure 26. Total seed yield of *rfs* pea mutants and Caméor cultivar in the field…… 99
Figure 27. Average 100-seed weight of *rfs* pea mutants and Caméor cultivar in the field…… 100
Figure 28. Percentage of insect damaged seeds in field trials of *rfs* pea mutants and Caméor cultivar in the field…… 101
Figure 29. Effect of pre-emergence herbicides on the germination of BCFN 1551 *rfs* pea mutant and Caméor cultivar pea plants in glasshouse conditions…… 102
Figure 30. Effect of pre-emergence herbicides on germination for BCFN 1551 *rfs* pea mutant and Caméor cultivar in the field…… 104
Figure 31. *R. leguminosarum* A3841-induced nodule formation in rfs pea mutants and Caméor control cultivar…… 105

Figure 32. Effect of RFOs, raffinose and verbascose, precursors, sucrose and galactinol, and pea flour and pea extracts on the alpha and beta diversity of the faecal microbiota composition in batch fermentations…….. 105
Figure 33. Effect of RFOs, raffinose and verbascose, precursors, sucrose and galactinol, and pea flour and pea extracts on faecal phylum composition in batch fermentations…… 114
Figure 34. Effect of RFOs (raffinose and verbascose), RFO precursors (sucrose and galactinol), pea flour and pea extracts (from Caméor and BCFN 1551) on faecal microbial composition in batch fermentations…….. 116
Figure 35. Effect of RFOs (raffinose and verbascose), RFO precursors (sucrose and galactinol), pea flour and pea extracts (from Caméor and BCFN 1551) on species from the *Bacteroidaceae* and *Oscillospiraceae* families in batch fermentations…….. 118
Figure 36. Functional prediction on the effect of RFO (raffinose and verbascose), RFO precursors (sucrose and galactinol), pea flour and pea extracts (from Caméor and BCFN 1551) based on genes present in the metagenome of batch fermentations…… 122
Figure 37. Effect of RFOs (raffinose and verbascose), RFO precursors (sucrose and galactinol), pea flour and pea extracts (from Caméor and BCFN 1551) on the relative abundance of *Bifidobacterium* shunt metabolic pathway in the metagenome of batch fermentations…….. 123
Figure 38. Effect of RFOs raffinose and verbascose, precursors sucrose and galactinol, pea flour and pea extracts on the proportion of SCFAs produced after 24 h batch fermentation…… 124
Figure 39. Effect of RFOs (raffinose and verbascose), RFO precursors (sucrose and galactinol), pea flour and pea extracts (from Caméor and BCFN 1551) on SCFA production. 125
Figure 40. Effect of RFOs (raffinose and verbascose), RFO precursors (sucrose and galactinol), pea flour and pea extracts (from Caméor and BCFN 1551) on valerate and lactate production…….. 126
Figure 41. PCA of the metabolites produced in the fermentations of RFO (raffinose), RFO precursors (sucrose, galactinol) and pea extracts and pea flour (from Caméor and BCFN 1551)…….. 127
Figure 42. Effects of 24 h batch fermentations with RFO (raffinose), RFO precursors (sucrose, galactinol) and pea flour and extracts (from Caméor and BCFN 1551) on bile acid synthesis and metabolism…….. 128
Figure 43. Effects of 24 h batch fermentations with RFO (raffinose), RFO precursors (sucrose, galactinol) and pea flour and pea extracts (from Caméor and BCFN 1551) on histidine metabolism…….. 130
Figure 44. Effects of 24 h batch fermentations with RFO (raffinose), RFO precursors (sucrose, galactinol) and pea flour and extracts (from Caméor and BCFN 1551) on tryptophan metabolism…… 131
Figure 45. Effect of RFOs (raffinose and verbascose), RFO precursors (sucrose and galactinol), pea flour and pea extracts (from Caméor and BCFN 1551) on the relative abundance of various genes of interest in the metagenome of batch fermentations. 133
Figure 46. Light microscopy image of organoids forming a monolayer on the apical compartment of the transwell…….. 134
Figure 47. Effect of fermented pea flour from Caméor or BCFN 1551 on the trans-epithelial electrical resistance (TEER) of human colon organoid monolayers…… 135
Figure 48. Cytokine production of intestinal organoids stimulated with fermented pea flour from Caméor or BCFN 1551…… 136
Figure 49. Cytokine production from intestinal organoids stimulated with fermented pea flour from Caméor or BCFN 1551 following a prior 24 h inflammatory stimulus…….. 138

Figure 50. Effect of fermented pea flour from Caméor or BCFN 1551 on the trans-epithelial electrical resistance (TEER) of human colon organoid monolayers following inflammatory cytokine stimulus…… 139
Figure 51. Schematic representation of a human TWIN SHIME® system and the timeline for baseline level and treatment samples collection…… 151
Figure 52. Effect of BCTILLRfs W521* and Caméor flour supplementation on alpha diversity of faecal microbiota composition from TWIN SHIME® fermentation…… 153
Figure 53. Effect of BCTILLRfs W521* and Caméor flour supplementation on beta diversity and analysis of similarities (ANOSIM) of faecal microbiota composition from TWIN SHIME® fermentation…… 154
Figure 54. Phylum microbial composition of the baseline microbiota prior to BCTILLRfs W521* and Caméor flour fermentation…… 155
Figure 55. Microbial composition of the baseline microbiota prior to BCTILLRfs W521* and Caméor flour fermentation…… 157
Figure 56. Longitudinal effect of BCTILLRfs W521* and Caméor flour fermentation on the relative abundance of the top ten bacterial families…… 159
Figure 57. Longitudinal effect of soluble potato starch and BCTILLRfs W521* and Caméor flour fermentation on the relative abundance of top ten bacterial species…… 161
Figure 58. Significant effects of BCTILLRfs W521* and Caméor flour fermentation on the species between ascending, transverse and descending colon…… 163
Figure 59. Effect of BCTILLRfs W521* and Caméor flour supplementation on PICRUSt2 Predictions of Biological Functions from identified Operational Taxonomic Units (OTUs). 164
Figure 60. Longitudinal effect of soluble potato starch at the baseline fermentation and pea flour on the production of SCFAs in TWIN SHIME® fermentations…… 165
Figure 61. Longitudinal effect of soluble potato starch at baseline fermentation and pea flour on the production of BCFAs in TWIN SHIME® fermentations…… 166
Figure 62. Effect of pea flour fermentation on cumulative gas production…… 167
Supplementary Figure S1. Rfs gene copy number identification for the genotyping of null mutants…… 182
Supplementary Figure S2. Amino acid sequences for Rfs (Psat0s2597g0160), Rfs-like (Psat1g055560) from pea and AtAkαGal3 (At5g20250) from *A. thaliana* …… 183
Supplementary Figure S3. LC-MS analysis of RFOs and precursors in Caméor pea extracts.. 184
Supplementary figure S4. *B. longum subesp. infantis* ATCC 15697 and *B. breve* UCC 2003 growth on individual RFOs (raffinose, stachyose, verbascose) or precursors (sucrose, galactinol) as sole carbon source…… 185
Supplementary Figure S5. *Bifidobacterium* raffinose utilisation clusters…… 186
Supplementary Figure S6. Microbial composition of faecal inoculum samples from healthy donors…… 188

List of Tables

Table 1. Nutrient values of cooked (boiled) mature seeds of various legumes per 100 g..... 25
Table 2. Total content of RFO in legumes..... 55
Table 3. TILLING mutants in Rfs and Rfs-like used in this study, including the nature of the mutation induced and the resulting amino acid change..... 61
Table 4. Primers and probes used to determine *Rfs* for gene copy number genotyping..... 62
Table 5. Primers used to target *Rfs* and *Rfs*-like genes for SNP genotyping..... 63
Table 6. Mobile phase gradient for the detection of RFOs..... 69
Table 7. Optimised LC-MS parameters optimised for the detection of RFOs..... 69
Table 8. Mobile phase gradient for quantification of SCFAs, BCFAs and lactate by LC-MS..... 77
Table 9. Optimised LC-MS operating parameters for SCFA, BCFA and lactate quantification..... 77
Table 10. Gene coordinates, identification and annotation of the 1.3 Mb deletion in the line FN 1551..... 85
Table 11. Genotyping results of the TILLING mutant seeds received from INRAE..... 86
Table 12. Total peak area under the curve from the fermentation of Caméor, TILLRfs W521* and BCFN 1551 pea flour using the ANKOM RF system..... 168

1 . Chapter One: Introduction

1.1 *Pisum sativum*

1.1.1 Global distribution and adaptation of pea and other legumes to the environment

1.1.1.1 Evolution from wild species to cultivated crops

The legume family, Leguminosae (or Fabaceae), the third largest angiosperm family, covers a vast diversity of plant species adapted to diverse ecological niches. An examination of 43 legumes, based on 71 conserved protein-coding genes in their plastosomes, was used to provide a maximum likelihood phylogeny tree (Choi and Choi, 2017) (Fig 1.). Sharing the Old-World clade are known species such as *Pisum sativum* (pea), *Lathyrus sativus* (grass pea), *Vicia faba* (faba beans) and *Lens culinaris* (lentil) which are part of the Fabeae tribe, whereas *Glycine max* (soybean) is in the Phaseoleae tribe.

Edible legumes include globally important food crops including soybean, culinary beans, peanut (*Arachis hypogaea*), chickpea (*Cicer arietinum*), clover (*Trifolium repens*), lentil, alfalfa (*Medicago sativa*) and pea (Group *et al.*, 2013). Legumes can be grown for consumption as vegetables or for their harvestable and edible seeds or grains and, when these edible seeds are mature and dry, they are called pulses (Wacker and Dresbøll, 2023). Since the 1980s, pea and soybean became the two most widely grown protein crops, initially for animal feed and, currently, soybean, pea and broad beans (*Vicia faba*) are the dominant grain legumes across Europe (Watson *et al.*, 2017). It is of note that since pea is both a grain legume and a vegetable (Santalla, Amurrio and DeRon, 2001), pea trait selection differs for these two uses. For instance, dry pea is used for whole seeds, as a basis for flour and protein isolates, while vegetable pea is used in its fresh form as shelled peas (Warkentin *et al.*, 2024) or as edible pods. Furthermore, legumes are an essential source of livestock feed and are an important crop for poverty alleviation and human nutrition (Goswami *et al.*, 2022). Key trait selection in pea is described in section 1.1.2.

Overall, there is a need to increase legume production in Europe, by intensifying production and expanding the cultivation area (van Loon *et al.*, 2023). According to the Food and Agriculture Organisation of the United Nations (FAO), updated data for 2022 (©FAO, 2023; Vahdat *et al.*, 2020), globally, major soybean producers are in Brazil and the United States, India tops chickpea production, Canada, India and Australia are top lentil producers and Russia, Canada and mainland China top producers of pea. Currently, only about 2% of European cropland is used for soybean cultivation, and approximately 1% is allocated for pea and faba bean production combined (van Loon *et al.*, 2023). Efforts are being made to adapt legumes to new environments, such as pea in eastern Scotland (Squire *et al.*, 2019) and chickpea in southern Australia and northern regions of South Asia (Rani *et al.*, 2020).

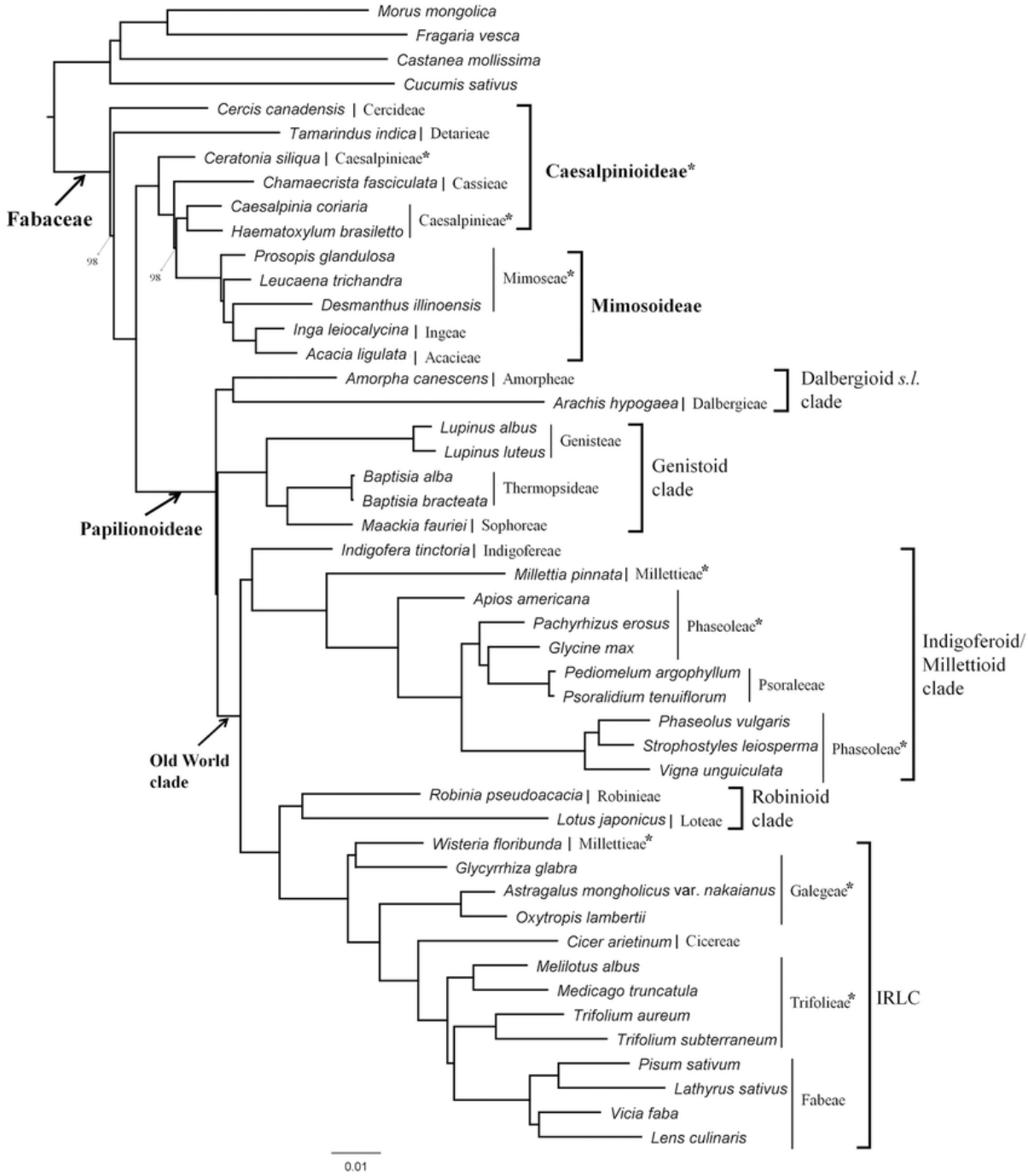


Figure 1. Fabaceae phylogeny tree. Maximum likelihood phylogeny tree of legume species generated from a concatenated matrix of 71 plastosomal protein-coding genes. Asterisks mark taxa that are not monophyletic, IRLC: Inverted Repeat-Lacking Clade. Reprinted from Choi and Choi (2017) under the Creative Commons CC-BY 4.0 license.

Legume crops, including pea, have been domesticated and used in the human diet for more than 10 000 years, where lentils, chickpeas and peas are considered Neolithic founder crops. Pea belongs to the ancient set of cultivated plants of the Near East domestication centre (Bohra *et al.*, 2022), and remains an important crop, for both human and animal nutrition across the globe (Lake *et al.*, 2021).

The domestication of crops involves a wide range of evolutionary changes that progressed through several continuous stages. The genes that have undergone positive selection display complete or near-complete fixation of selected mutations (Gross and Olsen, 2010), which is then observed for many crops in all lineages stemming from a single domestication event (Zhao *et al.*, 2021). Under these criteria, a gene that controls an important trait that segregates in a domesticated population is considered a diversification or improvement gene that played a lineage-specific role in the crop`s regional adaptation or improvement (Sedivy, Wu and Hanzawa, 2017). Over time, domesticated legumes have gradually lost characteristics of their wild ancestors, accumulating traits beneficial to human use (Chen *et al.*, 2021).

Domestication provides an important model for the study of evolution. For example, in soybean, the *de novo* assembly and whole-genome resequencing of wild and cultivated soybean genomes, provided a clearer picture of the modes of artificial selection that drove crop domestication and diversification (Sedivy, Wu and Hanzawa, 2017).

The origin of the progenitor species of domesticated pea has been studied through the exploration of genetic material from the Mediterranean Basin and Fertile Crescent region, both in terms of spatial and environmental patterns. The results suggest that the genetic diversity of wild pea may have been driven by Miocene-Pliocene events, while phylogenetic diversity centres may reflect Pleistocene-Holocene climatic changes (Smýkal *et al.*, 2017).

Crop domestication is shaped by relationships among three main factors: (i) the diversity of the crop itself, (ii) the human agronomic and cultural practices, and (iii) the ecological factors (Krug *et al.*, 2023). Crop domestication has been applied to select plants with increased suitability to human requirements, such as cultivation practices or taste (Chen, Gols and Benrey, 2015). Key traits related to legume crop improvement from domestication include pod shattering, seed hardness, shoot architecture, photoperiodic flowering, flower, seed coat and pod colour, and nematode resistance (Sedivy, Wu and Hanzawa, 2017). Central genes in flowering time control have also been recurrent targets of artificial selection, highlighting their importance in crop evolution and improvement (Sedivy, Wu and Hanzawa, 2017).

Many challenges faced nowadays require an increase in crop nutrient density, sustainability and climate resilience, in order to attain food security for the world population (as further discussed in 1.1.3.2). Modern agricultural methods have transformed crop production by enhancing yield, resilience, and biomass production. Despite this progress, human dietary needs rely heavily on just 15 crop species and factors such as rising food demand, climate change, and environmental degradation pose significant challenges to crops, where enhanced tolerance to various biotic and

abiotic stress is needed (Rubiales *et al.*, 2021). Particularly concerning is the loss of ancestral crops and the genetic diversity they offered during the domestication process (Scharff *et al.*, 2022).

1.1.1.2 Role of legumes in sustainable agriculture through nitrogen fixation

Legume farming plays a vital function in maintaining the qualities of ecosystems and could help model agricultural practices to support an enhanced usage of soil, water and other resources (Rani *et al.*, 2022). Indeed, legumes help improve soil quality through biological nitrogen fixation (BNF), thus reducing the use of nitrogen fertilisers. BNF is enabled through the development of root nodules which consist of symbiotic organs in which the *Rhizobium* bacteria fix nitrogen (Fig. 2). Root nodule formation requires the induction of a nodule meristem and the formation of a tubular structure (van Brussel *et al.*, 1992). The molecular dialogue between plants and bacteria represents a complex multi-staged process, with each step being crucial for the success of symbiosis (Sprent, 1989) (Sprent, 1989). Nodulation starts with an enhanced production of flavonoids from the plant, to attract rhizobia. The rhizobia respond by secreting nodulation factors (Nod factors) which penetrate the epidermis of the plant's roots and initiate infection. As the nodule develops, its further development and metabolism is primarily led by the host plant`s own growth and metabolism, including in the final senescence (Shumilina *et al.*, 2023).

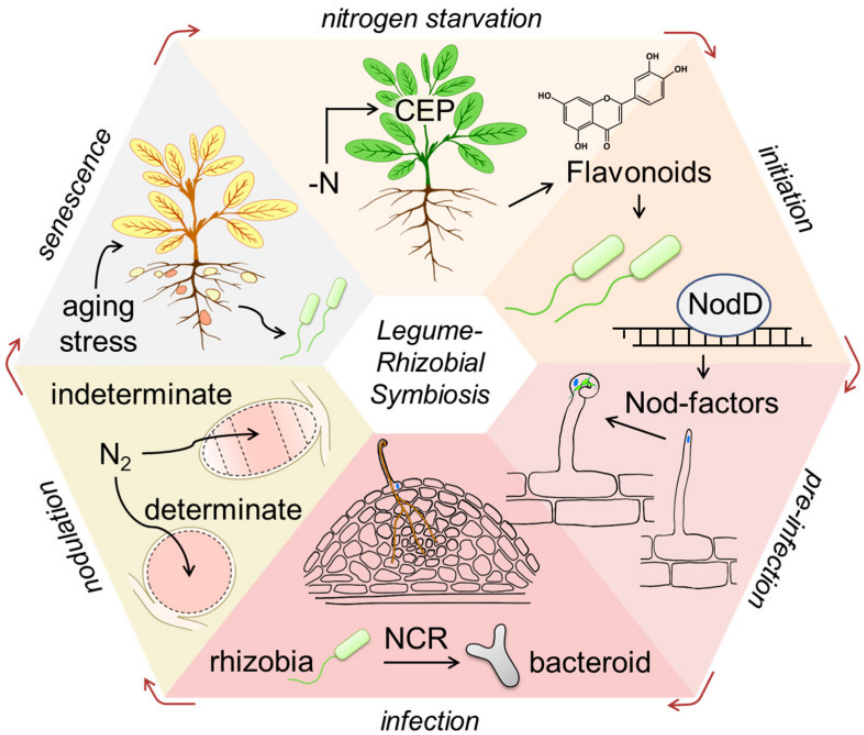


Figure 2. Stages of legume-rhizobia symbiosis. Nodulation is initiated with the detection of plant flavonoids by the rhizobia, followed by the expression of Nod factors that stimulate bacterial pre-infection of the plant. When the infection occurs, N2 is fixed from the nodule and transported into the plant. During the final stages of plant ageing and senescence, legume-rhizobial symbiosis comes to an end. CEP—C-

terminally encoded peptide; NodD—nodulation protein D; NCR—nodule-specific cysteine-rich peptides; N or N2—nitrogen. Taken from Shimulina et al. (2023), under Creative Commons CC BY 4.0 license.

BNF represents the major biological source of nitrogen input in agricultural soils, including in arid regions. Nonetheless, in symbiosis, the efficiency of nitrogen fixation is closely linked to the physiological condition of the host plant. Harsh environmental conditions can inhibit the growth and symbiotic functions of most rhizobia (Zahran, 1999).

However, some strains can form symbioses under salt and heat stresses (Zahran, 1999). For example, salt-tolerant rhizobia have been shown to provide drought resistance in *Phaseolus vulgaris* (common bean) (Mnasri, Aouani and Mhamdi, 2007) and, in *Vicia faba*, the rhizobia with higher salt tolerance showed higher nitrogen fixing capacity (Benmoussa *et al.*, 2022). Some mechanisms have been described that underpin the rhizobium symbiosis occurring under stress conditions. For instance, trehalose has been found to accumulate in rhizobia associated with peanut when grown under salt or osmotic stress conditions which helps the rhizobia cope with osmoregulation and remain in symbiosis (Zahran, 1999). Although the roles of the various trehalose biosynthetic pathways in rhizobia and their contribution to stress tolerance in bacteria require further characterisation (McIntyre *et al.*, 2007), there is evidence that, during bacterial cellular dehydration and stress encountered by the bacteria during nodulation, the enhanced trehalose concentration in rhizobia acts as a protector for bacterial proteins and membranes, and has been directly correlated with metabolism stabilisation in plants (Fernandez *et al.*, 2010; Lunn *et al.*, 2014).

Like other legumes, pea establishes symbiosis with several Rhizobium species, such as *R. leguminosarum*, *R. pisi*, *R. indicum* and *R. ruizarguesonis* (Flores-Félix *et al.*, 2020). This symbiosis is described as strain specific, here specific Rhizobium species would colonise the roots of different pea lines in a specific way (Young, Johnston and Brewin, 1982; Kneen and Larue, 1984). Under salinity stress, it was found that, while nodule formation persisted at up to 25 mM NaCl in pea roots, the nodules were considerably smaller than those of the untreated controls (Borucki and Sujkowska, 2008).

Modern-day pea cultivars exhibit reduced diversity of symbiotic rhizobia and fungi compared to their wild relatives (Provorov and Tikhonovich, 2003), while, in contrast, soybean`s ability to fix nitrogen benefited from domestication (Muñoz *et al.*, 2016). According to Liu et al. (Liu *et al.*, 2020a), the selection pressure on legume-associated soil microbes could lead to reduced or loss of diversity. Moreover, high nitrogen fertiliser application can disrupt the symbiotic relationship between legumes and rhizobia (Foley *et al.*, 2005).

1.1.1.3 Biotic and abiotic stressors in pea and other legumes

As with many other crops, the yield and productivity of legumes is threatened by biotic and abiotic stressors, mainly defined as stressors from microorganisms and insects, or from environmental conditions, respectively. There are numerous pathogens that

affect both the vegetative part as well as the root systems in legumes, with the effects of climate change increasing these threats in crops (for a detailed review on legume crop pathogens see ((Sharma *et al.*, 2021)). Key defence mechanisms against pathogen attacks include small RNAs as one of the major players in gene expression regulation in plants during developmental stages and under stress conditions (Garg *et al.*, 2017). Moreover, chemical defences mediated by phytohormone pathways and physical defence through callose depositions are also ways in which biotic stressors can be counteracted (Basu *et al.*, 2023).

In pea, one of the main biotic stressors is powdery mildew, caused by the fungal pathogen *Erysiphe pisi* (Sulima and Zhukov, 2022), followed by root-knot nematode (Sharma, Haseeb and Abuzar, 2006) and pea seed-borne mosaic virus (Congdon *et al.*, 2016), among many other pathogens that affect the roots, leaves and pods (Gossen *et al.*, 2020). Significant pests of peas include the pea leaf weevil, *Sitona lineatus* (Cárcamo *et al.*, 2018) and the pea moth, *Cydia nigricana* (Thöming and Saucke, 2011).

Osmotic stress caused by the amount of salt present in the soil (Habtamu Kide, 2022) is an example of abiotic stressors affecting crops. In addition to the effects on legume nodulation mentioned above (1.1.1.2.), drought stress directly affects intrinsic growth factors which, depending on the stage of plant development, can lead to cell death or affect later stages of seed formation and nutrient content (Singh, 2007). As a result, drought can negatively impact the nutritional composition and quality of seeds. For instance, in common beans, drought has been shown to lower the levels of nitrogen, phosphorus, iron, and zinc, which in turn affect the total protein content (Ghanbari *et al.*, 2013). In soybeans, drought has been shown to affect the fatty acid content as well as the stability and composition of the oil derived from the seeds (Bellaloui, Mengistu and Kassem, 2013). Legumes possess mechanisms to counteract drought stress including morphological changes, facilitated by their phenotypic plasticity, resulting in smaller plants with reduced leaf size (Varshney, Tuberosa and Tardieu, 2018), or with increased root development rates (Kavar *et al.*, 2008) or other root modifications. For example, in soybean, drought resistance has been associated with root modifications that enhance soil penetration and exploration (Jaganathan *et al.*, 2015). Antioxidant responses help regulate oxidative damage, and the accumulation of proline in roots has been shown to improve plant tolerance to drought stress (Nayyar and Walia, 2003). Additionally, increased solute assimilation (of carbohydrates and amino acids) in cells during drought maintains leaf turgidity (Savita, Tomer and Singh, 2020).

Extreme temperatures can also affect the metabolism of legume crops by damaging their respiration rate, resulting in the production of metabolites that can alter plant metabolism (Devasirvatham and Tan, 2018). Moreover, high temperatures can cause drought stress through increased transpiration or evaporation (Nadeem *et al.*, 2019).

Abiotic stressors, such as drought, extreme temperatures and soil salinity, can severely limit legume growth and yield. Ultimately, a comprehensive understanding of both abiotic and biotic factors is needed to select phenotypes carrying resilience genes and optimise legume production, contributing to more resilient agricultural systems and enhancing global food security.

1.1.2 Advancements and key milestones in legume and pea breeding

Legumes are one of the most domesticated groups of plants (Smýkal *et al.*, 2017) and progress in genomic methods has been instrumental in better understanding the phenomenon of speciation and in providing tools for the development of new varieties (Abbott and Fairbanks, 2016). However, overcoming challenges like the potential loss of genetic diversity during domestication is necessary to support the development of novel pea varieties.

Two important traits required for the domestication of legume crops are the elimination of seed dormancy and the elimination of seed dispersal mechanisms (Weeden, 2018). Seed dormancy and size reflect ecological advantages of favourable growth periods and are evolutionarily stable traits in temperate lineages with small seeds (Rubio de Casas *et al.*, 2017). The seed coat (testa) contains a hard, water-impermeable layer of cells that provides mechanical protection for the embryo (Baskin and Baskin, 2014), as shown in Fig. 3 (Weitbrecht, Müller and Leubner-Metzger, 2011). Moreover, the seed testa plays a key role in water uptake that initiates germination (Wojtyla *et al.*, 2006).

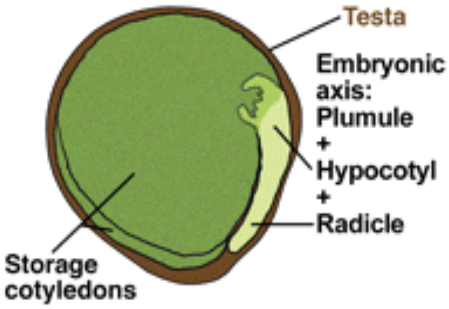


Figure 3. Morphology of a mature pea seed. The pea seed anatomy consists of an external coat that is the testa, the embryonic axis consists of the plumule at the top and the descending hypocotyl, which contains the radicle at its tip. Pea seeds contain two cotyledons, hence termed dicotyledonous. Image retrieved from Weitbrecht, Müller and Leubner-Metzger (2011), with permission from Oxford University Press.

The selection of desired traits in pea can range from traits important in terms of agriculture and crop yield to the selection of seed traits that change the seed nutritional composition (Robinson and Domoney, 2021). An example of an improved field trait is resistance to broomrape weed (Delvento *et al.*, 2023). An example of an improved seed quality trait is the optimised protein composition, which can enhance digestibility and amino acid availability, ultimately benefiting health (Olías *et al.*, 2023).

Reference collections have been established to support pea breeding research and the mapping of different traits (Holdsworth *et al.*, 2017). Advanced genetic analysis tools have enabled the precise identification of genetic loci and candidate genes linked to desirable traits in cultivated materials (Sawada *et al.*, 2022).

Fast neutron (FN) radiation induces genetic deletions of varying size upon irradiation (Kumawat *et al.*, 2019). The resulting populations have been described as having non-repairable lesions with higher delay in DNA repair (Hendry, 1991). As an example, a FN mutant population was developed in pea (Domoney *et al.*, 2013), using the *P. sativum* recombinant inbred line JI2822 as parent (Ellis *et al.*, 1992). Forward and reverse genetic screens on this population have identified a range of phenotypic mutants affecting plant and flower morphology, and seed protein profiles (Domoney *et al.*, 2013; Smýkal, von Wettberg and McPhee, 2020).

Additionally, a high-throughput forward and reverse genetics tool called Targeted Induced Local Lesions in Genomes (TILLING) has been used in pea. This approach was originally developed for *Arabidopsis thaliana* (McCallum *et al.*, 2000), the source of the first plant genome sequenced (Feldmann and Goff, 2014), but, since then, it has been applied to other plant species irrespective of their genome size and ploidy level. This approach is usually conducted through chemical mutagenesis, generating a high frequency of point mutations distributed randomly across the genome (Kurowska *et al.*, 2011). It is a non-transgenic approach (Barkley and Wang, 2008), which is based on the use of a mismatch-specific enzyme that identifies mutations in a target gene through heteroduplex analysis (Kurowska *et al.*, 2011; Gilchrist *et al.*, 2006). TILLING is characterised by the generation of allelic series of mutations, some of which show partial or complete loss of function, which can be useful for crop breeding (McCallum *et al.*, 2000).

The TILLING approach generates high quality mutant libraries (Lee *et al.*, 2005) and provides a useful and effective functional genomics tool to study genes responsible for desired phenotypes, with potential for crop improvement (Wang, Sun and Tao, 2006). Large populations can be screened for desirable phenotypes or specific gene mutations. This crop improvement strategy can be extended using techniques such as clustered regularly interspaced short palindromic repeats associated 9 (CRISPR-Cas9), that enable site-specific mutations (Grützner *et al.*, 2021; Wada *et al.*, 2020) which can be advantageous compared to the TILLING random mutagenesis approach (Jacob, Avni and Bendahmane, 2018). Recently, *Agrobacterium*-mediated CRISPR-Cas9 was used in pea to edit phytoene desaturase (Li *et al.*, 2023a) and lipoxygenase (Bhowmik *et al.*, 2023), the latter leading to changes in fatty acid composition and improvement of flavour profiles (Bhowmik *et al.*, 2023).
A pea TILLING mutant population was generated using the Caméor cultivar, an inbred cultivar developed by Seminor in 1973 in France (Kreplak *et al.*, 2019). The pipeline for the TILLING mutant population in pea is illustrated in Fig. 4 (Dalmais *et al.*, 2008). A database for this population was developed by the Plant Genomics Research Unit (URGV) at the National Research Institute for Agriculture, Food and Environment in France (INRAE, France), named the URGV TILLING database (UTILLdb) (Dalmais *et al.*, 2008). It contains phenotypic as well as sequence information on mutant genes and can be searched online for the identification of various locus using the BLAST (Basic Local Alignment Search Tool) tool. This population can be used to increase diversity (Dalmais *et al.*, 2008) and to study genes with unknown functions or roles in pea.

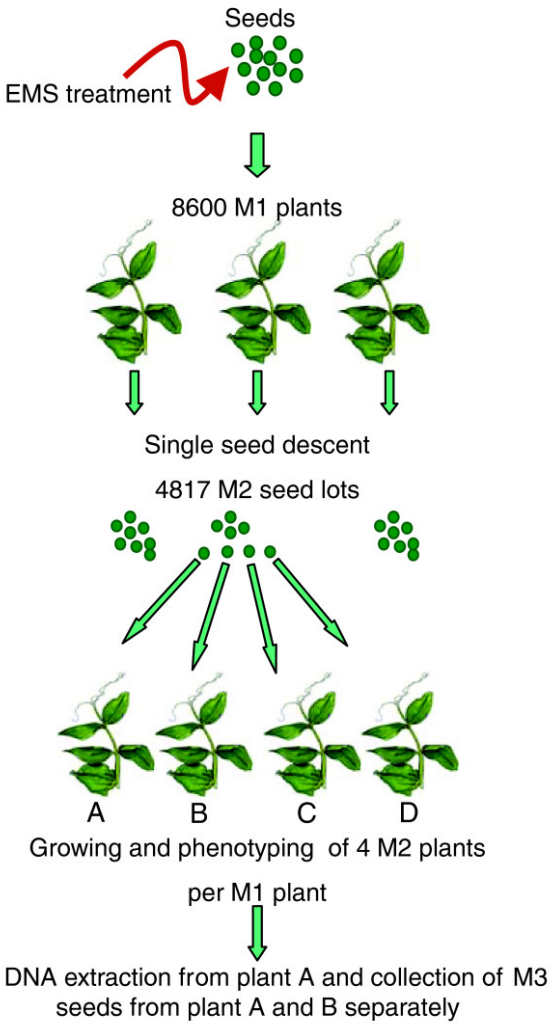


Figure 4. TILLING pipeline for UTILLdb pea population. A *P. sativum* TILLING mutant population was derived from ethylmethane sulphonate (EMS)-treated Caméor for the selection of mutations through reverse genetics. Briefly, after ethylmethane sulphonate (EMS) chemical treatment of seed, the modified plants (M1) were grown and seeds collected (M2). Four seeds (M2) were sown from each M1 plant. These plants (M2) were phenotyped and DNA extracted from one plant and the seeds collected (M3). Taken from Dalmais et al. (2008). Reproduced with permission from Springer Nature.

1.1.3 Nutritional value of pea and other legumes

1.1.3.1 Contribution of legumes to global food security

Pulses are commonly consumed in traditional diets in India, Central and South America (Leterme and Carmenza Muñoz, 2002), Middle East (Siddiq, Uebersax and Siddiq, 2022) and Africa (Ngigi *et al.*, 2022). In Western countries, legume consumption has historically been limited, partly due to the perceived flatulence and bloating effects associated with high consumption, but also due to societal perceptions that label pulse foods as a symbol of poverty and mainly value them as animal fodder (Henn *et al.*, 2022).

In the UK, the Eatwell guidelines recommend the consumption of beans, peas and lentils as good alternatives to meat because they are naturally very low in fat, and high in fibre, protein, vitamins and minerals (©Crown, last updated 29 November 2022). However, despite the recognised benefits for human and planetary nutrition and health, inconsistency in serving size recommendations for legume consumption suggests that dietary guidelines may need to be updated to better promote their consumption (Hughes, Pearson and Grafenauer, 2022).
Enhancing agricultural food security also requires modification of food systems, including crop diversification and maximising dietary benefits. For instance, in Ethiopia and Tanzania, the adoption of novel legume technologies contributed to creating a positive increase in their consumption (Asfaw *et al.*, 2012). Adopting new legume cultivars in Timor-Leste enhanced productivity and ecosystem diversity (Borges *et al.*, 2009). These examples underscore the importance of legumes in improving food security through better agricultural practices and increased dietary inclusion.
Overall, legumes are crucial in combating malnutrition worldwide, serving as a major source of plant-based proteins and are particularly important in regions where access to animal proteins is limited (Vissamsetti *et al.*, 2024).

1.1.3.2 Nutrient profile and bioactive compounds in legumes

Legumes are characterised by their high nutritional value. They provide fibre, protein, carbohydrates, B vitamins, iron, copper, magnesium, manganese, zinc and phosphorous, are naturally low in fat, and practically free of saturated fat and cholesterol (Polak, Phillips and Campbell, 2015). A 100-kilocalorie portion of pulses contains two to three times more fibre than even wholegrains (Didinger and Thompson, 2022), making legumes a way to enhance fibre intake and to 'close the fibre gap' between daily amounts of fibre recommended in the human diet and what is actually consumed (Thompson and Brick, 2016). The National Nutrient Database for Standard Reference Release 28 from the United States Department of Agriculture (USDA) (Haytowitz, 2015), provides the nutrient values of various legumes per 100 g edible portions of mature seed, cooked by boiling without salt (Table 1) (Çakir *et al.*, 2019). Here the detailed composition of minerals in addition to vitamins is shown, and differences can be observed between the different legume crops. Due to its nutritious content and given its low environmental impact, pea has been proposed as a key contributor to combat world hunger (Hacisalihoglu, Beisel and Settles, 2021). Moreover, the low glycaemic index of legumes is important for the prevention, treatment and nutrition of diabetic and obese individuals (Grdeń and Jakubczyk, 2023).

Table 1. Nutrient values of cooked (boiled) mature seeds of various legumes per 100 g. Obtained from Çakir *et al.* (2019).

	Unit	Soybeans	Peas	Lentils	Lupins	Chickpea	White bean	Black bean
Energy	kcal	172	118	116	119	164	139	132
Protein	g	18.21	8.34	9.02	15.57	8.86	9.73	8.86
Fat	g	8.97	0.39	0.38	2.92	2.59	0.35	0.54

Carbohydrate	g	8.36	21.1	20.13	9.88	27.42	25.09	23.71
Dietary Fiber	g	6	8.3	7.9	2.8	7.6	6.3	8.7
				Minerals				
Calcium, Ca	mg	102	14	19	51	49	90	27
Iron, Fe	mg	5.14	1.29	3.33	1.2	2.89	3.7	2.1
Magnesium, Mg	mg	86	36	36	54	48	63	70
Phosphorus, P	mg	245	99	180	128	168	113	140
Potassium, K	mg	515	362	369	245	291	561	355
Sodium, Na	mg	1	2	2	4	7	6	1
Zinc, Zn	mg	1.15	1	1.27	1.38	1.53	1.38	1.12
				Vitamins				
Vitamin C	mg	1.7	0.4	1.5	1.1	1.3	0	0
Thiamin	mg	0.155	0.19	0.169	0.134	0.116	0.118	0.244
Riboflavin	mg	0.285	0.056	0.073	0.053	0.063	0.046	0.059
Niacin	mg	0.399	0.89	1.06	0.495	0.526	0.14	0.505
Vitamin B-6	mg	0.234	0.048	0.178	0.009	0.139	0.093	0.069
Folate, DFE	µg	54	65	181	59	172	81	149
Vitamin B-12	µg	0	0	0	0	0	0	0
Vitamin A, IU	IU	9	7	8	7	27	0	6
Vitamin D	IU	0	0	0	0	0	0	0
Vitamin E	mg		0.03	0.11			0.94	0.87
Vitamin K	µg		5	1.7		4	3.5	3.3
				Lipids				
Fatty acids, total saturated	g	1.297	0.054	0.053	0.346	0.269	0.091	0.139
Fatty acids, total monounsaturated	g	1.981	0.081	0.064	1.18	0.583	0.031	0.047
Fatty acids, total polyunsaturated	g	5.064	0.165	0.175	0.73	1.156	0.152	0.231
Fatty acids, total trans	g	0	0	0	0	0	0	0

In addition to its nutritious properties, the bioactive components in pea exhibit a number of potential beneficial health effects, some of which require further *in vivo* validation. For instance, pea protein, when hydrolysed, may yield bioactive peptides such as angiotensin I- converting enzyme inhibitor with biological effect (Dahl, Foster and Tyler, 2012); ACE inhibitors are commonly indicated medications in the treatment of cardiovascular diseases and are used in medicine to treat and manage hypertension (Alcocer *et al.*, 2023).

There is also evidence that pea bioactive components can restore healthy levels of serum total cholesterol, low-density cholesterol and high-density lipoprotein cholesterol in rats fed a high-fat diet (Liu *et al.*, 2019). In addition, pea and its components have been reported to have anti-inflammatory effects *in vitro*. Pea polyphenols inhibited the release of nitric oxide, interleukin-6 (IL-6) and tumour necrosis factor-α (TNF- α) in co-cultures of Caco-2 and RAW 264.7 cells (Ndiaye *et al.*,

2012). Oral administration of pea pods to diabetic mice resulted in decreased serum glucose levels, hepatic enzymes, triglycerides and cholesterol (Mejri *et al.*, 2019). More recently, a short-term human dietary intervention study showed that consumption of pea seeds or flour with high resistant starch in dietary meals resulted in lower post-prandial glucose, changes in gut bacterial composition and increased excretion of propionate and butyrate in 24 h urinary samples (Petropoulou et al., 2020).
Finally, raffinose family of oligosaccharides (RFOs) in legumes have emerged as a new class of prebiotics by promoting the growth of health-beneficial bacteria as further described in section 1.4.3.
Taken together, these studies show the potential of pea to become a functional food with multiple applications in the food system.

1.2 The human gastrointestinal (GI) tract

1.2.1 Structure and function of the GI tract

The digestive system includes the alimentary canal encompassing the oral cavity, pharynx, oesophagus, stomach, small intestine, large intestine, rectum, anal canal, and anus (Cheng *et al.*, 2010). Macroscopically, the gut wall is composed of four distinct concentric layers: the mucosa, submucosa, muscularis externa and either adventitia (if above the diaphragm) or serosa (below the diaphragm) (Bannister, 1999; Mercado-Perez and Beyder, 2022) (Fig. 5).

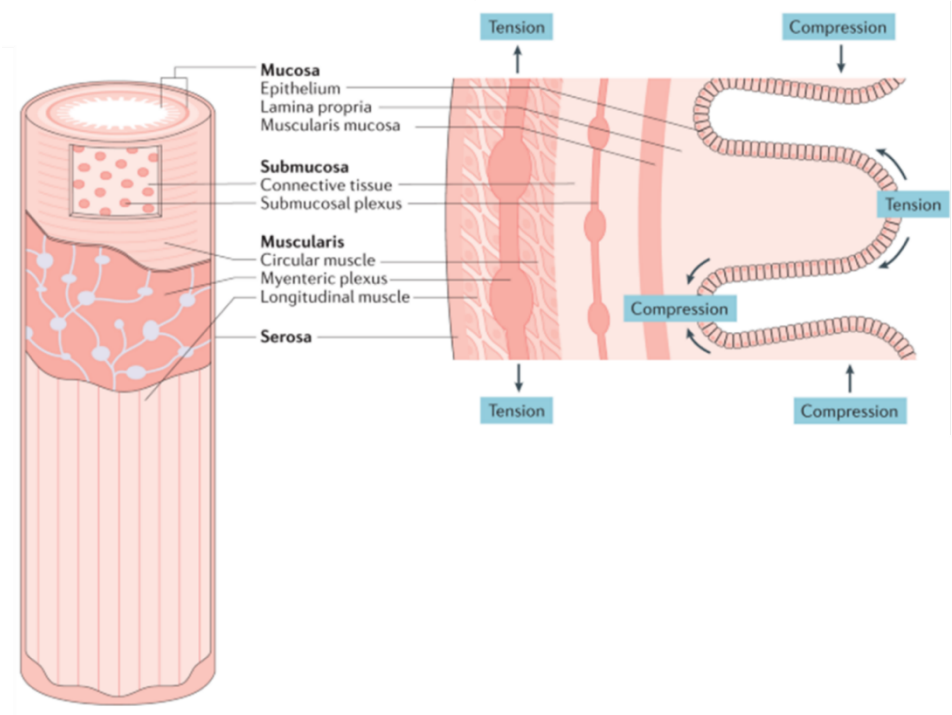


Figure 5. Gut layer organisation. Composite nature of the wall and layers of the human intestine, with a cross-section of the laminated structure and four layers of the gut wall, the inner serosa, muscularis, submucosa and most external mucosa. The figure also includes the compression and tension in the gut layers. Adapted from Mercado-Perez and Beyder (2022). Reproduced with permission from Springer Nature.

The GI tract fulfils many physiological functions encompassing the digestion of food, absorption of nutrients and waste elimination. Peristaltic movements aid in food digestion, nutrient absorption and bowel emptying, and induce shear stress and radial pressure on the epithelium, influencing intestinal development and homeostasis (Huizinga, 2018).

There are a number of secretions along the GI tract that allow its many functions, starting with salivary secretions, which are made of mucus, electrolytes, amylase and lipase, which help moisten and degrade food compounds such as starch or lipids (Hornbuckle, Simpson and Tennant, 2008). Gastric secretions include hydrochloric acid (HCl) and pepsin, the main enzyme of gastric digestion, capable of hydrolysing peptide bonds of most proteins (Hornbuckle, Simpson and Tennant, 2008). Biliary and exocrine pancreatic secretions enable the hydrolysis of dietary lipids, proteins and carbohydrates (Hornbuckle, Simpson and Tennant, 2008; Hedayat, Lapraz and Schuff, 2020; Murch, 2021).

Most of the nutrient absorption occurs in the small intestine. The large intestine, made of the caecum, appendix, colon, rectum and anal canal, reabsorbs water (Reed and Wickham, 2009) and is home to the gut microbiota which helps further degrade undigested foods reaching the colon (see section 1.3).

The GI tract also plays a critical role in immune surveillance via the dendritic cells, the mucosa-associated lymphoid tissue (MALT) and the gut-associated lymphoid tissue (GALT). The GALT comprises mainly the Peyer`s patches, in the small intestine and isolated lymphoid follicles, in the small intestine and large intestine. The microfold, or M cells, primarily found overlying GALT lymphoid follicles (Sauls, 2022), provide the main machinery for sampling the luminal microbes for mucosal immune surveillance (Dillon and Lo, 2019) and cover the lymphoid Peyer patches (Siebers and Finlay, 1996).

1.2.2 The intestinal epithelium

The intestinal epithelium contains a highly organised single layer of epithelial cells covered by mucus, providing protection against luminal compounds. The epithelium of the adult intestine is in constant contact with the underlying mesenchyme, determining progenitor cell proliferation and differentiation (Clevers, 2013).

The intestinal physiology is intimately linked to the microscopical details of its anatomy (Fig. 6) (Fellows and Varga-Weisz, 2020). The intestinal epithelium is folded to form a number of invaginations, which are called crypts of Lierbekühn (Dudás, 2023). The small and large intestine encompass different cell types with epithelial cells, creating the mucosal surface called the brush border through the projection of intestinal villi and microvilli which increase the luminal surface for absorption of nutrients (Crawley, Mooseker and Tyska, 2014). The gut epithelium is continuously renewed from stem cells located in the base of the intestinal crypts (Reynolds *et al.*, 2014). During tissue development and regeneration, transit-amplifying cells, which are a population of rapidly dividing progenitor cells that arise from intestinal stem cells, first change to a

multipotent and more proliferative state (Cancedda and Mastrogiacomo, 2023). Newly formed precursor cells then differentiate as they migrate away from the crypt towards the villus tip in the small intestine or to the luminal surface in the large intestine, where they are expelled into the lumen approximately every 3-4 days (Rees *et al.*, 2020). The exception to this is Paneth cells, which are long-lived and instead move towards the crypt base (Fig. 6) .

Absorptive enterocytes are the dominant differentiated cell types in the intestinal epithelium (Allaire *et al.*, 2018; Kong, Zhang and Zhang, 2018). These cells are columnar epithelial cells with microvilli in their apical cell membrane, which serve to increase the surface area available to effectively absorb nutrients from food (DeSesso and Jacobson, 2001; Walton *et al.*, 2016). Other cell differentiated types such as goblet cells, enteroendocrine cells and Paneth cells vary in abundance across the GI tract. The large intestine is mainly composed of enterocytes and goblet cells, with a small number of enteroendocrine cells, while the small intestine additionally contains Paneth cells (de Santa Barbara, van den Brink and Roberts, 2003).

Goblet cells are specialised in the secretion of mucus and their numbers increase with increased microbiota density from the proximal to the distal intestine, as the stool is compacted (Shroyer and Kocoshis, 2011). Goblet cells have recently been divided into new functional subpopulations: canonical goblet cells and non-canonical goblet cells, both producing type-2 mucin (MUC2), the main secreted mucin making up the mucus barrier (Liu *et al.*, 2020c). Canonical goblet cells are found along the crypt (Nyström *et al.*, 2021), while non-canonical goblet cells produce mucus with properties towards protecting the lower crypt and stem cells from microbial invasion (Nyström *et al.*, 2021). In addition to their secretory functions, goblet cells play a key role in the immune system as luminal sensors of antigens , through a pathway called goblet cell associated passage (GAP) (Knoop *et al.*, 2015; Zhang *et al.*, 2023), which allows the delivery of soluble antigens to dendritic cells located in the lamina propria (Pelaseyed *et al.*, 2014; Gustafsson *et al.*, 2021).

The mucus produced by goblet cells adds another layer of protection to the gut epithelium. The mucus layer organisation varies along the GI tract (Ermund *et al.*, 2013; Johansson, Larsson and Hansson, 2011; Johansson *et al.*, 2008). In the small intestine, where the majority of nutrient absorption occurs, the mucus layer is irregular and penetrable (Ermund *et al.*, 2013; Herath *et al.*, 2020), whereas, in the colon, where most of the gut microbiota is present, mucus shows a bilayer organisation with a thick outer layer facing the lumen and an inner layer attached to the epithelium (Juge, 2022; Johansson *et al.*, 2008; Johansson, Larsson and Hansson, 2011) (Fig. 6). While the colonic outer mucus layer is home to the gut microbiota, the inner layer keeps the gut microbiota at bay and restricts pathogenic bacteria to reach the epithelium (Hansson, 2012; Gustafsson and Johansson, 2022). Mucins are the main structural components of the mucus layer. These large glycoproteins play an important role in innate host immune defence against pathogens (Dharmani *et al.*, 2009; Inaba, Vujakovic and Bergstrom, 2023), as well as in shaping the gut microbiota with mucin glycans, providing a source of attachment and nutrients to gut bacteria (Peterson *et al.*, 2007; Bell and Juge, 2021; Luis and Hansson, 2023).

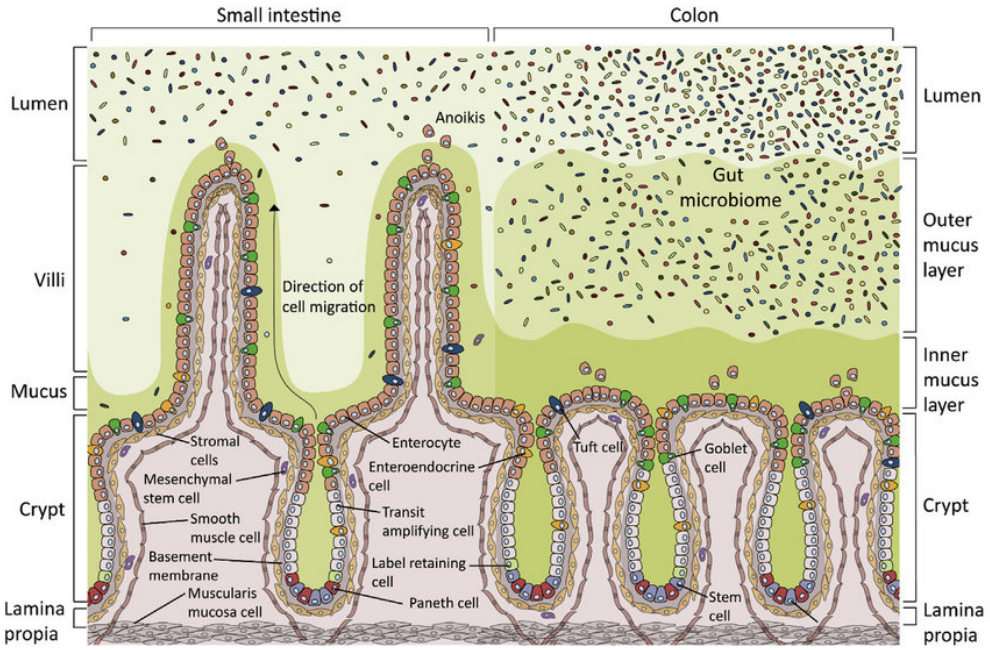


Figure 6. Cross-sectional structure of the small and large intestines and the major cell constituents of the epithelium. Enterocytes, the most abundant cell types in the inetsine, absorb nutrients from food; goblet cells are specialised in mucus secretion; transit-amplifying cells are proliferative cells prior to differentiation; enteroendocrine cells secrete hormones; tuft cells secrete prostanoids and opioids; and Paneth cells secrete anti-microbial peptides and support the stem cells. The small intestine is covered by a penetrable single mucus layer; the large intestine is covered by two layers of mucus, an inner compact layer attached to the epithelium, and a loose outer layer containing the gut microbiota. The lamina propia is a layer of connective tissue that supports epithelial and other cells and structures associated further, such as intestinal-associated immune cells, lymphatyc vessels and capillaries (not shown). Image reprinted from Fellow and Varga-Weisz (2020), under the Creative Commons CC-BY license.

Enteroendocrine cells and their subtypes are responsible for the secretion of over thirty different hormones in the GI tract (Gunawardene, Corfe and Staton, 2011). Hormone release in response to a meal-related stimuli allows to exert actions that range from local gut motility control to the regulation of insulin release and food intake (Houghton, 2021). These cells have been classified according to the principal hormone they produce (for a detailed review on cell subtypes please refer to (Atanga, Singh and In, 2023)). For example, the L-cell subtype, which have a cone-shaped appearance, with the base resting on the basal lamina of the intestinal epithelial lining (Kuhre *et al.*, 2021), are predominantly in the ileum and colon (Gutierrez-Aguilar and Woods, 2011), and can contact directly with luminal metabolites (Garcia-Gutierrez and Sayavedra, 2022); these cells release glucagon-peptide like 1 and 2 (GLP-1, GLP-2) and peptide YY (PYY), which are involved in appetite control, insulin release, slowing-down intestinal transit and stimulating cell proliferation (Drucker, 2018; Guan *et al.*, 2012). Moreover, enteroendocrine cells have an array of chemosensory mechanisms to detect stimuli beyond nutrient intake, sensing microbial metabolites and, in turn,

secrete peptide hormones and cytokines that influence directly the barrier function of the intestinal epithelium (Friedrich *et al.*, 2015).

Paneth cells are highly specialised secretory epithelial cells, located in the crypts of Lieberkühn. These pyramidal shaped, columnar cells (Bevins and Salzman, 2011) contain dense granules with abundant antimicrobial peptides and immunomodulating proteins that function to regulate the composition of the intestinal microbiota (Lueschow and McElroy, 2020). Paneth cells are known for the secretion of regenerating islet-derived 3 (Reg3) family of proteins with Reg3γ being active against Gram-positive bacteria and playing a critical role in the spatial separation of the intestinal bacteria and intestinal epithelia in the small intestine (Okumura and Takeda, 2017). In addition, Paneth cells have been shown to produce and release high levels of IL-17 during inflammation in response to TNF-α, in a coordinated interplay between innate and adaptive immunity responses (Takahashi *et al.*, 2008).

Another group of specialised intestinal epithelial cells are M cells, which can sample microbes from the lumen, and are located in organised mucosal lymphoid tissues such as Peyer patches (Dillon and Lo, 2019). M cells in Peyer`s patches release antigens that are taken up by dendritic cells (Sakhon *et al.*, 2015). Moreover, there is evidence that chronic inflammation may induce new M cell development in the absence of a fully formed or organised lymphoid tissue (Bennett *et al.*, 2016), thus highlighting their role beyond maintaining homeostasis, as a response to colonic inflammation.

Tuft cells are another group of cells found in the intestinal epithelium, but also in other tissues. These cells and are characterised by a flask-shaped body and a narrow neck with characteristic long microvilli that extend from the apical membrane of the cell towards the intestinal lumen (Hoover *et al.*, 2017). These chemosensory cells are present in small numbers in healthy conditions (McKinley *et al.*, 2017). However, their numbers dramatic change in response to inflammation, cancer and viral and parasitic infections (Banerjee *et al.*, 2020; Gerbe *et al.*, 2016), underscoring their role in barrier function of the epithelium.

At the bottom of the intestinal crypt are the intestinal stem cells (ISCs) where canonical signalling confines them to this niche (Seishima and Barker, 2019). These cells divide every 24 h to generate transit-amplifying cells that gradually migrate towards the top of the villi whilst differentiating into the previously mentioned lineages (Barker *et al.*, 2007). In the small intestine, Paneth cells serve as an epithelial niche component for ISCs providing stem cell factors such as human epidermal growth factor (EGF) or Wnt family member 3 (Wnt3) (Sato *et al.*, 2011) and protection from injury (Cui *et al.*, 2023). ISCs are instrumental to the epithelium regeneration process; two distinct types are distinguished by their position in the crypts and their fast or slow cycling properties (Takeda *et al.*, 2011). The crypt base columnar cells (CBCs) are the fast-cycling ISC population, while the reserve stem cells are capable of rescuing CBC loss during an injury (Breault *et al.*, 2008; Powell *et al.*, 2012; Karmakar *et al.*, 2020). In addition, there is new evidence suggesting de-differentiation of progeny cells (Murata *et al.*, 2020), a process more recently termed as `intestinal cell plasticity` to maintain barrier integrity and homeostatic stability (Meyer *et al.*, 2022).

The mucus and single layer of epithelium cells provide gut barrier integrity by separating subepithelial immune cells from the luminal microbiota (Ma, Nighot and Al-Sadi, 2018). The flux across the epithelial barrier is enabled by transcellular and paracellular transport, the latter being less selective and involving the movement of compounds via pore (charge selective) or leak pathways (non-charge-selective) (Horowitz *et al.*, 2023). The balance between paracellular permeability and barrier function is critical to preventing unregulated flux of material whilst allowing nutrient and water absorption (Wada *et al.*, 2013). This is enabled by tight junction proteins, namely zonula occludens (ZO) proteins ZO-1, ZO-2 and ZO-3 (Gumbiner, Lowenkopf and Apatira, 1991), occludin (Furuse *et al.*, 1993) and claudins (Furuse *et al.*, 1998; Günzel and Yu, 2013) which are expressed by intestinal epithelial cells and present intercellularly (Fig. 7) (del Rocio Encarnacion-Garcia and Nava, 2022).

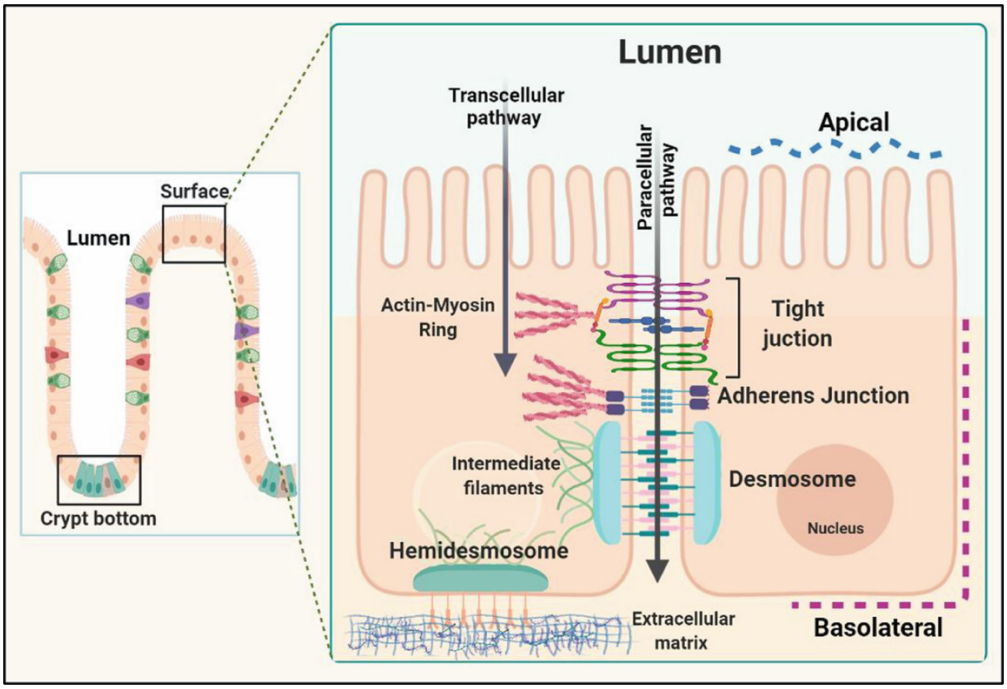


Figure 7. Flux across the epithelial barrier and depiction of intercellular junction. Intestinal epithelial cells regulate the unidirectional transcellular and paracellular flow of the luminal contents, from the apical side or lumen to the basolateral side of the cells towards the extracellular matrix. Intercellular junctions encompassing tight junctions, adherens junctions, and desmosomes bring cells together, and hemidesmosomes connect epithelial cells to the extracellular matrix. Actin and intermediate filaments constitute the cytoskeleton network in the intestinal epithelial cells. Reprinted from del Rocio Encarnacion-Garcia and Nava (2022). Reproduced with permission from Springer Nature.

Occludin contributes to the formation and disassembly of the tight junction and to the paracellular flux of large molecules (Buschmann *et al.*, 2013). There are various claudin proteins, but claudin-2 and claudin-15 are the major pore-forming claudins expressed within the intestinal epithelium, forming cation-selective channels, which control the paracellular flux of small molecules (Wada *et al.*, 2013). The passage of sodium or

water through claudin-2 channels has been shown to help pathogen clearance and reducing disease severity (Muto *et al.*, 2010)

In the absence of epithelial damage and unregulated flux, the tight junction eliminates intercellular spaces that adjoin cells, in conjunction with the presence of adherens juntions and desmosomes creating a cytoskeletal structure maintaining cell shape (Groschwitz and Hogan, 2009). On the other hand, defects in the intestinal tight junction barrier have been implicated in inflammatory diseases and infection. For example, patients with inflammatory bowel disease (IBD) have increased intestinal permeability and associated alterations in tight junction proteins (Čužić *et al.*, 2021) along with the production of inflammatory cytokines that regulate tight junction permeability (Edelblum and Turner, 2009).

Inflammation is a significant factor that can disrupt the integrity of the intestinal tight junctions. The production of inflammatory cytokines by immune cells can induce changes in tight junction protein expression and distribution, ultimately affecting the integrity of the gut barrier (Yoo and Oh, 2023; Mahapatro, Erkert and Becker, 2021). IL-1β (Landy *et al.*, 2016), TNF-α (Al-Sadi *et al.*, 2016) and interferon- γ (IFN-γ) (Bruewer *et al.*, 2005) contribute to the overall intestinal inflammatory process by increasing intestinal tight junction permeability, through various mechanisms that remain to be fully elucidated. Conversely, anti-inflammatory cytokine IL-10 has been shown to promote intestinal tight junction barrier function (Kennedy *et al.*, 2000; Mazzon *et al.*, 2002) as well as transforming growth factor β (TGF-β) (Planchon *et al.*, 1994; Howe *et al.*, 2005). Another important cytokine in the intestinal environment is IL-22, a member of the IL-10 superfamily of cytokines. This cytokine is mainly produced by immune cells and it can promote intestinal epithelium proliferation and play a role in tissue regeneration (Dudakov, Hanash and van den Brink, 2015). This cytokine connects immune functions with metabolic functions of the host (Gaudino *et al.*, 2024), it is induced by the gut microbiota and can have pro- and anti-inflammatory effects (Klotskova *et al.*, 2024). IL-22 induces epithelial cell proliferation aiding in rapid epithelium regeneration and wound healing (Patnaude *et al.*, 2021). Additionally, IL-22 induces IL-18 production from the epithelium, enhancing cell survival and induces Paneth cell differentiation (Chiang *et al.*, 2022). On the other hand, when chronically overexpressed, IL-22 can contribute to intestinal epithelium damage and reduce microbial diversity by inducing antimicrobial peptide production (Kamanaka *et al.*, 2011).

Intestinal epithelial cells also secrete cytokines and chemokines that modulate host immune responses. Epithelial cells produce IL-22, which stimulates ISC regeneration, promotes barrier integrity, and maintains intestinal homeostasis (Segrist and Cherry, 2020; Lindemans *et al.*, 2015) as well as IL-6 (Stadnyk, 2002), which performs a variety of roles, including preserving the integrity of the intestinal epithelium and mucosal barrier, and regulating the immune response to external microbes (Alhendi and Naser, 2023). Paneth cells produce inflammatory cytokines in a constitutive manner (e.g. TNF-α) or induced-upon-injury manner (e.g. IL-17) (Carneiro and Travassos, 2013). Intestinal epithelial cells also produce the anti-inflammatory cytokine IL-10, which likely contributes to tolerance to commensal bacteria (Andrews, McLean and Durum, 2018).

The complex organisation of the intestinal cell types and mucus layers play a pivotal role in the barrier function of the gut, with increased cellular exchange rates and regeneration upon mucosal injury as measures of barrier function to prevent sepsis (Blikslager *et al.*, 2007). There is also a crosstalk with underlying neurons, immune cells and other cell types and the intestine`s endogenous bacteria (Clark and Coopersmith, 2007).

As a result, there are many pathologies related to the disruption of one or more cellular or mucosal components in the gut. For example, dysfunction of the large intestine`s normal physiology can result in several medical conditions such as irritable bowel syndrome (IBS), diverticulosis and IBD including Crohn's disease (CD) and ulcerative colitis (UC) (Azzouz and Sharma, 2022).

1.3 The gut microbiota

1.3.1 Establishment and composition of the microbiota in the GI tract

The GI tract harbours a diverse community of microorganisms referred to as the gut microbiota. These microorganisms were originally identified through culture techniques (Finegold *et al.*, 1983) but the advent of next-generation sequence uncovered the diversity of microbes populating the gut, showing that only 24% of the bacterial species identified corresponded to previously-described organisms (Suau *et al.*, 1999). Successive metagenomic efforts led to more complete and detailed genomic catalogues of the gut microbiota (Almeida *et al.*, 2021; Qin *et al.*, 2010; Kraal *et al.*, 2014) and unravelled a range of interactions with the host (Lau *et al.*, 2016).

The gut microbiota changes significantly over the human lifetime. Colonisation of the infant gut begins at birth with transmissibility, acquisition and retention times of microbial species being influenced by maternal vaginal microbiome, the mode of delivery (vaginal vs caesarean section) and place of delivery (hospital vs home) (Reyman *et al.*, 2019) (Selma-Royo *et al.*, 2024). In the early years of the child's life, there is a period of change and fluctuation depending on feeding source, breast milk or formula (Greenhalgh *et al.*, 2016).

During this period, the infant gut microbiota is primarily defined by the presence of Bifidobacteria, which possess adaptative traits suited to the intestinal ecological niche and play an important role in the developmental processes of the infant, including the production of the gut mucus layer, protecting from opportunistic pathogens and incoming microorganisms (Turroni *et al.*, 2022). The genus Bifidobacterium encompasses numerous species and subspecies with *B. longum* showing a clear advantage over the other subspecies in the presence of fucosylated human milk oligosaccharides (HMOs) due to its adapted glycoside hydrolase (GH) repertoire (Selma-Royo *et al.*, 2024)

Further changes occur upon weaning due to the introduction of new nutrients in the infant diet (Schanche *et al.*, 2015; Greenhalgh *et al.*, 2016). Here, Bifidobacteria become outcompeted by adult-type microorganisms (Agans *et al.*, 2011; Derrien,

Alvarez and de Vos, 2019). During adolescence, the gut microbiota has not reached full maturity, and the fluctuation in hormonal levels influences its diversity (Yuan *et al.*, 2020). During adulthood, the microbiota composition reaches stability (King *et al.*, 2019; Radjabzadeh *et al.*, 2020), with species in the Firmicutes and Bacteroides phyla making up more than 90% of the total gut microbiota (Rios-Covian *et al.*, 2017; Consortium, 2012). The adult gut microbiota also contains less represented phyla, here referenced as the original source using the former nomenclature and in brackets the latest International Code of Nomenclature for Prokaryotes (ICNP) (Oren et al., 2023), such as Proteobacteria (now Pseudomonadota), Verrucomicrobia (now Verrucomicrobiota) or Actinobacteria (now Actinomycetota) (Consortium, 2012). The major genera of obligate anaerobes in the human gut microbiota include: *Bacteroides, Bifidobacterium, Clostridium, Eubacterium, Fusobacterium, Peptococcus, Peptostreptococcus* and *Ruminococcus.* The genera of facultative anaerobic bacteria include *Escherichia, Enterobacter, Enterococcus, Klebsiella, Lactobacillus* and *Proteus* (Vallès *et al.*, 2014; Tanaka and Nakayama, 2017). A seminal study suggested a classification of the human adult gut microbiota into three enterotypes identifiable by the proportion of *Bacteroides*, *Prevotella* and *Ruminococcus* (Arumugam *et al.*, 2011). This concept has since been revised, leading to proposed enterosignatures dominated by either *Bacteroides, Firmicutes, Prevotella, Bifidobacterium*, or *Escherichia* (Costea *et al.*, 2018; Frioux *et al.*, 2023). In the elderly, the gut microbiota is characterised by a reduced bacterial diversity and metabolic activity (Salazar *et al.*, 2017; Jeffery, Lynch and O'Toole, 2016).

Additionally, the composition of the gut microbiota has been shown to differ according to different biogeographic regions (Gaulke and Sharpton, 2018) and environmental factors with diet being one of the main modulators (Yatsunenko *et al.*, 2012; Rehman *et al.*, 2016; Filippo *et al.*, 2010; He *et al.*, 2018), as discussed in the following sections.

1.3.2 Biogeography of the gut microbiota in the gut

The composition of the human gut microbiota varies along and across the GI tract (Fig. 8). The highest microbial density is found in the human colon (Dieterich, Schink and Zopf, 2018) which contains approximately 2 to 5 x 10^{11} bacteria/g wet weight faeces and a total of several hundred grams of bacteria while the inhospitable stomach acid and the proximal duodenum with bile and pancreatic secretions limit colonisation (Dieterich, Schink and Zopf, 2018). There are a few bacteria that can survive highly extreme acidic environments, amongst them *Helicobacter pylori*, a known risk factor for chronic gastritis, gastric ulcers and stomach cancer (Camilo, Sugiyama and Touati, 2017).

Transversally, the gut microbiota differs in the lumen, mucus, or epithelium surface (Juge, 2012; Vuik *et al.*, 2019; Juge, 2022). Furthermore, the inter-fold regions in the large intestine, present a different microbiota composition compared to the lumen microbiota (Nava, Friedrichsen and Stappenbeck, 2011; Pédron *et al.*, 2012). These spatial compositional differences are closely related to the functions of the different anatomic regions in the GI tract (Nava, Friedrichsen and Stappenbeck, 2011) and are shaped by the environment with antimicrobials, pH and oxygen gradients playing a major role (Wang *et al.*, 2020; McCallum and Tropini, 2024) (Fig. 8).

The pH of the small intestine is constantly exposed to the strong acids of the gastric juice, and the average is 6.1 in the duodenum, rising to 7.1 in the middle part and reaching 7.5 in the distal part of the small intestine (Abuhelwa, Foster and Upton, 2016). A lower pH, together with a higher oxygen level, limits the bacterial density in the small intestine (Wang *et al.*, 2020; McCallum and Tropini, 2024). There is a pH of 6.0 near the caecum at the entrance of the large intestine which then increases throughout to reach approximately 7.0 near the exit of the large intestine (Pye *et al.*, 1990).

In the small intestine, despite the challenges associated with sample collection, core members of the microbiota have been identified, namely *Veillonella*, *Prevotella*, *Fusobacterium* and *Haemophilus;* there are members of the gut microbiota commonly found in the duodenum and jejunum including *Neisseria*, *Granulicatella*, *Gemella*, *Rothia* and *Actinomyces*, while the ileum also include *Bacteroides*, *Escherichia*, *Shigella*, *Ruminococcus*, *Bifidobacterium*, *Clostridium* and *Lactobacillus* (Yersin and Vonaesch). These pH gradients along the GI tract favour the colonisation with *Bacteroidaceae, Prevotellaceae* and *Rikenellaceae* in the proximal colon and *Lachnospiraceae* and *Ruminococcaceae* in the distal colon (Donaldson, Lee and Mazmanian, 2016).

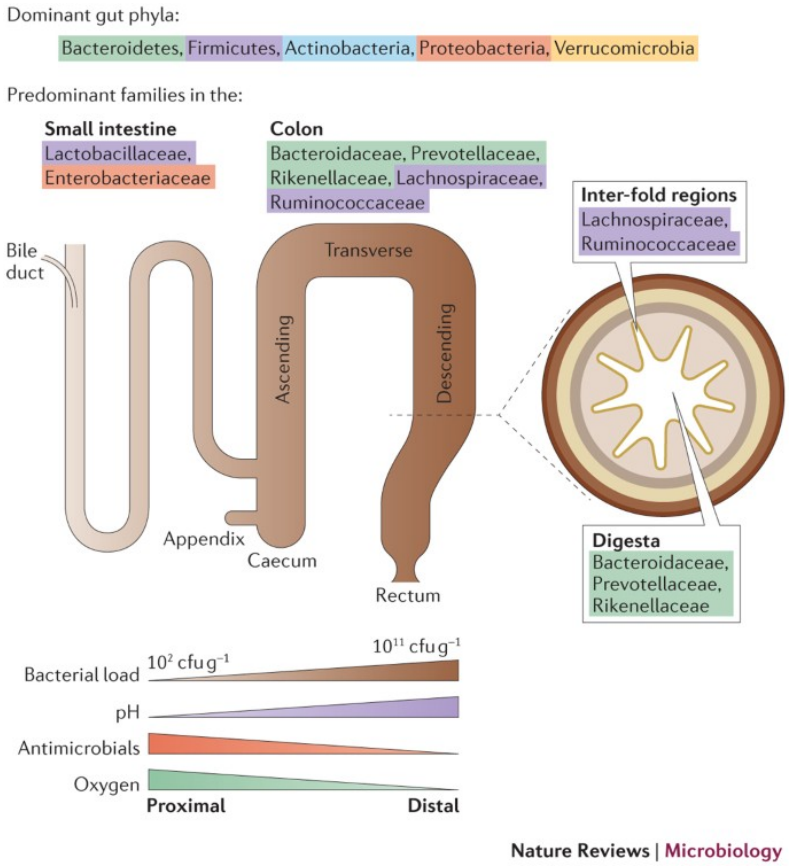


Figure 8. Dominant bacteria families in the small and large intestine. There are different compositions in the distribution of dominant bacteria across the small and large intestine, which also reflect physiological differences along the length of the GI tract. The cross-sectional view of the large intestine depicts differences in composition

between the interfolds and the lumen or digesta. Image taken from (Donaldson, Lee and Mazmanian, 2016). Reproduced with permission from Springer Nature.

The fermentation of dietary fibres and oligosaccharides by intestinal bacteria is particularly active in the proximal colon, including the caecum, where a large amount of short-chain fatty acids (SCFAs) and their metabolites are produced (den Besten *et al.*, 2013).

Insights into the impact of pH changes on the gut microbiota composition have been obtained from studies using *in vitro* models of fermentation.

For instance, a static *in vitro* experiment testing different pH levels showed an expansion of commensal *E. coli* at pH 5.5, while *Bacteroide*s spp. were predominant at pH 6.7 (Walker *et al.*, 2005). Moreover, butyrate-producing Gram-positive bacteria were not detected when pH was higher than 6.7 (Walker *et al.*, 2005). Altogether, this illustrates that even a shift of one pH unit can cause significant changes in the composition of the human colonic microbiota (Walker *et al.*, 2005). In a follow up study, *Bacteroides* were shown to grow at a wide range of pH values in *in vitro* fermentation models (Duncan *et al.*, 2009) whilst bacteria such as *Faecalibacterium* and *Roseburia* could grow at weakly acidic pH of 5.5 and produce more butyrate (Walker *et al.*, 2005). Lowering the colonic pH can also inhibit the growth of pathogenic bacteria, including *Enterobacteraciae*, which are sensitive to low pH (Hirshfield, Terzulli and O'Byrne, 2003). Using *in vitro* fermentation models each with mucosal carriers, it was found that an increase in pH from 7.0 to 7.5 led to an increase in luminal community richness, including *Ruminococcaceae*, but reduced mucosal community richness such as *Colinsella* and *Shigella*. Conversely, it was reported that lowering the pH to 6.5 in the same model decreased the evenness of both luminal and mucosal communities in a donor-independent manner (Firrman *et al.*, 2022).

The influence of pH conditions on SCFA production was previously reported using a pH-controlled fermenter, where it was found that a pH of 6.5 resulted in more acetate being produced while a lower pH led to increased butyrate production (Walker *et al.*, 2008). Additionally, *in vitro* cultures with mixed faecal microbiota showed higher lactic acid production at pH 5.9 compared to pH 6.4 (Belenguer *et al.*, 2007). These changes will have different effects depending on the original gut microbiota composition of the donor as species that convert lactate to butyrate or propionate such as *Anaerobutyricum* and *Anaerostipe*s spp. are also key to prevent excess acidity from lactate accumulation (Shetty *et al.*, 2020). In line with this, a recent fermentation study using a small scale *in vitro* colon model (CoMiniGut), showed that while colonic pH gradients significantly influenced the gut microbiota, with low pH increasing *Bifidobacterium* spp., and higher pH promoting the production of SCFAs and increased abundance of *Butyricimonas,* overall, the influence of the faecal donor and substrate was more pronounced than changes in pH (Xie *et al.*, 2024).

The fermentation of carbohydrates into SCFAs leads to a reduced osmotic pressure in the intestinal lumen, while carbohydrate and/or SCFAs malabsorption lead to an increase in the excretion of these acids in the faeces, elevating osmotic pressure,

resulting in osmotic diarrhoea (Hammer *et al.*, 1989; Hammer *et al.*, 1990). Although the significance of faecal pH in clinical practise is limited, it is currently used as an indicator of carbohydrate malabsorption in the colon and osmotic diarrhoea (Kasırga, 2019).

In addition to pH, the environment in the GI tract is also affected by oxygen, where an oxygen gradient is formed along the GI tract during the digestion process (Chikina and Matic Vignjevic, 2021) with oxygen levels being higher in the proximal small intestine and progressively decreasing toward the caecum (Donaldson, Lee and Mazmanian, 2016). In addition, measurements of the partial oxygen pressure have estimated that levels decrease across the colonic muscle wall, towards the crypt-lumen interface, being the lowest in the lumen of the ascending and sigmoid colon (He *et al.*, 1999).

In colonocytes, the energy metabolism towards oxygen consumption reinforces luminal hypoxia (Byndloss, Pernitzsch and Bäumler, 2018) and allows the survival of anaerobes. The response to hypoxia is mediated by hypoxia-inducible factors (HIF), which regulate the expression of a cohort of genes that promote adaptation to hypoxia (Singhal and Shah, 2020). Chronic HIF activation exacerbates disease conditions, leading to intestinal injury, inflammation and colorectal cancer (Shah, 2016).

The hypoxic intestinal microenvironment also varies with age. Newborn intestines are better oxygenated than those of adults, resulting in the prevalence of initial aerobic and facultative anaerobic bacteria (Walrath *et al.*, 2021). In adults, excess oxygen in the colon can disrupt the normal microbiota, potentially contributing to inflammation and infiltration by pathogens (Rivera-Chávez, Lopez and Bäumler, 2017).

NO and hydrogen sulphide (H_2S) contribute to maintaining luminal oxygen gradients due to their redox properties (Leclerc *et al.*, 2021). *In vitro,* NO exposure has been shown to increase the redox potential, altering the fermentation profile and gas production leading to less strict anaerobic conditions (Leclerc *et al.*, 2021). NO produced by facultative or anaerobic bacteria can diffuse into mucosal microaerobic zones, leading to oxidative modifications of fatty acids and influencing host mucosal immunity (Shah *et al.*, 2004). In the case of H_2S, sulphate-reducing bacteria use sulphate as a terminal electron acceptor, neutralising reactive oxygen species, a process dependent on oxygen and nutrient availability (Rey *et al.*, 2013).

The hypoxic environment in the gut is crucial for intestinal physiology and microbial ecology, with the microbiome playing a key role in maintaining it. This creates an ecological niche with various ecosystems along the GI tract, shaped by mucus, pH, and oxygen gradients.

In vitro fermentation models can recapitulate the anaerobic condition of the gut, by using anaerobic cabinets or nitrogen injection into the media or bespoke reactors (Donaldson, Lee and Mazmanian, 2016; Dupont *et al.*, 2019). The pH is usually set at 6.8 to model the distal colon, but can be modified or controlled depending on the complexity of the fermentation models to mimic more physiological conditions (Roupar *et al.*, 2021).

These models vary from small volume fermentations to fully controlled bioreactors, operating in batch, semi-continuous or continuous modes, with setups that can include multiple stages. Batch fermentation in closed tubes can be used to monitor treatment impact on microbial kinetics and gas production (Yao *et al.*, 2018). On the other hand, continuous culture systems allow a temporal monitoring of induced changes. The pioneer `Gibson' continuous model used three vessels corresponding to proximal, transverse and distal colon, with varying volumes and pH (Gibson, Cummings and Macfarlane, 1988). This fermentation system was designed to reproduce characteristics of the microbiota in the proximal and distal colons, and was validated on the basis of chemical and microbiological measurements on intestinal contents obtained from human sudden death victims (Gibson, Cummings and Macfarlane, 1988).

More recently continuous models have been developed to reproduce the entire GI tract with multichambered systems and flow of feed, where some fermenters are fed at an interval (Venema and van den Abbeele, 2013) like the EnteroMix model which allows the use of small volumes (Mäkivuokko *et al.*, 2006) or the highly standardised Simulator of the Human Intestinal Microbial System (SHIME®), recapitulating the stomach, small intestine, ascending, transverse and descending colon, which is used for long-term studies (Van de Wiele *et al.*, 2015). Here, the feeding follows a fill-and-draw approach where a defined nutritional medium is added three times a day to the gastric compartment, while pancreatic and bile liquid are added to the small intestine compartment (Van de Wiele *et al.*, 2015). Different SHIME® models have been developed for different applications. For example, the TWIN SHIME® contains duplicate vessels to allow to run two conditions in parallel. The M SHIME® includes a mucosal interface with mucin-covered microscosms (Van den Abbeele *et al.*, 2012) allowing the growth of mucin-associated species (Van de Wiele *et al.*, 2015). Finally, some of the continuous models can include immobilised microbiota to improve stabilisation and washout prevention such as the Polyfermentor intestinal model (PolyFermS) (Zihler Berner *et al.*, 2013), or peristaltic movements to mix the dietary compounds, such as the dynamic SIMulator of the GastroIntestinal tract (SIMGI®)(Barroso *et al.*, 2015). These *in vitro* fermentation systems also allow to measure the biological effect of intra- and inter- individual variations in the donor faecal samples (Thorman *et al.*, 2024).

1.3.3 Role of the gut microbiota in human health

The gut microbiota provides a range of beneficial properties to the host, helping maintain the integrity of the mucosal barrier, providing nutrients and vitamins to the host, contributing to the maturation of the host immune system and protecting against pathogens (Thursby and Juge, 2017). The combined metabolic activity of the resident gut microbiota creates the microbial metabolome which, in turn, impacts the host metabolism and physiology (Flint *et al.*, 2015). From an evolution perspective, it is generally admitted that the gut microbiota has coevolved with the host, resulting in the selection of microorganisms and diversity which benefit human health (Moeller *et al.*, 2016).

1.3.3.1 Carbohydrate fermentation and SCFA and gas production

The main dietary carbohydrates that reach the large intestine are resistant starches, plant cell wall polysaccharides and non-digestible oligosaccharides (Flint *et al.*, 2012a; Silva, Bernardi and Frozza, 2020). These complex carbohydrates are made up of insoluble structures that reach the colon undigested by the host due to the small number of GHs encoded by the human genome (Hansen *et al.*, 2020). These then become substrates to the gut microbiota which possesses a large repertoire of diverse carbohydrate-active enzymes (CAzymes) dedicated to carbohydrate degradation (Kaoutari *et al.*, 2013). A total of 189 families of GHs, 43 polysaccharide lyases and 20 carbohydrate esterases have been described to date (06/07/2024), with a large proportion of these encoded in microbial genomes (Drula *et al.*, 2022) (www.cazy.org). Through metagenomic, metabolomic, transcriptomic and metatranscriptomic analyses, it has been possible to mine for the enzymes encoded by the gut microbiota (Jia *et al.*, 2022). Phenotype-based clustering has also recently shown that members of the same species perform specific roles in polysaccharide metabolism within the microbial community; for example, the ability to utilise dietary polysaccharides and endogenous mucin glycans was negatively correlated, suggesting that nutritional niches are colonised by distinct species clusters (Pudlo *et al.*, 2022).

The fermentation of carbohydrates in the large intestine has metabolic consequences for the human host (Silva, Bernardi and Frozza, 2020). From carbohydrates, gut bacteria produce SCFAs that can account for up to 10% of the total daily caloric intake (Bergman, 1990). They consist of a subset of saturated fatty acids containing six or less carbon molecules, with acetate, propionate, butyrate being the major SCFAs released through fermentation (Fig. 9) (Wong *et al.*, 2006; Tan *et al.*, 2014) followed by valerate and caproate, produced in lower amount (Akhtar *et al.*, 2022).

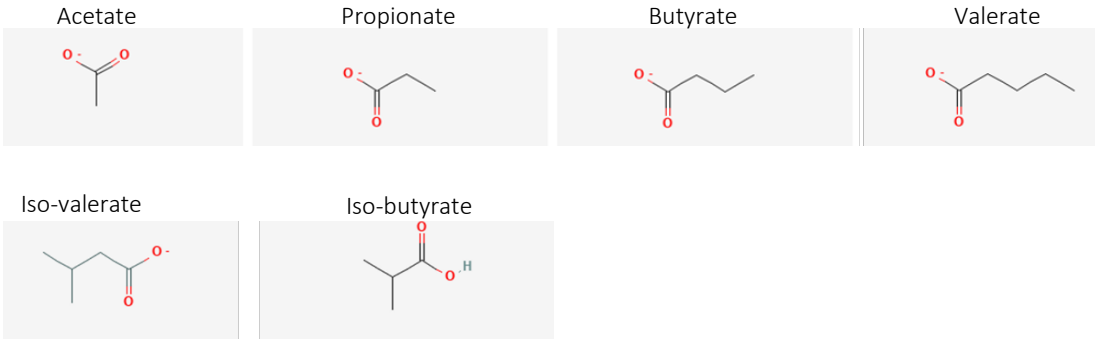


Figure 9. Chemical structures of acetate, propionate, butyrate and valerate. Retrieved from the PubChem Compound Summary for compound identifier (CID) 175, CID 104745, CID 104775, CID 114781, CID 3587356 and CID 6590 available from https://pubchem.ncbi.nlm.nih.gov (NCBI).

The effects of SCFAs on the function of the GI tract include intestinal tissue proliferation, enhanced absorption of minerals and water, and modulation of GI tract contractility (Ney *et al.*, 2023). SCFAs are a key energy source for colon and ileum cells,

they regulate the function of innate immune cells, regulate the differentiation of T and B cells, and play a role in regulating energy homeostasis and metabolism (Yao *et al.*, 2022). For example, butyrate via oxidative phosphorylation, which is an oxygen-consuming process, is critical for initiating and maintaining an hypoxic gradient in the intestine (Kelly *et al.*, 2015; Inai, Kobayashi and Shibata, 1999). SCFAs are absorbed by the host colonocytes and utilised as an energy source via G-couple protein free fatty acid receptors (Scheppach, 1994; Sleeth *et al.*, 2010b), with butyrate acting act as the main fuel for the colonic wall (Sleeth *et al.*, 2010a). Increased SCFAs lead to a pH reduction in the intestinal environment, which plays an important role in maintaining human health, by reducing the solubility of toxic cholic acids, promoting the absorption of minerals and inhibiting the absorption of ammonia (Wong *et al.*, 2006).

Moreover, SCFAs are involved in the regulation of cellular processes and signalling to other organs (Bugaut, 1987; Musso, Gambino and Cassader, 2010). For example, they influence the expression of host peptides and hormones related to appetite regulation (Psichas *et al.*, 2015; Chambers *et al.*, 2015). In recent years, SCFAs have been implicated as the main mediators of gut-brain-axis and neurodegenerative disorders, as extensively reviewed (Dalile *et al.*, 2019; Silva, Bernardi and Frozza, 2020; Guo *et al.*, 2022; O'Riordan *et al.*, 2022; Majumdar, Siva Venkatesh and Basu, 2023). Elevated levels of excreted and non-absorbed faecal SCFAs have been associated with gut microbiome dysbiosis, obesity, hypertension and cardiometabolic risk factors (De la Cuesta-Zuluaga *et al.*, 2019).

There is evidence that alterations in SCFA production can induce epigenetic changes and vice versa. SCFAs demonstrate the ability to inhibit the activity of histone deacetylases that affect gene expression by increasing histone acetylation, and this may have an effect on lymphocytes proliferation and function (Tan *et al.*, 2014; Cavaglieri *et al.*, 2003).

Cross-feeding of SCFAs plays a key role in maintaining gut homeostasis. Firmicutes such as *F. prausnitzii, Eubacterium rectale* and *Eubacterium hallii*, are dominant producers of butyrate in the colon (Louis *et al.*, 2010). These butyrate-producing bacteria can utilise acetate via cross-feeding and increase their butyrate production. For example, co-cultures of *F. prausnitzii* S3/L3 with *B. adolescentis* L2-32 resulted in an increase in butyrate production compared to *F. prausnitzii* monocultures (Rios-Covian *et al.*, 2015). Recently, lactate cross-feeding was described between *B. longum*, *B. pseudocatenulatum* and *Megasphera indica*, from faecal culture isolates, leading to butyrate production from *M. indica* (Zhao *et al.*, 2024).

Finally, the fermentation of complex carbohydrates can lead to the production of gases by the gut microbiota which include hydrogen, methane, carbon dioxide, hydrogen sulphide, and nitric oxide, which can modulate the physiology of hosts (Kalantar-Zadeh *et al.*, 2019; Rowland *et al.*, 2018) and can be eliminated from the GI tract by further metabolism into non-gaseous products, absorption into the bloodstream and exhalation via breath or through the anus as flatulence (Mego *et al.*, 2017; Mutuyemungu *et al.*, 2023).

1.3.3.2 Production of vitamins and other metabolites

Humans lack the biosynthetic capacity to produce most vitamins and these must thus be provided by exogenous sources such as the diet. In addition, members of the gut microbiota are able to synthesise vitamin K as well as most of the water-soluble B vitamins, such as biotin, cobalamin, folates, nicotinic acid, pantothenic acid, pyridoxine, riboflavin and thiamine (Morowitz, Carlisle and Alverdy, 2011). In contrast to dietary vitamins, which are absorbed in the proximal tract of the small intestine, the predominant uptake of microbially produced vitamins occurs in the colon (LeBlanc *et al.*, 2013).

Vitamin B is mainly supplied through diet and, to a lesser extent synthesised by gut microbes (Wan *et al.*, 2022). The term vitamin B_{12} is generally used to describe a type of cobalt corrinoid of the cobalamin group essential for red blood cell formation and neurological function (Bohrer, 2017). *Limosilactobacillus reuteri* (formerly *Lactobacillus reuteri*) CRL1098 was the first lactic acid bacterial strain identified as capable of producing a cobalamin-like compound (Santos *et al.*, 2007). Vitamin B_{12} is a cofactor of many enzymatic reactions and regulates bacterial genes through riboswitches and other mechanisms (Guetterman *et al.*, 2022). Recent evidence based on *in vitro* batch fermentation models suggests that a healthy gut microbiota can meet its own B_{12} requirements (Kundra *et al.*, 2022).

Vitamin K is involved in the function of many proteins that have ultimate effects on iron uptake, inflammation, vascular homeostasis, bone health and the coagulation system (Dai *et al.*, 2023). Members of the gut microbiota identified as vitamin K producers include *Enterobacter agglomerans, Serratia marcescens* and *Enterococcus faecium* extracted from the neonatal faecal microbiota (Cooke, Behan and Costello, 2006).

Gut bacteria also produce metabolites from the fermentation of amino acids. Tryptophan is catabolised by gut microorganisms resulting in a wide range of metabolites implicated in both beneficial and adverse host effects (Sinha *et al.*, 2024). For example, tryptophan can be metabolised by gut microbes into AhR aryl hydrocarbon receptor ligands such as indole-3-propionic acid (Hou, Li and Ying, 2023). A defective activation of AhR leads to decrease production of GLP-1 and IL-22 which contribute to intestinal permeability (Natividad *et al.*, 2018), enhancing epithelial barrier function (see section 1.2.2.). Microbial metabolites derived from dietary essential amino acids can also affect serotonin-dependent control of gut motility. For example, *Ruminococcus gnavus* can generate phenethylamine and tryptamine from the catabolism of dietary phenylalanine and tryptophan that directly stimulate serotonin biosynthesis in intestinal enterochromaffin cells, leading to increased GI transit and colonic secretion (Zhai *et al.*, 2023). Dietary choline and L-carnitine are essential for fatty acid translocation and cell membrane structures (Hongu and Sachan, 2003) and their metabolism by gut microbes produces trimethylamine N-oxide (TMAO) (Zhen *et al.*, 2023), which has been implicated in metabolic disorders when dysregulated (Org *et al.*, 2017).

Furthermore, metabolites produced by the host can be modified by gut bacteria as is the case of bile acids. Bile acids are essential for lipid and vitamin digestion absorption; they are synthesised from cholesterol in hepatocytes, to produce primary bile acids,

mainly chenodeoxycholic acid and cholic acid (Chiang, 2014). Primary bile acids are typically conjugated by hepatocytes into glycine or taurine before being secreted (Chen, 2023). These primary bile acids are secreted into small channels called canaliculi, which lead to bile ducts reaching the intestine (Tabibian *et al.*, 2013). Approximately 95 % of bile acids are reabsorbed actively from the terminal ileum and are recycled in the liver, the gut microbiota can also transform them into secondary bile acids and deconjugates them (Chiang, 2014). The most prevalent secondary bile acids are deoxycholic acid (DCA) and lithocholic acid (LCA) (Guzior and Quinn, 2021). These secondary bile acids can either be passively reabsorbed to re-enter the circulating bile acid pool or excreted in faeces (Martin *et al.*, 2007). Secondary bile acids have been suggested as regulators of metabolic host homeostasis. For example, a decrease in hepatic DCA and LCA concentrations following a short antibiotic treatment in mice led to a decrease in serum triglyceride levels (Kuno *et al.*, 2018). Furthermore, DCA was shown to inhibit vegetative growth and toxin production in *Clostridium difficile* life cycle (Usui *et al.*, 2020).

There is also some evidence that the gut microbiota can produce *de novo* metabolites or compounds affecting human health. For instance, some gut microbes such as members from the genus *Bacteroides* and species of *Bifidobacterium longum* (Starke *et al.*, 2023) have the capacity to synthesise branched amino acids (BCAAs) *de novo* (Amorim Franco and Blanchard, 2017). Essential BCAAs, namely valine, isoleucine and leucine, play a crucial role in maintaining homeostasis in mammals by regulating protein synthesis, glucose and lipid metabolism, insulin resistance, hepatocyte proliferation and immunity (Tajiri and Shimizu, 2018). The most abundant BCAA-producing bacteria are *Prevotella copri* and *Bacteroides vulgatus* (Li *et al.*, 2023b).

Polyamines, such as putrescine, spermidine, and spermine, play a significant role in modulating the gut microbiota composition and function (Tofalo, Cocchi and Suzzi, 2019) and influence the host`s metabolism and immune responses (Bui *et al.*, 2023). *Bacteroides thetaiotaomicron*, a dominant species in the human gut, produces spermidine through the enzymatic activity of N-carbamoylputrescine amidohydrolase, which is regulated by feedback inhibition to maintain intracellular polyamine homeostasis (Shimokawa *et al.*, 2023).

In summary, the metabolites produced by the gut microbiota are essential to various aspects of human health, including immune function, disease prevention, metabolic balance and mental health. Recently, metabolomic surveys highlighted the vast array of metabolites produced by the gut microbiota (Shaffer *et al.*, 2017; Van Treuren and Dodd, 2020; Sparagon *et al.*, 2022). Additionally, genome-scale metabolic models (GSMMs) predicted that approximately 1543 metabolites are uniquely formed by the gut microbiota, not found in human GSMMs, suggesting a significant number of microbial metabolites yet to be experimentally validated (Neveu *et al.*, 2023). Further supporting this, a study using a non-targeted metabolomics approach identified 331 metabolite ions in urine affected by the depletion of the faecal microbiome, including 100 modified metabolites, indicating the extensive metabolic output of the gut microbiota (Zheng *et al.*, 2023).

Understanding the intricate relationships between gut microbiota and their metabolites offers opportunities to develop therapeutic interventions and personalised nutrition strategies aimed at promoting health and preventing disease.

1.3.3.3 Modulation of the immune system

The intestinal immune system, being the largest immune organ in humans, begins to develop during the embryonic stage and continues to mature in response to the gut microbiota and dietary signals (Stras *et al.*, 2019; Al Nabhani and Eberl, 2020).

The maturation of the gut microbiota is associated with the development of regulatory T cells (Tregs) and the production of butyrate, which plays a protective role against allergies and other immune-mediated diseases (Ruohtula *et al.*, 2019). This correlated with the prevalence of *B. longum* at early life stage followed by increasing colonisation with butyrate-producing *Faecalibacterium* in the neonatal microbiota (Ruohtula *et al.*, 2019).

The gut microbiota interacts with both hematopoietic and non-hematopoietic cells, impacting immune response development, including the induction of immunoglobulin A (IgA) and regulation of CD4+ T cells (Lee, 2022).

The gut microbiota has a role in sustaining immune surveillance. For example, polysaccharide A (PSA) produced by *Bacteroides fragilis* plays an important role in immune regulation through the activation of Toll-like Receptor 2 on dendritic cells, which induces the secretion of IL-10 (Johnson, Jones and Cobb, 2015). Moreover, in mice, *B. fragilis* induction of IL-10 showed delay in the onset of experimental autoimmune encephalomyelitis (Ramakrishna *et al.*, 2019).

Conversely, both the innate and adaptive immune systems are involved in modulating a stable microbial ecosystem, managing the equilibrium between commensal and pathogenic microorganisms (Shao *et al.*, 2023). Alterations in the dynamics between the gut microbiota and the immune system can lead to undesirable adverse effect with gut dysbiosis recognised as a driver of various diseases, including cancer (Zhao *et al.*, 2023).

1.3.3.4 Protection against pathogens

The gut microbiome plays a crucial role in colonisation resistance, which is the ability of the gut to prevent the establishment and growth of pathogenic microorganisms. This resistance is primarily mediated through several mechanisms, including nutrient competition, production of antimicrobial compounds, and modulation of host immune responses. For example, Horrocks et al. (2023) showed that antibiotic treatment, by eliminating the gut microbiota, freed up nutrients becoming available to pathogens, facilitating their colonisation (Horrocks *et al.*, 2023). Similarly, Akritidou et al. (2023) demonstrated that the gut microbiota, particularly in the ileum, served as a barrier against foodborne pathogens like *Salmonella* Typhimurium and *Listeria monocytogenes*, with diet influencing the effectiveness of this resistance (Akritidou *et al.*, 2023).

In addition, some members of the gut microbiota can produce toxic substances such as bacteriocins to inhibit pathogens from intestinal colonisation (Cavera *et al.*, 2015). Bacteriocins have a protective effect on the gut microbiota by reducing the susceptibility of developing inflammatory processes and modifying the host immune system (Cardoso *et al.*, 2022). There are various types of bacteriocins each with different targets and they usually target bacterial membranes to exert their anti-infective properties (Pérez-Ramos *et al.*, 2021). For example, ruminococcin C1, an antimicrobial peptide, naturally produced by *R. gnavus* E1 in the gut, confers activity against *Clostridium perfringens* (Chiumento *et al.*, 2019). Moreover, bacteriocins from lactic acid bacteria can help the producer outcompete pathogenic bacterial species (Alvarez-Sieiro *et al.*, 2016).

Other chemicals can also be produced by the gut microbiota that inhibit pathogenic bacterial growth. For example, in *L. reuteri*, the anaerobic reduction of glycerol to 1,3-propanediol produces the intermediate 3-hydroxypropanal, which when accumulated, leads to the production of the anti-microbial compound reuterin (De Weirdt *et al.*, 2010).

Given the range of beneficial properties provided by the gut microbiota, it is not surprising that an alteration in the composition of the gut microbiota or 'dysbiosis', term first coined by (Scheunert, 1920), has been associated with a growing number of intestinal and extra-intestinal diseases (Hooks and O'Malley, 2017; Belizário and Faintuch, 2018).

1.3.4 Modulation of the gut microbiota by the diet

1.3.4.1 Effect of plant-rich diets on the gut microbiota

Substrate availability is a major contributor to the physiology and composition of the gut microbiota across lifetime (Yáñez *et al.*, 2021). Over the past decade, the increased popularity of vegetarian and vegan diets in Western countries (Alcorta *et al.*, 2021) has raised questions on how these changes affect the gut microbiota and whether they can be long-lasting.

The analysis of the faecal microbiota of over 240 long-term vegans and vegetarians and an equal number of omnivores, using classical bacteriological isolation and enumeration, showed that total bacteria counts of Bacteroides, *Bifidobacterium* and *E. coli* and *Enterobaceriaceace* were significantly lower in vegans, while other groups such as *Citrobacter* and *Clostridium* were significantly higher (Zimmer *et al.*, 2012). With the advent of next-generation sequencing, and more recently, machine learning, an increasing number of studies explored the interplay between diet and the gut microbiome as recently reviewed (Ross *et al.*, 2024). The effects of dietary changes on the gut microbiota can be determined by measurement of alpha and beta diversity indexes. In microbial ecology, alpha diversity refers to the diversity within a single sample, often measured by indices such as species richness and the Shannon-Weaver index, which account for both the number of species and their relative abundances (Finn, 2024). On the other hand, beta diversity measures the variation in microbial community composition between different samples or environments (Finotello,

Mastrorilli and Di Camillo, 2016) and can be assessed using methods like Bray-Curtis dissimilarity and principal coordinate analysis (PCoA) (Kers and Saccenti, 2021).

For example, statistically significant differences in the alpha diversity indices were found within the gut microbiota of vegetarians and omnivores, when on these diets for more than 12-months prior to the study, with the vegetarians showing greater bacterial richness (Losasso *et al.*, 2018).

Similarly, findings from the Baltimore Longitudinal Study of Aging (n=705) evidence that adherence to a defined `healthful plant-based diet` was positively associated with microbiome alpha diversity and the relative abundance of beneficial bacterial species like *F. prausnitzii* and *B. thetaiotaomicron*, while inversely associated with harmful species and metabolites like TMAO (Shen *et al.*, 2024). A longitudinal study of the gut microbiota of the hunter-gatherer Hadza community (n=188), consuming a plant-rich diet influenced by lifestyle practices of hunting and foraging, showed differences in the gut microbiota composition, compared to the gut microbiota from an American cohort from the Human Microbiome Project. Particularly, the results in the Hadza population showed a relative stability of Firmicutes across seasons, with the Hadza microbiota possessing higher levels of genes encoding plant-degrading enzymes, while the American cohort had genes encoding enzymes targeted to animal and mucin degradation (Fragiadakis *et al.*, 2019).

More recently, a cross-sectional analysis of faecal samples (n=1098) from participants of the Personalised Responses to Dietary Composition Trial (PREDICT 1) study based in the UK, identified significant associations between specific microbes and nutrients, foods, food groups, and general dietary indices, particularly driven by the presence and diversity of healthy and plant-based foods. Microbial biomarkers of obesity were reproducible across external cohorts and correlated with circulating blood metabolites indicative of cardiovascular disease risk, while certain microbes like the bacteria *Prevotella copri* and the parasite *Blastocystis* spp. were linked to favourable postprandial glucose metabolism (Asnicar *et al.*, 2021).

Dietary patterns or food types associate with distinct groups of bacteria. For example, a cross-sectional study of the faecal microbiome in healthy population and patients with GI disorders (n=1425) showed that the consumption of breads and legumes were associated with a lower abundance of *E. coli*, *B. fragilis* and *Parabacteroides* (Bolte *et al.*, 2021). On the other hand, processed foods and animal-derived foods were consistently associated with higher abundances of Firmicutes, *Ruminococcus* species and endotoxin pathways (Bolte *et al.*, 2021).

Fibre-rich plant-based food are critical in influencing the composition and metabolic activity of the gut microbiota, with SCFAs having an important impact on human health (see section 1.3.3.1) (Simpson and Campbell, 2015). This was demonstrated in early work showing that, in human intervention trials, reducing the overall intake of complex dietary carbohydrates resulted in decreased SCFA production with a disproportionate decrease in faecal butyrate (Flint *et al.*, 2012b).

The short-term consumption of diets composed entirely of animal or plant products has been shown to influence the microbial community structure in humans. For example, a two-week intervention (n=248) showed that a higher intake of vegetables and fruits was associated with increased levels of butyrate-producing Clostridiales and higher community richness depending on the initial microbiota state (Klimenko *et al.*, 2018). During a shorter (five-day) intervention, with a plant-based diet or an animal-based diet, the alpha diversity did not significantly change upon the diet type, but each individual's gut microbiota was significantly affected with a decrease in beta-diversity occurring one day after the animal-based diets reached the distal gut microbiota (David *et al.*, 2014).

However, these changes to the gut microbiota can be transitory, and short-term microbial changes appear to be dependent on the baseline microbial composition or `enterotypes' (Arumugam *et al.*, 2011). For example, a cross-sectional analysis of dietary and environmental variables in healthy volunteers (n=98) showed that *Bacteroides* `enterotype 1' was associated with animal protein and fat-rich diets while *Prevotella* `enterotype 2' was associated with carbohydrate-rich diets (encompassing 11 vegetarians and 1 vegan participant) (Wu *et al.*, 2011). In this work, another group of participants (n=10) underwent a short-term intervention (10 days) with a high fat/low fibre or low fat/ high fibre diet, here transitory changes in the microbiome composition were only observed after 24 hours of the intervention diet in the abundance of some species, however, at the end of the trial, the enterotype groups were found to be unchanged compared to prior to the short-term intervention, indicating that the short-term intervention did not overcome interindividual baseline microbial composition (Wu *et al.*, 2011).

A one-year long-term dietary intervention conducted on healthy overweight participants (n=49) showed that, while a high-fibre diet could influence the abundance of certain microbial species, with effects seen at three months, these changes were transient and no differences were found when comparing the microbial composition between baseline and after one year (Fragiadakis *et al.*, 2020).

These differences in the community of human faecal bacteria in response to diet or food components have also been thoroughly investigated using *in vitro* models of fermentation, as reviewed extensively (Moon *et al.*, 2016; Nissen, Casciano and Gianotti, 2020). For example, early work showed that a bacterial group encompassing *Roseburia* and *Eubacterium rectale*, plays a crucial role in mediating the butyrogenic effect of fermentable dietary carbohydrates *in vitro,* and this was further supported *in vivo* (Louis *et al.*, 2007). These early findings highlighted that the metabolic activities of the gut microbial community were affected not only by the presence of substrates, but also by pH reduction in the environment within the gut lumen due to SCFA production (Louis *et al.*, 2007), underscoring the benefits of conducting *in vitro* fermentation models in complement to human intervention studies.

Overall, plant-based diets are increasingly being recognised for their ability to enhance gut microbiota diversity and composition, contributing to better gastrointestinal and overall health.

1.3.4.2 Effect of prebiotics on the gut microbiota

The use of prebiotics is one of the strategies to modulate the gut microbiota towards health (Moles and Otaegui, 2020). According to the International Scientific Association for Probiotics and Prebiotics (ISAPP), prebiotics are defined "as a substrate that is selectively utilised by host micro-organisms conferring a health benefit" (Gibson *et al.*, 2017). Confirmed prebiotics include galacto-oligosaccharides (GOS), fructo-oligosaccharides (FOS), inulin and inulin-type fructans (ITF), and lactulose (Scott *et al.*, 2020). Candidate emerging prebiotics include soy/soybean oligosaccharides, pectin, cellulose, resistant starch, xylan, xylo-oligosaccharides (XOS), arabinoxylooligosaccharides (AXOS), mannose, maltose, malto-oligosaccharides, isomaltulose, palatinose, polydextrose, raffinose oligosaccharides (RFOs) and β-glucans (Scott *et al.*, 2020), and human milk oligosaccharides (HMOs) (Gibson *et al.*, 2017; Okburan and Kızıler, 2023).

Non-digestible carbohydrates can be grouped based on their sizes; for instance, resistant starches and arabinoxylans are large polysaccharides which are fermented at a slower rate compared to short oligosaccharides. Arabinoxylans are nondigestible carbohydrate considered as the main non-cellulosic polysaccharides in the cell walls of cereals, and have structural differences which can determine their biological effects, fermentation processes and influence on the gut microbiota (Paesani *et al.*, 2020). Extracted arabinoxylans from soft and hard wheat were shown to promote selective growth of *L. reuteri* and *Bifidobacterium breve* in monocultures (Paesani *et al.*, 2019) and the growth of *Bifidobacterium* and release of SCFAs in a colonic *in vitro* fermentation model (Paesani *et al.*, 2020).

Historically, prebiotics have been studied for their impact on bifidobacteria and lactobacilli (Gibson *et al.*, 2004). However, it soon became apparent that their effect was not restricted to these bacterial species. An early *in vitro* study showed that inulin derived from dahlia caused and increase in *R. flavefaciens*, *R. bromii* and *E. cylindroides*, three strictly anaerobic, gram-positive bacteria (Duncan *et al.*, 2003). Moreover, ITF supplementation led to the increased abundance of *Bifidobacterium*, *Lactobacillus* and *F. prausnitzii* in faecal samples, as thoroughly reviewed (Hughes *et al.*, 2022). Short-chain FOS have been shown to promote the growth of faecal isolates of *F. prausnitzii*, *Roseburia* spp., *Eubacterium* spp., *Anaerostipes caccae*, *Coprococcus* spp., *Anaerostipes hadrum*, *Bifidobacterium* spp. and *Bacteroides* spp. growing in monocultures (Scott *et al.*, 2014). Increasingly, evidence of the effect of prebiotics on the modulation of the gut microbiota came from metagenomic studies. For example, in a meta-analysis of six studies involving the analysis of 821 stool samples from 451 participants, it was found that GOS intervention increased the relative abundance of *Akkermansia muciniphila* in healthy individuals. Additionally, the presence of other beneficial microbes like *Eubacterium hallii* and *Bacteroides* showed enhanced correlation with *A. muciniphila* upon GOS intervention, suggesting a beneficial modulation of the gut microbiota composition (Tian *et al.*, 2024).

An *in vitro* fermentation of protein isolates from plant and animal source showed that the presence of high carbohydrate complexity in the medium (1 % mix of starch, inulin, arabinogalactan, xylan and pectin) resulted in differential bacterial abundances

depending on the origin of the protein fermented; beef protein resulted in an increase of Bifidobacterium, soy protein increased Desulfovibrionaceae and pea proteins in *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Weissella* (Behounek *et al.*, 2024).

The glycosidic bonds in complex polysaccharides can be highly diverse and their degradation require linkage-specific GHs. Gram-negative, obligate anaerobic members of the class Bacteroidia (formerly Bacteroidetes), exhibit remarkable proficiency in the degradation of complex polysaccharides due to the coordinated actions of enzymes encoded by polysaccharide utilisation locus (PUL) (Muramatsu and Winter, 2024; Bjursell, Martens and Gordon, 2006). PULs encode an array of cell surface glycan-binding proteins, TonB-dependent transporters, GHs, polysaccharide lyases, carbohydrate esterases and carbohydrate sensors or transcriptional regulators (Grondin *et al.*, 2017). The first best-studied example of a PUL is the starch utilisation system in *Bacteroides* (Martens *et al.*, 2009). Since then, a number of PULs have been identified in Bacteroidetes by transcriptomics for the utilisation of dietary complex polysaccharides including arabinoxylan, xyloglycan, beta-glucans, hemicellulose or pectin (Pereira *et al.*, 2021; Foley *et al.*, 2019; Tamura *et al.*, 2019; Ndeh *et al.*, 2017; Martens *et al.*, 2011). There is therefore the potential to use PULs as predictive biomarkers of prebiotic responses (Kok *et al.*, 2024). In gram-positive bacteria, GHs are often found as part of cluster of genes dedicated to the catabolism of carbohydrates (Ndeh and Gilbert, 2018). For example, *Roseburia inulinivorans* A2-194 showed differentially induced gene clusters when grown on inulin or fructose (Scott *et al.*, 2011). These PULs or gene clusters encode for various proteins involved in transport and metabolism of polysaccharide breakdown products, and the released products benefit the bacterial community (Rogowski *et al.*, 2015).

Prebiotics have been shown to modulate health through their fermentation products such as SCFAs. For example, L-sorbose and xylitol have been shown to promote butyrate production using *in vitro* fermentation with human faecal cultures, by increasing the abundance of butyrate-producers, *Anaerostipes hadrus* and *A. caccae,* (Sato *et al.*, 2016). Gram-positive bacteria of the Clostridia class also contribute to the production of SCFAs through polysaccharide degradation (Muramatsu and Winter, 2024). For example, *R. bromii* generates formate, acetate, and ethanol when provided resistant starch. When co-cultured with *Blautia hydrogenotrophica*, formate was consumed and additional acetate was produced (Laverde Gomez *et al.*, 2019). More recently, a study conducted in mice found that xylitol enhances propionate synthesis via cross-feeding among *L. reuteri*, *B. fragilis* and *E. coli* and this promoted the transcription of phosphate acetyltransferase in *Bifidobacterium,* increasing propionate production (Xiang *et al.*, 2021). The effect of prebiotics on particular species can be strain specific. For example, an early study found that in monoculture, 54 out of 55 Bifidobacterium strains fermented FOS, while only eight grew when inulin was used as the carbon source. In the same study, batch fermentations with faecal samples resulted in bifidogenic effect on both prebiotics, FOS and inulin, which can be explained by the cross-feeding of Bifidobacteria on the monosaccharides released by primary degraders of inulin, resulting in production of butyrate as the main SCFA (Rossi *et al.*, 2005).

As a result of prebiotic fermentation by specific group of bacteria, SCFAs are not end products of metabolism but can be cross fed to other bacteria, leading to broader changes in microbial community structure. This has been shown through supplementation of *in vitro* fermentation models with individual SCFAs (formate, acetate, propionate, butyrate, lactate, succinate, valerate, isobutyrate, and isovalerate) where, compared to control cultures, butyrate-supplemented cultures showed a reduced relative abundance of Firmicutes and Bacteroidetes but increased relative abundance of Proteobacteria, whereas propionate-supplemented cultures led to a decrease in the relative abundance of Bacteroidetes (Peterson *et al.*, 2022).

Other examples of prebiotic-induced cross-feeding interactions that led to SCFA production in the gut microbiota are depicted in Fig. 10 (Saa *et al.*, 2022).

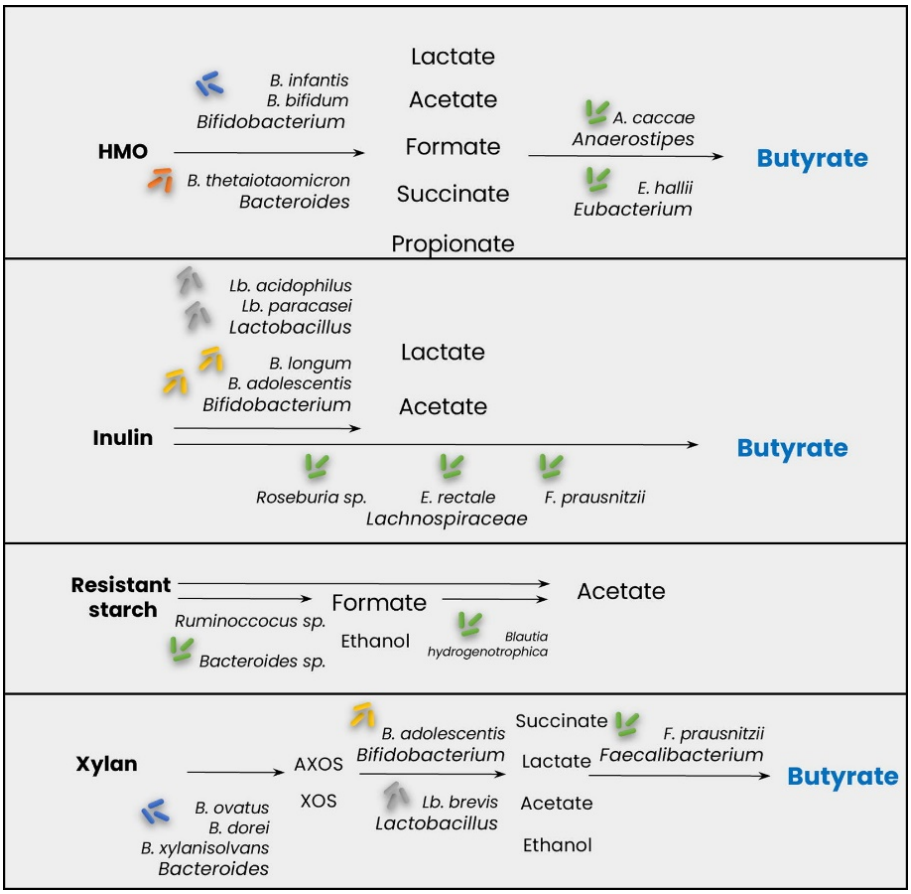


Figure 10. Effect of bacterial cross-feeding from prebiotic dietary sources on SCFA production. The figure shows the overview of some cross-feeding interactions in the gut microbiota, from the initial dietary source (HMO: human milk oligosaccharides; inulin, resistant starch and xylan, AXOS: arabinoxylanooligosaccharides; XOS: xyloligosaccharides), leading to the production of SCFAs. Taken from Saa et al. (2022). Reproduced under Creative Commons Attribution-NonCommercial-No Derivatives License (CC BY NC ND).

An important consideration in the definition of prebiotics is that they must fulfil the requirement of providing health benefits to the host, and not solely generate microbiota changes. The effect on health has been widely studied using animal

models, particularly to understand the protective effect of prebiotics against pathogen infection. For example, feeding mice with inulin on inulin in combination with *Lactobacillus acidophilus* MTCC 447, led to decreased translocation of *Salmonella* Typhimurium to the liver. This effect was more pronounced when inulin was supplemented on its own, suggesting a role of prebiotics through a different mechanism than symbiotic supplementation (Rishi *et al.*, 2009). Another study conducted in mice showed increased immunoglobulin levels and macrophage phagocytic activity one week post immunisation with attenuated *S.* Typhimurium combined with a FOS/inulin mix, followed by improved mouse survival upon a further challenge with a virulent *Salmonella* (Benyacoub *et al.*, 2008). Available data on the mechanisms by which prebiotics promote the growth of microorganisms involved in SCFA is often limited to *in vitro* fermentation using human faecal samples (see section 1.3.3.1) or animal experiments, and there is a need for long-term human SCFA intervention studies investigating their effects on intestinal inflammation and various parameters of metabolic health as highlighted in a detailed review (Blaak *et al.*, 2020).

Research in humans indicates that the appropriate dosage of prebiotics can significantly influence their effectiveness. For example, the supplementation of 15 g daily of inulin-type fructans in patients (n=13) with mild to moderately active UC, showed increased colonic butyrate production and reduction of colitis compared to patients consuming a lower daily dose of 7.5 g (n=12) (Valcheva *et al.*, 2019). However, these prebiotic effects appear to be dependent on the severity of the disease. For example, 2.8 g/day of GOS supplementation for 6 weeks led to an increase in faecal *Bifidobacterium* proportions in UC patients with less active disease (n=6) as compared to patients not in remission (n=7), and although there were no changes in the clinical score or inflammation, the treatment contributed to normalised stools form and frequency (Wilson *et al.*, 2021).

The role of prebiotics has also been studied in patients suffering from metabolic diseases closely linked to obesity. A meta-analysis of twenty-six randomised controlled trials showed that the consumption of dietary prebiotic supplements, between 2 days and 28 weeks, reduced postprandial glucose and insulin concentration in healthy and overweight individuals (Kellow, Coughlan and Reid, 2014). Recently, the supplementation of corn bran arabinoxylans at 25-35 g/day in overweight volunteers (n= 38) showed that self-reported flatulence, bloating and stomach aches increased during the first three weeks but were reverted to baseline during the weeks four to six. This adaptation varied between individuals and was related to their baseline diet . These effects correlated with the relative abundance of *B. longum* at baseline and shift in acetate production after arabinoxylan fermentation (Deehan *et al.*, 2024).

The benefits of FOS and GOS on human health have been extensively reviewed, and shown to restore intestinal bacterial balance, enhance bioavailability of minerals, lower risk factors of cardiovascular disease and cancer, and prevent obesity (Nobre *et al.*, 2022; Wang *et al.*, 2024b). In addition, a recent study showed that a 14.2 g/day GOS and FOS supplementation in women from less than 21 weeks gestation to six months post-partum (n=74 mother-infant pairs) resulted in significant differences in faecal microbiota profiles in both maternal and infant samples. There was an increased relative abundance of *Bifidobacteria* in women at 28 and 36 weeks of gestation while

12-month old infants showed increased abundance of *Peptostreptococcaceae*, *Acetatifactor*, *Clostridium*, and *Romboutsia* (Jones *et al.*, 2024). Moreover, these prebiotics have been shown to help with the prevention and management of intestinal conditions such as IBS or IBD. For example, supplementation with 3.5 or 7 g/day of GOS relieved symptoms of flatulence, abdominal pain and discomfort in patients with IBS (n=44) and increased *Bifidobacterium* spp. in their faeces (Silk *et al.*, 2009). Prebiotics have also been tested *in vitro* for their effects in addressing microbial dysbiosis in patients. For example, *in vitro* human colonic fermentation of FOS and SCFA esters using samples from patients with UC, characterised by a high abundance of *R. gnavus, Escherichia, Shigella, Klebsiella* and other potential pathogens, showed that the addition of butyrylated and propionylated FOS induced the proliferation of *Bifidobacteria* and inhibition of *Clostridium* (Chen *et al.*, 2023).

There is also recent evidence that prebiotics can improve iron absorption in adults and infants, as recently reviewed (Husmann, Zimmermann and Herter-Aeberli, 2022). A recent study showed that daily administration of infant cereal formula supplemented with GOS and FOS (7.5 g) for three weeks was associated with improved iron absorption on infants with iron-deficient anaemia (n=191). After the intervention, the proportion of iron absorbed from meals containing the prebiotics was 60% higher than from the meals without prebiotics used before intervention. Additionally, there was an increase in Lactobacillus abundance in prebiotic-iron supplemented groups, along with a reduction in Enterobacteriaceae and pathogens (Mikulic *et al.*, 2024).

Lately, a few studies explored the influence of prebiotics on mood, neurobiology and behaviour, but these led to inconclusive results (Kao, Harty and Burnet, 2016; Johnstone *et al.*, 2021; Freijy *et al.*, 2023).

New emerging prebiotics such as HMOs are manufactured on a large scale and added to infant formula milk (Barile and Rastall, 2013). A systematic review including 26 clinical trials, where HMOs were tested as individual structures or as blends, showed beneficial gut health and immune system effects in response to HMO supplementation (Schönknecht *et al.*, 2023). For example, patients with IBS (n=58) consuming a daily dose of 10 g of a mix with 2`-fucosyllactose (2'-FL) and lacto-N-neotetraose (LNnT) (in 4:1 proportion) showed a 50% increase in Bifidobacteria after four weeks (Iribarren *et al.*, 2020). Moreover, HMOs have been found to help reduce some of the symptoms of IBS; 5 g/day of the same HMO mix (4:1 2'FL and LNnT) led to a reported decline in the severity and duration of abdominal pain and bloating over 12 weeks in adults with IBS (n=317) (Palsson *et al.*, 2020). This recent evidence suggests HMOs as potential therapeutic options for IBS, as recently reviewed (Sanz Morales *et al.*, 2022).

Candidate prebiotic RFOs such as raffinose and verbascose are being considered as novel foods for their beneficial effects (Pacifici *et al.*, 2017; Cardoso *et al.*, 2021), as further described in section 1.4.2.

Based on these studies, it is clear that the type of prebiotic, the dose administered, and administration length, all play a critical role in influencing health outcome as reviewed for GI diseases (Davani-Davari *et al.*, 2019). Moreover, inter individual differences in bacterial species could explain responders and non-responders within

studies (Ojima *et al.*, 2022), and bacterial cross-feeding must also be considered (Culp and Goodman, 2023). These considerations led to a shift towards personalised nutrition or precision nutrition, where individual microbiome characteristics and associated metabolic phenotypes could be matched more optimally with specific prebiotics (Cunningham *et al.*, 2021). A significant portion of the work to date has focused on the role of purified prebiotics provided as supplements, but there is also the potential to develop novel food products to increase fibre consumption.

1.4 Raffinose Family Oligosaccharides (RFOs)

1.4.1 Biosynthesis and accumulation of RFOs in plants

RFOs are a group of sugars consisting of three oligosaccharides, raffinose, stachyose and verbascose (Fig. 11 A), which are found in plants and particularly abundant in legumes such as pea, but also present in whole grains, some cruciferous vegetables and fruits (Ahnen *et al.*, 2020). Raffinose is a trisaccharide formed by a galactosyl extension of sucrose, and further galactosyl extensions lead to the tetrasaccharide stachyose and the pentasaccharide verbascose (Fig.11 B).

In plants, RFOs can be synthesised via two biosynthetic pathways, the galactinol-dependent pathway and the galactinol-independent pathway. The most common one is the galactinol-dependent pathway, which is well-characterised in legumes (Fig. 11 B). Pea utilises the galactinol-dependent pathway, where galactinol is produced from myo-inositol by the galactinol synthase enzyme (GolS) (Liu *et al.*, 2024). Next, galactinol and sucrose act as the precursors of RFOs, where they are converted by the enzyme raffinose synthase (International Union of Biochemistry and Molecular Biology enzyme nomenclature: enzyme commission, EC 2.4.1.82) (Lehle and Tanner, 1973) into raffinose; this enzyme will herein be referred to as Rfs. The biosynthesis of RFOs is controlled by the levels of initial substrates, particularly myo-inositol followed by sucrose and galactinol (Karner *et al.*, 2004). Sucrose is the first galactosyl residue acceptor during synthesis of raffinose (Peterbauer *et al.*, 2002b). Moreover, sucrose acts as a source of uridine diphosphate (UDP)-glucose, which becomes epimerised to UDP-galactose; the latter is used for the synthesis of galactinol, the donor of galactosyl residues in the RFO biosynthetic pathway (Peterbauer and Richter, 2001).

In pea, Rfs, belonging to GH36 family, is the key enzyme in the biosynthesis of raffinose from sucrose and has been demonstrated to act as a transglycosidase at pH 7.0 (Peterbauer *et al.*, 2002a). The hydrolytic activity of Rfs is suggested to be inhibited by the sucrose present in plant tissues (Peterbauer *et al.*, 2002a). In pea, raffinose chain elongation to stachyose and verbascose is carried out by a multifunctional stachyose synthase (Peterbauer *et al.*, 2002b; Vahdat *et al.*, 2020). Moreover, in some crops such as soybean, Rfs isoforms have been characterised. For example, in soybean Rfs isoforms 2 (RS2) and 3 (RS3) are responsible for raffinose synthesis and mutations in genes encoding these isoforms were found associated with increased sucrose and decreased RFO levels in seeds (Bilyeu and Wiebold, 2016; Hagely *et al.*, 2020).

The galactinol-independent pathway is less well studied and has only been described in *Ajuga reptans* and *Coleus blumei* (non-legumes) (Bachmann, Matile and Keller,

1994; Gilbert, Wilson and Madore, 1997). Moreover, this pathway has only been reported to occur in leaves, and hypothesised to be absent in seeds (Yan *et al.*, 2022).

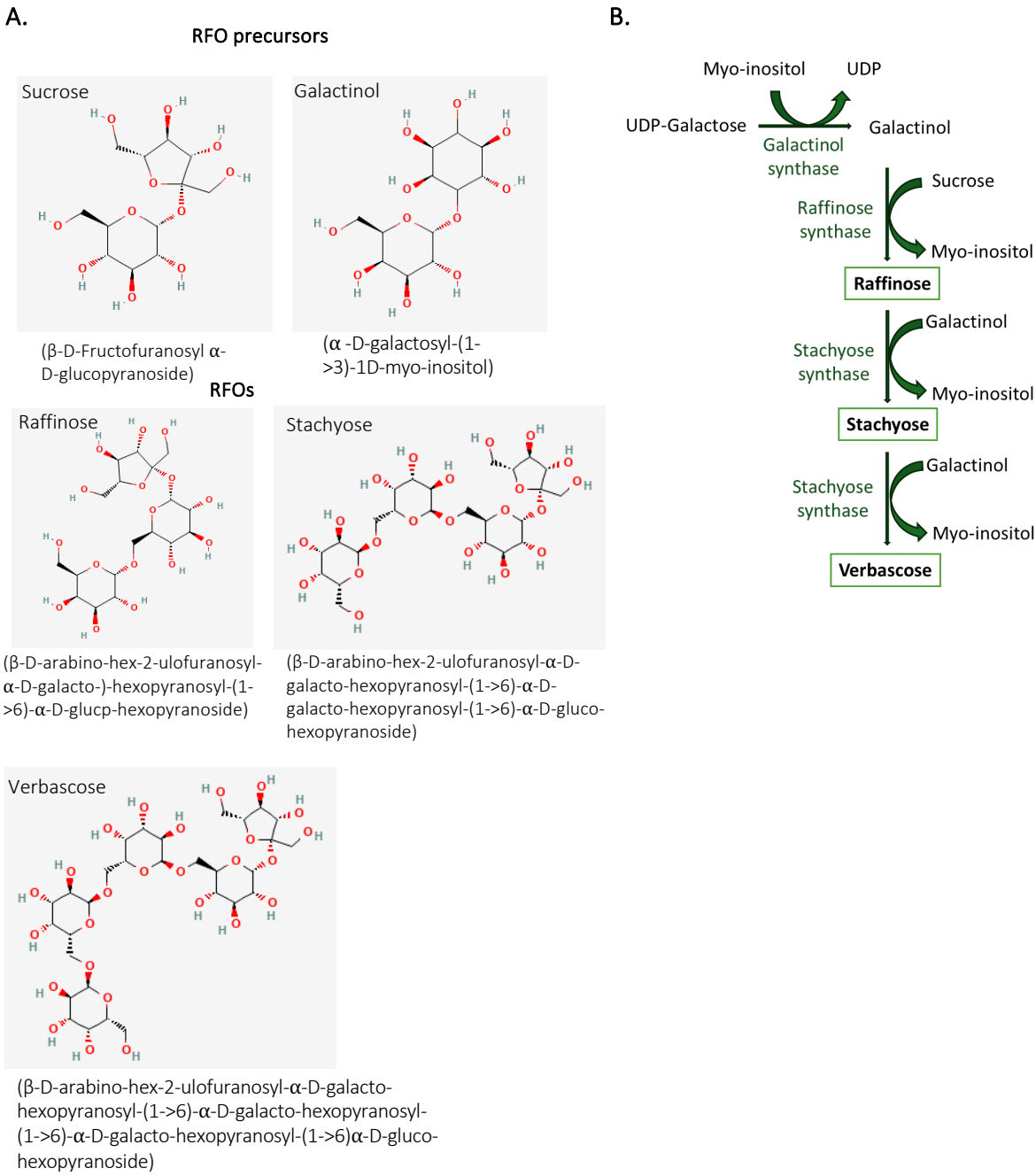


Figure 11. Chemical structures of RFO precursors (sucrose and galactinol) and RFOs (raffinose, stachyose and verbascose), with detail of the galactinol-dependent biosynthesis pathway. (**A**). Chemical structures of RFO precursors (sucrose and galactinol), and RFOs (raffinose, stachyose and verbascose) (Varki et al., 2015; Neelamegham et al., 2019). Structures retrieved from PubChem (CIDs: 11727586, 5988, 439242, 439531, 441434) (Kim et al., 2022). (**B**). Galactinol-dependent RFO biosynthetic pathway. Pathway adapted from Elango et al (2022).

The accumulation of sucrose occurs during the early stages of seed development, as shown in soybean (Kambhampati *et al.*, 2021), whilst RFOs usually accumulate in the

late stages during physiological maturation and the phase of seed drying (Obendorf *et al.*, 2009). Although RFOs are mainly stored in the seeds of legumes (Salvi, Varshney and Majee, 2022), they can be present in various plant tissues, such as roots, tubers, leaves and stems (Hannah *et al.*, 2006; Chibbar and Båga, 2003).However, the mechanisms by which RFOs accumulate in the seeds are less understood.
RFOs are present in variable concentrations in the seeds of legumes depending on the crop, as determined in various cultivars in Poland (Table 2). Faba bean and soybean showed the lowest total RFO content, stachyose was most abundant in soybean and lupin while verbascose was the most abundant in pea and faba bean (Kasprowicz-Potocka, Gulewicz and Zaworska-Zakrzewska, 2022).

Table 2. Total content of RFO in legumes.

Crop	Cultivars (n)	Raffinose	Stachyose	Verbascose
			(mg/g)	
Soybean	27	8.8 ± 1.13	40 ± 7.94	1.7 ± 0.51
Lupin (white, yellow and narrow-leaved)	41	9.5 ± 1.25	48.5 ± 8.22	26.8 ± 9.38
Pea	25	8.9 ± 1.42	27.7 ± 5.58	29.0 ± 5.06
Faba bean	6	2.5 ± 0.22	9.2 ± 0.67	38.5 ± 2.58

The data are expressed as mean ± standard deviation. Data are extracted from Kasprowicz-Potocka e*t al.* (2022).

1.4.2 Role of RFOs in plants

To understand the roles of RFOs and carbohydrates in the physiology of the plant, it is important to understand the source-sink interaction that follows carbon fixation from photosynthesis (Rosado-Souza *et al.*, 2023). This source-sink term is used to describe the interaction between a source tissue, defined as a net producer and a sink tissue, the net importer of photosynthetic product (Ho, 1988; Rosado-Souza *et al.*, 2023). Therefore, source-sink interactions comprise the carbon metabolism network, where the integration of sucrose and trehalose pathways act in the regulation of plant growth and signalling in addition to balancing energy provision and utilisation throughout the plant (Griffiths, Paul and Foyer, 2016).

The RFO precursor sucrose plays a role in glycolysis, or carbon and energy supply for plant growth and development where it is mobilised through the plant phloem (Lemoine *et al.*, 2013). Phloem unloading is a process by which the nutrients in the plant, including sugar, and water move to sink parenchymal tissue, where sugar acts as a signal molecule (Braun, 2022). In addition to sucrose, in some plants such as cucumber, RFOs can act as main transport compounds transported through the phloem (Hu *et al.*, 2011). Mutations in genes that encode proteins involved in RFO precursor biosynthesis can affect RFO accumulation. Here it is important to note that triose-phosphates, the exportable products from the Calvin cycle, are used for two

major products of photosynthesis: starch and sucrose (Raghavendra, Sunil and Bapatla, 2017). As a result, alterations in the synthesis of triose-phosphates are associated with changes in the ratio of accumulation of these two major products in the plant, resulting in some level of dependency between starch and RFO biosynthesis. For example, a decrease in the accumulation of pea starch in *r* and *rb* mutants (so-called because the mutant genes confer wrinkled or `rugosus` appearance to seeds) (Wang and Hedley, 1991) led to a higher concentration of sucrose in cotyledon tissues and, in turn, resulted in accumulation of higher amounts of RFOs (Gawłowska *et al.*, 2017). On the other hand, for example, CRISPR-Cas9 knockout of galactinol synthase led to a reduction of RFOs in soybean seeds (Le *et al.*, 2020).

RFOs have been reported to play a role in the protection of plants from drought (Sharma *et al.*, 2014), freezing or cold-triggered reactions (Bachmann, Matile and Keller, 1994; Gu *et al.*, 2018), indicating a general role in stress-induced changes in plants. For example, in maize, there is a down-regulation of starch and sucrose biosynthesis and up-regulation of their degradation in drought-stressed ovaries, although these changes do not occur to the same extent in stressed meristems, indicating organ-specific responses of two carbohydrate pathways associated with abiotic stress responses in plants (Kakumanu *et al.*, 2012). Moreover, in maize, these tissue-specific drought-stress responses and their regulators have been shown to be highly correlated with developmental and physiological adaptation of the plant (Wang *et al.*, 2019a).

Dehydration in pea seedlings led to increased *Rfs* expression, indicating a role for raffinose in dehydration stress conditions (Lahuta *et al.*, 2014). More recently, raffinose was identified as one of the metabolic markers of stress response under soil drought conditions in pea (Lahuta, Szablińska-Piernik and Horbowicz, 2022). Following a 5-day irrigation interruption period resulting in a reduction in the plant water content, pea showed an inhibition of shoot growth and an increase in raffinose accumulation, while raffinose levels decreased after rewatering (Lahuta, Szablińska-Piernik and Horbowicz, 2022). This defence reaction during osmotic disturbance was found to be largely dependent on the adaptation of the root system, including symbiosis with rhizobia (Lahuta, Szablińska-Piernik and Horbowicz, 2022). Moreover, in chickpeas, increased levels of sugars and sugar alcohols, such as inositol, were shown to aid osmoregulation during drought conditions (Amede, Schubert and Stahr, 2003).

RFOs have also been implicated in the response to other environmental stressors. For example, a study on potato showed that under osmotic stress caused by salt treatment, the genes encoding GolS and Rfs were upregulated (Jing *et al.*, 2023), in line with previous findings showing that osmolytes, including raffinose (Slama *et al.*, 2015), commonly accumulate in soybean and cucumber in response to salt stress (Gul *et al.*, 2022). Additionally, RFOs have emerged as compounds that can ameliorate seed vigour and plant longevity, as shown in Arabidopsis (Gangl and Tenhaken, 2016).

The possible effects of environmental conditions on RFO accumulation have been studied in soybean (Dierking and Bilyeu, 2009), where two major mutants of RS2, the

main Rfs isoform in soybean (Dierking and Bilyeu, 2009), characterised by reduced RFO levels, showed relatively low changes in RFO levels across plants grown in different environments (Valentine *et al.*, 2017), suggesting that not all isoforms are involved in responses to environmental conditions (Dierking and Bilyeu, 2009). In addition to RS2, two other Rfs isoforms have been identified in soybean, namely RS3 and RS4, however, their roles in RFO synthesis remains to be elucidated (Maeda, Toda and Kaga, 2024).

Another environmental stress where RFOs could play a role is frost tolerance. A study in pea, using a quantitative trait loci (QTL) approach to identify chromosomal regions linked to frost tolerance, found that a raffinose gene locus colocalised with a QTL for frost damage (Dumont *et al.*, 2009).

Although the role of RFOs in legumes has been associated to environmental stressors, the direct protective role of RFOs in pea remains to be investigated, for example through knockouts of RFO biosynthesis pathway genes. This approach will provide valuable insights into the metabolic pathways that influence seed quality, stress responses and overall plant health, and will ultimately contribute to advancements in agricultural practices and crop improvement. In addition, due to the high nutritional value and significance of pea as a low carbon food crop, it is important to understand the effects of RFOs in the context of sustainable agriculture (Powers and Thavarajah, 2019) and climate change, for example for the development of drought resistant crops (Kumar *et al.*, 2019).

1.4.3 Effect of RFOs on animal and human health

Historically, RFOs have been associated with anti-nutrient properties (Vidal-Valverde *et al.*, 2003; De Angelis *et al.*, 2021) due to their resistance to digestion by human enzymes (Peterbauer *et al.*, 2001) and their role in promoting flatulence (Martínez-Villaluenga, Frias and Vidal-Valverde, 2008). Moreover, the reduction of RFOs (and associated increase in sucrose) from soybean in animal feed, has been shown to increase digestibility and metabolisable energy. Therefore, it has been suggested that eliminating seed RFOs through plant breeding, using directed molecular selection for alleles responsible for RFO accumulation, could improve the nutritional content of pea by improving the metabolisable energy (Neus, Fehr and Schnebly, 2005).

However, recent studies have shown that RFOs have prebiotic potential by promoting the growth of beneficial bacteria and reducing pathogen burden in the large intestine (Elango *et al.*, 2022).
An *in vitro* study using RFOs from guar bean (*Cyamopsis tetragonoloba*) showed that raffinose and stachyose were metabolised by 75% of the species used in monoculture (namely *Lactobacili, Bifidobacteria, Bactococcus lactis, Streptococcus salivarius* ssp*. thermophilus, Pediococcus* and *Enterococcus faecium*), with limited verbascose fermentation, while *L. reuteri*, *Pseudomonas pentosaceus* and *Bacillus lactis* HNO19 ™ were able to ferment raffinose, stachyose and verbascose (Zartl *et al.*, 2018). Another study, using faecal samples from two healthy adult donors, showed that both lactulose and raffinose (10 g/L) resulted in increased relative abundance of well-known beneficial bacteria species, namely *Bifidobacteria* and *Lactobacillus* and reduced

significantly the relative amount of *Proteobacteria* (Amorim *et al.*, 2020). This contrasts with an earlier *in vitro* colon fermentation showing that 5 g/L raffinose supplementation resulted in an increase of the *Proteobacteria* population in a pooled faecal sample from healthy adults (Adamberg *et al.*, 2018). Interestingly, a study on patients with CD, suggested that the accumulation of less complex sugars such as raffinose may contribute to dysbiosis and inflamed gut (Zeng, Inohara and Nuñez, 2017).

Moreover, a 3-week randomised crossover intervention study where 12 healthy adults were given diets supplemented with either 200 g/day of canned chickpeas or 5 g/day of individual raffinose showed a marginal increase in Bifidobacterium with the raffinose diet and a reduced number of the putrefactive and pathogenic bacteria *Clostridium histolyticum* and *C. lituseburense* with the chickpea diet, compared to the control, showing the potential of raffinose to modulate the intestinal microbial composition in healthy humans (Fernando *et al.*, 2010).

In addition to their prebiotic potential, RFOs have been shown *in vitro* to have anti-allergic, anti-obesity- anti-diabetic properties in human cells (Muthukumaran *et al.*, 2018; Borges *et al.*, 2019) and RFO supplementation led to improved gut histomorphology and duodenum structure as well as resistance to opportunistic pathogens in animals (Stadnicka *et al.*, 2020).

Taken together, these studies reveal the complex interactions between RFOs and the host. Therefore, achieving the right balance of RFO content may be the way forward to promote human health (Elango *et al.*, 2022).

1.5 Aim and objectives

The overall aim of this work is to investigate the role of RFOs in plants and their influence on the structure and function of the human gut microbiota and gut health, through the study of novel pea mutants with altered RFO content.
Specific objectives are:

i. To characterise the RFO content on pea seeds with mutations in the *raffinose synthase* and putative *raffinose synthase* gene.
ii. To evaluate the performance of pea mutants with reduced RFO content in the field and under glasshouse conditions.
iii. To determine the impact of control and mutant peas on the structure and function of the gut microbiota through metabolomics, metagenomic analyses and gas production.
iv. To investigate the effect of fermented pea samples on gut barrier function using human colonic organoid-based models.

2 . Chapter Two: Materials and methods

2.1 Plant-based assays

2.1.1 Pea seeds used in this study

The commercial cultivar of pea, Caméor, used in this work is available from the John Innes *Pisum* Germplasm collection as accession JI3253 at the John Innes Centre (JIC, Norwich Research Park, Norwich, UK; https://www.seedstor.ac.uk/).

The mutants used in this study were selected primarily based on the availability of seed material, which determined their inclusion in the experimental work. While this practical constraint shaped the experimental design, the selected lines provided a suitable basis for evaluating responses under various environmental and treatment conditions. The critical mutants used in the various experiments were as follows:

- OCFN 1551: Used in the first-year field trial (Chapter 3).
- BCFN 1551: Used in the second-year field trial, water deficit experiment in the glasshouse, herbicide trials (both field and glasshouse), batch fermentation experiments (Chapter 4), and gas production experiments (Chapter 5.
- BC2FN 1551: Used in the third-year field trial and *Rhizobium* nodulation experiment (Chapter 3).
- TILLRfs W521*: Used in the gas production experiment (Chapter 5).
- BCTILLRfs W521*: Used in the *Rhizobium* nodulation experiment (Chapter 3) and SHIME® dynamic fermentation (Chapter 5).

The genetic origin and development of these mutants are described as follows in Section 2.1.1.1.

2.1.1.1 Mutant origin

2.1.1.1.1 Fast Neutron mutagenesis

The *rfs* mutant pea used in this work, FN 1551/11, is derived from a fast-neutron population generated in the accession, JI2822 (Hofer *et al.*, 2009). The FN1551/11 mutant was one of two identified by Carol Moreau (unpublished data, Domoney group, JIC) as lacking a major *Rfs* gene. To remove other background mutations, FN1551/11 was backcrossed to JI2822 (the progenitor line of the fast neutron population) twice by Carol Moreau (unpublished data, Domoney group, JIC) to generate FN1551-BC2. During the backcross, plants carrying the *Apulvinic* locus associated with a pulvinus phenotype were selected. The FN1551-BC2 was crossed to Caméor by Gabriel Robinson and will be referred here as mutant OCFN 1551 (OC: outcross).

2.1.1.1.2 TILLING mutagenesis

Seeds from a mutant population, previously generated through using ethyl methane sulfonate (EMS) mutagenesis in Caméor (Dalmais *et al.*, 2008), were screened for mutations in *Rfs* and a putative *Rfs*; the latter selected by Andy Chen (unpublished

data, Domoney group, JIC), from gene expression data (Alves-Carvalho *et al.*, 2015) and is referred to onwards as a *Rfs*-like gene. The mutant pea seeds were obtained from INRAE (Institute of Plant Sciences Paris Saclay, France) (Table 3). A total of 4-6 seeds were received for each TILLING mutation.
Commercial cultivar seeds of *P. sativum* Caméor were used as controls, as the background in this line is closest the background to where the backcrossed mutants originated.

Table 3. TILLING mutants in *Rfs* and *Rfs*-like used in this study, including the nature of the mutation induced and the resulting amino acid change.

Gene	Families	Nature	AA change	Mutant name
Rfs	2891	NONSENSE	W530*	TILLRfs W530*
Rfs	3281	MISSENSE	G464S	TILLRfs G464S
Rfs	3392	MISSENSE	G129E	TILLRfs G129E
Rfs	3463	MISSENSE	G509D	TILLRfs G509D
Rfs	3482	MISSENSE	H543Y	TILLRfs H543Y
Rfs	3929	MISSENSE	W521*	TILLRfs W521*
Rfs	4615	MISSENSE	G479D	TILLRfs G479D
Rfs	4641	MISSENSE	A544V	TILLRfs A544V
Rfs-like	2811	NONSENSE	W339*	TILLRfs-like W339*
Rfs-like	2836	MISSENSE	P146L	TILLRfs-like P146L
Rfs-like	2885	MISSENSE	S397F	TILLRfs-like S397F
Rfs-like	3203	MISSENSE	G252D	TILLRfs-like G252D
Rfs-like	3411	MISSENSE	G341E	TILLRfs-like G341E
Rfs-like	3794	MISSENSE	E118K	TILLRfs-like E118K
Rfs-like	3904	MISSENSE	G280R	TILLRfs-like G280R
Rfs-like	3971	NONSENSE	W265*	TILLRfs-like W265*
Rfs-like	4023	MISSENSE	D231N	TILLRfs-like D231N
Rfs-like	4080	MISSENSE	G150S	TILLRfs-like G150S

The nature of the mutation induced and resulting amino acid (AA) change is included. Families refers in every case to the mutant line number in INRAE resources. *STOP codon.

2.1.1.2 Mutant identification

2.1.1.2.1 Pea growth conditions

Plants of the mutant lines and the commercial cultivar Caméor used as a control were grown in the JIC glasshouse facilities, using a compost mix called John Innes cereal mix (65% peat, 25 % loam, 10% grit, 3kg/m^3 dolomic limestone, 1.3 kg/m^3 PG mix, 3 kg/m^3 osmocote exact fertiliser), with the temperature set to 18 °C during the day and 12 °C at night, and lighting to provide a 16 h photoperiod.

2.1.1.2.2 DNA extraction and quantification from plant leaflets

Plant leaflets, consisting of one leaf-like part of a compound leaf morphology (see Fig. 12) (Constance, 1955), from 2 to 3-week-old plants were collected for the isolation of DNA. Briefly, DNA was extracted from leaflet tissue from each plant using the QIAmp 96 DNA QIAcube HT KIT (QIAGEN, UK) on the Qiacube HT machine (QIAGEN, UK), according to the suppliers' instructions at JIC genotyping facility. DNA was quantified using a NanoDrop 1000 UV-Vis spectrophotometer (Thermo Fischer Scientific, Wilmington, DE).

2.1.1.2.3 Genotyping

Genomic DNA from the pea plant leaflets was genotyped using two distinct assays. Gene copy number quantification was used for the genotyping of OCFN 1551, BCFN 1551 and BC2FN 1551 -derived mutants. Identification of single nucleotide polymorphisms (SNPs) was conducted for the genotyping of the TILLING mutants.

2.1.1.2.2.1 Gene copy number quantification

The determination of gene copy numbers was carried out by quantitative polymerase chain reaction (qPCR)-based assay (TaqMan) using the primers and fluorescent probes listed in Table 4. The TaqMan probes used to detect *Rfs* gene and the *Agps2* housekeeping gene, were dually labelled with a reporter dye (Fam or Vic) and a quencher dye (Tamra). The reactions contained: 5 µL TaKaRa Ex Taq® polymerase (TaKaRa, Seoul, Korea), 1 µL of 10 mM forward and reverse primer mix, 3 µL of water and either 1 µL of DNA template or water for the non-template control. The PCR conditions used were: one cycle at 95 °C for 15 min, followed by 39 cycles of 95 °C for 15 s, 60 °C for 1 min. The plate was read using a Bio-Rad CFX96 qPCR (BioRad, UK) set to read FAM™ (fluorescence emission 517 nm) and VIC™ (fluorescence emission 551 nm) fluorochromes.

The gene copy number was then calculated using Δ cycle threshold (Ct) (ΔCt = PeaRFSP2 Ct - PeaAgps2TmP Ct) and samples sorted in a scatter plot, where a homozygous wild-type had two copies of the *Rfs* gene, hemizygous segregants had just one copy of the gene and homozygous nulls only showed amplification of the Agps2 internal standard only.

Table 4. Primers and probes used to determine *Rfs* for gene copy number genotyping.

Probes		Sequence
PeaRFSP2		Fam-CGAGCATATTCCGGTTCAAAGTTTGGTG-Tamra
PeaAgps2TmP		Vic-AGCGTGCAACTGCTTTCGGTTTGATG-Tamra
Gene	**Primer**	**Sequence**
Pea*Agps2*Tm	Forward	GCTGCGTTGCCAATGGA
	Reverse	ACTCRATTATACGCCCCTCTTCAT
Rfs	Forward	CTCGTCTCTGACTACGTCATCTACC
	Reverse	TGCTGAGATGGAGGTAAAGACAGGC

2.1.1.2.2.2 SNP Identification of TILLING mutants

For SNP identification, DNA was amplified by PCR using *Rfs* and *Rfs*-like specific primers (Table 5). Amplification was performed in 10 µL reactions containing: 5 µL HotStart Taq polymerase (QIAGEN, UK), 0.2 µL 5 µM forward and 0.2 µL 5 µM reverse primers, 1 µL genomic DNA and 3.6 µL water. The PCR conditions were: one cycle at 95 °C for 15 min, followed by 39 cycles of 94 °C for 30 s, 62 °C for 30 s and 72 °C for 1 min each, and finally one cycle of 15 min at 72 °C. The PCR products were analysed using a 2 % agarose gel and quantified using a NanoDrop 1000 UV-Vis Spectrophotometer (Thermo Fischer Scientific, Wilmington, DE).

The mutations were confirmed by Sanger sequencing of the corresponding genomic amplicons from the PCR products. Briefly, in a Eurofins Mix2Seq Kit tube (Eurofins Genomics, Ebensburg, Germany), the PCR products (5 µL of 1ng/µL) were mixed with with 5 µL of forward or reverse primer at 5 µM forward or reverse primers listed in Table 5 in 10 µL final reaction volume. Sequencing was carried out at (Eurofins Genomics, Ebensburg, Germany) . Following identification of the TILLING mutation based on the genotyping of the SNP using the Chromas software (Technelysium, Australia), the mutants were classified as homozygous mutant, homozygous wild-type or heterozygous.

Table 5. Primers used to target *Rfs* and *Rfs*-like genes for SNP genotyping.

Gene	Primer	Sequence
Rfs Pair 1	Forward	CTCGTCTCTGACTACGTCATCTACC
	Reverse	TGCTGAGATGGAGGTAAAGACAGGC
Rfs Pair 2	Forward	GTCGTCTTGTACAACAAACAATAGC
	Reverse	AGTTGAATAGACCCAAAACACCAGC
Rfs-like	Forward	GCTTCAAAGCCACCGATTCG
	Reverse	ACTTTGACCCCATCAATTCC

Pair 1 was used to identify the SNP in mutant TILLRfs G129E, Pair 2 was used to identify all other *Rfs* TILLING mutants.

2.1.1.2.4 Crossing programme to derive backcrossed seeds

To reduce the effects of additional background mutations on the effect of *Rfs* on RFO content, the *Rfs* mutant OCFN 1551 and the TILLRfs W521* were selected for crossing to the commercial cultivar Caméor. The OCFN 1551 previously generated by the Domoney group (see 2.1.1.1.1.) was backcrossed to Caméor giving rise to BCFN 1551 and a further backcross called BC2FN 1551. The mutants were selected by genotyping and inspected visually according to the apulvinic phenotype (which has altered leaflet attachment) arising from the closely linked gene also deleted in the mutant. In Fig. 10, a schematic representation of the crossing process and mutant selection is described, where homozygous mutants (*rfs/rfs*), BCFN 1551 and BC2FN 1551, are highlighted in the yellow boxes. The same process was followed to obtain the BCTILLRfs

W521*mutant, however, in this case, there was no visual confirmation of the mutation.

Briefly, the first cross was conducted by taking pollen from a homozygous mutant plant (OCFN 1551 or TILLRfs W521*) and brushing it onto the stigma of a Caméor flower which had emasculated anthers. The seeds in the pod which developed from the cross-pollinated flower were sown to produce F1 hybrid plants. Heterozygous F1 plants, containing the mutant (*rfs*) and the wild-type gene (*Rfs*) were identified as *Rfs/rfs* F1. These plants were allowed to self-pollinate and produce F2 progeny in order to obtain a segregating population. The F2 seeds were sown and genotyped and plants which were homozygous for the *rfs* mutation (in the yellow boxes) were used for seed multiplication.

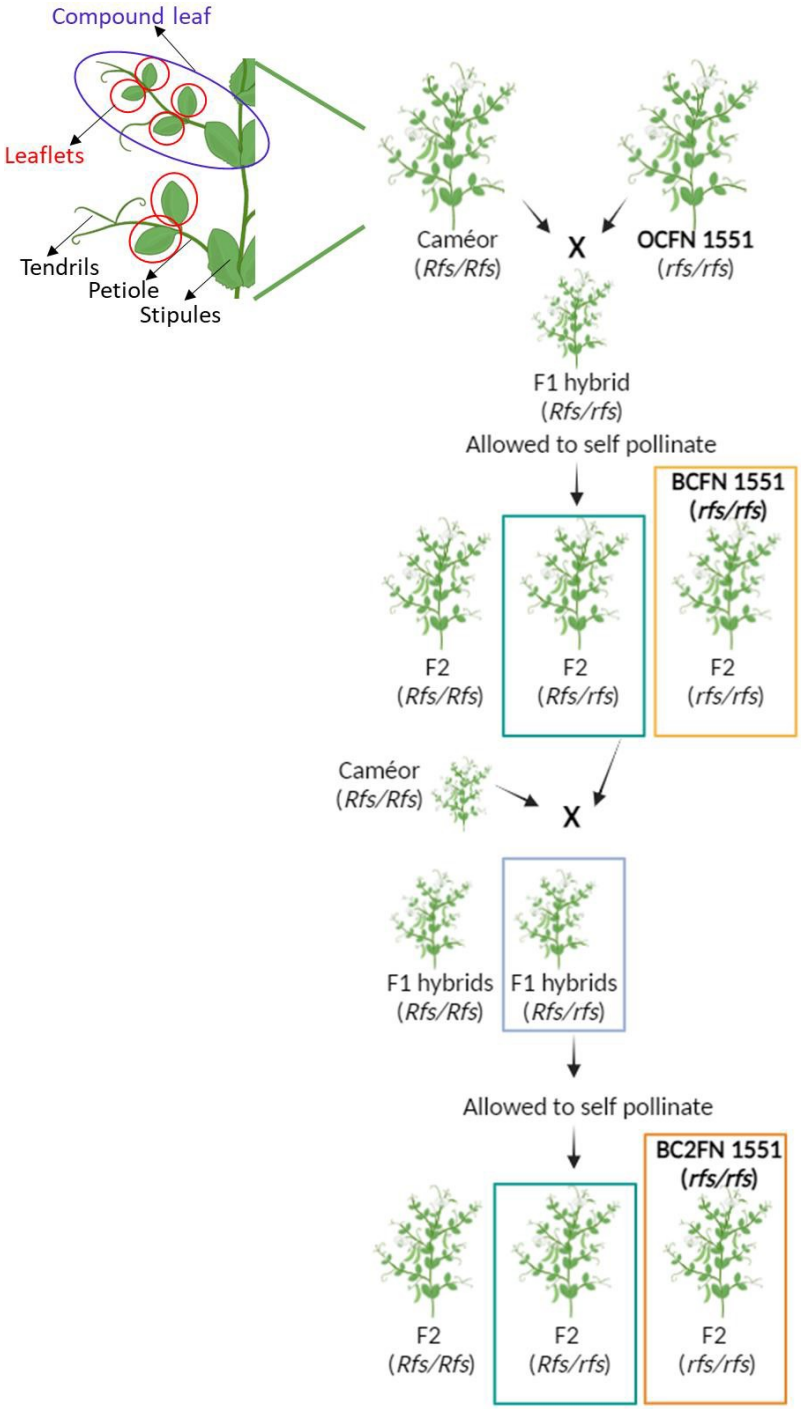


Figure 12. Schematic showing introgression of a donor locus (*rfs/rfs)* into the commercial cultivar Caméor using backcrossing, and close-up of pea plant compound leaf morphology. The genotypes are denoted in brackets for the two alleles of Rfs. X symbolises the crossing between plants with different genotypes. The green box shows the genotype selected for further crossing to Caméor, the yellow box represents the backcrossed mutant plants. Red circles highlight the leaflets. Created in BioRender scientific illustrator.

2.1.2 Field trial

2.1.2.1 Experimental plots

The study was conducted at the Dorothea de Winton Field Station, located at Church Farm, Bawburgh, NR9 3PY (Norwich, UK).
The plot (experimental unit) area was 1 m^2 with 10 rows of seeds (100 seeds per plot). Pre-emergence herbicides Nirvana™ (Pendimethalin+Imazamox) mixed with Centium™ were applied by spraying after seed sowing, at a concentration of 4.0 L per hectare and 0.25 L per hectare, respectively.

2.1.2.2 Plot harvest

In 2022, the seeds were sown on April 8th, 2022, and pods were harvested in August 2022.
In 2023, the seeds were sown on April 10th and pods were harvested in July 2023.
In 2024, the seeds were sown on the 26th of March and the pods were harvested in July 2024.

2.1.2.2.1 Description of traits observed in seeds

The total seed yield per plot was determined by counting total seeds with an automated seed counter (Contador, Pfeuffer, Germany), using the feed container number three, designed for peas.

The average weight per seed was determined by weighing 100 intact seeds (in triplicate) per plot.

The proportion of insect-damaged seeds was determined by first separating visually the damaged seeds from the rest of the seeds and counting them individually by hand. Seeds were considered to be damaged when holes from insect larvae were identified in the seeds (Supplementary Fig. S1). The proportion of damaged seeds was calculated relative to the total number of seeds per plot.

2.1.3 Controlled water deficit in the glasshouse

The Caméor cultivar and BCFN 1551 pea lines were grown in the JIC glasshouse using a compost mix called John Innes cereal mix (65% peat, 25 % loam, 10% grit, 3kg/m^3 dolomic limestone, 1.3 kg/m^3 PG mix, 3 kg/m^3 osmocote exact fertiliser).
During the first 26 days post sowing (dps), plants were watered automatically in the glasshouse, by bench flooding daily. On 27 dps, plants for each genotype were split into two groups (n=25): the plants that were bench-watered throughout the experiment (hereafter referred to as controls) and the water-deficit (WD) plants subjected to a drought period (no water) for 9 days.
After the water-deficit period, the WD plants were rewatered, first for 15 days by the addition of 20 mL of water daily (recovery watering) and then returned to daily automated bench flooding watering (Fig. 13).
Watering was the only condition tested, whereas light, temperature and other environmental conditions remain constant, and all plants were held on the same bench space in the glasshouse.

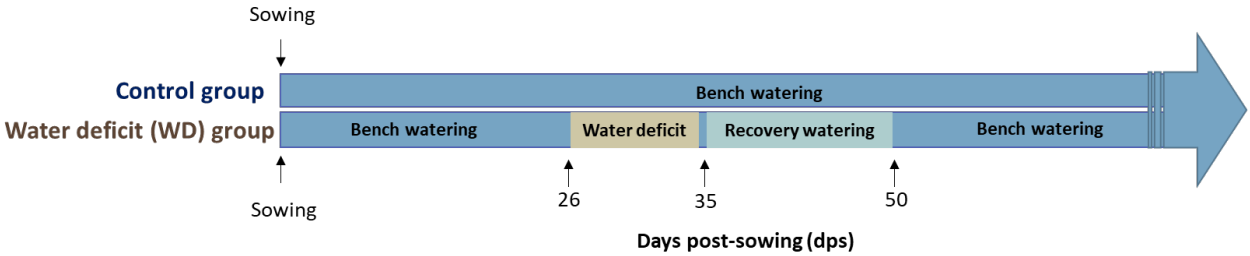


Figure 13. Experimental design used to study plant responses to water deficit (WD). Caméor and BCFN 1551 seeds were sown in compost in the glasshouse. Control groups were bench-watered throughout (n=25, per genotype). Time is expressed in days-post sowing (dps). Water deficit (WD) was imposed during the vegetative stage for 9 days, starting at 27 dps followed by a 16 day-controlled re-watering period, and final return to bench watering (n=25, per genotype).

The plant development, flowering and pod formation were recorded. After harvesting, the number of seeds per pod, the total seed yield and mean seed weight were determined for each treatment condition and genotype as described above (2.1.2.2.1.).

2.1.4 Pre-emergence herbicide trials

Two pre-emergence herbicide trials were conducted. The first trial was conducted in the JIC glasshouse. Caméor and OCFN 1551 seeds were sown a day prior to herbicide application, either in JIC cereal mix compost or using soil imported from the field. Three seeds of each genotype were sown in pots of 13 cm diameter.
A second trial was conducted in the field in 2023. Ten seeds from BCFN 1551 and Caméor, each, were used for each herbicide condition sown in the field.
For both trials, the herbicides applied were Nirvana™ and Centium™, which are routinely used in combination in the field (see section 2.1.2.1). The effect of herbicides on germination was addressed at three weeks post sowing, where the number of plants that had germinated per pot or in the field were counted manually to calculate the germination percentage.

2.1.5 *Rhizobium* nodulation

2.1.5.1 Pea root inoculation and nodule quantification

Pea seeds were surface sterilised using 70% ethanol for 30 s, then immersed in 10% bleach for 5 min with shaking and rinsed thoroughly with sterile water (four times, 500 mL each time). Sterile seeds were germinated at room temperature (RT), in darkness for 3 days, on sterile filter paper soaked with sterile water inside of a Petri dish (5 seeds/plate).

Sprouting seeds were individually transferred into 250 mL flasks containing 100 mL Fahraeus agar medium [per litre: CaCl2.2H2O 0.1g, MgSO4.7H2O 0.12g, KH2PO4 0.1g, Na2HPO4.12H2O 0.15g, FeC6H2O7.5H2O, Oxoid No 3 Agar 5.0g], 1.0 mL Gibson's trace element solution [per litre: H3BO3 2.86g, MnSO4.4H2O 2.03g, ZnSO4.7H2O 220mg, CuSO4.5H2O 80mg and H2MoO4.H2O 80mg], adjusted at pH 6.5.

Flasks were covered and plants grown in the dark at room temperature (RT) for 7 days. After 7 days the elongated hypocotyl was passed through the neck of the flask and each plant was inoculated with 1 mL of *Rhizobium*. Flasks were covered with a black plastic bag to keep the roots dark. Plants were grown in an environmentally controlled room at the JIC, with 16 h light and controlled temperature at 20° C. Total nodules per plant were quantified after 3 weeks of plant growth.

2.1.5.2 *Rhizobium leguminosarum* culture conditions

Rhizobium leguminosarum strain A3841 (streptomycin resistant) was cultured in tryptone-yeast extract (TY) minimal medium [Tryptone 5.0 g, Yeast extract 3.0 g, CaCl2 x 2 H2O 0.9 g, per L] and grown for 3 days at 28 °C.
The number of cells was calculated by first adjusting the optical density (OD) at 600 nm to 1 (equivalent to 5 x 10^8 cells). Then the cells were diluted in water to a concentration of 200 cells/mL, and 1 mL of 200 cells/mL used to inoculate each flask containing pea plants.

2.1.6 Pea flour and extract preparation

Pea flour was produced by milling whole dry seeds using the Retsch Cyclone Mill Twister (Verder Scientific, UK) at a 14 000-rpm speed. Flour was passed through a 125 µM pore sized stainless steel sieve (Endecotts, UK).

Pea extracts were obtained by weighing 5 mg of previously prepared flour and added into a 10 mL grade A volumetric flask. Flour was mixed with 50 % ethanol (in MilliQ water) and the suspension sonicated for 15 min. The samples were centrifuged at 11 000 rpm to pellet solids, and the pea extract supernatants were transferred to empty vials.

To carry out batch fermentation assays (see 2.2.2.1.), the pea extract samples were then subjected to centrifugal vacuum evaporation using the Genevac EZ-2 Elite Personal Evaporator (Genevac, UK) at low boiling point (BP) programme, in order to obtain a dry powder that was reconstituted with batch media.

For analytical measurements (see 2.1.7.), the 50% ethanol used to extract the flour contained additionally 50 mg/L of melezitose, used as internal standard. Moreover, after the centrifugation step at 11 000 rpm, the supernatants were directly used for analysis without further steps.

2.1.7 Analytical measurements

2.1.7.1 Liquid chromatography–mass spectrometry (LC–MS) RFO quantification

Pea flour extracts were obtained as described (section 2.1.6.) using 50 g/L meletizose in 50% ethanol. A standard solution was prepared with 0.025 mg/mL myo-inositol, 0.5 mg/mL sucrose, 0.05 mg/mL galactinol, 0.12 mg/mL raffinose, 0.5 mg/mL stachyose and 0.5 mg/mL verbascose, as individual commercial standards (Sigma-Aldrich Gillingham, UK). The standard solution was then diluted sequentially 1:2 to give five calibration levels as previously described (Rayner et al., 2017).

LC-MS was conducted by Paul Brett at the JIC Metabolite Services Platform using a Shimadzu single quadrupole, with a 100 mm x 2.1mm 150-amide - Hydrophilic Interaction Liquid Chromatography (HILIC) column (Accucore™), and a gradient of two mobile phases (Table 6). The injection volume used was 1 µL and the gradient was based on 0.1% formic acid in water and 0.1% formic acid in acetonitrile. The total run time per sample was 15 min (Table 6).

Table 6. Mobile phase gradient for the detection of RFOs.

Time (min)	% formic acid In water	% formic acid in acetonitrile	Flow (µL/min)
0	20	80	400
8	40	60	400
10	40	60	400
10.5	20	80	400
15	20	80	400

Identification of RFOs and precursors was achieved based on retention time of authentic RFO standards (Table 7).

Table 7. Optimised LC-MS parameters optimised for the detection of RFOs.

Compound	Retention time (min)	SIM mass	Polarity
Sucrose	3.1	386.9	Negative
Myo-inositol	3.8	224.9	Negative
Internal standard (melezitose)	4.4	549	Negative
Raffinose	4.9	549	Negative
Galactinol	5.5	386.9	Negative
Stachyose	6.7	711	Negative
Verbascose	8.1	873	Negative

SIM: selected ion monitoring.

2.1.7.2 Total starch assay

Pea flour (see 2.1.6.) was used for quantification of total starch using the Megazyme® total starch assay (α-amylase/amyloglucosidase) following a modified version scaled to 1:20 (Moreau *et al.*, 2022; Perez-Moral *et al.*, 2018) of the manufacturer's protocol (Megazyme International, Ireland) as detailed below.
A total of 5 mg of pea flour was mixed with 250 µL of 80% (v/v) aqueous ethanol transferred into a 1.5 mL Eppendorf tube. After incubation at 50 °C for 5 min, the mixture was then vortexed, and an additional 250 µL of 80% (v/v) aqueous ethanol was added. The tube was centrifuged for 10 min at 3,000 rpm, and the supernatant was discarded.

The pellet was resuspended in 500 µL of 80% (v/v) aqueous ethanol and vortexed. The sample was centrifuged again for 10 min at 3,000 rpm, and the supernatant was removed. The pellet was then first resuspended in 20 µL KOH solution, to prevent lump formation, followed by addition of 80 µL 2 M KOH. The total starch was then dissolved by stirring the mixture for approximately 20 min on an ice/water bath using a magnetic micro-stirrer (7x2 mm) in a cold room.
A volume of 400 µL of 1.2 M sodium acetate buffer (pH 3.8) was added to the suspension, followed by vortexing. Subsequently, 5 µL of thermostable α-amylase (100 U/mL, diluted 1:30) and 5 µL of amyloglucosidase (AMG, 3300 U/mL), both provided by the kit, were added and mixed by vortexing. The tubes were incubated at 50 °C for 30 min with intermittent mixing using a vortex mixer or an Eppendorf thermomixer with gentle agitation.
The samples were diluted 10-fold by adding 100 µL of the samples to 900 µL of distilled water, followed by thorough mixing by pipetting. The diluted solution was centrifuged at 3,000 rpm for 10 min. Aliquots of 10 µL of the diluted solution were transferred into a 96-well microplate for colorimetric and absorbance assays. Blanks (10 µL water), and D-glucose controls (10 µL, 1 mg/mL) were transferred into the 96-well plate in quadruplicates.
Finally, to each well, including the D-glucose controls and water blanks, a total of 300 µL of GOPOD reagent (provided by the kit) was added. The plate was sealed with an adhesive sheet and incubated at 50 °C for 20 min in an Eppendorf Thermomixer C with a plate adaptor, under shaking at 300 rpm. After the incubation, the adhesive film was removed, and the absorbance measured at 510 nm against the reagent blank using a SpectraMax® 340PC (Medical Devices, UK) plate reader and the data collected using the SoftMax Pro® 4.8 software. The determination of resistant starch was conducted following the manufacturer's instruction for solid samples, where the absorbance was converted to µg following adjustments for the conversion of free D-glucose to anhydro D-glucose.

2.2 Microbiology

2.2.1 *Bifidobacteria* growth assays

2.2.1.1 Bacterial strains and culture conditions

B. longum subesp *infantis* ATCC 15697 / DSM 20088 and *B. breve* UCC2003 / NCIMB 8807 used in this study were from the National Collection of Industrial, Food and Marine Bacteria (NCIMB, UK). Growth assays were performed in an anaerobic cabinet (VAC Genesis Glovebox) having a humidified atmosphere of N_2 (oxygen level maintained below 0.3 ppm by oxygen purifier throughout the experiment). The strains were first cultured in De Man–Rogosa–Sharpe (MRS) media [peptone 10.0 g/L, yeast extract 5.0 g/L, beef extract 10.0 g/L, glucose 20.0 g/L, potassium phosphate 2.0 g/L, sodium acetate 5.0 g/L, magnesium sulfate 0.2 g/L, manganese sulfate 0.05 g/L, Tween 80 1.08 g/L, ammonium citrate 2.0 g/L] for two days.
To test the growth of bacteria on individual sugars as sole carbon source, sucrose, galactinol, raffinose, glucose, or verbascose (Merck Life Science UK Ltd), were added at a final concentration of 2 mg/mL to modified MRS (mMRS) which has the same

composition as MRS but lacking glucose and then the whole media was filter-sterilised using sterile 0.22 µm Corning® syringe filters (Corning Limited – Life Sciences. United Kingdom). The bacteria cultures were diluted to an optical density (OD 600nm) of 1.0 in mMRS and 5 µL was added to 200 µL medium in 96 well-plates and grown in the Whitley A95 anaerobic workstation (Don Whitley Scientific Limited, England) for up to 24 h. Uninoculated mMRS medium was used as a negative control.

2.2.2 Fermentation assays

2.2.2.1 Batch fermentation assays

For batch fermentation assays, a faecal slurry was prepared from a faecal sample of donors participating in the QIB Colon model study. The study was approved by the Quadram Institute Bioscience Human Research Governance committee (IFR01/2015), and London – Westminster Research Ethics Committee (15/LO/2169). The informed consent of all participating subjects was obtained, and the trial is registered at http://www.clinicaltrials.gov (NCT02653001). Participants included men and women over the age of 18 living or working within 10 miles of the Norwich Research Park. Participants within this study displayed normal bowel habits (defecation between 3 times a day and 3 times a week), with a Bristol stool chart classification of 3-5 and no prior diagnosis of chronic GI health problem. Individuals were excluded from the study if they had used antibiotics in the past two months or had recently taken other medication or food supplements, were pregnant or breastfeeding, recently had any operations or surgeries requiring general anaesthetic or experienced GI complications such as vomiting or diarrhoea in the past 72 h.
Prior to the cultures, fresh faecal samples (10 g) were diluted 1:10 in pre-reduced phosphate buffer solution (PBS) containing 10% glycerol, and homogenised using a Stomacher 400 (Seward, UK) at 230 rpm for 45 s. The processed samples were stored at -80 °C.

Individual sugars, sucrose, galactinol, raffinose, verbascose, glucose (Merck Life Science UK Ltd), and pea flour and pea extracts previously obtained through ethanol extraction (see section 2.1.6.) were diluted to 2 mg/mL in batch medium [1 L batch culture media contained 2 g peptone, 2 g Yeast extract, 0.1 g NaCl, 0.04 g K_2HPO_4, 0.04 g KH_2PO_4, 0.01 g $MgSO_4.6H_2O$, 0.01 g $CaCl_2.2H_2O$, 2 g $NaHCO_3$, 0.5 g Cysteine-HCl, 0.5 g Bile salts, 2 ml Tween 80, 0.02 g Hemin, 10 µl 0.5% v/v Vitamin K and 0.37 nM vitamin B12] and filter-sterilised using 0.22 µm Corning® syringe filters (Corning Limited – Life Sciences, United Kingdom). The medium was pre-reduced in the anaerobic chamber (Don Whitley Scientific) two days prior carbohydrate supplementation.
The medium was inoculated with the faecal suspension at a ratio 1:10 in a final volume of 3.3 mL. The culture was incubated at 37 °C under anaerobic conditions for 24 h.
At the end of the 24 h fermentation, 1 mL of the culture was centrifuged at 8,000 rpm for 5 min, the supernatant was separated and kept at -80 °C for metabolomics analysis (see 2.2.5.), and the cell pellet stored at -20 °C prior to DNA extraction for metagenomic analysis (see 2.2.3.1.1.).

2.2.2.2 Simulator of the Human Intestinal Microbial Ecosystem (SHIME®)

To investigate the effect of the fermentation of pea flour from two different genotypes (Caméor wild-type and BCRfsTILL W521* mutant) on the gut microbiota structure and function, the TWIN SHIME® (registered name from Ghent University and ProDigest) was setup as a by operating two systems in parallel, as previously described (Eeckhaut *et al.*, 2008). The TWIN SHIME® reactors include a stomach, small intestine and three colon regions: namely ascending colon (AC), transverse colon (TC), and descending colon (DC). Here, the three colon units of each SHIME® unit were inoculated with human faecal microbiota from a healthy donor participating into the QIB Colon model study (as described in section 2.2.1).
Following inoculation, the standard SHIME® basal medium was used for feeding the model for 33 days. The medium containing 1.0 g/L arabinogalactan, 2.0 g/L pectin, 1.0 g/L xylan, 4.0 g/L potato starch, 0.4 g/L glucose, 3.0 g/L yeast extract, 3.0 g/L peptone,1.0 g/L mucin, 0.5 g/L cysteine and pancreatic juice (made of 12.5 g/L $NaHCO_3$, 6.0 g/L bile salts and 0.9 g/L pancreatin) was prepared in the laboratory and autoclaved at 121 °C for 15 min. Analysis of samples in the control period supplementing with 1.68 g/day of potato starch (days 28, 30 and 33), were used as the baseline activity in the different reactors and as a control to compare with the results from the treatment period where the diet was supplemented with 1.68 g/day of pea flour for 19 days (Caméor wild-type and BCRfsTILL W521* mutant) (see section 2.1.6. for flour preparation).
The TWIN SHIME® reactors utilise the fill-and-draw method to simulate different phases of food uptake and digestion. Here, peristaltic pumps added nutritional medium (140 mL, three times daily) and pancreatic and bile liquids (60 mL, three times daily) into the stomach and small intestine compartments and carried out the emptying of the small intestine suspension into the AC, followed by transfer to TC and finally DC compartments after specified intervals of 3 h. All vessels were kept anaerobic by flushing them with N_2, and they were continuously stirred and kept at 37 °C.
Samples were collected three times a week from the AC, TC and DC colon vessels. Aliquots of 1 mL of sample were centrifuged for 10 min at 14 000 rpm at 4 °C. Supernatants were removed and kept at - 80 °C for SCFA analysis (see 2.2.5.1.) and cell pellets were processed for DNA extraction and full-length 16S rRNA gene sequencing (see 2.2.3.1.2.).

2.2.2.3 Gas quantification

Gas production from the fermentation of the pea flours (section 2.1.6.) was measured using the ANKOM RF gas production system (ANKOM Technology, Macedon, New York, USA). Briefly, the basal medium was prepared with [0.7134 g/L of KCl and NaCl, 0.2378 g/L $CaCl_2{\cdot}2H_2O$, 0.5945 g/L $MgSO_4{\cdot}7H_2O$, 1.567 g/L piperazine-N,N′-bis(2-ethanesulfonic acid) buffer, 0.642 g/L NH_4Cl and 1.189 g/L trypticase], trace minerals [11.891 mL of trace mineral solution/L contained 25 mg/L $MnCl_2{\cdot}4H2O$, 20 mg/L $FeSO_4{\cdot}7H_2O$, 25 mg/L $ZnCl_2$, 25 mg/L $CuCl{\cdot}2H_2O$, 50 mg/L $CoCl_2{\cdot}6H_2O$, 50 mg/L SeO_2, 250 mg/L $NiCl_2{\cdot}6H_2O$, 250 mg/L $Na_2MoO_4{\cdot}2H2O$, 31.4 mg/L $NaVO_3$ and 250 mg/L H_3BO_3], haemin [0.05 g haemin in 25 mL of 0.05 NaOH in 500 mL boiled deionised water] and fatty acid solution [1.37 mL acetic acid, 0.6 mL propionic acid, 0.368 mL butyric acid, 0.094 mL isobutyric acid, 0.11 mL 2-methylbutyric acid, 0.11 mL valeric acid and 0.11 mL isovaleric acid in 200 mL of 0.2 mM NaOH]. The basal pH of this

solution was adjusted to 6.8 with concentrated KOH and 76 mL medium were dispensed into 100 mL bottles and autoclaved.
On the day preceding the faecal sample inoculation, the basal medium was supplemented with 5 mL of a vitamin solution [0.0204 g/L biotin, 0.0206 g/L folic acid, 0.164 g/L calcium d pantothenate, 0.164 g/L nicotinamide, 0.164 g/L riboflavin, 0.164 g/L thiamine HCl, 0.164 g/L Pyridoxine HCl, 0.0204 g/L p-aminobenzoic acid and 0.0206 g/L cyanocobalamin], previously diluted (1:4) in 82 g/L Na_2CO_3 solution. Finally, the pH was adjusted to 7.0 and 1 mL of reducing solution [1 g L-Cysteine HCl and 1 g Na2S·9H2O in 50 mL boiling deionised water] was added to complete the medium.
A total of 0.5 g of pea flour from each pea variant (Caméor, BCFN 1551 or BCTILLRfs W521*) was added to the fermentation bottles with the complete medium (final volume 82 mL). To reduce the medium in each bottle, CO_2 was bubbled 1 h through the solution until it became clear. Bottles were sealed to allow flour hydration and media pre-warming overnight at 37 °C.
The bottles were then inoculated with 3 mL of diluted faecal samples (frozen at −80 °C and defrosted at RT prior to testing) in a microbiology safety cabinet under sterile conditions. Five human faecal samples from the QIB colon model study, namely HD1, HD2, HD3, HD4 and HD5, were used individually, one in each replicate. Each substrate was fermented in triplicate per volunteer faecal sample. After inoculation the bottles were sealed to remain anaerobic and incubated at 37 °C in a shaking incubator for 24 h. During this time, gas pressure was automatically measured hourly using the ANKOM RF system (ANKOM Technology, Macedon, New York, USA).
To remove any discrepancies in gas produced from sources other than the test substrates, a non-substrate blank was included. Results are reported as cumulative gas volume produced during fermentation (Rotbart *et al.*, 2017). Using Prism, the area under the curve (AUC) was calculated. For this the parameters to calculate the total area under the curve of gas production were the following: all peaks have to be above zero cumulative pressure level, and the minimum peak height cannot be less than 10% of the distance from minimum pressure level to the maximum level.

2.2.3 Metagenomics analyses

2.2.3.1 DNA extraction from batch fermentations

2.2.3.1.1 DNA extraction using the FastDNA™ Spin Kit for Soil

The bacterial DNA from batch fermentation assays (section 2.2.2.1.) was extracted from the cell pellet using the FastDNA™ SPIN Kit for Soil (MP Biomedicals). Briefly, the cell pellet was resuspended in PBS (978 µL) and incubated at 4 °C for 1 h following addition of MT Buffer (122 µL) supplied in the kit. The suspensions were then homogenised in the FastPrep® Instrument (MP Biomedicals) 3 times for 40 s at a speed setting of 6.0 with 5 min on ice between each bead-beating step. The FastDNA™ SPIN Kit for Soil (MP Biomedicals) protocol was then followed as recommended by the supplier.

2.2.3.1.2 DNA extraction using the Maxwell® RSC Fecal microbiome DNA kit

DNA from TWIN SHIME® fermentation assay (section 2.2.2.2) and from the faecal donor inoculum for gas quantification (section 2.2.2.3) was extracted from the cell pellet using the Maxwell RSC Fecal Microbiome DNA Kit (Promega Corporation, UK) (MP Biomedicals) and the Maxwell RSC instrument for automated nucleic acid extraction. Briefly, the cell pellet was resuspended in 1 mL of lysis buffer and 40 µL of proteinase K, and vortexed for 30 s. The tube was placed into a heat block at 95 °C for 5 min, allowed to cool for 2 min, vortexed for 1 min, and incubated at 56 °C for 5 min. The suspension (1100 µL) was then homogenised in the FastPrep® Instrument (MP Biomedicals) 3 times for 40 s at a speed setting of 6.0 with 5 min on ice between each bead-beating step. The protocol was then followed as recommended by the manufacturer using the Maxwell® RSC 48 Instrument (Promega Corporation, UK).
The purity, quantity and integrity of the extracted DNA were assessed with NanoDrop 1000 UV-Vis Spectrophotometer (Thermo Fischer Scientific, Wilmington, DE) and Qubit® Fluorometer (Thermo Fischer Scientific).

2.2.3.1.3 Shotgun metagenomics sequencing

Following DNA extraction (see section 2.2.3.1.1.), the concentration of DNA was normalised to 5 ng/µL and library preparation was conducted in house by David Baker and Rhiannon Evans at the Quadram Institute Core Sequencing department, as described below.
Firstly, the pool was end-repaired, A-tailed and ligated with P7 and P5 Illumina 9bp barcodes (Illumina Catalogue No. FC-131-2001 to 2004) for Illumina sequencing. Libraries were then pooled following quantification in equal quantities. The final pool was quantified on a Qubit 3.0 instrument and run on a D5000 ScreenTape (Agilent Cat.No. 5067-5579) using the Agilent Tapestation 4200 to calculate the final library pool molarity. A total of 20 µL of the library at 15 nM was used for shotgun metagenomic sequencing using the Illumina HiSeq platform paired end sequencing (2x150 bp) coverage (4.5 Gb for 10-15M read pairs) at Genewiz (Azenta Life Sciences, UK).

2.2.3.1.3.1 Bioinformatics analysis of shotgun metagenomic sequencing data

All analyses described below, including taxonomic classification and functional analysis were conducted in house at the Quadram Institute Bioscience (QIB) by Dr Alise Ponsero.

<u>Sequencing data processing</u>

Sequencing reads were analysed for quality control using FastQC v0.11.9 (available at https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). TrimGalore! v0.6.10 (available at https://github.com/FelixKrueger/TrimGalore) was used to trim possible sequencing primers. Potential human read contamination was removed using Hostile v1.1.0 (Constantinides, Hunt, and Crook 2023) against the human-t2t-hla reference database.

<u>Community composition profiling and functional profiling</u>

Metaphlan v4.0.1 (Blanco-Míguez et al. 2023) against the Jun23_CHOCOPhlAnSGB_202307 database was run on the sequences after quality control in order to profile the samples. Humann3 v3.8.0 (Beghini et al. 2021) was run against the uniref90_201901b_full database. Gene counts were grouped into MetaCyc pathways and normalised into copy per million reads (cpm) using the humann_renorm_table utility script.

Data visualisation

For the taxonomic profiles, the PCA plots and biplots were generated on the species counts after centred log ratio transformation (CLR). On the other hand, the principal component analysis (PCA) plots for the functional pathways were generated using the cpm pathway counts directly.

2.2.3.1.4 Analysis of full-length 16S rRNA gene sequencing

Following DNA extraction from cell pellets (see section 2.2.3.1.2.) the concentration of DNA was determined using NanoDrop 1000 UV-Vis Spectrophotometer (Thermo Fischer Scientific, Wilmington, DE) and Qubit® Fluorometer (Thermo Fischer Scientific) and normalised to 10 ng/µL using deionised water, before sending to Novogene (China) for sequencing and analysis.
Amplified DNA fragments were end-repaired, and A-tailed. The sequencing adapters were ligated to the ends of the DNA fragments using DNA-binding enzyme, and the DNA fragments were purified using AMpure PB magnetic beads to construct a SMRTbell™ template. Finally, sequencing primers were annealed to the SMRTbell™ templates, followed by binding of the sequence polymerase to the annealed templates. The library was checked with Qubit for quantification. Quantified libraries were pooled and sequenced on PacBio Sequel II/IIe systems, according to effective library concentration and data amount required. Full length 16S rRNA gene sequencing was carried out using the PacBio Revio platform with HiFi reads with primers 27 F and 1514 R, for 0.03 M Clean Reads per sample.

2.2.3.1.4.1 Bioinformatics analysis of full-length 16S rRNA gene sequencing

This was conducted by Novogene Bioinformatic Technology, Beijing, China, as described below.

Sequencing data processing

The PacBio BAM file was split according to barcode and filtered to get clean data. Then, the effective data is used to do Amplicon Sequence Variants (ASVs) analysis and species annotation. Denoising, species annotation, alpha diversity analysis, beta diversity analysis, and ANCOM analysis were performed using QIIME2 ™.

Denoise and species annotation of ASVs

For the effective tags, the DADA2 or deblur module in QIIME2 ™ software was used to perform data denoise, or quality control. The scikit-learn machine learning module in QIIME2 ™ software was used to compare ASVs with the database and to obtain the species annotation of each ASV.

Alpha diversity

QIIME2™ was used to calculate alpha diversity indices including observed operational taxonomic units (OTUs), Shannon, and dominance indices. The rarefaction curve and species accumulation boxplot were drawn. In case of grouping, the differences between groups of alpha diversity were analysed by default.

Beta diversity

Firstly, the UniFrac distance was calculated by QIIME2 ™ software, and the dimensionality reduction maps of Principal component analysis (PCA), Principal Coordinate Analysis (PCoA) and non-metric multidimensional scaling (NMDS) were drawn by R software. Among them, Ade4 and ggplot2 packages in R software were used to display PCA and PCoA. Then, Adonis and Anosim functions in QIIME2 ™ software were used to analyse the significance of community structure differences among groups. Finally, the Linear Discriminant Analysis Effect Size (LEfSe) was performed using the LEfSe software, and linear discriminant analysis score threshold was set to 4 by default. In MetaStat analysis, R software was used to test the differences between the two groups at the level of phylum, class, order, family, genus and species, and p value was obtained. The species with p value less than 0.05 were selected as the significant differences between the two groups; in t-test, R software was also used to analyse the significant differences of species at each taxonomic level.

Function prediction

Phylogenetic Investigation of Communities by Reconstruction of Unobserved Stats 2 (PICRUSt2) was used for predicting functional abundance based only on marker gene sequences. Gene family copy numbers of ASV were inferred using castor, and the gene functional spectrum of their common ancestor, was defined based on the ASV tree and gene information of ASVs in the Greengenes database. This allowed the predicted sample gene family profiles to be used to infer pathway abundances using MinPath so as to predict the metabolic function of bacteria group.

2.2.4 Analytical measurements

2.2.4.1 Liquid chromatography–mass spectrometry (LC–MS) for quantification of SCFAs, branched-chained fatty acids (BCFAs) and lactate

Quantification of SCFAs in the supernatants from the fermentation assays (see 2.2.2.1 and 2.2.2.2.) was conducted by direct LC-MS using isotopically labelled internal standards, at QIB by Shikha Saha (Saha *et al.*, 2021). SCFA standards were purchased from Sigma (Dorset, UK) and reconstituted in acidified (with phosphoric acid) Milli-Q water to prepare 100 mM stock solutions kept at -20 °C. A standard curve was produced from the 100 mM stock solutions using serial dilutions (10 mM to 0.001 mM), and prepared fresh prior to each run.
An Agilent 6490 Triple Quad MS mass spectrometer equipped with an Agilent 1290 HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a

Thermofisher PGC 3 µm column (50 x 2.1 mm) with a guard column was used for the analysis. The column temperature and autosampler were maintained at 40 °C and 4 °C, respectively. The injection volume was 2 µL. Samples were analysed using a compound mobile phase consisting of a gradient of 0.1% formic acid in water and 0.1% formic in acetonitrile as specified per time interval (Table 8). The total run was 10 min.

Table 8. Mobile phase gradient for quantification of SCFAs, BCFAs and lactate by LC-MS.

Time (min)	% formic acid in water	% formic acid in acetonitrile	Flow (mL/min)
0	0	0	0.15
4	0	60	0.15
6	0	100	0.15
10	100	0	0.15

Nitrogen gas was used for nebulisation, desolvation and collision. The analytes were monitored in multiple-reaction monitoring (MRM) mode. The MRM precursor, product ions and collision energy were optimised by the Agilent optimiser software and the transitions of precursor ions (m/z) and the main optimised MS operating parameters are described in Table 9. The source parameters were as follows: a gas temperature of 200 °C with a gas flow of 16 L/min, a sheath gas temperature of 300 °C with a sheath gas flow of 11 L/min, a nebuliser pressure of 50 psi and capillary voltage of 3500 V for positive polarity, and a Nozzle Voltage 1000 V. The iFunnel parameters were high pressure radio frequency of 150 V and low-pressure RF of 60 V. The LC eluent flow was sprayed into the mass spectrometer interface without splitting. Identification was achieved based on retention time of authentic standards (Table 9).

Table 9. Optimised LC-MS operating parameters for SCFA, BCFA and lactate quantification.

Analyte	Retention Time (min)	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Collision Energy	Cell Accelerator Energy	Polarity
Acetate	1.5	61.1	43	16	4	Positive
D4-Acetate	1.5	65.1	47	14	4	Positive
Butyrate	3.7	89.1	43.1	14	4	Positive
13C2-Butyrate	3.7	91.1	44	14	4	Positive
Isobutyrate	2.9	89.1	43.1	14	4	Positive
D6-Isobutyrate	2.9	95	49	14	4	Positive
Iso-Valerate	4.2	103.1	43	14	4	Positive
D9-Isovalerate	4.2	112.2	50.2	18	4	Positive
Lactate	1.7	89	42.9	10	5	Negative
13C3-Lactate	1.7	92	46	10	4	Negative
Propionate	2.3	75	29	18	4	Positive
D2-Propionate	2.3	77	31.1	14	4	Positive

Valerate	4.7	103.1	75	10	4	Positive
D9-Valerate	4.7	112.1	80	10	4	Positive

2.2.4.2 Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectroscopy (UPLC-MS/MS) for untargeted metabolomics

The supernatants from the 24 h batch fermentation (obtained as described in 2.2.2.1.) were analysed at Metabolon Inc. (Morrisville, NC, USA) by Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectroscopy (UPLC-MS/MS using a Waters ACQUITY UPLC and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization source and Orbitrap mass analyser operated at 35,000 mass resolution.
Six biological replicates were analysed per condition. The data were visualised using PCA.

2.3 Intestinal organoid cultures

2.3.1 Organoid culture expansion

Colonic organoids (from Human Organoid Donor 2= HOD2) generated from distal colonic biopsies obtained from participants under the MOTION Study Ethics (IRAS project ID 241617) were banked in liquid nitrogen, defrosted by Dr Tanja Šuligoj (Juge Group, Quadram Institute Bioscience, UK) using Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) (ThermoFisher Scientific, UK) with 1% bovine serum albumin (BSA). Organoids were seeded onto Corning® Matrigel® Matrix domes (Corning, NY, USA) (from now onwards referred to as Matrigel® domes) as described below.

First, organoid Matrigel® domes were grown in IntestiCult™ Organoid Growth Medium for human-derived organoids (OGM) (STEMCELL Technologies, Cambridge, UK) and passaged every 7-10 days for cell expansion. For organoid culture expansion, the Matrigel® domes were first disrupted and all pieces of Matrigel® with media were pooled into 15 mL tubes (3-4 blobs/tube) and the volume topped up with cold PBS. To avoid losses, the wells were washed to collect any remaining Matrigel® which were then mixed on a roller for 1 h at 4 °C. The tubes were then centrifuged at 250 x g for 5 min at 4 °C and the supernatant was removed. This was followed by the addition of 1 mL of trypsin into each tube and organoids were resuspended and incubated at 37 °C for 2 min and disrupted by pipetting up and down vigorously 40 times. After the final vigorous pipetting, ice-cold Dulbecco's Modified Eagle Medium (DMEM) (ThermoFisher Scientific, UK), containing 1% BSA was added to each tube and the contents were centrifuged at 290 x g for 5 min at 4 °C. The supernatant was discarded and ice-cold Matrigel® (in 50% PBS) required for the number of domes to be seeded (40 µL/dome) was added to the cell pellet.

After soft resuspension avoiding the formation of bubbles or solidification of Matrigel®, the domes containing organoids were pipetted into each well of the plate a 24-well tissue culture-treated plate, pre warmed in a 37 °C incubator for at least 1 h

prior to passage. OGM was replaced every 2 days, and cultures passaged every 7-10 days for further expansion or transwell seeding.

ROCK-inhibitor Y-27632 dihydrochloride (ROCKi) (STEMCELL Technologies, Cambridge, UK) was added at 10 µM to the organoid culture media to prevent stress in the cultures during the first 48 h after: organoid thawing and any organoid passage.

2.3.2 Culture of organoids on transwells

To obtain an intestinal organoid monolayer, a transwell model, with a porous membrane was used. Prior to seeding, the transwells (diameter 6.5 mm with 0.4 µm pore size) (Corning, NY, USA) were coated with collagen IV [1X] and kept at 37 °C for 2 h.

For the seeding of organoids on the transwells, organoid growing in Matrigel were disrupted as described for cell expansion (see 2.3.1.) with the following modifications. To obtain single cells for transwell seeding, after the vigorous pipetting of organoids with Matrigel®, these were incubated for a second time for 1-2 min. Finally, at the final step of seeding, instead of resuspending the organoid in Matrigel®, organoid-derived single cells were resuspended directly into OGM medium supplemented with ROCKi for the first 48 h post-seeding. The number of transwells to be seeded (100 µL/transwell) was calculated prior to seeding (using 2 domes/transwell).

Organoids seeded on transwells were allowed to expand for 7 days in OGM, after this, the medium was changed from OGM to IntestiCult™ Organoid Differentiation Medium (ODM) for human-derived organoids (STEMCELL Technologies, Cambridge, UK) for another 7 days. During the first 48 h of OGM change, the medium was supplemented with ROCKi to prevent stress from the change of medium.

2.3.3 Cytokine-induced inflammatory challenge on organoid monolayers

To model a compromised epithelium, established monolayers (obtained as described in 2.3.2) were treated with a cytokine cocktail consisting of TNF-α, IL-1a and IFN-γ (each at a concentration of 1 ng/mL) (PeproTech, London, UK) added to both the apical and basal compartments and incubated for a period of 24 h. Transepithelial electrical resistance (TEER) was measured at baseline prior to cytokine addition as described in section 2.3.5. Following the 24 h inflammatory stimulus, both apical and basolateral media were removed and treated described in 2.3.3.

2.3.4 Fermented pea flour treatment on organoid monolayers

Supernatants from batch fermentation (2.2.2.1.) were filter-sterilised using 0.22 µm Corning® syringe filters (Corning Limited – Life Sciences. United Kingdom) and were diluted 1:2 with ODM. A total of 100 µL was used as treatment on the intestinal organoid monolayer growing on the apical side of the transwell for 24 h, while the basolateral side medium was refreshed with ODM. After 24 h incubation with the supernatant from the faecal fermentation, the effluents from the apical and basolateral compartments were collected and kept at - 80 °C for cytokine analysis (see section 2.4.1).

2.3.5 Transepithelial electrical resistance (TEER) measurement assays

TEER was measured using an Epithelial Voltohmmeter (EVOM ®) instrument (World Precision Instruments (WPI), Sarasota, FL, USA) with STX2 electrode (World Precision Instruments, Sarasota, FL, USA). By passing a constant alternating current through the outer electrodes and measuring the voltage drop across the inner electrodes, the ohmic resistance of the tissue barrier sample is calculated (Raut *et al.*, 2021). TEER was measured on day 7 post-seeding (prior to ODM media change), on day 14 (treatment day) and 24 h after any treatment. Only monolayers with TEER > 1000 Ω·cm^2 were used on day 14 post seeding for treatment with faecal fermentation samples. The TEER reported values were as follows= Tissue resistance (Ω) x Transwell (cm^2).

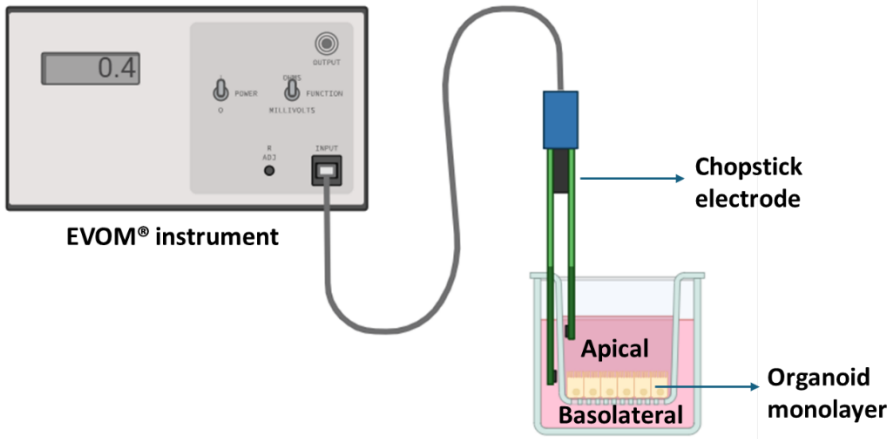


Figure 14. Transepithelial/endothelial electrical resistance (TEER) measurement using EVOM ® instrument. Created in BioRender scientific illustrator.

2.4 Immunological assays

2.4.1 LEGENDplex™ flow cytometry-based multiplex

The LEGENDplex™ bead-based immunoassays (Biolegend, San Diego, CA) was used as multiplex platform to quantify inflammatory cytokines and chemokines, namely, IP-10, TNF-α, MCP-1, IL-12, TGF-β1, IL-8 and IFN-γ, from cell culture effluents, following the manufacturer`s protocol using a v-bottom plate. All reagents and standards were provided by the kit.

To conduct this sandwich immunoassay, samples and standards (25 µL each) were first individually mixed with 25 µL of assay buffer in a 96-well plate, as per protocol recommendation for cell supernatant samples. The beads, which are conjugated to analyte-specific antibodies and have different sizes and internal fluorescence intensities, were vortexed for 20 s and 25 µL added onto each well for a final volume of 75 µL. The plate was sealed and covered with aluminium foil to protect from light. The pate was then put on a shaker at 800 rpm for 2 h. After the incubation period, the plate was centrifuged at 1050 rpm for 5 min, followed by discarding the supernatant

in one forceful motion. The plate was then washed with 200 µL of 1 X wash buffer (provided in the kit) and incubated for one min. The plate was then centrifuged as above and the supernatant discarded.

Detection antibodies (25 µL) were added to each well. The plate was then sealed from light and put in the shaker for 800 rpm for 1 h. After the incubation period, 25 µL streptavidin-phycoerythrin was added to each well directly, and the plate was sealed and covered with aluminium foil again and put in the shaker at 800 rpm for 30 min. The plate was centrifuged and the supernatant discarded. After this, the plate was washed by dispensing 200 µL of 1X was buffer and centrifuging, to help reduce the background. After the washing step, 150 µL of 1X wash buffer was added to each empty well and the beads were resuspended by pipetting and ready to be read for analysis. The signal from each sample and control was read using the ID7000™ Spectral Cell Analyzer (Sony, Tokyo) with autosampler.

Prior to the analysis, the Sony ID Software was setup in the ID7000™ instrument. For this, the instrument settings were selected for lasers 355 nm, 405 nm, 561nm and 637 nm, following the Sony ID7000™ Spectral Cell Analyzer setup guide for LEGENDplex™ Panels provided by BioLegend. The sample flow rate was set to 1. To set up the worksheet, on forward scatter (FSC) vs side scatter (SSC) density plot the A, B, and A+B gates were drawn, after this, each density plot was opened to display PE on X-axis and APC on Y-axis. The A+B gate was selected and 300 events per analyte were selected, with a 3000-event maximum. The gate and gain set up was conducted by loading a plate with the standard curve, the top standard was selected, the FSC was adjusted to maximum 17 and the SSC to 3.78. A, B and A+B gates were adjusted around the main populations and used to synchronise gates across all wells.

The concentration of analytes was determined using a standard curve provided in the kit and the LEGENDplex™ Data Analysis Software.

2.5 *In silico* analyses

2.5.1. AlphaFold modelling

AlphaFold 2 modelling was performed using UniProt protein sequence for Rfs (Peterbauer et al., 2002) (UniProt ID: Q8VWN6) (UniProt: a worldwide hub of protein knowledge, 2019), using the last updated AlphaFold DB version 2022-11-01. The 3D figure was created with the AlphaFold Monomer v2.0 pipeline (Varadi et al., 2023; Jumper et al., 2021).

2.5.2. Analysis of raffinose utilisation clusters in Bifidobacteria

The data repository of prokaryotic CAZyme-containing gene clusters that have been experimentally validated as polysaccharide utilisation loci (PUL), dbCAN-PUL (dbCAN-PUL (unl.edu) version 2 (Ausland et al., 2020; Zhang et al., 2018), was used to search for RFO PULs in Bifidobacteria. The search terms of carbohydrate substrate were: raffinose, stachyose and verbascose. The sequences of four PUL (0138,0232,0276 and 0455) were selected as clusters for comparative cluster alignment of homologous loci

within all Bifidobacteria using BLAST and plotted as an identity heatmap using R (R Core Team, 2022).

2.6 Statistical analyses

The RFO and starch levels were compared between the mutants and Caméor, using two- way ANOVA test and Bonferroni multiple comparisons using GraphPad Prism 5.04 (GraphPad software Inc.). The same software and statistical analysis were used to calculate differences between the growth curves of Bifidobacteria monocultures and cytokine levels in the organoid cultures.

Species richness and taxa relative abundances means in metagenomic data were compared across the experimental conditions using ANOVA test on the relative abundance counts. After checking the test's assumptions using the Levene test and a visual inspection data distribution using quantile-quantile (QQ) plots, an ANOVA model was created excluding the HD1 control samples (see section 2.2.2.1.). The post-hoc tests were carried out using a Tukey HSD test. The tests were conducted using the rstatix R package v0.7.2 and the plots were generated using the ggplot2 R package v3.5.0.

For differences in agronomical traits unpaired t-test was performed using GraphPad Prism 5.04 (GraphPad software Inc.). For the analysis of untargeted metabolomics, the Welch's two-sample t-test was used to test whether two unknown means were different from two independent populations. Standard statistical analyses were performed in ArrayStudio/Jupyter Notebook on log transformed data.

3 . Chapter Three: Characterisation and agronomical traits of *P. sativum* raffinose synthase mutants

3.1 Introduction

The production of *P. sativum* (pea) is increasing globally (©FAO, 2023) due to its nutritional quality and being an ecologically sustainable crop along with other legumes (Yanni *et al*, 2023). Amongst the fermentable carbohydrates present in pea seeds are a group of small sugars RFOs comprised of raffinose, stachyose and verbascose (Elango *et al*, 2022).

The biosynthesis of RFOs in pea occurs via the galactinol dependent pathway, where raffinose synthase (Rfs) leads to the formation of raffinose via a galactosyl extension of sucrose, which leads to subsequent α-1,6-galactosyl extensions of glucose, resulting in the formation of the trisaccharide raffinose, the tetrasaccharide stachyose and the pentasaccharide verbascose (Elango, 2022). However, a less explored pathway, the galactinol independent pathway, has been identified in *Ajuga reptans* (a non-legume) via the galactan-galactan galactosyl transferase (Bachmann, 1994). In pea, a galactinol-independent enzyme activity, similar to GGT, has been shown to catalyse the transfer of galactose residue from one stachyose to another (Peterbauer *et al*, 2001).

For many years, RFOs have been considered to be undesirable components in the human food and animal feed industries, due to flatulence promotion and, therefore, efforts have been made to reduce their content in legume crops (Gupta, 1987). Yet, RFOs have been suggested to protect plants from various stressors. In different plant species, an increase of RFOs has been observed under the effects of abiotic stressors such as heat (Panikulangara *et al*, 2004), cold (Bachmann *et al*, 1994), salinity or drought (Farrant *et al*, 2007). Despite the identification of genes involved in RFO accumulation in several plants (Li *et al*, 2020; Cui *et al*, 2021; Peterbauer *et al*, 2001), much remains to be understood about the mechanisms and enzymatic reactions involved in the synthesis of RFOs and their potential role under stress conditions in pea.

In this chapter, we investigated the role of RFOs in pea using *P. sativum* plants from a) the commercial pea cultivar, Caméor, b) a fast-neutron (FN) mutant of pea (FN 1551/11), with a deletion mutation impacting the major *rfs* gene and backcrossed with Caméor, and c) TILLING-derived Caméor mutants, with mutations in either the major *raffinose synthase* gene (*Rfs*) or a *raffinose synthase* (*Rfs-like*) gene. We hypothesised that the depletion of RFOs in mutant peas could be detrimental to plant development and that this could affect how they survive and produce offspring under environmental stressors, rhizobia nodulation or natural field conditions. The potential role of RFOs in stress conditions was addressed in the glasshouse by induction of extreme water deficit to the FN derived mutant, a common environmental stressor in pea crops (Bagheri *et al*, 2023). The performance of the pea RFO mutants was also tested in three field trials. RFOs in plants present both challenges and opportunities for enhancing crop resilience and productivity. This study aims to provide information about the role of genetic changes in RFO synthesis in the environmental adaptation of pea plants as potential novel varieties.

3.2 Results

3.2.1 Identification of *rfs* pea mutants

A mutant line, FN 1551/11, was selected from a fast neutron mutagenised population generated in the line JI2822 (Domoney *et al.*, 2013). FN 1551/11 was identified as lacking a major *Raffinose synthase (Rfs)* gene, as described in the methods section (2.1.1.1.). As a result of the mutagenesis, this line lacks not only *Rfs,* but also other genes within a 1.3 Mb deletion in chromosome 5.
Recent efforts of the Pulse Crop Genetic Improvement Network (www.pcgin.org) in combination with the Pea genomics supporting the UK Environment and Agri-food Sectors (PEAS) group at the John Innes Centre, have produced a high-quality genome assembly of the JI2822 line (Rayner *et al,* in preparation). Sequence analysis of the FN 1551/11 has been mapped to the JI2822 assembly and results of this analysis identified the deleted genes as shown in Table 10.

Table 10. Gene coordinates, identification and annotation of the 1.3 Mb deletion in the line FN 1551.

Genomic coordinates	Gene identification number	Gene annotation
chr5: 390185873-390186472	N/A	*Apulvinic* (elongated petiolule, *elp-1 like*)
chr5: 390434908-390435300	PsatJI2822v1chr5G66400	*Aminopeptidase*
chr5: 390544256-390549417	PsatJI2822v1chr5G66420	*Tryptophan aminotransferase-related protein*
chr5:390573422-390577028	PsatJI2822v1chr5G66440	*Galactinol--sucrose galactosyltransferase-like (**Rfs**)*
chr5: 390725497-390728707	PsatJI2822v1chr5G66460	*G-patch domain*
chr5: 390734568-390741085	PsatJI2822v1chr5G66480	*FKBP12-interacting protein of 37 kDa*
chr5: 390742454-390742924	PsatJI2822v1chr5G66500	*Ribosomal protein-like protein*
chr5: 390782021-390782917	PsatJI2822v1chr5G66520	*Ribosomal protein-like protein*
chr5: 390783231-390784238	PsatJI2822v1chr5G66540	*No apical meristem-associated C-terminal domain (LONG1)*
chr5: 390929027-390931756	PsatJI2822v1chr5G66560	*Zinc-binding RING-finger*

The FN 1551/11 line was crossed to Caméor; the first cross to Caméor was an outcross from the JI 2822 background (OCFN 1551, previously developed at the Domoney Group, JIC), the second cross was a further backcross to Caméor (BCFN 1551), and a further backcross to Caméor (BC2FN 1551) has been derived). The gene copy number of *Rfs* in the daughter plants resulting from the crosses, has been determined using gene copy number quantification assays (Supplementary Fig. S1). Here, the plants

resulting from the cross had one copy number of the gene. The plants identified as hemizygous from the first outcrossing were grown in the glasshouse, allowed to self-pollinate, and F2 plants genotyped to identify homozygous mutants. When the homozygous mutants were identified, as having zero gene copy numbers, the seeds from these plants were grown in the glasshouse to multiply seed numbers and to cross to Caméor (BCFN 1551). The F1 plants identified as hemizygous, having one copy number of the gene, were cross-pollinated with Caméor, to produce the following backcross generation (BC2FN 1551).

The Caméor TILLING mutants were obtained from the pea collection (Dalmais *et al.*, 2008) maintained by the National Research Institute for Agriculture, Food and Environment (INRAE) in France (Institute of Plant Sciences Paris Saclay); genotyping of the delivered mutants was conducted by DNA sequence analysis of *Rfs* and *Rfs*-like genes followed by single nucleotide polymorphism (SNP) identification. Briefly, the selected seeds were sown in the glasshouse and genomic DNA was extracted from young leaves of 2-3 weeks old plants. Primers targeting the mutation in *Rfs* or *Rfs*-like were designed using Primer-BLAST tool using the sequences for both targets.

Following analysis of the sequence reads and chromatogram peaks, the TILLING mutant seeds were classified as homozygous, homozygous wild-type or heterozygous mutants (Table 11; between four and six seeds were supplied per mutant line).

Table 11. Genotyping results of the TILLING mutant seeds received from INRAE.

Mutant name	Gene	Genotype result
TILLRfs A544V	Rfs	Heterozygous
		Homozygous mutant
		Heterozygous
		Homozygous mutant
		Homozygous mutant
		Heterozygous
TILLRfs G129E	Rfs	Wild-type homozygous
		Heterozygous
		Wild-type homozygous
		Wild-type homozygous
TILLRfs W530*	Rfs	Heterozygous
		Heterozygous
		Homozygous wild-type
		Heterozygous
TILLRfs G464S	Rfs	Heterozygous
		Heterozygous
		Heterozygous
		Heterozygous

TILLRfs G509D	Rfs	No germination
		No germination
		No germination
		No germination
TILLRfs H543Y	Rfs	Homozygous wild-type
		Heterozygous
		Heterozygous
		Homozygous mutant
TILLRfs W521*	Rfs	Heterozygous
		Homozygous wild-type
		Homozygous mutant
		Heterozygous
TILLRfs G479D	Rfs	Homozygous wild-type
		Homozygous wild-type
		Homozygous wild-type
		Homozygous wild-type
		Homozygous wild-type
		Homozygous wild-type
		Homozygous wild-type
TILLRfs-like G280R	Rfs-like	Homozygous wild-type
		Homozygous wild-type
		No germination
		No germination
TILLRfs-like D231N	Rfs-like	Homozygous wild-type
		Heterozygous
		Heterozygous
		Heterozygous
TILLRfs-like G150S	Rfs-like	Homozygous wild-type
		Homozygous wild-type
		No germination
		No germination
TILLRfs-like W339*	Rfs-like	Homozygous wild-type
		Homozygous mutant
		Homozygous mutant
		No germination
TILLRfs-like P146L	Rfs-like	Homozygous wild-type
		Homozygous wild-type
		Homozygous wild-type
		Homozygous wild-type
TILLRfs-like E118K	Rfs-like	Homozygous mutant
		Homozygous mutant
		Homozygous mutant

		Homozygous mutant
TILLRfs-like S397F	Rfs-like	Homozygous wild-type
		Homozygous wild-type
		Homozygous wild-type
		No germination
TILLRfs-like G341E	Rfs-like	Homozygous mutant
		Homozygous mutant
		Homozygous wild-type
		Homozygous mutant

*Corresponds to amino acid substitution for a stop codon.

Amongst the lines tested, six pea mutant lines, TILLRfs A544V, TILLRfs H543Y, TILLRfs W521*, TILLRfs-like W339*, TILLRfs-like E118K and TILLRfs-like G341E, had at least one homozygous mutant plant. For the rest of the lines, their genotypes were distributed between heterozygous and homozygous wild type. Moreover, none of the seeds from line TILLRfs G509D germinated.

Out of these six mutant lines, four lines were selected for quantification of RFOs, two with mutations in the *Rfs* gene, TILLRfs A544V and TILLRfs W521*, and two with mutations in the *Rfs*-like gene, TILLRfs-like G341E and TILLRfs-like E118K, based on the protein variation effect analyser (PROVEAN) score (Choi and Chan, 2015). This score predicts the biological effect of the amino acid substitutions. The software cut-off for a neutral mutation is from 0 to -2.5, and the scores for lines TILLRfs A544V, TILLRfs-like G341E and TILLRfs-like E118K, were -3.751, -7.549 and -3.704, respectively, indicating a deleterious effect of the mutations.

There was no PROVEAN score for line TILLRfs W521* because the nucleotide change predicts an early stop codon. Fig. 15 reports the structural model of Rfs as predicted by AlphaFold, here W521 (Trp521) is shown in pink and the interacting residues in green (Ile 113, Gln 137, Ile 138, Leu 155, Val 206, Val 210, Asn473, Phe 476, Leu 477, Tyr517, Asn 518, Ser 519, Leu 520, Met 522, Gly 523, Asn 524, Phe 525).

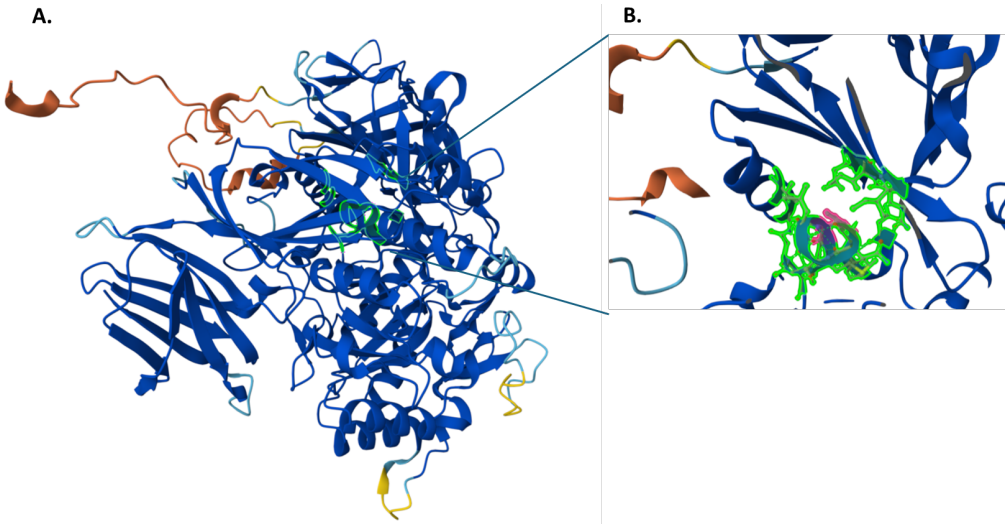


Figure 15. Structural model of Rfs predicted by AlphaFold. (A) Overview of Rfs domain fold. (B) Close-up of Tryptophan (Trp) residue in position 521 (W521) (pink) using a ball-and-stick model and the 17 surrounding residues (green). This model was created based on the predicted Rfs protein sequence from JI2822, which is 100% identical to galactinol—sucrose galactosyltransferase (UniProt: Q8VWN6 (RFS_PEA)).

In pea Rfs, Asp426 (D426) has been demonstrated to be one of the catalytic residues (Peterbauer *et al.*, 2002a) and recent crystallography studies of an α-galactosidase, AtAkαGal3, from *A. thaliana* identified other catalytic residues, namely lysine (position 424), aspartate (position 426), arginine (position 487), aspartate (position 490), and aspartate (position 491) in the catalytic domain of the enzyme (positions here are based on the amino acid residues of the Rfs sequence from pea and not *A. thaliana* for clarity) (Chuankhayan *et al.*, 2023). A protein sequence alignment of AtAkαGal3 with pea Rfs and Rfs-like amino acid sequence showed that these residues were also conserved in the pea enzymes (Fig. 16), indicating that the catalytic domain of Rfs is within these positions, and conserved in both pea Rfs and Rfs-like. The complete protein sequences of Rfs, Rfs-like and AtAkαGal3 are provided in Supplementary Fig. 2.



Figure 16. Alignment of the catalytic region of α-galactosidase AtAkαGal3 from *A. thaliana* and raffinose synthases Rfs and Rfs-like from pea. Representation of the previously identified conserved motifs (Chuankhayan et al., 2023) of conserved amino acids 424KVD426 and 487RVGDD491. The blue arrow indicates the position of the residues based on the pea Rfs sequence.

3.2.2 Characterisation of RFOs in pea *rfs* mutants

The RFO profile in the seeds of the selected pea mutants was analysed by LC-MS as described in the methods section (see 2.5.1.2.). The analytical approach was conducted by comparing the concentrations of each RFO, precursor and myo-inositol for each mutant against the reference values for the Caméor commercial cultivar (Fig. 17). An example of LC-MS chromatogram is shown in Supplementary Fig. S1 and the quantification reported in Table 1.

The OCFN 1551 mutant showed statistically significant differences as compared to Caméor across all sugars analysed. This included increases in the levels of precursors, sucrose and galactinol, with 2.6 and 12.7-fold changes, respectively (p value < 0.0001). Moreover, the mutant was depleted in raffinose and stachyose and significantly reduced in verbascose (0.05-fold change) (p values < 0.0001).

For the TILLRfs W521*, there was also a significant increase in the levels of the precursors sucrose and galactinol (p value < 0.0001), with 1.9 and 5.8-fold changes, respectively. For this mutant, all RFOs were detected, but significantly reduced (p value < 0.0001) compared to Caméor (raffinose: 0.2-fold change, stachyose: 0.2-fold change and verbascose: 0.4-fold change). For the TILLRfs A544V mutant, there was also a significant increase in the precursors sucrose and galactinol compared to Caméor (p value <0.00001), with 1.5 and 5.4-fold changes respectively; moreover, there was a significant reduction (p value < 0.0001) in RFOs compared to Caméor (raffinose: 0.12-fold change, stachyose: 0.24-fold change and verbascose: 0.62-fold change).

The TILLRfs-like G341E showed higher levels of sucrose (1.21-fold change, p value < 0.0001) and lower raffinose levels (0.60-fold change, p value < 0.0001). Finally, TILLRfs-like E118K showed significantly reduced sucrose, raffinose and stachyose (0.80-fold change, 0.44-fold change and 0.74-fold change respectively, p value < 0.0001).

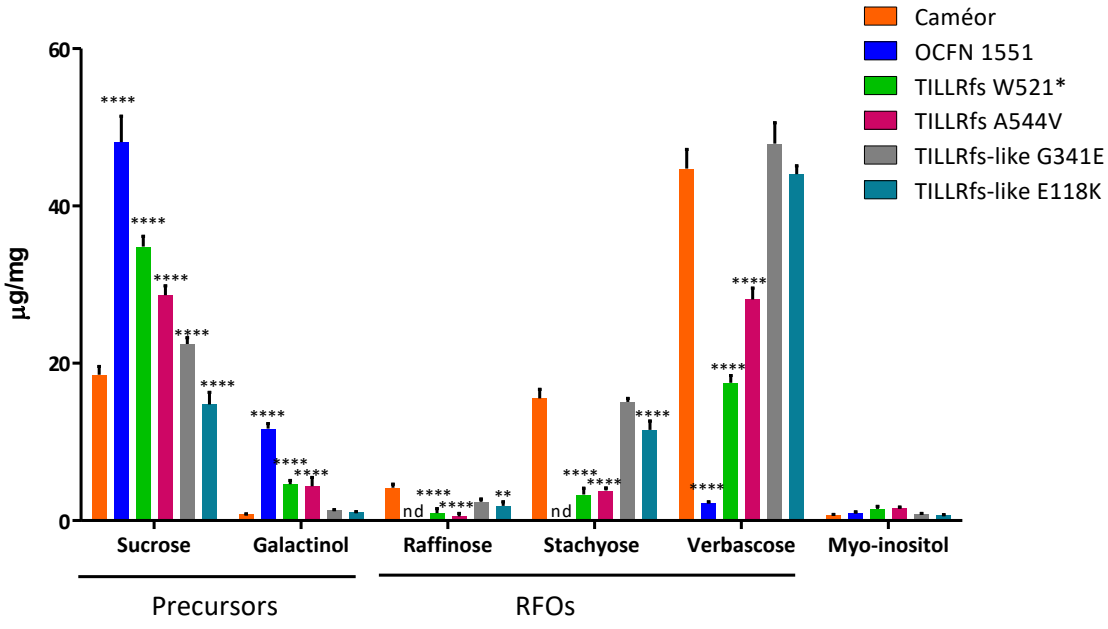


Figure 17. LC-MS analysis of RFOs and their precursors in pea mutants and control lines. Sugars were extracted from dry seeds of TILLING mutants for Rfs (TILLRfs W521* and TILLRfs A544V), and Rfs-like genes (TILLRfs-like G341E and TILLRfs-like E118K), the OCFN 1551 FN mutant and the Caméor line. Three biological replicates were analysed, each performed in technical duplicates. The results are represented as means with respective standard errors. Two-way ANOVA with Bonferroni`s pairwise comparison against Caméor (****) p value < 0.0001, (**) p value < 0.01. nd= not detected.

Together these data showed that the pea mutants obtained from both mutational approaches, TILLING or Fast Neutron, have distinct RFO and precursor content, with most of their differences being statistically significant as compared to the commercial cultivar Caméor.

Due to the higher sucrose accumulation in the mutant pea lines, and the link between carbohydrate transport and biosynthesis in plants (see section 1.4.2.), total starch was quantified in the pea seeds to determine the influence of the mutations on starch content and to gain information on the level of this fermentable carbohydrate (compared to RFO).

The results showed that the total starch concentration (expressed as % w/w) from the R*fs* and *Rfs*-like TILLING mutants and the OCFN 1551 mutant were not significantly different to the commercial cultivar Caméor (Fig. 18), with the average values across all lines ranging between 31-35 % starch.

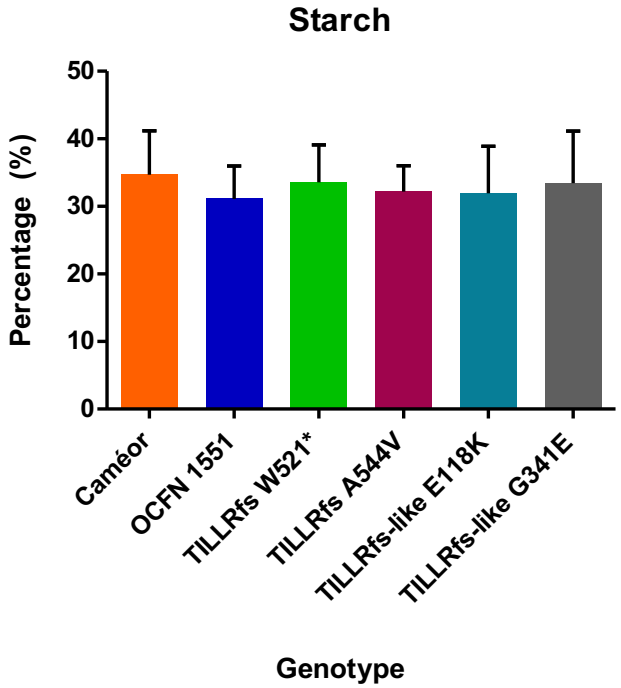


Figure 18. Starch concentrations (% w/w) in *rfs* pea mutants and Caméor cultivar. Concentrations of total starch were determined using the Megazyme assay of TILLING mutants for Rfs (TILLRfs W521* and TILLRfs A544V), and Rfs-like genes (TILLRfs-like G341E and TILLRfs-like E118K), the OCFN 1551 FN mutant and the Caméor line. Means

and standard deviations of three technical replicates from two biological replicates for each seed sample were analysed.

3.2.3 Effect of water deficit on agronomical traits of *rfs* pea mutants in the glasshouse

Given that the RFOs have been implicated in drought stress, the BCFN 1551 mutant was selected to determine the effect of RFO depletion in seeds on the plant fitness under water deficit (WD) conditions. Here, 25 plants of the Caméor cultivar and 25 plants of BCFN 1551 at 26 days post sowing (dps), were not watered for nine days in the glasshouse. After this period, a recovery watering regime was implemented during the following seven days, where the plants were watered with 20 mL daily. After the seven days, which corresponds to 42 dps in the plant development timeline, the plants were watered daily alongside their controls until plant senescence occurred. The control group of plants for both BCFN 1551 (n=25) and Caméor (n=25) was grown under a normal bench watering regime throughout the whole experiment until senescence.

3.2.3.1 Effect of controlled water deficit on plant phenotype

Plants of the Caméor cultivar and BCFN 1551 mutant were subjected to a WD for nine days (diagram of watering scheme can be found in Figure 13) (Fig. 19 A). At the end of this period, the WD BCFN 1551 plants appeared more affected than Caméor, as shown by the senescence of leaflets and stem (Fig. 19 A, right panel). Furthermore, following the recovery watering period, BCFN 1551 plants did not appear to recover at the same rate compared to Caméor, as the phenotype in their early leaf nodes and stem showed progression towards senescence even before recovery watering period (Fig. 19 B, right panel).

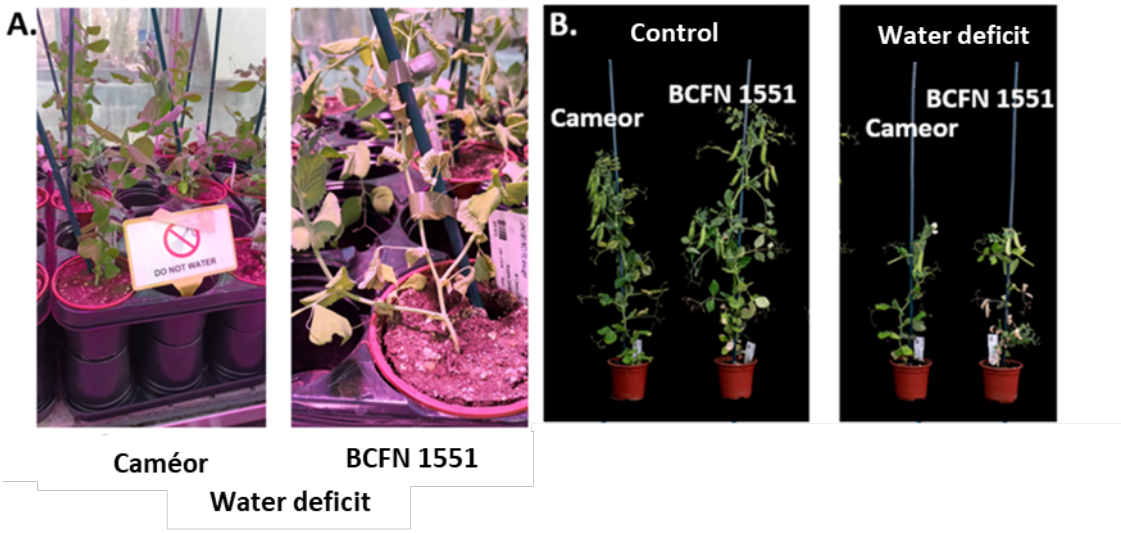


Figure 19. Development of Caméor and BCFN 1551 pea plants under water deficit (WD) conditions in the glasshouse. (**A**) Caméor and BCFN 1551 plants during the last day of WD period. (**B**) Control Caméor and BCFN 1551 plants compared to WD plants, at the end of the watering recovery period.

Following complete senescence, the seeds were harvested from each plant by collecting dry pods. As an initial measure of agronomical traits affected, the number of pods produced per plant was quantified (Fig. 20 A). There was a significant reduction in the pods produced by WD Caméor compared to Caméor controls (41.5 % reduction, p value < 0.0001) and the same occurred when comparing WD BCFN 1551 to BCFN 1551 controls (46.5 % reduction, p value <0.0001). Moreover, there was a significant difference in pod numbers in controls, between the BCFN 1551 plants compared to Caméor (15.8% increase in number in BCFN 1551, p value <0.005); this difference was not observed between the two genotypes in WD condition.

In addition, when the total seed yield, defined as total seed number per plant, was quantified (Fig. 20 B), a significant reduction was observed in WD Caméor compared to Caméor controls (37.7 % reduction, p value < 0.0001), and in WD BCFN 1551 compared to BCFN 1551 controls (49.4 % reduction, p value <0.0001). There was also a significant decrease in total seed yield for WD BCFN 1551 compared to WD Caméor (21.9% decrease, p value <0.0001). Despite BCFN 1551 control plants having produced more pods compared Caméor controls, no significant differences were found in the seed yield of the two genotypes in the watered controls.

Furthermore, all the seeds from each replicate were weighed and divided by the total number of seeds to obtain the average seed weight. No significant difference was observed between controls and WD conditions within the genotypes. However, in both controls and WD, BCFN 1551 had a significantly lower mean seed weight compared to Caméor seeds (9.8 % and 16.7 % reduction, with p values < 0.05 and < 0.01, respectively) (Fig. 20 C).

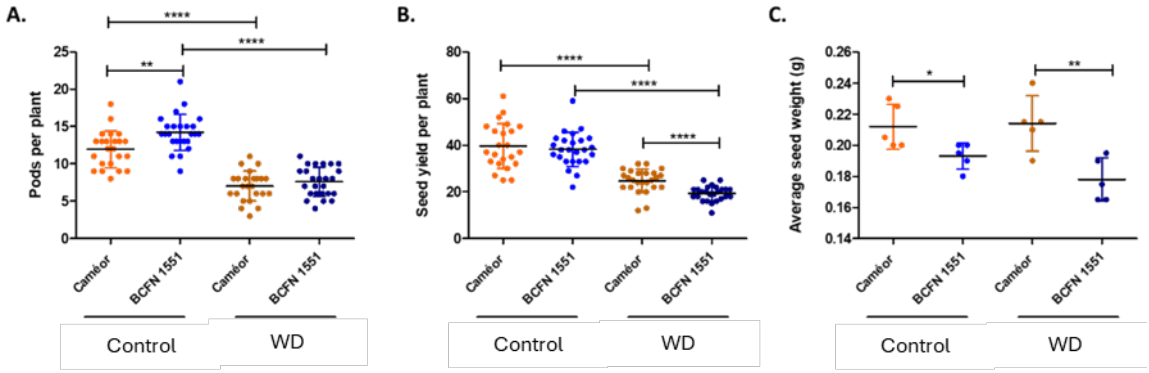


Figure 20. Seed yield traits in BCFN 1551 mutant and Caméor cultivar pea plants grown under controlled and WD conditions in the glasshouse. (**A**) Total pods produced per plant, (**B**) Total seed yield per plant, (**C**) Average individual seed weight measured in groups of five plants for each condition. Unpaired t-test (*) p value < 0.05, (**) < 0.01, (****) p value <0.0001. WD= water deficit.

Together these data indicated that the effect of WD was more pronounced in BCFN 1551 for total seed yield, which was significantly lower during WD compared to Caméor (Fig. 20 B) and showed an earlier senescence prior to recovery watering (Fig. 19 A).

Next, we evaluated the effect of WD condition on flowering completion as a measure of stress response in plants (Wada and Takeno, 2010). At 41 days post sowing (dps), 100% WD Caméor plants and ~80% of WD BCFN 1551 plants had flowers, respectively, while 100% WD BCFN 1551 plants had flowers at 42 dps (Fig. 21). In contrast, no Caméor and BCFN 1551 control plants had flowers at 41 dps, and plants from both genotypes started flowering on 42 dps with all plants flowering at 43 dps.

Pod formation also occurred earlier for WD Caméor and WD BCFN 1551 with no difference between genotypes. Here, at 46 dps, more that 50% of WD Caméor and WD BCFN 1551 plants had one or more pods, in contrast to control plants where fewer than 10% of plants had at least one pod on the same day (Fig. 21 B).

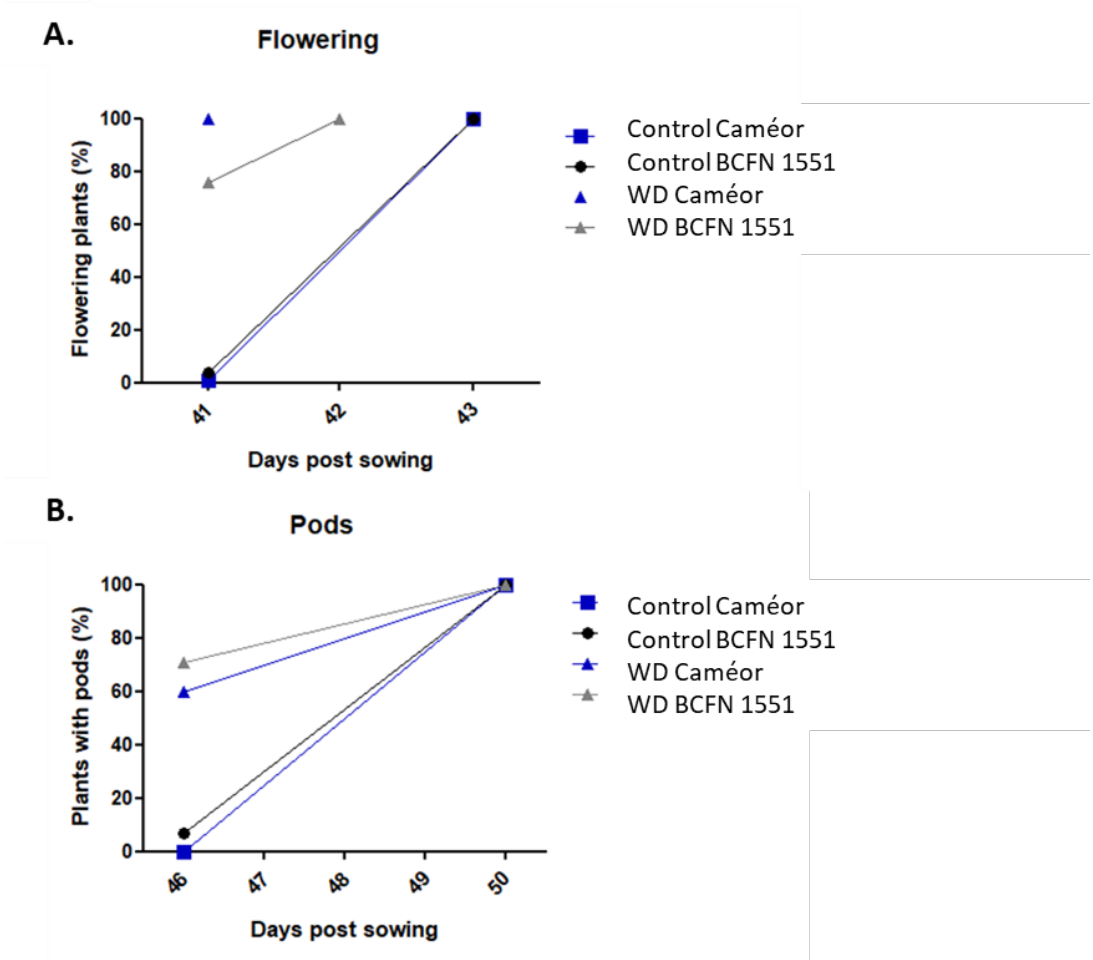


Figure 21. Flowering production and pod formation onset during the development of BCFN 1551 and Caméor cultivar under controlled watering and WD conditions. (**A**) Flowering onset on days post sowing, and (**B**) pod formation on days post sowing. Percentages calculated from observations of 25 plants per genotype under each condition. WD= water deficit.

Together, these data showed that although Caméor and BCFN 1551 grew to similar seed yield in the glasshouse, the induction of WD had a severe impact on pea plants, reflected in reduced pod production and seed yield when compared to control plants, and this effect was exacerbated in the BCFN 1551 mutans. Moreover, WD also induced

earlier flowering (by 1-2 days) and pod formation for both Caméor and BCFN 1551 (by 3 days), compared to control plants.

3.2.3.2 Effect of water deficit on RFOs in pea seeds

The same approach as described in section 3.2.1 was used to analyse RFOs in seeds harvested from the WD experiment. BCFN 1551 showed significantly higher levels of sucrose and galactinol, reduced level of verbascose and a complete depletion of raffinose and stachyose when compared to Caméor (Fig. 22), in agreement with data obtained previously from glasshouse-grown plants (Fig. 20).

Two main changes in RFO or precursor content were observed when comparing the seeds produced for each genotype, in control and WD plants: (1) a significant increase in verbascose levels in WD Caméor seeds compared to the seeds produced by Caméor controls (1.13-fold change, p value < 0.05) and (2) a significant increase in sucrose levels in WD BCFN 1551 seeds compared to the seeds produced by BCFN 1551 controls (1.10-fold change, p value < 0.05).

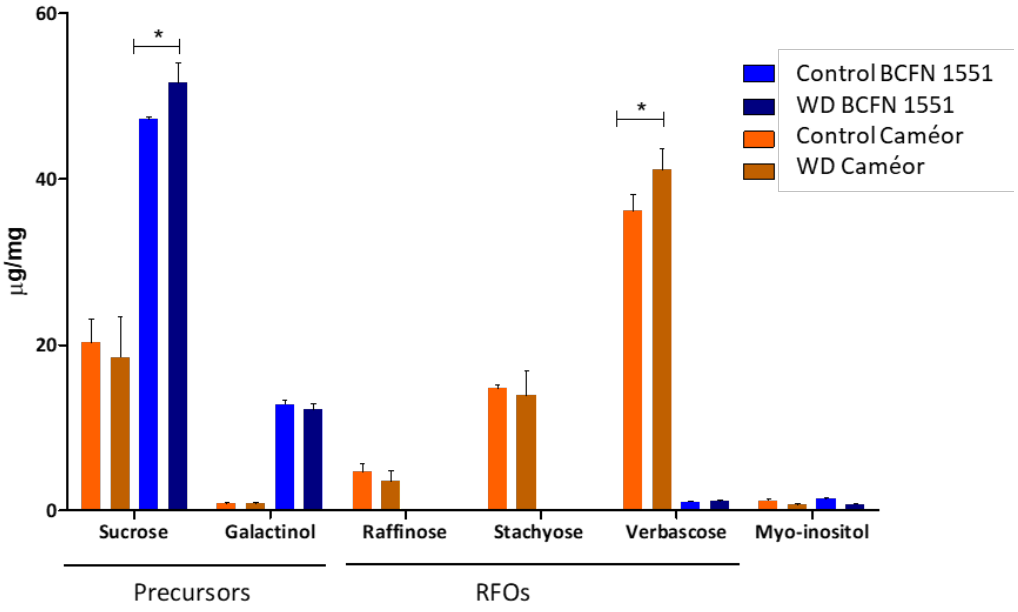


Figure 22. LC-MS analysis of RFOs and their precursors in mature seeds from pea mutants under controlled watering or WD conditions in the glasshouse. Sugars were extracted from dry seeds of Caméor and BCFN 1551. Three biological replicates were analysed by LC-MS, each in technical triplicates. The results show the means with respective standard deviations. WD= water deficit. Two-way ANOVA (*) p < 0.05.

These results suggests that RFOs and RFO precursor sucrose, may contribute in the plant responses to protection against drought stress.

3.2.4 Evaluation of agronomical traits of pea RFO mutants in the field

The mutants derived from the FN 1551/11-crosses in Caméor, namely OCFN 1551 (sown in 2022), BCFN 1551 (sown in 2023) and BC2FN 1551 (sown in 2024), were grown in the field to assess their performance under natural conditions. Each line was sown in triplicate using 100 seeds per microplot. Several agronomic traits were

evaluated, including germination, plant developmental traits and seed yield and quality, as presented below.

3.2.4.1 Effect on seed germination

The first parameter measured was the germination of the *rfs* mutant seeds sown in the field compared to Caméor seed germination. Here, we observed a significant reduction in the germination of OCFN 1551 compared to Caméor in 2022 (9.1 % reduction, p value < 0.01), the same occurred with the first backcross BCFN 1551 in 2023 (17.7 % reduced, p value < 0.005) and with the second backcross BC2FN 1551 in 2024 (27.4 % reduced, p value < 0.005).

In 2024, the first backcross of RfsTILL W521*, BCRfsTILL W521*, was also grown in the field, and the germination percentage for this mutant showed no significant difference compared to the Caméor cultivar (Fig. 23).

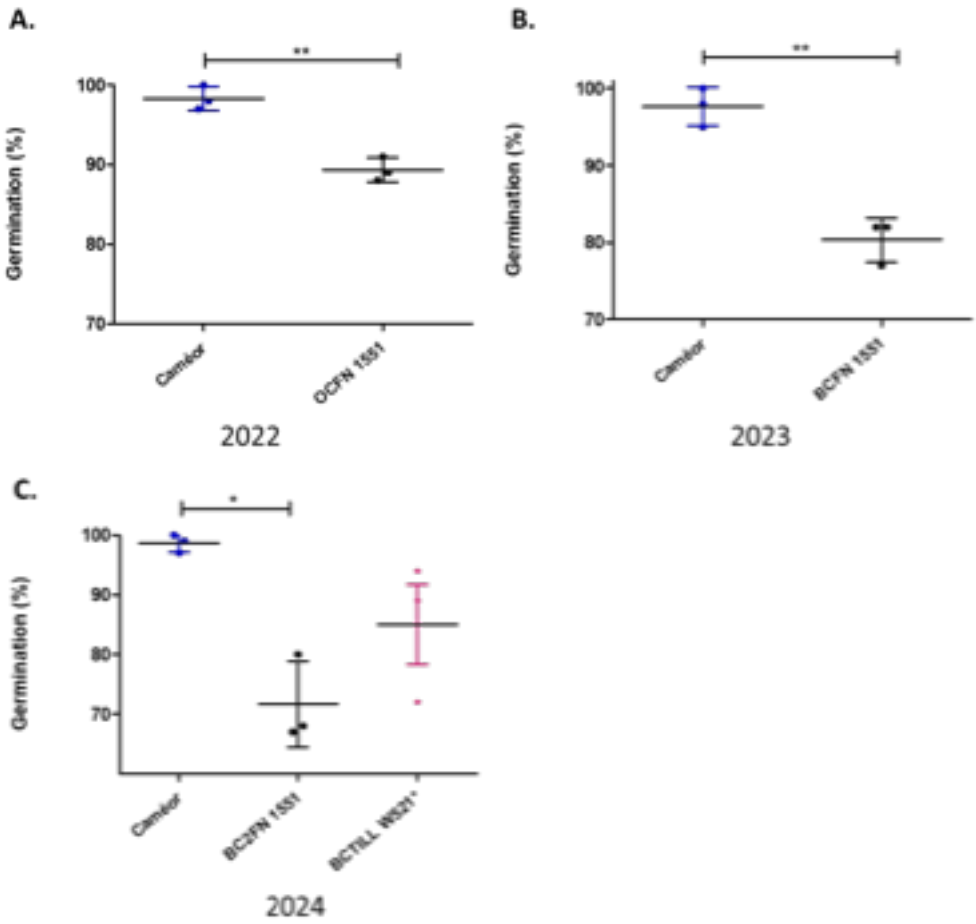


Figure 23. Germination percentage of *rfs* pea mutants and Caméor cultivar in the field. (**A**) 2022 field trial with OCFN 1551, (**B**) 2023 field trial with BCFN 1551 and (**C**) 2024 field trial with BC2FN 1551 and BCRfsTILL W521*. Each panel shows the mean germination percentage with standard deviations for three microplots, acting as technical replicates, per line per year. (*) p value< 0.01, (**) p value< 0.005.

3.2.4.2 Effect on plant development traits

The seedling development was monitored and one of the main differences observed between the FN *rfs* mutants and Caméor was the presence of yellow leaflets (in all years, involving: OCFN 1551, BCFN 1551 and BC2FN 1551), especially in the early stages between 2-4 weeks post sowing (wps) (Fig. 24). In 2024, the BCRfsTILL W521* was introduced in the field, and this mutant did not develop any leaflet yellowing or visible abnormality (Fig. 24 C).

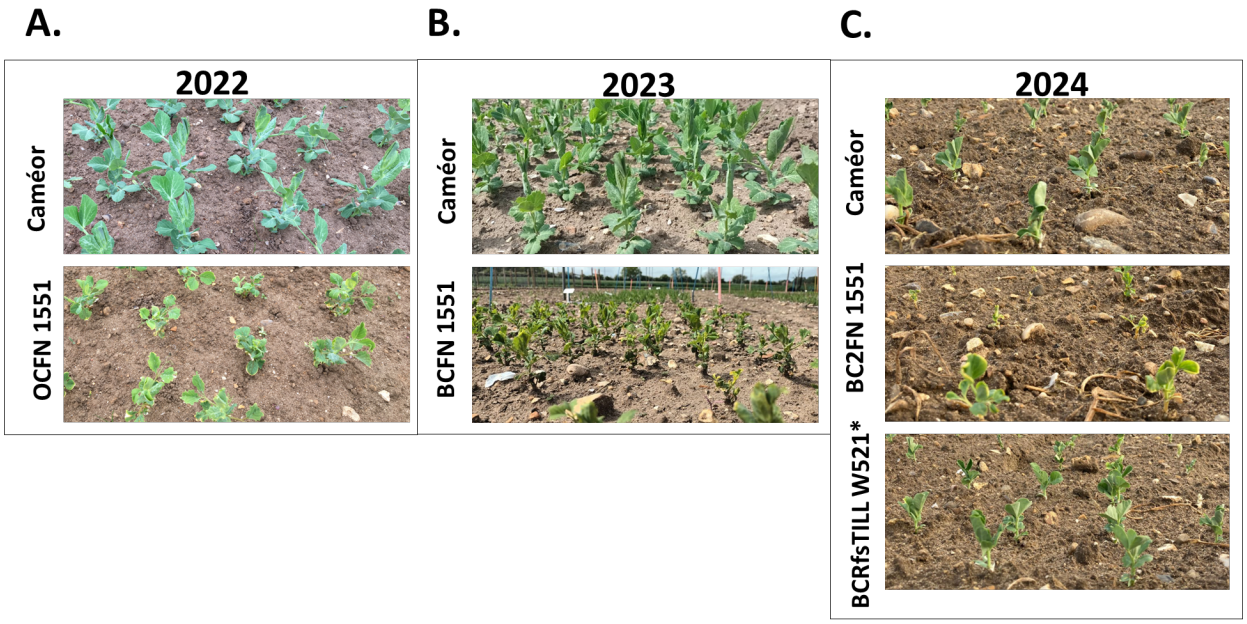


Figure 24. Close-up of plant leaflets of *rfs* pea mutants and Caméor cultivar in the field. The panels correspond to one representative view of the three microplots for each genotype. (**A**) 2022 field trial with Caméor (top) and OCFN 1551(bottom); (**B**) 2023 field trial with Caméor (top) and BCFN 1551 (bottom); (**C**) 2024 field trial with Caméor (top), BC2FN 1551 (middle) and BCRfsTILL W521* (bottom). All photos were taken at two weeks post sowing.

Moreover, it was observed that, between 4 and 7 wps, the FN-derived mutant plants developed more slowly and with a smaller phenotype in size compared to Caméor (Fig. 25). In contrast, the mutant BCRfsTILL W521* continued to show a phenotype more similar to that of the Caméor cultivar (Fig. 25 C), indicating that this mutant was less affected by the mutation.

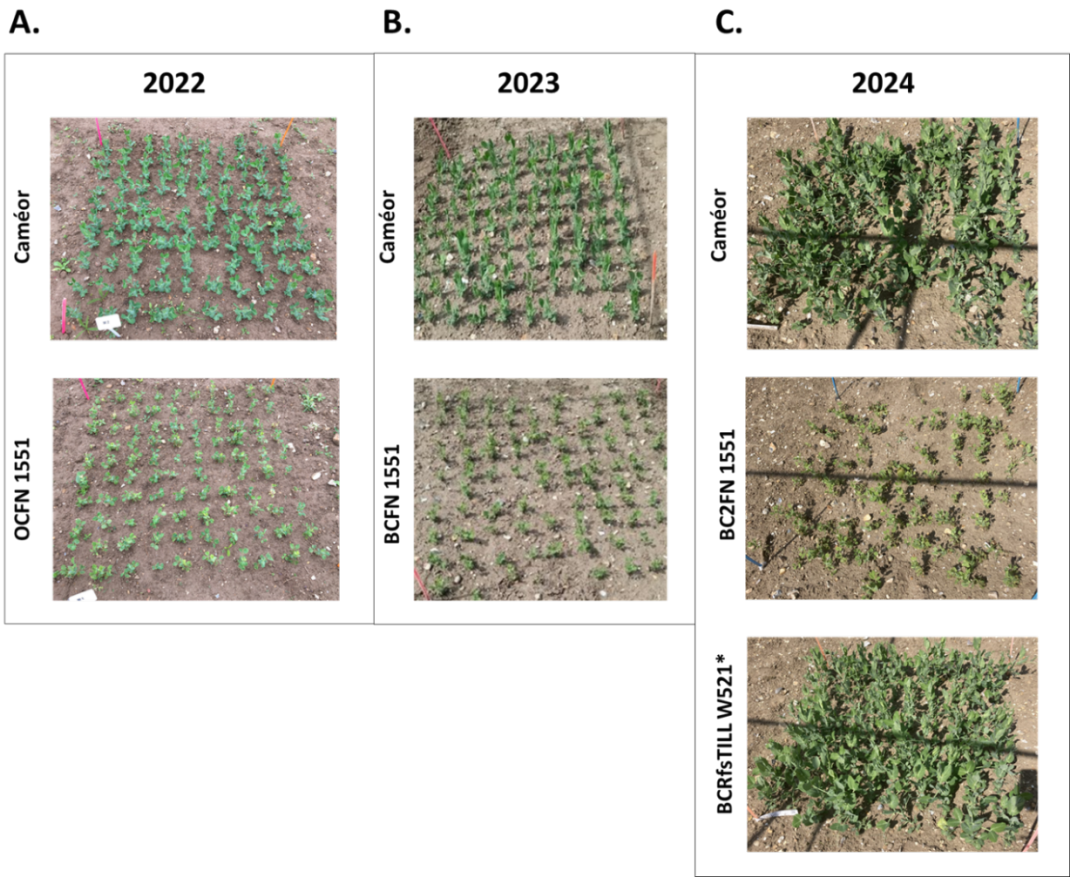


Figure 25. Development of *rfs* pea mutants and Caméor cultivar pea plants in the field. The panels correspond to one microplot representative of the three microplots for each genotype. (**A**) 2022 field trial with Caméor (top) and OCFN 1551 (bottom) at 4 weeks post sowing; (**B**) 2023 field trial with Caméor (top) and BCFN 1551 (bottom) at 4 weeks post sowing; (**C**) 2024 field trial with Caméor (top), BC2FN 1551 (middle) and BCRfsTILL W521* (bottom) at 7 weeks post sowing.

3.2.4.3 Effect on total seed yield and seed weight

Following harvesting of the plants at the end of the season, dry seeds were collected from the mature plants by mechanical sorting, and the total seeds produced in each microplot counted.

In 2022, the OCFN 1551 mutant showed a significant decrease in the average total seed yield (76.0 % reduction, p value < 0.005) compared to Caméor (Fig. 26 A). This also occurred in the year 2023 with BCFN 1551 (85.4 %, p value < 0.005) and in 2024 BC2FN 1551 (94.6 %, p value < 0.005). Further, the BCRfsTILL W521* showed a significant decrease in the average total seed yield (12.2 %, p value < 0.05) compared to Caméor.

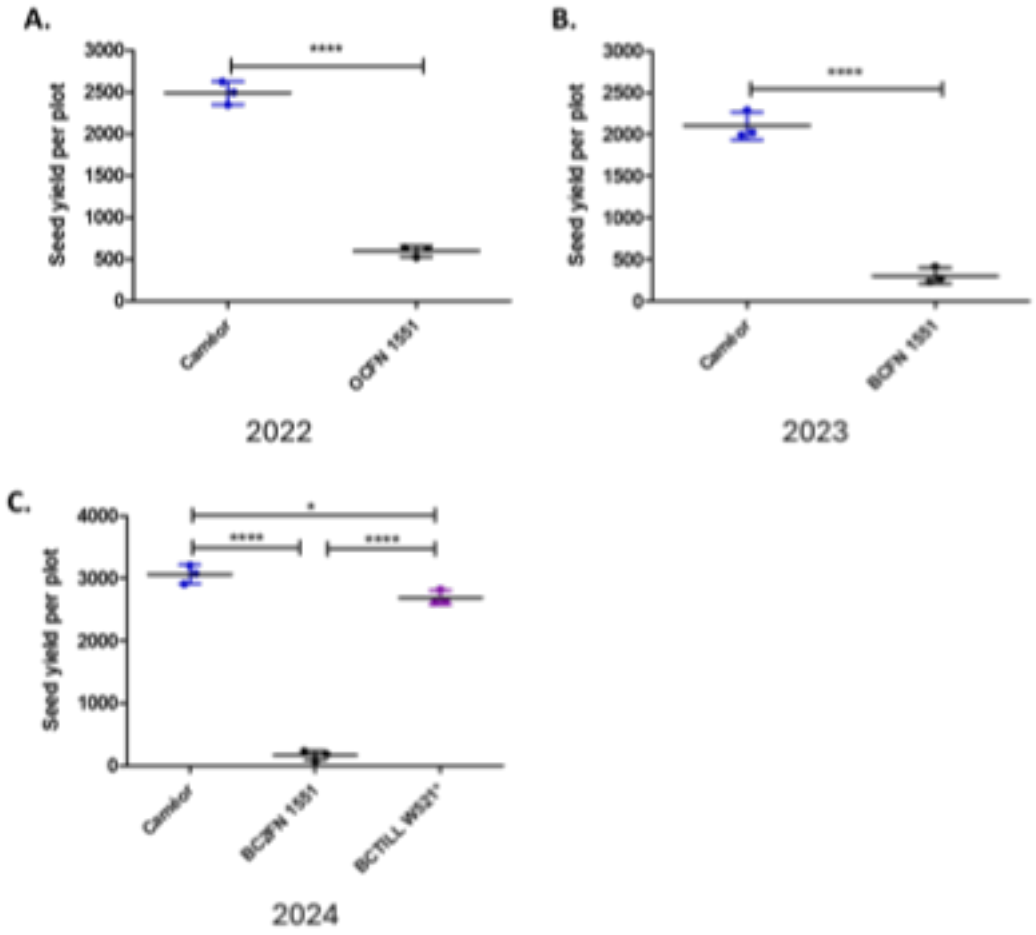


Figure 26. Total seed yield of *rfs* pea mutants and Caméor cultivar in the field. The total seeds produced in each microplot for each genotype were counted. (**A**) 2022 field trial with Caméor and OCFN 1551, (**B**) 2023 field trial with Caméor and BCFN 1551 and (**C**) 2024 field trial with Caméor, BC2FN 1551 and BCRfsTILL W521*. Means and standard deviations of three microplots, acting as technical replicates per pea line, per line per year. T-test, (*) p value < 0.05, (****) p value < 0.005.

In addition to lower seed yield, the FN-derived mutants (OCFN 1551, BCFN 1551 and BC2FN 1551) produced a reduced mass of seeds measured as 100-seed weight. This was quantified by measuring the weight of 100 seeds per genotype per microplot, in triplicate, and the average was used as a representative value for each year (Fig. 27). In 2022, the seeds produced by OCFN 1551 weighed significantly less than those of Caméor (25.5 % reduction, p value < 0.01). This was also reflected in 2023, with significantly lower seed weight in the seeds produced by BCFN 1551 compared to Caméor (28.6 % reduction, p value < 0.005) while in 2024 BC2FN 1551 compared to Caméor was reduced, not significantly (28.3 %).

Further, the BCRfsTILL W521* showed no difference in the average 100-seed weight compared to Caméor.

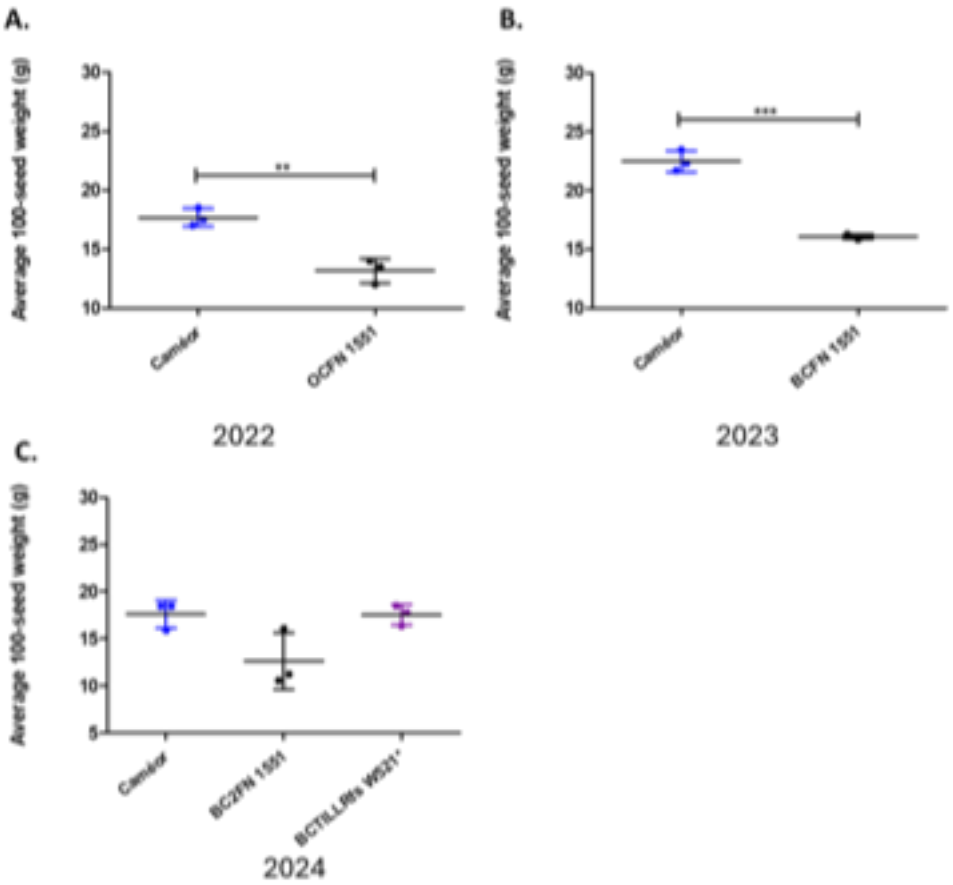


Figure 27. Average 100-seed weight of *rfs* pea mutants and Caméor cultivar in the field. Average of the weight of 100 seeds measured in triplicate for each microplot of each genotype. (A) 2022 field trial with Caméor and OCFN 1551, (B) 2023 field trial with Caméor and BCFN 1551 and (C) 2024 field trial with Caméor, BC2FN 1551 and BCRfsTILL W521*. Means and standard deviations of three microplots, acting as technical replicates, per line per year. T-test (**) p value< 0.01, (***) p value< 0.005.

3.2.4.4 Effect on insect-damaged seeds

We next aimed to assess insect damage in pea seeds to better understand its influence of the mutations on seed quality and overall crop performance. Here, seed damage by insects was evaluated by counting the seeds that had any form of insect damage, which was visualised by holes on or through the pea seeds. No statistical difference was found between the two genotypes in 2022 or 2023. Although there was more insect damage observed in the year 2022 (Fig. 28 A) than in 2023 (Fig. 28 B), this was a characteristic of the particular year rather than the genotype, as shown by the level of damage in both Caméor and OCFN 1551. Finally, in 2024, a significant increase in insect damage was observed in the seeds of Caméor and BCRfsTILL W521* when compared to BC2FN 1551 (p value <0.05 and p value < 0.01 respectively), while no significant difference was found between Caméor and BCRfsTILL W521*.

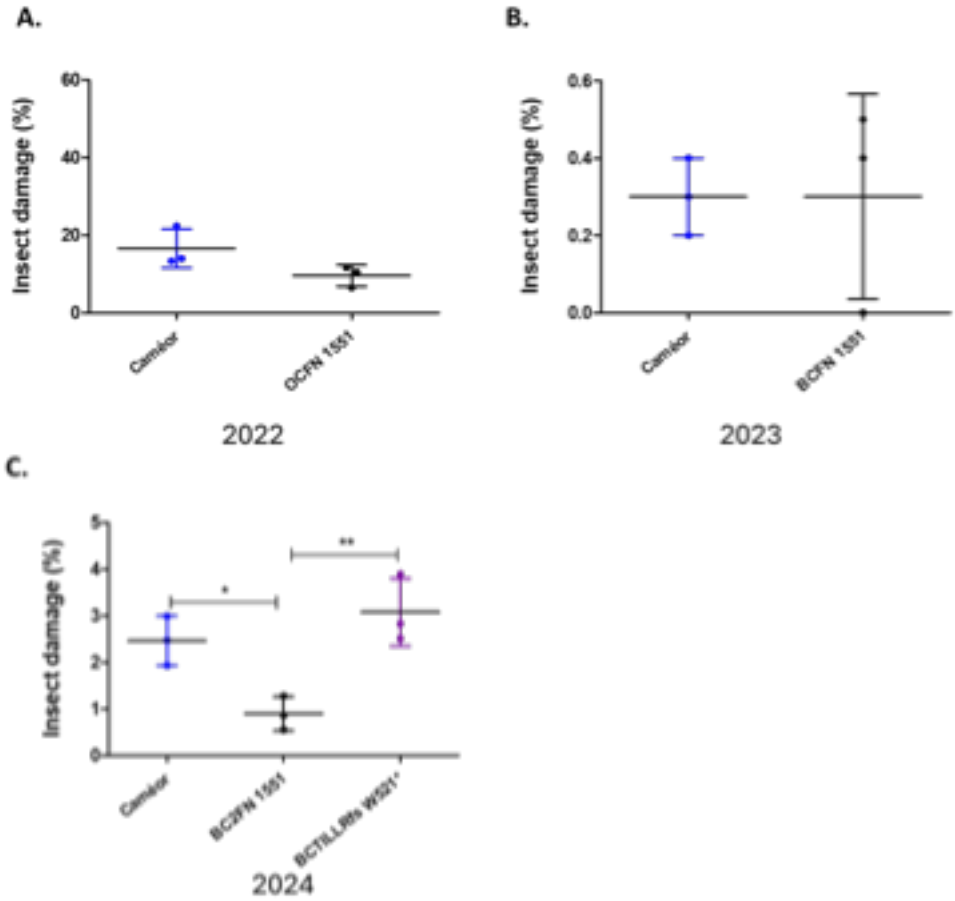


Figure 28. Percentage of insect damaged seeds in field trials of *rfs* pea mutants and Caméor cultivar in the field. Seeds with any insect damage were counted from each microplot of each genotype and expressed as % of the total seed number. (**A**) 2022 field trial with Caméor and OCFN 1551, (**B**) 2023 field trial with Caméor and BCFN 1551 and (**C**) 2024 field trial with Caméor, BC2FN 1551 and BCRfsTILL W521*. Means and standard deviations of three microplots, acting as technical replicates, per line per year. (*) p value <0.05, (**) p value <0.01.

Together these data indicate that in the field, the FN 1551/11 mutants had a lower performance in terms of germination and seed yield compared to Caméor, while BCRfsTILL W521*, grown in the field only one year, performed more similarly to Caméor.

3.2.5 The effects of pre-emergence herbicides on FN *rfs* mutant and control pea plants

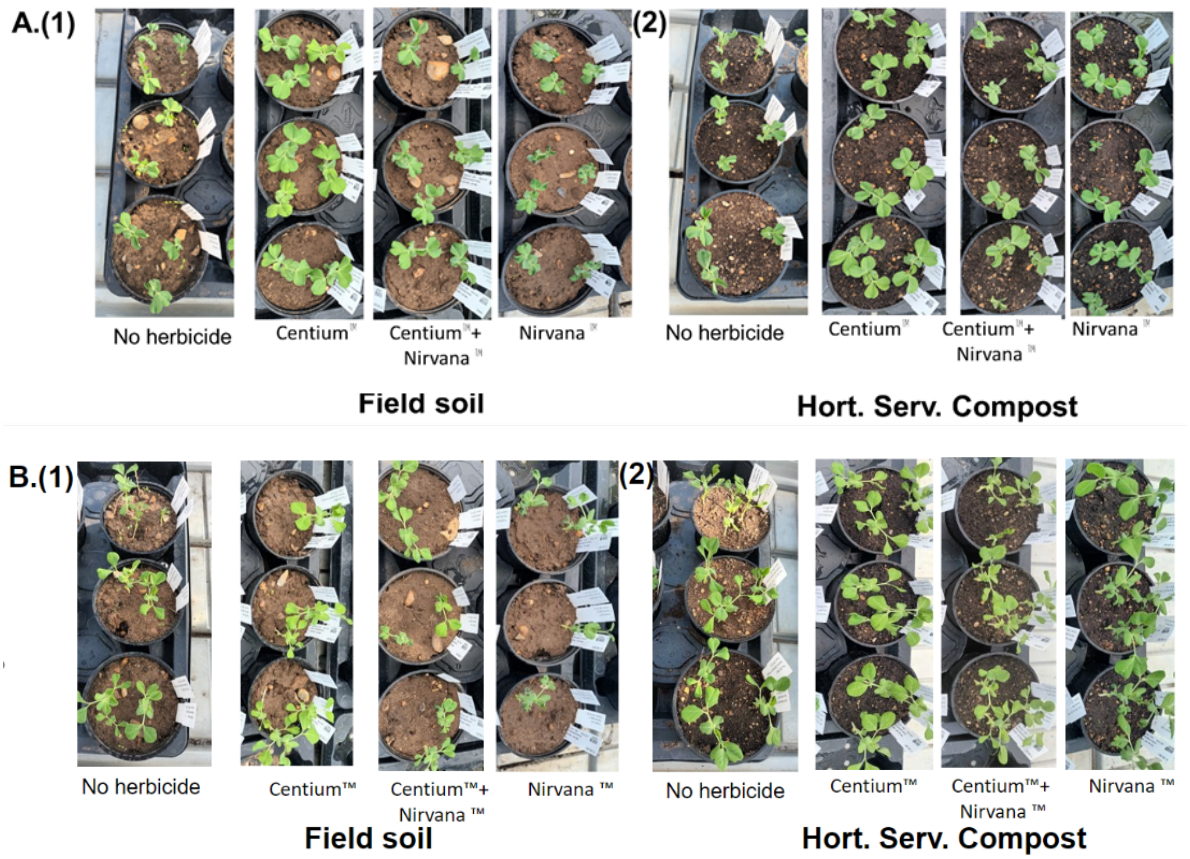
The reduced germination, yellowing plant phenotypes, and poorer plant performance observed for the OCFN 1551 and BCFN1551 mutants grown in the field in 2022 and 2023, respectively, was in marked contrast with the performance of these mutants in the glasshouse, which consistently grew comparably to the controls (see sections 3.2.3.1. and 3.2.3.2.). Therefore, we tested whether the field soil and/or the application of pre-emergence herbicides could have contributed to the impaired performance of the mutant lines in the field.

3.2.5.1 Herbicide trial in the glasshouse

Given the results observed in the field trial, particularly the FN 1551-derived mutants, seeds from the OCFN 1551 and Caméor (three seeds per pot in triplicates) were sown in the glasshouse using field soil or Horticultural Services compost cereal mix, the latter being the regular glasshouse compost mix; and subjected to herbicide treatment.

The pre-emergence herbicides used in the field to prevent weed growth were applied by spraying the soil on the day post sowing to replicate the conditions in the field. In the field, these consist of Centium™ (FMC Agro Ltd., UK) and Nirvana™ (BASF Ireland Ltd.). Here a combination of Centium™ and Nirvana™ was tested, as well as each herbicide individually.

All seeds germinated in the glasshouse and no yellowing of seedlings was observed upon herbicide treatments (Fig 29 A, B). We found a significant reduction in the germination in the field soil for the BCFN 1551 treated with Nirvana™ only, compared to the BCFN 1551 in the untreated field soil (p value < 0.05) (Fig. 29 C); however, this difference was not replicated for this genotype when sown in compost, suggesting the effect is more likely due to soil type than the application of pre-emergence herbicides.



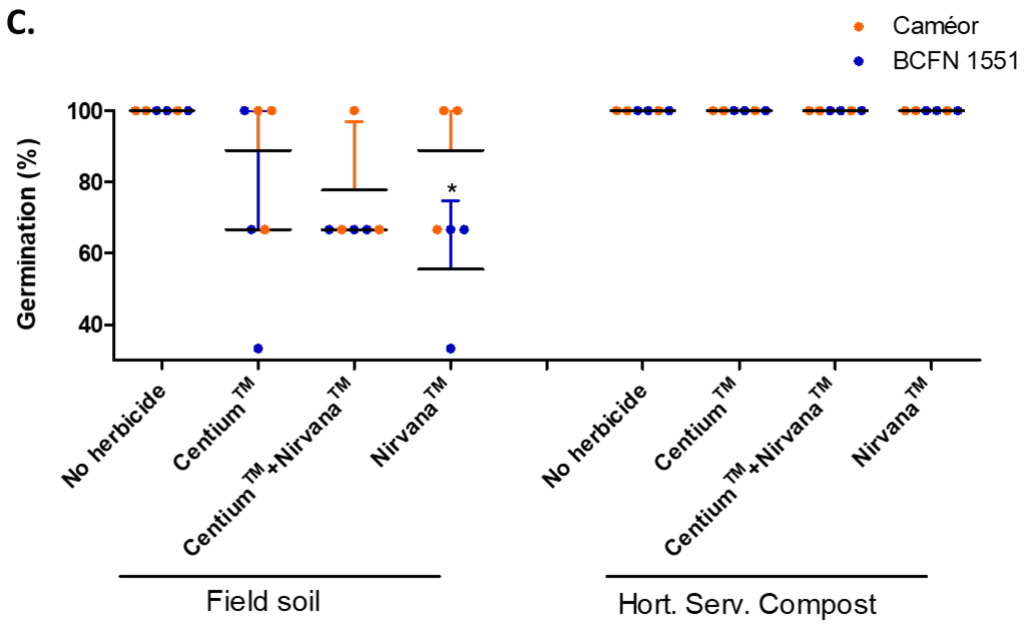


Figure 29. Effect of pre-emergence herbicides on the germination of BCFN 1551 *rfs* pea mutant and Caméor cultivar pea plants in glasshouse conditions. (**A**). Caméor plant germination in the absence (No herbicide) or presence of Centium™ (clomazone), a combination of Centium™ and Nirvana™ (clomazone+pendimethalin+imazamox), or Nirvana™ (pendimethalin+imazamox) using (1) field soil or (2) compost used by JIC horticultural services. (**B**) BCFN 1551 plant germination in the absence (No herbicide) or presence of Centium™, a combination of Centium™ and Nirvana™, or Nirvana™ using (1) field soil or (2) compost used by JIC horticultural services. (**C**) Germination percentage of BCFN 1551 and control plants in each pot growing in field soil or compost used by JIC horticultural services. Means and standard deviation plotted, statistical comparisons were made between each herbicide condition and no herbicide, within each soil type. (*) p value < 0.05.

3.2.5.2 Herbicide trial in the field

In the year 2023, an additional small pre-emergence herbicide trial was conducted in the field using the BCFN 1551 seeds (Fig. 30). Here, no difference in the phenotype of the BCFN 1551 plants was observed upon treatment with the pre-emergence herbicides (Centium™, Centium™ + Nirvana™ and Nirvana™) (Fig. 30 B, C, D) compared to BCFN 1551 control plants from the same genotype without herbicide application (Fig. 30 A). Furthermore, BCFN 1551 showed 10%, 0% and 20% germination difference compared to Caméor germination upon treatment with Centium™, Centium™ + Nirvana™ and Nirvana™, respectively (Fig. 30 E), which is similar to the germination rate difference obtained in the absence of herbicide treatment, which was 20%.

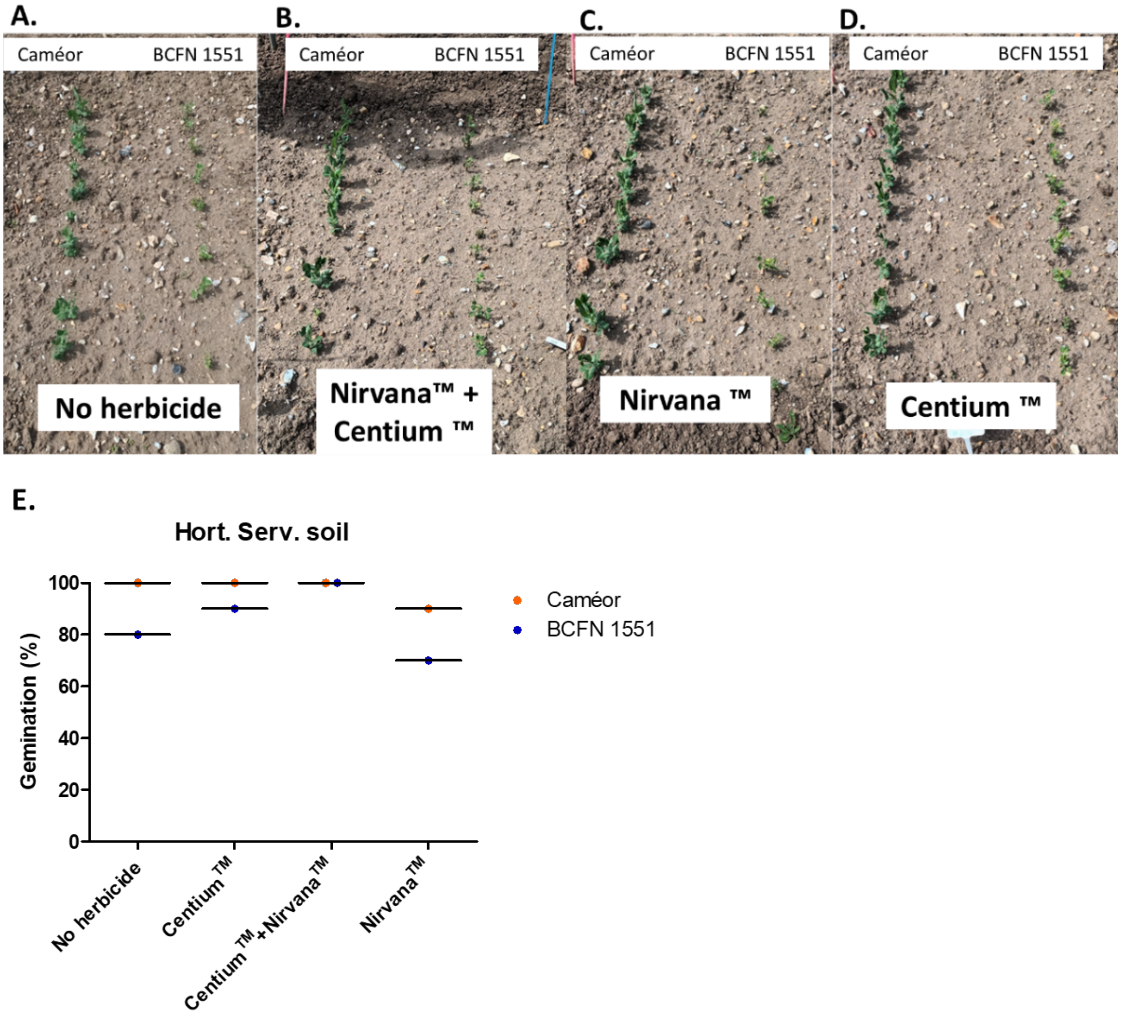


Figure 30. Effect of pre-emergence herbicides on germination for BCFN 1551 *rfs* pea mutant and Caméor cultivar in the field. A total of ten seeds per genotype, BCFN 1551 and Caméor cultivar were sown for each row where pre-emergence herbicide or no herbicide conditions were tested. (**A**) no herbicide control, (**B**) Nirvana™ + Centium ™, (**C**) Nirvana™ and (**D**) Centium ™. (**E**) Germination percentage of BCFN 1551 and Caméor plants in each row.

Together, these field trials suggested that the phenotypes observed for OCFN 1551 and BCFN 1551 in the field were not attributable to the application of pre-emergence herbicides, as no significant differences between BCFN 1551 and Caméor were found when the combination of Centium™+Nirvana™ was applied under glasshouse conditions, nor between no herbicide and Centium™+Nirvana application ™ in the field.

3.2.6 Nodulation of *R. leguminosarum* A3841 on *rfs* mutants and control pea plants

The effect of *rfs* genetic variation on RFOs and RFO precursors composition in seeds on the interaction with symbiotic root bacteria, was investigated through the root nodulation. *Rhizobium* nodulation was evaluated in the pea mutants BC2FN 1551 and BCRfsTILL W521*, along with Caméor control plants (Fig. 31). Inoculation with *R.*

leguminosarum A3841, a known bacterial symbiont in pea (Johnston and Beringer, 1975), was performed on pea seedlings grown in low nutrient media (see 2.1.5). At four weeks post inoculation, the total nodules were counted manually for ten plants per mutant. The results showed a significantly higher number of nodules in both BC2FN 1551 and BCRfsTILL W521* compared to Caméor (p value < 0.0001 and p value < 0.01 respectively).

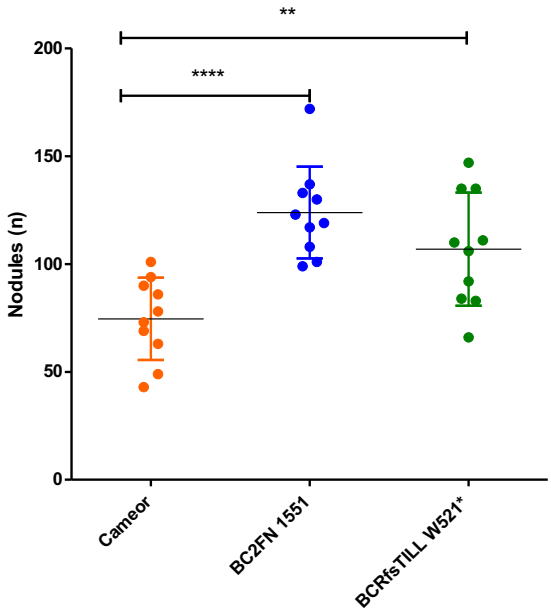


Figure 31. *R. leguminosarum* A3841-induced nodule formation in *rfs* pea mutants and Caméor control cultivar. Ten plants were analysed per mutant and control line to determine the nodulation efficiency in a unique experiment. Results represented as mean and standard deviation bars. (**) p value < 0.01, (****) p value < 0.0001.

3.3 Discussion

RFOs, a group of soluble tri-, tetra- and pentasaccharides abundant in the seeds of legumes, have been associated with numerous functions in plants, such as storage and nutrient transport (Sengupta *et al*, 2015). There is evidence for the role of RFOs in plant health and development during biotic and abiotic stresses (Nishizawa-Yokoi *et al*, 2008) and seed germination (Blöchl *et al*, 2007). However, little is known about the mechanisms underpinning their functional roles in abiotic stress tolerance in pea.

In this work, we first analysed the levels of RFOs and their precursors in the seeds of pea mutants with mutations in *Rfs* and *Rfs*-like genes, compared to the commercial cultivar Caméor. Our analysis showed that the total RFOs in Caméor were comprised of 8.4 % raffinose, 26.5 % stachyose and 65.1 % verbascose, in line with previous relative RFO values reported for pea seeds (Gawłowska *et al*, 2017; Kasprowicz-Potocka *et al*, 2022).

The OCFN 1551 mutant, lacking the entire *Rfs* gene, showed the most extreme RFO phenotype in seeds, with undetectable levels of raffinose and stachyose, and a significant reduction in verbascose, which correlated with increased concentrations of the precursors, sucrose and galactinol. The other *Rfs* mutants analysed, TILLRfs W521* and TILLRfs A544V, with targeted mutations in the *Rfs* gene, also showed significant reductions in RFOs. TILL RfsW521*, with a nonsense mutation, had a lower verbascose content than TILLRfs A544V compared to Caméor, and a higher accumulation of precursors, sucrose and galactinol. We observed a difference in the relative accumulation of the precursors, sucrose and galactinol, in the different mutants; these compounds were more abundant in the FN 1551/11 mutants, reflecting the more severe effect of the FN *rfs* mutation on the RFO pathway.

The mutation in TILLRfs W521* is a nonsense mutation, leading to the replacement of Trp with a premature stop codon in position 521. This type of mutation often results in non-functional proteins due to their premature termination. However, our RFO analysis showed that the TILLRfs W521 pea mutant had detectable levels of all RFOs in the seeds, in contrast to the FN 1551-derived crosses where the entire *Rfs* gene was deleted, along with other genes within the 1.3 Mb deletion. This may suggest that the mutation led to the synthesis of a truncated Rfs protein with partial function or that another gene (*Rfs-like*) was able to synthesise the RFOs in this mutant. Supporting the latter possibility, we showed that the TILLRfs-like E118K mutant resulted in reduced RFO content in seeds. However, RFO content in TILLRfs-like E118K seeds was not as reduced as with the *rfs* mutants. Although RFO content in *TILLRfs*-like *E118K* seeds was not as strongly reduced as in *rfs* mutants, the substantial (>50%) reduction observed in the *TILLRfs W521** line supports the conclusion that *Rfs* is the major isoform involved in seed RFO biosynthesis. However, due to uncertainty regarding the functional impact of the *E118K* mutation, the contribution of *Rfs*-like to RFO biosynthesis cannot be excluded. Future work is needed to include the backcrossing of *Rfs-like* mutants, and further evaluation of their performance in the field.

Rfs is part of the α-amylase protein family (GH13), comprising the largest GH (Stam *et al*, 2006). This family is characterised by a TIM-barrel fold made of three domains (A,

B, C) with the active site cleft being located between domains A and B, where a triad of catalytic residues (Asp, Glu and Asp) are required for catalysis (Wang, 2023). We showed that Rfs and Rfs-like sequences of pea share the conserved catalytic region of AtAkαGal3, recently characterised in *A. thaliana* (Chuankhayan, 2023). In pea, part of this domain (Asp residue in position 426) has also been previously described as catalytic for pea Rfs (Peterbauer, 2002), however the recent crystallography complexes found on AtAkαGal3 (Chuankhayan, 2023) provide a model for the understanding of pea Rfs, were other residues are involved on its catalytic domain. Importantly, our sequence alignment indicates that the W521* mutation occurred downstream of this domain. It is therefore possible that the truncated Rfs enzyme retained some of the required catalytic machinery for RFO biosynthesis, although we cannot exclude that the RFOs detected are attributable to Rfs-like enzymes or to another pathway, e.g. the galactinol-independent pathway, less studied in pea.

Only *A. reptans* and *Coleus blumei* are confirmed so far to use a galactinol-independent pathway for the synthesis of RFOs (Bachmann, 1994; Gilbert, 1997). This pathway has been implicated in the synthesis of larger RFOs, stachyose and verbascose and the hexasaccharide ajugose (only described in *A. reptans*) via the enzyme galactan-galactan galactosyl transferase (GGT) (Bachmann, 1994), which catalyses the transfer of a galactosyl moiety from one RFO, such as raffinose, to another RFO. However, there is evidence that the galactinol-independent pathway of RFO biosynthesis is active in pea seeds, via an uncharacterised enzyme, different from GGT, which requires raffinose to be present to synthesise stachyose and verbascose (Peterbauer, 2001). Thus, it is tempting to speculate that, in the FN-1551/11-derived mutants where only the largest of RFOs, verbascose, is detected albeit at low levels, Rfs-like or another putative Rfs (not explored here) may lead to the synthesis of low quantities of raffinose, that could be used in the galactinol-independent pathway towards the formation of verbascose, hence raffinose becoming undetectable. Interestingly, the activity of GGT has been found to be significantly less compared to verbascose synthase during the seed development of another legume, *Vicia villosa* (winter vetch) (Lahuta, 2006). RNA-sequencing data would also allow us to monitor the gene expression of *Rfs*, *Rfs*-like and *GGT* across pea plant and seed development.

Another possibility that could lead to the partial function of Rfs in the TILLRfs W521* mutant, could be that a non-canonical translational event occurs, in which an amino acid is incorporated in the stop codon site. This process is known as stop codon readthrough (SCR), and it occurs when a ribosome reads a stop codon as a sense codon which can generate a protein isoform with novel biological functions that alter its activity, stability or subcellular localization (Rodnina, 2019; Palma, 2021). SCR events have been recently described to be commonly present in monocots and dicots, where proteogenomic strategies have allowed the detection of 179 SCR events in soybean and 265 SCR events in *Arabidopsis* (Zhang, 2024). However, the understanding of the occurrence of SCR events is incomplete (Blanchet, 2018 ;Aerni, 2015), and known mechanisms cannot explain some of the amino acids recorded at stop codons (Zhang, 2024). This reinforces the need for in-depth investigations of SCR events in plants, and particularly in pea, to help elucidate the mechanism of stop mutations. In addition, further work is needed to characterise the enzymatic activities of Rfs and Rfs-like

enzymes in pea to discriminate between the different hypotheses, one way to achieve this could be through transient expression experiments.

Drought stress is a factor known to limit crop production worldwide, by depriving plants of water required for their growth (Cruz de Carvalho, 2008). Raffinose is thought to play a role in plant drought stress tolerance, based on i) its accumulation in leaves when plants encounter drought stress (Liu et al., 2023), and ii) the induction of *Rfs* expression in pea seedlings under dehydration conditions (Lahuta et al., 2014). Following the low performance of the OCFN 1551 in the field in 2022 (when extreme drought was experienced during early seedling growth), a water deficit experiment was conducted in the glasshouse using BCFN 1551 and Caméor plants, to understand whether deletion of the major *Rfs* in pea could be responsible for this effect. The water deficit led to an earlier senescence in the BCFN 1551 plants, and a significant increase in sucrose in the seeds, with no changes in RFOs, compared to the control BCFN 1551. Drought conditions have been shown to increase sucrose synthesis, causing an increase in the accumulation of sucrose, as previously shown in rice subject to drought stress during grain filling (Yang et al., 2001). It was only in the water deficit Caméor seeds, where verbascose was observed to be slightly but significantly increased compared to the control; this suggests that this RFO, the longest carbohydrate in the family, may have a role in the response of pea plants to environmental stress. Similarly, in maize, a major raffinose synthase (ZmRAFS) was found to enhance drought tolerance by synthesising more raffinose (Li et al., 2020). Moreover, the fact that the RFO content in the BCFN 1551 seeds (lacking *Rfs)* was not affected by controlled water deficit conditions suggests that Rfs has more impact in RFO production during seed formation than the putative *Rfs*-like, which is present in both Caméor and BCFN 1551, possibly leading to a baseline production of RFOs. Similarly, in the model plant organism, *A. thaliana*, it has been demonstrated that out of six identified putative *Rfs* genes (Nishizawa-Yokoi, Yabuta and Shigeoka, 2008), only one has been functionally linked to raffinose biosynthesis in leaves under water deficit (Egert, Keller and Peters, 2013). Furthermore, the mutant of this raffinose synthase isoform in *A. thaliana* produced seeds with detectable raffinose levels, suggesting the existence of at least another specific raffinose synthase expressed in seeds (Egert, Keller and Peters, 2013). Such scenario may occur with the FN 1551/11-derived mutants, where verbascose remains detectable, further underscoring the need to study the range of Rfs and Rfs-like isoforms in pea.

Under biotic or abiotic stresses, crop yield has been directly related to carbon fluxes in the plant, where a significant part of the carbon fixed by photosynthesis is diverted to different organs such as the roots (Durand *et al.*, 2016). In soybean, when plants were under stress during flowering, seeds showed a significant increase in sucrose (Du *et al.*, 2020). Moreover, when drought stress occurs, leaf senescence is accelerated (Rane *et al.*, 2021). It is therefore possible that the sucrose increase in BCFN 1551 under water deficit could be a result of changes in carbon flux, while in Caméor, sucrose can be directed to the formation of more complex oligosaccharides for carbon reserve, such as verbascose. However, carbon fluxes in pea are highly dependent on time and length of the environmental changes or stressors, and, even under highly controlled conditions, the carbon allocation in source-sink dynamics is different during the plant`s developmental stages (Morin *et al.*, 2022). This underscores the complexity

of compensatory mechanisms of carbon flux, which under water deficit conditions in pea remain to be further explored.

While the FN 1551 mutant grew in the glasshouse under standard conditions similarly to Caméor, its performance was drastically impaired in the field over three consecutive years, which is in marked contrast with the BCRfsTILL W521*mutant which growth was unaffected (though the latter was only tested during one season in the field and backcrossed once). This highlights BCRfsTILL W521* as a promising candidate for future investigations and underscores the importance of testing mutants in the field.

It cannot be excluded that the different phenotypes associated with FN derived mutants in the glasshouse and in the field are due to disruptions in other metabolic or regulatory pathways. The *Apu* (*elp-1 like*) gene is an orthologous gene to *Elp1* in *Medicago truncatula* (Chen *et al.*, 2012). Mutations in *Elp1* have shown an abnormal leaf movement and effects on plant hormone signalling, particularly auxin-related genes (Bai *et al.*, 2022). In addition, *Tryptophan aminotransferase-related protein (TAR)* which is also within the deleted region of FN-backcrossed mutants, is involved in auxin biosynthesis in several plants including *Lotus japonicus* and rice (Bai *et al.*, 2022; Kakei *et al.*, 2017). Auxin is a key plant growth hormone, and these two deleted genes, within the 1.3 Mb deletion, can have an impact leading to deficient auxin signalling and biosynthesis. In pea it has been shown that a mutation in *PsTAR2* gene reduces the auxin content in seeds, leading to smaller seed size (McAdam *et al.*, 2017). Furthermore, the role of auxin has also been studied in drought stress, for example in rice and potato, where auxin overproduction has been found along with an enhanced drought tolerance (Colebrook *et al.*, 2014). Moreover, changes in auxin also suggest a possible explanation as to why the chlorosis phenotype (leaf yellowing) of the FN 1551/11-backcrossed mutants is present in the field, where soil temperature and rainfall are variable throughout the trial. Previous studies exist in which auxins have been identified in regulating iron deficiency in roots (Lin *et al.*, 2016; Roman *et al.*, 2023), and iron deficiency has also been linked to leaf chlorosis (Therby-Vale *et al.*, 2022). It thus seems plausible that the combination of environmental stressors in addition to fewer nutrients present in the field soil, compared to the glasshouse compost, could alter further the phytohormonal response in the FN 1551/11-backcrossed mutants.

Our preliminary nodulation experiment revealed a significantly higher *R. leguminosarum* A3841-induced nodule formation in the *rfs* mutants, BCFN 1551 and BCRfsTILL W521*, compared to Caméor. This rhizobium strain induces root nodule formation in legumes such as pea and lentil (Karunakaran *et al*, 2009). This is important when considering rhizobium strains for infecting roots, as nodulation depends on pea genotype and rhizobium strain compatibility, which are also influenced by soil conditions and the microbial ecology present (Clúa *et al*, 2018). Studies have shown that sucrose stimulates nodule formation (Yoshida *et al*, 1978) and that sucrose synthase (SucS) is crucial for nodulation and nitrogen fixation (Anthon *et al*, 1990), as shown in pea mutants with severely reduced SucS activity (Gordon *et al*, 1999). Moreover, dicarboxylates and sucrose are the main carbon sources within nodules, and in ineffective nitrogen fixing mutant nodules low levels of sucrose have been found (Pini *et al*, 2017). Since FN-derived and TILLRfs W521* pea mutants accumulate

sucrose because of reduced RFO production, this may influence the observed changes in nodulation. However, increased nodule numbers do not necessarily result in higher total nitrogen fixation (Kassaw *et al*, 2015). In the future, in addition to nodule counts, the effects of *rfs* mutation on nodulation, could be further investigated by quantifying nodule and plant biomass, along with the nitrogen-fixing capacity of the formed nodules.

Moreover, although the BCRfsTILL W521* mutant has only been tested in the field during one season, it has shown a better adaptability to natural conditions than the FN 1551/11-derived mutants. For comparison, a soybean mutant with significantly reduced raffinose synthase activity in developing seeds, and reduced levels of raffinose and stachyose and increases in the precursor sucrose (Hitz *et al.*, 2002; Kerr, 2000), did not show differences in germination, seed yield and plant maturation under field conditions (Neus, Fehr and Schnebly, 2005). The BCRfsTILL W521* mutant shows that it is possible to produce pea seeds from a mutant that has significantly reduced RFO content in seeds and that can perform well when growing in field conditions. In addition, this mutant will be more suitable to study the effects of environmental stressors such as water deficit in pea in the future, where the effects will be directly related to the *Rfs* mutation.

In the future, further backcrossing of the different *rfs* mutants and monitoring of their agronomical traits under field conditions will allow near-isogenic lines to be obtained which can be more meaningfully compared and could be tested commercially. Moreover, mutants for the putative *Rfs*-like gene (TILLRfs-like E118K and TILLRfs-like G341E) provide a resource to further evaluate the mechanisms involved in RFO synthesis in pea and its different organs.

4 . Chapter Four: Effects of RFOs and RFOs in pea on the human gut microbiota and barrier function

4.1 Introduction

The human gut microbiota is a highly diverse microbial community that influences host health by metabolising dietary components and promoting intestinal homeostasis (Hou *et al.*, 2022) (see section 1.3.).

As shown in Chapter 3, RFOs accumulate in the seeds of the commercial pea cultivar Caméor at different concentrations i.e. raffinose (4.2 µg/mg), stachyose (15.6 µg/mg) and verbascose (44.7 µg/mg). Pea seeds can be used for human consumption under the form of vegetable or pulses as part of the dietary intake of legumes. Legumes are a sustainable food supply due to their nutritional quality, health properties and low carbon footprint (Tulbek, Wang and Hounjet, 2024) (see section 1.1.1.2.).

However, the fermentation of RFOs by the gut microbiota has been associated with intestinal discomfort and bloating in humans, limiting increased legume consumption (Padalkar *et al.*, 2023). Indeed, while the fermentation of fibre and large carbohydrates can lead to the production of gas, small carbohydrates, like RFOs, which are rapidly-fermented are often implicated rapid production of gas (Mutuyemungu *et al.*, 2023).

However, in recent years, RFOs have gained increasing attention due to their potential prebiotic effects (Elango *et al.*, 2022) (see section 1.4.3). For example, raffinose, verbascose and stachyose have been shown to sustain the growth of probiotic strains such as *L. reuteri*, *P. pentosaceus* and *B. lactis* NO19™ (Hufnagel *et al.*, 2018). Moreover, raffinose fermentation using human faecal microbiota resulted in increased lactate and SCFAs, and the relative abundance of *Bifidobacterium* and *Lactobacillus* (Amorim *et al.*, 2020).

Regarding their health-promoting potential, the influence of fermented prebiotics and SCFAs on intestinal permeability has been extensively studied *in vitro* using epithelial cells such as Caco-2 cells (Peng *et al.*, 2007; Wang *et al.*, 2012; Isayama *et al.*, 2023) with SCFAs shown to improve barrier function in enterocytes (Zheng *et al.*, 2020; Pearce, Karl and Weber, 2022) and, more recently, using advanced tissue models based on intestinal organoids grown on transwells or gut-on-chips, as shown with fermented HMOs (Šuligoj *et al.*, 2020). Faecal supernatants from an *in vitro* fermentation with raffinose, inulin, lactulose, partially hydrolysed guar gum, indigestible dextrin or alginate have been shown to protect and maintain intestinal barrier functions during an inflammation-induced model, compared to cells treated with un-supplemented fermentation (Kono *et al.*, 2023).

Although the study of individual RFOs provides relevant mechanistic information on the induced microbial changes, metabolites produced and associated role in gut health, there is a need for research focusing on the prebiotic effect of RFOs within the food matrix.

This chapter explores the effects of RFOs within the pea flour and extract of the BCFN 1551 and Caméor cultivar characterised in Chapter 3, and as individual compounds, on the modulation of the human gut microbiota composition and metabolite production using an *in vitro* batch fermentation. Moreover, the effects of the fermentation of Caméor and BCFN 1551 pea on gut barrier function were explored using a human colonic organoid monolayer model.

4.2 Results

4.2.1 Effect of RFOs on gut microbiota structure and function

The following two sections (4.2.1.1. and 4.2.1.2.) outline the results of the investigation into the effects of individual RFOs and their precursors, sucrose and galactinol, as well as pea flour and extracts from Caméor and BCFN 1551 on the gut microbiota using an *in vitro* batch fermentation model.

Prior to fermentation, the individual RFOs and precursors, and pea flour were added as carbon sources in the medium at a concentration of 2 mg/mL. Pea extracts used for the supplementation were obtained by extracting RFOs from 2 mg/mL pea flour (see 2.1.6.). These were added to batch models seeded with a human faecal sample (HD1) and fermentations were conducted for 24 h. A medium-only control (with no supplementation) was also included.

It should be noted that the concentrations of RFOs and precursors in the pea material (flour and extract) as determined by LC-MS (see 3.2.2.) are different to that used here for the individual sugars. Consequently, while statistical comparisons were made between different individual sugars and between different pea derived material (flour or extracts) treatments, no statistical comparisons were performed between individual sugar and pea treatments.

4.2.1.1 Effect of RFOs on the human gut microbial composition in an *in vitro* batch fermentation

We first determined the effects of fermentations with individual RFO precursors (sucrose and galactinol), RFOs (raffinose and verbascose) and pea (flour and extract) from Caméor and BCFN 1551 on alpha and beta diversity of the faecal microbiota composition.

The alpha diversity of species richness was evaluated using Shannon Index (Fig. 32 A). The original inoculum HD1 showed the highest species richness compared to any of the supplemented fermentations. Moreover, the observed species richness was significantly different between the treatments (ANOVA, $p = 0.01$).

Based on the taxonomic composition between samples, or beta diversity, three clusters were identified using Principal Coordinate Analysis (PCoA) (Fig. 32 B). A first cluster encompassing fermentations with sucrose, raffinose and verbascose (C1), a second cluster for the galactinol condition (C2) and a third cluster comprising pea flour and pea extract treatments (C3).

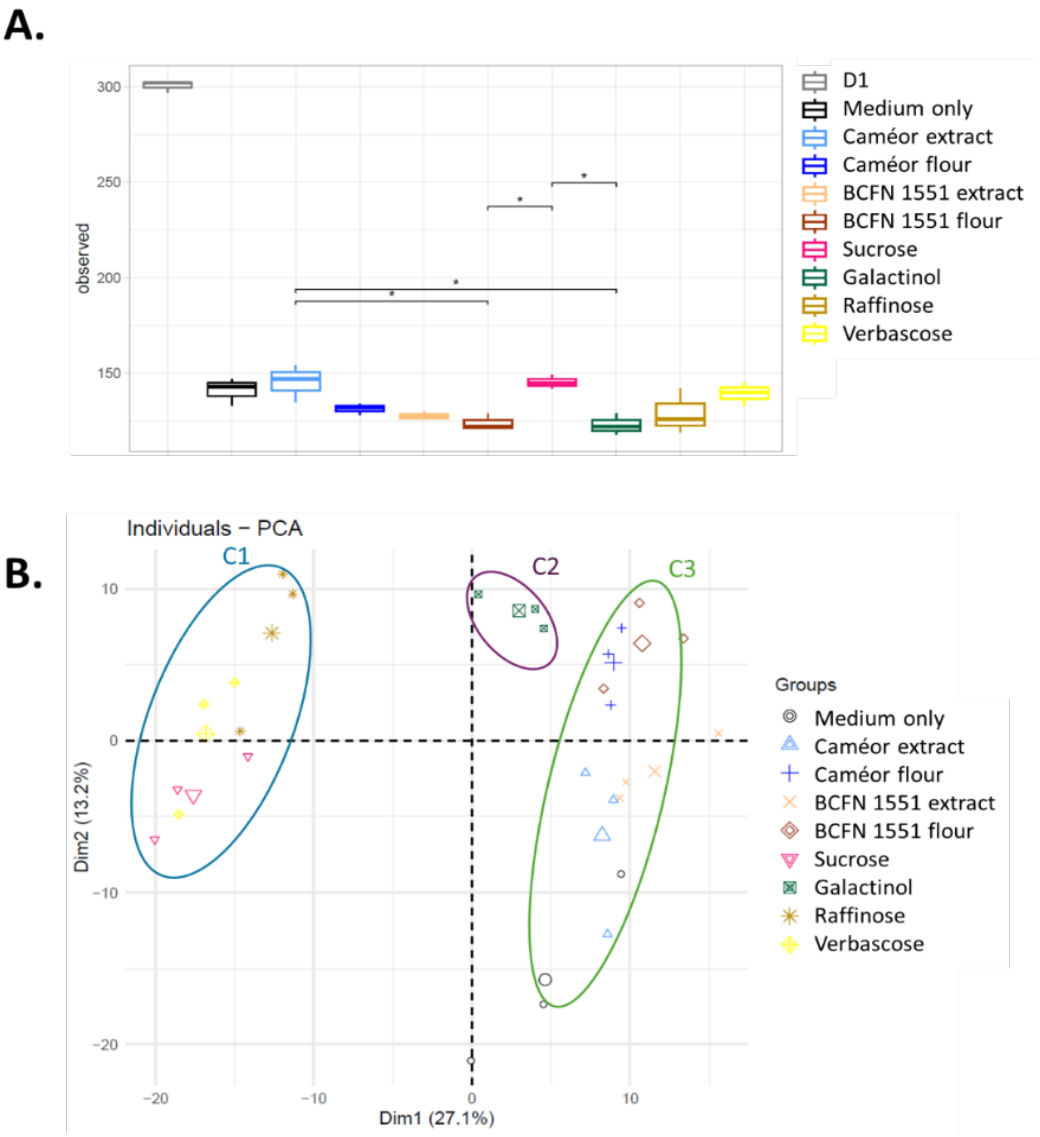


Figure 32. Effect of RFOs, raffinose and verbascose, precursors, sucrose and galactinol, and pea flour and pea extracts on the alpha and beta diversity of the faecal microbiota composition in batch fermentations. (**A**) Observed species richness across the samples measured by Shannon Index. An ANOVA model was obtained on all samples, excluding the donor faecal sample (HD1). The post-hoc tests were carried using a Tukey HSD test. (*) p value < 0.05. (**B**) PCoA using Aitchinson distances between the samples. The species counts were CLR transformed and a Euclidean distance was computed between the samples. The samples are coloured by treatment type, and the donor faecal sample (HD1) was excluded from this plot for readability. C1: cluster 1, comprised by grouped samples from sucrose, raffinose and verbascose fermentations; C2: cluster 2, comprised by grouped samples from galactinol fermentation and C3: cluster 3, comprised by grouped pea flour and pea extract fermentations.

The community composition was globally consistent across the different replicates for the same conditions.

The faecal donor sample (HD1) had a clearly distinct composition compared to the rest of the samples (Figs. 33 and 34). The phylum classification utilises the latest International Code of Nomenclature for Prokaryotes (ICNP) (Oren *et al.*, 2023), however, for clarification purposes, the former names are included. At the phylum level (Fig. 33 A), HD1 was dominated by Bacillota (formerly Firmicutes) (57 %), followed by Bacteroidota (formerly Bacteroidetes) (14 %), Euryarchaeota (5 %), Actinomycetota (formerly Actinobacteria) (3 %), and Pseudomonadota (formerly Proteobacteria) (1 %). Following 24 h fermentation, the composition of the faecal inoculum changed to Pseudomonadota (56 %), followed by Bacillota (14 %), Bacteroidota (11 %), Euryarchaeota (2 %), and Actinomycetota (5 %) (Fig. 33 B).

The fermentation with RFO extracts and flour from Caméor and BCFN 1551, and the precursor galactinol led to a shift in the dominant phylum towards Pseudomonadota (53 %, 50% 55 %, 53 % and 48 %, respectively) and Bacillota (14 %, 15% 14 %, 14 % and 21 %, respectively) (Fig. 33 C,D,E and F).

The fermentation of the individual sugars, sucrose, raffinose and verbascose, was characterised by a phylum composition dominated by Actinomycetota (29 %, 35 % and 34 %, respectively), followed by Pseudomonadota (29 %, 31 % and 24 %, respectively) (Fig. 33 G,H and I).

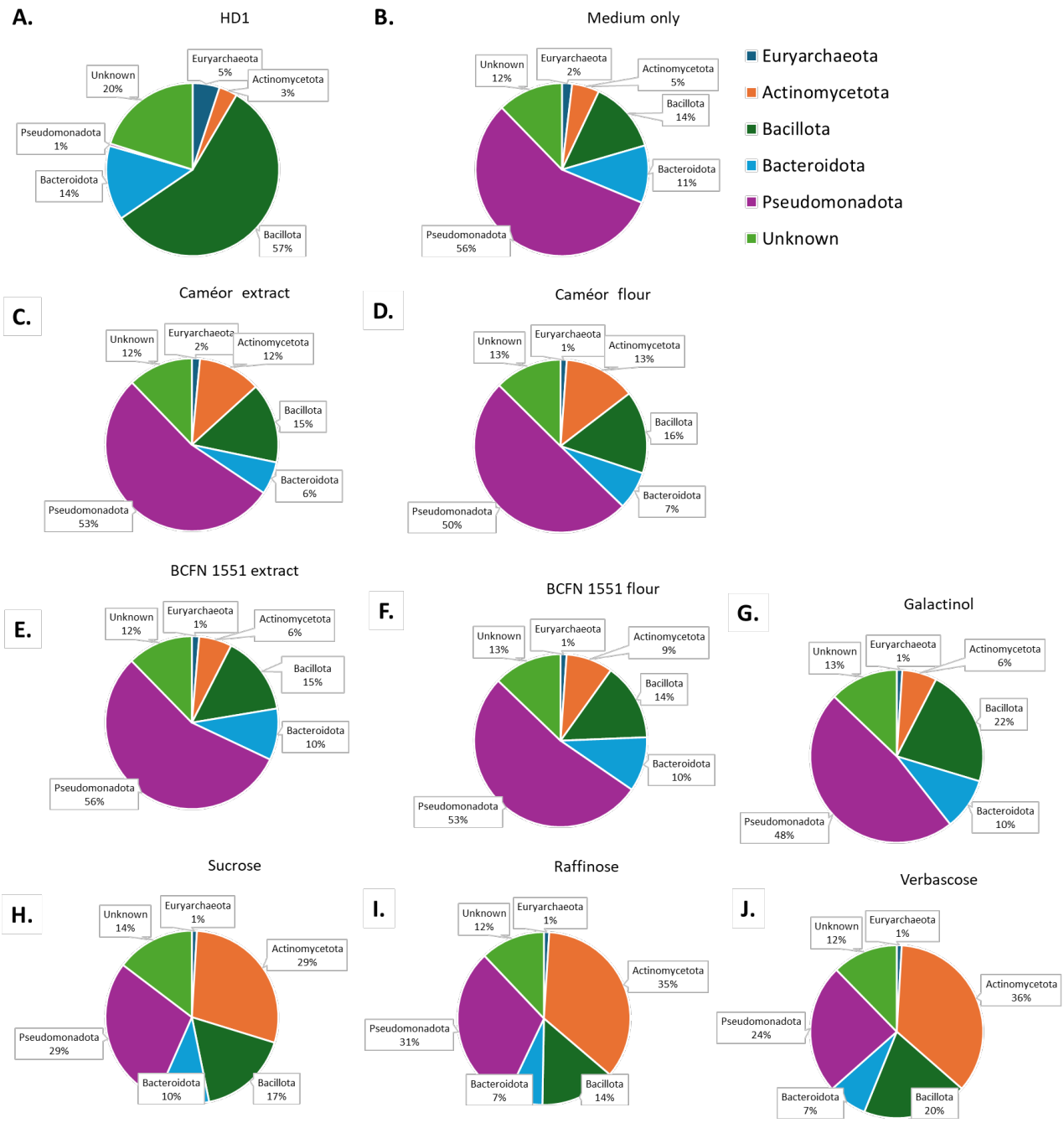


Figure 33. Effect of RFOs, raffinose and verbascose, precursors, sucrose and galactinol, and pea flour and pea extracts on faecal phylum composition in batch fermentations. Relative abundance of all taxa at the phylum level in the faecal sample from a healthy individual before fermentation (HD1), and after 24 h batch fermentation with sucrose, galactinol, raffinose and verbascose, pea flour and extracts from Caméor and the BCFN 1551 or medium-only. (**A**) Human donor 1 (HD1) faecal inoculum, (**B-J**) 24 h fermentations with (**B**) medium-only, (**C**) Caméor extract, (**D**) Caméor flour, (**E**) BCFN 1551 extract, (**F**) BCFN 1551 flour, (**G**) galactinol, (**H**) raffinose, (**I**) sucrose and (**J**) verbascose. Mean relative abundance calculated from triplicate faecal samples, for HD1, and triplicate fermentations for all other conditions.

At the family level (Fig. 34 A), the mean composition of HD1 consisted of: Oscillospiraceae (14 %), Lachnospiraceae (11 %), Bacteroidaceae (5.3 %), Methanobacteriaceae (5 %), Rikenellaceae (4 %), Clostridiaceae (2.9 %), Veillonellaceae (2.7 %), Bifidobacteriaceae (2.25 %), Peptostreptococcaceae (1.5 %), Streptococaceae (1 %), Actinomycetaceae (0.4 %), Sutterellaceae (0.24 %) Eubacteriales incertae sedis (0.22 %), Tannerellaceae (0.14 %), Coriobacteriaceae (0.09 %) and Enterobacteriaceae (0.001 %).

Sucrose, raffinose and verbascose fermentations showed similar effects on the relative abundance of community composition, with a majority of Enterobacteriaceae (24 %, 27 % and 20 %, respectively), followed by Bifidobacteriaceae (20 %, 29 % and 32%, respectively) and Corionibacteriaceae (7.3 %, 5.8% 2.1 %, respectively). On the other hand, the pea flour and extract from Caméor and BCFN 1551 fermentations clustered together and were characterised by a majority of Enterobacteriaceae (49%, 46%, 51% and 47 % respectively) followed by Bifidobacteriaceae (9.2%, 10.5%, 3.9 % and 6.0 % respectively). Finally, the galactinol fermentation was characterised by a higher relative abundance of Enterobacteriaceae (43 %) and Lachnospiraceae (7 %).

The top ten species identified across all samples (Fig. 34 B) were: *Alitipes onderdonkii*, *Alistipes shahii*, *Bifidobacterium adolescentis*, *Bifidobacterium longum*, *Citrobacter freundii*, *Clostridium fessum*, *Collinsella aerofaciens*, *Dialister succinatifilus*, *Methanobrevibacter smithii* and *Sutterella wadsworthensis.* HD1 was dominated by unidentified species, followed by *M. smithii* (5.0%), *D. succinatiphilus* (2.4 %), *B. longum* (1.9 %), *A. shahii* (0.6 %) and *B. adolescentis* (0.4 %).

Significant differences in the top ten species were found across fermentation conditions tested. *B. adolescentis* (Fig. 34 B 3) was found significantly higher in batch fermentations supplemented with sucrose, raffinose and verbascose compared to galactinol (6.3-fold change, 5.6-fold change and 7.6-fold change respectively, p value < 0.05). *B. longum* (Fig. 34 B 2) was significantly increased in the fermentation of Caméor extract and flour compared to BCFN 1551 extract and flour (3.8-fold change and 2.9-fold change, respectively, p value < 0.05). *B. longum* was also significantly increased in the fermentations with sucrose, raffinose and verbascose compared to galactinol (3.9-fold change, 8.9-fold change and 8.7-fold change, respectively, p value < 0.05). Finally, *B. longum* was significantly increased in the fermentations with raffinose and verbascose compared to sucrose (2.3-fold change and 2.2-fold changes, respectively, p value < 0.05). The capacity of Bifidobacteria strains to utilise RFOs was confirmed i*n vitro* with anaerobic growth assays showing growth of *B. longum* subsp. *infantis* and *B. breve* UCC 2003 on raffinose, stachyose and verbascose (Supplementary Fig. S4). Bioinformatics analyses confirmed the presence of a cluster dedicated to raffinose utilisation in the genome of *Bifidobacterium* strains, characterised by either the presence of GH13 or GH36 within the cluster (Supplementary Fig. S5).

The relative abundance of *C. fessum* (Fig. 34 B 5) was significantly increased upon Caméor flour fermentation compared to fermentations with Caméor extract or BCFN 1551 flour (1.5-fold change and 1.8-fold change, respectively, p value < 0.05). The relative abundance of *C. freundii* (Fig. 34 B 1) was significantly increased in galactinol fermentation compared to sucrose, raffinose and verbascose fermentations (1.7-fold

change, 1.5-fold change and 2.0-fold change, respectively, p value < 0.05). The relative abundance of *C. aerofaciens* (Fig. 34 B 4) was significantly increased in sucrose fermentation compared to galactinol, raffinose and verbascose fermentations (4.5-fold change, 1.5-fold change and 4.0-fold change, respectively, p value < 0.05), and in raffinose fermentation compared to verbascose fermentation (2.7-fold change, p vale< 0.0001).

Finally, the relative abundance of *Sutterella wadsworthensis* (Fig. 34 B 8) was significantly higher in sucrose fermentation compared to galactinol fermentation (3.2-fold change, p value < 0.05).

There were no significant differences in *A. onderdonkii*, *A. shahii. M. smithii* nor *D. succinatiphilus* among the conditions tested.

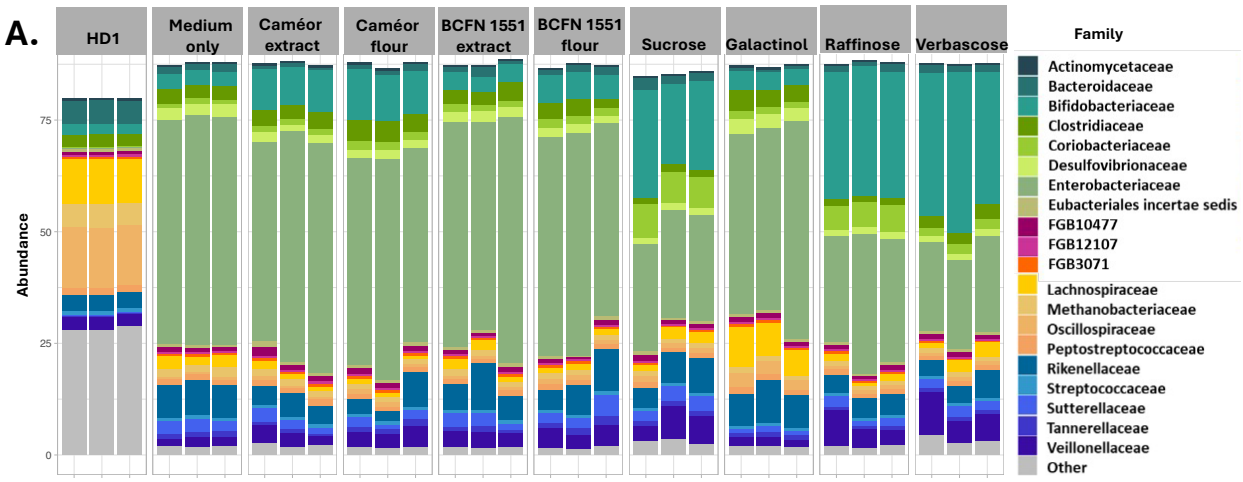


Figure 34. Effect of RFOs (raffinose and verbascose), RFO precursors (sucrose and galactinol), pea flour and pea extracts (from Caméor and BCFN 1551) on faecal microbial composition in batch fermentations. Relative abundance of the microbial composition in the faecal sample from a healthy individual before fermentation (HD1), and after 24 h batch fermentation with sucrose, galactinol, raffinose and verbascose, pea flour and extracts from Caméor and BCFN 1551 or medium-only. The top bacteria taxa are classified by (**A**) family and (**B**) species levels. Top ten species identified across all groups: (1) *C. freundii*, (2) *B. longum*, (3) *B. adolescentis*, (4) *C. aerofaciens*, (5) *C. fessum*, (6) *D. succinatifilus*, (7) *M. smithii*, (8) *S. wadsworthensis*, (9) *A. shahii* and (10) *A. onderdonkii*. ANOVA with post-hoc Tukey HSD test, p values < 0.05. Mean relative abundance calculated from triplicate faecal samples.

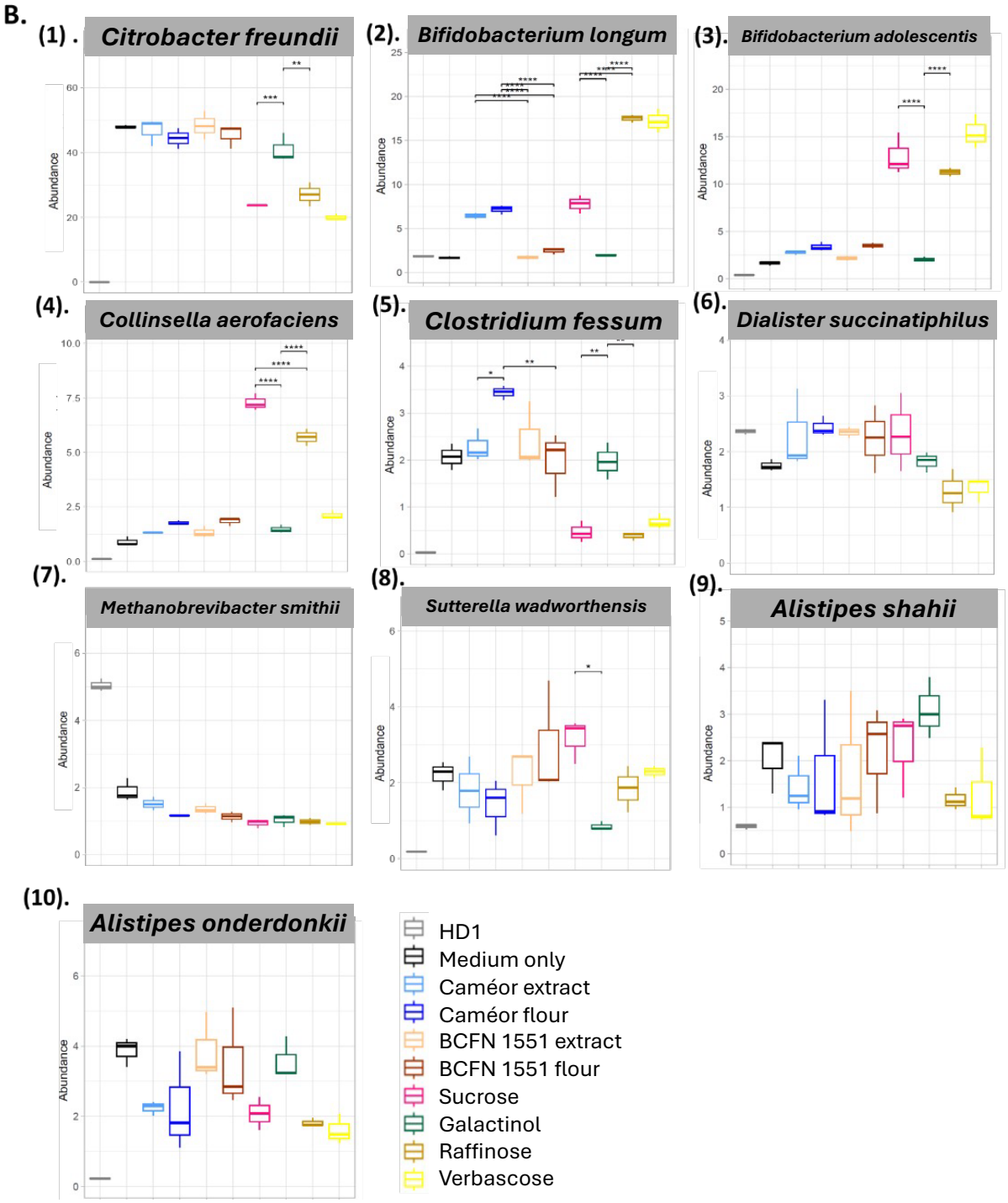


Figure 34 (continued). Effect of RFOs (raffinose and verbascose), RFO precursors (sucrose and galactinol), pea flour and pea extracts (from Caméor and BCFN 1551) on faecal microbial composition in batch fermentations. Relative abundance of the microbial composition in the faecal sample from a healthy individual before fermentation (HD1), and after 24 h batch fermentation with sucrose, galactinol, raffinose and verbascose, pea flour and extracts from Caméor and BCFN 1551 or medium-only. The top bacteria taxa are classified by (**A**) family and (**B**) species levels. Top ten species identified across all groups: (1) *C. freundii*, (2) *B. longum*, (3) *B.*

adolescentis, (4) *C. aerofaciens*, (5) *C. fessum*, (6) *D. succinatifilus*, (7) *M. smithii*, (8) *S. wadsworthensis*, (9) *A. shahii* and (10) *A. onderdonkii*. ANOVA with post-hoc Tukey HSD test, p values < 0.05. Mean relative abundance calculated from triplicate faecal samples.

Furthermore, the species within the families Bacteroidaceae and Oscillospiraceae, present in HD1, were also identified in the fermentation treatments (Fig. 36). *B. ovatus*, *B. uniformis* (Fig. 35 C and E) were significantly increased in sucrose fermentation compared to galactinol fermentation (4.7-fold change and 4.8-fold change, respectively, p value < 0.05). *B. caccae* and *R. bromii* (Fig. 35 G and H) were significantly increased in sucrose fermentation compared to raffinose fermentation (4.7-fold change, 2.3-fold change, respectively, p value < 0.05).

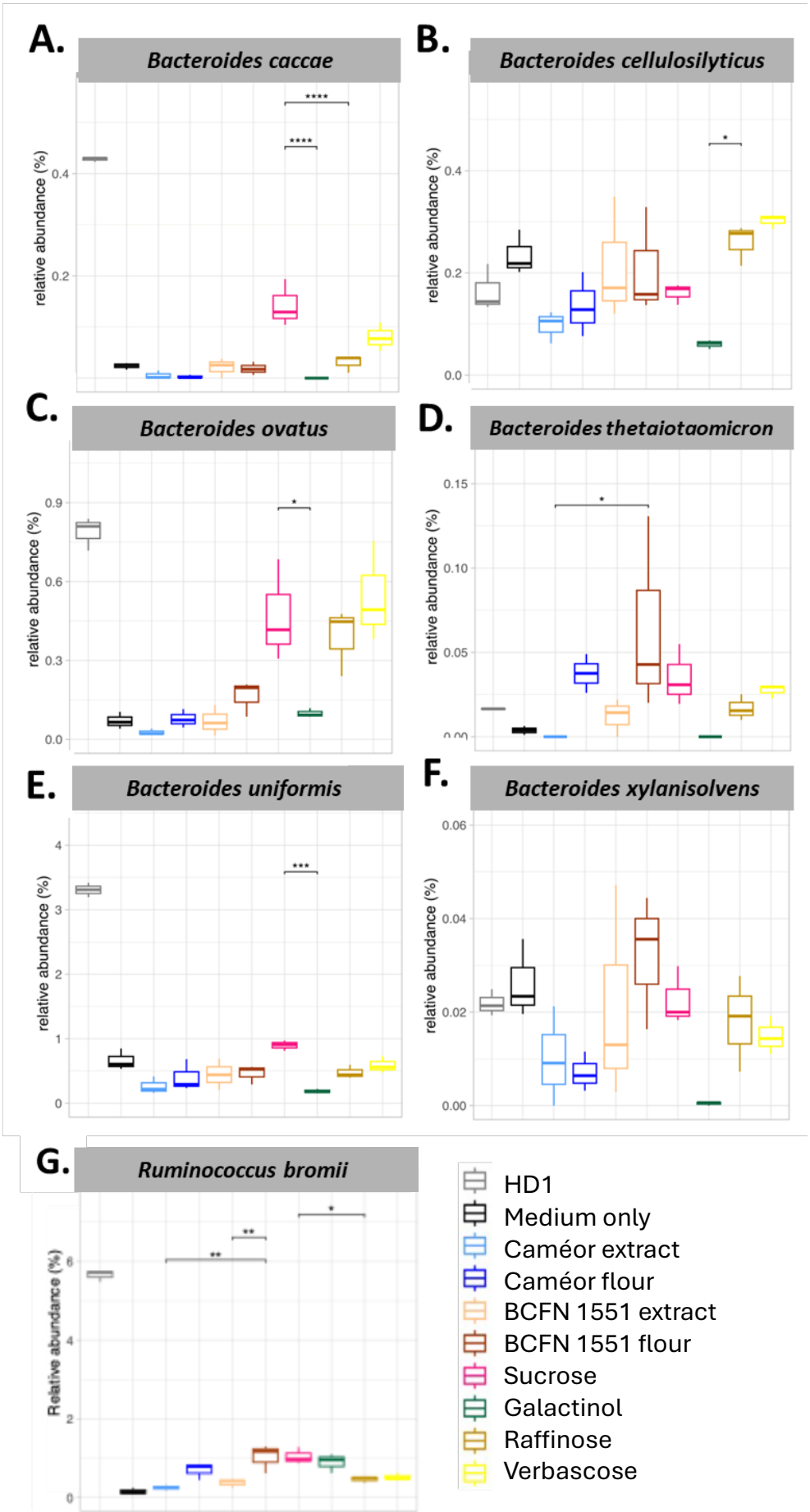

A.
Bacteroides caccae
relative abundance (%)
B.
Bacteroides cellulosilyticus
relative abundance (%)
C.
Bacteroides ovatus
relative abundance (%)
D.
Bacteroides thetaiotaomicron
relative abundance (%)
E.
Bacteroides uniformis
relative abundance (%)
F.
Bacteroides xylanisolvens
relative abundance (%)
G.
Ruminococcus bromii
Relative abundance (%)
HD1
Medium only
Caméor extract
Caméor flour
BCFN 1551 extract
BCFN 1551 flour
Sucrose
Galactinol
Raffinose
Verbascose

Figure 35. Effect of RFOs (raffinose and verbascose), RFO precursors (sucrose and galactinol), pea flour and pea extracts (from Caméor and BCFN 1551) on species from the Bacteroidaceae and Oscillospiraceae families in batch fermentations. Relative abundance of the microbial composition in the faecal sample from a healthy individual before fermentation (HD1), and after 24 h batch fermentation with sucrose, galactinol, raffinose and verbascose, pea flour and extracts from Caméor and BCFN 1551 or medium-only. The species identified were (**A**) *B. caccae*, (**B**) *B. cellulosilyticus*, (**C**) *B. ovatus*, (**D**) *B. thetaiotaomicron*, (**E**) *B. uniformis* and (**F**) *B. xylanisolvens*, (**G**) *Prevotella marsellensis* and (**H**) *R. bromii*. An ANOVA model was obtained on all samples, excluding the donor faecal sample (HD1). The post-hoc tests were carried using a Tukey HSD test. Mean relative abundance calculated from triplicate faecal samples.

Taken together, these data showed that sucrose, raffinose and verbascose supplementations led to an increase in *B. adolescentis* relative abundance compared to galactinol, and that *B. longum* and *B. cellulosilyticus* were increased in raffinose and verbascose fermentations compared to sucrose and galactinol fermentations. Moreover, BCFN 1551 fermentation was characterised by a significant reduced relative abundance of *B. longum* and *C. fessum* compared to Caméor, suggesting a potential role of these species in RFO utilisation.

Next, the functional potential of the microbial community was investigated at the pathway level, normalised as counts per million (cpm). The functional potential profiles identified a clustering of the samples similar to that obtained for the taxonomic composition, with sucrose, raffinose and verbascose fermentations clustering in the same cluster (C1m), while galactinol, pea flour and pea extract fermentations formed a distinct cluster (C2m) (Fig. 36).

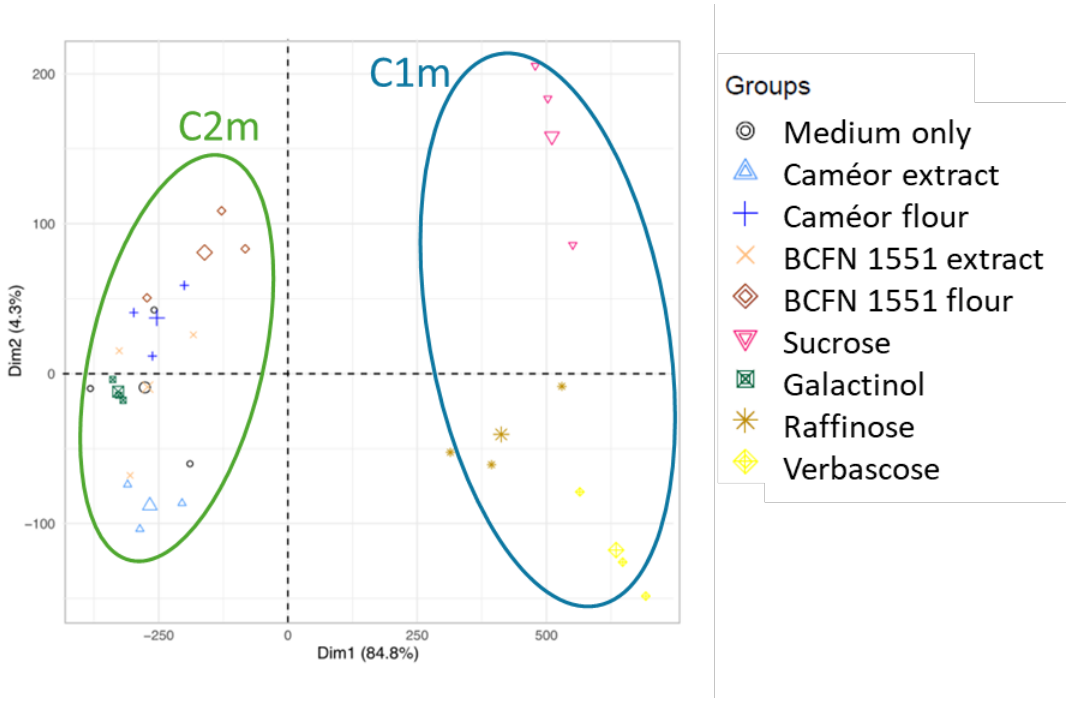


Figure 36. Functional prediction on the effect of RFO (raffinose and verbascose), RFO precursors (sucrose and galactinol), pea flour and pea extracts (from Caméor and BCFN 1551) based on genes present in the metagenome of batch fermentations. PCA of the functional potential predicted from the microbial composition after 24 h batch fermentation with sucrose, galactinol, raffinose and verbascose, pea flour and extracts from Caméor and BCFN 1551 or medium-only. Gene counts were aggregated at the pathway level and normalised as cpm before being used in a PCA. The donor faecal inoculum (HD1) was excluded from this plot for readability. Two clusters, C1m and C2m, were identified as shown on the Figure.

Based on the findings above showing a consistent increased abundance of Bifidobacteria in fermentations supplemented with sucrose, raffinose, verbascose, or Caméor flour and extract, we next determined the abundance in cpm of the "Bifidobacterium shunt" which allows Bifidobacteria to efficiently utilise various carbohydrates, including RFOs (Devika and Raman, 2019). This pathway was found in higher relative abundance in the sucrose, raffinose and verbascose fermentations compared to galactinol fermentation (5.1-fold change, 5.5-fold change and 6.3-fold change, respectively p value < 0.0001), and in the fermentation of Caméor extract and flour compared to BCFN 1551 (2.0-fold change and 1.5-fold change, respectively, p value < 0.01 and p value < 0.05, respectively) (Fig. 37), suggesting a role in RFO utilisation.

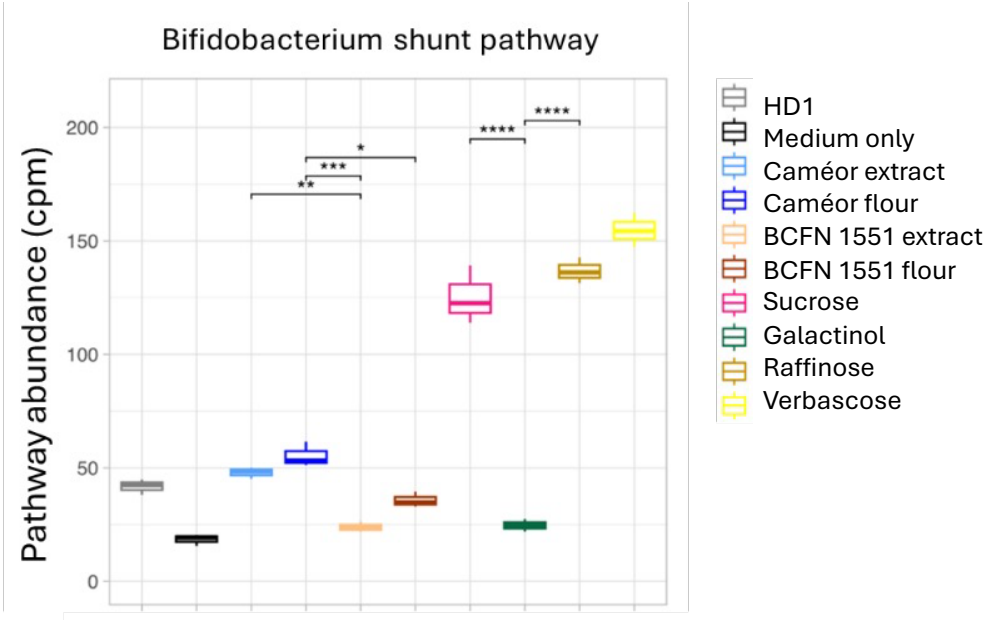


Figure 37. Effect of RFOs (raffinose and verbascose), RFO precursors (sucrose and galactinol), pea flour and pea extracts (from Caméor and BCFN 1551) on the relative abundance of Bifidobacterium shunt metabolic pathway in the metagenome of batch fermentations. Pathway abundance as counts per million (cpm) of the Bifidobacterium shunt microbial pathway identified in the metagenome of the 24 h batch fermentations with sucrose, galactinol, raffinose or verbascose, pea flour and extracts from Caméor and BCFN 1551, or medium-only. An ANOVA post-hoc Tukey HSD test model was obtained on all samples, excluding the unfermented control condition

HD1. * p value < 0.05, ** p value < 0.01, *** p value < 0.001 and **** p-value < 0.0001. Mean relative abundance calculated from triplicate faecal samples.

4.2.1.2 The impact of RFOs on metabolite production by the human gut microbiota

4.2.1.2.1 Effect of treatments on SCFAs, BCFAs and lactate production

The supernatants from the 24 h batch fermentation with individual RFOs (raffinose and verbascose), precursors (galactinol and sucrose), and pea flour and extract from Caméor and BCFN 1551 were analysed in-house by UPLC-MS/MS for the production of SCFAs, BCFAs and lactate.

Analysis of total SCFAs showed the overall production trend acetate > propionate > butyrate in all batch fermentation conditions (Fig. 38).

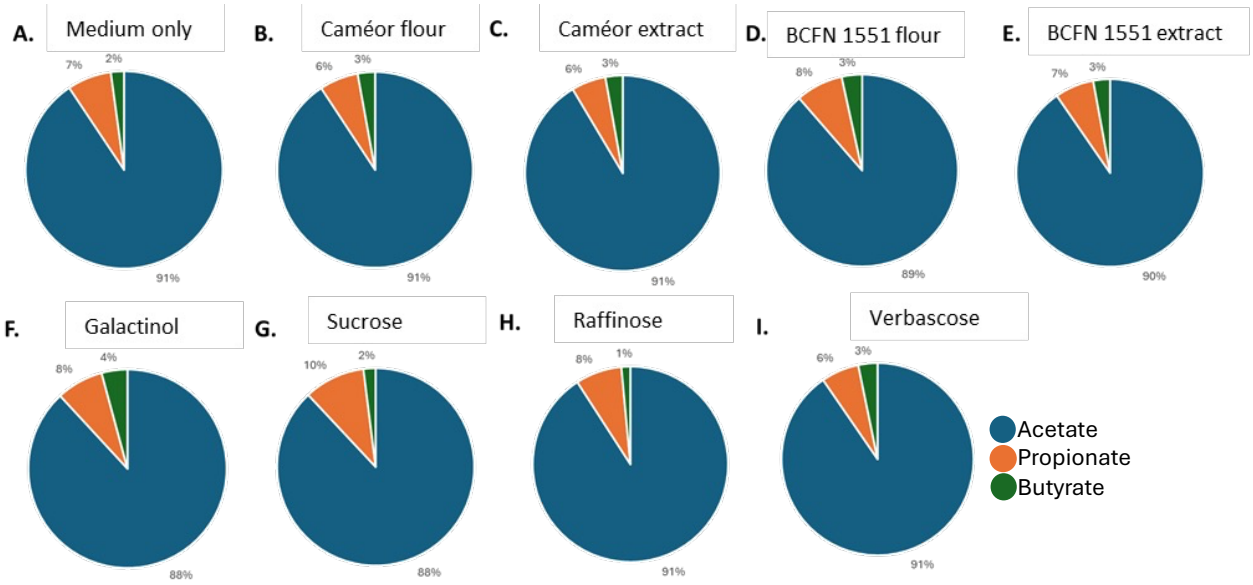


Figure 38. Effect of RFOs raffinose and verbascose, precursors sucrose and galactinol, pea flour and pea extracts on the proportion of SCFAs produced after 24 h batch fermentation. SCFAs (acetate, propionate and butyrate) were quantified by LC-MS from 24 h batch fermentations with (**A**) medium-only, (**B**) Caméor flour, (**C**) Caméor extract, (**D**) BCFN 1551 flour, (**E**) BCFN 1551 extract, (**F**) galactinol, (**G**) sucrose, (**H**) raffinose and (**I**) verbascose. The values represent averages of 3 replicates.

Acetate was significantly reduced in Caméor extract compared to Caméor flour fermentation (1.16-fold change, p < 0.001). However, no differences were observed between Caméor and BCFN 1551 (Fig 39 A).

Similarly for propionate production, in Caméor extract fermentation, propionate was significantly reduced compared to flour fermentation (1.32-fold change, p <0.01) (Fig. 39 B). There was no difference in propionate production between Caméor and BCFN 1551.

Butyrate levels were significantly lower in sucrose and raffinose fermentations when compared to galactinol fermentation (2.1-fold change and 2.9-fold change, respectively p < 0.05) and when compared to verbascose fermentation (1.5-fold

change and 2.0-fold change, $p < 0.05$). There was an increased production of butyrate in BCFN 1551 fermentation compared to Caméor fermentation, with both extract and flour, however this was not statistically significant (Fig. 39 C).

Finally, the fermentation of verbascose led to significantly lower levels of total SCFAs compared to sucrose and raffinose fermentation (0.91-fold change, for both respectively, $p < 0.05$) while no differences were found between Caméor and BCFN 1551 in the total production of SCFAs, neither flour nor extracts (Fig. 39 D).

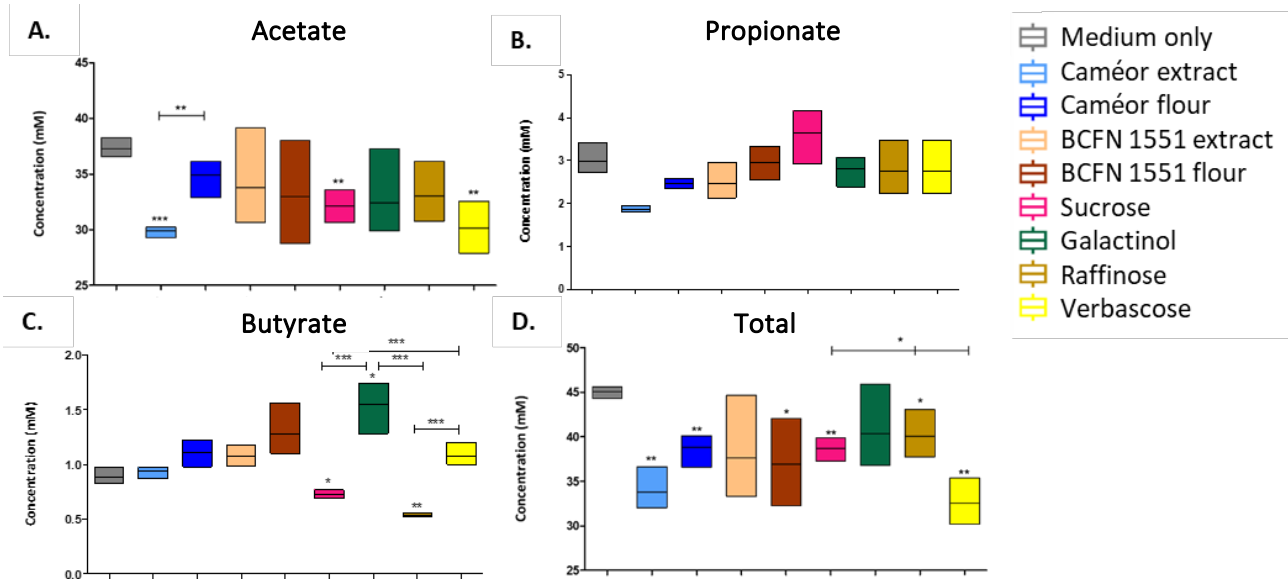


Figure 39. Effect of RFOs (raffinose and verbascose), RFO precursors (sucrose and galactinol), pea flour and pea extracts (from Caméor and BCFN 1551) on SCFA production. SCFAs were quantified by LC-MS from batch fermentations with individual galactinol, sucrose, and verbascose, pea flour and extract from Caméor and BCFN 1551 or medium-only: (**A**) acetate, (**B**) propionate and (**C**) butyrate and (**D**) total SCFAs (the sum of A, B and C) (*) p value<0.05, (**) p value < 0.01, (***) p value < 0.001, (****) p value < 0.001. Stars that are on top of bars without connecting lines correspond all to statistical differences between the respective fermentation condition compared to the fermentation of medium-only.

Analysis of BCFAs, iso-butyrate, iso-valerate and valerate, revealed only the presence of valerate across all conditions, including the fermentation with medium-only (Fig. 40 A), no significant differences were observed in valerate production in any of the conditions tested.

With regards to lactate, the results revealed its presence after fermentation of Caméor flour, sucrose, galactinol and raffinose, whereas none was detected in the fermentations with Caméor extract, BCFN 1551 extract and flour, and verbascose (Fig. 40 B). There was a significant reduction in lactate production in fermentations with sucrose and Caméor flour compared to medium-only (11.7-fold change and 1.8-fold change, respectively, p value < 0.05), and no lactate was detected in Caméor extract,

BCFN 1551 flour and extract. Lactate was not detected from the fermentation of Caméor extract, BCFN 1551 flour and extract, or verbascose.

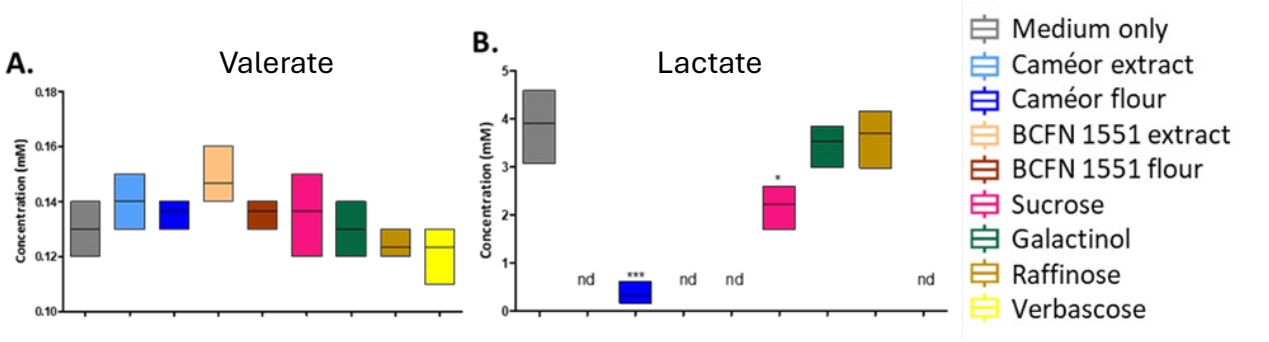


Figure 40. Effect of RFOs (raffinose and verbascose), RFO precursors (sucrose and galactinol), pea flour and pea extracts (from Caméor and BCFN 1551) on valerate and lactate production. Batch cultures supplemented with individual galactinol, sucrose, or verbascose, or pea flour and extract from Caméor or BCFN 1551 or with medium-only. Quantification of (**A**) valerate and (**B**) lactate. (*) p value <0.05, (**) p value <0.01, (***) p value <0.001, (****) p value <0.001. Stars that are on top of bars without connecting lines correspond all to differences when the respective fermentation condition had compared to the fermentation of medium-only.

Taken together, these data showed that RFOs, RFO precursors and pea materials differentially affected SCFA production but no difference in SFCA production was observed between the fermentation of Caméor and BCFN 1551, suggesting that RFO changes within the two genotypes were not enough to induce an effect above that generated by the fermentation of pea materials.

4.2.1.2.2 Effect of treatments on overall metabolites using an untargeted metabolomics approach

To gain further insights into the effect of RFOs on metabolite production, based on microbiome data from section 4.2.1.1., untargeted metabolomics was performed using the supernatants collected from the 24 h fermentations with raffinose, its two precursors, galactinol and sucrose, and pea seed material (flour and extract) from Caméor and BCFN 1551.

PCA showed clustering into four distinct clusters (M0, M1, M2, M3 and M4) with the medium-only cluster (M0) segregating away from the groups associated with the fermentation of individual sugars or pea flour and pea extracts (Fig. 41). The treatment groups separated into three clusters: cluster 1 included fermentations with sucrose and raffinose (M1); cluster 2 corresponded to galactinol fermentation (M2); and cluster 3 encompassed fermentations with Caméor extract, Caméor flour, BCFN 1551 extract, and BCFN flour (M3) (Fig. 41).

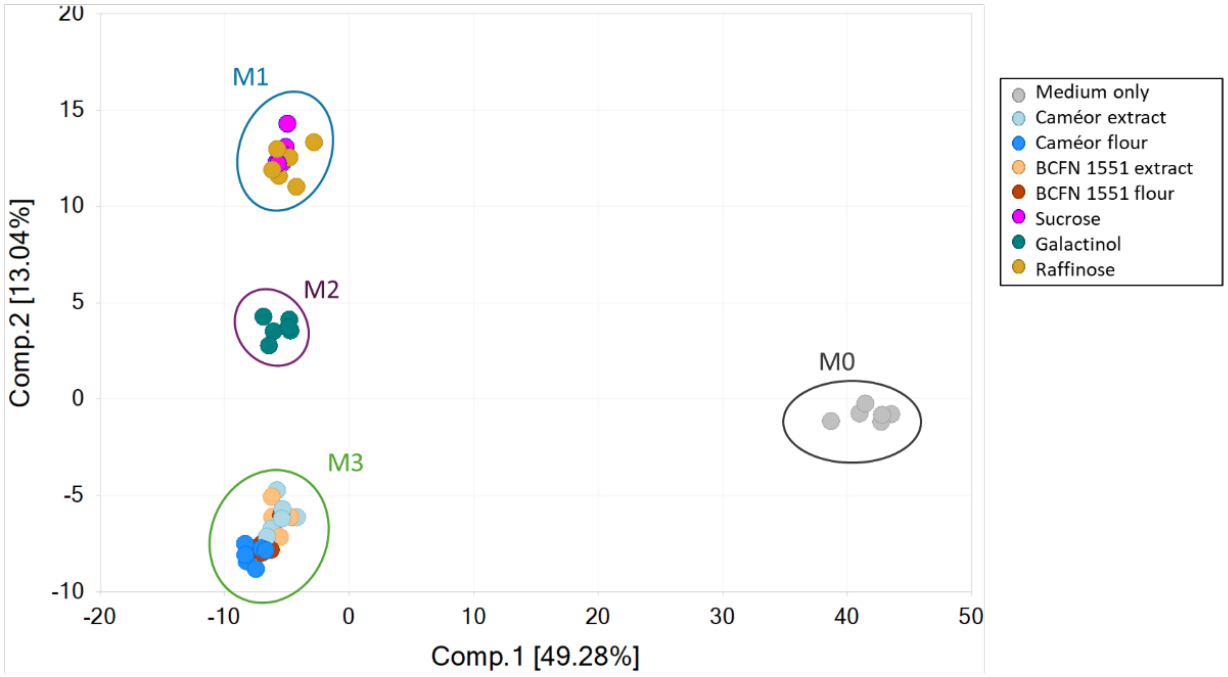


Figure 41. PCA of the metabolites produced in the fermentations of RFO (raffinose), RFO precursors (sucrose, galactinol) and pea extracts and pea flour (from Caméor and BCFN 1551). Analysis of the metabolites produced after 24 h anaerobic batch fermentation with individual sugars, raffinose and precursors, galactinol and sucrose, and pea material (flour and extract) from Caméor and BCFN 1551 and medium-only. M0: cluster 0, M1: cluster 1, M2: cluster 2 and M3: cluster 3.

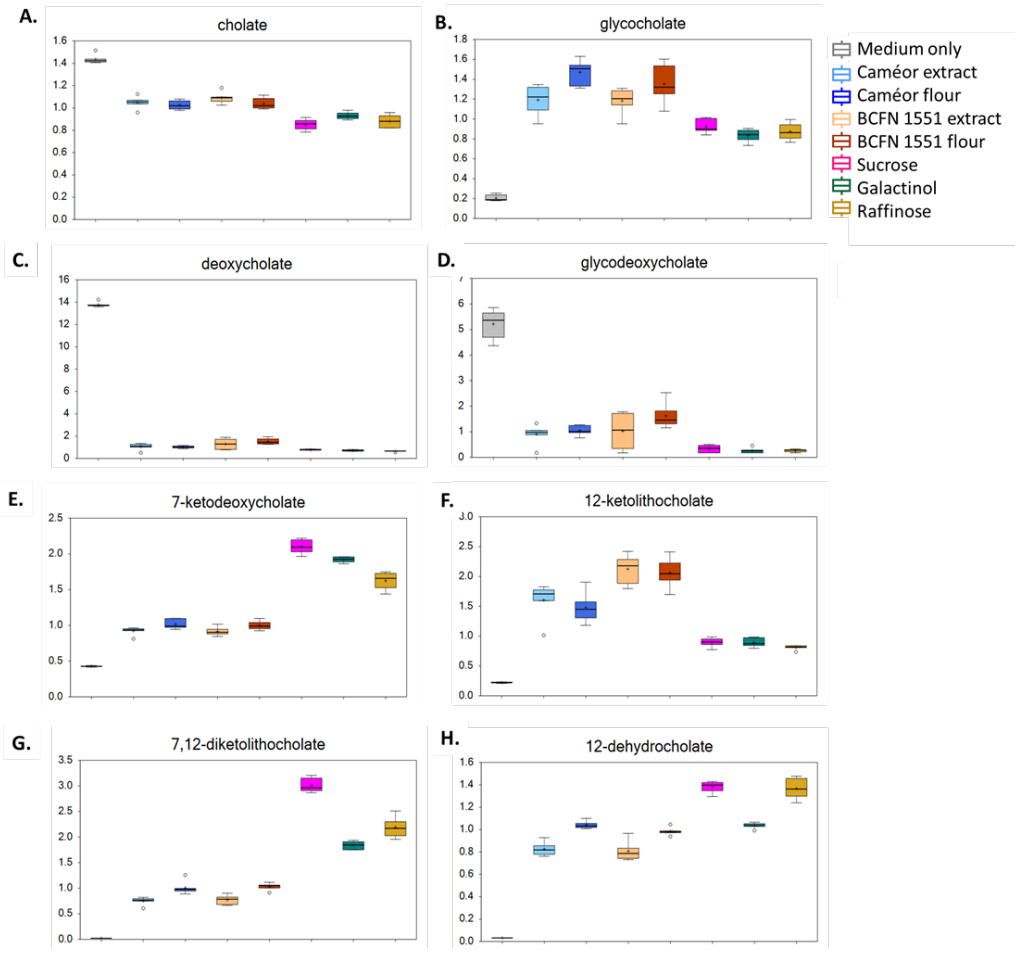
Next, specific metabolites were identified in various metabolic pathways, highlighting treatment-dependent differences in metabolites being catabolised, converted or modified, as further described below.

Bile acids showed significant changes between the different conditions tested. A summary of all fold changes (with associated values) is provided in the heatmap (Fig. 42 J), and all the differences mentioned below correspond to statistically significant fold changes (Fig. 42 J, red boxes).

In addition to bile acid depletion, specific secondary bile acids were found to be produced upon treatments with pea treatments or individual sugars. For example, 7,12-diketolithocholate and 12-dehydrocholate were increased upon sucrose and raffinose fermentations compared to galactinol fermentation (Fig. 42 G and H).

Glycocholate levels were higher in the pea flour fermentation compared to the pea extract fermentation for both Caméor and BCFN 1551(Fig. 42 B).

The levels of 12-ketolithocholate were higher in the BCFN 1551 fermentation, compared to the Caméor, both flour and extract. Moreover, this metabolite was produced in higher levels in the fermentation with pea extract compared to fermentation pea flour, from both Caméor and BCFN 1551, respectively (Fig. 42 F).



J.

		Comparisons between extracts and flour		Comparisons between pea genotypes		Comparisons between individual sugars		
		Caméor extract / Caméor flour	BCFN 1551 extract / BCFN 1551 flour	BCFN 1551 flour / Caméor flour	BCFN 1551 extract / Caméor extract	Sucrose / Galactinol	Sucrose / Raffinose	Raffinose / Galactinol
Compound	cholate	1.02	1.05	1.01	1.04	0.92	0.97	0.95
	glycocholate	0.81	0.87	0.92	0.99	1.11	1.06	1.05
	deoxycholate	1.03	0.85	1.49	1.23	1.10	1.20	0.91
	glycodeoxycholate	0.86	0.63	1.55	1.14	1.26	1.27	0.99
	7-ketodeoxycholate	0.91	0.92	0.99	0.99	1.09	1.29	0.85
	12-ketolithocholate	1.09	1.03	1.39	1.32	1.00	1.11	0.91
	7,12-diketolithocholate	0.74	0.75	1.02	1.03	1.64	1.38	1.19
	12-dehydrocholate	0.79	0.82	0.95	0.98	1.34	1.01	1.32

I.

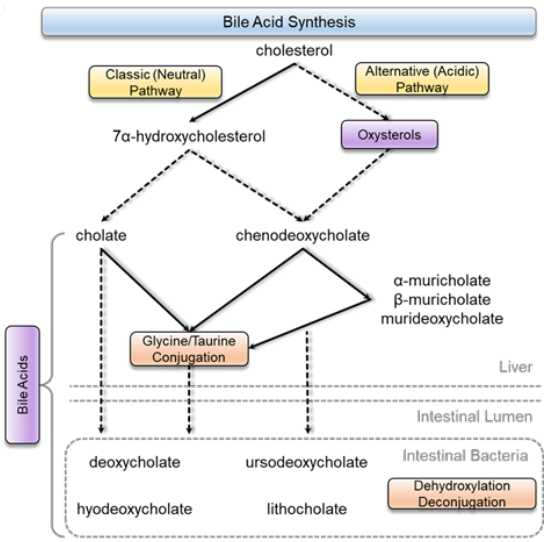
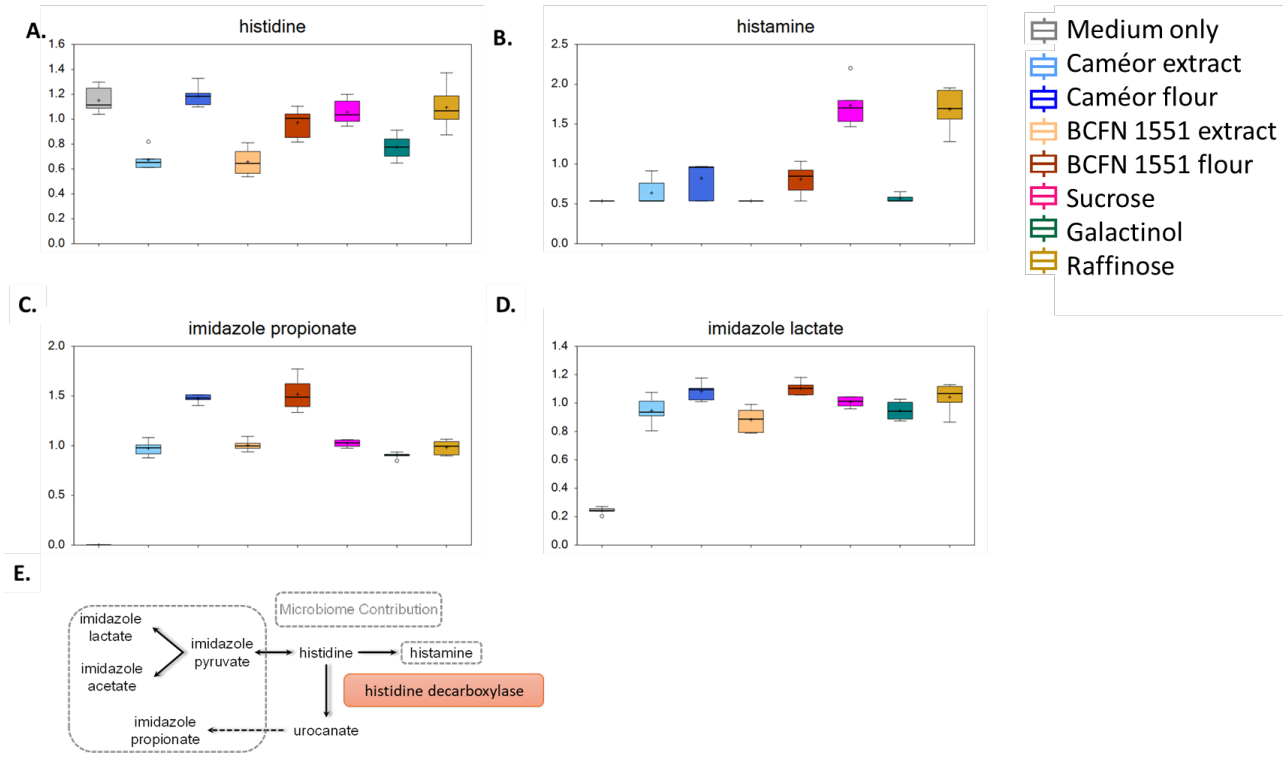


Figure 42. Effects of 24 h batch fermentations with RFO (raffinose), RFO precursors (sucrose, galactinol) and pea flour and extracts (from Caméor and BCFN 1551) on bile acid synthesis and metabolism. Metabolites produced from 24 h anaerobic batch fermentation with individual sugars, raffinose and precursors, galactinol and sucrose, pea material (flour and extract) from Caméor and BCFN 1551 and medium-only: (**A**) cholate, (**B**) glycocholate, (**C**) deoxycholate, (**D**) glycodeoxycholate, (**E**) 7-ketodeoxycholate, (**F**) 12-ketolithocholate, (**G**) 7,12-diketolithocholate and (**H**) 12-dehydrocholate. (**I**) Bile acid synthesis and secondary bile acid production by intestinal bacteria. Means from triplicate faecal samples. (**J**) Heatmaps of Welch`s two-sample t-test fold of change in bile acid production. Data in each column are the results of comparisons between two fermentation conditions for each metabolite. Here comparisons of bile acids levels between fermentations with pea flour and extract (from Caméor or BCFN 1551) or between Caméor and BCFN 1551 (pea flour or extract), or between individual sugars, sucrose, galactinol and raffinose are represented. Colour coding red and green shaded cells indicate p value ≤ 0.05 (red indicates that the mean values are significantly higher for that comparison; green values significantly lower). Light green shaded cells indicate 0.05 < p value < 0.10.

Metabolic changes were also related to histidine metabolism. Histamine production (Fig. 43 B) was observed in fermentations with sucrose, raffinose compared to galactinol. Moreover, histamine production was also observed increased from Caméor flour and BCFN 1551 flour fermentation, compared to the respective extracts (Fig. 43 B).

Other microbiome-specific metabolites of histidine included imidazole propionate (Fig. 43 C) and imidazole lactate (Fig. 43 D) which were found in higher levels in pea flour fermentations compared to pea extract fermentations from both Caméor and BCFN 1551.



F.

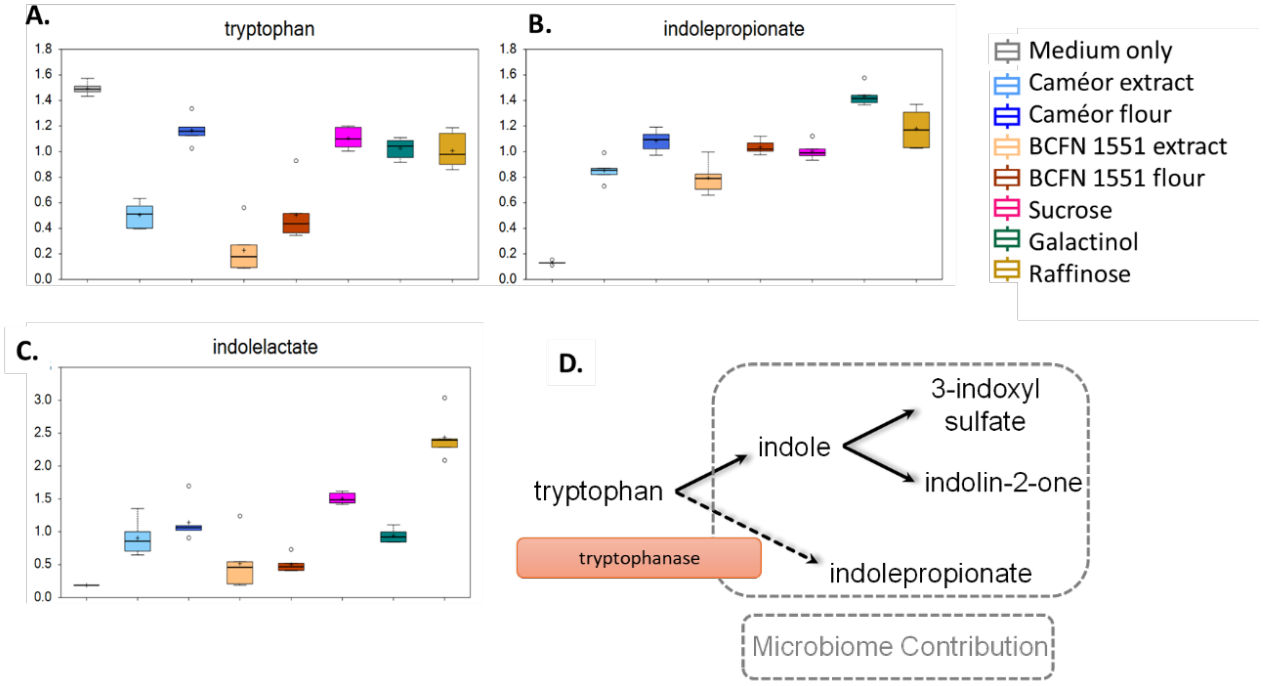
		Comparisons between extracts and flour		Comparisons between pea genotypes		Comparisons between individual sugars		
		Caméor extract / Caméor flour	BCFN 1551 extract / BCFN 1551 flour	BCFN 1551 flour / Caméor flour	BCFN 1551 extract / Caméor extract	Sucrose / Galactinol	Sucrose / Raffinose	Raffinose / Galactinol
Compound	histidine	0.57	0.68	0.82	0.98	1.36	0.97	1.41
	imidazole propionate	0.66	0.66	1.03	1.03	1.14	1.04	1.09
	imidazole lactate	0.87	0.80	1.02	0.93	1.07	0.97	1.10
	histamine	0.78	0.66	0.99	0.84	3.08	1.03	2.99

Figure 43. Effects of 24 h batch fermentations with RFO (raffinose), RFO precursors (sucrose, galactinol) and pea flour and pea extracts (from Caméor and BCFN 1551) on histidine metabolism. Metabolites produced from 24 h anaerobic fermentations with individual sugars, raffinose and precursors, galactinol and sucrose, pea material (flour and extract) from Caméor and BCFN 1551 or medium-only: (**A**) histidine, (**B**) histamine, (**C**) imidazole propionate, (**D**) imidazole lactate. (**E**) Histidine metabolism by the gut microbiota. Means from triplicate faecal samples. (**F**) Heatmaps of Welch`s two-sample t-test fold of change on histidine metabolism. Data in each column are the results of comparisons between two conditions for each metabolite. Here comparisons of bile acids levels between fermentations with pea flour and extract (from Caméor or BCFN 1551) or between Caméor and BCFN 1551 (pea flour or extract), or between individual sugars, sucrose, galactinol and raffinose are represented. Colour coding red and green shaded cells indicate p value ≤ 0.05 (red indicates that the mean values are significantly higher for that comparison; green values significantly lower). Light red shaded cells indicate 0.05 < p value < 0.10.

Differences were also observed in metabolites generated from the microbial-specific catabolism of aromatic amino acids, particularly tryptophan. Differential levels of tryptophan were observed between BCFN 1551 and Caméor fermentations, with lower tryptophan levels detected in BCFN 1551 fermentation compared to Caméor

(Fig. 44 A). For both pea genotypes, there were lower tryptophan levels in fermentations with pea extract compared to pea flour.

Tryptophan-derived metabolites such as indolepropionate (Fig. 44 B) and indolelactate (Fig. 44 C) were increased in the fermentations with pea (flour or extract) from Caméor compared to BCFN 1551 (Fig. 44 E). Moreover, indolelactate (Fig. 44 C) was also significantly increased in sucrose and raffinose fermentation compared to galactinol.



E.

		Comparisons between extracts and flour		Comparisons between pea genotypes		Comparisons between individual sugars		
		Caméor extract / Caméor flour	BCFN 1551 extrac / BCFN 1551 flour	BCFN 1551 flour / Caméor flour	BCFN 1551 extrac / Caméor extract	Sucrose / Galactinol	Sucrose / Raffinose	Raffinose / Galactinol
Compound	tryptophan	0.34	0.78	0.15	0.34	0.74	0.69	0.67
	indolelactate	4.87	6.15	2.78	2.70	8.11	5.06	13.13
	indolepropionate	6.51	8.27	6.06	7.89	7.66	10.93	8.99

Figure 44. Effects of 24 h batch fermentations with RFO (raffinose), RFO precursors (sucrose, galactinol) and pea flour and extracts (from Caméor and BCFN 1551) on tryptophan metabolism. Metabolites produced from 24 h anaerobic batch fermentation with individual sugars, raffinose and precursors, galactinol and sucrose, pea material (flour and extract) from Caméor and BCFN 1551 or medium-only: (**A**) tryptophan, (**B**) indolepropionate and (**C**) indolelactate. (**D**) Microbiome contribution to tryptophan metabolism. (**E**) Heatmaps of Welch`s two-sample t-test fold of change on tryptophan metabolism. Data in each column are the results of comparisons between two conditions for each metabolite. Here comparisons of bile acids levels between fermentations with pea flour and extract (from Caméor or BCFN 1551) or between Caméor and BCFN 1551 (pea flour or extract), or between individual sugars,

sucrose, galactinol and raffinose are represented. Colour coding red and green shaded cells indicate p≤0.05 (red indicates that the mean values are significantly higher for that comparison; green values significantly lower).

Finally, sucrose, raffinose, and galactinol were undetected in the metabolomics analysis across all conditions tested, indicating that they were fully consumed following 24 h fermentation.

Taken together, these data showed that raffinose and sucrose fermentations were characterised by higher levels of 7,12-diketolithocholate, 12- dehydrocholate, histamine, indolelactate, and lower indolepropionate compared to the precursor galactinol; while BCFN 1551 fermentation was characterised by higher levels of secondary bile acids and lower histidine and tryptophan levels within the human gut microbial community compared to Caméor flour fermentation.

To gain molecular insights into the metabolomics, findings described above, the shotgun metagenomic sequencing data (section 4.2.1.1.) were analysed to explore the occurrence of genes implicated in the various metabolic pathways found significantly different between fermentation conditions. Functional classification was performed based on Kyoto Encyclopedia of Genes and Genomes Orthology (KO). The following genes: *bile salt hydrolase* (*bsh), sucrose-6-phosphatehydrolase* (*cscA*), *aldehyde dehydrogenase* superfamily *(ALDH)* and *lactate dehydrogenase* (*ldh*) (Fig. 45 C,D,E and F) were significantly increased in sucrose, raffinose and verbascose fermentations compared to galactinol fermentation (p value < 0.001). The occurrence of the alpha-galactosidase *melA* (Fig. 45 B) was significantly decreased in the sucrose, raffinose and verbascose fermentations compared to galactinol (p value < 0.001, p value < 0.01 and p value < 0.0001, respectively). Overall, no significant differences were observed in the occurrence of *tryptophanase* (*tnaA)* between the conditions tested (Fig. 45 A).

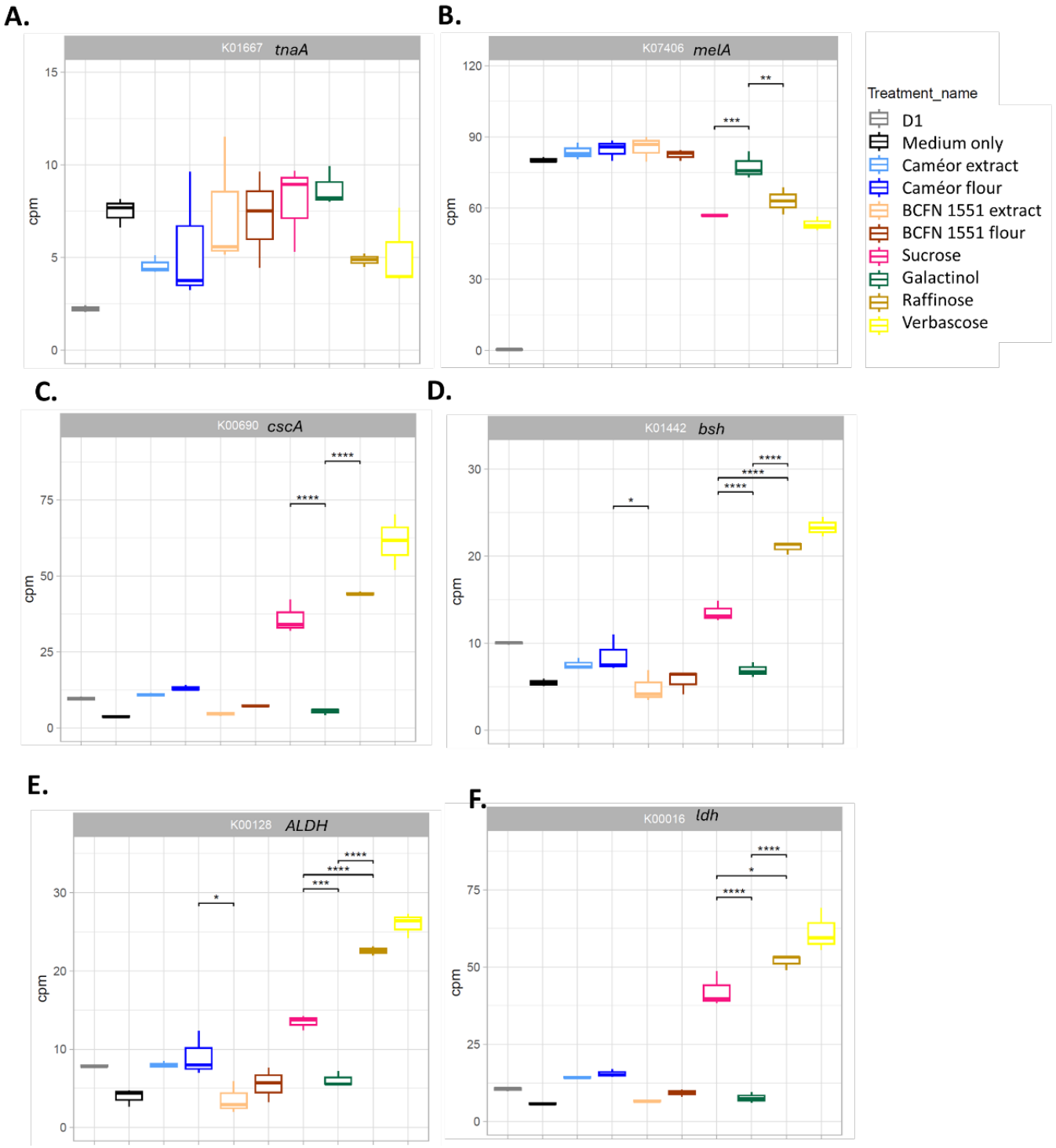


Figure 45. Effect of RFOs (raffinose and verbascose), RFO precursors (sucrose and galactinol), pea flour and pea extracts (from Caméor and BCFN 1551) on the relative abundance of various genes of interest in the metagenome of batch fermentations. Relative abundance at the genus level of the gut microbiota after 24 h batch fermentations with sucrose, galactinol, raffinose or verbascose, pea flour and extracts from Caméor and BCFN 1551 or medium-only. The genes of interest were (**A**) tryptophanase (tnaA, KO number:1667), (**B**) alpha-galactosidase (melA, KO number:7406), (**C**) sucrose phosphorylase (cscA, KO number:0690), (**D**) bile salt hydrolase (bsh, KO number:1442), (**E**) aldehyde dehydrogenase (ALDH, KO

number:0128), and (**F**) L-lactate dehydrogenase (ldh, KO number:0016). An ANOVA post-hoc Tukey HSD test model was obtained on all samples, excluding the unfermented control condition HD1. * p value < 0.05, ** p value < 0.01, *** p value < 0.001 and **** p-value < 0.0001. Mean relative abundance calculated from triplicate faecal samples.

4.2.2. Effects of RFO fermentation from pea on gut barrier function using *in vitro* human-derived colonic organoid model

The effects of fermented pea flour from BCFN 1551 and Caméor on gut barrier function were investigated using a model of human colonic organoids grown on transwells for two weeks, until they differentiate and form a monolayer (Fig. 46).

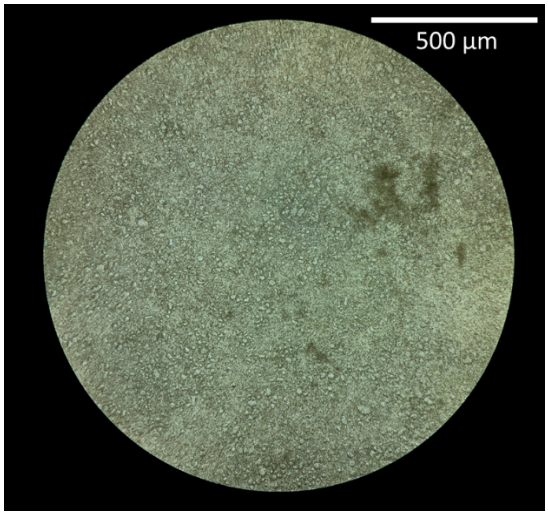


Figure 46. Light microscopy image of organoids forming a monolayer on the apical compartment of the transwell. Representative image of organoids seeded on the apical compartment grown for 14 days, prior to treatment with fermentation or cytokines. Organoid monolayer imaged using an ECHO Revolve microscope on brightfield mode. Scale bar= 500 µm.

The fermented products from the 24 h batch fermentations with pea flour from Caméor and BCFN 1551, or medium-only were filter-sterilised and applied to the apical side of the intestinal monolayer as described in section 2.3.4.

The integrity of the organoid-derived epithelial monolayer was evaluated by measuring the trans-epithelial electrical resistance (TEER) prior to (pre-treatment) and 24 h after incubation with the fermentation media (post-treatment) (Fig. 47). TEER determines the integrity of tight junction dynamics in cell culture models of epithelial monolayers and is determined by applying a direct current voltage to the electrodes and measuring the resulting current (Srinivasan *et al.*, 2015). No differences in TEER were observed between the conditions tested compared to their baseline (pre-treatment).

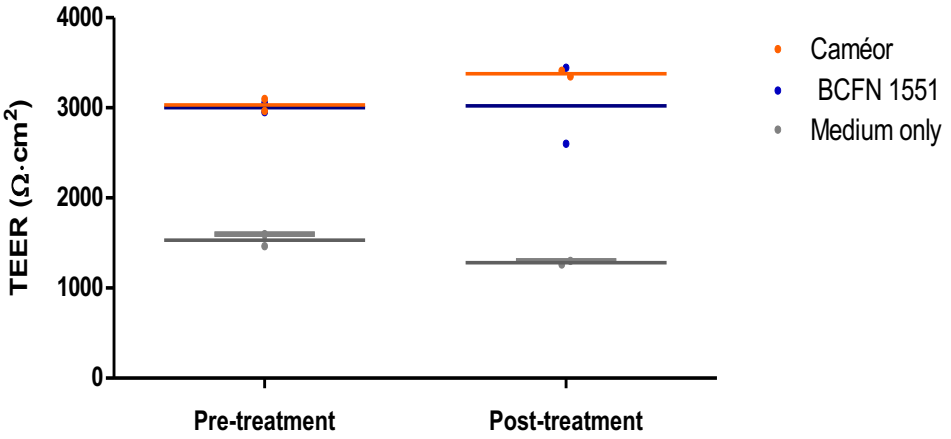


Figure 47. Effect of fermented pea flour from Caméor or BCFN 1551 on the trans-epithelial electrical resistance (TEER) of human colon organoid monolayers. TEER was measured prior and after 24 h incubation with the supernatant from batch fermentations with pea flour from Caméor or BCFN 1551, or medium-only . The TEER was measured as Ω and multiplied by the surface area of the transwell, resulting in $\Omega \cdot cm^2$.

We next assessed the effect of the treatments on the production of the following cytokines and chemokines: Interferon γ-induced protein 10 kDa (IP-10), IL-8, TNF-α, TGF-β1, Monocyte chemoattractant protein-1 (MCP-1), IL-1β and IL-12, using the LEGENDplex™ human inflammation panel immunoassay. The cytokines and chemokines were measured from the cell culture medium collected from both the apical and basolateral compartments after 24 h incubation with supernatants from the batch fermentations (Fig. 48).

There was an increase of MCP-1 production in the basolateral compartment upon incubation with fermented medium-only compared to the treatment with the fermented pea flours of Caméor and BCFN 1551 (2.5-fold change and 2.4-fold change, respectively, p value < 0.05). Moreover, in the basolateral compartment, fermented Caméor flour led to an increase in production of IP-10 and TGF-β1 (12.1-fold change and 2.9-fold change, respectively p value < 0.05), compared to the medium-only control.

Both fermented Caméor and BCFN 1551 flours led to an increase in IL-8 compared to the medium-only fermentation in both apical (11.9-fold change and 8.8-fold change, respectively, p value < 0.001) and basolateral (12.8-fold change and 7.3-fold change, respectively, p value < 0.001). There was also a detection of basolateral TNF-α and IFN-γ upon incubation with fermented Caméor and BCFN 1551 flours, whereas these cytokines were not detected in the basolateral compartment of the medium-only treatment.

IL-1β and IL-12 were only detected apically, with no significant differences found between the fermented treatments.

Finally, in terms of pea genotypes, there was a significant increase in IL-8 production, in both apical and basolateral compartments, when the organoids were treated with fermented Caméor compared to BCFN 1551 (1.35-fold change and 1.75-fold change, respectively, p value < 0.001).

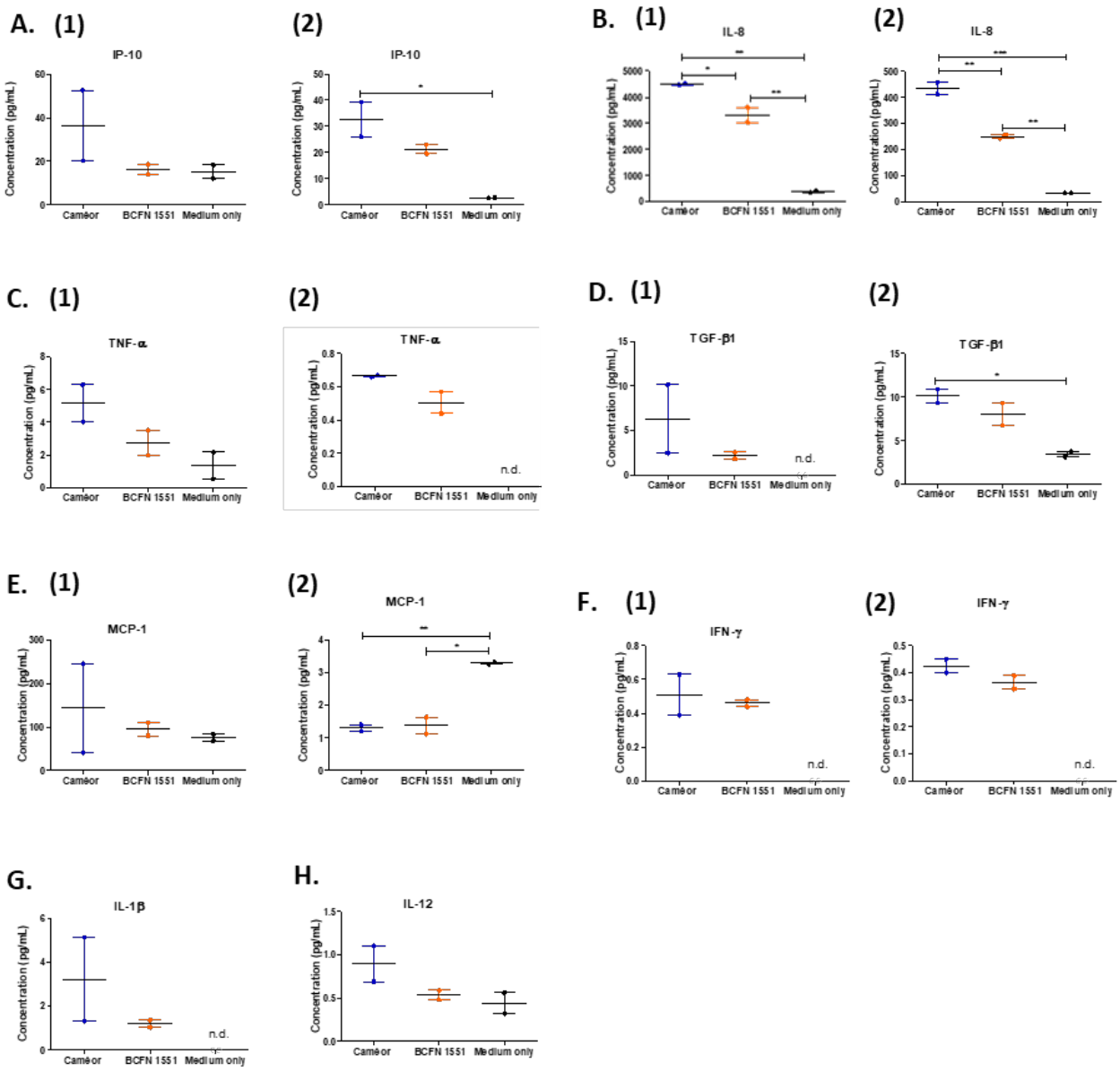


Figure 48. Cytokine production of intestinal organoids stimulated with fermented pea flour from Caméor or BCFN 1551. Monolayers of colonic organoids were treated on the apical compartment with supernatants from 24 h fermentations with pea flour from Caméor (blue) or BCFN 1551 (orange), or with medium-only (black) as control. Cytokines and chemokines quantified using LEGENDplex™ bead-based immunoassays from the media supernatants collected in the apical (1) and basolateral (2) compartments were (**A**) IP-10, (**B**) TNF-α, (**C**) MCP-1, (**D**) IFN-γ, (**E**) IL-8, (F) TGF-β1, (**G**) IL-12 (apical data only) and (**H**) IL-1β (apical data only). Data shown are the mean of biological duplicates. Statistics: (*) p value < 0. 05, (**) p value < 0.001, (***) p value < 0.0001.

Together, these results showed that the intestinal epithelium maintained its integrity with minor effects of fermented pea treatments in terms of cytokine/chemokine production.

To determine whether RFOs in peas may strengthen gut barrier function, we next attempted to develop a colonic organoid model recapitulating a compromised intestinal monolayer. Here, we first treated the organoid monolayer with a cocktail of TNF-α, IL-1a and IFN-γ (each at a concentration of 1 ng/mL), applied in both apical and basolateral compartments for 24 h following published methodology (Boj *et al.*, 2021). After 24 h, the medium with cytokine cocktail was removed and fresh medium (without cytokines) was added to the basolateral compartment, whereas supernatants from fermented BCFN 1551, Caméor and medium-only were applied to the apical compartment, as above. The cytokines and chemokines were measured from the cell culture medium collected from both the apical and basolateral compartments after 24 h incubation (Fig. 49).

Our preliminary data showed that IP-10 and IL-8 production in the basolateral compartment was significantly decreased with both Caméor and BCFN 1551 fermentations compared to medium-only (2.2-fold change and 2.0-fold change, respectively, for IP-10, p value < 0.0001 and 2.0-fold change and 1.9-fold change, respectively for IL-8, p value <0.001)

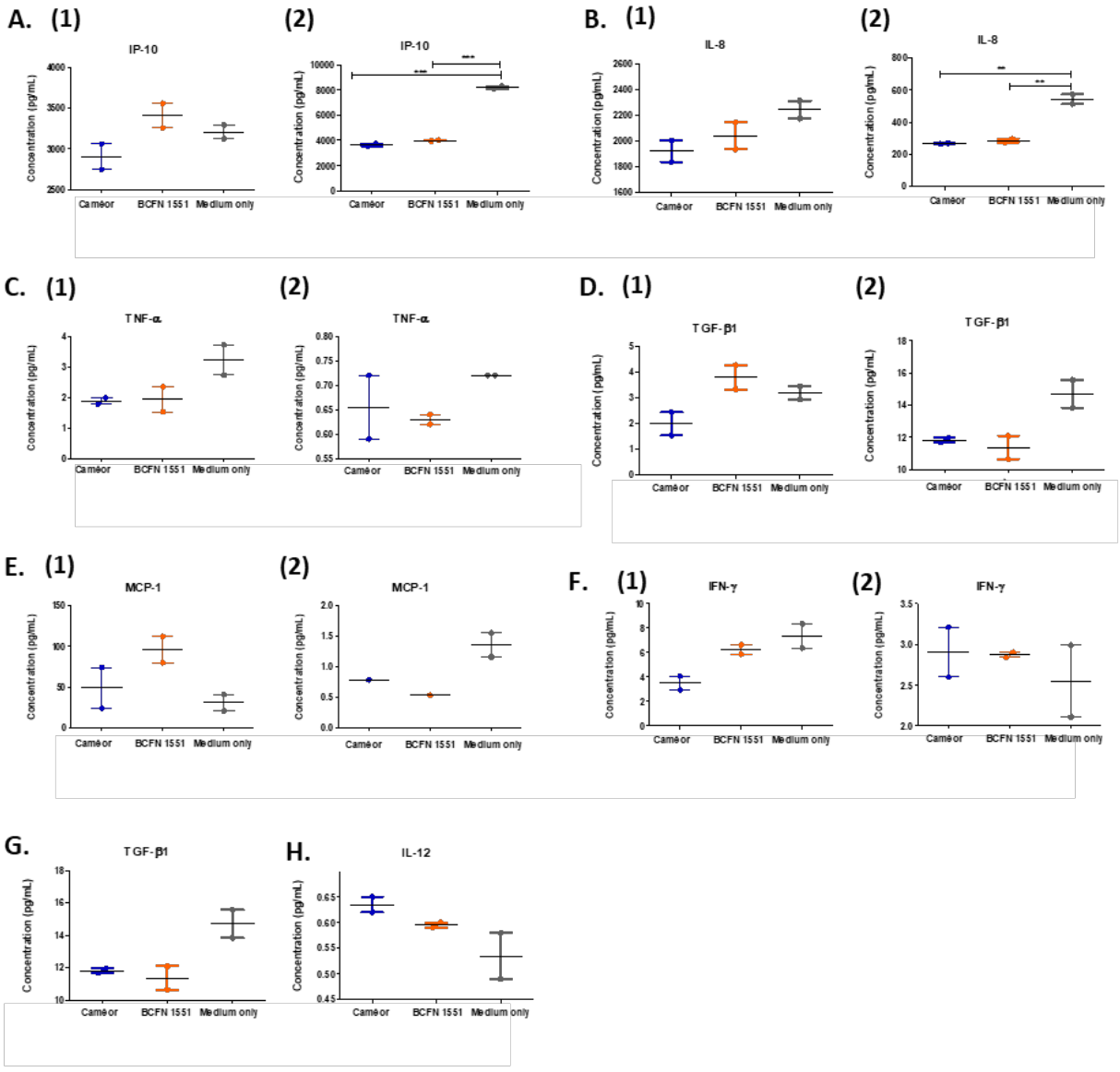


Figure 49. Cytokine production from intestinal organoids stimulated with fermented pea flour from Caméor or BCFN 1551 following a prior 24 h inflammatory stimulus. Monolayers of colonic organoids were treated with an inflammatory stimulus for 24 h on the apical compartment, after this the stimulus was removed and monolayers were treated with supernatants from 24 h fermentations with pea flour from Caméor (blue) or BCFN 1551 (orange), or with medium-only (grey) as control. Cytokines and chemokines quantified using LEGENDplex™ bead-based immunoassays from the media supernatants collected in the apical (1) and basolateral (2) compartments were: (**A**) IP-10, (**B**) TNF-α, (**C**) MCP-1, (D) IFN-γ, (**E**) IL-8, (**F**) TGF-β1, (**G**) IL-12 (apical data only) and (**H**) IL-1β (apical data only). Data shown are the mean of biological duplicates. Statistics: (*) p value < 0. 05, (**) p value < 0.001, (***) p value < 0.0001.

However, the inflammatory challenge did not appear to lead to changes in barrier permeability as shown by TEER values which were not significantly affected compared

to the baseline, after the inflammatory stimulus or following fermented pea flour or medium-only treatment (Fig. 50), so more work is needed to optimise this inflammation model.

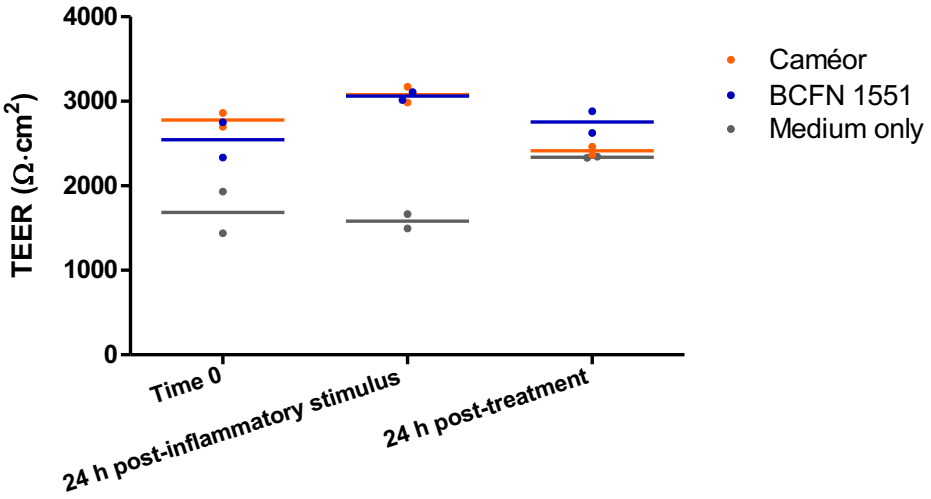


Figure 50. Effect of fermented pea flour from Caméor or BCFN 1551 on the trans-epithelial electrical resistance (TEER) of human colon organoid monolayers following inflammatory cytokine stimulus. TEER was measured prior addition of inflammatory cytokine cocktail (Time 0), 24 h after the inflammatory cytokine stimulus (TNF-α, IL-1a and IFN-γ, each at a concentration of 1 ng/mL), and after 24 h incubation with the supernatant from batch fermentations with pea flour from Caméor (orange) and BCFN 1551 (blue) or medium-only (grey). The TEER was measured as Ω and multiplied by the surface area of the transwell, resulting in $\Omega \cdot cm^2$.

4.3 Discussion

In Chapter 3, we showed that the mutant BCFN 1551 crossed to Caméor cultivar was depleted in RFOs, raffinose, stachyose, and significantly reduced in verbascose with elevated levels of precursors, sucrose and galactinol. This mutant was therefore selected to assess the influence of RFOs in pea on the gut microbiota composition and function in comparison to Caméor using a 24 h batch fermentation model. This closed and static model is inoculated with faecal microbiota suspensions to assess microbial changes over short-term periods under anaerobic conditions (Gagnon and Brubaker, 2015). Here, we tested the influence of both pea flour and pea extracts from Caméor and BCFN 1551 as well as individual RFOs (raffinose and verbascose) and RFO precursors (sucrose and galactinol). Pea extracts were used with the aim to reduce the matrix effect of the pea flour, providing RFOs undigested how they would reach the colon. However, it should be noted that RFO flours and extracts did not include a pre-digestion step, and the presence of other nutritional components such as protein and starch was not evaluated in these samples.

PCA analysis of microbiome sequencing data showed a clear clustering of fermentations with sucrose, raffinose and verbascose segregating together, away from the galactinol treatment, and from the pea treatments. The fact that the pea treatments clustered together irrespective of the phenotype can be explained by the presence of other fermentable carbohydrates e.g. starch in the samples, while raffinose and verbascose and sucrose share commonalities in structure (see Fig. 11).

On the other hand, the chemical structure of galactinol on the other hand is characterised by an α-D-galactoside moiety with a 1D-myo-inositol substituent at the anomeric position (see Fig. 11), which fermentation by the gut microbiota may rely on different bacterial catabolism pathways. Here our PCA analysis suggests that galactinol has a distinct influence (unrelated to RFO or starch) on the alpha and beta diversity of the faecal microbiota composition. The myo-inositol substituent in its structure is an ancient molecule (Nishihara *et al.*, 1992) and myo-inositol has been shown to increase propionate and butyrate and several known probiotic strains in monoculture fermentations, suggesting that this compound could have prebiotic effects (Bui *et al.*, 2021). In addition, myo-inositol has been found to inhibit duodenal glucose absorption and reduce glucose rise in blood (Chukwuma, Ibrahim and Islam, 2016). Inositol-deficient diets or alterations in its metabolism are potentially associated with several diseases, particularly metabolic disorders where inositol is implicated in insulin function (Caputo *et al.*, 2020). However, the microbial metabolism pathway of galactinol and myo-inositol intake remains to be fully elucidated.

The microbial composition of the donor inoculum was dominated by Bacillota (formerly Firmicutes) (56 %) and Bacteroidota (formerly Bacteroidetes) (14 %),

consistent with the typical composition of a healthy human adult, with Firmicutes and Bacteroidota representing ~ 90% of the gut microbiota (Arumugam *et al.*, 2011). The batch fermentations showed a clear shift in microbiota composition at the phylum level, characterised by the dominant abundance of Pseudomonadota (formerly Proteobacteria) in the fermentation with pea and galactinol, and by the dominant abundance of Actinomycetota (formerly Actinobacteria) in the fermentations with sucrose, raffinose and verbascose. However, the predominance of Pseudomonadota was also observed when the faecal inoculum was incubated in medium-only, suggesting that this change was not necessarily a result of the treatments. Since the faecal inoculum prior to fermentation (HD1) showed the highest species richness compared to all fermentation conditions, it is possible that transportation and/or sample preparation may have affected its microbial composition through oxygen exposure (Thorman et al., 2024) as a large proportion of microbes are highly sensitive to oxygen (Rinninella *et al.*, 2019) and hyperoxia can provoke gut dysbiosis (Xing *et al.*, 2020). Although the specific effects on Proteobacteria are not consistently reported across studies, it has been hypothesised that increased availability of oxygen could generate Proteobacteria blooms in the gut (Knights *et al.*, 2014). Additionally, although not dominant, there was an increase in Proteobacteria in the individual sugar fermentations; here the dominance of Actinomycetota could be due to the concentration of RFO and RFO precursors used in the fermentations with individual sugars, which was higher than the concentration of these sugars in the pea treatments. This phylum includes genera such as Bifidobacterium and Collinsella, known to ferment dietary carbohydrates (Flint *et al.*, 2012a). In line with our data, fermentation of raffinose (10 g/L) has been shown to decrease the relative abundance of Proteobacteria while increasing the populations of Bifidobacterium and Lactobacillus (Amorim *et al.*, 2020). The strong similarities observed in the phylum-level profiles between pea extracts and flours likely arise from several factors. Both forms contain common fermentable substrates, such as starch and possibly protein, which were not removed prior to fermentation. The absence of a digestion step means that these substrates would have been similarly available to the microbiota in both treatments. Furthermore, the relatively coarse resolution of phylum-level analysis may mask more subtle differences in microbial responses that could be influenced by variations in RFO content. Overall, these findings suggest that the composition of the broader nutrient matrix, rather than RFO content alone, plays a key role in shaping the gut microbiota at this taxonomic level.

Several differences were found at the species level in the batch fermentations supplemented with individual RFOs, precursors and pea material. Overall, the most abundant species identified were *B. adolescentis*, *B. longum*, *C. fessum*, *C. freundii*, *C. aerofaciens*, *S. wadsworthensis*, *A. onderdonkii*, *A. shahii*, *M. smithii* and *D. succinatiphilus,* the first five significantly increased in various treatments. Moreover,

differences were also identified for *B. caccae*, *B. cellosilyticus*, *B. ovatus*, *B thetaiotaomicron*, *B. uniformis* and *R. bromii*.

B. adolescentis and *B. longum* were found significantly increased in sucrose, raffinose and verbascose fermentations, in line with previous studies. Several species of the human gut microbiota have been shown to benefit from RFOs including Bifidobacteria (Gänzle and Follador, 2012; O'Callaghan and van Sinderen, 2016; Oliver *et al.*, 2021; Kanwal *et al.*, 2023). Such bifidogenic effect has been reported with raffinose using *in vitro* fermentation of infant (Adamberg *et al.*, 2018) and adult human faecal samples (Kan et al., 2020). Moreover, the fermentation of RFOs, raffinose and verbascose, when compared to their precursor sucrose, led to significantly higher abundance of *B. adolescentis* which was not observed for *B. longum*, underscoring the role of *B. adolescentis* in RFO metabolism while *B. longum* appears to be more adept at utilising sucrose, also in line with our *in vitro* growth assays. We confirmed that sucrose was the best substrate to promote the growth of *B. longum* in monoculture, followed by raffinose, stachyose and verbascose, in agreement with the presence of a catabolism pathway characterised by the presence of *cscA* (encoding for a β-fructofuranosidase) and *scrP* (encoding for a sucrose phosphorylase) genes in *B. longum* NCIMB 702259T (Kullin, Abratt and Reid, 2006). In line with this *in vitro* and *in silico* analyses, we found that the cpm in the dataset for the *cscA* gene cluster, belonging to the GH32 family (Kullin, Abratt and Reid, 2006), was also increased in sucrose, raffinose and verbascose fermentations, and that the cpm abundance in the "Bifidobacterium shunt" or fructose-6-phosphate shunt pathway (De Vries, Gerbrandy and Stouthamer, 1967) was increased in these conditions.

Moreover, in fermentations with sucrose and raffinose, there was an increase in histamine production, which in the gut is primarily facilitated by bacterial histidine decarboxylase enzymes, that convert the amino acid L-histidine into histamine (del Valle *et al.*, 2018). The taxonomic distribution of histamine-secreting bacteria in the gut is diverse, spanning multiple phyla such as Bacillota, Bacteroidota, Actinomycetota, Pseudomonadota, and Fusobacteriota suggests that histamine production is a widespread capability among gut bacteria (Engevik *et al.*, 2024b; Engevik *et al.*, 2024a). Key bacterial species identified as histamine producers in the human gut include *F. varium*, *C. perfringens*, *L. reuteri*, *M. morganii*, and *K. aerogenes* (Engevik *et al.*, 2024b; Engevik *et al.*, 2024a; De Palma *et al.*, 2022). However, none of these species were identified in the batch fermentations, and histidine decarboxylase genes were not identified by bioinformatics analysis. In the future, it will be interesting to investigate the mechanisms underpinning the potential effect of raffinose on histamine production in the gut and identify the species involved during cross-feeding interactions within the gut microbiota.

C. aerofaciens, *B. caccae*, *B. ovatus*, were found to be significantly increased in fermentations with sucrose compared to fermentations with the precursor galactinol.

B. ovatus was also higher abundance in fermentations with raffinose and verbascose fermentation, however to a lesser extent than with sucrose. This is consistent with the capacity of *B. ovatus* to utilise sucrose, and to a lesser extent raffinose and stachyose (Gherardini, Babcock and Salyers, 1985; Fultz *et al.*, 2021). *B. ovatus* is considered beneficial to the host through the production of SCFAs and other metabolites that regulate host metabolism (Tan, Zhai and Chen, 2019). Moreover, the increase in secondary bile acids 7-ketodeoxycholate, 7,12-diketolithocholate and 12-ketodehydrocholate upon sucrose fermentation could be associated with the increase in *C. aerofaciens* and *B. adolescentis* in sucrose fermentation as described above. In line with a faecal microbiota transplant study whereby *B. adolescentis*, *C. aerofaciens*, and *F. prausnitzii* were found to be positively correlated with increased secondary bile acid levels (Bustamante *et al.*, 2022). Moreover, it has been shown that the conversion of deoxycholate to hyodeoxycholate is catalysed by another species from this genus, *C. stercoris* (Lucas *et al.*, 2021). ferments various plant- and animal-derived carbohydrates, producing H_2, ethanol, SCFAs, and lactate in the human colon (Kageyama, Benno and Nakase, 1999). However, *Collinsella* has been associated with pro-inflammatory functions and is often increased in obesity and non-alcoholic fatty liver disease (Purohit *et al.*, 2024).

B. cellulosilyticus, a common species in the human gut (McNulty *et al.*, 2013), was found to be significantly increased in raffinose and verbascose fermentations and has been reported to colonise 77% of adult in Europe (n=124) and 62% of adults in the United States (n=139) (Qin *et al.*, 2010; Consortium, 2012). Moreover, this species grows well on wheat arabinoxylans due to its xylan-degrading enzyme arsenal (McNulty *et al.*, 2013; Centanni *et al.*, 2017) and can outcompete other prevalent *Bacteroides* spp. in arabinoxylan-rich conditions (Patnode *et al.*, 2019). There is also evidence that adherence to a plant-based diet was associated with a higher abundance of *B. cellulosilyticus* (Li *et al.*, 2021). In gnotobiotic mice colonised with *B. cellulosilyticus* fed a plant polysaccharide-rich diet, the most highly expressed CAZyme was BWH2_1228t, a putative α-galactosidase (McNulty *et al.*, 2013). This suggests that *B. cellulosilyticus* may be able to utilise RFOs from plant-based foods, though this requires experimental confirmation.

R. bromii was significantly increased in the batch fermentation with both sucrose and galactinol. It degrades starch to produce glucose and malto-oligosaccharides (Crost *et al.*, 2018). *R. bromii* and *B. adolescentis* act as primary degraders of starch in the gut (Dobranowski and Stintzi, 2021) and cross-feeding has been reported between these two species (Kovatcheva-Datchary *et al.*, 2009), suggesting the increase of *R. bromii* may be the result of cross-feeding from *B. adolescentis* sucrose metabolism; however, this would have to be further validated, as well as its increase in galactinol fermentation.

C. freundii was significantly increased in galactinol fermentation, alongside an increase in cpm abundance of *melA* and *tnaA* genes. The *melA* gene encodes a GH4 α-galactosidase, previously shown to catalyse hydrolysis of aryl substrates (Chakladar *et al.*, 2011). Although direct utilisation of galactinol by *C. freundii* has not been demonstrated, multiple enteric bacteria can utilise sugar alcohols such as mannitol as a sole carbon source following transport via a hexitol phosphoenolpyruvate-dependent phosphotransferase system (Lengeler, 1975a; Lengeler, 1975b), including *C. freundii* (Anderson *et al.*, 2018). The observed increase of cpm counts of *tnaA,* which encodes a tryptophanase, aligns with the untargeted metabolomics data, showing increased levels of indolepropionate, a tryptophan-derived metabolite in the fermentation of galactinol. Raffinose and sucrose fermentations produced more indolelactate but less indolepropionate than galactinol. Both are products of bacterial tryptophan metabolism via the indolic pathway (Konopelski and Mogilnicka, 2022), and have demonstrated anti-inflammatory effects in murine models (Wang *et al.*, 2024a). Aryl substrates, such as indolepropionate, have been found to decrease intestinal permeability through activation of AhR (Scott, Fu and Chang, 2020). Moreover, the presence of CAZyme-encoding genes in the C. freundii genome supports its potential role in carbohydrate metabolism (Wang *et al.*, 2024c). However, the pathogenic potential of *C. freundii* cannot be overlooked, as it is known to cause a range of infections, including diarrhoea, particularly in immunocompromised individuals (Liu *et al.*, 2020b; Jabeen *et al.*, 2023).

In the present study, *B. longum* was significantly increased in fermentations with Caméor flour and extract compared to BCFN 1551, suggesting a potential role for RFOs in this response. However, *B. adolescentis* levels did not differ between genotypes or sample types. These findings indicate that *B. longum* may act as a primary degrader of raffinose and verbascose in the presence of RFOs, while *B. adolescentis* likely targets starch, regardless of pea genotype. Legumes have previously demonstrated bifidogenic properties. For instance, *in vitro* fermentation with cooked and pre-digested cowpea and black bean flour, or raw lupin and broad bean flour, showed increased Bifidobacterium abundance, although changes were not resolved at species level (Teixeira-Guedes *et al.*, 2020; Gullón *et al.*, 2015). Moreover, short-term consumption (4 weeks) of yellow pea noodles, led to an increase in *B. longum* and *R. bromii* which decreased to baseline level two weeks after the treatment ended (Yamada *et al.*, 2023).

Consistently, *R. bromii* levels were comparable across fermentations, though a trend toward higher abundance was noted in pea flour relative to extracts. This suggests that *R. bromii* proliferation is more closely linked to starch content in the flour. Additionally, *C. fessum* was significantly increased in Caméor flour fermentation compared to BCFN 1551. As *C. fessum* has been shown to lack the capacity to utilise carbohydrates such as raffinose and sucrose (Seo *et al.*, 2020), this difference may

reflect cross-feeding interactions within the microbial community rather than direct substrate utilisation. Broader studies in humans have shown mixed effects of legume diets on gut microbiota. For instance, lupin fibre increased *Bifidobacterium* spp. and a reduced Clostridium species, namely *C. ramosum, C. cocleatum*, and *C. spiroforme*a bundance (Smith *et al.*, 2006). In contrast, an 8-week pilot legumes diet intervention (involving the replacement of one lunch meal with a vegetarian legume-based meal on five weekdays) showed increased *C. leptum* subgroup, linked to amino acid metabolism (Ferreira *et al.*, 2024). The biological implications of *C. fessum* expansion in the gut also remain to be explored.

In this study, fermentation with both flour and extract forms of Caméor and BCFN 1551 peas led to elevated levels of the secondary bile acid 12-ketolithocholate. This secondary bile acid has been found to suppress IL-17A secretion, contributing to an anti-inflammatory phenotype in dextran-sulphate sodium-induced colitis in mice (Li *et al.*, 2023) suggesting a potential immunomodulatory role of pea-derived substrates. While secondary bile acids are known to support metabolic homeostasis, including regulation of glucose metabolism, and energy expenditure (Funabashi *et al.*, 2020; Makki *et al.*, 2023), their overproduction, particularly DCA, has been linked to impaired glucose metabolism and a worsened lipid profile, contributing to the development of cardiometabolic diseases (Wahlström *et al.*, 2024; Lu *et al.*, 2024). Notably, we observed no significant differences in the bile salt hydrolase (*bsh*) cpm abundance across pea treatments or between sucrose and raffinose, this could be because other secondary bile acids including 7-ketodeoxycholate and 7,12-diketolithocholate showed increased production in both sucrose and raffinose fermentations, which require the action of bsh for their synthesis. Previous studies have related gut dysbiosis to an altered, mainly reduced, production of secondary bile acids (Duboc *et al.*, 2013; Wang *et al.*, 2019). However, the implications of 12-ketolithocholate in pea fermentation requires further investigation to assess the effects of RFOs on human health.

Finally, there were no differences in SCFA production between Caméor and BCFN 1551 treatments, or between sucrose and raffinose fermentations. However, butyrate production was significantly increased in verbascose fermentation compared to raffinose fermentation, potentially due to increased abundance in several *Clostridium* species, a cluster comprised with key butyrate (Singh *et al.*, 2022).

Our data are in line with studies showing a bifidogenic effect from raffinose using *in vitro* fermentation with faecal samples (Adamberg *et al.*, 2018; Trindade, Abratt and Reid, 2003; Kan *et al.*, 2020; Dinoto *et al.*, 2006). In addition, we showed an increased abundance of other bacterial species, particularly carbohydrate degraders, in line with studies showing an association between vegetarian diet and the enrichment of pathways related to carbohydrate metabolism (De Angelis *et al.*, 2020; Koh *et al.*, 2016). Moreover, the presence of non-carbohydrate degraders was also identified,

which may be due to cross-feeding interactions (Xiao *et al.*, 2024). Overall, the batch fermentation results highlighted compositional and metabolic shifts in the gut microbiota following exposure to individual RFOs and pea treatments. While bifidogenic effect was observed with raffinose, and carbohydrate degraders increased across conditions, the effects of Caméor and BCFN 1551 remained similar. This likely reflects the relatively minor contribution of RFOs to pea composition, overshadowed by starch and sucrose content, as described in section 1.4.1., which may have driven much of the microbial response.

While the batch model used here provided first insights into the effects of pea RFOs on gut microbial composition and function, its static nature and accumulation of fermentation end-products limit *in vivo* relevance over extended timeframes (Isenring *et al.*, 2023). For longer-term studies that better reflect physiological conditions, dynamic, multistage systems are preferred (Isenring *et al.*, 2023) as they allow for stable microbial ecosystems and spatial–temporal adaptation of the colonic microbiota to dietary inputs (Gagnon and Brubaker, 2015).

In addition, to their effects on the gut microbiota, fermentation products of prebiotic fibres, such as oat β-glucan and inulin, have been found to increase the strength of the gut barrier *in vitro* (Pham *et al.*, 2018). Building on this, we assessed the impact of fermented Caméor and BCFN 1551 flours on human colonic organoid-derived intestinal monolayers. These organoid models offer a physiologically relevant platform to explore how microbial metabolites, and dietary inputs influence epithelial cell homeostasis (Rubert et al., 2020) and inflammation (Varani et al., 2023). Moreover, they provide a tool for screening interventions that may mitigate pro-inflammatory responses, supporting the development of personalised nutritional strategies (Crawford et al., 2023).

The results showed that 24 h stimulation of colonic organoids isolated from healthy donors with fermented pea flour led to an IL-8 production, a crucial mediator in the enhancement of the inflammatory response in IBD, as it functions as a potent chemoattractant of neutrophils (Harada *et al.*, 1994), which was increased in Caméor compared to BCFN 1551. The main function of IL-8 is to induce chemotaxis and facilitate the migration of neutrophils and other inflammatory cells toward and infection site (Cotton *et al.*, 2016; Martins-Green, Petreaca and Wang, 2013; Rostami Nejad *et al.*, 2011). Fermented Caméor also induced higher levels of IP-10 and TGF-β1 relative to the medium-only control. IP-10 is a recognised biomarker for disease severity (Liu *et al.*, 2011), while TGF-β1 acts as a critical regulator of immune tolerance in the gut, suppressing pro-inflammatory responses (Letterio and Roberts, 1998) and contributing to intestinal immune homeostasis (Konkel and Chen, 2011). Dysregulations of TGF-β signalling has been implicated in IBD pathogenesis (Feagins, 2010; Biancheri *et al.*, 2014). In contrast, the treatments with Caméor and BCFN 1551 fermentations led to significantly less production of MCP-1 compared to medium-only control. Although the exact recruitment mechanisms of monocytes in IBD remain unclear (Ott *et al.*, 2023), MCP-1 is known to be upregulated in inflamed tissues via pro-inflammatory stimuli such as TNF-α, IL-1 and endotoxins (Strieter and Kunkel, 1993; Proost, Wuyts and Van Damme, 1996). Notably, the expression of IP-10, IL-8 and MCP-1 in the lamina propria of colonic tissue biopsies have been found to be elevated

in patients with UC compared with healthy controls (Uguccioni *et al.*, 1999). Here, these three chemokines were present at variable levels across the fermentation conditions tested. Additionally, both fermented flours increased IFN-γ and TNF-α compared to the medium control. IFN-γ is often used to assess intestinal inflammation severity (Pabst and Cerovic, 2024), and although not commonly used in IBD, anti-IFN-γ therapy can be effective in the treatment of CD (Cui *et al.*, 2013). TNF-α, a central inflammatory cytokine, can induce IL-1β; however, IL-1β levels remained low across all conditions.

The effects of diets rich in legumes on pro- and anti-inflammatory cytokine production have been previously reported in intervention studies. For example, cooked whole-bean diets were shown to exert both beneficial and adverse effects during experimental colitis in mice, by reducing the levels of inflammatory biomarkers such as IFN-γ and increasing the production of anti-inflammatory cytokine IL-10 (Zhang *et al*, 2014). In addition, legumes also contain various other bioactive compounds, such as phenols, polysaccharides (pectin and xylans), proteins and peptides, have been identified in legumes that have been reported to display anti-inflammatory effects *in vivo* and *in vitro*, as previously reviewed (Juárez-Chairez *et al.*, 2022). For example, peptide fractions of navy bean and light red kidney bean milk and yogurt subjected to *in vitro* gastrointestinal digestion were found to limit IL-8 production in TNF-*α*-stimulated Caco-2 and HT-29 cell lines (Chen *et al.*, 2019).

Our results suggest that, overall, the treatment with fermented Caméor led to an induction more towards inflammatory responses than BCFN 1551 fermentation; however, the mechanisms and biological relevance of these observations remain to be explored. One possibility could be linked to the increase in secondary bile acids in the fermentation of BCFN 1551, which may offer protection. Indeed, using a pro-inflammatory intestinal model consisting of a co-culture of Caco-2 and HT29-MTX-E12 cells in the apical compartment and activated dendritic cells in the basolateral compartment, it was shown that apical treatment with LCA and DCA restored TEER and increased the expression of genes related to intestinal epithelial integrity and mucus layer (although not significantly) (van der Lugt *et al.*, 2022).

Under the conditions tested here using a healthy intestinal epithelium, TEER was not affected by the treatments, which is consistent with the similar SCFA profiles obtained across fermentation treatments (section 4.2.1.2.2.). In addition, the integrity of the organoid monolayers may be such that it may not be possible to further increase the TEER, in contrast to monolayers formed by Caco2 cells which are less tight (Kollmann *et al.*, 2023). To address this issue, we have attempted to disrupt the gut barrier formed by colonic organoids using a cocktail of inflammatory cytokines prior to the pea fermentation treatments. However, we could not observe a decrease in TEER under the conditions tested, so more work is needed to optimise this model.

These preliminary findings in human-derived colonic organoids underscore the complexity of gut immune responses and the importance of using physiologically relevant models to assess the effects of fermented dietary components. Organoids offer a closer approximation to the in vivo intestinal environment than conventional cell lines. Future studies should incorporate immune cells, such as peripheral blood mononuclear cells, to better capture inflammatory cascades, and include known pro- and anti-inflammatory compounds as controls to contextualise cytokine responses. It

would also be valuable to assess the impact of fermented RFOs and pea in inflamed organoid models mimicking a compromised gut barrier (Meir *et al.*, 2020; Xing *et al.*, 2023; Takashima *et al.*, 2019).

5. Chapter Five: Effects of BCTILLRfs W521* pea mutant on gut microbiota structure, function and gas production

5.1 Introduction

The discomfort associated with the consumption of RFOs from the diet has led to previous suggestions to reduce or eliminate these from legumes through breeding techniques (Gupta, Gupta and Kumar, 2021). However, this would not only compromise their prebiotic effects (as explored in Chapter 4), but the plant capacity to respond to stress, as shown in Chapter 3 where the depletion of RFOs in the BCFN 1551 mutant was associated with higher susceptibility to drought stress and poor performance in field trials.

Therefore, developing novel pea varieties with variable RFO levels such as the TILLRfs W521* mutant characterised in Chapter 3 could provide a trade-off for increasing pea human consumption as a sustainable food while maintaining prebiotic properties and competitive agricultural traits.

The composition of RFOs in the TILLRfs W521* pea mutant was 0.9 µg/mg raffinose, 3.3 µg/mg stachyose and 17.5 µg/mg verbascose as determined by LC-MS which compares to 4.2 µg/mg raffinose, 15.5 µg/mg stachyose and 44.7 µg/mg verbascose for the commercial cultivar Caméor. There was also an increase in RFO precursors in the TILLRfs W521*, containing 34.8 µg/mg sucrose and 4.6 µg/mg galactinol, as compared to 18.5 µg/mg sucrose and 0.8 µg/mg galactinol in Caméor (see Chapter 3).

The TILLRfs W521* pea mutant was shown to retain plant fitness and yield in the field trial, making it a promising candidate for future research. Here, the effects of the fermentation from the BCTILLRfs W521* mutant on the gut microbiota were explored using a dynamic *in vitro* colon model. The TWIN SHIME® system represents a gut model that mimics the entire GI tract incorporating stomach, small intestine and different colon regions (Van de Wiele et al., 2015).

Finally, we investigated the *in vitro* effects of TILLRfs W521* flour fermentation on gas production as compared to Caméor and BCFN 1551 using the ANKOM RF fermentation system.

5.2 Results

5.2.1. The effect of BCTILLRfs W521* and Caméor pea flour on the human gut microbiota structure and function using a dynamic fermentation system

5.2.1.1 TWIN SHIME® experimental design

The TWIN SHIME® design was utilised with two arms running in parallel, each simulating the stomach, small intestine, and colon with its three main sections: ascending (A), transverse (T), and descending (D) regions (Fig. 51 A).

The TWIN SHIME® was inoculated with a human faecal sample (HD2) obtained from a healthy donor and the microbiota allowed to stabilise for 33 days in basal medium in both arms. The microbiota composition of the HD2 inoculum, was determined by full-length 16S rRNA gene sequencing (see Supplementary Fig. S6 A). Stabilisation was monitored by sampling each colon vessel every two to three days, once a day at the same time and measuring SCFA contents by LC-MS.

On day 33, the potato starch from the basal the medium was replaced by pea flour at a concentration of 4 g/L, which is equivalent to 1.68 g/day of flour. Arm 1 received Caméor flour and arm 2 BCTILLRfs W521* flour. Following the introduction of the pea flour, the changes in microbial composition and metabolic activity were monitored daily for up to day 52 (Fig. 51 B). Here, the microbiota was measured by full-length 16 rRNA sequencing and SCFA were measured by LC-MS.

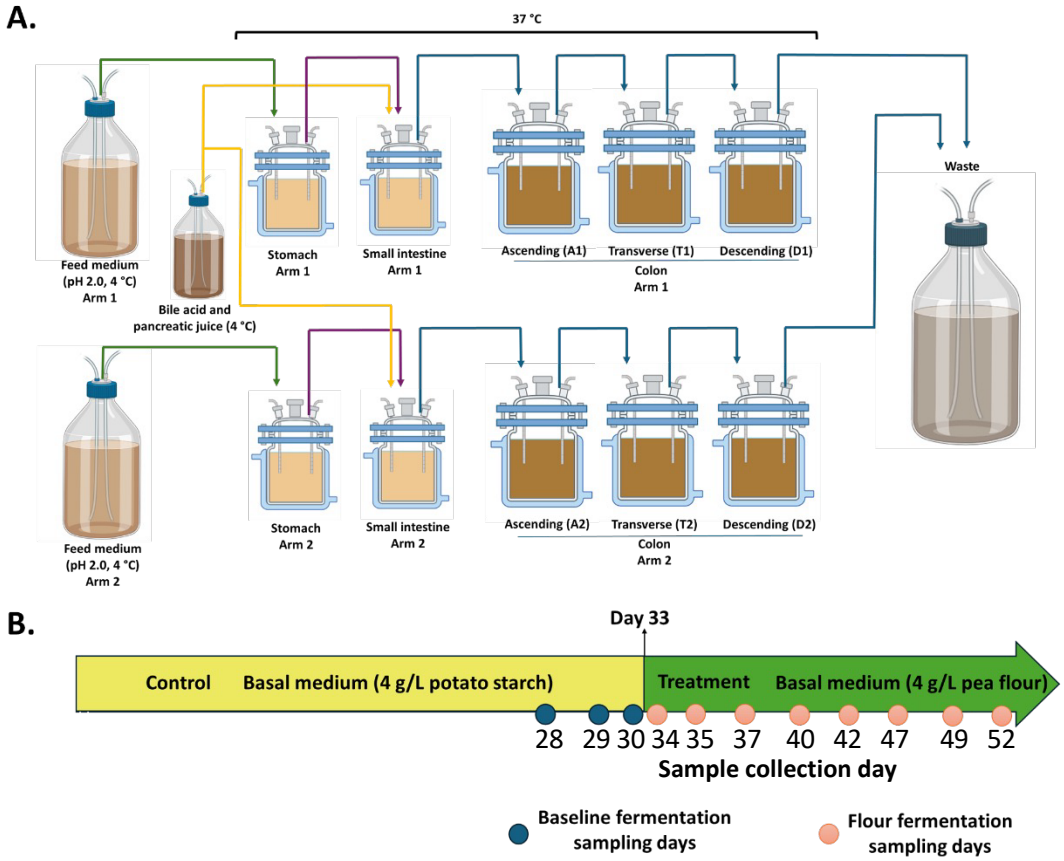


Figure 51. Schematic representation of a human TWIN SHIME® system and the timeline for baseline level and treatment samples collection. (**A**) The bioreactors for arm 1 and arm 2 are represented by the stomach, the small intestine and the three colon vessels: ascending (A1, A2), transverse (T1, T2) and descending (D1, D2). Green lines: connections between the feed medium and the stomach bioreactors; purple lines: connections between the stomach and small intestine bioreactors; blue lines: connections between the different bioreactors for transfers (till the waste); yellow lines: connections from pancreatic juice media for digestion simulation. (**B**) Timeline of the TWIN SHIME® experiment. After the inoculation of human faeces in the ascending (A1 and A2), transverse (T1 and T2) and distal (D1 and D2) colon vessels, the microbiota was left to stabilise for 33 days. Created in BioRender scientific illustrator.

5.2.1.2 Effect of BCTILLRfs W521* and Caméor pea flour on the human gut microbiota composition

To determine the effect of the treatments on the composition of the gut microbiota, DNA was extracted from samples collected at days 28, 30 and 33 (baseline microbiota before addition of pea flour) and days 34, 35, 37, 40, 42, 47, 49, 52 (following pea flour treatment) and analysed by full-length 16 rRNA sequencing. This technique has recently been implemented as an improvement to traditional 16 rRNA sequencing which relies on the sequencing of variable regions that may introduce a bias to specific bacterial groups, while this approach sequences the full-length rRNA, therefore improving the accuracy of strain assignment (Buetas *et al.*, 2024).

To assess the richness and evenness of species within the fermentation vessels, several key metrics were used to study alpha diversity, including the Shannon index, OTUs, and dominance (Fig. 52). These analyses showed no statistically significant differences among the samples in each pair of vessels of the simulated colon vessel (A1, vs. A2, T1 vs. T2 and D1 vs. D2). This finding suggests a consistent level of species diversity across these samples, indicating that the fermentation process did not lead to notable changes in species composition or richness.

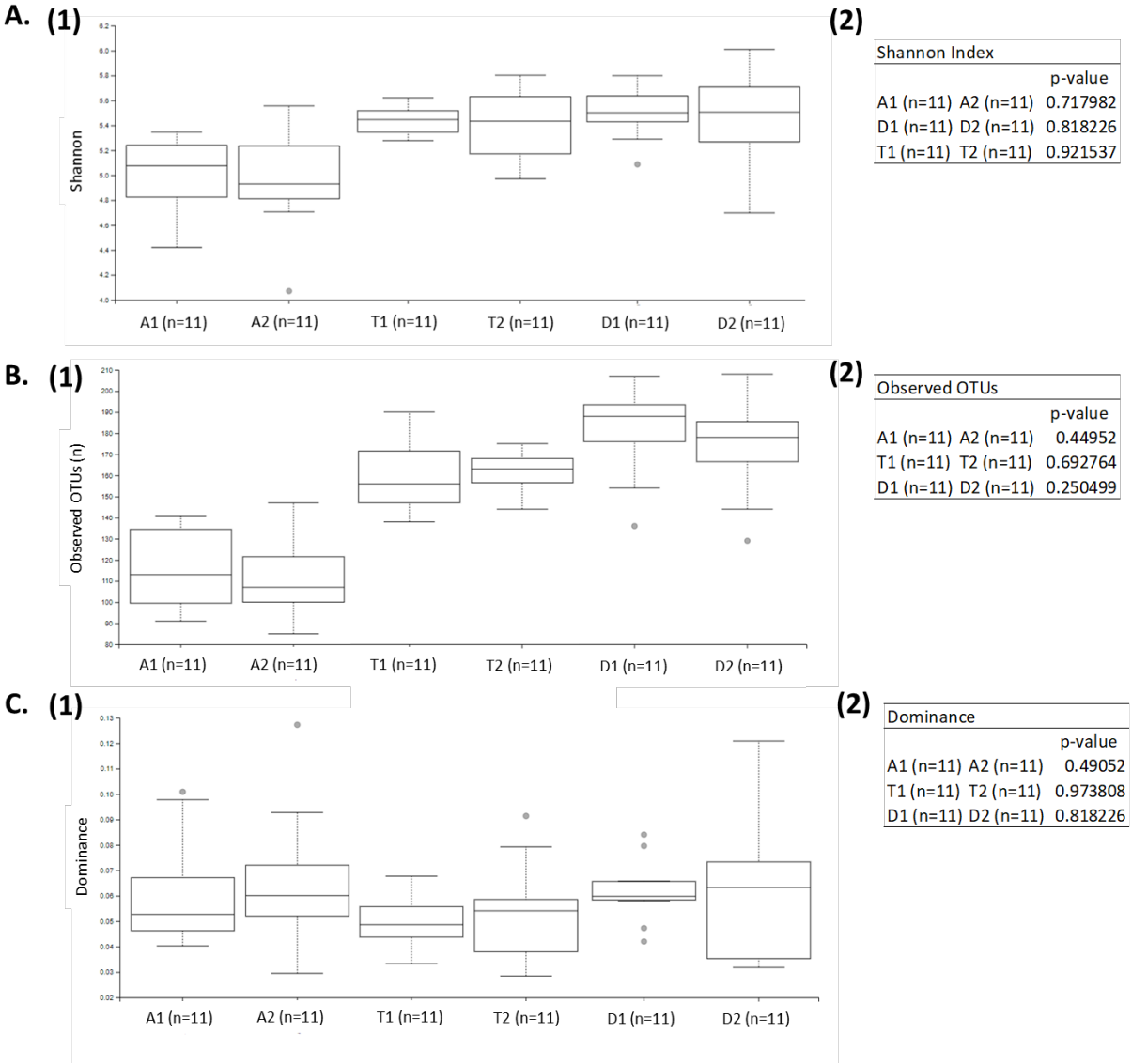
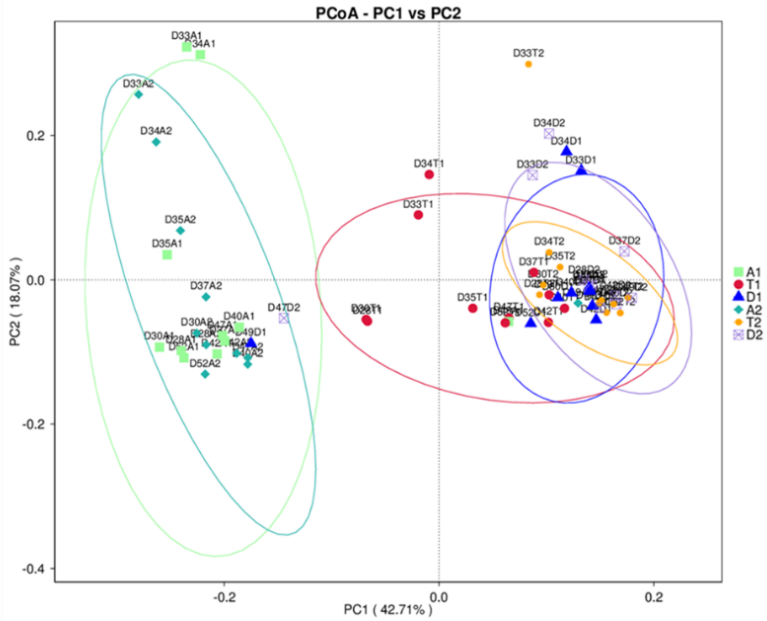


Figure 52. Effect of BCTILLRfs W521* and Caméor flour supplementation on average alpha diversity of faecal microbiota composition from TWIN SHIME® fermentation. The box plots (1) depict alpha diversity of microbial communities from TWIN SHIME® fermentation of pea flour as measured by (**A**) the Shannon Index, (**B**) distribution of OTUs and (**C**) species dominance. A1: ascending colon, arm 1; A2: ascending colon, arm 2; T1: transverse colon, arm 1; T2: transverse colon, arm 2; D1: descending colon, arm 1; D2: descending colon, arm 2. Error bars represent standard deviation across biological samples. (2) Statistical significance between groups was assessed using a one-way non-parametric ANOVA and p value for each group is described. The data is averaged across all timepoints (n=11), before and after pea flour addition.

When investigating the effect of pea flour on the overall faecal microbiota composition, no significant differences were observed between vessels corresponding to the same colonic region, as confirmed by the beta diversity analysis using Bray-Curtis distance matrices or analysis of similarities (ANOSIM) statistical tests (Fig. 53). However, across the colonic vessels, it could be observed that the data from the

ascending colon in both arms (A1 and A2) clustered together, whereas there was an overlap in the transverse and descending colon clusters in both arms (T1, T2, D1 and D2), indicating that the microbiota compositions of the transverse and descending colon were similar, and distinct from that of the ascending colon, independently of the treatment.

A.



B.

		R	p value	q value
A1	A2	-0.01608	0.597015	0.597015
T1	T2	0.411871	0.004975	0.005741
D1	D2	0.248385	0.004975	0.005741

Figure 53. Effect of BCTILLRfs W521* and Caméor flour supplementation on beta diversity and analysis of similarities (ANOSIM) of faecal microbiota composition from TWIN SHIME® fermentation. (A) PCoA of beta-diversity based on Bray Curtis distances for microbial community composition in samples collected from the ascending, transverse, and descending colon. The analysis compared the effects of Caméor flour and BCTILLRfs W521* flour fermentations. Samples were collected every two or three days, over a period of 19 days, specifically on days 28, 29, 30, 34, 35, 37, 40, 42, 47, 49, and 52, with one sample per vessel per day. Each point on the plot represents a distinct sample, color-coded according to the vessel type and condition tested. (B) ANOSIM analysis with R-values ranging from -1 to 1. R-values close to 0 indicate no significant differences between inter-group and intra-group variations. An R-value close to 1 indicates that the differences between groups are greater than the differences within groups. P value provides the statistical significance of the observed R-value. A1: ascending colon, arm 1; A2: ascending colon, arm 2; T1: transverse colon, arm 1; T2: transverse colon, arm 2; D1: descending colon, arm 1; D2: descending colon, arm 2.

The baseline phylum overview (prior pea flour treatment), calculated as the mean of the microbiota composition at days 28, 30 and 33, showed that:
In the ascending colon, arm 1 was primarily defined by the presence of Actinomycetota (43 %), Bacillota (37 %), Bacteroidota (11 %) and Desulfobacterota (8.5 %) (Fig. 48 A 1). Similarly, the ascending colon in arm 2 was comprised of Actinomycetota (40 %), Bacillota (32 %), Bacteroidota (14 %) and Desulfobacterota (13 %) (Fig. 54 A 2).

In the transverse colon, arm 1 was primarily characterised by the presence of Bacillota (44 %), Actinomycetota (24 %), Bacteroidota (21 %) and Desulfobacterota (8 %) (Fig. 48 B 1) while arm 2 was characterised by the presence of Bacteroidota (38 %), Bacillota (36 %), Desulfobacterota (11 %) and Actinomycetota (8 %) (Fig. 54 B 2).
Finally, the descending colon arm 1 was comprised predominantly of Bacillota (46 %), Bacteroidota (23 %), Actinomycetota (15 %) and Desulfobacterota (10 %) (Fig. 53 C 1), whereas arm 2 was characterised by the presence of Actinomycetota (43 %), Bacillota (37 %), Bacteroidota (11 %) and Desulfobacterota (8 %) (Fig. 54 C 2).

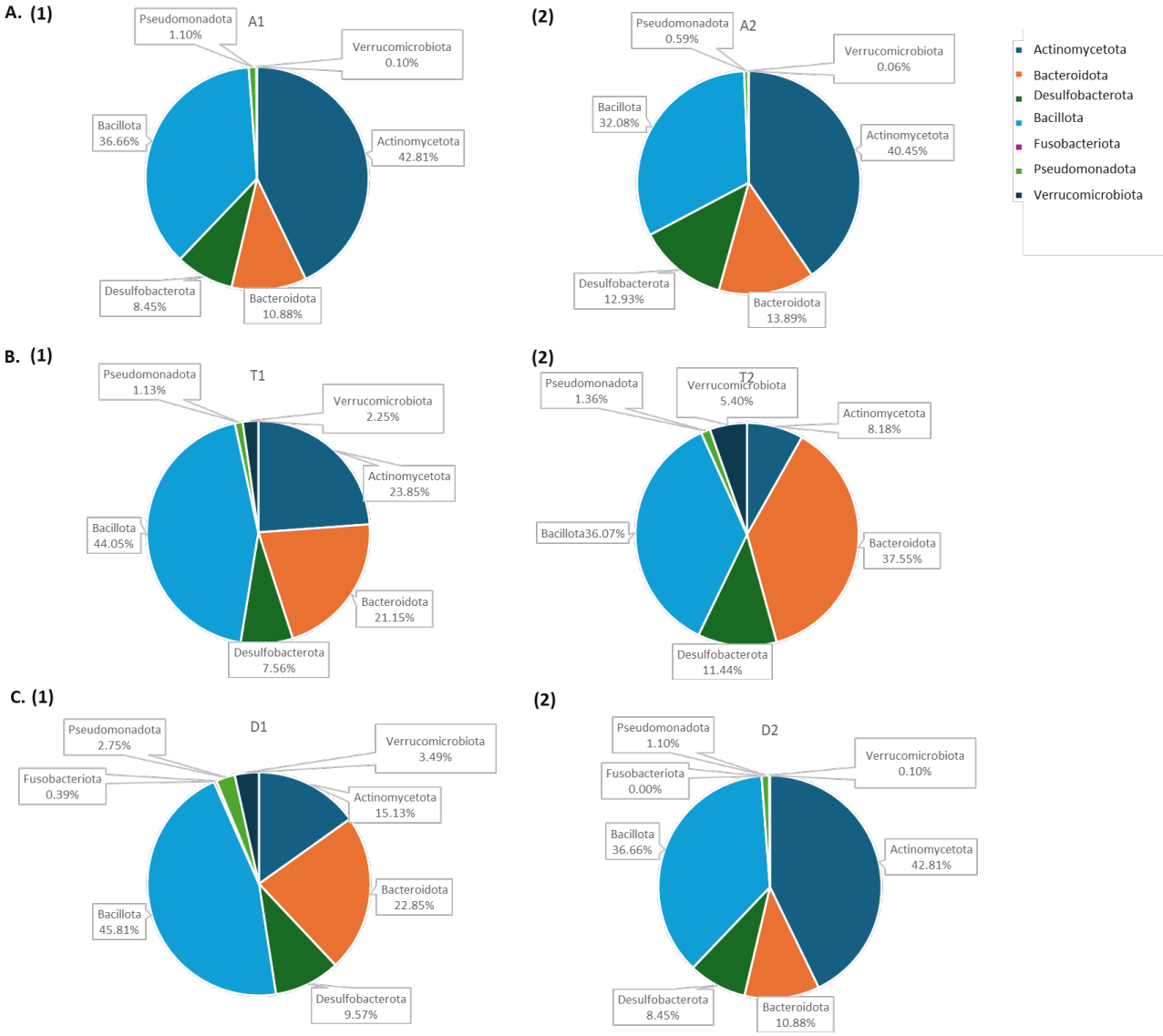


Figure 54. Phylum microbial composition of the baseline microbiota prior to BCTILLRfs W521* and Caméor flour fermentation. Pie charts illustrating the mean relative abundance of baseline microbial phyla in the three simulated colonic regions: (**A**) ascending, (**B**) transverse, and (**C**) descending for each TWIN SHIME® arm (1) and (2) at days 28, 30 and 33. Each chart displays the percentage composition of all detected phyla present prior to the introduction of pea flour.

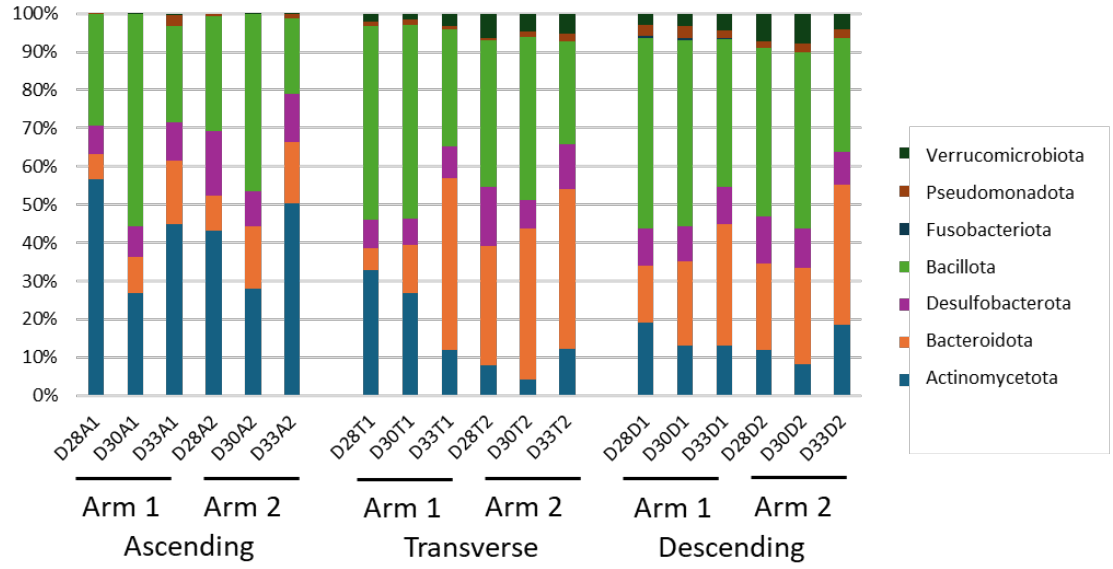
The mean phylum overview provided a general evaluation of the gut microbiota status in both arms, however, there were fluctuations in the microbial community

composition for each day, which can be seen in more detail at the family and species levels (Fig. 55 A and B), as described below.

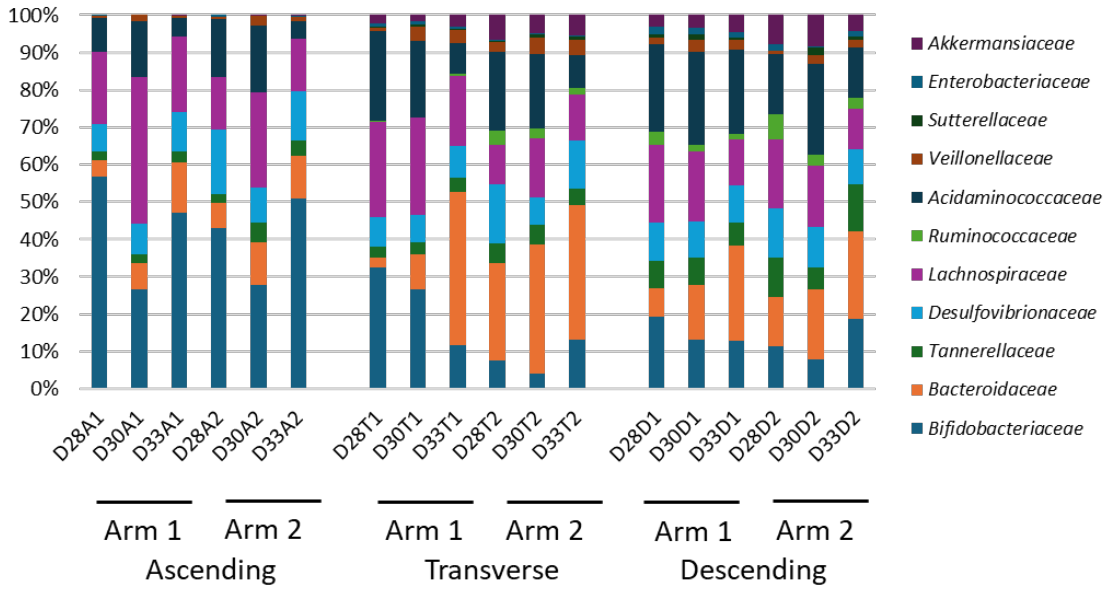
The ten most prevalent families throughout all simulated sections of the colon included Akermansiaceae, Enterobacteriaceae, Sutterellaceae, Veillonellaceae, Acidaminococcaceae, Ruminococcaceae, Lachnospiraceae, Desulfovibrionaceae, Tannerellaceae, and Bifidobacteriaceae (Fig. 54 B). The ascending colon was primarily characterised by Bifidobacteriaceae, in both arms, while the transverse colon was mainly marked by Bifidobacteria in arm 1 and Bacteroidaceae in arm 2. Finally, the descending colon was mostly represented by Acidaminococcaceae in both arms.

The ten most prevalent species throughout all simulated sections of the colon included *A. muciniphila*, *Acidaminococcus instestini*, *Clostridium bolteae*, *Bilophila wadsworthia*, *B. uniformis, Parabacteroides distasonis*, *B. vulgatus*, *B. thetaiotaomicron, B. ovatus* and *B. adolescentis* (Fig. 55 C). The ascending colon was primarily characterised by *B. adolescentis* in both arms, while the transverse colon was mainly characterised by *B. adolescentis* in arm 1 and by *A. instestini* in arm 2, respectively, and lastly, the descending colon was largely represented by *A. instestini* in both arms.

A.


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
D28A1 D30A1 D33A1 D28A2 D30A2 D33A2 D28T1 D30T1 D33T1 D28T2 D30T2 D33T2 D28D1 D30D1 D33D1 D28D2 D30D2 D33D2
Arm 1 Arm 2 Arm 1 Arm 2 Arm 1 Arm 2
Ascending Transverse Descending
Verrucomicrobiota
Pseudomonadota
Fusobacteriota
Bacillota
Desulfobacterota
Bacteroidota
Actinomycetota

B.


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
D28A1 D30A1 D33A1 D28A2 D30A2 D33A2 D28T1 D30T1 D33T1 D28T2 D30T2 D33T2 D28D1 D30D1 D33D1 D28D2 D30D2 D33D2
Arm 1 Arm 2 Arm 1 Arm 2 Arm 1 Arm 2
Ascending Transverse Descending
Akkermansiaceae
Enterobacteriaceae
Sutterellaceae
Veillonellaceae
Acidaminococcaceae
Ruminococcaceae
Lachnospiraceae
Desulfovibrionaceae
Tannerellaceae
Bacteroidaceae
Bifidobacteriaceae

C.

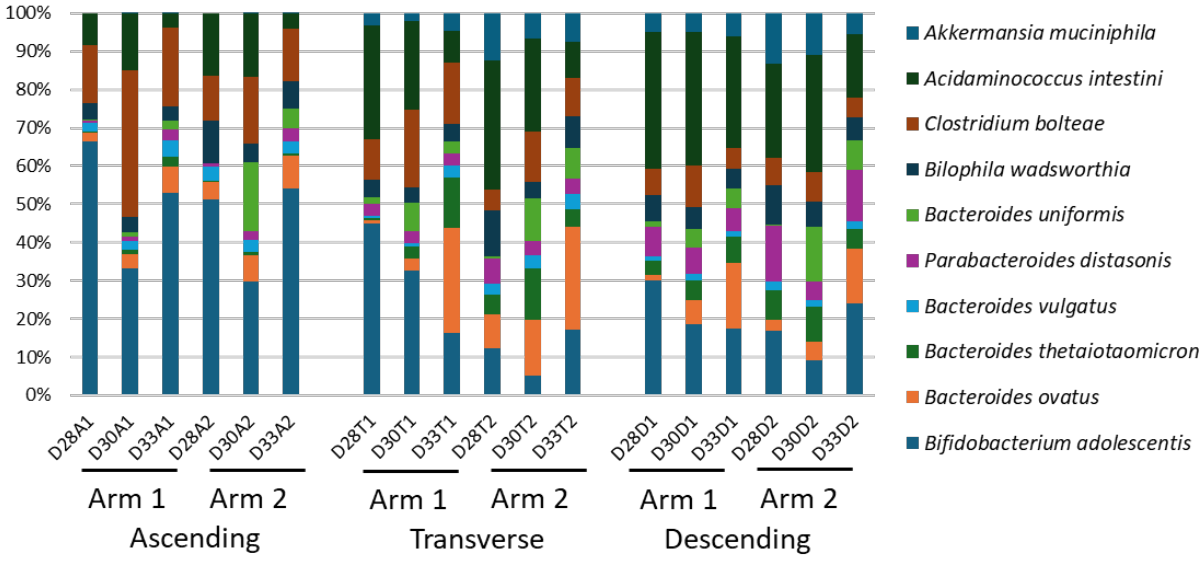
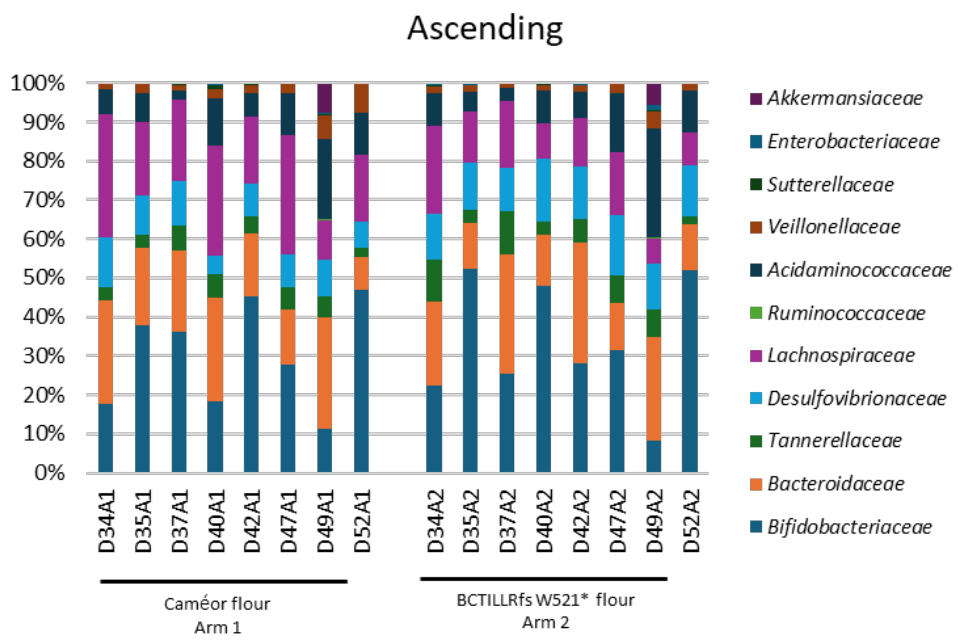

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D28A1 D30A1 D33A1 D28A2 D30A2 D33A2 D28T1 D30T1 D33T1 D28T2 D30T2 D33T2 D28D1 D30D1 D33D1 D28D2 D30D2 D33D2
Arm 1 Arm 2 Arm 1 Arm 2 Arm 1 Arm 2
Ascending Transverse Descending
Akkermansia muciniphila
Acidaminococcus intestini
Clostridium bolteae
Bilophila wadsworthia
Bacteroides uniformis
Parabacteroides distasonis
Bacteroides vulgatus
Bacteroides thetaiotaomicron
Bacteroides ovatus
Bifidobacterium adolescentis

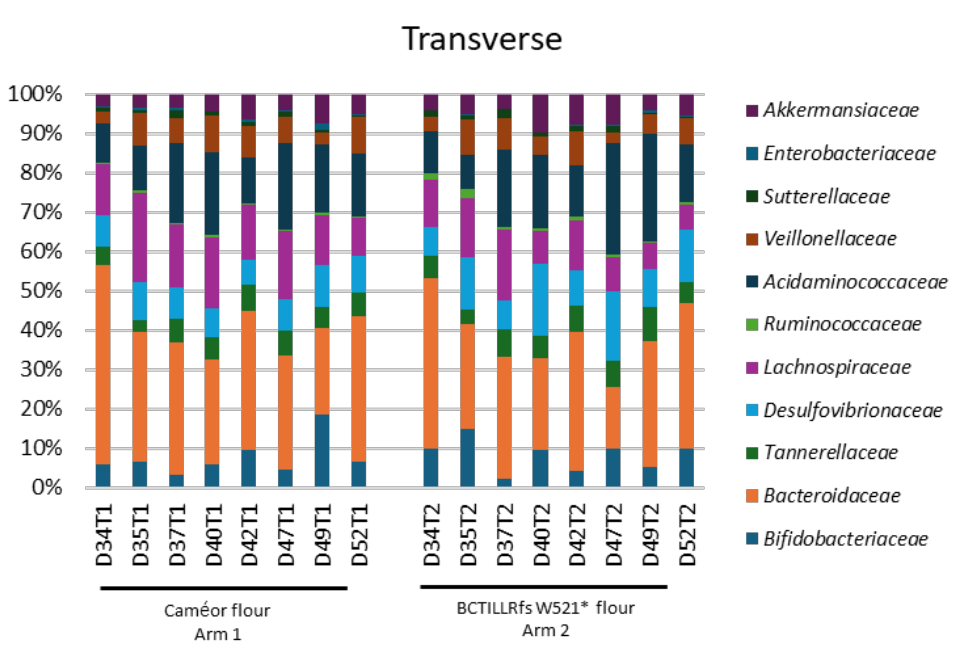
Figure 55. Microbial composition of the baseline microbiota prior to BCTILLRfs W521* and Caméor flour fermentation. The relative abundance of the top ten taxa at the (**A**) phylum level, (**B**) family level and (**C**) the top ten taxa displayed at the species level. Sample annotation: D## indicates the day of sample collection, followed by a letter and number that correspond to the colonic segment (A for ascending colon, T for transverse colon, D for descending colon) and the treatment arm of the TWIN SHIME® model (1 or 2). Each bar represents a single sample for that timepoint.

When investigating taxonomic family changes over time across all vessels, the most dominant families observed across both arms throughout the fermentation of pea flour were Bifidobacteriaceae in the ascending and transverse colon, and Acidaminococcaceae in the descending colon following addition of the pea flour substrate (Fig. 56).

A.


Ascending
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
D34A1
D35A1
D37A1
D40A1
D42A1
D47A1
D49A1
D52A1
D34A2
D35A2
D37A2
D40A2
D42A2
D47A2
D49A2
D52A2
Caméor flour
Arm 1
BCTILLRfs W521* flour
Arm 2
Akkermansiaceae
Enterobacteriaceae
Sutterellaceae
Veillonellaceae
Acidaminococcaceae
Ruminococcaceae
Lachnospiraceae
Desulfovibrionaceae
Tannerellaceae
Bacteroidaceae
Bifidobacteriaceae

B.


Transverse
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
D34T1
D35T1
D37T1
D40T1
D42T1
D47T1
D49T1
D52T1
D34T2
D35T2
D37T2
D40T2
D42T2
D47T2
D49T2
D52T2
Caméor flour
Arm 1
BCTILLRfs W521* flour
Arm 2
Akkermansiaceae
Enterobacteriaceae
Sutterellaceae
Veillonellaceae
Acidaminococcaceae
Ruminococcaceae
Lachnospiraceae
Desulfovibrionaceae
Tannerellaceae
Bacteroidaceae
Bifidobacteriaceae

C.

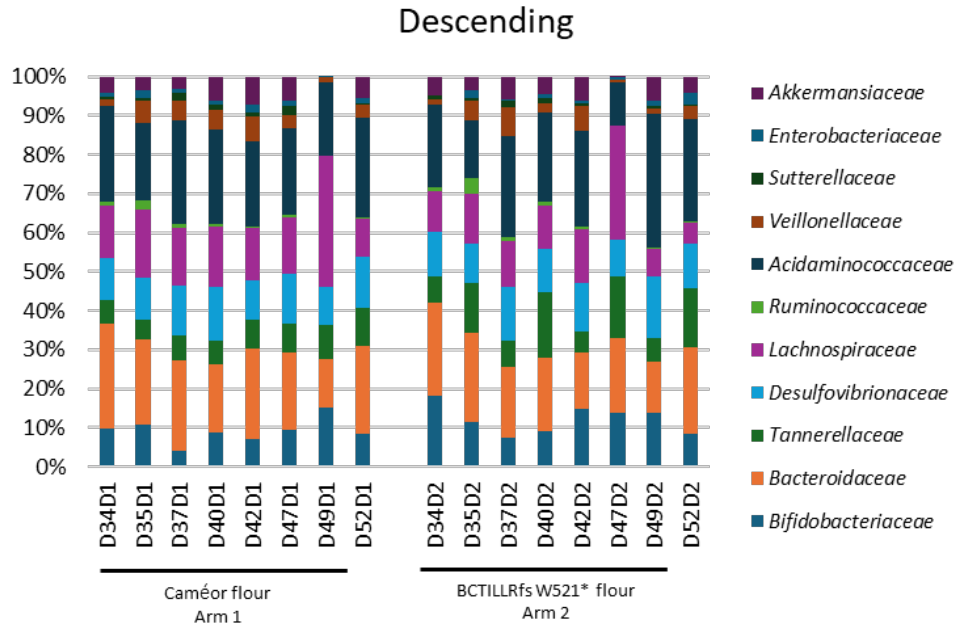

Descending
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
D34D1
D35D1
D37D1
D40D1
D42D1
D47D1
D49D1
D52D1
D34D2
D35D2
D37D2
D40D2
D42D2
D47D2
D49D2
D52D2
Caméor flour
Arm 1
BCTILLRfs W521* flour
Arm 2
Akkermansiaceae
Enterobacteriaceae
Sutterellaceae
Veillonellaceae
Acidaminococcaceae
Ruminococcaceae
Lachnospiraceae
Desulfovibrionaceae
Tannerellaceae
Bacteroidaceae
Bifidobacteriaceae

Figure 56. Longitudinal effect of BCTILLRfs W521* and Caméor flour fermentation on the relative abundance of the top ten bacterial families. (**A**) Ascending colon, (**B**) transverse colon and (**C**) descending colon. Sample annotation: D## indicates the day of sample collection, followed by a letter and number that correspond to the colonic segment (A for ascending colon, T for transverse colon, D for descending colon) and the treatment arm of the TWIN SHIME® model (1 or 2).

The most dominant species observed across both arms throughout the fermentation of pea flour were *B. adolescentis* in the ascending colon, in both arms of the transverse colon and descending colon, both dominated by *A. intestini* (Fig. 57).

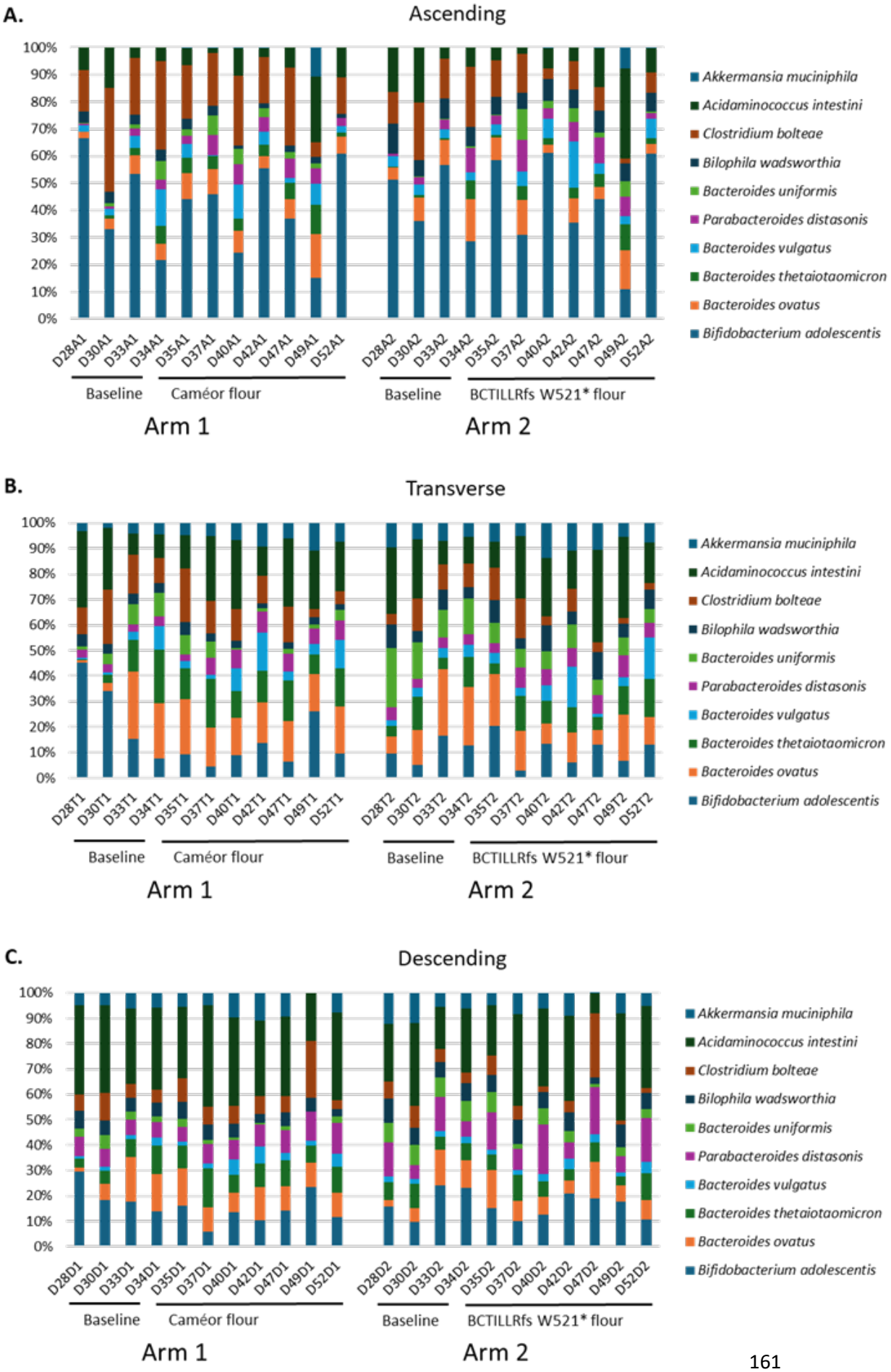

A.
Ascending
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
D28A1 D30A1 D33A1 D34A1 D35A1 D37A1 D40A1 D42A1 D47A1 D49A1 D52A1
D28A2 D30A2 D33A2 D34A2 D35A2 D37A2 D40A2 D42A2 D47A2 D49A2 D52A2
Baseline
Caméor flour
Baseline
BCTILLRfs W521* flour
Arm 1
Arm 2
Akkermansia muciniphila
Acidaminococcus intestini
Clostridium bolteae
Bilophila wadsworthia
Bacteroides uniformis
Parabacteroides distasonis
Bacteroides vulgatus
Bacteroides thetaiotaomicron
Bacteroides ovatus
Bifidobacterium adolescentis
B.
Transverse
D28T1 D30T1 D33T1 D34T1 D35T1 D37T1 D40T1 D42T1 D47T1 D49T1 D52T1
D28T2 D30T2 D33T2 D34T2 D35T2 D37T2 D40T2 D42T2 D47T2 D49T2 D52T2
Baseline
Caméor flour
Baseline
BCTILLRfs W521* flour
Arm 1
Arm 2
C.
Descending
D28D1 D30D1 D33D1 D34D1 D35D1 D37D1 D40D1 D42D1 D47D1 D49D1 D52D1
D28D2 D30D2 D33D2 D34D2 D35D2 D37D2 D40D2 D42D2 D47D2 D49D2 D52D2
Baseline
Caméor flour
Baseline
BCTILLRfs W521* flour
Arm 1
Arm 2

Figure 57. Longitudinal effect of soluble potato starch and BCTILLRfs W521* and Caméor flour fermentation on the relative abundance of top ten bacterial species. (**A**) Ascending colon, (**B**) Transverse colon, (**C**) Descending colon. Sample annotation: D## indicates the day of sample collection, followed by a letter and number that correspond to the colonic segment (A for ascending colon, T for transverse colon, D for descending colon) and the treatment arm of the TWIN SHIME® model (1 or 2). Days 28, 29, 30 and 33 correspond to baseline fermentation with potato starch, days 34, 35, 37, 40, 42, 47, 49 and 52 correspond to fermentation with pea flour. Results are based on a single replicate for each timepoint.

Although our analyses showed that the microbial composition in both arms was different prior to the fermentation of pea flour (as averaged from three samples taken on days 28, 30 and 33), t-tests were performed to determine species with significant variation between the two arms, following pea flour treatments (Fig. 58 A 1, B 1 and C 1) (for statistical purposes all timepoints during the treatment in each arm were used to do the comparisons). In the ascending colon, notable species included *C. bolteae* (higher in A1, 1.8-fold change), *B. wadsworthia* (higher in A1, 2.4-fold change), *Ruminococcus lactaris* (higher in A1, 5.0-fold change) and *C. aerofaciens* (higher in A1, 2.9-fold change). In the transverse colon, significant species included *B. uniformis* (higher in T2, 2.4-fold change), *A. muciniphila* (higher in T2, 1.4-fold change), *B. wadsworthia* (higher in T2, 2.1-fold change), *B. intestinalis* (higher in T1, 2.5-fold change), *C. scindens* (higher in T1, 4.3-fold change), *Bacteroides massiliensis* (higher in T2, 2.3-fold change), *R. lactaris* (higher in T1, 3.6-fold change) and *C. aerofaciens* (higher in T1, 15-fold change). In the descending colon, a wider variety of genera was identified, including *P. distasonis* (higher in D2, 1.6-fold change), *B. wadsworthia* (higher in D2, 1.4-fold change), *B. uniformis* (higher in D2, 2.5-fold change), *B. intestinalis* (higher in D1, 2.9-fold change), *Desulfovibrio piger* (higher in D1, 1.6-fold change), *C. scindens* (higher in D1, 2.5-fold change), *B. longum* (higher in D2, 2.6-fold change), *Parabacteroides faecis* (higher in D2, 4.8-fold change).
The significant differences in species representation between Caméor and BCTILLRfs W521 treatments (at days: 34, 35, 37, 40, 42, 47, 49 and 52) were also found between the two arms at the baseline level (days 28, 30 and 33) between the arms (1 or 2), so these cannot be attributed to the Caméor and BCTILLRfs W521* treatments (Fig. 58 A 2, B 2 and C 2). The only exception was for *C. scindens* which, in the descending colon of arm 1, was consistently elevated following Caméor fermentation, compared longitudinally to the mean of its baseline level (fold changes: 2.2, 2.5, 3.6, 2.4, 3.6, 1.9 and 1.1 for days: D34, D35, D37, D40, D42, D47, D49 and D52, respectively, statistics were not possible as these are single point samples with no technical replicates).

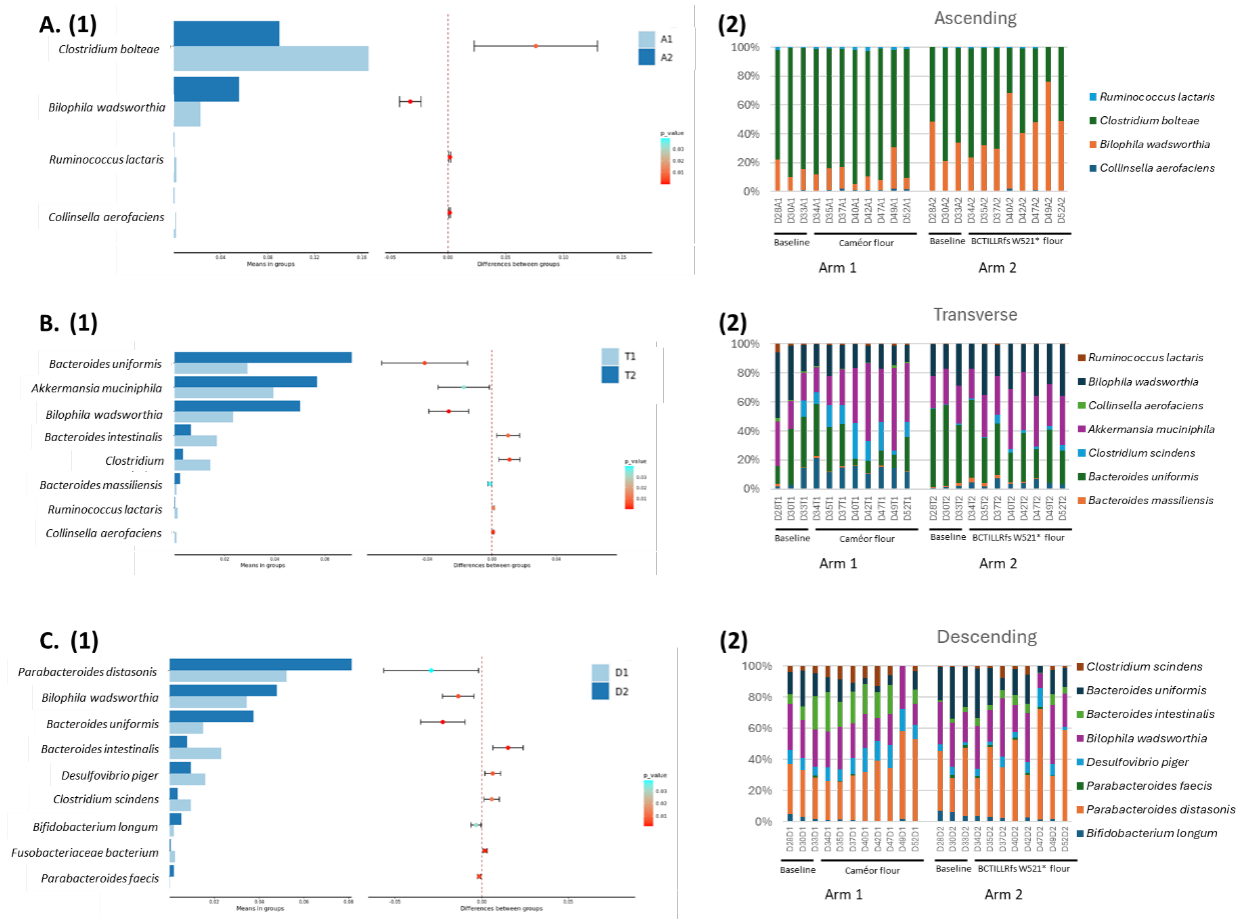


Figure 58. Significant effects of BCTILLRfs W521* and Caméor flour fermentation on the species between ascending, transverse and descending colon. Identified species with significant variation between the two arms after t-test. (**A**) Ascending colon, (**B**) Transverse colon, (**C**) Descending colon. (1) Species abundance differences between groups, and each bar in the figure represents the mean abundance of the species in different groups, this is followed by the confidence of inter-group differences. Here the leftmost point of each circle in the figure represents the lower limit of 95% confidence interval, and the rightmost point of the circle represents the upper limit of 95% confidence interval. The centre of the circle represents the differences of the mean value, and the colour of the circle represents the p value (significance heatmap: blue < 0.05, red < 0.001) of differences significance test between groups of the corresponding species. (2) Longitudinal relative species abundance across the treatment duration. A1: ascending colon, arm 1; A2: ascending colon, arm 2; T1: transverse colon, arm 1; T2: transverse colon, arm 2; D1: descending colon, arm 1; D2: descending colon, arm 2.

Finally, a Phylogenetic Investigation of Communities by the Reconstruction of Unobserved States (PICRSt2) was used for predicting biological functions from OTUs based on their 16S rRNA gene sequences. This analysis provided an estimation of differences in the metabolic functions that might be present in microbial communities. The PCA plot showed an overlap in the T1, T2, D1 and D2 clusters and an overlap in the A1 and A2 clusters, with no differences arising from the pea flour fermentation treatment (Fig. 59), consistent with the analysis of microbiome sequencing data.

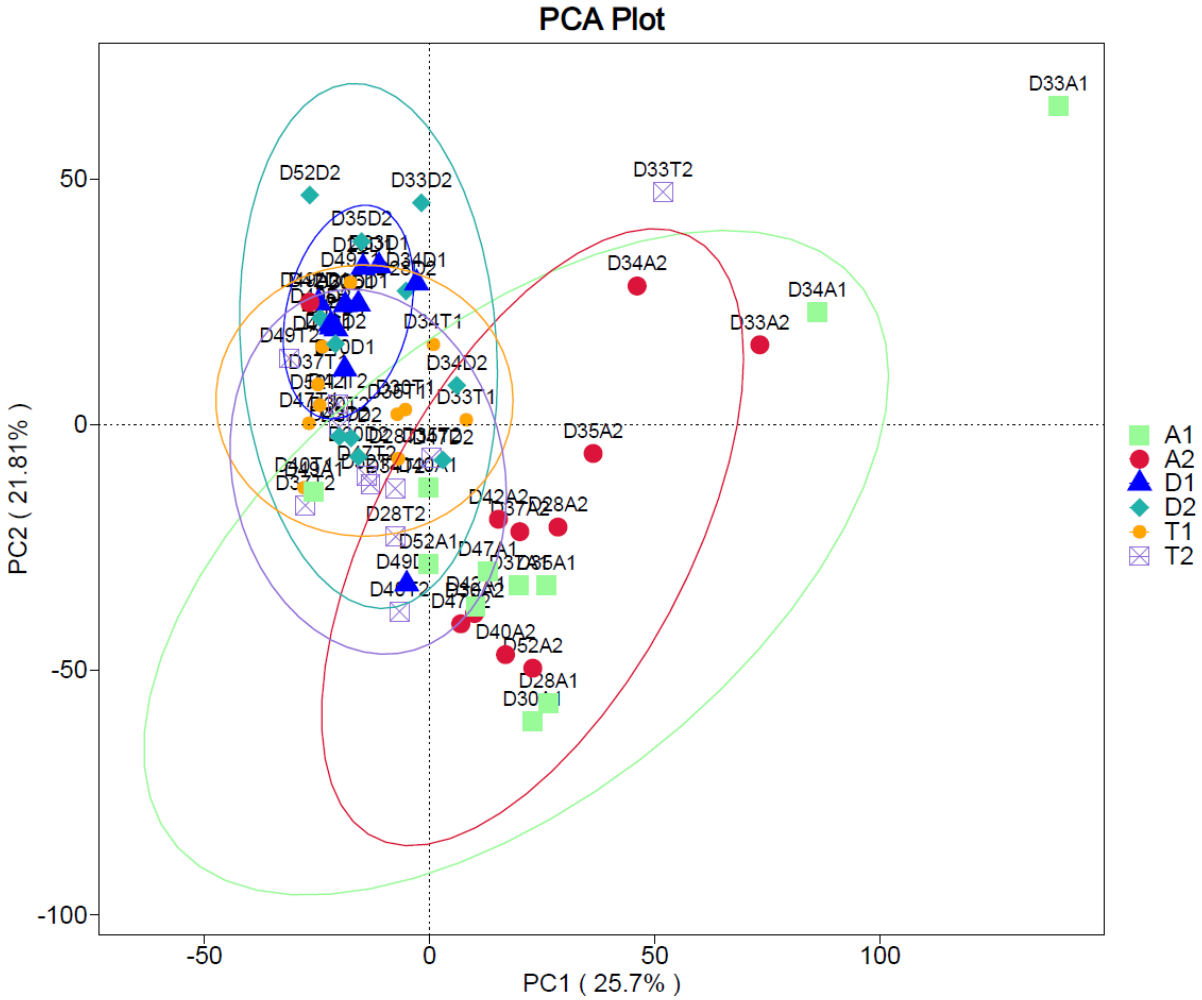


Figure 59. Effect of BCTILLRfs W521* and Caméor flour supplementation on PICRUSt2 Predictions of Biological Functions from identified Operational Taxonomic Units (OTUs). The PCA plot illustrates the variance in biological function predictions across different samples, highlighting the clustering of samples based on their functional profiles. Each point represents a sample, while the axes indicate the principal components that capture the most significant variance in the dataset. The results suggest potential functional similarities and differences among the samples, providing insights into the ecological roles of the microbial communities represented by the OTUs. Samples are grouped into clusters according to the vessel. A1: ascending colon, arm 1; A2: ascending colon, arm 2; T1: transverse colon, arm 1; T2: transverse colon, arm 2; D1: descending colon, arm 1; D2: descending colon, arm 2.

5.2.1.3. Effect of BCTILLRfs W521* and Caméor pea flour fermentation on the production of SCFAs and BCFAs

To monitor the effect of pea flour supplementation on the production of SCFAs and BCFAs by the human gut microbiota, the supernatant of samples collected across the longitudinal fermentation with Caméor or BCTILLRfs W521* (days 34 to 52) were analysed by UPLC-MS/MS and compared with the stabilisation period (days 28 to 33).

The results showed the production of acetate, followed by butyrate and propionate (Fig. 60). Throughout the fermentation period, acetate was the most abundant SCFA produced across both arms (Fig. 60 A).

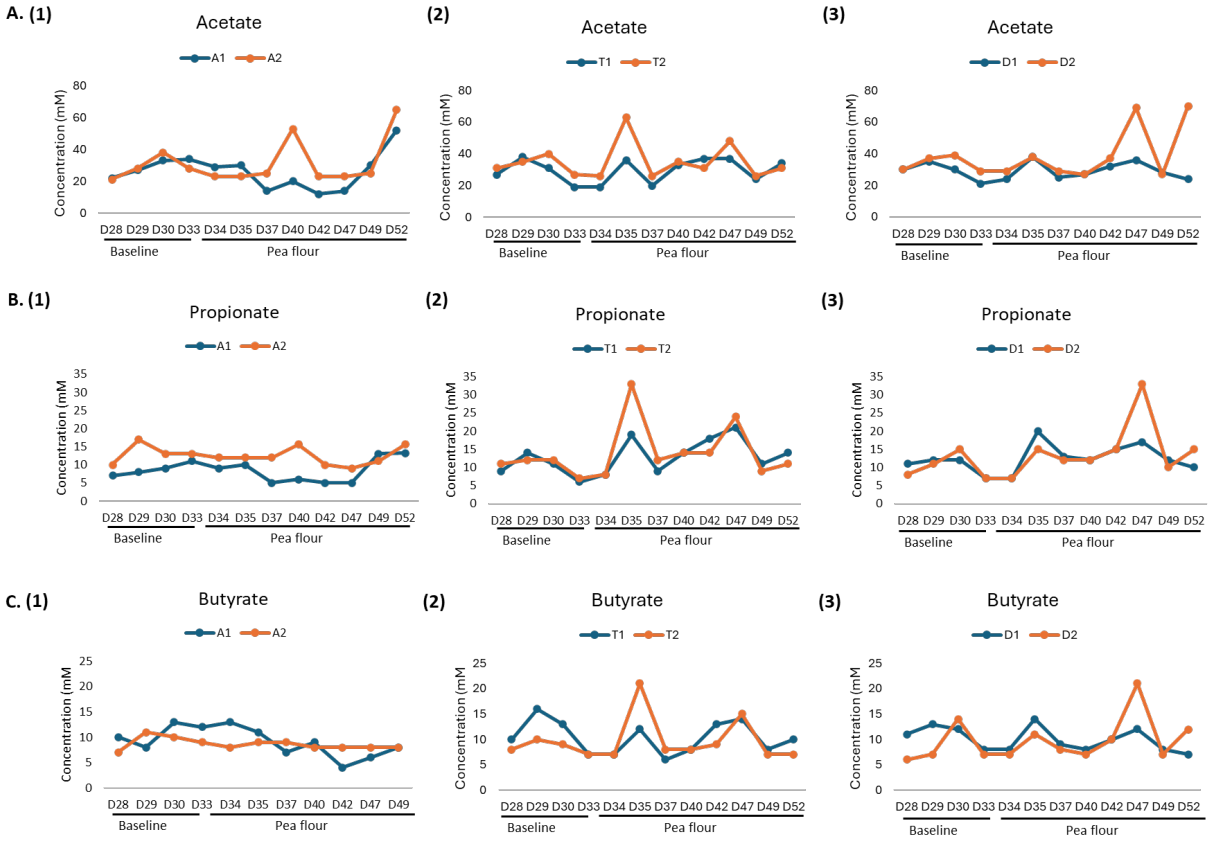


Figure 60. Longitudinal effect of soluble potato starch at the baseline fermentation and pea flour on the production of SCFAs in TWIN SHIME® fermentations. (**A**) acetate, (**B**) propionate, and (**C**) butyrate, in (1) ascending colon (A1= arm 1, A2 = arm 2), (2) transverse colon (T1= arm 1, T2 = arm 2) and (3) descending colon (D1= arm 1, D2 = arm 2), during TWIN SHIME® fermentation. Days 28, 29, 30 and 33 correspond to baseline fermentation with potato starch, days 34, 35, 37, 40, 42, 47, 49 and 52 correspond to fermentation with pea flour. Results are based on a single replicate for each timepoint.

The BCFAs detected were iso-valerate, iso-butyrate and valerate (Fig. 61). The chemical structures are shown in Supplementary Fig. S7. Iso-butyrate and iso-valerate were present in all vessels across both arms while valerate was only detected in the supernatants from the fermentation in the transverse colon of arm 1, and in the transverse and descending colon in arm 2. However, it should be noted that the data represented are based on a single replicate and do account for the variability that may occur between replicates. Overall, the results did not show any differences in the levels of SCFAs and BCFAs produced upon pea flour fermentation (days 34 to 52) compared to the baseline fermentation (analysed on days 28, 29, 30 and 33).

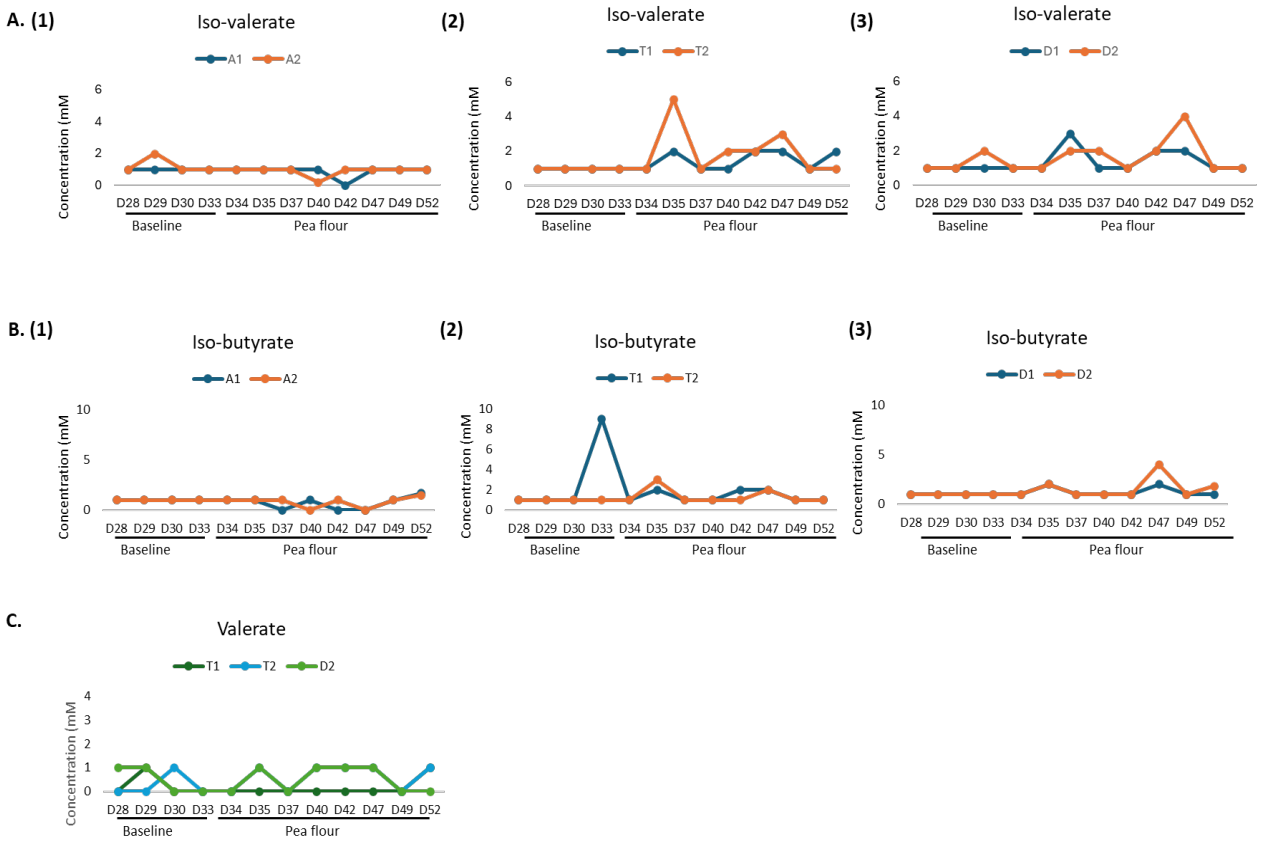


Figure 61. Longitudinal effect of soluble potato starch at baseline fermentation and pea flour on the production of BCFAs in TWIN SHIME® fermentations. (**A**) iso-valerate, (**B**) iso-butyrate, and (**C**) valerate, in (1) ascending colon (A1= arm 1, A2 = arm 2), (2) transverse colon (T1= arm 1, T2 = arm 2) and (3) descending colon (D1= arm 1, D2 = arm 2), during TWIN SHIME® fermentation. Days 28, 29, 30 and 33 correspond to baseline fermentation with potato starch, days 34, 35, 37, 40, 42, 47, 49 and 52 correspond to fermentation with pea flour. Results are based on a single replicate for each timepoint.

5.2.2. Effect of BCTILLRfs W521*, BCFN 1551 and Caméor pea flour on cumulative gas pressure using the ANKOM^{RF} fermentation system

The effects of BCFN 1551 and TILLRfs W521* mutants and Caméor on gas production were investigated using the ANKOM RF® fermentation system which allowed measurements of the cumulative pressure of gas production over a 24 h period. Five fermentations were conducted with faecal samples obtained from five different individual donors, HD1 used in section 4.2.1.1., HD2 used in section 5.2.1., and HD3, HD4 and HD5. The faecal microbiota composition of each donor was determined by full length 16S rRNA gene sequencing and the analysis is provided in Supplementary Fig. S6.

The overall gas production was normalised to medium-only controls (Fig. 62 A). Interindividual variations were evident as seen with a start of cumulative pressure production after 10 h in HD1 and HD3 compared to HD2, HD4 and HD5; and low gas

production in HD4 (< 2 psi cumulative gas pressure) compared to HD1, HD2, HD3 and HD5 reaching 15-20 psi cumulative gas pressure (irrespective of the flour genotype). Despite these interindividual variations, there was a trend for increased mean cumulative pressure from Caméor fermentation (Fig. 62 B) compared to pea fermentations with mutant BCFN 1551 and TILLRfs W521*, suggesting a potential contribution of RFOs in gas production.

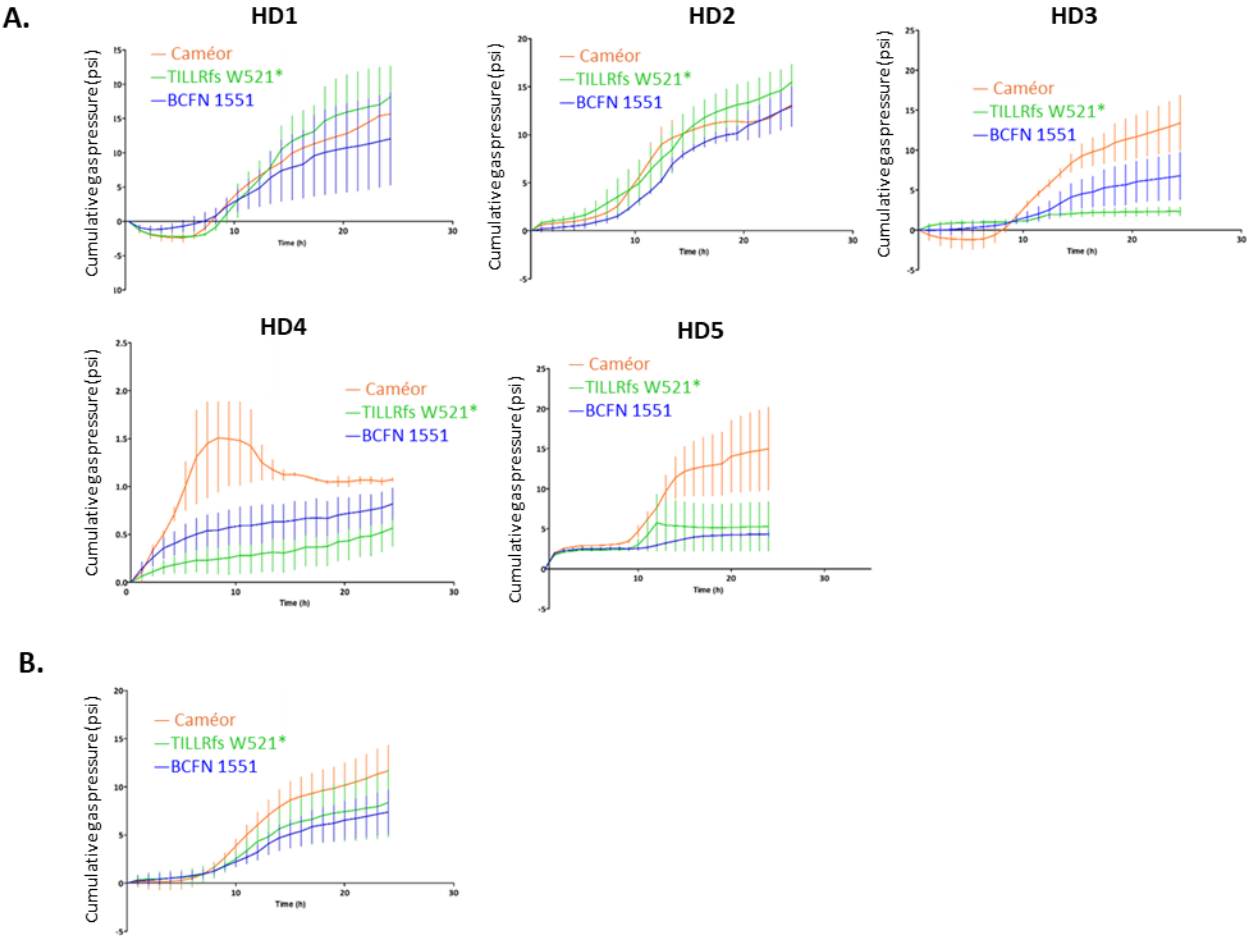


Figure 62. Effect of pea flour fermentation on cumulative gas production. Separate vessels were inoculated with faecal samples (HD1, HD2, HD3, HD4 and HD5) from five healthy donors, each of the five donor samples (HD1, HD2, HD3, HD4 and HD5) was run in triplicate vessels. Each vessel was supplemented with pea flour from the variants Caméor, BCFN 1551, and TILLRfs W521* and incubated in anaerobic conditions for 24 h. All conditions led to increased gas production and are represented as a normalisation with medium-only control vessels inoculated with faecal samples. (**A**) corresponds to the data obtained from each individual vessel, (**B**) represents the average of the data obtained with HD1, HD2, HD3, HD4 and HD5.

In addition, we measured the area under the curve (AUC) covering the cumulative pressure over the 24 h period.
No statistically significant changes could be observed between treatments across all 5 fermentations. However, Caméor fermentation produced the highest peak areas (dark

grey, Table 5) for three replicates (HD3, HD4 and HD5); and in all five samples, Caméor fermentation peak areas were larger compared to BCFN 1551, although not statistically significant.
The gas production from the fermentation of the TILLRfs W521* was less consistent showing variable peak areas across the 5 fermentations. For instance, in HD1 and HD2, the fermentation of this mutant produced higher peak areas compared Caméor, whereas in HD3 and HD4, the total area of gas produced from this fermentation was lower than the one produced from the BCFN 1551.

Table 12. Total peak area under the curve from the fermentation of Caméor, TILLRfs W521* and BCFN 1551 pea flour using the ANKOM^{RF} system.

Faecal sample donor	Area under the curve (total peak area)		
	Caméor	TILLRfs W521*	BC2FN 1551
HD1	155.9	**175.5**	125.7
HD2	165.3	**180.9**	136.3
HD3	**138.4**	37.96	71.47
HD4	**25.05**	7.213	13.59
HD5	**193.9**	93.49	75.71
Average of all fermentations	**135.7**	99.01	84.55

Data are highlighted from dark grey (the largest peak area) to white (lowest peak area) for visualisation purposes.

5.3 Discussion

The TWIN SHIME® was used to assess the effect of changes in RFO content in pea on the gut microbiota composition and function by comparing the effects of BCTILLRfs W521* and Caméor in the two arms of the model for a period of 19 days. The microbiota composition was assessed using full-length 16S rRNA gene sequencing, which provides improved taxonomic information compared to traditional 16S rRNA sequencing but lacks functional insight of shotgun metagenomics (used in Chapter 4). Shotgun metagenomics provides enhanced detection of bacterial diversity and prediction of genes (Liao *et al.*, 2023).

The system was first run for a 33-day stabilisation period in the TWIN SHIME® basal medium containing potato starch. This led to a baseline microbiota composition dominated at the phylum level by Bacteroidota, Bacillota and Actinomycetota in both arms, which is in line with the typical microbiota composition of human healthy adults (Arumugam *et al.*, 2011).

Moreover, the microbiome of the faecal inoculum differentiated into GI region-specific clusters, as shown by the beta diversity distribution. The transverse colon region shared similarities with the descending colon region (for both arms), where the clusters were overlapping, while the ascending colon (for both arms) segregated into a separate cluster. In previous studies using TWIN SHIME®, the transverse colon has been shown to act as an intermediate between the ascending and the descending colon sharing common microbial composition with these two regions of the colon (Liu *et al.*, 2018). The segregation of the ascending colon microbiome into a separate cluster, underscores the uniqueness of the microbial environment in this region, potentially driven by factors such as pH or nutrient availability.

The pea flour treatments did not affect the overall microbiota composition from the baseline, as shown by measures of alpha and beta diversity. However, further statistical analyses revealed differences at the species level between Caméor fermentation and BCTILLRfs W521* fermentation, however these were also found significantly different between the two arms during the baseline fermentation, so these differences cannot be attributed to changes in RFO content. Interestingly, from the species differing significantly between the two arms, *C. scindens* was found to be increased from baseline level in the descending colon in arm 1, perhaps suggesting a role of *C. scindens* in Caméor fermentation. One of the key functions of *C. scindens* is the transformation of primary bile acids into secondary bile acids, such as DCA (Ridlon, Kang and Hylemon, 2010). Although the role of *C. scindens* in carbohydrate fermentation is not well studied, an animal experiment showed a decrease in *C. scindens* upon supplementation with resistant starch (Reuter *et al.*, 2024) while elevated levels of DCA from *C. scindens* were correlated with impaired glucose metabolism in mice colonised with a defined microbial community containing *C. scindens* as the only DCA producer (Wahlström *et al.*, 2024). This suggests that the role of *C. scindens* in bile acid metabolism during the fermentation process, could influence other metabolic outcomes. However, the exact mechanism through which *C.scindens* interacts with dietary components such as RFOs remains unclear.

The lack of significant microbial changes upon pea treatment may be due to the composition of the diet and the duration of treatment. For instance, a study by Jie *et al.* (2021) suggested that dietary changes may not be sufficient to induce significant shifts in microbial communities, particularly if the baseline diet was already well-adapted to the existing microbial population (Jie *et al.*, 2021). Here, it is possible that the baseline microbiota shaped by the presence of soluble potato starch in the TWIN SHIME® basal medium, may have masked the potential effects of RFOs in pea flour treatments. Indeed, previous work showed that supplementation of potato starch at 11.11 mg/mL using an *in vitro* fermentation system inoculated with an adult faecal inoculum, led to increased Bifidobacterium relative abundance and the production of acetate and lactate (Klostermann *et al.*, 2024). Moreover, in a human intervention study (n=10), participants consuming raw potato starch (12 g to 48 g/day) presented consistent increased *B. adolescentis* levels in faecal samples (Nagara *et al.*, 2022). Here, *B. adolescentis*, which we showed in Chapter 4 was one of the top ten species involved in RFO utilisation, was significantly more abundant than *B. longum* throughout baseline and treatment period in both arms, and therefore the effect of the pea flour treatments may not be detectable due to prior soluble potato starch fermentation. In addition, GH13 are the most represented CAZyme-encoding genes in the gut microbiome, suggesting the wide capacity of gut microbes to ferment soluble starch (Kaoutari *et al.*, 2013) and GH13 enzymes have been reported to act in combination with GH36 α-galactosidase for the degradation of raffinose (Mao *et al.*, 2018). Future work should include a detailed examination of the synergistic interactions between GH13 and GH36 enzymes during the fermentation of both RFOs and soluble starch. Metaproteomic and metabolomic approaches could provide insights into the specific metabolic pathways most active in *B. adolescentis* and *B. longum* under different dietary conditions, particularly in response to pea flour treatments with variable RFO content. These studies could help clarify whether the abundance of *B. adolescentis*, potentially driven by prior soluble potato starch fermentation, overshadow the detectable effects of pea interventions *in vitro*.

Metabolically, the analysis of function prediction with PICRUSt2 aligned with the results of the beta diversity analysis, showing analogous functional profiles in clusters of similar microbial compositions, likely due to the presence of common taxa contributing to specific metabolic processes (Schoeps et al., 2024). This relationship is supported by studies showing that microbial diversity is closely linked to ecosystem functions, and functional redundancy can stabilise ecosystem functions (Vieira-Silva *et al.*, 2016). However, PICRUSt only predicts conserved functional traits and cannot distinguish strain-specific functionality (Douglas et al., 2020).

We next measured SCFAs and BCFAs in the fermentation vessels of the two arms. No differences were found in the production of acetate, butyrate and propionate between the two arms before and after the treatment with pea flour, consistent with the microbiome data. However, there were days characterised by an increase in specific SCFAs and BCFAs. For example, on day 33 (prior to pea flour fermentation), there was an increase in iso-butyrate in the transverse colon of arm 1, which correlated with an increase in *B. ovatus*; the capacity of *B. ovatus* to produce iso-butyrate was previously reported in monocolonised mice (Horvath *et al.*, 2022). On day 35 (48 h after pea flour fermentation started), there was an increase in acetate,

butyrate and propionate production in the transverse colon of arm 1, which coincided with an increase in *C. bolteae* and *B. ovatus*. Moreover, on the same day, there was an increase in acetate, butyrate, propionate and iso-valerate in the transverse colon of arm 2, coinciding with an increase in *B. adolescentis* and *B. ovatus*. As previously discussed in Chapter 4, *B. ovatus* has been described to generate acetic acid, propionic acid, iso-butyric acid and iso-valeric acid (Horvath *et al.*, 2022). These changes were transitory; however, it is possible that they may have been linked to the flour treatments. On day 47, in the descending colon of arm 2, there was an increase in acetate, butyrate, propionate and iso-valerate, going in hand with an increase in *C. bolteae*. BCFAs, predominantly iso-valerate and iso-butyrate, have been described to be synthesised in small quantities, they have been posited as potential indicators of protein fermentation, however, their faecal concentrations and implications on health remain to be understood, as previously reviewed (Rios-Covian *et al.*, 2020). Very little is known on the capacity of *C. bolteae* to utilise carbohydrates (Song *et al.*, 2003). Increases in *C. bolteae* abundance have been associated with certain autism spectrum disorders (Pequegnat *et al.*, 2013). Moreover, the presence of *C. bolteae* has been correlated with the expression of inflammatory genes in neuromyelitis optica spectrum disorders (Pandit *et al.*, 2021). However, these are association studies and, in the future, it will be interesting to study the role of *C. bolteae* in RFO utilisation/fermentation and associated impact on host health.

Together, these changes in microbial composition and SCFA and BCFA production were transitory and further work is required to assess the effect BCTILLRfs W521* and Caméor fermentations on other metabolites using untargeted metabolomics.

Finally, we measured the effect of RFO modulation in pea on gas production, as this is one of the main perceived barriers limiting pulse consumption (Winham and Hutchins, 2011; Mutuyemungu *et al.*, 2023). Indeed, a randomised controlled trial, showed that 50 % of healthy subjects reported increased flatulence in the first week of consuming pinto or baked beans, but the reported percentage dropped to 38 % after the second week of the trial (Winham and Hutchins, 2011). Elevated levels of hydrogen in the breath due to the fermentation of undigested carbohydrates by the gut microbiota, can be used to quantify gas production (Hammer *et al.*, 2022).

In vitro, the effect of reducing RFO levels in green pea by physical and chemical treatments (including boiling and germination) on gas production has been previously measured in *in vitro* fermentation using Hungate tubes; the results showed that 24 h fermentation of pre-digested and treated pea led to a reduction in gas production associated with a reduction in the abundance of Bacteroides and propionate levels (Rose et al., 2021). Here, we used the *in vitro* ANKOM^{RF} fermentation system to quantify cumulative gas pressure from *in vitro* fermentation of Caméor, TILLRfs W521 * and BCFN 1551. No significant differences were reported between the fermentation of the pea flours. However, the trend was towards a highest cumulative gas pressure from the fermentation of Caméor, followed by TILLRfs W521 * and BCFN 1551, suggesting that their differences in RFO content may contribute to the amount of gas produced. It should also be noted that AUC measurements showed a maximum peak area of 193.9 (in Caméor flour fermentation), which is well under that reported for inulin (7230-peak area) or psyllium (1035-peak area) in *in vitro* fermentations using

faecal samples from patients with IBS (Gunn *et al.*, 2022). This suggests that, at the concentration of pea flour used in this work, the differences in RFO content between the pea mutants and Caméor may not be sufficient to generate significant changes in gas production. In the future, it will be important to include a pea starch extract as a control for baseline gas production, and inulin at a dose relevant to human consumption as a positive control for elevated gas production.

Moreover, the increased levels of RFO precursors in the BCFN 1551 and BCRfsTILL W521* pea mutants (shown to alter microbial composition and function in Chapter 4), raises the possibility that these precursors may obscure the effects of RFO reduction on overall gas pressure accumulation. Future experiments to address gas production by pea should include both commercial RFOs and RFO precursors, as well as pre-treated (for example cooked) and pre-digested flour.

Altogether, the pea mutants BCFN 1551 and TILLRfs W521* provided first insights into the role of RFOs within pea seeds in modulating the human gut microbiota composition, metabolite and gas production. However, more studies are required to correlate this effect to their RFO content.

6. Chapter Six: Conclusions, limitations and future perspectives

Overview

RFOs, including raffinose, stachyose, and verbascose, are present in various plant species, particularly in legume seeds such as peas (Ahnen et al., 2020). These compounds have been suggested as implicated in providing protection against environmental stressors such as drought (Sharma et al., 2014) and cold (Bachmann, Matile and Keller, 1994; Gu et al., 2018), highlighting their role in stress responses. Moreover, sucrose, the precursor metabolite to RFOs, is integral to the glycolytic pathway in plants, contributing to the carbon and energy supply essential for growth and development (Lemoine et al., 2013).

Historically, RFOs have been associated with anti-nutrient effects in humans, due to their resistance to digestion (Peterbauer et al., 2001), which can lead to flatulence (Martínez-Villaluenga, Frias and Vidal-Valverde, 2008). However, recent studies suggest that RFOs have prebiotic potential, promoting the growth of beneficial bacteria and inhibiting the growth of pathogenic microorganisms in the large intestine (Elango et al., 2022).

In the context of addressing climate change and food security, legumes like peas are being considered as sustainable crops, given their positive effects on soil fertility, minimal fertiliser requirements, and high nutritional content. However, gastrointestinal discomfort remains a significant barrier to increased pulse consumption (Winham and Hutchins, 2011; Mutuyemungu et al., 2023).

This study aimed to investigate the role of RFOs in both plant and gut health using pea lines with varying RFO contents, specifically the Caméor cultivar, FN 1551/11-derived mutants (crossed to Caméor), and the BCRfsTILL W521* mutant, generated in Caméor.

6.1 The FN 1551/11 mutant showed altered plant resistance to stress and poor performance in the field compared to Caméor, while the BCRfsTILL W521* mutant performed well in field conditions

Following the selection and back-crossing of *rfs* pea mutants, seed RFO content differed significantly among Caméor and the FN 1551/11-derived and BCRfsTILL W521* mutants; FN 1551/11 showed a complete depletion of raffinose and stachyose and a significant reduction in verbascose, while BCRfsTILL W521* had a less significant reduction in raffinose, stachyose and verbascose. Both mutants accumulated sucrose and galactinol, RFO precursors.

The FN 1551/11 mutant showed a significantly lower seed yield and early senescence, under drought conditions in the glasshouse. Moreover, the FN 1551/11 mutants performed poorly in field trials over three consecutive years. The data indicated that the deletion negatively impacted plant development under field environmental conditions, despite the FN mutants performing as well as controls under standard glasshouse conditions. These results suggest a potential role of RFOs, or RFO precursors, sucrose and galactinol, implicated in pea plant responses to drought, with potential implications for improving drought tolerance in crops. Moreover, both BCRfsTILL W521* and BC2FN 1551 developed more Rhizobium-induced nodules than

Caméor. This may be linked to elevated sucrose levels in seeds, as sucrose supports redox homeostasis in nodules, though its concentration in nodules remains unclear. Future studies should measure sucrose transport from roots to nodules, assess BNF activity, quantify RFOs in other tissues like roots, and test a broader range of *Rhizobium* strains.

Furthermore, future work should include a more detailed investigation into the metabolic and regulatory pathways affected by the 1.3 Mb deletion in the FN 1551/11-backcrossed mutants. Potential links between auxin-related genes and observed phenotypes were identified, suggesting a broader impact on metabolic and regulatory pathways in peas. The impact of *apu* deletion has been explored in studying abnormal leaf movement and effects on plant hormone signalling (Chen *et al*, 2012), particularly auxin-related genes (Bai *et al*, 2022). In addition, *tryptophan aminotransferase-related protein (TAR)*, which is also within the deleted region of FN 1551/11-backcrossed mutants, is involved in auxin biosynthesis in several plants including *Lotus japonicus* and rice (Bai *et al*, 2022; Kakei *et al*, 2017). The role of auxin has also been studied in drought stress, where auxin overproduction was found to be associated with enhanced drought tolerance (Colebrook *et al*, 2014). These findings open up new avenues for exploring the intersection of hormone signalling, stress responses, and metabolic pathways in plants, which could lead to more targeted genetic modifications for crop improvement.

Additionally, the complexity of the phenotypic outcomes in the FN 1551/11-backcrossed mutants in the field (and not in the glasshouse), suggests that other genetic or environmental factors are at play. The interaction between these factors and their contribution to the observed phenotypes needs more research to be fully understood, with further mutant backcrossing to obtain isogenic lines. Another FN mutant (FN 1765) with a deletion of *rfs* but differing in the extent of the deletion (where *Long1* is not deleted, Domoney et al., personal communication), provides a valuable resource for further study in this area as *Long1* encodes a transcription factor with a central role in mediating the effects of light on gibberellin biosynthesis in pea (Weller *et al*, 2009).

The TILLRfs W521* mutant showed detectable levels of all RFOs despite a nonsense mutation, suggesting partial functional redundancy, which may be conferred by i) the production of a partially active truncated Rfs, ii) the presence of a galactinol-independent pathway, iii) the expression of an active Rfs-like enzyme or (iv) the production of full-length Rfs through putative stop codon readthrough (SCR). A comprehensive transcriptomics analysis would allow us to explore the expression patterns of *Rfs* and *Rfs*-like genes, during different stages of pea development and under various stress conditions. This could clarify the specific roles of these enzymes and their relative contributions to RFO synthesis in different plant organs and their impact on plant stress responses.

Further exploration into the role of RFOs in plant growth or development is needed, especially give reports of reduced photosynthesis and delay in fruit development in cucumber α-galactosidase mutants, the enzymes involved in RFO catalysis (Hua *et al*, 2021; Liu *et al*, 2022). Field performance remains an essential yet often underexplored aspect in the study of novel mutants (Zaidem *et al*, 2019; Plessis *et al*, 2015; Purugganan et al, 2021). Our field trials BCTILLRfs W521* mutant as a promising

candidate for further research on the role of RFOs in abiotic stress tolerance and as a potential contributor to developing stress-resilient, nutritionally enhanced pea varieties. This line is being multiplied for future human dietary studies.

6.2 RFOs and precursors impact the gut microbiota composition and function *in vitro*

The gut microbiota composition is influenced by the type of dietary fibres and carbohydrates consumed, with shifts in microbial communities resulting from changes in fermentation substrates (Cronin *et al*, 2021). RFOs are emerging prebiotics due to their ability to support beneficial gut microbiota, prior studies have shown the bifidogenic effects of raffinose and other RFOs, where certain bacterial species thrive on these sugars (Adamberg *et al*, 2018; Trindade *et al*, 2003; Kullin *et al*, 2006; Dinoto *et al*, 2006). Here, we showed that specific species such as *B. longum*, *B. adolescentis*, and *C. aerofaciens* showed significant increases upon supplementation with RFOs and the precursor sucrose. Notably, raffinose and verbascose fermentations led significant increases in *B. longum* abundance, while sucrose led to significant increases in *C. aerofaciens* abundance. We confirmed the capacity of Bifidobacteria species to utilise RFOs in anaerobic monocultures and showed that *B. longum* subsp. *infantis* ATCC 15697 grew significantly more in sucrose supplementation compared to *B. breve* UCC2003, whereas both strains utilised raffinose, stachyose or verbascose equally. This was further supported by *in silico* analyses, showing the occurrence of GH13 and/or GH36 in all publicly available Bifidobacteria genomes.

Beyond microbial shifts, RFOs and their precursors also altered metabolic outputs. Verbascose uniquely increased butyrate levels, while sucrose and raffinose elevated secondary bile acids and metabolites with potential anti-inflammatory properties. Interestingly, galactinol fermentation produced distinct metabolites, such as indolepropionate, highlighting functional differences among RFO precursors. Studies investigating the prebiotic potential of RFOs in plant foods or extracts have primarily focused on soy-derived flour or soybean soluble sugar pools often referred to as soybean oligosaccharides which include raffinose and stachyose but are low in verbascose (Valentine *et al*, 2017). Similarly, research into RFOs in chickpea, is largely centred on raffinose and stachyose, as verbascose is either present in very small amounts or absent (Tosh *et al*, 2013; Han *et al*, 2006). As a result, very little is known about the contribution of verbascose in the prebiotic properties of RFOs (Kumar *et al*, 2010). Here, we showed that pea seeds accumulate RFOs predominantly in the form of verbascose, which sets them apart in terms of oligosaccharide profile. In addition, our data suggest that verbascose is a main contributor of the beneficial outputs associated with the fermentation of RFOs such as butyrate production, underscoring the use of pea as a healthy food crop, although more work is needed to ascertain the effects of verbascose in pea on human health.

Importantly, we showed that fermentation with sucrose had a metabolic impact similar to that of raffinose, suggesting that this RFO precursor may may also function as prebiotics with potential health benefits. In contrast, the fermentation of galactinol had a distinct effect on the gut microbiota structure and function, underscoring the need to consider both RFOs and their precursors when altering RFO biosynthesis in plants.

According to their definition, prebiotics consist of a group of substrates selectively metabolised by host microorganisms, that in adequate amounts confer a health benefit to the host (Gibson et al., 2017; Salminen et al., 2021; Gibson and Roberfroid, 1995). Several of the metabolites identified in this study, such as SCFAs, secondary bile acids, histamine, and tryptophan derivatives, have been associated with health effects; however, their specific impact on host health remains to be demonstrated. Here, we used human-based intestinal organoid models to explore the potential of RFO in peas to modulate gut barrier function, but future experiments should also allow include individual RFOs and their precursors.

6.3 *In vitro* fermentation of Caméor and FN 1551/11 and BCRfsTILL W521 pea flours led to differences in microbial composition and metabolism but cannot be attributed to RFO content

Our work provided first insights into the effects of pea mutants with altered RFO contents on gut microbiota composition and metabolic activity. Two *in vitro* fermentation models were used: a static batch system to compare the RFO-depleted BCFN 1551 mutant with Caméor, and the dynamic TWIN SHIME™ model to assess the effects of BCRfsTILL W521* supplementation as compared to Caméor.

In the batch fermentation, all pea treatments, using both flour and pea extracts, irrespective of genotypes, resulted in a shift from a *Firmicutes*-dominated community to one dominated by *Proteobacteria* or *Actinobacteria*, whereas in the TWIN-SHIME™ both Caméor and BCRfsTILL W521* treatments retained *Firmicutes*-dominated community after pea flour treatment compared to baseline levels. These discrepancies likely reflect the contrasting nature of the models. The static batch system, while straightforward, allows the accumulation of waste products and lacks environmental control. The TWIN SHIME™ model, by providing continuous flow and waste removal, better replicates in vivo fermentation dynamics. Other contributing factors include differences in the faecal inoculum, media composition, and the use of a baseline microbial fermentation with soluble potato starch in the TWIN SHIME™ model. These variables influence microbial community structure and may explain the divergent outcomes observed.

In the batch fermentation model, Caméor increased *B. longum* abundance, while *B. adolescentis* levels remained similar across Caméor and BCFN 1551 fermentations. This suggests *B. longum* as a primary RFO degrader, whereas *B. adolescentis*, known as a resistant starch degrader (Jung *et al*, 2023), acts independently of pea genotype. BCFN 1551 fermentation also showed higher secondary bile acids and lower histidine, and tryptophan levels compared to Caméor. Further research is needed to explore microbial interactions and cross-feeding during pea fermentation.

In the dynamic fermentation model, our data suggest that the baseline diet, containing potato starch by shaping the microbiota composition during the stabilisation period, may mask the effects of RFOs within the pea flours on the microbiota structure and function. In addition, the difference in the amount of RFOs between BCFN 1551, BCTILLRfs W521* and Caméor may not be sufficient to lead to detectable changes in the levels of SCFAs. Moreover, in the future, using a pre-cooked pea material (rather than raw flour) in the TWIN-SHIME™ model may help better recapitulate *in vivo*

digestion before reaching the gut microbiota for fermentation (Cherta-Murillo *et al*, 2023).

Finally, we showed that differences in RFO content in pea might influence gas production, but not to the extent reported with pure prebiotics like inulin (Gunn *et al*, 2022). This may be due to the comparative low RFO amount in pea, which effect will then be overshadowed by other fermentable carbohydrates in pea such as starch. Additionally, these findings suggest that, when consumed as part of the pea, the RFO content in legumes may have a lesser impact on gas production than previously estimated using purified RFOs (Elango *et al*, 2022). In addition, the increased levels of RFO precursors, sucrose and galactinol, in BCFN 1551 and BCRfsTILL W521* mutants, may contribute to mask the effect of RFO depletion on gas production compared to Caméor as we showed that these precursors were also fermented by the gut microbiota. Future experiments quantifying the gas production from fermentation with individual RFOs and precursors would allow to further elucidate this.

To assess the potential of fermented pea RFOs to modulate gut barrier function, we used human intestinal organoid models. While no differences in epithelial integrity were observed between fermented Caméor and BCFN 1551 flours, Caméor fermentation increased IL-8 production. Under inflammatory conditions, both fermentations reduced IP-10 and IL-8 levels compared to control, suggesting potential anti-inflammatory effects. However, model limitations may have masked impacts on barrier integrity, likely due to insufficient disruption of the epithelium. Future studies using gut-on-chip technology could better replicate physiological conditions, enabling real-time monitoring of barrier function and inflammatory responses. This would provide deeper insight into how fermented pea components affect gut health.

6.4 Concluding remarks and future perspectives

The selection of pea mutants with altered RFO contents provided an opportunity for exploring the role of RFOs and their precursors in plant and gut health.

The 1.3 Mb deletion in the FN 1551/11 mutant was shown to affect plant performance under drought conditions in the glasshouse. While this had a drastic impact on performance in the field, it seems likely that this may involve Rfs and/or other genes within the deleted region, which do not affect plant growth and development in the glasshouse; though any impact these genes may have on plant growth and development in the glasshouse remains unclear. Another FN deletion mutant lacking the major seed RFOs but differing in the additional genes deleted should be investigated further. The BCTILLRfs W521* mutant appears to be a good candidate for producing pea seeds with reduced RFOs, without compromising plant growth in field conditions. Future research should explore the differences in the responses to abiotic and biotic stressors on the different mutants.

Additional pea mutants characterised in this work, with mutations in genes encoding Rfs and Rfs-like isoform, showed variable RFO content in their seeds; these provide a valuable resource for further exploration of the genetic and biochemical mechanisms regulating RFO accumulation, and plant development under environmentally

challenging conditions. Future work on metabolomic analysis of RFOs and precursors across different organs and developmental stages in the plant and stress conditions would allow to understand dynamic changes in RFOs and precursors and their role in the plant. Moreover, further backcrossing of pea mutants by introgressing the mutations into Caméor cultivar to develop near-isogenic pea lines, and their subsequent field testing will be required to elucidate the full range of functions that RFOs and their biosynthetic pathways play in pea development, stress response, and plant health.

Furthermore, our research revealed the presence of verbascose in the *rfs* null mutant FN 1551/11, underscoring the further investigations needed for the galactinol-independent pathway of RFO biosynthesis. The large difference in sucrose levels in the seeds of FN 1551/11, compared to the RfsTILL W521* and Caméor, and its effect on the plant requires further investigation.

Batch fermentation studies confirmed the prebiotic potential of RFOs and suggested that the RFO precursor sucrose may may also function as prebiotics with potential health benefits. The use of raw pea flour in dynamic fermentation studies allowed to provide an initial overview of potential effects of RFOs in pea on the gut microbiota structure and function. While the TWIN-SHIME™ is a more physiological model than batch fermentations, more diverse baseline diets could be tested to determine their impact on the pea intervention. It will also be important to assay the effect of pea treatments using faecal samples from different donors. Multiomics data integration using existing computational tools such as MixOmics (Rohart *et al*, 2017) or via emerging machine learning tools (reviewed in (Li, 2022)) would allow to further explore the association between metagenomics, metabolomics and gas production data. A deeper exploration of the strain-specific functionality and cross-feeding in the gut microbiota could be performed in the future via metatranscriptomics, as well as more detailed metabolic profiling beyond SCFAs in the dynamic colon model, to understand the broader impact of pea flour fermentation.

Future kinetics experiments could be conducted to understand the longitudinal consumption of RFOs with regards to the other fermentable carbohydrates in pea flour, including starch and RFO precursors. To this effect, a proximate composition analysis of the pea flour derived from the pea mutants BCFN 1551, BCTILLRfs W521* and Caméor should be conducted, this will also allow to have a better understanding of their nutritional quality (Millar *et al*, 2019), which will include details on its protein composition, likely to have influenced the fermentation models used here. Our preliminary results using human colonic organoids, showed the need to optimise the models and the conditions used to compromise the gut barrier. The inclusion of immune cells, such as peripheral blood mononuclear cells in co-culture with the intestinal organoids, will help understand further the inflammatory cascade induced by fermented pea flour.

Remaining gaps include understanding how the findings translate to human physiology. Expanding the scope of the study using long-term *in vitro* dynamic fermentation simulations combined with gut-on-chip models or through human

intervention studies is needed to explore the potential health implications of RFOs in pea lines developed in this work.

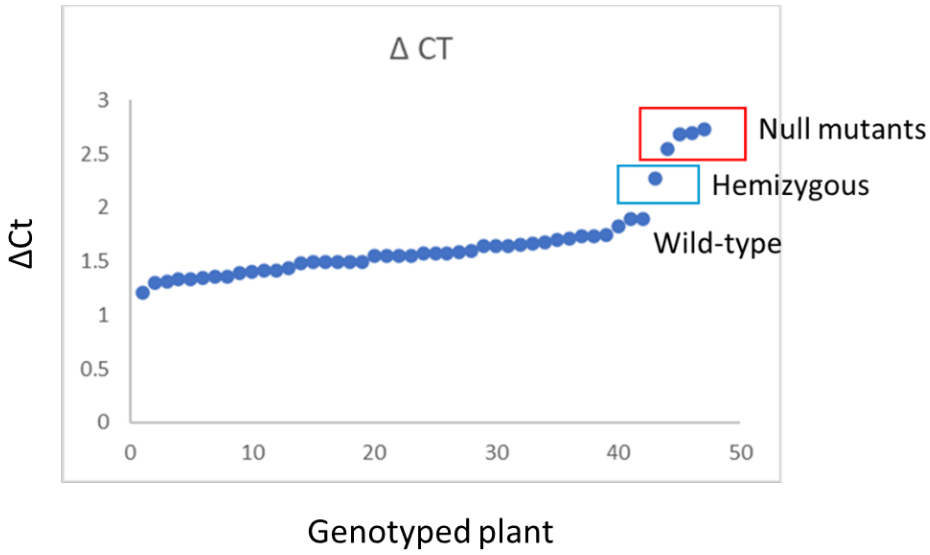
Future Directions

To build upon the findings of this study and further elucidate the role of Rfs isoforms in seed RFO biosynthesis and their impact on gut microbiota, several avenues for future research are proposed:

- Dissect the role of RFOs in stress tolerance and plant development: Conduct targeted physiological and metabolomic studies on FN 1551/11 and BCRfsTILL W521* mutants, and other TILLING mutants, under drought, cold, and other abiotic stresses to clarify the contribution of RFOs and precursors (e.g., sucrose, galactinol) to stress responses. Quantifying RFOs across plant tissues and growth stages, including roots and nodules, will help determine their dynamic role.
- Investigate the broader genetic impact of the 1.3 Mb deletion in FN 1551/11: Further functional characterisation of auxin-related genes and other disrupted pathways is needed, especially under field conditions. Additional mutants, such as FN 1765, should be studied to separate the effects of *rfs* deletion from those of co-deleted loci like *Long1*.
- Clarify the biochemical basis for residual RFOs in Rfs mutants: Explore mechanisms such as partial enzymatic activity, alternative biosynthetic pathways, or translational readthrough in BCRfsTILL W521* via transcriptomics and enzyme assays, to define redundancy between Rfs and Rfs-like.
- Develop near-isogenic pea lines for controlled field evaluations: Backcrossing mutant lines at least six more times into the Caméor background will enable more precise assessments of RFO function in field performance and stress tolerance, removing confounding background variation.
- Elucidate the specific effects of verbascose on gut microbiota and host health: Verbascose was a key component of pea RFOs in this study yet remains under-investigated. Targeted fermentations and *in vivo* models should assess its specific prebiotic and metabolic effects.
- Advance *in vitro* fermentation models and integrate multi-omics approaches: Future studies should use cooked pea flours and include varied baseline diets and donor microbiotas in dynamic models like TWIN-SHIME™. Coupling metagenomics, metabolomics, and gas production data with tools like MixOmics (Rohart *et al,* 2017) or machine learning will help unravel strain-specific and cross-feeding interactions.
- Optimise human gut models to assess functional impacts: Refinement of colonic organoid models—potentially via inflammatory challenges, inclusion of immune cells, and use of gut-on-chip platforms—will allow more accurate simulation of gut barrier responses to fermented pea products.

- Validate findings through human dietary interventions: Ultimately, controlled human studies using selected low-RFO pea lines (e.g., BCRfsTILL W521*) are required to confirm the physiological relevance of *in vitro* findings and establish health claims related to gut function and gas production.

Supplementary figures



Supplementary Figure S1. *Rfs* gene copy number identification for the genotyping of null mutants. ΔCt value (Fam™ Ct minus Vic™ Ct) and plot on scatter graph-data of FN 1551/11 *rfs* mutants and controls: homozygous with two copies, hemizygous with one copy and homozygous nulls (mutants in the red box), when there is no amplification of the target gene but amplification of the *Agps*2 internal standard occurs.

Rfs

MAPPSITKTATQQDVISTVDIGNSPLLSISLDQSRNFLVNGHPFLTQVPPNITTTTTSTPSPFLDFKSNKDTIANNNNTL
QQQGCFVGFNTTEAKSHHVVPLGKLKGIKFTSIFRFKVWWTTHWVGTNGHELQHETQILILDKNISLGRPYVLLLPIL
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YKFREYENGDNGGKKGLVGFVRDLKEEFRSVESVYVWHALCGYWGGVRPKVCGMPEAKVVVPKLSPGVKMTME
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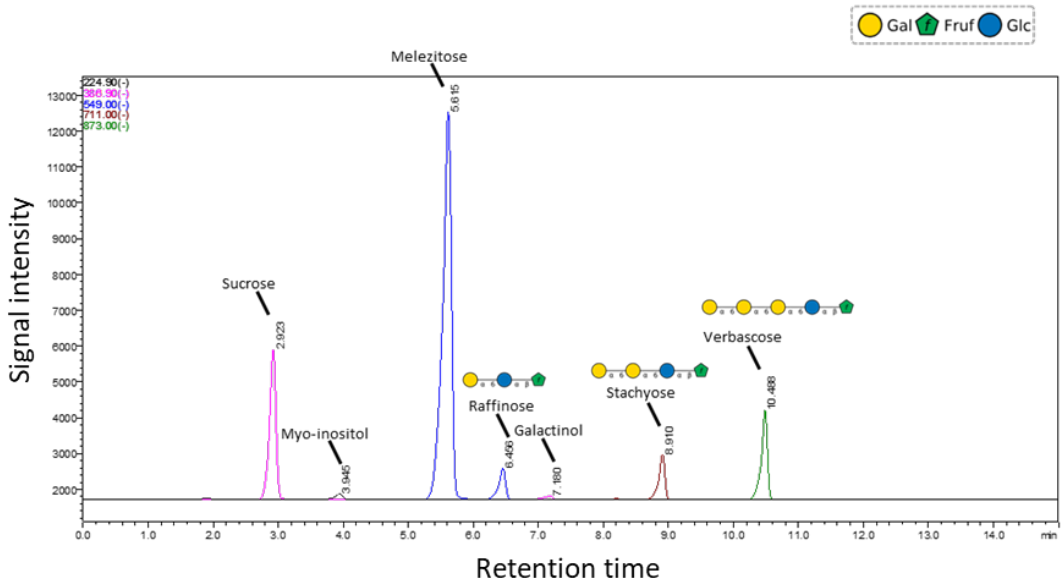
Rfs-like

MSPPNPTLNSNKNNITTTKHLNSCSNSPFTFTLSESTLKVNNHIILSDVPKNITTTTTSTTTTTTNGCFLGFKATDSKSR
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SGGPIYISDTVGNHNFELLKTLALPDGTILRCEYYALPTKDCLFADPLHDGKTMLKIWNLNKYTGVLGVFNCQGGGW
FRDIRSNKCASEFSHLVSTKINIKDIEWESGKNPISIESVQLFALYFSQAKKLKLFSPSDTEEISLEPFNFELITVSPITLFPKK
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SKFSSVQYIF

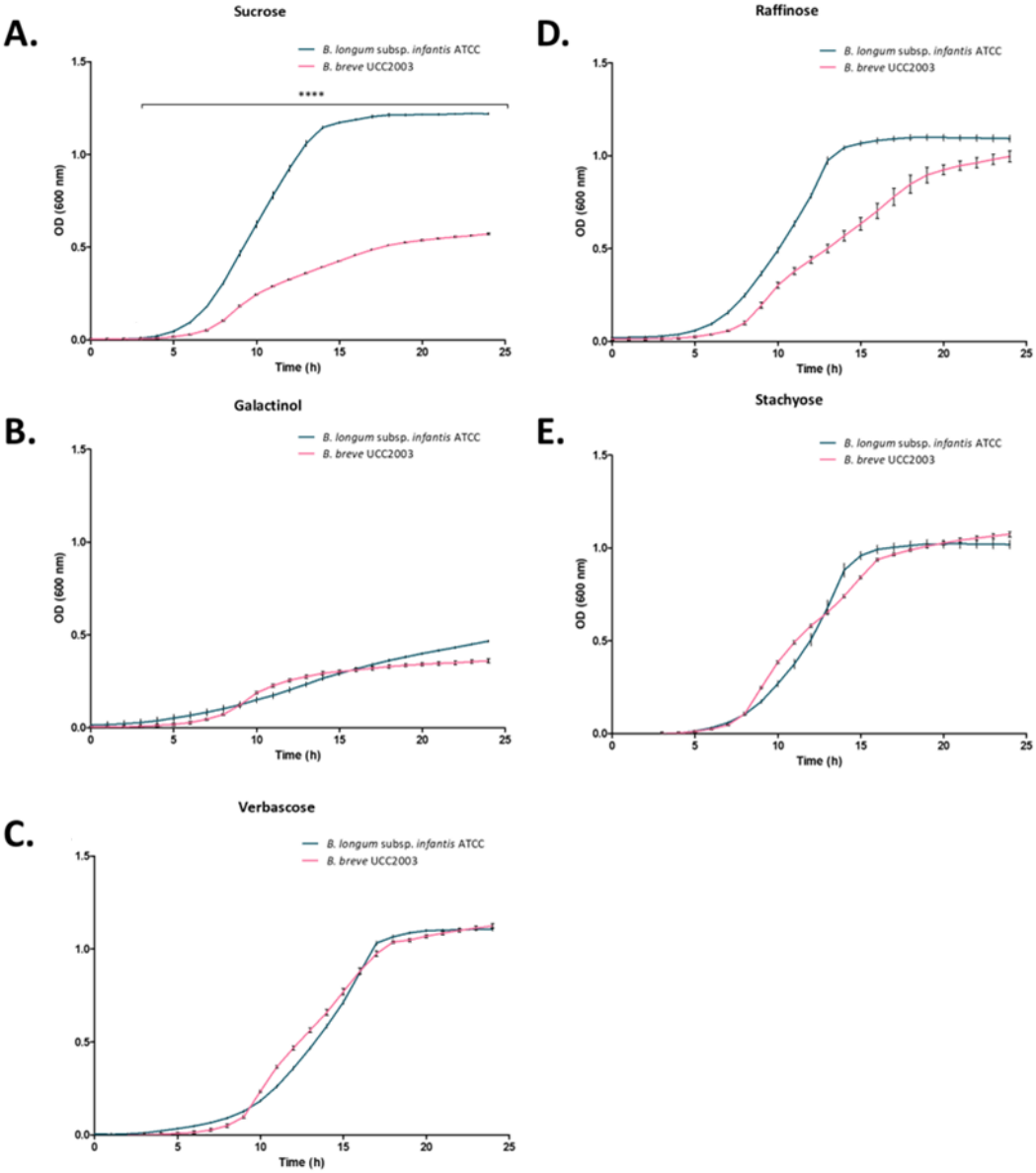
AtAkαGal3

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MAQRMGEMGRDIPYETQFLLVESNDGSHLESDGANGVECNQKVYTVFLPLIEGSFRSCLQGNVNDEVELCLESGD
VDTKRSSFTHSLYIHAGTDPFQTITDAIRTVKLHLNSFRQRHEKKLPGIVDYFGWCTWDAFYQEVTQEGVEAGLKSLA
AGGTPPKFVIIDDGWQSVERDATVEAGDEKKESPIFRLTGIKENEKFKKKDDPNVGIKNIVKIAKEKHGLKYVYVWHA
ITGYWGGVRPGEEYGSVMKYPNMSKGVVENDPTWKTDVMTLQGLGLVSPKKVYKFYNELHSYLADAGVDGVKV
DVQCVLETLGGGLGGRVELTRQFHQALDSSVAKNFPDNGCIACMSHNTDALYCSKQAAVIRASDDFYPRDPVSHTI
HIASVAYNSVFLGEFMQPDWDMFHSVHPAAEYHASARAISGGPLYVSDSPGKHNFELLRKLVLPDGSILRARLPGRP
TRDCLFADPARDGVSLLKIWNMNKYTGVLGVYNCQGAAWSSTERKNIFHQTKTDSLTGSIRGRDVHSISEASTDPTT
WNGDCAVYSQSRGELIVMPYNVSLPVSLKIREHEIFTVSPISHLVDGVSFAPIGLVNMYNSGGAIEGLRYEAEKMKVV
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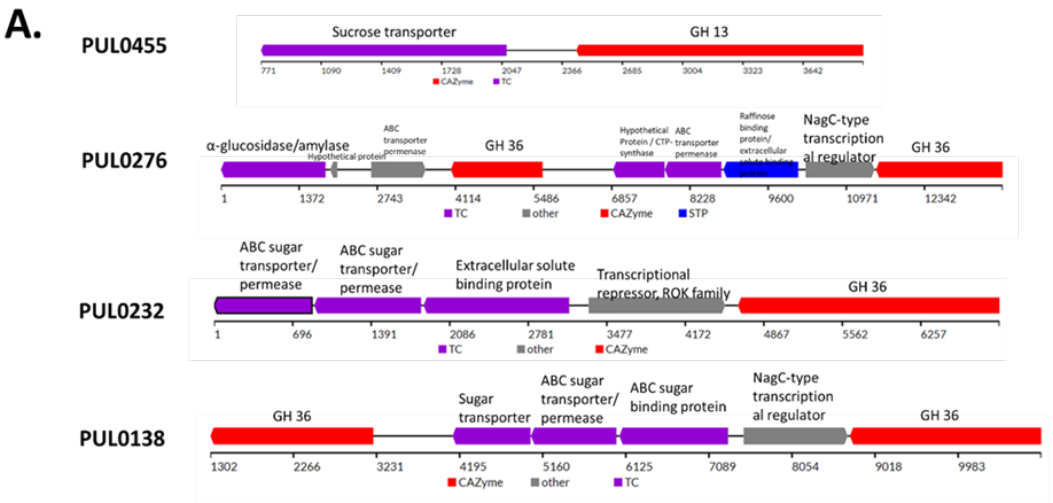
Supplementary Figure S2. Amino acid sequences for Rfs (Psat0s2597g0160), Rfs-like (Psat1g055560) from pea and AtAkαGal3 (At5g20250) from *A. thaliana* . Amino acid sequences used to identify active site residues (highlighted in yellow) in Rfs and Rfs-like compared to AtAkαGal3 (Chuankhayan et al., 2023).



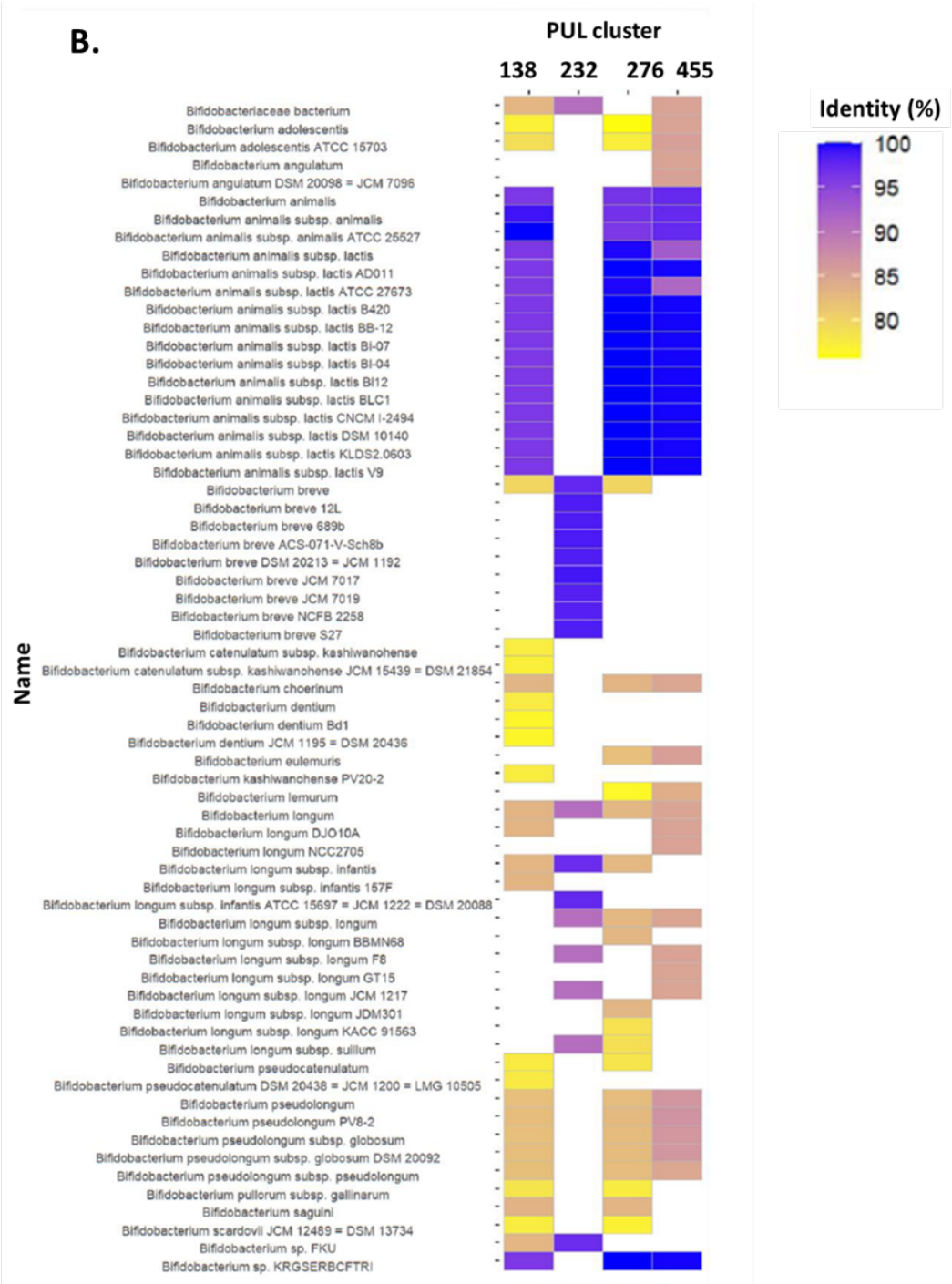
Supplementary Figure S3. LC-MS analysis of RFOs and precursors in Caméor pea extracts. Chemical structures of RFOs: raffinose, stachyose and verbascose according to the symbol nomenclature for glycans (Varki et al., 2015; Neelamegham et al., 2019) Gal= galactose, Fruf= fructose , Glc= glucose. Structures retrieved from PubChem (CIDs: 439242, 439531, 441434) (Kim et al., 2022).



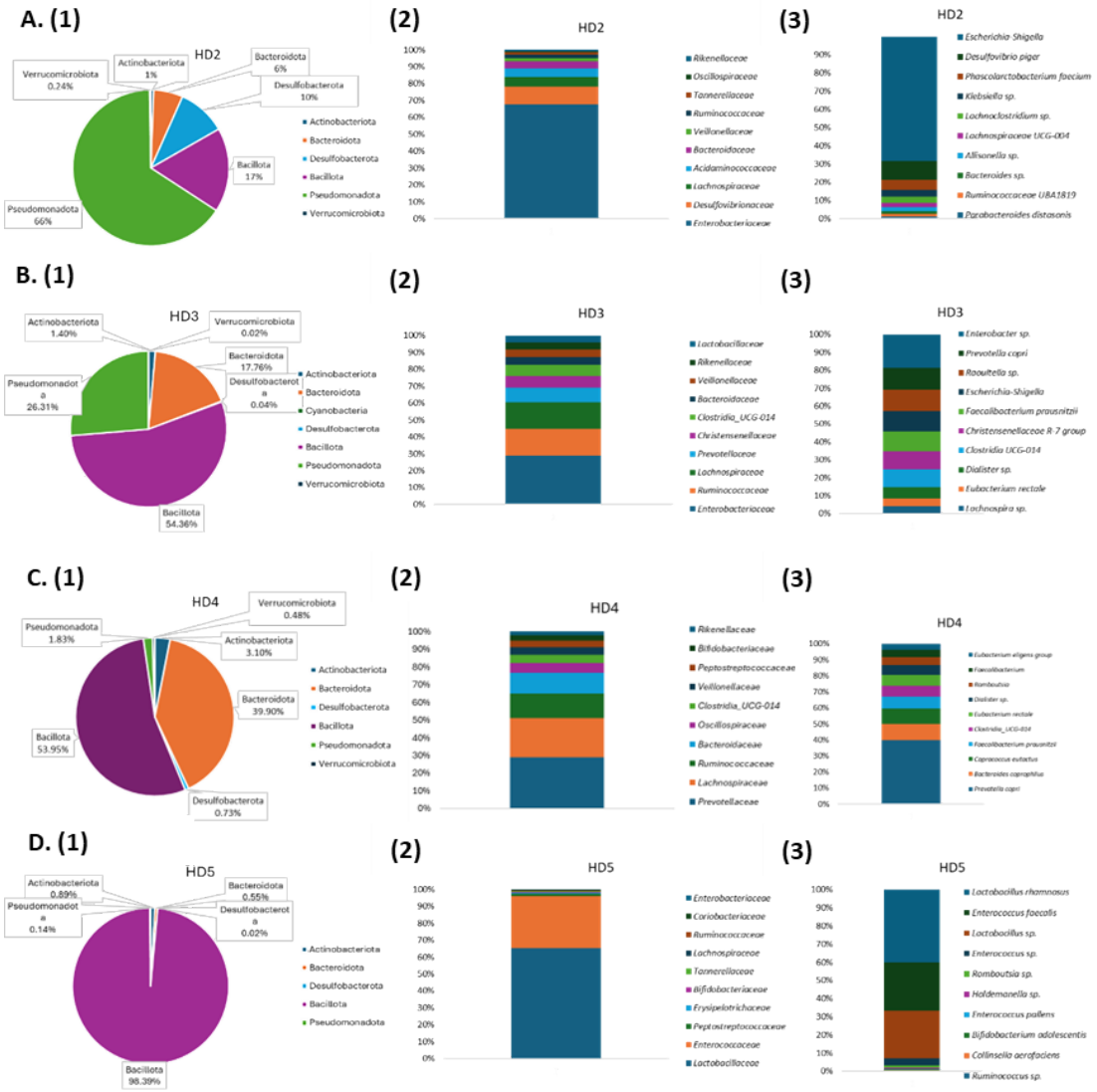
Supplementary figure S4. *B. longum* subesp. *infantis* ATCC 15697 and *B. breve* UCC 2003 growth on individual RFOs (raffinose, stachyose, verbascose) or precursors (sucrose, galactinol) as sole carbon source. *B. longum* subesp. *infantis* and *B. breve* were grown under anaerobic conditions in mMRS supplemented with each individual RFO or precursor. (**A**) sucrose, (**B**) galactinol, (**C**) verbascose, (**D**) raffinose and (**E**) verbascose. Each timepoint represents the average optical density (OD) at 600nm of three replicates per each sugar tested. Error bars correspond to standard deviations. Two-way ANOVA results *p value < 0.05 , **** p value < 0.0001.



Supplementary Figure S5. *Bifidobacterium* raffinose utilisation clusters. (A). Annotated dbPUL raffinose utilisation clusters, (B) heatmap of BLASTn identities across publicly available Bifidobacteria genomes and raffinose utilisation clusters from the CAZy dbCAN2-PUL database. Percentage of identity is indicated as a colour gradient from yellow (75%) to blue (100%), as shown by the colour key; blank spaces represent no identity.



Supplementary Figure S5 (continued). *Bifidobacterium* raffinose utilisation clusters. (**A**). Annotated dbPUL raffinose utilisation clusters, (**B**) heatmap of BLASTn identities across publicly available Bifidobacteria genomes and raffinose utilisation clusters from the CAZy dbCAN2-PUL database. Percentage of identity is indicated as a colour gradient from yellow (75%) to blue (100%), as shown by the colour key; blank spaces represent no identity.



Supplementary Figure S6. Microbial composition of faecal inoculum samples from healthy donors. Relative abundance of all taxa at the phylum level in the faecal sample from healthy individuals before fermentation (**A**) HD2: faecal donor 2, (**B**) HD3: faecal donor 3, (**C**) HD4: faecal donor 4 and (**D**) HD5: faecal donor 5; at the (1) phylum, (2) top ten family and (3) top ten species taxonomic level. For HD1, see Fig. 34. HD4 gut microbiota analysis is not yet available.

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