

Title	Effect of dairy based food ingredients on the gut microbiota of older consumers
Authors	Ntemiri, Alexandra
Publication date	2019
Original Citation	Ntemiri, A. 2019. Effect of dairy based food ingredients on the gut microbiota of older consumers. PhD Thesis, University College Cork.
Type of publication	Doctoral thesis
Rights	© 2019, Alexandra Ntemiri. - http://creativecommons.org/licenses/by-nc-nd/3.0/
Download date	2026-08-18 20:07:26
Item downloaded from	https://hdl.handle.net/10468/7423



**Effect of dairy based food ingredients on the gut
microbiota of older consumers**

A thesis presented to the National University of Ireland for the
degree of

Doctor of Philosophy

by

Alexandra Ntemiri, MRes, BSc

(113222522)

School of Microbiology

National University of Ireland, Cork

January 2019

Under the supervision of:

Professor Paul O'Toole

Professor Catherine Stanton and Professor Reynolds Paul Ross

Head of School of Microbiology

Professor Gerald Fitzgerald

Table of Contents

Table of Contents	ii
List of Figures	v
List of Supplementary Figures	xi
List of Tables	xiii
List of Supplementary Tables	xvi
List of Abbreviations	xx
Declaration	xxiv
Abstract	xxvi
1 Chapter 1 – Literature Review	1
1.1 From birth to old age: Factors that shape the human gut microbiome	2
1.1.1 Abstract	3
1.1.2 Introduction	5
1.1.3 Establishment of the gut microbiota	8
1.1.4 Shaping factors of gut microbiota composition	13
1.1.4.1 Diet	13
1.1.4.2 Exercise	17
1.1.4.3 Antibiotics	22
1.1.4.4 Extra-intestinal disorders: Metabolic syndrome and related obesity, type 2 diabetes, liver disease	23
1.1.4.5 Functional gastrointestinal disorders: Inflammatory bowel disease (IBD), Irritable bowel syndrome (IBS)	28
1.1.5 Ageing and microbiota alterations	32
1.1.6 Concluding remarks	37
1.1.7 References	39
1.2 The gut microbiota is a modifiable factor for maintaining health	68
1.2.1 Introduction	68
1.2.2 Prebiotics, Probiotics, and next-generation approaches	70
1.2.3 Live bacteriotherapy alternatives to FMT	73

1.2.4	<i>In vitro</i> and <i>in vivo</i> models for the development of gut microbiota therapeutics	78
1.3	The effect of milk and glycomacropeptide on the gut microbiota	89
1.3.1	The effect of milk on human health and the gut microbiota	89
1.3.1.1	Cardio-metabolic disease and all-cause mortality	90
1.3.1.2	Frailty	93
1.3.1.3	Other potential health benefits of milk consumption.....	96
1.3.1.4	Strengths and weaknesses of cohort observation studies assessing the effect of milk on various-cause mortality	99
1.3.1.5	Lactose-intolerance	101
1.3.1.6	Milk and the gut microbiota.....	103
1.3.1.7	The prebiotic potential of the dairy product Glycomacropeptide (GMP)	106
1.4	Aims and objectives	109
1.5	References	110
2	Chapter 2 - Glycomacropeptide sustains microbiota diversity and promotes specific taxa in an artificial colon model of elderly gut microbiota.....	147
2.1	Abstract	148
2.2	Introduction	149
2.3	Material and Methods.....	151
2.4	Results	156
2.5	Discussion	174
2.6	Funding Sources	178
2.7	Acknowledgements	178
2.8	References	179
3	Chapter 3-Retention of microbiota diversity by lactose free milk-supplemented diet was comparable to soy protein diet in a mouse model of healthy and frail elderly gut microbiota.	186
3.1	Abstract	187
3.2	Introduction	189
3.3	Materials and Methods	191
3.4	Results	197
3.5	Discussion	221
3.6	Conclusions	226
3.7	Funding Sources	226

3.8	Acknowledgements	227
3.9	References	228
3.10	Supplementary Chapter 3	239
4	Chapter 4 - Effect of blueberry on the composition and metabolism activity of gut microbiota and artificial microbiota community from the elderly.....	251
4.1	Abstract	252
4.2	Introduction	254
4.3	Materials and Methods	256
4.4	Results	260
4.5	Discussion	278
4.6	Conclusion.....	282
4.7	Funding Sources	283
4.8	Acknowledgements	283
4.9	References	284
4.10	Supplementary Chapter 4	295
5	Chapter 5- General discussion.....	307
5.1	References	325
	Acknowledgements.....	333

List of Figures

Figure 1 Overview of the Overview of the factors affecting the composition of the gut microbiota throughout life. C-section: Caesarean section, *E. coli*: *Escherichia coli*..... 8

Figure 2 A schematic representation of how extrinsic factors like diet and antibiotics influence normal gut microbiota leading to dysbiosis. The Wiggum plots at the bottom represent normal gut microbiota and gut microbiota in dysbiosis. Coloured circles represent correlated genera and their interactions are schematically indicated by lines. T2D: Type-2 diabetes, NAFLD: non-alcoholic fatty liver disease, NASH: non-alcoholic steatohepatitis, IBS: irritable bowel syndrome, IBD: inflammatory bowel disease..... 38

Figure 3 Principal coordinates analysis of UniFrac Unweighted (A) and Weighted (B) distances. “■” time points 0 hrs and “□” time points 24 hrs, “▲” stool microbiota before faecal slurry preparation. 160

Figure 4 Composition of the baseline (0 hrs) faecal microbiota at Phylum level. The relative abundance of the various phyla is shown for aggregated community (COM; EM425, EM278, EM604 and EM703) and aggregated longstay (LS; EM297, EM704) faecal microbiota. Only phyla with relative abundance $\geq 0.5\%$ are shown. 161

Figure 5 Composition of the baseline (0hrs) faecal microbiota at Family level. The relative abundance of the various families is shown for (A) aggregated community (EM425, EM278, EM604 and EM703) and (B) aggregated longstay type faecal microbiota (EM297, EM704). 162

Figure 6 Effect of the fermentation substrates on the alpha diversity (Shannon diversity index) of the faecal microbiota. Effect on diversity of aggregated microbiota composition across all donors (**A**) and microbiota separated by donor residential location, i.e. community (COM) and longstay (LS) (**B**). Stool sample represents the faecal microbiota before the preparation of inoculum faecal slurry. Wilcoxon test result: “*” $p < 0.05$; “a” $p < 0.1$ 165

Figure 7 Faecal microbiota profile after 24 hrs fermentation. Community type (COM) and longstay type aggregated (LS) microbiotas are presented at family level (**A**) and genus level (**B**). 170

Figure 8 Heat-map of the changes in bacterial genus abundance in the faecal microbiota after 24 hrs fermentation with selected substrates. Comparisons were performed pairwise between 0hrs and 24 hrs for aggregated microbiota types by residential location as defined in main text. The logarithmic ($\log_2$) fold change results were generated using the DeSeq2 package. COM and LS correspond to community type and longstay type aggregated faecal microbiotas respectively. Significance is denoted by “*” for p values adjusted for multiple testing using Benjamini-Hochberg, $\text{padj} < 0.05$ 171

Figure 9 Short chain fatty acid (SCFA) levels in the faecal microbiota after 24 hrs fermentation in media supplemented as indicated. Concentration ($\mu\text{mol/g}$ of faecal pellet) of acetate, propionate and butyrate in aggregated community (**A**) and longstay (**B**) microbiotas. Kruskal-Wallis test with Dunn’s post hoc test was performed to compare SCFA production at 24 hrs after supplementation with the various substrates against glucose supplementation in the community type microbiota; “*” $p < 0.05$, “**” $p < 0.005$ 173

Figure 10 Timeline of the 11 weeks mouse trial. Time-points of samples collection: T0 during the acclimatisation, T1 and T2 during the Abx treatment, T3, T4 and T5 during the dietary intervention. The FMT source was used for the “humanisation” of the Abx- treated animals. The isocaloric and isonitrogenous diets were refined diets with 20% supplementation with the shown ingredients. 193

Figure 11 Animal body weight during the 11 weeks of the trial. The body weight from the first week of Abx treatment (W0) till the completion of the trial (W11), is shown. Body weight change between baseline and end of trial is shown at the bottom. **A** and **B**: COM: community type humanisation and LS: longstay type humanisation, respectively. 200

Figure 12 Composition of the human and murine baseline faecal microbiota at phylum level. Phyla that were present at ≥ 1 % relative abundance are presented. LS: longstay type faecal microbiota; COM: community type faecal microbiota; murine: aggregated faecal microbiota across all mice at baseline..... 201

Figure 13 Alpha diversity indices at the end of the trial (T5) for mice humanised with community (COM) type faecal microbiota. LAC_FREE: lactose-free milk supplemented diet, WMILK: whole milk supplemented diet, GMP: glycomacropeptide supplemented diet, CONTROL: soy-protein based control diet. Only tests that passed the $p_{adj} \leq 0.05$ threshold for the Kruskal Wallis test are presented. Asterisks refer to the Dunn’s post hoc test: * $p_{adj} \leq 0.05$, ** $p_{adj} \leq 0.005$, *** $p_{adj} \leq 0.0005$. Details on the values are given in S Table 4..... 203

Figure 14 Alpha diversity indices at the end of the trial (T5) for the faecal microbiota of mice humanised with longstay (LS) type faecal microbiota. LAC_FREE: lactose-free milk supplemented diet, WMILK: whole milk

supplemented diet, GMP: glycomacropeptide supplemented diet, CONTROL: soy-protein based control diet. Only tests that passed the $p_{adj} \leq 0.05$ threshold for the Kruskal Wallis test are presented. Asterisks refer to the Dunn's post hoc test: * $p_{adj} \leq 0.05$, ** $p_{adj} \leq 0.005$, *** $p_{adj} \leq 0.0005$. Details on the values are given in S Table 5.....204

Figure 15 Principal coordinates analysis (PCoA) of the murine faecal microbiota at the end of the trial (T5). The mice were humanised with community (COM) type faecal microbiota. **A:** Weighted UniFrac distances; **B:** Unweighted UniFrac distances. LAC_FREE: lactose-free milk supplemented diet, WMILK: whole milk supplemented diet, GMP: glycomacropeptide supplemented diet, CONTROL: soy-protein based control diet.”●” denotes female mouse microbiota; “▲” denotes male mouse microbiota.206

Figure 16 Composition of the murine faecal microbiota at family level at the end of the trial (T5) in mice humanised with community (COM) type human faecal microbiota. LAC_FREE: lactose-free milk supplemented diet, WMILK: whole milk supplemented diet, GMP: glycomacropeptide supplemented diet, CONTROL: soy protein based control diet. Families present at ≥ 1 % are shown. .210

Figure 17 Composition of the murine faecal microbiota at species level at the end of the trial (T5) in mice humanised with community (COM) type human faecal microbiota. LAC_FREE: lactose-free milk supplemented diet, WMILK: whole milk supplemented diet, GMP: glycomacropeptide supplemented diet, CONTROL: soy protein based control diet. Only species present at ≥ 1 % are shown.211

Figure 18 Principal coordinates analysis (PCoA) of the murine faecal microbiota at the end of the trial (T5). The mice were humanised with long stay

(LS) type faecal microbiota. **A:** Weighted UniFrac distances; **B:** Unweighted UniFrac distances. LAC_FREE: lactose-free milk supplemented diet, WMILK: whole milk supplemented diet, GMP: glycomacropeptide supplemented diet, CONTROL: soy protein based control diet. “●” denotes female mouse microbiota; “▲” denotes male mouse microbiota.215

Figure 19 Composition of the murine faecal microbiota at family level at the end of the trial (T5) in mice humanised with longstay (LS) type human faecal microbiota. LAC_FREE: lactose-free milk, WMILK: whole milk, GMP: glycomacropeptide, CONTROL: soy-protein based control diet. Only families present at ≥ 1 % relative abundance are shown.218

Figure 20 Composition of the murine faecal microbiota at family level at the end of the trial in mice humanised with longstay (LS) type human faecal microbiota. LAC_FREE: lactose-free milk, WMILK: whole milk, GMP: glycomacropeptide, CONTROL: soy-protein based control diet. Only families present at ≥ 1 % relative abundance are shown.219

Figure 21 Order level development of the faecal microbiota from COM donors (EM278, EM425) and LS donors (EM704, EM297). Orders present with relative abundance $\geq 1\%$ are shown. Substrate conditions are basal medium supplemented with: BB: blueberry powder; BB.MIX: blueberry powder and prebiotic mix of carbohydrates; MIX: mix of prebiotic carbohydrates. Time points: 0 h, 16 h and 24 h of fermentation.266

Figure 22 Family level development of the faecal microbiota from COM donors (EM278, EM425) and LS donors (EM704, EM297). Families present at $\geq 1\%$ are shown. Substrate conditions are basal medium supplemented with: BB: blueberry

powder; BB.MIX: blueberry and prebiotic mix of carbohydrates; MIX: mix of prebiotic carbohydrates. Time points: 16 h and 24 h of fermentation.267

Figure 23 Development of the MCC100 composition after 24 h fermentation.

The relative abundance of the dominant phyla Bacteroidetes, Firmicutes and Proteobacteria (**A**) and the relevant families (**B**) is shown for time points 0 h, 16 h and 24 h. BB: blueberry powder supplementation; BB.MIX: blueberry powder and prebiotic carbohydrate mix supplementation; MIX: prebiotic carbohydrate mix supplementation. Only families present with $\geq 1\%$ relative abundance are shown. “*” statistically significant: Kruskal-Wallis test result $p \leq 0.05$, $p_{adj} \leq 0.05$, Dunn’s test $q_{adj} \leq 0.05$. “a” statistical trend: Kruskal-Wallis test result $p \leq 0.05$, $p_{adj} \leq 0.1$, Dunn’s test $q_{adj} \leq 0.1$275

Figure 24 Short chain fatty acid (SCFA) production by the faecal microbiota and artificial consortium MCC100 after 24 h fermentation with selected substrates.

The concentration ($\mu\text{mol/g}$ of faecal pellet) of acetate, propionate and butyrate in the fermentation fluid from the COM type (EM278 and EM425) and LS type (EM297, EM704) faecal microbiotas and consortium MCC100 is shown in **A**, **B** and **D** respectively. The combined SCFA production in the aggregated (i.e. across all donors faecal microbiotas) is shown in panel **C**. Kruskal-Wallis test with Dunn’s post hoc test was applied to compare the SCFA levels at 24 h. *** $p \leq 0.0005$, ** $p \leq 0.005$, * $p \leq 0.05$. BB: blueberry powder supplementation; BB.MIX: blueberry powder and prebiotic supplementation; MIX: prebiotic carbohydrate mix supplementation.277

List of Supplementary Figures

S Figure 1 Principal coordinates analysis (PCoA) of the UniFrac distances one week after “humanisation” (T3). A. Weighted UniFrac distances; B. Unweighted UniFrac distances; “▲” community (COM) type faecal microbiota; “●” longstay (LS) type faecal microbiota; **red**: baseline murine faecal microbiota; **blue**: T3 time point. The inocula of human faecal microbiota are denoted as COM and LS.241

S Figure 2 Principal coordinates analysis (PCoA) of the UniFrac distances at end of the trial (T5). A. Weighted UniFrac distances; B. Unweighted UniFrac distances; “▲” community (COM) type faecal microbiota; “●” longstay (LS) type faecal microbiota; **red**: baseline murine faecal microbiota; **blue**: T5 time point. The inocula of human faecal microbiota are denoted as COM and LS.243

S Figure 3 Differentially abundant taxa at family level at the end of the trial (T5) for aggregated female and male mouse faecal microbiota. A: community (COM) type colonisation; B: longstay (LS) type colonisation. Asterisks next to taxa denote the result of Kruskal-Wallis comparison; markings above boxplots denote the Dunn’s post hoc result. Significant values: “●” $p_{adj} < 0.1$; “*” $p_{adj} < 0.05$; “**” $p_{adj} < 0.01$; “***” $p_{adj} < 0.001$; “****” $p_{adj} < 0.0001$. COM type humanisation:247

S Figure 4 Composition of the murine faecal microbiota at phylum level at the mid part of the dietary intervention (T4). COM: community type microbiota, LS: longstay type microbiota. LAC_FREE: lactose free milk, WMILK: whole milk, GMP: glycomacropeptide, CONTROL: soy protein based control diet. Only phyla present at ≥ 1 % relative abundance are presented.248

S Figure 8 Principal Coordinates Analysis (PCoA) of the faecal microbiota and MCC100 at baseline (t0 h). A: Weighted UniFrac distances; B: Unweighted UniFrac distances. “●” Community (COM) type microbiota; “▲” Longstay (LS) type microbiota; red: BB supplementation; blue: BB.MIX supplementation; green: MIX supplementation.....296

S Figure 9 Alpha diversity of the community (COM) type faecal microbiota at 24 h. The results refer to the aggregated faecal microbiotas EM278 and EM425. BB: blueberry supplementation; BB_MIX: blueberry and prebiotic carbohydrate mix supplementation; MIX: carbohydrate mix supplementation.....297

S Figure 10 Alpha diversity of the longstay (LS) type faecal microbiota at 24 h. The results refer to the aggregated faecal microbiotas EM704 and EM297. BB: blueberry supplementation; BB_MIX: blueberry and prebiotic carbohydrate mix supplementation; MIX: carbohydrate mix supplementation.....298

S Figure 11 Principal Coordinates Analysis (PCoA) of the faecal microbiota and MCC100 at 24 h. A: Weighted UniFrac distances; B: Unweighted UniFrac distances. “●” Community (COM) type microbiota and MCC100; “▲” Longstay (LS) type microbiota; red: BB supplementation; blue: BB.MIX supplementation; green: MIX supplementation.300

List of Tables

Table 1 Culture-independent studies associating changes in gut microbiota to diet.	19
Table 2 Diagnostic criteria for IBS adapted from Rome III (http://www.theromefoundation.org/criteria/).....	32
Table 3 Selected studies employing murine models in the study of gut microbiota, diet, health and disease.....	87
Table 4 Bovine milk components. The data is taken from the Society of Dairy Technology (https://www.sdt.org/pages/) and O’Riordan <i>et al.</i> (2014). The table is not exhaustively presenting all milk components, but the main ones.....	90
Table 5 Chemical composition of the two glycomacropeptide products used in this study, Lacprodan CGMP-10 and semi-purified tGMP.	157
Table 6 Composition of the customized experimental diets (ssniff Spezialdiäten GmbH).	194
Table 7 Differences in the female and male murine microbiota composition associated with separation in the PCoA grouping by whole milk (WMILK) and control diet at the end of the trial (T5). Only species present at ≥ 1 % relative abundance are presented. The humanisation of the mice was performed with community (COM) type microbiota.....	212
Table 8 Differences in the female and male murine microbiota composition associated with separation in the PCoA grouping by lactose-free milk and whole	

milk diets. Only species that were present at ≥ 1 % relative abundance are presented.

The humanisation was performed with longstay (LS) microbiota.....220

Table 9 Development of the composition and comparison between time points of the relative abundance and comparison between time points of the relative abundance of dominant families after 24 h fermentation with COM faecal microbiota (EM278, EM425). BB: blueberry powder supplementation; BB.MIX: blueberry powder and prebiotic carbohydrate mix supplementation; MIX: prebiotic carbohydrate mix supplementation. Missing values indicate that the relevant taxon had <1% abundance in all vessels (two or three) summed for mean abundance. The mean value of the relative abundance per time point is shown.....268

Table 10 Development of the composition and comparison between time points of the relative abundance of dominant families after 24 h fermentation with LS faecal microbiota (EM704, EM297). BB: blueberry powder supplementation; BB.MIX: blueberry powder and prebiotic carbohydrate mix supplementation; MIX: prebiotic carbohydrate mix supplementation. Missing values indicate that the relevant taxon had <1% abundance in all vessels (two or three) summed for mean abundance. The mean value of the relative abundance per time point is shown.269

Table 11 MCC100 alpha diversity indices after 24 h fermentation with the selected substrates. BB: blueberry powder supplementation; BB.MIX: blueberry powder and prebiotic carbohydrate mix supplementation; MIX: prebiotic carbohydrate mix supplementation.273

Table 12 Development of the relative abundance at species level in the MCC100 after 24 h fermentation and comparison by time point. BB: blueberry powder supplementation; BB.MIX: blueberry powder and prebiotic carbohydrate mix

supplementation; MIX: prebiotic carbohydrate mix supplementation. Missing values indicate that the relevant taxon had <1% abundance in all vessels (two or three) summed for mean abundance.....274

List of Supplementary Tables

S Table 1 Mouse treatment groups tested by diet, gender and the human microbiota type. The number of mice at the end of the trial per group is indicated. The experimental arms of groups A, B, C and D were performed simultaneously followed by the experimental arms of groups E, F, G and H. The animals that received HydroGel treatment during Abx period, is indicated.	239
S Table 2 Composition of the baseline murine and the human faecal microbiota. EM297; longstay type faecal microbiota (LS); EM425 community type faecal microbiota (COM). Only species present at ≥ 1 % relative abundance in at least one group are presented.	240
S Table 3 Alpha diversity indices of the faecal microbiotas of the human donors and the murine baseline faecal microbiota. EM297: longstay type faecal microbiota (LS); EM425: community type faecal microbiota (COM); murine: aggregated faecal microbiota across all mice at baseline.....	241
S Table 4 Alpha diversity statistical analysis for community (COM) type microbiota at the end of the trial (T5) LAC_FREE: lactose free diet; WMILK: whole milk diet; GMP: GMP diet; CONTROL: control diet.....	243
S Table 5 Alpha diversity statistical analysis for longstay (LS) type microbiota at the end of the trial (T5) LAC_FREE: lactose free diet; WMILK: whole milk diet; GMP: GMP diet; CONTROL: control diet.....	244
S Table 6 Differences in the female and male murine microbiota composition associated with separation in the PCoA grouping by whole milk and control	

diet. Only families that were present at ≥ 1 % relative abundance in at least one group are presented. WMILK: whole milk diet; CONROL: control diet; community (COM) type microbiota.....245

S Table 7 Differences in the female and male murine microbiota composition associated with separation in the PCoA grouping by the lac-free and whole milk diet. Only families that were present at ≥ 1 % relative abundance in at least one group are presented. LAC_FREE: lactose free diet; WMILK: whole milk diet; longstay (LS) type microbiota.247

S Table 8 Alpha diversity analysis for community (COM) type microbiota at the mid part of the trial (T4) LAC_FREE: lactose free diet; WMILK: whole milk diet; GMP: GMP diet; CONTROL: control diet.....249

S Table 9 Alpha diversity analysis for longstay (LS) type microbiota at the mid part of the trial (T4) LAC-FREE: lactose free diet; WMILK: whole milk diet; GMP: GMP diet; CONTROL: control diet.....250

S Table 10 Alpha diversity indices for the faecal microbiotas and MCC100 at baseline (0 h).....296

S Table 11 Development of the composition and comparison between time points of the relative abundance of orders after 24 h fermentation with community (COM) type faecal microbiota (EM278, EM425). BB: blueberry powder supplementation; BB.MIX: blueberry powder and prebiotic carbohydrate mix supplementation; MIX: prebiotic carbohydrate mix supplementation. Missing values indicate that the relevant taxon had <1% abundance in all vessels (two or

three) summed for mean abundance. The mean value of the relative abundance per time point is shown.300

S Table 12 Development of the composition and comparison between time points of the relative abundance of orders after 24 h fermentation with longstay (LS) type faecal microbiota (EM704, EM297). BB: blueberry powder supplementation; BB.MIX: blueberry powder and prebiotic carbohydrate mix supplementation; MIX: prebiotic carbohydrate mix supplementation. Missing values indicate that the relevant taxon had <1% abundance in all vessels (two or three) summed for mean abundance. The mean value of the relative abundance per time point is shown.301

S Table 13 Development of the composition and comparison between time points of the relative abundance of species after 24 h fermentation with community (COM) type faecal microbiota (EM278, EM425). BB: blueberry powder supplementation; BB.MIX: blueberry powder and prebiotic carbohydrate mix supplementation; MIX: prebiotic carbohydrate mix supplementation. Missing values indicate that the relevant taxon had <1% abundance in all vessels (two or three) summed for mean abundance. The mean value of the relative abundance per time point is shown.302

S Table 14 Development of the composition and comparison between time points of the relative abundance of species after 24 h fermentation with longstay (LS) type faecal microbiota (EM704, EM297). BB: blueberry powder supplementation; BB.MIX: blueberry powder and prebiotic carbohydrate mix supplementation; MIX: prebiotic carbohydrate mix supplementation. Missing values indicate that the relevant taxon had <1% abundance in all vessels (two or three)

summed for mean abundance. The mean value of the relative abundance per time
point is shown.305

List of Abbreviations

AA	amino acids
AAD	antibiotic associated diarrhoea
Abx	antibiotics
AG	arabinogalactan
α -GOS	α -galactooligosaccharides
α -lac	alpha-lactalbumin
BCAA	branched chain amino acids
BMO	bovine milk oligosaccharides
CD	Crohn's disease
CDI	<i>Clostridium difficile</i> infection
CDV	cardiovascular disease
CLA	conjugated linoleic acid
CMP	caseinomacropeptide
COM	community
CONS	coagulase negative staphylococci
CRC	colorectal cancer
CRP	C-reactive protein
C-section	caesarean section
EAA	essential amino acids
eCB	endocannabinoid system
FGID	functional gastrointestinal disorders
FISH	fluorescent <i>in situ</i> hybridisation
FMT	faecal material transplantation

FOS	fructo-oligosaccharides
FSR	fraction synthetic rates
GABA	γ -aminobutyric acid
Gal	galactose
GBA	gut-brain axis
gDNA	genomic DNA
GF	germ free
GIT	gastrointestinal tract
GlcNAc	<i>N</i> -acetylglucosamine
GMP	glycomacropeptide
GOS	galacto-oligosaccharides
HMA	human microbiota associated
HMO	human milk oligosaccharides
HPA	hypothalamic-pituitary-adrenal
HPLC	high performance liquid chromatography
IBD	inflammatory bowel disease
IBS	irritable bowel syndrome
IBS-C	constipation-predominant
IBS-D	diarrhoea-predominant
IS	immune system
κ -CN	kappa casein
LC-AX	long-chain arabinoxylan
LDL-C	low-density lipoprotein cholesterol
LNP	lactose non-persistent
LOS	late onset sepsis

LPS	lipopolysaccharides
LS	longstay
MAMPs	microbial-associated molecular patterns
MCC	microbiome culture collection
MetS	metabolic syndrome
MFGM	milk fat globule membranes
MPB	muscle protein breakdown
MPS	muscle protein synthesis
mTOR	mechanistic target of rapamycin
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
NEC	necrotising colitis
NeuAc	<i>N</i> -acetylneuraminic acid
NeuGc	<i>N</i> -glycolylneuraminic acid
NF- κ B	nuclear factor κ B
NGP	next-generation probiotics
NSP	non-starch polysaccharides
OS	oligosaccharides
OTU	operational taxonomic unit
PAG	phenylacetylglutamine
PBS	phosphate buffer saline
PCoA	principal coordinates analysis
PCS	p-cresol sulphate
Phe	phenylalanine
PKU	phenylketonuria

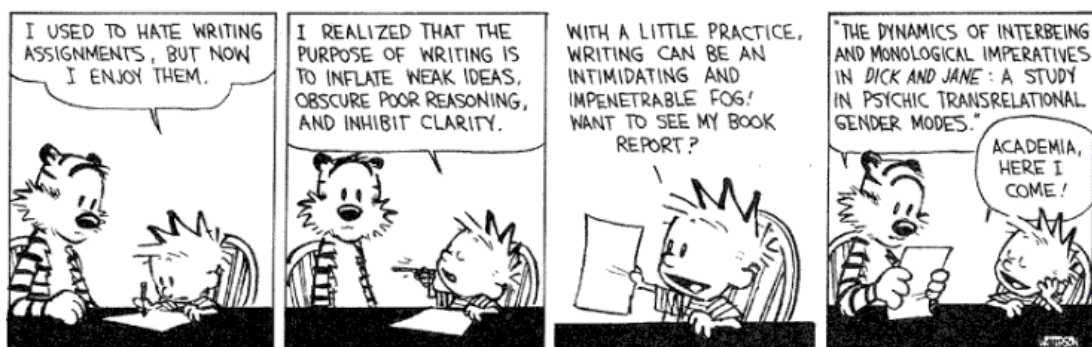
PUFA	polyunsaturated fatty acids
RS	resistant starch
SCFA	short chain fatty acids
SFA	saturated fatty acids
SIBO	small intestinal bacterial overgrowth
sWPI	sweet whey protein
T2D	type 2 diabetes
tGMP	semi-purified GMP
TNF- α	tumour necrosis factor α
Trp	tryptophan
Tyr	tyrosine
UC	ulcerative colitis
WHO	world health organisation
WT	wild type
XOS	xylo-oligosaccharides

Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism

Signed:

Alexandra Ntemiri, January 2019



Calvin and Hobbs, by Bill Watterson

Abstract

The proportion of the world population aged over 60 yrs is steadily increasing. Non-communicable disease and cognitive decline are prevalent in the elderly population which present a significant burden for national health care systems and economies. Malnutrition is prevalent in many longterm care unit-residing older people and importantly, it is also common among community-living elderly people. The deficiency in the aged population in certain nutrients, such as vitamins and proteins, may be associated with dementia and loss of bone and muscular health. Importantly, frailty that is accompanied by sarcopenia (i.e. deregulation of muscular homeostasis) is prevalent among older adults and may gradually lead to functional disability. Many studies have shown the importance of dietary management of aged-related conditions.

In the last decade, our knowledge of the importance of the gut microbiota in health and disease has significantly increased. Importantly, shifts in the composition and diversity of the gut microbiota of older people compared to healthy young adults are being identified and associated with ageing and age-related conditions, such as frailty. Similarly to other conditions for which gut microbiota modulation may lead to significant improvement of health, microbiota modulation may be a novel strategy for preventing or ameliorating ageing. This proposed gut microbiota modulation could be achieved through the use of prebiotics, probiotics or live bacteriotherapy among others. Importantly, diet can be a means of general (not targeted) gut microbiota modulation due to its documented significance in the shaping of the gut microbiota and to the fact that common dietary components may be a source of novel prebiotics.

25 In the ElderFood project, we investigated the prebiotic potential of milk and milk-
26 derived components to modulate the gut microbiota of older subjects. Using *in vitro*
27 and *in vivo* colon models, we generated an in-depth insight into the effect of
28 glycomacropeptide (GMP), a milk-derived peptide that contains mucin-type glycans,
29 and milk (whole and lactose free) on the elderly gut microbiota. We also performed
30 exploratory work for the investigation of fibre and polyphenol-rich foods on the
31 elderly gut microbiota and on artificial bacterial communities. The use of artificial
32 bacterial communities can increase our understanding of the microbiota dynamics
33 and how microbiota members respond to diet.

34

Chapter 1

35

Literature Review

36 **1.1 From birth to old age: Factors that shape the human gut**
37 **microbiome**

38 Chapter 1 was published as a book chapter:

39 Ntemiri, A., Stanton, C., Ross, R. P., O'Toole, P. W. From birth to old age: Factors
40 that shape the human gut microbiome. In "The Human Microbiome Handbook"
41 Tetro JA, Allen-Vercoe E, eds. Lancaster, PA, **2016**. pp 35-72.

42 **1.1.1 Abstract**

43 The term microbiome generally refers to the microbiota and the collective genetic
44 information those organisms carry. This genetic information supplements the
45 genomic and metabolic potential of the host. A common approach to analysing the
46 microbiome is to monitor compositional and functional changes in the microbiota
47 and then attempting to correlate those changes with health or disease symptoms. This
48 is usually a prelude to attempting to identify mechanisms and effectors.

49 Largely based on culture-independent techniques, the core composition of the
50 intestinal microbiota has recently been identified. The same phyla, taxa and genera
51 are generally found in the microbiota of the gastrointestinal tract of humans
52 irrespective of the condition of the subjects under testing, that is, healthy or non-
53 healthy young or older subjects. Thus, selective pressure and co-evolution between
54 the host and the intestinal commensals have apparently established a functionally
55 stable microbiota composition in the gut. Differences in the relative abundance of
56 bacterial subgroups in the gut microbiota, i.e. dysbiosis, indicate changes in the
57 health status of the host, or major environmental perturbations such as diet or
58 antibiotics.

59 The changes in the microbiota from early colonisation events to those that occur
60 during ageing are summarised here. Habitual diet is postulated to be the most
61 determinative factor in shaping the human gut microbiome. “Westernised diet” has
62 been identified as a major link between nutrient and gut microbiota signalling,
63 inflammation and metabolic disease. Dysbiosis also characterises functional
64 disorders of the gastrointestinal track like irritable bowel syndrome and
65 inflammatory bowel disease. The microbiota of the gastrointestinal tract of older

66 subjects differs from that of young healthy adults. A key aspect in future research is
67 to identify the exact molecular mechanisms that link dysbiosis and disease in order
68 to develop therapeutic strategies based on microbiota manipulation or administration
69 of microbial products.

70 **1.1.2 Introduction**

71 The term microbiome refers to the microbiota collection of microorganisms or in a
72 particular site and more specifically to the collective genetic information of those
73 organisms. Investigation of the association of gut microbiota to phenotypes of health
74 or disease is a major feature of contemporary microbiome research (Cho and Blaser,
75 2012). Culture-independent techniques and massive parallel next-generation
76 sequencing has generated data that enabled the profiling of the gut microbiota of
77 various healthy and disease cohorts ranging from infancy to the extremities of life
78 span as discussed in following chapters.

79 The phyla Firmicutes and Bacteroidetes are the most predominant in the healthy
80 adult gut microbiota and around 90 % of the bacterial groups found in the intestine
81 typically belong to these phyla, whereas Actinobacteria, Proteobacteria and
82 Verrucomicrobia comprise up to around 2 %, 1 % and 0.1 % respectively of the
83 intestinal microbiota composition (Hold *et al.*, 2002; Wang *et al.*, 2003; Eckburg *et*
84 *al.*, 2005; Rajilic-Stojanovic *et al.*, 2009; Tap *et al.*, 2009; Qin *et al.*, 2010).
85 Dominant bacterial groups in the gut microbiota belong to the genera
86 *Faecalibacterium*, *Ruminococcus*, *Eubacterium*, *Dorea*, *Bacteroides*, *Alistipes* and
87 *Bifidobacterium* (Tap *et al.*, 2009). Eckburg *et al* (2005) also identified substantial
88 representation of butyrate-producing Firmicutes belonging mostly to clostridia
89 (clusters IV, XIVa) and of *Bacteroides thetaiotaomicron* in the intestinal microbiota,
90 both subgroups known for exerting beneficial effects upon the host (Wrzosek *et al.*,
91 2013).

92 The gut microbiota composition as described above is based on studies on the
93 intestinal microbiota of westernised urban cohorts. There are exceptions to this

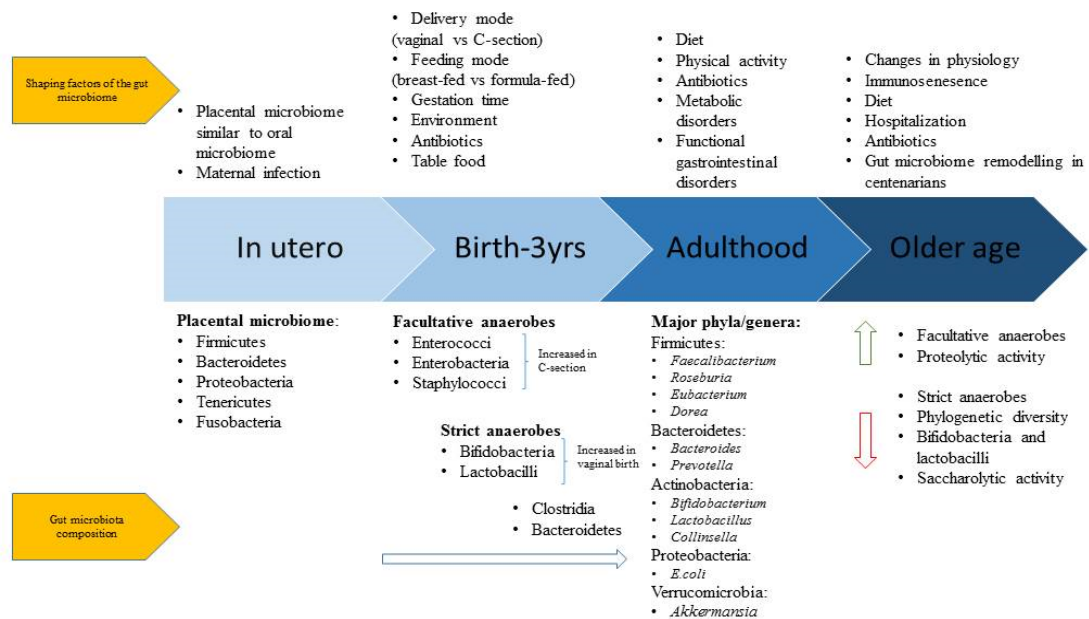
94 general picture of the composition of the gut microbiota. A recent study on the gut
95 microbiota of healthy hunter-gatherers in Hadza of Tanzania revealed the absence of
96 bifidobacteria and other major compositional differences compared to a European
97 urban cohort (Schnorr *et al.*, 2014). Strong selective pressure during co-evolution of
98 commensal gut bacteria and the mammalian host (Ley *et al.*, 2009) apparently
99 resulted in the establishment of symbiotic relationships that allow the host to extract
100 the maximum energy from the available habitual diet sources. It seems that the gut
101 microbiota of the Hadza cohort is adapted to a certain diet which is significantly
102 different to the westernised urban diet.

103 Host genetics seems to play an important role in shaping the microbiome profile
104 (approximately 2% contribution of genetics; Rothschild *et al.*, 2018) and potentially
105 explains some of the inter-individual variations in predominant species, subgroups
106 and phylotypes (Eckburg *et al.*, 2005; Benson *et al.*, 2010). However, a more
107 determinative role in the composition of the gut microbiota has been attributed to
108 habitual diet compared to host-genetics (Zhang *et al.*, 2010). Rothschild *et al.* (2018)
109 reported that environmental factors such as diet and lifestyle contribute over 20% to
110 the microbiota variability observed between individuals. Recently, bacterial groups
111 have been identified that are stably either absent or abundant over time in most
112 individuals (based on a cohort of a thousand western adults) without being affected
113 by short-term diet intervention and which correlated to health status such as
114 overweight and ageing (Lahti *et al.*, 2014).

115 It is becoming accepted that there is a functional redundancy in the gut microbiota
116 (Mahowald *et al.*, 2009; Lozupone *et al.*, 2012) which explains how variant bacterial
117 groups can result in the same functional core of the human gut microbiome

118 (Turnbaugh *et al.*, 2009; Qin *et al.*, 2010) Lessons from microbial ecology support
119 the notion that bacterial community structure is rather explained from the point of
120 functional genes than by species composition (Burke *et al.*, 2011).

121 The gut microbiome shifts that occur throughout the lifespan and the factors driving
122 these changes are reviewed here, including the early colonisation events of the
123 gastrointestinal track and the changes observed during ageing. The important role of
124 diet in modulating the intestinal microbiota is also discussed, summarising the
125 existing evidence indicating that diet can underpin metabolic disorders and
126 inflammation via the microbiota. Furthermore, we present the existing evidence
127 linking gut microbiota and exercise. Finally, dysbiosis associated with antibiotic
128 intake and functional disorders of the gastrointestinal track are reviewed. In **figure 1**
129 an overview of the factors affecting the composition of the gut microbiota
130 throughout life is presented.



131

132 **Figure 1 Overview of the factors affecting the composition of**
 133 **the gut microbiota throughout life.** C-section: Caesarean section, *E. coli*:
 134 *Escherichia coli*.

135 1.1.3 Establishment of the gut microbiota

136 Several studies on germ-free (GF) mice demonstrate the importance of the gut
 137 microbiota in the development of gut physiology and the priming of the innate and
 138 adaptive immune systems (reviewed in Sekirov *et al.*, 2010; Sommer and Backhead,
 139 2013). Disrupted colonisation patterns of the GIT in early life may have an
 140 immediate or future impact on health (Salminen *et al.*, 2004; Penders *et al.*, 2007;
 141 Cahenzli *et al.*, 2013; El Aidy *et al.*, 2013; Penders *et al.*, 2013; Ardeshtir *et al.*,
 142 2014).

143 *In utero*, the foetus is generally considered sterile although some recent studies
 144 suggest the opposite. Bacteria such as *Escherichia coli*, *Enterococcus faecium* and
 145 *Streptococcus epidermidis* have been isolated from the meconium of healthy term
 146 infants (Jimenez *et al.*, 2008). Bacteria have also been isolated from the amniotic

147 fluid of preterm neonates which could however be indicative of a hidden infection
148 and preterm delivery risk (DiGiulio *et al.*, 2008). In a first study on the placental
149 microbiome, Aagaard *et al.* (2014) conducted 16S rRNA sequencing and shot-gun
150 metagenomics on placental samples from 320 individuals. Comparing the findings to
151 other body microbiota data they reported the presence of a distinct community
152 comprised of non-pathogenic Firmicutes, Tennericutes, Proteobacteria, Bacteroidetes
153 and Fusobacteria. The placental microbiota was found to be similar to the microbiota
154 of the oral cavity. Importantly, an association between placental microbiota and
155 infection during the first pregnancy months and risk of preterm birth was observed.
156 Despite data suggestive of prenatal presence of bacteria in the uterus environment,
157 the role of these bacteria in the colonisation of the gastrointestinal track (GIT)
158 remains largely unclear and debateable (Matamoros *et al.*, 2013). Recently, Perez-
159 Muñoz (2017) concluded that the existing data cannot support the pre-birth existence
160 of fetal microbiota.

161 Colonisation of the human GIT is a dynamic and complex event and begins with
162 facultative anaerobes that prime the niche for the strict anaerobes that will follow
163 and ultimately prevail in the healthy adult microbiota. After birth the first bacteria
164 that colonise the infant gut are Enterococci, Enterobacteria (*E. coli*, *E. faecium*,
165 *Enterococcus faecalis*) and some staphylococci, and when these organisms deplete
166 the gut of oxygen anaerobes such as bifidobacteria, lactobacilli, clostridia and
167 Bacteroidetes emerge (Palmer *et al.*, 2007; Alderberth *et al.*, 2009). In the
168 repopulation of a GF murine model with normal murine gut microbiota, fermentation
169 metabolites and cross-feeding between early colonising bacteria and successors
170 facilitated the establishment of the latter (El Aidy *et al.*, 2013). The colonisation
171 event is regarded as leading to a dynamic eubiotic balance between the commensal

172 bacteria and the host inhibiting the growth of pathobionts, a phenomenon called
173 colonization resistance (Lawley and Walker, 2012).

174 It has been observed that in the first days of life the microbiota not only in the gut
175 but in all body niches (skin, oral mucosa, nasopharyngeal cavity) is similar and
176 reflects the mode of delivery (Dominguez-Bello *et al.*, 2010). Samples taken from
177 new-borns within 24 h of birth showed that vaginally born neonates harboured a
178 microbiota close to the maternal vaginal microbiota with *Lactobacillus*, *Prevotella*,
179 *Atopobium* and *Sneathia* spp. as prevalent taxa, whereas in neonates delivered by
180 Caesarean section (C-section) the microbiota of various body niches including the
181 gut resembled that of maternal skin (Dominguez-Bello *et al.*, 2010). A few days after
182 birth bifidobacteria are among the most dominant taxa in the gut of naturally
183 delivered babies (Biasucci *et al.*, 2008) but they seem to be absent or in low
184 representation in the gut of new-borns delivered by C-section (Aires *et al.*, 2011).

185 Jost *et al.* (2012) found that from the fourth day of life till the end of the first month
186 the gut microbiota of vaginally delivered breast-fed babies was dominated by
187 bifidobacteria. It was also reported that *Bacteroides* species showed variant patterns
188 of establishment that correlated to low counts of *Bifidobacterium* species, that
189 clostridia of the clusters IV and XIV were undetectable and that the presence of
190 *Lactobacillus* was not stable (Jost *et al.*, 2012). Other studies also confirm that
191 *Bifidobacterium* and *Lactobacillus* dominate the infant gut (Turroni *et al.*, 2012).
192 Aspects of the colonisation of the human gut by bifidobacteria have been
193 comprehensively reviewed in Ventura *et al.* (2012).

194 Interestingly, gestation time seems to affect the colonisation patterns of the GIT.
195 Vaginally delivered preterm babies seem to have an intestinal microbiota of low

196 phylogenetic diversity compared to full-term infants (Arboleya *et al.*, 2012). In
197 comparison to full-term babies preterm children were found to harbour more
198 facultative and fewer strict anaerobes and these differences could be attributed to
199 exposure to intensive medical treatment, hospitalisation and insufficient organ
200 function (Arboleya *et al.*, 2012).

201 The gut microbiota of preterm infants seems to be a risk factor for the development
202 of necrotising enterocolitis (NEC) / late onset sepsis (LOS). Both culture and non-
203 culture based analysis of stool samples from infants born around the 27th week of
204 pregnancy showed that those diagnosed with NEC and LOS had higher faecal
205 abundance of Enterobacteria and coagulase negative staphylococci (CONS)
206 respectively (Stewart *et al.*, 2012). As observed in another study on the gut
207 microbiota of preterm babies (Mshvildadze *et al.*, 2010) meconium was not sterile.
208 Dysbiosis rather than the presence of pathobionts, for example CONS, was
209 postulated to be significant in the onset of LOS in preterm patients (Mai *et al.*, 2013).
210 The group also observed low abundance of bifidobacteria in stool samples of LOS
211 patients. Interestingly, it has been recently suggested that irrespective of mode of
212 birth, feeding and antibiotic treatment the colonisation of the preterm GIT follows a
213 certain pattern from Bacilli to γ -Proteobacteria to Clostridia mainly influenced by
214 time of birth (La Rosa *et al.*, 2014).

215 It has been controversially proposed that during the final pregnancy stages maternal
216 symbiotic gut bacteria could translocate to the mammary gland (Fernandez *et al.*,
217 2013). Studies identified *Streptococcus*, *Staphylococcus* and *Corynebacterium*, and
218 lactic acid bacteria and bifidobacteria as dominant bacteria in the breast-milk
219 microbiota which however, seems to change with lactation towards a community

220 composition more rich in *Veillonella*, *Leptotrichia* and *Prevotella* (Hunt *et al.*, 2010;
221 Cabrera-Rubio *et al.*, 2012; Soto *et al.*, 2014). The same strains of bifidobacteria,
222 lactobacilli and staphylococci have been isolated from both the faeces of new-borns
223 and the maternal breast-milk of their mothers (Martin *et al.*, 2012) demonstrating
224 that the two bacterial communities are interrelated.

225 Many studies have confirmed that the intestinal microbiota of breast-fed new-born
226 children harbours more bifidobacteria than formula-fed e.g. Harmsen *et al.* (2000),
227 Martin *et al.* (2008), Roger *et al.* (2010), Bezirtzoglou *et al.* (2011). Others failed to
228 report significant differences in the bifidobacteria population in infant stool samples
229 between breastfed and formula-fed babies but abundance of *E. coli* and *Clostridium*
230 *difficile* was higher in formula-fed subjects (Penders *et al.*, 2005).

231 Breast milk contains human milk oligosaccharides (HMO). New-born babies are not
232 able to fully digest those oligosaccharides but infant-type bifidobacteria like
233 *Bifidobacterium longum* subsp. *infantis* are genetically endowed with the ability to
234 efficiently metabolise various short chain HMOs, an ability not detected in adult-
235 type bifidobacteria like *B. longum* subsp. *longum* (LoCassio *et al.*, 2010; Sela and
236 Mills, 2010; Garrido *et al.*, 2011). A variety of pathways for HMO metabolism are
237 distributed among the infant-type bifidobacteria revealing the selective pressure
238 breast feeding has put on certain bacteria groups (Zivkovic *et al.*, 2011). In all cases,
239 research agrees that after weaning, the introduction of solid food marks clear
240 changes in the microbiota although pre-weaning colonisation patterns are still
241 detectable (Fallani *et al.*, 2011; Koenig *et al.*, 2011).

242 As discussed below, antibiotics have an impact on the gut microbiota at every age.
243 Analysis of faecal microbiota of infants exposed to antibiotic treatment during the

244 first days after birth showed that the microbiota had limited numbers of
245 bifidobacteria and high abundance of Enterobacteria and Proteobacteria (Brunser *et*
246 *al.*, 2006; Tanaka *et al.*, 2009; Persaud *et al.*, 2014). In early life antibiotics can
247 disturb normal colonisation patterns which in turn can affect mammalian immunity
248 and metabolism in later life (reviewed in Rautava *et al.*, 2012). Cho *et al.* (2012)
249 observed that infant mice treated with antibiotics developed a gut microbiota that
250 was correlated to increased body fat. Cox *et al.* (2014) showed that in weaning mice
251 antibiotic treatment resulted in an altered gut microbiota capable of inducing obesity,
252 a permanent trait of the microbiota that could be transmitted through faecal transfer
253 to GF mice. The group postulated that the microbiota-related obesity was a result of
254 the altered gut microbiota composition due to antibiotic treatment. This has profound
255 implications for human weight management at population levels because paediatric
256 antibiotic usage is very frequent.

257 Studies suggest that the microbiota begins to stabilise to a more adult-like profile
258 from year one till 2.5 years of age (Palmer *et al.*, 2007; Koenig *et al.*, 2011). More
259 recent research shows that the microbiota undergoes changes till the 36th month of
260 age and that an enterotype establishment can be observed between the 9th month and
261 the third year of age (Yatsunenko *et al.*, 2012; Bergstrom *et al.*, 2014).

262 **1.1.4 Shaping factors of gut microbiota composition**

263 **1.1.4.1 Diet**

264 Habitual diet plays a significant role in the development of the composition of the
265 intestinal microbiota (Scott *et al.*, 2013, Simoes *et al.*, 2013). The intestinal
266 microbiota rapidly responds to diet variations which is a characteristic of the gut
267 microbiota of many mammalian groups (Muegge *et al.*, 2011; David *et al.*, 2014).

268 Most of the dietary nutrient absorption occurs in the duodenum and small intestine
269 whereas undigested molecules like complex polysaccharides reach the large intestine
270 (reviewed in Wong and Jenkins, 2007). In the colon a phylogenetically diverse and
271 metabolically active bacterial community salvages energy from the undigested food
272 particles which is subsequently used for the maintenance of the microbiota and by
273 the host (Blaut and Clavel, 2007).

274 Fermentation of undigested polysaccharides as well as dietary and endogenous
275 proteinaceous substrates and polyphenols results in various end-products with
276 tremendous impact on colonic health and homeostasis (Cummings and Macfarlane,
277 1997; Nyangale *et al.*, 2012, Windey *et al.*, 2012). These fermentation compounds
278 are predominantly the short chain fatty acids (SCFA) propionate, acetate and
279 butyrate, and gasses like H₂, H₂S and CO₂ as well as potentially toxic phenolic and
280 indolic compounds, ammonia, amines and sulphur molecules (reviewed in
281 Macfarlane and Macfarlane, 2012).

282 Different non-digestible carbohydrates like resistant starch (RS), non-starch
283 polysaccharides (NSP) and prebiotics can selectively enhance the growth of certain
284 bacterial groups in the GIT (Martinez *et al.*, 2010; Walker *et al.*, 2011). Modulated
285 microbial fermentation can yield beneficial end-products for the host. One such
286 fermentation product is butyrate that is extensively studied for the beneficial effect it
287 exerts on colonic epithelial cells (reviewed in Hamer *et al.* 2008). *Clostridium*
288 clusters XIVa and IV, with representatives like *Eubacterium* / *Roseburia* species and
289 *F. prausnitzii* respectively, are major saccharolytic players in butyrate generation in
290 the colon (reviewed in Louis and Flint, 2009) and potentially prominent regulators of

291 innate immunity through the production of SCFAs (Atarashi *et al.*, 2011; Atarashi *et*
292 *al.*, 2013; Smith *et al.*, 2013).

293 A statistical model was generated by Faith *et al.* (2011) that allowed for up to 60%
294 accuracy in the prediction of shifts in species abundance as a response to host diet.
295 The team isolated ten bacteria species from the human colon, sequenced their
296 genomes and introduced them in gnotobiotic mice. They subsequently conducted
297 detailed measurements on species abundance and gene expression in response to host
298 diet, from which the statistical model was developed. Animal models are valuable
299 tools in studying shifts in the microbiome but there is need for more extensive
300 human studies to better simulate the complex conditions occurring naturally in the
301 GIT (Walker *et al.*, 2010; Muegge *et al.*, 2011). Importantly, a recent study by
302 Korpela *et al.* (2014) developed models to predict the gut microbiota responses to
303 diet intervention based on the intestinal microbiota composition, predominantly on
304 the Firmicutes abundance, of certain obese individuals.

305 A summary of culture-independent studies correlating habitual diet to gut microbiota
306 is presented in **Table 1**. From metagenomic studies on human faecal data sets from
307 different countries Arumugam *et al.* (2011) observed that collectively, the grouping
308 of the data formed three clusters called “enterotypes”. Analytically, enterotype
309 number 1 was *Bacteroides*-dominated and positively co-related with
310 *Parabacteroides*, *Lactobacillus*, Clostridiales and *Alkaliphilus* species, and was
311 characterised by enrichment in metabolism of a broad spectrum of carbohydrate
312 utilization and proteolytic activity. In enterotype 2 *Prevotella* was predominant and
313 co-occurred with *Desulfovibrio* probably in a synergistic consortium for mucin
314 degradation. The *Ruminococcus* enterotype correlated with *Akkermansia* abundance

315 which is a mucin-degrading bacterium. The “enterotype” classification was based on
316 the high abundances of certain genera and this grouping should be regarded as
317 metabolic variations in the way gut microbiota utilises available nutrients.

318 Consumption of a vegetarian diet leads to changes in the microbiota of the GIT. Low
319 abundance of *Clostridium* cluster IV and higher but not significant abundance of
320 *Bacteroides* was observed by qPCR analysis of faecal samples of vegetarians
321 compared to omnivores (Liszt *et al.*, 2008). Another culture-independent study
322 reported low abundance of *Clostridium* cluster XIVa and the Roseburia / E. rectale
323 group in the faecal samples of vegetarians compared to omnivores (Kabberdoss *et al.*,
324 2012). Diet intervention with strict vegetarian diet on obese individuals for one
325 month led to changes in the intestinal microbiota composition of patients without
326 any impact on enterotypes and improved inflammatory markers in blood (Kim *et al.*,
327 2013). In that study, the diet intervention reduced the Firmicutes / *Bacteroides* ratio
328 and the proportion of Enterobacteria in the intestinal microbiota, and increased the
329 abundances of *Clostridium* cluster XIVa and IV.

330 Based on diet interventions and habitual diet of 98 individuals Wu *et al.* (2011)
331 reported that colonic communities clustered according to the aforementioned
332 enterotypes 1 and 2 and that number 3 demonstrated a tendency to fuse with
333 enterotype 1. The *Bacteroides*-dominated cluster co-occurred with *Alistipes* and
334 *Parabacteroides* and associated with an animal protein and saturated fat-based
335 habitual diet. In contrast, the *Prevotella*-cluster correlated with *Paraprevotella* and
336 *Catenibacterium* and was related to fibre intake. Importantly, enterotypes as
337 monitored by Wu *et al.* (2011) were driven by habitual diet and overall, remained
338 stable during the diet intervention, although changes in the microbiome did occur as

339 a response to the new diet. The group also concluded that the two clusters mirrored a
340 westernised and a more agrarian kind of diet respectively.

341 Similar conclusions were drawn when high-through put sequencing was conducted
342 on 16S rDNA from faecal samples of children of two geographically distinct areas:
343 urban in Florence, Italy and rural in Boulpon, Burkina Faso (De Filippo *et al.*, 2010).
344 The rural diet was almost vegetarian and enriched in starch, fibre and plant
345 polysaccharides and low in animal fat and protein. The faecal microbiota of subjects
346 residing in rural areas was enriched in bacteria enabling maximum energy harvest
347 from fibre and high rates of SCFAs production. Genera like *Xylanibacter*, *Prevotella*,
348 *Butyrivibrio* and *Treponema* were characteristic of the intestinal microbiota of
349 people living in rural areas and indicative of the potential to harvest energy from
350 complex polysaccharides. By contrast, the urban diet sustained a much less diverse
351 microbiota.

352 Taken together, the results of the two studies above reveal the impact on gut
353 microbiota of two kinds of diet, one “rural” enriched in fibre and low in fat and the
354 other more “westernised”, rich in animal protein and saturated fat. The global
355 convergence of dietary habits towards a westernised high fat / low fibre diet is an
356 increasing phenomenon in modern society and is accompanied by the escalating
357 prevalence of inflammatory and metabolic disease. The interplay between
358 Westernised dietary habits, commensal bacteria and inflammation are
359 comprehensively reviewed by Thorburn *et al.* (2014).

360 **1.1.4.2 Exercise**

361 No extensive studies have been dedicated so far to the exploration of the effect of
362 exercise on the human microbiota. There are however some indications that exercise

363 affects the gut environment based on a murine model. Matsumoto *et al.* (2008)
364 observed that rats that regularly exercise on the wheel-run had an intestinal
365 microbiota distinct from the microbiota of sedentary animals and notably the former
366 had higher colonic butyrate concentration compared to sedentary littermates. Choi *et*
367 *al.* (2013) observed exercise-induced changes in the intestinal microbiota of healthy
368 mice. Another study showed that exercise can lead to shifts in the gut microbiota of
369 obese mice (Evans *et al* 2013). Recently, Clarke *et al.* (2014) studied the effect of
370 exercise on the intestinal microbiota of the members of a professional rugby team.
371 The study showed that athletes had a distinct faecal microbiota and lower
372 inflammatory status in blood compared to controls. Athletes and low body mass
373 index (BMI) controls had higher abundance of *Akkermansia* in the faecal samples
374 compared to high BMI controls. The group correlated the gut microbiota changes not
375 only with regular intense exercise but with the high protein diet regime the athletes
376 consumed. However, it was impossible to rule out the effect of potential confounding
377 factors. The existing evidence indicate that gut-microbiota is responsive to exercise
378 but more research including intervention studies is necessary to elucidate the
379 mechanisms of such responses. Recently, Barton *et al.* (2018) reported that after
380 reexamining the microbiome of the Clarke *et al.* (2014) study-participants using
381 shotgun metagenomics analysis, the microbiome of athletes at taxonomic and mostly
382 at metabolomic level had significant differences compared to the more sedentary
383 controls; the athletes' microbiome profile was associated with high fibre and protein
384 diet and exercise.

385 **Table 1 Culture-independent studies associating changes in gut microbiota to diet.**

Cohort	Analysis Method	Diet	Main findings	Reference
29 healthy adults	qPCR, PCR-DGGE	Habitual vegetarians, omnivores	↓ <i>Clostridium</i> cluster IV ↑ <i>Bacteroides</i> spp. (non-significant)	Liszt <i>et al.</i> (2009)
46 healthy adults	qPCR	Habitual vegetarians, omnivores	↓ <i>Clostridium</i> cluster XIVa ↓ <i>Roseburia</i> / <i>E. rectale</i> group	Kabberdoss <i>et al.</i> (2012)
6 obese with type 2 diabetes (T2D)	16S rRNA sequencing, biochemical analysis	Strict vegetarian diet/ 1 month	↓Firmicutes/ <i>Bacteroides</i> ratio ↓No effect on enterotypes ↓Pathobionts, Enterobacteria ↑ <i>Bacteroides fragilis</i> , <i>Clostridium</i> cluster XIVa, IV species ↓Body weight, blood triglycerides, cholesterol	Kim <i>et al.</i> (2013)
14 healthy Mossi ethnic group children (rural community), 15 healthy Europeans (urban community)	16S rRNA sequencing	-Habitual rural diet: low fat, protein, sugar, high fibre	-↑ <i>Bacteroidetes</i> , unique presence of <i>Xylanibacter</i> , <i>Prevotella</i> , <i>Butyrivibrio</i> , <i>Treponema</i>	De Filippo <i>et al.</i> (2010)
		-Habitual urban diet: high fat, protein, sugar, low fibre	-↑Firmicutes, ↑Enterobacteria	

Cohort	Analysis Method	Diet	Main findings	Reference
-98 healthy subjects	16S rRNA sequencing	-Habitual fat-rich diet -Habitual fibre-rich diet	-↑ <i>Bacteroides</i> , <i>Alistipes</i> , <i>Parabacteroides</i> -↑ <i>Prevotella</i> , <i>Paraprevotella</i> , <i>Catenibacterium</i>	Wu <i>et al.</i> (2011)
-10 healthy <i>Bacteroides</i> enterotype		Diet intervention: -high fat/protein -carbohydrates	- <i>Bacteroides</i> enterotype correlated - <i>Prevotella</i> enterotype related -No permanent enterotype switch	
14 overweight subjects	16S rRNA sequencing, qPCR	-Resistant starch diet intervention	-↑ <i>Roseburia</i> / <i>E. rectale</i> related Firmicutes	Walker <i>et al.</i> (2011)
18 lean subjects, 33 mammalian species	16S rRNA sequencing, qPCR	-Reduced carbohydrates diet intervention Proteins, insoluble fibre	-↓ <i>Roseburia</i> / <i>E. rectale</i> related Firmicutes -Similar enzymatic activity and adaptation to diet across species	Muegge <i>et al.</i> (2011)
178 older subject: community, long-stay	16S rRNA sequencing	-Community: moderate fat/high fibre, diverse diet -Long-stay: high fat/protein/sugar, low fibre, less diverse diet	-↑Phylogenetic diversity, ↑Firmicutes, <i>Roseburia</i> , <i>Coprococcus</i> -↓Phylogenetic diversity, ↑Bacteroidetes, <i>Parabacteroides</i> , ↓ <i>Eubacterium</i> , <i>Anaerotruncus</i> , <i>Coprobacillus</i>	Claesson <i>et al.</i> (2012)
20 monozygotic twins	16S rRNA sequencing, DGGE	Habitual diet	<i>Bacteroides</i> spp. and bifidobacteria most affected by habitual diet	Simoes <i>et al.</i> (2013)

Cohort	Analysis Method	Diet	Main findings	Reference
40 professional athletes	16S rRNA sequencing, biochemical	Strict habitual diet, high protein	↑ <i>Akkermansia</i> sp., ↓inflammatory markers in blood	Clarke <i>et al.</i> (2014)
10 healthy individuals	16S rRNA sequencing, qPCR, RNA-sequencing, biochemical	Short-term intervention: -Animal-based diet -Plant-based diet:	-↑Bile-tolerant bacteria: <i>Alistipes</i> , <i>Bilophila</i> , <i>Bacteroides</i> , ↓plant fibre metabolising Firmicutes -↑ <i>Roseburia</i> / <i>Eubacterium</i> , Ruminococci	David <i>et al.</i> (2014)

386 1.1.4.3 Antibiotics

387 Antibiotic treatment can reduce the compositional diversity and the metabolic
388 potential of the gut microbiome, with an effect that attenuates with treatment
389 cessation (Pérez-Cobas *et al.*, 2013). Jakobsson *et al.* (2010) studied subjects
390 receiving clarithromycin and metronidazole treatment and monitored microbiota
391 over a period of four years. After a week of treatment distinct changes in the
392 composition of the intestinal microbiota of all subjects were observed. Interestingly,
393 the group also observed that although the general trend was gut microbiota recovery
394 in individuals after completion of treatment, some cases failed to fully recover the
395 pre-treatment microbiota. The same pattern of inter-individual responses to
396 antibiotics, recovery after cessation of treatment but with cases among subjects
397 studied with reduced to absent restoration of taxa in the intestinal microbiota, was
398 reported in other studies (Jernberg *et al.*, 2007; Dethlefsen *et al.*, 2008; Dethlefsen
399 and Relman, 2011). Inter-individual responses reflected the variations of the healthy
400 status microbiota among individuals. The faecal metabolome associated with the
401 microbiota seems to be affected. Streptomycin was shown to exert multiple effects
402 on murine intestinal metabolic activity (Caetano *et al.*, 2011) implying a potential
403 effect on the microbiota involved in certain metabolic pathways

404 Low diversity of the gut microbiota makes the intestinal environment susceptible to
405 entero-pathogens. In a murine model after two days of treatment with
406 vancomycin/streptomycin a dose-dependent effect was apparent upon the
407 composition of the gut microbiota (Sekirov *et al.*, 2008). The group observed that
408 although total bacterial numbers were not affected, the composition of the microbiota
409 was altered making the animals vulnerable to *Salmonella* serovar *Typhimurium*
410 infection.

411 Another common example of infection susceptibility and breach in the colonisation
412 resistance phenomenon is that of *Clostridium difficile* infection (CDI). *C. difficile* is
413 a common nosocomial pathogen, causing antibiotic-associated diarrhoea (AAD) and
414 affecting mostly hospitalised older patients. Epidemiology and risk factors of CDI
415 were reviewed by Ananthakrishnan (2011). Cephalosporins, macrolides, clindamycin
416 and predominantly fluoroquinolones are some of the associated infection-risk
417 antibiotics (Pépin *et al.*, 2005). The successful treatment of CDI in a mouse model
418 with a mixture of bacteria isolated from healthy subjects (Lawley *et al.*, 2012), and
419 in humans with a stool substitute (Petrof *et al.*, 2013) showed that a phylogenetically
420 diverse mix of faecal bacteria is sufficient to displace *C. difficile* from the gut more
421 effectively than any known antibiotic.

422 Overall, antibiotic administration results in mostly short-term disturbances in the
423 microbiota and these disturbances tend to revert with the end of antibiotic use. The
424 dominant taxa in the gut microbiota tend to recover soon after short-term
425 administration ends showing that balancing ecological mechanisms drive the
426 microbiota back to its pre-treatment status whereas long-term use may result in
427 permanent alterations in the intestinal microbiota (De La Cochetiere *et al.*, 2005) and
428 increase risk of AAD.

429 **1.1.4.4 Extra-intestinal disorders: Metabolic syndrome and related obesity,** 430 **type 2 diabetes, liver disease**

431 The metabolic syndrome is mainly characterised by insulin resistance and visceral
432 obesity as well as raised blood pressure, atherogenic dyslipidaemia and pro-
433 inflammatory status (Grundy *et al.*, 2004). The syndrome is a risk factor for the
434 interrelated development of cardiovascular disease (CDV) and type 2 diabetes (T2D)
435 (Huang, 2009). Dysbiosis seems to play a role in this syndrome. The intermediate

436 step is the interplay between the gut microbiota, nutrient absorption, fat storage and
437 host metabolism (reviewed in Musso *et al.*, 2011). The metabolic syndrome also
438 affects hepatic physiology with the potential for development of the non-alcoholic
439 fatty liver disease (NAFLD) and the more severe non-alcoholic steatohepatitis
440 (NASH) (Abu-Shanab and Quigley, 2010).

441 **Obesity and type 2 diabetes**

442 There is an accumulation of evidence that commensal bacteria play a significant role
443 in modulating fat storage. When lean GF mice were conventionalised with normal
444 gut microbiota they gained body fat although food intake was not increased
445 (Backhed *et al.*, 2004). Cani *et al.* (2007; 2008) used a murine model to propose a
446 sequence of events leading from high-fat diet to gut microbiota-controlled metabolic
447 disorders. High-fat feeding was correlated with increased intestinal permeability and
448 high lipopolysaccharides (LPS) plasma concentrations leading to metabolic
449 endotoxemia, inflammation and eventually metabolic conditions. Obesity and
450 diabetes reflecting the metabolic deregulation were attenuated in a mouse strain
451 lacking the main LPS-receptor CD14, demonstrating the importance of LPS in the
452 initiation of the metabolic deregulation (Cani *et al.*, 2007). In humans, plasma LPS
453 levels increase after increase of fat intake (Erridge *et al.*, 2007) and translocation of
454 bacterial components across the gut barrier seem to occur in obese and diabetic
455 subjects (Gummesson, *et al.*, 2011; Hawkesworth *et al.*, 2013).

456 Importantly, LPS interacts with the endocannabinoid system (eCB) which emerges
457 as an important player in the metabolic deregulation in obesity (Lamber and
458 Muccioli, 2007). The endocannabinoid system is implicated in gut barrier integrity
459 as blocking the G protein-coupled receptor of eCB CB₁ resulted in reduced plasma

460 LPS and an increase in tight-junction proteins in murine model (Muccioli *et al.*
461 2010).

462 Ley *et al.* (2005) showed that the establishment of the diet-induced obesity
463 phenotype in mice was accompanied by low intestinal microbiota diversity and
464 altered relative abundances of the major taxa with increased abundance of Firmicutes
465 and reduction to 50% of phylum Bacteroidetes compared to lean mice. The group
466 also demonstrated that the potential to gain energy from nutrients was improved in
467 this “obese microbiome”: more enzymes involved in starch and carbohydrate
468 metabolism, more fermentation-end products like butyrate and reduced energy load
469 in faeces compared to lean littermates (Turnbaugh *et al.*, 2006). Importantly, the
470 increased energy uptake trait could be transmitted to germ-free mice by faecal
471 transplantation (Turnbaugh *et al.*, 2006). Studying obesity-associated shifts in gut
472 microbiota in humans, Ley *et al.* (2006) obtained results analogous to data from the
473 mouse studies. Accordingly, in obese humans Bacteroidetes relative abundance was
474 lower compared to Firmicutes. However, studies by other groups in humans have
475 been contradictory and report different changes in the ratios of the Firmicutes and
476 Bacteroidetes (Duncan *et al.*, 2008; Schwartz *et al.*, 2010). In a Danish cohort of
477 obese and non-obese people, metagenomics analysis showed that the number of the
478 gut microbiota genes used as a measure of bacterial richness in the microbiota, was
479 associated with obesity phenotypes (Le Chatelier *et al.* 2013). In individuals with
480 low gene counts demonstrated associated obesity phenotypes such adiposity and
481 insulin resistance, and had increased abundance of *Bacteroides*, *Parabacteroides*,
482 certain *Ruminococcus* taxa and Proteobacteria among others, whereas in the gut
483 microbiota of individuals with high gene counts “anti-inflammatory” taxa such as
484 *Faecalibacterium* were dominant (Le Chatelier *et al.* 2013). Cotillard *et al.* (2013)

485 reported that low gene richness in obese and overweight individuals was associated
486 with higher low-grade inflammation and deregulated metabolism.

487 Like in obesity, research on the microbiota of patients with type 2 diabetes (T2D)
488 reveals significant alterations compared to healthy controls. The “diabetic microbiota”
489 had high proportions of *Bacteroidetes-Prevotella* group whereas the abundance of
490 the butyrate-producer group *C. coccoides-E. rectale* was reduced (Larsen *et al.*,
491 2010). A metagenomic analysis on the faecal microbiome of Chinese T2D patients
492 showed decreased butyrate biosynthesis and metabolism of vitamins, enrichment in
493 metabolism of xenobiotics, branched chain amino acids (BCAA) and methane, and
494 sulphate reduction to mention some (Qin *et al.*, 2012). The group also reported
495 opportunistic pathogens, the mucin-degrader *Akkermansia muciniphila* and sulphur
496 reducers like *Desulfovibrio* sp. to be higher in abundance in T2D faecal microbiota.
497 A metagenome-wide study on a cohort of 145 healthy and with risk for diabetes
498 European women also revealed composition and function alterations in the gut
499 microbiota of the at-risk subjects (Karlsson *et al.*, 2013). The group developed a
500 diabetes risk predicting tool which however failed to predict diabetes when applied
501 on the aforementioned Chinese cohort suggesting that the metagenomic markers for
502 T2D between the two cohorts were cohort-specific and could not be generalised.
503 Importantly, Forslund *et al.* (2015) reported that when controlling for the use of
504 antidiabetes drug use i.e. metformin, a certain “diabetic” gut microbiota profile
505 emerged characterised by significant reduction in butyrate producers. Metformin is
506 widely used for T2D treatment and there is data supporting that part of its
507 antidiabetic activity is mediated by acting on the gut micorbiota (Wu *et al.*, 2017).

508 **Non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH)**

509 A range of hepatic conditions related to obesity are grouped under the term NAFLD
510 including the inflammatory steatosis NASH (Brunt and Tiniakos, 2010). In obesity
511 accompanied by co-morbidity the NAFLD prevalence is 95% and prevalence for
512 NASH is 25%, whereas in diabetic patients NASH may reach 63% prevalence (Bajaj
513 *et al.*, 2012). The gut microbiota of patients with either NAFLD or NASH have not
514 yet been extensively studied.

515 Mouzaki *et al* (2013) conducted one of the first culture-independent studies on 50
516 adults with steatosis, non-alcoholic steatohepatitis and healthy controls. The group
517 observed a diet-independent reduced relative abundance of Bacteroidetes in the stool
518 of NASH patients. A previous study on the intestinal microbiota of obese, healthy
519 children and children with NASH showed that obese and NASH microbiomes could
520 cluster in a *Prevotella*-enriched enterotype diversifying from the healthy controls
521 (Zhu *et al.*, 2012). *Bacteroides* abundance was higher in obese and NASH subjects
522 compared to healthy controls, Actinobacteria had relatively lower abundance in non-
523 healthy subjects and Proteobacteria abundance increased significantly from healthy
524 to obese individuals with higher scores in NASH subjects (Zhu *et al.*, 2012). The
525 study also associated the elevated ethanol measurements in NASH patients blood
526 samples to a gut microbiota enriched in ethanol-producing bacteria like *Escherichia*
527 *coli*.

528 Data on the gut microbiota of NAFLD patients is still limited. A clear link between
529 gut microbiota, diet and metabolic disorders exist. However, these two studies on
530 NAFLD and intestinal microbiota yielded diet-independent results implying a causal
531 role of the gut microbiota in the pathogenesis of the condition. Serino *et al.* (2012)
532 investigating metabolic disorders and gut microbiota in a murine model showed that

533 irrespective of diet and host genetics gut microbiota and the naturally occurring
534 metabolic variations of the intestinal microbiome among individuals predisposed the
535 animals for metabolic conditions. Future work is expected to further elucidate both
536 the mechanisms through which diet controls gut microbiota-driven host metabolism
537 disorders and the existence of a diet-independent role of the gut microbiota in the
538 progression of metabolic conditions.

539 **1.1.4.5 Functional gastrointestinal disorders: Inflammatory bowel disease** 540 **(IBD), Irritable bowel syndrome (IBS)**

541 Functional gastrointestinal disorders (FGIDs) are a broad group of commonly
542 occurring gastrointestinal conditions with irritable bowel syndrome (IBS) being the
543 most prevalent (Talley, 2008). Using Rome III criteria similar symptoms in IBS and
544 IBD could be identified (Bryant *et al.*, 2011).

545 **Inflammatory bowel disease (IBD)**

546 The two major forms of IBD are ulcerative colitis (UC) and Crohn's disease (CD)
547 and both are influenced by host genetics and deregulated immunological response to
548 the intestinal microbiota (Abraham and Cho, 2009). Culture-independent studies
549 show abnormalities in the microbiome of individuals with IBD. Frank *et al.* (2007)
550 compared the microbiota in biopsies of UC and CD to those of healthy controls.
551 Although the abundances of the predominant taxa were not altered, significant
552 variations in the relative abundances of subgroups distinguished the healthy from the
553 non-healthy microbiotas and health-promoting butyrate-producers belonging to
554 Bacteroidetes and Lachnospiraceae were significantly reduced in abundance in the
555 IBD samples. Conversely, Proteobacteria and *Bacillus* species were increased in
556 proportion.

557 Manichah *et al* (2006) applied metagenomics to the faecal microbiota of CD patients
558 and observed reduced phylogenetic diversity. Patient faecal samples demonstrated
559 low abundance of the butyrate-producer groups *Clostridium leptum* and *Clostridium*
560 *coccoides* and were enriched in Gram negative species and the family
561 *Porphyromonadaceae*. *F. prausnitzii* is a major butyrate-producer and the dysbiosis
562 and symptomology characterized by the significant reduction of this Firmicutes in
563 CD could be reversed by oral administration of this organism in a murine model
564 (Sokol *et al.*, 2008).

565 Another bacterium implicated in IBD is *Bacteroides fragilis*. Transcriptional
566 analysis of colonic mucosal biopsies showed that the phylum Bacteroidetes was the
567 most active based on rRNA gene libraries and *B. fragilis* was dominant in CD based
568 on proportion of sequences in rRNA library/percentage of sequences in rRNA gene
569 library (AIR) (Rehman *et al.* 2010). Previously, Swidsinski *et al.* (2005) had
570 demonstrated the prevalence of this species in gut microbiota of IBD patients and
571 especially CD and they suggested the potential role of the high abundance of this
572 bacterium in increased barrier permeability.

573 Risk factors for IBD have been identified, and host genetics and dysbiosis have been
574 associated to the progression of the condition. However, the exact mechanisms
575 leading to IBD remain elusive thus making the unravelling of IBD causation a
576 paradigm for the understanding of the complex interplay between microbiota, host
577 and inflammation (Huttenhower *et al.*, 2014). Furthermore, the complex cross-talk
578 between the causative factors of the condition, the inter-individual variations in gut
579 microbiota and the fact that no universal colitogenic bacteria have been identified

580 indicate the need for more personalised IBD therapeutic strategies (Stephens and
581 Round, 2014).

582 **Irritable bowel syndrome (IBS)**

583 A common FGID disorder is IBS and unlike IBD it is not associated with
584 histopathological conditions and it is believed to be related to the deregulation of the
585 gut-brain axis (GBA) (Ringel and Maharshak, 2013). The cross-talk between gut and
586 brain and the association with intestinal homeostasis and progression of FGID have
587 been recently comprehensively reviewed by Collins and Bercik (2009) and Collins
588 (2014). IBS progression can be outlined as follows: extrinsically-induced dysbiosis,
589 for example after severe infection, triggers mucosal immunity in genetically
590 predisposed subjects resulting in enteric nervous system deregulation caused by
591 intestinal permeability (Collins *et al.*, 2009; Simren *et al.* 2009; Steck *et al.*, 2013).

592 The diagnostic criteria set by the Rome Foundation, Rome criteria III
593 (<http://www.theromefoundation.org/criteria/>), are widely used for FGID diagnosis
594 (Soares, 2014); outlined here in **Table 2**. Three subtypes of the condition exist based
595 on stool appearance: diarrhoea-predominant (IBS-D), constipation-predominant
596 (IBS-C), mixed (IBS-M) (Longstreth *et al.*, 2006).

597 Using culture-based techniques and PCR - denaturing gradient gel electrophoresis
598 (PCR-DGGE) analysis Matto *et al.* (2005) observed temporal instability of the gut
599 microbiota of IBS patients but failed to associate shifts in bacterial groups with
600 symptoms of the condition. Other culture-independent studies also showed temporal
601 instability of the intestinal microbiota of IBS subjects, lower abundance of
602 *Lactobacillus* species in stool samples of patients with IBS-D, increase in abundance

603 of *Veillonella* species in IBS-C individuals and a reduction in Clostridia species
604 abundance (Malinen *et al.*, 2005; Kassinen *et al.*, 2007).

605 Other culture-independent studies showed that in the faecal microbiota of IBS
606 patients compared to controls, the Firmicutes to Bacteroidetes ratio was increased,
607 Actinobacteria had lower abundance and Proteobacteria were more abundant
608 (Krogus-Kurikka *et al.*, 2009; Rajilic-Stojanovic *et al.*, 2011). Similar results were
609 published by Jeffrey *et al.* (2012), that is, significant differences between gut
610 microbiota of IBS patients and healthy controls and increased Firmicutes to
611 Bacteroidetes ratio. Subgroups were formed based on the faecal microbiota of
612 patients and importantly, one of these was characterised by normal-resembling faecal
613 microbiota. These patients had high scores for anxiety and depression, suggesting a
614 subclass of IBS on non-microbial aetiology.

615 In stool samples of IBS-C patients the fermentation potential of the microbiota was
616 altered: fluorescent *in situ* hybridisation (FISH) revealed that the abundance of H₂-
617 consuming sulphur-reducing bacteria and butyrate producing *Roseburia* / *E. rectale*
618 group was significantly higher and lower respectively compared to control subjects
619 (Chassard *et al.* 2012). The altered fermentation activity of the intestinal microbiota
620 in IBS patients was also indicated by abnormal hydrogen and methane breath
621 measurements in patients (King *et al.*, 1998). Interestingly, the commensal
622 methanogenic Archaea with the production of H₂ may have a role in IBS
623 pathogenesis (Triantafyllou *et al.*, 2014) but studies to date yield contradictory
624 results (Rajilic-Stojanovic *et al.*, 2011; Kim *et al.* 2012).

625 Studies so far have not fully revealed the exact mechanism through which the
626 microbiota influences the onset and progression of IBS. The fact that probiotics seem

627 to ameliorate the symptoms of the dysbiotic condition of this multifaceted syndrome
 628 further promotes the idea of microbiota disturbances implicated in the disease
 629 (Santos and Whorwell, 2014). IBS is an interesting field of research as the
 630 unravelling of the disease mechanisms will enable further insight in the brain-gut
 631 axis communication and how mediators from the intestinal microbiota can influence
 632 cognitive function in subjects with intestinal disorders (Bercik *et al.*, 2011).

633 **Table 2 Diagnostic criteria for IBS adapted from Rome III**
 634 (<http://www.theromefoundation.org/criteria/>).

Criteria for diagnosis of IBS	
• Intermittent abdominal pain and discomfort related to at least two of the conditions below: 1. <i>Improvement with defecation</i> 2. <i>Alterations in the defecation habits</i> 3. <i>Alterations in stool form</i>	• Frequency of symptoms: 3 days per month for the last 3 months • Symptom detection at least 6 months before diagnosis.

635 1.1.5 Ageing and microbiota alterations

636 There is growing scientific interest in the connection between low-grade
 637 inflammation observed in older age and the role of gut microbiota. According to this
 638 concept, accumulation of antigenic stimulation and stressors throughout life leads to
 639 declined immune responses and low immune adaptiveness resulting in inflamm-
 640 ageing (Franceschi *et al.*, 2000). An overview of how the protective activity of the
 641 immune system (IS) is deregulated with immuno-senescence, that is the ageing of
 642 the IS, is presented by De Martinis *et al.* (2005). The Nuclear Factor κ B (NF- κ B)
 643 seems to be at the core of this process by being the major signalling pathway in the
 644 innate IS (Salminen *et al.*, 2008).

645 It is now accepted that this low-grade inflammatory status with characteristic
646 markers circulating in the plasma of older people is associated with the degenerative
647 conditions that characterise old age (Howcroft *et al.*, 2013). Among the most usual
648 pro-inflammatory markers correlated with muscle tissue damage and sarcopenia,
649 neuro-degeneration and obesity are C-reactive protein (CRP), tumour necrosis
650 factor- α (TNF- α), IL-6 and IL-18 (Howcroft *et al.*, 2013).

651 Antigenic stimuli associated with the microbiota composition shifts observed in
652 older age may contribute to inflamm-ageing. The exact correlation and mechanisms
653 that link the microbiome to ageing remain unclear and this presents a promising field
654 for future research and new therapeutic strategies.

655 **Changes in the gut microbiota in older age**

656 Changes in the GIT physiology, in the functionality of the IS, in lifestyle and
657 nutritional habits, and hospitalisation and medical treatment can impact the
658 composition of the gut microbiota in older age and consequently health status
659 (reviewed in Cusack and O'Toole, 2013).

660 The combined data from older mainly culture-dependent studies suggest that age is
661 accompanied by an increase in the proportion of facultative anaerobes, with a
662 simultaneous decline of beneficial anaerobes such as bifidobacterial and lactobacilli
663 and importantly, an overall decline in species diversity in several bacterial groups
664 (Woodmansey, 2007). During inflammation pathobionts - potentially harmful
665 bacteria that are in low abundance in the healthy gut, increase in abundance
666 displacing commensals (Pédron and Sansonetti, 2008). The example of the
667 proliferation of the pathogenic *Salmonella enterica* serotype *Typhimurium* in the gut
668 shows how a pathogen can compete with commensals and prevail in the dysbiotic

669 condition of the inflamed ecosystem and further promote inflammation (Winter *et al.*,
670 2010). Furthermore, an alarming correlation exists between the low diversity profile
671 of the intestinal microbiota of older subjects and infection or non-symptomatic
672 colonization by the common nosocomial pathogen *C. difficile* (Rea *et al.*, 2012).

673 Studies based on culture-independent techniques offered more comprehensive
674 information about the shifts occurring in the gut microbiome of older subjects.
675 Overall, studies do not yield homogenous results and there are variations based on
676 the cohorts and the methods employed. However, it seems that compared to controls,
677 the intestinal microbiota of older people is characterised by differences in the
678 abundance of specific bacterial groups and the composition and species diversity of
679 these groups is altered (Tiihonen *et al.*, 2010). Variations in the reported composition
680 and significant inter-individual variability in the microbiota could be attributed to the
681 fact that ageing alone is not enough to alter the generally stable microbiota (Brussow,
682 2013) and the alterations should be viewed and explained accompanied by data on
683 the cohort diet, physical activity, drug and antibiotic intake, and even geographical
684 and socio-economical profile.

685 When the faecal bacterial population of older community-dwellers, hospitalised and
686 hospitalised on antibiotic treatment were compared, Bartosch *et al.* (2004) noted that
687 the basic difference in the faecal microbiota between healthy and hospitalized
688 subjects was the significant reduction in the *Bacteroides* / *Prevotella* group. In the
689 same study, bifidobacteria, *Desulfovibrio* spp., *Clostridium clostridioforme* and *F.*
690 *prausnitzii* also declined in abundance after hospitalisation. However, the relative
691 abundance of the aforementioned bacteria remained stable as the total bacterial load
692 in stool of hospitalised subjects also decreased. Antibiotic treatment had an

693 additional negative impact on the faecal microbiota. Antibiotics lowered the
694 diversity in the stool microbiota and even eliminated certain bacterial groups,
695 promoting the growth of opportunistic species and enterococci like *E. faecalis*
696 (Bartosch *et al.*, 2004).

697 Mueller *et al.* (2006) studied the gut microbiota composition of 230 healthy
698 individuals from four European countries. A significant increase in Enterobacteria
699 abundance in the stool samples of the older volunteers was observed irrespective of
700 country of origin whereas for most of the other dominant bacterial groups like *F.*
701 *prausnitzii*, bifidobacteria, *Bacteroides* / *Prevotella* no generalised conclusions could
702 be made that would unify the results from the stool samples of all individuals tested.
703 However, the *Bacteroides* / *Prevotella* group was more abundant in male subjects
704 compared to female. Zwielehner *et al.* (2009) comparing the faecal microbiota of
705 long-term residential elderly to healthy adults reported an overall decline in diversity
706 of the dominant phyla and significantly low representation of *Clostridium* cluster IV
707 and bifidobacteria along with inter-individual variations in *Bacteroides*. Comparing
708 the faecal microbiota of 161 Irish subjects aged >65 years and 9 younger controls
709 identified significant differences and remarkable inter-individual variability was
710 reported for Bacteroidetes and Firmicutes abundances (Claesson *et al.*, 2011)

711 When faecal microbiota of healthy adults (around 30 years), older (around 70 years)
712 and centenarian subjects was analysed the intestinal microbiota of centenarians was
713 distinct from the other subject categories (Biagi *et al.*, 2010). With Firmicutes and
714 Bacteroidetes remaining the major phyla throughout life, certain re-arrangements
715 were observed in the microbiota of centenarians with extensive remodelling in the
716 population of *Clostridium* cluster XIVa (Biagi *et al.*, 2010).

717 Data from the ELDERMET project was able to demonstrate a clear correlation
718 between diet, gut microbiota and health status in a large cohort of older subjects. The
719 participants were stratified in community-dwelling and short-term and long-term
720 hospitalized. The profiling of the intestinal microbiota was accompanied by data
721 collection on habitual diet, inflammation status, cognitive function and frailty.
722 Overall, subjects frequenting or residing in long-stay care units had poor diet which
723 correlated with higher frailty scores, higher inflammation indicators like IL-6, IL-8,
724 TNF α and CRP in the serum and a distinct microbiota in comparison to community-
725 dwellers of the same ethno-geographic region (Claesson *et al.*, 2012). Bacteroidetes-
726 related operational taxonomic units (OTUs) were more abundant in faecal samples of
727 long-stay subjects whereas in community-residing subjects the phylum Firmicutes
728 was at higher levels with dominant genera *Coprococcus* and *Roseburia* for the latter
729 group of subjects and *Parabacteroides*, *Eubacterium*, *Anaerotruncus*, *Lactonifactor*
730 and *Coprobacillus* for the former (Claesson *et al.*, 2012).

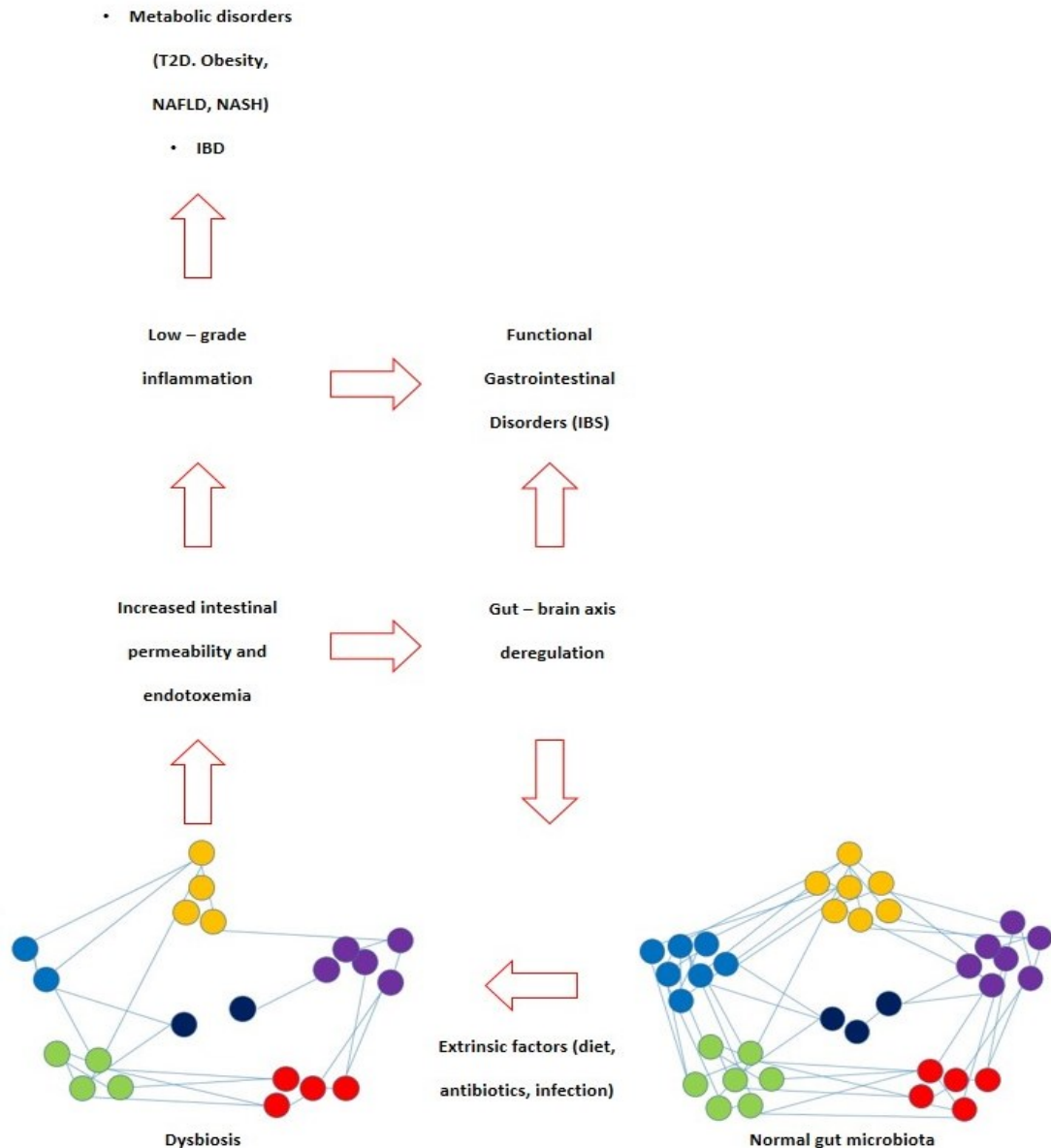
731 The gut microbiota in older age is characterised by both compositional and
732 functional alterations expressed in centenarians in the marked shift from a
733 saccharolytic metabolism towards putrefaction with significant loss of genes
734 involved in SCFA production (Rampelli *et al.*, 2013). Given the importance of these
735 metabolites in gut homeostasis (Smith *et al.*, 2013) this shift could increase the risk
736 for disease. Protein fermentation metabolites can also be detrimental for health by
737 affecting gut homeostasis (Windey *et al.*, 2012). However, in people reaching the
738 hundredth decade of life changes in the microbiome could be seen as an evolutionary
739 advantage. In centenarians high phenylacetylglutamine (PAG) and p-cresol sulphate
740 (PCS) secretion indicates shifts in the microbiome which could reflect an overall

741 remodelling of structure and functionality in order for the human organism to survive
742 in the extremities of life-span (Collino *et al.*, 2013).

743 **1.1.6 Concluding remarks**

744 As summarised here there is accumulating evidence predominantly from studies
745 based on high throughput parallel sequencing which underlines the significance of
746 gut microbiota in modulating health and disease. The gut microbiota is affected by
747 the host life-style, with a major factor being habitual diet. Intestinal microbiota
748 profiling conducted on faecal samples of various cohorts ranging from the Hazda
749 hunter-gatherers of Tanzania to subjects residing in westernised urban areas has
750 revealed how diet shapes the composition and function of the mammalian
751 microbiome. Nutrient products generated by commensal bacterial fermentation in the
752 colon affect fat storage, metabolism and even the gut-brain axis. In **Figure 2** a
753 schematic overview of how extrinsic factors like diet and antibiotics influence
754 normal gut microbiota leading to dysbiosis is presented. Interestingly, the gut
755 microbiota is significantly associated with the rate of ageing of the human organism
756 (Heintz and Mair, 2014).

757 Thus the gut microbiota is a promising target for novel therapeutics for a wide range
758 of conditions and for the manipulation of the progression of ageing itself (Föxx-
759 Orenstein *et al.*, 2010; Cani and Delzenne, 2011; Candela *et al.*, 2014). Culturomics
760 and phylogeny are indispensable for the development of novel microbial therapeutics
761 as they offer a tool for a more detailed study of bacterial physiology and metabolism
762 (Walker *et al.*, 2014). Understanding gut bacterial physiology will also facilitate the
763 development of *in silico* simulation models of the host-microbe / microbe-microbe
764 interactions (Thiele *et al.*, 2013).



765

766 **Figure 2 A schematic representation of how extrinsic factors like diet and**
 767 **antibiotics influence normal gut microbiota leading to dysbiosis.** The Wiggum
 768 plots at the bottom represent normal gut microbiota and gut microbiota in dysbiosis.
 769 Coloured circles represent correlated genera and their interactions are schematically
 770 indicated by lines. T2D: Type-2 diabetes, NAFLD: non-alcoholic fatty liver disease,
 771 NASH: non-alcoholic steatohepatitis, IBS: irritable bowel syndrome, IBD:
 772 inflammatory bowel disease.

773 1.1.7 References

- 774 Aagaard, K., Ma, J., Antony, K. M., Ganu, R., Petrosino, J., Versalovic, J. (2014).
775 The placenta harbours a unique microbiome. *Sci Transl Med*, 6(237), 237-65.
- 776 Abraham, C., Cho, J., H. (2009). Inflammatory Bowel Disease. *N Engl J Med*,
777 (361), 2066–2078.
- 778 Abu-Shanab, A., Quigley, E. M. M. (2010). The role of the gut microbiota in
779 non-alcoholic fatty liver disease. *Nat Rev Gastroenterol Hepatol*, 7(12), 691-701.
- 780 Adlerberth, I., Wold, A E. (2009). Establishment of the gut microbiota in
781 Western infants. *Acta Paediatr*, 98(2), 229–38.
- 782 Aires, J., Thouverez, M., Allano, S., Butel, M. J. (2011). Longitudinal analysis
783 and genotyping of infant dominant bifidobacterial populations. *System Appl*
784 *Microbiol*, 34(7), 536–41.
- 785 Ananthakrishnan, A. A., (2011). *Clostridium difficile* infection: epidemiology,
786 risk factors and management. *Nat Rev Gastroenterol Hepatol*, 8(1), 17-26.
- 787 Antunes, L. C. M., Han, J., Ferreira, R. B. R., Lolic, P., Borchers, C. H., Finlay,
788 B. B. (2011). Effect of antibiotic treatment on the intestinal metabolome.
789 *Antimicrob Agents Chemother*, 55(4), 1494-1503.
- 790 Arboleya, S., Ang, L., Margolles, A., Yiyuan, L., Dongya, Z., Liang, X., Solis,
791 G., Fernandez, N., de los Reyes-Galvin, C. G., Gueimonde, M. (2012). Deep 16S
792 rRNA metagenomics and quantitative PCR analyses of premature infant fecal
793 microbiota. *Anaerobe*, 18, 378-380.

794 Arbolea, S., Binetti, A., Salazar, N., Fernandez, N., Solís, G., Hernandez-
795 Barranco, A., Margolles, A., de los Reyes-Gavilan, C. G., Gueimonde, M. (2012).
796 Establishment and development of intestinal microbiota in preterm neonates.
797 *FEMS Microbiol Ecol*, 79(3), 763-772.

798 Ardeshir, A., Narayan, N. R., Méndez-Lagares, G., Lu, D., Rauch, M., Huang, Y.,
799 Van Rompay, K. K. A., Lynch, S. V., Hartigan-O'Connor, D. J. (2014). Breast-
800 fed and bottle-fed infant rhesus macaques develop distinct gut microbiotas and
801 immune systems. *Sci Transl Med*, 6(252), 252ra120.

802 Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D. R.,
803 Fernandes G. R., *et al.* (2011). Enterotypes of the human gut microbiome. *Nature*,
804 473(7346), 174–180.

805 Atarashi, K., Tanoue, T., Oshima, K., Suda, W., Nagano, Y., Nishikawa, H.,
806 Fukuda, S., Saito, T., Narushima, *et al.* (2013). Treg induction by a rationally
807 selected mixture of Clostridia strains from the human microbiota. *Nature*,
808 500(7461), 232-6.

809 Atarashi, K., Tanoue, T., Shima, T., Imaoka, A., Kuwahara, T., Momose, Y.,
810 Honda, K. (2011). Induction of colonic regulatory T cells by indigenous
811 *Clostridium* species. *Science*, 331(6015), 337–341.

812 Backhed, F., Ding, H., Wang, T., Hooper, L. V, Koh, G. Y., Nagy, A., Gordon, J.
813 I. (2004). The gut microbiota as an environmental factor that regulates fat storage.
814 *Proc Natl Acad Sci U S A*, 101(44), 15718–23.

815 Bajaj, J. S., Hylemon, P. B., Younossi, Z. (2012). The intestinal microbiota and
816 liver disease. *Am J Gastroenterol Suppl*, 1(1), 9-14.

817 Barton, W., Penney, N. C., Cronin, O., *et al.* (2018). The microbiome of
818 professional athletes differs from that of more sedentary subjects in composition
819 and particularly at the functional metabolic level. *Gut*, 67(4):625-633.

820 Bartosch, S., Fite, A., MacFarlane, G. T., and McMurdo, M. E. T. (2004).
821 Characterization of bacterial communities in faeces from healthy elderly
822 volunteers and hospitalized elderly patients by using Real-Time PCR and effects
823 of antibiotics treatment on the fecal microbiota. *Appl Environ Microbiol*, 70(6),
824 3575-3581.

825 Benson, A. K., Kelly, S. A., Legge, R., Ma, F., Low, S. J., Kim, J., Pomp, D.
826 (2010). Individuality in gut microbiota composition is a complex polygenic trait
827 shaped by multiple environmental and host genetic factors. *Proc Natl Acad Sci U*
828 *SA*, 107(44), 18933–18938.

829 Bercik, P., Denou, E., Collins, J., Jackson, W., Lu, J., Jury, J., Deng, Y.,
830 Blennerhassett, P., *et al.* (2011). The intestinal microbiota affect central levels of
831 brain-derived neurotrophic factor and behaviour in mice. *Gastroenterol*, 141(2),
832 599-609.

833 Bergstrom, A., Skov, T. H., Bahl, M. I., Roager, H. M., Christensen, L. B.,
834 Ejlerskov, K. T., Licht, T. R. (2014). Establishment of intestinal microbiota
835 during early life: a longitudinal, explorative study of a large cohort of Danish
836 infants. *Appl Environ Microbiol*, 80(9), 2889–900.

837 Bezirtzoglou, E., Tsitsias, A., Welling, G. W. (2011). Microbiota profile in
838 feces of breast- and formula-fed newborns by using fluorescence in situ
839 hybridization (FISH). *Anaerobe*, 17(6), 478-82.

840 Biagi, E., Cnadel, M., Turrone, S., Garagnani, P., Franceschi, C., Brigidi, P.
841 (2013). Ageing and gut microbes: Perspectives for health maintenance and
842 longevity. *Pharmacol Res*, 69, 11-20.

843 Biagi, E., Nylund, L., Candela, M., Ostan, R., Bucci, L., Pini, E., De Vos, W.
844 (2010). Through ageing, and beyond: gut microbiota and inflammatory status in
845 seniors and centenarians. *PloS One*, 5(5), e10667.

846 Biasucci, G., Benenati, B., Morelli, L., Bessi, E., Boehm, G. (2008). Cesarean
847 delivery may affect the early biodiversity of intestinal bacteria. *J Nutr*, 138(9),
848 1796S–1800S.

849 Blaut, M., Clavel, T. (2007). Metabolic diversity of the intestinal microbiota :
850 Implications for health and disease. *J. Nutr.*, 137(3), 751–755.

851 Brunser, O., Gotteland, M., Cruchet, S., Figueroa, G., Garrido, D., Steenhout, P.
852 (2006). Effect of a milk formula with prebiotics on the intestinal microbiota of
853 infants after an antibiotic treatment. *Pediatr Res.*, 59(3), 451-6.

854 Brunt, E. M., Tiniakos, D. G. (2010). Histopathology of non-alcoholic fatty liver
855 disease. *World J Gastroenterol*, 16(42), 5286-96.

856 Brussow, H. (2013). Microbiota and healthy ageing: observational and
857 nutritional intervention studies. *Microbial Biotechnology*, 6(4), 326–34.

858 Bryant, R. V, van Langenberg, D. R., Holtmann, G. J., Andrews, J. M. (2011).
859 Functional gastrointestinal disorders in inflammatory bowel disease: impact on
860 quality of life and psychological status. *J Gastroenterol Hepatol.*, 26(5), 916–
861 923.

862 Burke, C., Steinberg, P., Rusch, D., Kjelleberg, S., & Thomas, T. (2011).
 863 Bacterial community assembly based on functional genes rather than species.
 864 *Proc Natl Acad Sci USA*, 108(34), 14288–14293.

865 Cabrera-Rubio, R., Collado, M. C., Laitinen, K., Salminen, S., Isolauri, E., Mira,
 866 A. (2012). The human milk microbiome changes over lactation and is shaped by
 867 maternal weight and mode of delivery. *Am J Clin Nutr*, 96(3), 544-51.

868 Cahenzli, J., Koller, Y., Wyss, M., Geuking, M. B., McCoy, K. D. (2013).
 869 Intestinal microbial diversity during early-life colonization shapes long-term IgE
 870 levels. *Cell Host Microbe*, 14(5), 559-570.

871 Candela, M., Biagi, E., Brigidi, P., O'Toole, P. W., de Vos, W.M. (2014).
 872 Maintenance of a healthy trajectory of the intestinal microbiome during aging: a
 873 dietary approach. *Mech Ageing Dev*, 136-137, 70-75.

874 Cani, P. C., Amar, J., Iglesias, M. A., Poggi, M., Knauf, C., Bastelica, D.,
 875 Neyrinck, A. M., Fava, F., Tuohy, K. M., *et al.* (2007). Metabolic endotoxemia
 876 initiates obesity and insulin resistance. *Diabetes*, 56(7), 1761-72.

877 Cani, P. C., Bibiloni, R., Knauf, C., Waget, A., Neyrinck, A. M., Delzenne, N.
 878 M., Burcelin, R. (2008). Changes in the gut microbiota control metabolic
 879 endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes
 880 in mice. *Diabetes*, 57(6), 1470-1481.

881 Cani, P. D., Delzenne, N. M. (2011). The gut microbiome as therapeutic target.
 882 *Pharmacol Therap*, 130(2), 202–12.

883 Chassard, C., Dapoigny, M., Scott, K. P., Crouzet, L., Del'homme, C., Marquet,
884 P., Maritn, J. C., Pickering, G. *et al.* (2012). Functional dysbiosis within the gut
885 microbiota of patients with constipated-irritable bowel syndrome. *Aliment*
886 *Pharmacol Ther*, 35(7), 828–38.

887 Cho, I. Blaser, M. J. (2012). The human microbiome: at the interface of health
888 and disease. *Nat Rev Genet*, 13(4), 260-270.

889 Cho, I., Yamanishi, S., Cox, L., Methé, B. A., Zavadil, J., Li, K., *et al.* (2012).
890 Antibiotics in early life alter the murine colonic microbiome and adiposity.
891 *Nature*, 488(7413), 621–626.

892 Choi, J. J., Eum, S. Y., Rampersaud, E., Daunert, S., Abreu, M. T., Toborek, M.
893 (2013). Exercise attenuates PCB-induced changes in the mouse gut microbiome.
894 *Environ Health Perspect*, 121(6), 725-730.

895 Clarke, S. F., Murphy, E. F., O'Sullivan, O., Lucey, A. J., Humphreys, M, Hogan,
896 Aileen, Hayes, P., O'Reilly, M., *et al.* (2014). Exercise and associated dietary
897 extremes impact on gut microbial diversity. *Gut*, [Epub ahead of print].

898 Claesson, M. J., Cusack, S., O'Sullivan, O., Greene-Diniz, R., de Weerd, H.,
899 Flannery, E., *et al.* (2011). Composition, variability, and temporal stability of the
900 intestinal microbiota of the elderly. *Proc Natl Acad Sci USA*, 108 (Suppl 1),
901 4586–4591.

902 Claesson, M. J., Jeffrey, I. B., Conde, S., Power, S. E., O'Connor, E. M., Cusack,
903 S., Harris, H. M. B., Coakley, M., Lakshminarayanan, B., *et al.* (2012). Gut
904 microbiota composition correlates with diet and health in elderly. *Nature*, 488,
905 178-184.

906 Collino, S., Montoliu, I., Martin, F.-P. J., Scherer, M., Mari, D., Salvioli, S.,
 907 Rezzi, S. (2013). Metabolic signatures of extreme longevity in northern Italian
 908 centenarians reveal a complex remodelling of lipids, amino acids, and gut
 909 microbiota metabolism. *PloS One*, 8(3), e56564.

910 Collins, S. M., Bercik, P. (2009). The relationship between intestinal microbiota
 911 and the central nervous system in normal gastrointestinal function and disease.
 912 *Gastroenterology*, 136(6), 2003-2014.

913 Collins, S., Verdu, E., Denou, E., Bercik, P. (2009). The role of pathogenic
 914 microbes and commensal bacteria in irritable bowel syndrome. *Dig Dis*, 27(suppl
 915 1), 85-89.

916 Cotillard, A., Kennedy, S. P., Kong, L. C., *et al.* (2013). Dietary intervention
 917 impact on gut microbial gene richness. *Nature*, 500(7464):585-588.

918 Cox, L. M., Yamanishi, S., Sohn, J., Alekseyenko, A. V., Leung, J. M., Cho, I.,
 919 *et al.*, (2014). Altering the intestinal microbiota during a critical developmental
 920 window has lasting metabolic consequences. *Cell*, 158(4), 705–721.

921 Cummings, J. H., Macfarlane, G. T. (1997). Role of intestinal bacteria in nutrient
 922 metabolism. *JPEN J Parenter Enteral Nutr*, 21(6), 357–365.

923 Cusack, S., O'Toole, P. W. (2013). Diet, the gut microbiota and healthy ageing:
 924 How dietary modulation of the gut microbiota could transform the health of older
 925 populations. *Agro FOOD Ind Hi Tech*, 24(2), 54-57.

926 David, L. A, Maurice, C. F., Carmody, R. N., Gootenberg, D. B., Button, J. E.,
 927 Wolfe, B. E., Ling, A. V., Devlin, A. S., *et al.* (2014). Diet rapidly and
 928 reproducibly alters the human gut microbiome. *Nature*, 505(7484), 559–63.

929 De Filippo, C., Cavalieri, D., Di Paola, M., Ramazzotti, M., Poullet, J. B.,
 930 Massart, S., Collini, S., Pieraccini, G., Lionetti, P. (2010). Impact of diet in
 931 shaping gut microbiota revealed by a comparative study in children from Europe
 932 and rural Africa. *Proc Nantl Acad Sci USA*, 107(33), 14691–14696.

933 De La Cochetiere, M. F., Durand, T., Lepage, P., Bourreille, A., Galmiche, J. P.,
 934 Dore, J. (2005). Resilience of the dominant human fecal microbiota upon short-
 935 course challenge. *J Clin Microbiol*, 43(11), 5588-5592.

936 De Martinis, M., Franceschi, C., Monti, D., Ginaldi, L. (2005). Inflamm-ageing
 937 and lifelong antigenic load as major determinants of ageing rate and longevity.
 938 *FEBS Lett*, 579(10), 2035–2039.

939 Dethlefsen, L., Huse, S., Sogin, M. L., Relman, D. A. (2008). The pervasive
 940 effect of an antibiotic on the human gut microbiota, as revealed by deep 16S
 941 rRNA sequencing. *PLoS Biol*, 6(11), e280

942 Dethlefsen, L., Relman, D. A. (2011). Incomplete recovery and individualized
 943 responses of the human distal gut microbiota to repeated antibiotic perturbation.
 944 *Proc Nantl Acad Sci USA*, 108(suppl. 1), 4554-4561

945 DiGiulio, D. B., Romero, R., Amogan, H. P., Kusanovic, J. P., Bik, E. M.,
 946 Gotsch, F., Kim, C. J., Erez, O., Edwin, S., Relman, D. A. (2008). Microbial
 947 prevalence, diversity and abundance in amniotic fluid during preterm labour: a
 948 molecular and culture-based investigation. *PloS One*, 3(8), e3056.

949 Dominguez-Bello, M. G., Costello, E. K., Contreras, M., Magris, M., Hidalgo, G.,
 950 Fierer, N., Knight, R. (2010). Delivery mode shapes the acquisition and structure
 951 of the initial microbiota across multiple body habitats in newborns. *Proc Natl*
 952 *Acad Sci USA*, 107(26), 11971–5.

953 Duncan, S. H., Lobley, G. E., Holtrop, G., Ince, J., Johnstone, A M., Louis, P.,
 954 Flint, H. J. (2008). Human colonic microbiota associated with diet, obesity and
 955 weight loss. *Int J Obes (Lond)*, 32(11), 1720–1724.

956 Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent,
 957 M., Gill, S. R., Nelson, K. E., Relman, D. A. (2005). Diversity of the human
 958 intestinal microbial flora. *Science*, 308(5728), 1635-1638.

959 El Aidy, S., Derrien, M., Merrifield, C. A, Levenez, F., Dore, J., Boekschoten, M.
 960 V, Deker, J., Holmes, E., Zoetendal, E. G., *et al.* (2013). Gut bacteria-host
 961 metabolic interplay during conventionalisation of the mouse germfree colon.
 962 *ISME J* 7(4), 743–755.

963 El Aidy, S., Hooiveld, G., Tremaroli, V., Backhed, F., Kleerebezem, M. (2013).
 964 The gut microbiota and mucosal homeostasis: colonized at birth or at adulthood,
 965 does it matter? *Gut Microbes*, 4(2), 118-124.

966 Erridge, C., Attina, T., Spickett, C. M., Webb, D. J. (2007). A high-fat meal
 967 induces low-grade endotoxemia: evidence of a novel mechanism of postprandial
 968 inflammation. *Am J Clin Nutr*, 86(5), 1286-1292.

969 Evans, C. C., LePard, K. J., Kwak, J. W., Stancukas, M. C., Laskowski, S., *et al.*
 970 (2014). Exercise prevents weight gain and alters the gut microbiota in a mouse
 971 model of high fat diet-induced obesity. *PLoS One*, 9(3), e92193.

972 Fallani, M., Amarri, S., Uusijarvi, A., Adam, R., Khanna, S., Aguilera, M., Gil,
 973 A., Vieites, J. M., Norin, E. *et al.* (2011). Determinants of the human infant
 974 intestinal microbiota after the introduction of first complementary foods in infant
 975 samples from five European centres. *Microbiology*, 157(Pt 5), 1385–1392.

976 Fernandez, L., Langa, S., Martin, V., Maldonado, A., Jimenez, E., Martin, R.,
 977 Rodriguez, J. M. (2013). The human milk microbiota: origin and potential roles
 978 in health and disease. *Pharmacol Res*, 69(1), 1-10.

979 Forslund, K., Hildebrand, F., Nielsen, T., Falony, G. (2016). Disentangling the
 980 effects of type 2 diabetes and metformin on the human gut microbiota. *Nature*,
 981 528(7581):262-266.

982 Foxx-Orenstein, A. E., Chey, W. D. (2012). Manipulation of the gut microbiota
 983 as a novel treatment strategy for gastrointestinal disorders. *Am J Gastroenterol*
 984 *Suppl*, 1(1), 41–46.

985 Franceschi, C., Bonafe, M., Valensin, S., Benedictis, G. D. E. (2000). Inflamm-
 986 ageing: an evolutionary perspective on immunosenescence. *Ann NY Acad. Sci.*,
 987 (908), 244–254.

988 Frank, D. N., Amand, A. L. ST., Feldman, R. A, Boedeker, E. C., Harpaz, N.,
 989 Pace, N. R. (2007). Molecular-phylogenetic characterization of microbial
 990 community imbalances in human inflammatory bowel diseases. *Proc Natl Acad*
 991 *Sci USA*, 104(34), 13780–13785.

992 Garrido, D., Kim, J. H., German, J. B., Raybould, H. E., Mills, D. A. (2011).
 993 Oligosaccharide binding proteins from *Bifidobacterium longum* subsp. *infantis*
 994 reveal a preference for host glycans. *PLoS One*, 6(3), e17315.

995 Gummesson, A., Carlsson, L. M., Storlien, L. H., Backhed, F., Lundin, P.,
 996 Lofgren L., Stenlof, K., Lam, Y. Y., Fagerberg, B., Carlsson, B. (2011).
 997 Intestinal permeability is associated with visceral adiposity in healthy women.
 998 *Obesity(Silver Spring)*, 19(11), 2280-2282.

999 Grundy, S. M., Brewer, H. B. Jr, Cleeman, J. I., Smith, S. C., Lenfant, C. (2004).
 1000 Definition of metabolic syndrome: Report of the National Heart, Lung, and
 1001 Blood Institute/American Heart Association conference on scientific issues
 1002 related to definition. *Circulation*, 109(3), 433–438.

1003 Hamer, H. M., Jonkers, D., Venema, K., Vanhoutvin, S., Troost, F. J., Brummer,
 1004 R.-J. (2008). Review article: the role of butyrate on colonic function. *Aliment*
 1005 *Pharmacol Ther*, 27(2), 104–119.

1006 Harmsen, H. J., Wildeboer-Veloo, A. C., Raangs, G. C., Wagendorp, A. A., Klijn,
 1007 N., Bindels, J. G., Welling, G. W. (2000). Analysis of intestinal flora
 1008 development in breast-fed and formula-fed infants by using molecular
 1009 identification and detection methods. *J Pediatr Gastroenterol Nutr*, 30(1), 61-67.

1010 Hawkesworth, S., Moore, S. E., Fulford, A. J. C., Barclay, G. R., Darboe, A. A.,
 1011 Mark, H., Nyan, O. A., Prentice, A. M. (2013). Evidence for metabolic
 1012 endotoxemia in obese and diabetic Gambian women. *Nutr Diabetes*, 3, e83.

1013 Heinz, C., Mair, W. (2014). You are what you host: microbiome modulation of
 1014 the aging process. *Cell*, 156(3), 408-411.

1015 Hold, G. L., Pryde, S. E., Russell, V. J., Furrie, E., Flint, H. J. (2002).
 1016 Assessment of microbial diversity in human colonic samples by 16S rRNA
 1017 sequence analysis. *FEMS Microbiol Ecol*, 39(1), 33-39.

1018 Howcroft, T. K., Campisi, J., Louis, G. B., Smith, M. T., Wise, B., Wyss-Coray,
 1019 T., Augustine, A. D., McElhaney, J. E., Kohanski, R., Sierra, F. (2013). The role
 1020 of inflammation in age-related disease. *Aging*, 5(1), 84–93.

1021 Huang, P. L. (2009). A comprehensive definition for metabolic syndrome. *Dis*
 1022 *Model Mech*, 2(5-6), 231–237.

1023 Hunt, K. M., Foster, J. A., Forney, L. J., Schutte, U. M. E., Beck, D. L., Williams,
 1024 J. E., Collier, N. N., McGuire, M. K., McGuire, M. A. (2010). The human milk
 1025 microbiome: a potential influence on mammary health and bacterial colonization
 1026 of infant gut. *FASEB J*, 206.5.

1027 Huttenhower, C., Kostic, A. D., Xavier, R. J (2014). Inflammatory bowel disease
 1028 as a model for translating the microbiome. *Immunity*, 40(6), 843-854.

1029 Jakobsson, H. E., Jernberg, C., Andersson, A. F., Sjolund-Karlsson, M., Jansson,
 1030 J. K., Engstrand, L. (2010). Short-term antibiotic treatment has differing long-
 1031 term impacts on the human throat and gut microbiome. *PloS One*, 5(3), e9836.

1032 Jeffrey, I. B., O'Toole, P. W., Ohman, L., Claesson, M. J., Deane, J., Quigley, E.
 1033 M., Simren, M. (2012). An irritable bowel syndrome subtype defined by specie-
 1034 species alterations in faecal microbiota. *Gut*, 61(7), 997-1006.

1035 Jernberg, C., Lofmark, S., Edlund, C., Jansson, J. K. (2007). Long-term
 1036 ecological impacts of antibiotic administration on the human intestinal
 1037 microbiota. *ISME J*, 1(1), 56-66.

1038 Jimnez, E., Marin, M. L., Martin, R., Odriozola, J. M., Olivares, M., Xaus, J.,
1039 Fernandez, L., Rodriguez, J. M. (2008). Is meconium from healthy newborns
1040 actually sterile ? *Res. Microbiol*, 159(3), 187–193.

1041 Jost, T., Lacroix, C., Braegger, C. P., Chassard, C. (2012). New insights in gut
1042 microbiota establishment in healthy breast fed neonates. *PLOS One*, 7(8), e44595

1043 Kabeerdoss, J., Devi, R. S., Mary, R. R., Ramakrishna, B. S. (2012). Faecal
1044 microbiota composition in vegetarians: comparison with omnivores in a cohort
1045 of young women in southern India. *Br J Nutr*, 108(6), 953–957.

1046 Karlsson, F. H., Tremaroli, V., Nookaew, I., Bergstrom, G., Behre, C. J.,
1047 Fagerberg, B., Nielsen, J., Backhed, F. (2013). Gut metagenome in European
1048 women with normal, impaired and diabetic glucose control. *Nature*, 498(7452),
1049 99-103.

1050 Kassinen, A., Krogius-Kurikka, L., Makivuokko, H., Rinttila, T., Paulin, L.,
1051 Corander, J., Malinen, E., Apajalahti, J., Palva, A. (2007). The fecal microbiota
1052 of irritable bowel syndrome patients differs significantly from that of healthy
1053 subjects. *Gastroenterology*, 133(1), 24-33.

1054 Kim, G., Deepinder, F., Morales, W., Hwang, L., Weitsman, S., Chang, C.,
1055 Gunsalus, R., Pimentel, M. (2012). *Methanobrevibacter smithii* is the
1056 predominant methanogen in patients with constipation-predominant IBS and
1057 methane on breath. *Dig Dis Sci*, 57(12), 3213-3218.

1058 Kim, M.-S., Hwang, S.-S., Park, E.-J., Bae, J.-W. (2013). Strict vegetarian diet
1059 improves the risk factors associated with metabolic diseases by modulating gut

1060 microbiota and reducing intestinal inflammation. *Environ Microbiol Rep*, 5(5),
1061 765–75.

1062 King, T. S., Elia, M., Hunter, J. O. (1998). Abnormal colonic fermentation in
1063 irritable bowel syndrome. *Lancet*, 352(9135), 1187-1189.

1064 Koenig, J. E., Spor, A., Scalfone, N., Fricker, A. D., Stombaugh, J., Knight, R.,
1065 Angenent, L. T., Ley, R. E. (2011). Succession of microbial consortia in the
1066 developing infant gut microbiome. *Proc Nat Acad Sci U S A*, 108 (Suppl 1),
1067 4578–4585.

1068 Korpela, K., Flint, H. J., Johnstone, A. M., Lappi, J., Poutanen, K., Dewulf,
1069 E., Delzenne, N., de Vos, W. M., Salonen, A. (2014). Gut microbiota signatures
1070 predict host and microbiota responses to dietary interventions in obese
1071 individuals. *PloS One*, 9(6), e90702.

1072 Krogius-Kurikka, L., Lyra, A., Malinen, E., Aarnikunnas, J., Tuimala, J., Paulin,
1073 L., Makivuokko, H., *et al.* (2009). Microbial community analysis reveals high
1074 level phylogenetic alterations in the overall gastrointestinal microbiota of
1075 diarrhoea-predominant irritable bowel syndrome sufferers. *BMC Gastroenterol*,
1076 9(1), 95.

1077 Le Chatelier, E., Nielsen, T., Qin, J., *et al.* (2013). Richness of human gut
1078 microbiome correlates with metabolic markers. *Nature*, 500(7464):541-546.

1079 Lahti, L., Salojarvi, J., Salonen, A., Scheffer, M., de Vos, W. M. (2014). Tipping
1080 elements in the human intestinal ecosystem. *Nat Commun*, 5, 4344.

1081 Lambert, D. M., Muccioli, G. G. (2007). Endocannabinoids and related N-
 1082 acylethanolamines in the control of appetite and energy metabolism: emergence
 1083 of new molecular players. *Curr Opin Clin Nutr Metab Care*, 10(6), 735–744.

1084 La Rosa, P. S., Warner, B. B., Zhou, Y., Weinstock, G. M., Sodergren, Hall-
 1085 Moore, C. M., Stevens, H. J., *et al.* (2014). Patterned progression of bacterial
 1086 populations in the premature infant gut. *Proc Nat Acad Sci U S A*, 111(34),
 1087 12522-12527.

1088 Larsen, N., Vogensen, F. K., van den Berg, F. W. J., Nielsen, D. S., Andreasen,
 1089 A. S., Pedersen, B. K., Al-Saoud, W. A., *et al.* (2010). Gut microbiota in human
 1090 adults with type 2 diabetes differs from non-diabetic adults. *PloS One*, 5(2),
 1091 e9085.

1092 Lawley, T. D., Clare, S., Walker, A. W., Stares, M. D., Connor, T. R., Raisen, C.,
 1093 Goulding, D., Rad, R., Schreiber, F. *et al.* (2012). Targeted restoration of the
 1094 intestinal microbiota with a simple, defined bacteriotherapy resolves relapsing
 1095 *Clostridium difficile* disease in mice. *PLoS Pathog*, 8(10), e1002995.

1096 Lawley, T. D., Walker, A. W. (2013). Intestinal colonization resistance.
 1097 *Immunology*, 138(1), 1–11.

1098 Ley, R. E., Backhed, F., Turnbaugh, P., Lozupone, C. A, Knight, R. D., Gordon,
 1099 J. I. (2005). Obesity alters gut microbial ecology. *Proc Nat Acad Sci U S A*,
 1100 102(31), 11070–11075.

1101 Ley, R. E., Lozupone, C. A., Hamady, M., Knight, R., Jeffrey, I. (2009). Worlds
 1102 within worlds: evolution of the vertebrate gut microbiota. *Nat Rev Microbiol*,
 1103 6(10), 776–788.

1104 Ley, R. E., Turnbaugh, P. J., Klein, S., Gordon, J. I. (2006). Human gut microbes
1105 associated with obesity. *Nature*, 444, 1022-23.

1106 Liszt, K., Zwieler, J., Handschur, M., Hippe, B., Thaler, R., Haslberger, A. G.
1107 (2009). Characterization of bacteria, clostridia and Bacteroides in faeces of
1108 vegetarians using qPCR and PCR-DGGE fingerprinting. *Ann Nutr Metab*, 54(4),
1109 253–257.

1110 LoCasio, R. G., Desai, P., Sela, D. A., Weimer, B., Mils, D. A. (2010). Broad
1111 conservation of milk utilization genes in *Bifidobacterium longum* subsp. *infantis*
1112 as revealed by comparative genomic hybridization. *Appl Environ Microbiol*,
1113 76(22), 7373-7381.

1114 Longstreth, G. F., Thompson, W. G., Chey, W. D., Houghton, L. A., Mearin, F.,
1115 Spiller, R. C. (2006). Functional bowel disorders. *Gastroenterology*, 130(5),
1116 1480-1491.

1117 Louis, P., Flint, H. J. (2009). Diversity, metabolism and microbial ecology of
1118 butyrate-producing bacteria from the human large intestine. *FEMS Microbiol*
1119 *Lett*, 294(1), 1–8.

1120 Lozupone, C. A., Stombaugh, J. I., Gordon, J. I., Jansson, J. K., Knight, R.
1121 (2012). Diversity, stability, and resilience of the human gut microbiota. *Nature*,
1122 489(7415), 220-230.

1123 Macfarlane, G. T., Macfarlane, S. (2012). Bacteria, colonic fermentation, and
1124 gastrointestinal health. *J AOAC Int*, 95(1), 50-60.

1125 Mahowald, M. A., Rey, F. E., Seedorf, H., Turnbaugh, P. J., Fulton, R. S.,
 1126 Wollam, A., Shah, N., Wang, C., Magrini, V. *et al.*, (2009). Characterizing a
 1127 model human gut microbiota composed of members of its two dominant bacterial
 1128 phyla. *Proc Natl Acad Sci U S A*, 106(14), 5859-5864.

1129 Mai, V., Torrazza, R. M., Ukhanova, M., Wang, X., Sun, Y., Li, N., Shuster, J.,
 1130 Sharma, R., Hudak, M. L., Neu, J. (2013). Distortions in development of
 1131 intestinal microbiota associated with late onset sepsis in preterm infants. *PLOS*
 1132 *One*, 8(1), e52876.

1133 Malinen, E., Rinttilä, T., Kajander, K., Matto, J., Kassinen, A., Krogius, L.,
 1134 Korpela, R., Palva, A. (2005). Analysis of the fecal microbiota of irritable bowel
 1135 syndrome patients and healthy controls with real-time PCR. *Am J Gastroenterol*,
 1136 100(2), 378-382.

1137 Manichanh, C., Rigottier-Gois, L., Bonnaud, E., Gloux, K., Pelletier, E.,
 1138 Frangeul, L., Nalin, R., Jarrin, C. *et al.* (2006). Reduced diversity of faecal
 1139 microbiota in Crohn's disease revealed by a metagenomic approach. *Gut*, 55(2),
 1140 205–11.

1141 Martin, V., Maldonado-Barragan, A., Moles, L., Rodriguez-Banos, M., Campo,
 1142 R. D., Fernandez, L., Rodriguez, J. M., Jimenez, E. (2012). Sharing of bacterial
 1143 strains between breast milk and infant feces. *J Hum Lact*, 28(1), 36-44.

1144 Martin, R., Jimenez, E., Heilig, H., Fernandez, L., Marin, M., Zoetendal, G.,
 1145 Rodriguez, J. M. (2008). Isolation of bifidobacteria from breast milk and
 1146 assessment of the bifidobacterial population by PCR-denaturing gradient gel

1147 electrophoresis and quantitative real-time PCR. *Appl Environ Microbiol*, 75(4),
 1148 965-969.

1149 Martinez, I., Kim, J., Duffy, P. R., Schlegel, V. L., Walter, J. (2010). Resistant
 1150 starches types 2 and 4 have differential effects on the composition of the fecal
 1151 microbiota in human subjects. *PloS One*, 5(11), e15046.

1152 Matamoros, S., Gras-Leguen, C., Le Vacon, F., Potel, G., de La Cochetiere, M. F.
 1153 (2013). Development of intestinal microbiota in infants and its impact on health.
 1154 *Trends Microbiol*, 21(4), 167–173.

1155 Matsumoto, M., Inoue, R., Tsukahara, T., Ushida, K., Chiji, H., Matsubara, N.,
 1156 Hara, H. (2008). Voluntary running exercise alters microbiota composition and
 1157 increases n-butyrate concentration in the rat cecum. *Biosci Biotechnol Biochem*,
 1158 72(2), 572–576.

1159 Matto, J., Maunuksela, L., Kajander, K., Palva, A., Korpela, R., Kassinen, A.,
 1160 Saarela, M. (2005). Composition and temporal stability of gastrointestinal
 1161 microbiota in irritable bowel syndrome - a longitudinal study in IBS and control
 1162 subjects. *FEMS Immunol Med Microbiol*, 43(2), 213–222.

1163 Mouzaki, M., Comelli, E. M., Arendt, B. M., Bonengel, J., Fung, S. K., Fischer,
 1164 S. E., Allard, J. P. (2013). Intestinal microbiota in patients with nonalcoholic
 1165 fatty liver disease. *Hepatology*, 58(1), 120–127.

1166 Mshvildadze, M., Neu, J., Shuster, J., Theriaque, D., Li, N., Mai, V. (2010).
 1167 Intestinal microbial ecology in premature infants assessed with non-culture-based
 1168 techniques. *J Pediatr*, 156(1), 20-5.

1169 Muccioli, G. G., Naslain, D., Backhed, F., Reigstad, C. S., Lambert, D. M.,
1170 Delzenne, N. M., Cani, P. D. (2010). The endocannabinoid system links gut
1171 microbiota to adipogenesis. *Mol Syst Biol*, 6, 392.

1172 Muegge, B. D., Kuczynski, J., Knights, D., Clemente, J. C., Gonzalez, A.,
1173 Fontana, L., Henrissat, B., Knight, R., Gordon, J. I. (2011). Diet drives
1174 convergence in gut microbiome functions across mammalian phylogeny and
1175 within humans. *Science*, 332(6032), 970-974

1176 Mueller, S., Saunier, K., Hanisch, C., Norin, E., Alm, L., Midtvedt, T., Cresci, A.,
1177 Silvi, S., Orpianesi, C., *et al.* (2006). Differences in fecal microbiota in different
1178 European study populations in relation to age, gender, and country: a cross-
1179 sectional study. *Appl Environ Microbiol*, 72(2), 1027–33.

1180 Musso, G., Gambino, R., Cassader, M. (2011). Interactions between gut
1181 microbiota and host metabolism predisposing to obesity and diabetes. *Annu*
1182 *Review Med*, 62, 361–380.

1183 Nyangale, E., Mottram, D.S., Gibson, G.R. (2012). Gut microbial activity,
1184 implications for health and disease: The potential role of metabolic analysis. *J*
1185 *Proteome Res*, 11(12), 5573-5585.

1186 O'Toole, P. W., Claesson, M. J. (2010). Gut microbiota: Changes throughout the
1187 lifespan from infancy to elderly. *Intl Dairy J*, 20(4), 281–291.

1188 Palmer, C., Bik, E. M., DiGiulio, D. B., Relman, D. A., Brown, P. O. (2007).
1189 Development of the Human Infant Intestinal Microbiota. *PLoS Biol*, 5(7), e177.

1190 Pedron, T., Sansonetti, P. (2008). Commensals, bacterial pathogens and intestinal
1191 inflammation: an intriguing ménage à trois. *Cell Host Microbe*, 3(6), 344-347.

1192 Penders, J., Gerhold, K., Stobberingh, E. E., Thijs, C., Zimmermann, K., Lau, S.,
1193 Hamelmann, E. (2013). Establishment of the intestinal microbiota and its role for
1194 atopic dermatitis in early childhood. *J Allergy Clin Immunol*, 132(3), 601–607.

1195 Penders, J., Thijs, C., van den Brandt, P. A, Kummeling, I., Snijders, B., Stelma,
1196 F., Adams, H., van Ree, R., Stobberingh, E. E. (2007). Gut microbiota
1197 composition and development of atopic manifestations in infancy: the KOALA
1198 Birth Cohort Study. *Gut*, 56(5), 661–667.

1199 Penders, J, Thijs, C., Vink, C., Stelma, F. F., Snijders, B., Kummeling, I., van
1200 den Brandt, Stobberingh, E. E.. (2006). Factors influencing the composition of
1201 the intestinal microbiota in early infancy. *Paediatrics*, 118(2), 511-521.

1202 Penders, J, Vink, C., Driessen, C., London, N., Thijs, C., Stobberingh, E. E.
1203 (2005). Quantification of *Bifidobacterium* spp., *Escherichia coli* and *Clostridium*
1204 *difficile* in faecal samples of breast- fed and formula-fed infants by real time PCR.
1205 *FEMS Microbiol Lett*, 243(1), 141-147.

1206 Pepin,J., Saheb, N., Coulombe, M. A., Alary, M. E., Corriveau, M. P., Authier,
1207 S., Leblanc, M., Rivard, G., *et al.* (2005). Emergence of fluoroquinolones as the
1208 predominant risk factor for *Clostridium difficile*-associated diarrhea: a cohort
1209 study during an epidemic in Quebec. *Clin Infect Dis*, 41(9), 1254-60.

1210 Perez-Cobas, A. E., Gosalbes, M. J., Friedrichs, A., Knecht, H., Artacho, A.,
1211 Eismann, K., Moya, A. (2013). Gut microbiota disturbance during antibiotic
1212 therapy: a multi-omic approach. *Gut*, 62(11), 1591–601.

1213 Perez-Muñoz, M., E., Arrieta, M., C., Ramer-Tait, A., E., Walter, J. (2017) A
1214 critical assessment of the “sterile womb” and “in utero colonization” hypotheses:
1215 Implications for research on the pioneer infant microbiome. *Microbiome*, 5(1):1-
1216 19.

1217 Persaud, R., Azad, M. B., Konya, T., Guttman, D. S., Chari, R. S., Sears, M. R.,
1218 Becker, A. B., Scott, J. A., Kozyrskyj, A. L., the CHILD Study Investigators
1219 (2014). Impact of perinatal antibiotic exposure on the infant gut microbiota at
1220 one year of age. *Allergy Asthma Clin Immunol*, 10(Suppl 1), A31.

1221 Petrof, E. O., Gloor, G. B., Vanner, S. J., Weese, S. J., Carter, D., Daigneault, M.
1222 C., Brown, E. M., Schroeter, K., Allen-Vercoe, E. (2013). Stool substitute
1223 transplant therapy for the eradication of *Clostridium difficile* infection:
1224 “RePOOPulating” the gut. *Microbiome*, 1(1), 3.

1225 Qin, J., Li, Y., Cai, Z., Li, S., Zhu, J., Zhang, F., Liang, S., Zhang, W, Guan, Y.,
1226 Shen, D., Peng, Y. *et al.* (2012). A metagenome-wide association study of gut
1227 microbiota in type 2 diabetes. *Nature*, 490(7418), 55–60.

1228 Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K. S., Manichanh, C., *et al.*
1229 (2010). A human gut microbial gene catalogue established by metagenomic
1230 sequencing. *Nature*, 464(7285), 59–65.

1231 Rajilic-Stojanovic, M., Biagi, E., Heilig, H. G., Kajander, K., Kekkonen, R. A.,
1232 Tims, S., de Vos, W. M. (2011). Global and deep molecular analysis of
1233 microbiota signatures in fecal samples from patients with irritable bowel
1234 syndrome. *Gastroenterology*, 14(5), 1792-1801.

1235 Rajilic-Stojanovic, M., Heilig, H. G., Molenaar, D., Kajander, K., Surakka, A.,
 1236 Smidt, H., de Vos, W. M. (2009). Development and application of the human
 1237 intestinal tract chip, a phylogenetic microarray: analysis of universally conserved
 1238 phylotypes in the abundant microbiota of young and elderly adults. *Environ*
 1239 *Microbiol*, 11(7), 1736-1751.

1240 Rampelli, S., Candela, M., Turrone, S., Biagi, E., Collino, S., Franceschi, C.,
 1241 O'Toole, P.W., Brigidi, P. (2013). Functional metagenomic profiling of intestinal
 1242 microbiome in extreme ageing. *Aging*, 5(12), 902–912.

1243 Rautava, S., Luoto, R., Salminen, S., Isolauri, E. (2012). Microbial contact
 1244 during pregnancy, intestinal colonization and human disease. *Nat Rev*
 1245 *Gastroenterol Hepatol*, 9(10), 565–576.

1246 Rea, M.C., O'Sullivan, O., Shanahan, F., O'Toole, P. W., Stanton, C., Ross, R.
 1247 P., Hill, C. (2012). *Clostridium difficile* carriage in elderly subjects and
 1248 associated changes in the intestinal microbiota. *J Clin Microbiol*, 50(3), 867-875.

1249 Rehman, A., Lepage, P., Nolte, A., Hellmig, S., Schreiber, S., Ott, S. J. (2010).
 1250 Transcriptional activity of the dominant gut mucosal microbiota in chronic
 1251 inflammatory bowel disease patients. *J Med Microbiol*, 59(Pt 9), 1114–1122.

1252 Roger, L. C., Costabile, A., Holland, D. T., Hoyles, L., McCartney, A. L. (2010).
 1253 Examination of faecal *Bifidobacterium* populations in breast - and formula - fed
 1254 infants during the first 18 months of life. *Microbiology*, 156(11), 3329-3341.

1255 Rothschild, D., Weissbrod, O., Barkan, E., *et al.* (2018). Environmental factors
 1256 dominate over host genetics in shaping human gut microbiota composition.
 1257 *Nature*, 555(25973):210-215.

1258 Salminen, S., Gibson, G. R., McCartney, A. L., Isolauri, E. (2004). Influence of
 1259 mode of delivery on gut microbiota composition in seven year old children. *Gut*,
 1260 53(9), 1388-1389.

1261 Salminen, A., Huuskonen, J., Ojala, J., Kauppinen, A., Kaarniranta, K., Suuronen,
 1262 T. (2008). Activation of innate immunity system during aging: NF- κ B signalling
 1263 is the molecular culprit of inflamm-aging. *Ageing Res Rev*, 7(2), 83–105.

1264 Santos, A. R., Whorwell, P. J. (2014). Irritable bowel syndrome: the problem and
 1265 the problem of treating it - is there a role for probiotics? *Proc Nutr Soc*, 26, 1–7.

1266 Sekirov, I., Russell, S. L., Antunes, L. C. M., Finlay, B. B. (2010). Gut
 1267 microbiota in health and disease. *Physiol Rev*, 90(3), 859–904.

1268 Schwiertz, A., Taras, D., Schafer, K., Beijer, S., Bos, N. A, Donus, C., Hardt, P.
 1269 D. (2010). Microbiota and SCFA in lean and overweight healthy subjects.
 1270 *Obesity*, 18(1), 190–195.

1271 Schnorr, S. L., Candela, M., Rampelli, S., Centanni, M., Consolandi, C., Basaglia,
 1272 G., Turrioni, S., Biagi, E., *et al.* (2014). Gut microbiome of the Hadza hunter-
 1273 gatherers. *Nat Commun*, 5, 3654.

1274 Scott, K. P., Gratz, S. W., Sheridan, P. O., Flint, H. J., Duncan, S. H. (2013). The
 1275 influence of diet on the gut microbiota. *Pharmacol Res*, 69(1), 52–60

1276 Sela, D. A, Mills, D. A. (2010). Nursing our microbiota: molecular linkages
 1277 between bifidobacteria and milk oligosaccharides. *Trends Microbiol*, 18(7), 298–
 1278 307.

1279 Serino, M., Luche, E., Gres, S., Baylac, A., Bergé, M., Cenac, C., *et al.* (2012).
 1280 Metabolic adaptation to a high-fat diet is associated with a change in the gut
 1281 microbiota. *Gut*, 61(4), 543–553.

1282 Simoes, C. D., Maukonen, J., Kaprio, J., Rissanen, A., Pietilainen, K. H., Saarela,
 1283 M. (2013). Habitual dietary intake is associated with stool microbiota
 1284 composition in monozygotic twins. *J Nutr*, 143(4), 417–423.

1285 Simren, M., Barbara, G., Flint, H. J., Spiegel, B. M. R., Spiller, R. C., Vanner, S.,
 1286 Verdu, E. F., Whorwell, P. J., Zoetendal, E. G., Rome Foundation Committee
 1287 (2013). Intestinal microbiota in functional bowel disorders: a Rome foundation
 1288 report. *Gut*, 62(1), 159–176.

1289 Smith, P. M., Howitt, M. R., Panikov, N., Michaud, M., Gallini, C. A., Bohlooly-
 1290 Y, M., Glickman, J. N., Garrett, W. S. (2013). The microbial metabolites, short
 1291 chain fatty acids, regulate colonic Treg cell homeostasis. *Science*, 341(6145),
 1292 569-573.

1293 Soares, R. L. (2014). Irritable bowel syndrome: A clinical review. *World J*
 1294 *Gastroenterol*, 20(34), 12144-12160.

1295 Sokol, H., Pigneur, B., Watterlot, L., Lakhdari, O., Bermudez-Humaran, L. G.,
 1296 Gratadoux, J.-J., Langella, P. (2008). *Faecalibacterium prausnitzii* is an anti-
 1297 inflammatory commensal bacterium identified by gut microbiota analysis of
 1298 Crohn disease patients. *Proc Natl Acad Sci U S A*, 105(43), 16731–6.

1299 Sommer F., Backhed, F. (2013). The gut microbiota-masters of host development
 1300 and physiology. *Nat Rev Microbiol*, 11(4), 227-238.

1301 Soto, A., Martin, V., Jimenez, E., Mader, I., Rodriguez, J. M., Fernandez, L.
1302 (2014). Lactobacilli and bifidobacteria in human breast milk: influence of
1303 antibiotherapy and other host and clinical factors. *J Pediatr Gastroenterol Nutr*,
1304 59(1), 78-88.

1305 Steck, N., Mueller, K., Schemann, M., Haller, D. (2013). Republished: bacterial
1306 proteases in IBD and IBS. *Postgrad Med*, 89(1047), 25-33.

1307 Stephens, W. Z., Round, J. L. (2014). IgA targets the troublemakers. *Cell Host*
1308 *Microbe*. 16(3), 265-267.

1309 Stewart, C. J., Marrs, E. C. L., Magorrian, S., Nelson, A., Lanyon, C., Perry, J.
1310 D., Embleton, N. D., Cummings, S. P., Berrington, J. E. (2012). The preterm gut
1311 microbiota: changes associated with necrotizing enterocolitis and infection. *Acta*
1312 *Paediatr*, 101(11), 1121-1127.

1313 Swidsinski, A., Ladhoff, A., Pernthaler, A., Swidsinski, S., Loening-Baucke, V.,
1314 Orther, M., Weber, J., *et al.* (2002). Mucosal flora in inflammatory bowel disease.
1315 *Gastroenterol*, 122(1), 44-54

1316 Talley, N. J. (2008). Functional gastrointestinal disorders as a public health
1317 problem. *Neurogastroenterol Motil* , 20 (Suppl 1), 121–129.

1318 Tanaka, S., Kobayashi, T., Songjinda, P., Tateyama, A., Tsubouchi, M.,
1319 Kiyohara, C., Shirakawa, T., Sonomoto, K., Nakayama, J. (2009). Influence of
1320 antibiotic exposure in the early postnatal period on the development of intestinal
1321 microbiota. *FEMS Immunol Med Microbiol*, 56(1), 80-87.

1322 Tap, J., Mondot, S., Levenez, F., Pelletier, E., Caron, C., Furet, J. P., Ugarte, E.,
1323 Munoz-Tamayo, R., *et al.* (2009). Towards the human intestinal microbiota
1324 phylogenetic core. *Environl Microbiol*, 11(10), 2574–2584.

1325 Thiele, I., Heinken, A., Fleming, R. M. T. (2013). A systems biology approach to
1326 studying the role of microbes in human health. *Curr Opin Biotechnol*, 24(1), 4-
1327 12.

1328 Thorburn, A. N., Macia, L., Mackey, C. R. (2014). Diet, metabolites, and
1329 “Western-Lifestyle” inflammatory diseases. *Immunity*, 40(6), 833-842.

1330 Tiihonen, K., Ouwehand, A. C., Rautonen, N. (2010). Human intestinal
1331 microbiota and healthy ageing. *Ageing Res Rev*, 9(2), 107–116.

1332 Triantafyllou, K., Chang, C., Pimentel, M. (2014). Methanogens, methane and
1333 gastrointestinal motility. *J Neurogastroenterol Motil*, 20(1), 31–40.

1334 Turnbaugh, P. J., Hamady, M., Yatsunenko, T., Cantarel, B. L., Duncan, A., Ley,
1335 R. E., Sogin, M. L., *et al.* (2009). A core gut microbiome in obese and lean twins.
1336 *Nature*, 457(7228), 480–484.

1337 Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V., Mardis, E. R.,
1338 Gordon, J. I. (2006). An obesity-associated gut microbiome with increased
1339 capacity for energy harvest. *Nature*, 444(7122), 1027–1031.

1340 Turrioni, F., Peano, C., Pass, D. a, Foroni, E., Severgnini, M., Claesson, M. J.,
1341 Ventura, M. (2012). Diversity of bifidobacteria within the infant gut microbiota.
1342 *PloS One*, 7(5), e36957.

1343 Ventura, M., Turrone, F., Motherway, M. O., MacSarry, J., van Sinderen, D.
 1344 (2012). Host-microbiome interactions that facilitate gut colonization by
 1345 commensal bifidobacteria. *Trends Microbol*, 20(10), 467-476.

1346 Walker, A. W., Duncan, S. H., Louis, P., Flint, H. J. (2014). Phylogeny, culturing,
 1347 and metagenomics of the human gut microbiota. *Trends Microbiol*, 22(5), 267–
 1348 274.

1349 Walker, A. W., Ince, J., Duncan, S. H., Webster, L. M., Holtrop, G., Ze, X.,
 1350 Brown, D., Stares, M. D., *et al.* (2011). Dominant and diet-responsive groups of
 1351 bacteria within the human colonic microbiota. *ISME J.*, 5(2), 220–230.

1352 Wang, X., Heazlewood, S. P., Krause, D. O., Florin, T. H. (2003). Molecular
 1353 characterization of the microbial species that colonize human ileal and colonic
 1354 mucosa by using 16S rDNA sequence analysis. *J Appl Microbiol*, 95(3), 508-520.

1355 Windey, K., De Preter, V., Verbeke, K. (2012). Relevance of protein
 1356 fermentation to gut health. *Mol Nutr Food Res*, 56(1), 184–96.

1357 Winter, S. E., Thiennimitr, P., Winter, M. G., Butler, B. P., Huseby, D. L.,
 1358 Crawford, R. W., Russell, J. M. *et al.* (2010). Gut inflammation provides a
 1359 respiratory electron acceptor for Salmonella. *Nature*, 467(7314), 426–429.

1360 Wong, J. M., Jenkins, D. J. (2007). Carbohydrate digestibility and metabolic
 1361 effects. *J Nutr*, 137(11 Suppl), 2539S-2546S.

1362 Woodmansey, E. J. (2007). Intestinal bacteria and ageing. *J Appl Microbiol*,
 1363 102(5), 1178–1186.

1364 Wrzosek, L., Miquel, S., Noordine, M.-L., Bouet, S., Chevalier-Curt, M. J.,
 1365 Robert, V., Philippe, C., *et al.* (2013). *Bacteroides thetaiotaomicron* and
 1366 *Faecalibacterium prausnitzii* influence the production of mucus glycans and the
 1367 development of goblet cells in the colonic epithelium of a gnotobiotic model
 1368 rodent. *BMC Biol*, 11, 61.

1369 Wu, G. D., Chen, J., Hoffmann, C., Bittinger, K., Chen, Y. Y., Keilbaugh, S. A.,
 1370 Bewtra, M., Knights, D., Walters, W. A. *et al.* (2011). Linking long-term dietary
 1371 patterns with gut microbial enterotypes. *Science*, 334(6052), 105–108.

1372 Wu, H., Esteve, E., Tremaroli, V., Khan, M. T., Caesar, R., Manneras-Holm, L.,
 1373 Stahlman, M., *et al.* (2017). Metformin alters the gut microbiome of individuals
 1374 with treatment-naïve type 2 diabetes, contributing to the therapeutic effects of the
 1375 drug. *Nat Med*, 23(7):850-858.

1376 Yatsunenko, T., Rey, F. E., Manary, M. J., Trehan, I., Dominguez-Bello, M. G.,
 1377 Contreras, M., Magris, M., Hidalgo, G. *et al.* (2012). Human gut microbiome
 1378 viewed across age and geography. *Nature*, 486(7402), 222–227.

1379 Zhang, C., Zhang, M., Wang, S., Han, R., Cao, Y., Hua, W., Mao, Y., Zhang, X.,
 1380 Pang, X. *et al.*, 2010. Interactions between gut microbiota, host genetics and diet
 1381 relevant to development of metabolic syndromes in mice. *ISME J*, 4(2), 232-241

1382 Zhu, L., Baker, S. S., Gill, C., Liu, W., Alkhouri, R., Baker, R. D., Gill, S. R.
 1383 (2013). Characterization of gut microbiomes in nonalcoholic steatohepatitis
 1384 (NASH) patients: a connection between endogenous alcohol and NASH.
 1385 *Hepatology*, 57(2), 601–9.

1386 Zivkovic, A. M., German, J. B., Lebrilla, C. B., Mills, D. A. (2011). Human milk
1387 glycobiome and its impact on the infant gastrointestinal microbiota. *Proc Natl*
1388 *Acad Sci U S A*, 108 (Suppl 1), 4653–4658.

1389 Zwielehner, J., Liszt, K., Handschur, M., Lassl, C., Lapin, A., Haslberger, A. G.
1390 (2009). Combined PCR-DGGE fingerprinting and quantitative-PCR indicates
1391 shifts in fecal population sizes and diversity of Bacteroides, bifidobacteria and
1392 *Clostridium* cluster IV in institutionalized elderly. *Exp Gerontol*, 44(6-7), 440–
1393 446.

1394 **1.2 The gut microbiota is a modifiable factor for maintaining** 1395 **health**

1396 **1.2.1 Introduction**

1397 In the previous section I reviewed the factors that shape the human gut microbiome
1398 throughout life and I discussed how GIT and extra-intestinal disease, and ageing
1399 affect and are affected by the gut microbiota. The colonisation process of the GIT
1400 tract begins shortly after birth; the first colonisers are facultative anaerobes
1401 originating from vaginal or skin microbiota depending on delivery mode, and are
1402 gradually succeeded by anaerobic microorganisms selected by host genetics, diet and
1403 the natural environment of the individual, including other people such as family
1404 members (Messer *et al.*, 2017). The gut microbiota of individuals can be viewed as
1405 part of a wider “local metacommunity” due to the microbial exchange that occurs
1406 among individuals and with their environment (Adair and Douglas, 2017).

1407 Although host-associated factors such as immunity and GIT physiology remain
1408 important (Kurilshikov *et al.*, 2017), environment is emerging as the most
1409 determinant one in the long-term shaping of the microbiome. Recently, Rothschild *et*
1410 *al.* (2018) showed that in spite of some degree of heritability, the gut microbiome is
1411 largely shaped by external environmental factors that prevail on host traits.

1412 Studies on the gut microbiota of urban and pre-agricultural modern populations have
1413 shown the significant impact of the environment (that is, an array of environmental
1414 factors including diet and life-style) on the shaping of the gut microbiota (reviewed
1415 in Mancabelli *et al.*, 2017). A clear example of environmental impact (available diet,
1416 microbial communities of their habitat) on the microbiota is that of the microbiota
1417 (gut, skin, oral) of the highly isolated Yanomami Ameridians (Clemente *et al.*, 2015).

1418 Isolation and lack of communication with external microbial communities
1419 contributed to the retention of the compositional robustness of the Ameridians
1420 microbiome which is not only the richest in diversity and functions so far studied-
1421 compared to both Westernised and other non-Westernised cohorts, but also one
1422 demonstrating little inter-individual variability (Clemente *et al.*, 2015).

1423 As discussed in Chapter 1.1., habitual dietary patterns impact the individual's
1424 longitudinal microbiota compositional profile whereas dietary shifts lead to
1425 reproducible microbiota responses (Flint *et al.*, 2017). For example, O'Keefe *et al.*
1426 (2015) showed that a 2-weeks switch of habitual diet between African Americans
1427 that were in high CRC risk due to their high-fat/low fibre diet, and low cancer risk
1428 rural Africans regularly consuming a low-fat/high-fibre diet, resulted in changes
1429 associated with the switched diets in both the gut microbiota composition and
1430 metabolome, and in colon mucosa CRC biomarkers.

1431 The association of the gut microbiota and dysbiosis with a plethora of disease that
1432 span all aspects of human physiology (reviewed in Chapter 1.1), and the
1433 responsiveness of the microbiota to external stimuli such as diet, makes the gut
1434 microbiota a promising therapeutic target (Levy *et al.*, 2017). Importantly, recent
1435 studies on animal models and humans have offered more evidence for the association
1436 of dysbiotic microbiota with ageing (Jackson *et al.*, 2016; Li *et al.*, 2016;
1437 Thevaranjan *et al.*, 2017) Therefore, gut microbiota modulation is a promising
1438 strategy towards ameliorating disease and also improving the life of the elderly in
1439 whom gut microbiota compositional and metabolic changes correlate with age-
1440 related conditions and co-morbidity (Duncan and Flint, 2013). In the following
1441 sections I will review the gut microbiota modulation approaches used to date.

1442 **1.2.2 Prebiotics, Probiotics, and next-generation approaches**

1443 The term prebiotic was first coined two decades ago and its definition has been
1444 repeatedly updated in order to encompass and reflect our increasing understanding of
1445 how the interplay between diet and gut microbiota affects host health (Bindels *et al.*
1446 2015). Prebiotics have moved from being a “non-digestible food ingredient that
1447 beneficially affects the host by selectively stimulating the growth and/or activity of
1448 one or a limited number of bacteria in the colon, and thus improves host health”
1449 described by Gibson and Roberfroid (1995) to a “substrate that is selectively utilized
1450 by host microorganisms conferring a health benefit” (Gibson *et al.*, 2017). This latest
1451 definition of the term prebiotic brought a consensus to the various definition updates
1452 of the last 20 years. According to this definition, selectivity is important and not
1453 restricted to bifidobacteria and lactobacilli that have been extensively used and
1454 studied as the main prebiotic targets (Turrone *et al.*, 2014). Importantly, a prebiotic
1455 may act on body parts other than the GIT that are niches of microorganisms and
1456 microbiota-mediated health effects must be demonstrated in the relevant host
1457 (humans or other animals) (Gibson *et al.*, 2017). If some evidence of causality is
1458 provided between administration and health effect, other non-carbohydrate substrates
1459 may qualify as prebiotics (Gibson *et al.*, 2017).

1460 Based on the existing scientific evidence, fructo-oligosaccharides (FOS) and galacto-
1461 oligosaccharides (GOS) are considered the substrates with most supportive data to fit
1462 the description of prebiotics whereas other oligosaccharides (OS) including human
1463 and bovine milk OS and other substrates such as dietary fibre, phenolic compounds,
1464 conjugated linoleic acid (CLA) and polyunsaturated fatty acids (PUFA) are prebiotic
1465 candidates but further research is required in order to confirm the prebiotic activity
1466 (Gibson *et al.*, 2017). The health benefits of prebiotic administration is likely to be

1467 mediated through microbial metabolite release and homeostatic signalling as a result
1468 of the selective prebiotic fermentation by target microorganisms (Gibson *et al.*,
1469 2017). The selectivity is relevant to specialised bacterial hydrolases for complex
1470 carbohydrates and mechanisms for substrate transfer into the cytoplasm which has
1471 been extensively described for the prototypical prebiotic taxa lactobacillus and
1472 bifidobacteria and the metabolism of OS (Goh and Klaenhammer, 2015).

1473 Probiotics are organisms that when administered alive and at a certain dose confer a
1474 health benefit to the recipient organism (Gareau *et al.*, 210). As mentioned before,
1475 bifidobacteria and lactobacilli are model probiotics and this can be attributed to
1476 certain aspects of their metabolism related to host colonisation and adaptation, such
1477 as dietary polysaccharide degradation, host-mucus utilization and bile salt hydrolysis
1478 (Turroni *et al.*, 2014). Certain physiological aspects such as the ability to survive
1479 acidic conditions and bile salt exposure in the GIT after administration, and oxygen
1480 tolerance, make the typical probiotics bifidobacteria and lactobacilli technologically
1481 manageable, partly explaining why probiotic research and development has focused
1482 on these taxa (Duncan and Flint, 2013). Importantly, the dominant notion that
1483 probiotic activity is restricted to certain strains within a taxon is evolving: in spite
1484 the fact that some strains may not have been thoroughly tested in human trials,
1485 minimum probiotic potential is being recognised in these strains due to the fact that
1486 they belong in well-established probiotic species and they demonstrate certain
1487 species-wide characteristics related to probiotic activity (Sanders *et al.*, 2018). Such
1488 characteristics are cell-wall structure including mucin-binding proteins, pili and S-
1489 layer proteins, exopolysaccharides, complex polysaccharide metabolic pathways,
1490 bile salt hydrolases and various primary and secondary metabolites such as SCFAs
1491 and AA (reviewed in Lebeer *et al.*, 2018; Sanders *et al.*, 2018).

1492 Probiotics may contribute to health through various ways. The integrity of the gut
1493 barrier is essential for host homeostasis (Köning *et al.*, 2016) and administration of
1494 probiotic *Lactobacillus* strains has been reported to improve tight junctions and
1495 barrier function *in vitro*, in murine models and humans (Karczewski *et al.*, 2010;
1496 Miyauchi *et al.*, 2012; Mujagic *et al.*, 2017; Paveljšek *et al.*, 2018). The SCFA
1497 butyrate is essential for gut barrier function and immunity, as discussed in previous
1498 sections, and probiotic administration can contribute to its production in the colon.
1499 The metabolites lactate and acetate produced by prototypical probiotics such as
1500 strains of bifidobacteria, can serve as substrates in cross-feeding interactions with
1501 butyrate producing colonic bacteria (reviewed in Rivière *et al.*, 2016). Probiotics can
1502 regulate host homeostasis through microbial-associated molecular patterns (MAMPs)
1503 recognised by host receptors (e.g. Toll-like receptors) and through secreted
1504 molecules triggering immunity host responses like secretion of anti-inflammatory
1505 cytokines (van Baarlen *et al.*, 2013; Sanders *et al.*, 2018). Importantly, these
1506 responses vary and are defined by inter-individual genetic differences and the
1507 probiotic strain (van Baarlen *et al.*, 2013; Sanders *et al.*, 2018). Probiotics may
1508 contribute to regulation of metabolism (Simon *et al.*, 2015) and may improve
1509 cognition through eliciting neurotransmitter and anti-inflammatory cytokine release,
1510 by being involved in γ -aminobutyric acid (GABA) and tryptophan regulation and in
1511 the regulation of the hypothalamic-pituitary-adrenal (HPA) axis among other
1512 mechanisms (reviewed in Sarkar *et al.*, 2016).

1513 ***Next-generation probiotics:*** Lactobacilli and bifidobacteria are the model probiotic
1514 organisms that research, regulatory authorities and industry have focused on to date
1515 (Duncan and Flint, 2013). Other bacteria such as *E. coli*, *Bacillus* spp., various
1516 enterococci, *Weissella* spp. and the fungus *Saccharomyces* have been also used as

1517 probiotics (O'Toole *et al.*, 2017). Importantly, next-generation sequencing and
1518 metagenomics that have enabled a better understanding of the role of certain GIT
1519 bacterial consortia in health and disease (Gilbert *et al.*, 2016), and the latest
1520 culturomics interest that has enlarged the list of isolated and routinely maintained in
1521 the laboratory GIT strict anaerobes (Lagier *et al.*, 2015), are contributing towards the
1522 development of novel probiotics.

1523 Candidate probiotics can be strictly anaerobic health-relevant taxa that are dominant
1524 or subdominant members of the “healthy” gut microbiota, as opposed to the
1525 traditionally used probiotics that do not reach dominant abundances in the healthy
1526 gut, and have a record of reduced abundance in certain disease (Duncan and Flint,
1527 2013). Next-generation probiotics must comply with the probiotic definition and the
1528 traditional regulatory requirements for probiotics, that is, safety record and a well-
1529 established pre-clinical and clinical record for the health-benefit conferred to the host
1530 by its administration (O'Toole *et al.*, 2017). *Bacteroides fragilis* and *B. xylanisolvens*,
1531 *F. prausnitzii*, *A. muciniphila*, and *Clostridium butyricum* are candidates for next-
1532 generation probiotics (NGPs) (reviewed in O'Toole *et al.*, 2017). Importantly, given
1533 the effort to develop NGPs based on gut microbiota members for which significantly
1534 reduced abundances have a causal link to targeted disease, NGPs or live
1535 bacteriotherapeutics, including isolated bacteria or bacterial consortia, may be likely
1536 to be regulated and delivered as pharmaceutical products rather than food products as
1537 the traditional probiotics (O'Toole *et al.*, 2017).

1538 **1.2.3 Live bacteriotherapy alternatives to FMT**

1539 ***Faecal Microbiota Transplantations:*** Faecal microbiota transplantation (FMT) is
1540 the gastrointestinal administration of faecal material in suspension from a donor to a

1541 recipient, aiming to confer a health benefit by altering the recipient's gut microbiota
1542 composition and potentially eliminating pathogenic colonization (Gupta *et al.*, 2016).
1543 Faecal microbiota transplantation has been successfully used to treat *Clostridium*
1544 *difficile* infection (CDI) and it is recommended for clinical practice that FMT be
1545 used in relapsing CDI when antibiotic treatment fails and when the risk of morbidity
1546 is prevalent (Gupta *et al.*, 2016). *Clostridium difficile* exploits the inflammatory state
1547 of the "dysbiotic" GIT often but not exclusively related to antibiotics exposure, in
1548 order to colonize the colon either asymptotically or causing diarrhoea of variant
1549 severity due to toxin release that confers an advantage to the pathogen against
1550 commensals (Hryckowian *et al.*, 2017). Importantly, CDI correlates to reduced
1551 phylogenetic diversity in the gut microbiota as observed in hospitalized CDI patients
1552 (Vincent *et al.*, 2013).

1553 Faecal microbiota transplantation introduces GIT commensals that compete for
1554 nutrients and niche with pathogens (Kelly *et al.*, 2015). Increase in the post-FMT
1555 phylogenetic diversity of the recipient's gut microbiota plays a significant role in *C.*
1556 *difficile* colonisation resistance and CDI resolution (Schubert *et al.*, 2015; Khanna *et*
1557 *al.*, 2017). Increase in the conversion of primary bile salts to secondary and in the
1558 abundance of relevant bacterial taxa involved in the bile salts metabolism such as
1559 *Clostridium scindens* of the Lachnospiraceae in the recipient faecal microbiota
1560 correlates with resolution of CDI (Buffie *et al.*, 2015; Staley *et al.*, 2016).
1561 Introduction of gut microbiota commensals that are able to utilize mucosa-derived
1562 sugars like sialic acids is reported as another mechanism of CDI resolution through
1563 FMT (Ng *et al.*, 2013). Antimicrobials released by members of the "healthy" donor
1564 faecal microbiota and the increase in abundance of butyrate-producing bacteria are

1565 other routes through which FMT may ameliorate CDI (Rea *et al.*, 2011; Antharam *et*
1566 *al.*, 2013).

1567 Given that antibiotic exposure may lead to reduced resilience to
1568 pathogens/pathobionts colonisation and expansion in the GIT of taxa such as *C.*
1569 *difficile*, enterococci, *Klebsiella* and *E. coli* (Lewis *et al.*, 2015), and the fact that
1570 other than *C. difficile* bacteria such as *Clostridium perfringens* and *Staphylococcus*
1571 *aureus* are associated with antibiotic-associated diarrhoea (AAD) (Larcombe *et al.*,
1572 2016), FMT could be efficient in treating AAD of various bacterial aetiology.
1573 Importantly, it is postulated that the therapeutic potential of FMTs can reach beyond
1574 the treatment of AAD and it can be extrapolated in other disease where dysbiosis is
1575 strongly associated with the condition. For example, faecal microbiota
1576 transplantation has been used to treat UC and CD (Moayyedi *et al.*, 2015) and FMT
1577 from lean to obese individuals resulted in amelioration of insulin resistance (Vrieze
1578 *et al.*, 2012; He *et al.*, 2017). In spite of the promising results from recent case
1579 studies of FMT treatment of conditions like IBD and metabolic disease, and contrary
1580 to CDI FMT treatment for which some scientific consensus exists allowing for
1581 recommendations and guidelines to clinicians, more research is necessary to
1582 establish similar workflow for other conditions such as IBD, IBS and metabolic
1583 disease (Cammarota *et al.*, 2017).

1584 Faecal microbiota transplantation is highly efficient in the treatment of recurrent CDI
1585 and holds some promise for the treatment of other dysbiosis-related conditions.
1586 Importantly, adverse outcomes have been reported for FMT including IBD flare and
1587 aspiration, and the long-term effect of FMT on the metabolic status of the recipient is
1588 yet unknown (Kelly *et al.*, 2015). There is scientific interest in refining the FMT

1589 approach in order for its implementation to be more clinician- and patient- friendly,
1590 safer and more standardized (Cammarota *et al.*, 2017). Importantly, research on how
1591 donor strains establish in the recipient gut microbiota environment is pointing
1592 towards the development of defined gut microbiota consortia that will allow for a
1593 highly personalised therapeutic strategy where recipient GIT ecology and immunity
1594 are taken into consideration for the development of the therapeutic consortium (Li *et*
1595 *al.*, 2016).

1596 ***Examples of live bacteriotherapy promoting health:*** Lawley *et al.* (2012)
1597 demonstrated in a murine model of recurrent CDI that a cocktail of six faecal
1598 bacteria (MixB) isolated from healthy mice was as efficient in resolving infection as
1599 FMT from healthy to diseased mice. The MixB was composed of the taxa
1600 *Staphylococcus warneri*, *Enterococcus hirae*, *Lactobacillus reuteri* and the newly
1601 then isolated *Anaerostipes* sp., *Bacteroides* sp. and *Enterorhabdus* sp. that were
1602 isolated from faecal samples used for the comparison FMT. The study underlined the
1603 importance of the phylogenetic diversity of the bacterial combination in resolving
1604 CDI and restoring the murine faecal microbiota to pre-infection composition and
1605 further showed that a mix of *Bacteroides/Lactobacillus* representing a probiotic-
1606 based treatment was not able to restore dysbiosis and resolve infection. Using a
1607 murine models Atarashi *et al.* (2013) isolated 17 strains from a human faecal
1608 microbiota donor that were able to induce Treg cell proliferation and anti-
1609 inflammatory components when introduce in germ-free mice. These strains that
1610 belonged to *Clostridium* clusters XIVa, IV and XVIII were efficient in attenuating
1611 inflammation in a chemically induced colitis murine model (Atarashi *et al.*, 2013).

1612 Petrof *et al.* (2013) introduced the “RePOOPulate” stool substitute that was used to
1613 treat refractory CDI in two patients with a highly infectious *C. difficile* strain. The
1614 bacterial mix consisted of 33 strains isolated from the faecal sample of a healthy
1615 adult and belonged to various phylogenetic groups that are abundant in the gut
1616 microbiota (Petrof *et al.*, 2013). Six months after the administration of
1617 “RePOOPulate” stool the patients were free of CDI symptoms and sequences (as
1618 revealed by 16S rRNA gene sequencing) not abundant pre-treatment were still
1619 present post-treatment suggesting a perseverance of the introduced bacteria in the
1620 recipient microbiota (Petrof *et al.*, 2013). This consortium referred to as microbial
1621 ecosystem therapeutic-1 (MET-1) was subsequently used in a murine model of
1622 *Salmonella typhimurium* infection (Martz *et al.*, 2015). MET-1 could not resolve
1623 infection but was found to be protective of tight-junctions activity which prevented
1624 the pathogen from establishing systemic infection (Martz *et al.*, 2015). The microbial
1625 ecosystem therapeutic-2 (MET-2) was developed from 33 strains isolated from the
1626 faeces of a healthy donor representing the minimal gut microbiota of the derivative
1627 donor (Yen *et al.*, 2015) and is currently being used in a clinical trial for the
1628 resolution of refractory CDI
1629 (<https://clinicaltrials.gov/ct2/show/study/NCT02865616>). In another recent clinical
1630 trial, Khanna *et al.* (2016) successfully treated CDI patients with SER-109, a
1631 therapeutic mix of encapsulated spores from 50 spore-forming Firmicutes species
1632 isolated from healthy donors.

1633 **1.2.4 *In vitro* and *in vivo* models for the development of gut microbiota**

1634 **therapeutics**

1635 *In vitro* GIT models and *in vivo* animal models are facilitating research on the
1636 therapeutic manipulation of the gut microbiota before applying these therapeutic
1637 approaches in clinical trials.

1638 ***In vitro* models:** Single- and multi-stage chemostats have been used for decades for
1639 temperature and pH controlled anaerobic faecal fermentations of selected substrates
1640 allowing for the simulation and study of basic aspects of faecal microbiota metabolic
1641 activity (Allison *et al.*, 1989; Macfarlane *et al.*, 1989). Model design varies from a
1642 single stage chemostat operating as batch or continuously, to multistage
1643 interconnected chemostats that operate in a continuous flow, simulating one or
1644 various parts from proximal to distal colon depending on the set pH of the different
1645 vessels (Payne *et al.*, 2012). More advanced continuous models such as SHIME and
1646 TIM aim at incorporating the host digestion factor (Payne *et al.*, 2012). TIM-2 was
1647 developed based on the stomach and small intestine TNO TIM-1 *in vitro* GIT model,
1648 and is a computer-controlled model for the large intestine that can simulate
1649 peristaltic movements and dialysis (reviewed by Venema, K., 2015). The simulator
1650 of the human intestinal microbial ecosystem SHIME is composed of five vessels
1651 mimicking the GIT from the stomach to the colon parts (reviewed by Van de Wiele
1652 *et al.*, 2015). Other *in vitro* models that are being used are the 4-vessel simulator of
1653 colon parts EnteroMix and the Lacroix model that allows for better growth of both
1654 lumen-living and attached to surfaces microbiota (reviewed in Venema and Van den
1655 Abbeele, 2013).

1656 *In vitro* models present inherent limitations such as the lack of the host immune and
1657 metabolic responses, the lack of epithelial cell barriers and mucin adhesion sites, and

1658 the lack of the high level of compartmentalisation that is achieved in the host GIT
1659 (Macfarlane and Macfarlane, 2007). Researchers have developed various *in vitro*
1660 model set-ups aiming at tackling these limitations; for example the multistage
1661 systems that have been developed in order to simulate various GIT tract parts as
1662 discussed previously. The introduction of mucin surfaces in two-stage systems
1663 offered an attachment site for sessile faecal bacteria such as *Bacteroides fragilis*-
1664 related taxa, clostridia and enterobacteria (Macfarlane *et al.*, 2005). Cinquin *et al.*
1665 (2006) applied a faecal microbiota immobilization technique in order to prevent
1666 wash-out of faecal microbiota in a three-stage continuous system. Modifications of
1667 the SHIME system allowed for incorporating a mucin environment (M-SHIME) that
1668 deterred wash-out of sessile members of the faecal microbiota such as *Clostridium*
1669 cluster XIVa belonging taxa leading to more *in-vivo* relevant conditions (Van den
1670 Abbeele *et al.*, 2012; Van den Abbeele *et al.*, 2013). In a more sophisticated set-up,
1671 SHIME was combined with a platform of host cells and microbiota interaction (HMI)
1672 (Marzorati *et al.*, 2014).

1673 In spite of the limitations, *in vitro* systems allow for a relatively low-cost,
1674 straightforward and high-throughput approach to study the effect of various
1675 substrates from candidate probiotics and prebiotics to novel drugs on the
1676 compositional and metabolic profile of the faecal microbiota deriving from healthy
1677 or diseased donors (Macfarlane and Macfarlane, 2007). The compositional effect on
1678 the faecal microbiota is analysed with various molecular methods such as 16S rRNA
1679 gene next-generation sequencing, qPCR and fluorescent *in situ* hybridisation (FISH)
1680 and analytical techniques such as HPLC are being used for SCFA production.

1681 For example, Van den Abbeele *et al.* (2013) compared the effect of long-chain
1682 arabinoxylan (LC-AX) and inulin on the human faecal microbiota in SHIME and
1683 TIM and reported that in both systems LC-AX demonstrated similar to inulin
1684 prebiotic effects on distinct bifidobacteria population and SCFA production.
1685 Compared to the FOS faecal fermentation pattern that was utilized in the simulated
1686 proximal colon area, arabinogalactan (AG) was available for fermentation in distal
1687 parts of the simulated colon in the SHIME system revealing a potential role of AG in
1688 reducing the distal colonic pH by increasing carbohydrate fermentation in the distal
1689 colon and promoting the growth of butyrate-producers such as *F. prausnitzii*
1690 (Terpend *et al.*, 2013).

1691 Chung *et al.* (2016) using a three-vessel *in vitro* system with variant fixed-pH
1692 conditions showed that the prebiotic effect of non-digestible substrates like pectin
1693 and inulin is determined by the faecal microbiota inter-individual compositional
1694 variations, the fluctuations in pH that naturally occur in the colon as opposed to the
1695 stable conditions of the simulated colonic fermentations, and that responses to
1696 substrates cannot be easily extrapolated to the whole taxonomic structure based on
1697 individual taxon-members responsiveness. Poeker *et al.* (2018) investigated the
1698 prebiotic effect of the dietary fibres β -glucan, α -galacto-oligosaccharides (α -GOS)
1699 and xylo-oligosaccharides (XOS) on the faecal microbiota of healthy individuals
1700 using a three-vessel continuous system (PolyFermS). They reported that the *in vitro*
1701 response of the faecal microbiota was largely dependent on the inter-individual
1702 variations of the baseline microbiota and proposed that the metabolic responses of
1703 the microbiota such as the SCFA production can be the most comparable measure of
1704 the prebiotic potential of test substrates on microbiotas from different subjects
1705 (Poeker *et al.*, 2018).

1706 Other potentially prebiotic substrates tested in *in vitro* systems are wheat dextrin, α -
1707 gluco-oligosaccharides, fructo-, galacto- and iso- malto- oligosaccharides, lactulose
1708 and lactosucrose (Hobden *et al.*, 2013; Sarbini *et al.*, 2013; Takagi *et al.*, 2016), as
1709 well as food ingredients such as whey, fruits and phenolic compounds (Sanchez-
1710 Patan *et al.*, 2015; Koutsos *et al.* 2017; Sanchez-Moya *et al.*, 2017). Probiotics have
1711 been tested in *in vitro* systems alone or combined with prebiotic substrates and in
1712 combination with pathobionts in pre-clinical tests investigating the potential of
1713 probiotics to prevent pathogen establishment in the GIT tract (Martinez *et al.*, 2013;
1714 Cardarelli *et al.*, 2016; van den Abbeele *et al.*, 2016).

1715 *In vitro* systems simulating colonic fermentation in studies of prebiotic and probiotic
1716 modulation of the faecal microbiota of healthy older subjects have been used but to a
1717 limited extent (Likotrafiti *et al.*, 2014; Liu *et al.*, 2016). Fehlbaum *et al.* (2015)
1718 proposed the use of the adjusted PolyFermS multi-stage *in vitro* system as a
1719 reproducible set-up for the study of various factors such as diet, on the faecal
1720 microbiota of older donors. The PolyFermS employed immobilized faecal
1721 microbiota to continuously seed connected fermentation vessels and medium turn-
1722 over period was regulated in order to represent colonic transit times observed in
1723 healthy older people (Fehlbaum *et al.* 2015). Faecal microbiota from healthy elderly
1724 has been used in *in vitro* studies and to my knowledge no study has explored the *in*
1725 *vitro* modulatory effect of prebiotic candidates on the low-diversity faecal microbiota
1726 from frail elderly.

1727 ***Murine models in gut microbiota research:*** Mouse models such as germ-free (GF)
1728 and gnotobiotic mice, human microbiota associated (HMA) and conventional mice,
1729 offer an *in vivo* environment to study interactions between the gut microbiota and

1730 exogenous factors like diet and therefore, circumvent to some extent the
1731 aforementioned limitations of the *in vitro* environment. In spite of caveats in
1732 translating observations from mice to humans, murine models have largely
1733 facilitated pre-clinical research on the host-microbiota interactions and causality with
1734 regards to health and disease, diet and novel therapeutics (Nguyen *et al.*, 2015).

1735 Germ-free (GF) mice are reared aseptically in order to prevent the development of
1736 their indigenous gut microbiota, they are kept under sterile conditions throughout
1737 their life and they allow the researcher to define the gut microbiota of the
1738 experimental animal by selected colonisation (Martin *et al.*, 2016).
1739 Conventionalisation can be performed by transplanting selected microbiota strains or
1740 whole faecal microbiota from healthy or diseased donors, mice or humans
1741 (“humanisation”). Due to shared host responses and colonisation mechanisms,
1742 microbiotas from ecologically divergent hosts can survive and establish in other than
1743 their indigenous GIT niche (Rawls *et al.*, 2006; Seedorf *et al.*, 2014).

1744 The primary concern with gnotobiotic models is the successful establishment of the
1745 donor microbiota and the potential metabolic impact on the recipient animal.
1746 Bäckhed *et al.* (2004) using GF C57BL/6 adult mice reported that microbiota
1747 transplantation from conventional to GF mice resulted in increased energy harvest
1748 mediated by the transplanted microbiota showing not only the importance of the gut
1749 microbiota in energy harvesting for the host but also that the transplantation could
1750 alter the recipient metabolic profile. Turnbaugh *et al.* (2006) demonstrated that
1751 metabolic profiles like obesity that are associated with certain gut microbiota
1752 composition (Ley *et al.*, 2005) can be transmitted from conventional obese mice to
1753 GF. The potential of metabolic profile transfer through conventionalisation is an

1754 essential tool in metabolic disease and obesity pre-clinical research allowing for
1755 causality and mechanistic observations on how the whole microbiota composition,
1756 certain taxa and the metabolome respond to Western diet mediating adiposity
1757 (Turnbaugh *et al.*, 2009).

1758 Due to rearing in the absence of the indigenous microbiota, the GF mouse has certain
1759 GIT physiology alterations and immunity impairments compared to conventional
1760 animals such as fewer and smaller Peyer patches and mesenteric lymph nodes,
1761 alterations in the villus formations of the epithelial cells and lower expression levels
1762 of Toll-like receptors (reviewed in Round and Mazmanian, 2009). These
1763 physiological differences compared to conventional animals and constraints
1764 regarding the laborious process of generation and maintenance of GF murine strains,
1765 have led to the development of methods for the depletion of the indigenous gut
1766 microbiota of conventional mice with antibiotic (Abx) treatment (Bereswill *et al.*,
1767 2011; Hintze *et al.*, 2014). The metabolic profile transfer demonstrated in GF murine
1768 models was partly demonstrated in ampicillin-treated C57BL/6 adult and weaning
1769 mice that received microbiota transplants from obese and lean mice (Ellekilde *et al.*,
1770 2015). The donors microbiota compositional profile was maintained and some
1771 obesity-related clinical outcomes were observed for a certain post-transplantation
1772 period before the microbiota composition begun to revert back to the murine baseline
1773 (Ellekilde *et al.*, 2015).

1774 Antibiotic treatment of conventional mice prior to microbiota transplantation
1775 presents certain advantages and disadvantages compared to GF mice (summarised in
1776 Lundberg *et al.*, 2016). The relatively lower maintenance cost and the fact that the
1777 Abx approach can be applied to the desired mouse strain by the researcher as

1778 opposed to the limited available GF mouse strains that are prepared in specialised
1779 laboratories are some advantages of the Abx method. The fully mature immune
1780 system of the Abx mice is another comparative advantage. Overgrowth of
1781 pathobionts, antibiotic resistance expansion and re-emergence of the indigenous
1782 baseline microbiota are some of the negative aspects of the Abx approach that may
1783 lead to spurious reproducibility. Furthermore, it has been reported that
1784 conventionalisation of GF C57BL/6 adult mice resulted in “conventionalisation” of
1785 the innate and adaptive immune system 30 days post microbiota transplantation (El
1786 Aidy *et al.*, 2012; El Aidy *et al.*, 2014). Importantly, Chung *et al.* (2013)
1787 demonstrated that it is the mouse-related (or host-related) gut microbiota that can
1788 “normalise” the immature GF mouse immunity. The potential of achieving
1789 normalisation of the immune system of GF mice within a certain time should be
1790 taken into account when designing experiments on host-immunity-microbiota
1791 interactions.

1792 ***Efficacy of human microbiota colonisation of the murine gut and use of***
1793 ***conventional mice in gut microbiota studies:*** Another point of consideration on
1794 gnotobiotic mouse models is the efficacy of the establishment of the xenomicrobiota
1795 in the recipient gut. It is well documented that the host selective pressure that shapes
1796 the indigenous microbiota will be also applied to the transplanted microbiota
1797 reshaping it in order to match the host physiology, immunity and diet (Messer *et al.*,
1798 2016). Therefore, certain donor species such as taxa that depend on host-specific
1799 niches like the mucosa, may be proven unable to establish in the recipient gut
1800 rendering the transplanted microbiota significantly dependant on the given diet
1801 (Arrieta *et al.*, 2016). Wos-Oxley *et al.* (2012) comparing the colonisation patterns of
1802 GF rats, GF mice and ciprofloxacin-treated mice with human faecal microbiota

1803 under a low-fat high-polysaccharide diet, reported that mice favoured the
1804 Bacteroidetes establishment whereas major Firmicutes taxa belonging to *Clostridium*
1805 cluster IV, XIVa and the *Clostridium* leptum group did not reach a high level of
1806 colonisation efficacy.

1807 Arrieta *et al.* (2016) proposed that, at least in studies focusing on the overall
1808 ecological effect on the gut microbiota of an exogenous factor such as diet,
1809 conventional mice with their intact gut microbiota could be a more informative pre-
1810 clinical approach. Caveats in the extrapolation of conclusions from studies with
1811 conventional mice to humans that to some extent can be generalised for the
1812 aforementioned murine models, are the differences in physiology and anatomy of the
1813 murine and human GIT tract including size, mucosal surfaces, the existence or not of
1814 forestomach and the caecum size, that define specific niches for host-adapted taxa
1815 (Nguyen *et al.*, 2015). The taxa shared between murine and human gut microbiota
1816 significantly differ in relative abundances and distinct bacterial groups dominate the
1817 two microbiotas; for example, *Faecalibacterium*, *Ruminococcus* and *Prevotella* are
1818 dominant in the human gut microbiota whereas *Lactobacillus*, *Alistipes* and
1819 *Turicibacter* dominate the murine gut (Nguyen *et al.*, 2015).

1820 Importantly, the murine microbiota differs between the various available mouse
1821 strains and even within strains, the provider company, the maintenance facilities, co-
1822 housing and coprophagy may significantly contribute to the baseline murine
1823 microbiota, thus affecting reproducibility of experiments and comparisons between
1824 studies (Hugenholtz and de Vos, 2018). Given the importance of mouse models in
1825 host-gut microbiota research, the determinative role of the baseline murine
1826 microbiota in the study outcomes and the lack of extensive studies focusing on the

1827 indigenous murine microbiota, Lagkourdos *et al.* (2016) established a collection of
1828 isolated murine bacterial strains, analogous to human culturomics.

1829 So far, I have discussed major mouse models i.e. GF and Abx-treated mice
1830 conventionalised with murine microbiota or “humanised” with human microbiota,
1831 and points of consideration for each approach such as immune responses in GF mice
1832 and efficacy of colonization with xenomicrobiota. Importantly, conventional mice
1833 are a valuable tool in the study of host-microbiota interactions since every model is
1834 accompanied by certain considerations when it comes to translating observations
1835 from mouse to humans. With regards to reproducibility, gnotobiotic mice remain a
1836 well-controlled, reproducible animal model used in many studies that have
1837 significantly contributed to our increased understanding of the complicated
1838 interactions between gut microbiota-host-dysbiosis-exogenous factors. The selected
1839 *in vivo* studies shown in **Table 3** demonstrate the breadth of the scientific questions
1840 concerning gut microbiota diet, health and disease that have been investigated
1841 through employing mouse models and it is not an exhaustive review of the existing
1842 murine model studies.

1843 **Table 3 Selected studies employing murine models in the study of gut microbiota, diet, health and disease.**

Mouse model	Donor microbiota	Condition	Test substrate	Outcomes	Reference
C57BL6/J GF	Human	Effect of diet on the microbiome	Low-fat polysaccharide rich diet	– human faecal microbiota establishment in GF mice	Turnbaugh <i>et al.</i> 2009
			High-fat sugar rich “Western” diet	– microbiota transmission from gnotobiotic mice to offspring	
				– rapid and reproducible responses in composition metabolome and gene expression of the microbiota due to switch to “Western” diet altered	
				– transmission of host diet-induced adiposity to gnotobiotic mice	
				– isolation of obesity associated <i>Erysipelotrichia</i> taxa	
C57BL6/J conventional	-	Diet-induced obesity	High-fat diet (HF)	AX restored gut microbiota changes due to HF diet:	Neyrinck <i>et al.</i> , 2011
			Arabinoxylan (AX)	– ↑ <i>Bacteroides-Prevotella</i> spp., <i>Roseburia</i> spp. – ↑ caecal bifidobacteria AX decreased adiposity, improved tight junctions function	
C57BL6/J conventional	-	Diet-induced obesity	High-fat diet (HF)	AXOS restored obesity measurements:	Neyrinck <i>et al.</i> , 2012
			Arabinoxylan OS (AXOS)	– ↓ insulin resistance	
				– ↓ metabolic endotoxemia	
				– ↓ macrophage infiltration	
				– ↓ IL6 in plasma	
				AXOS improved gut microbiota factors: – ↑ caecal bifidobacteria – Up-regulations of tight junction proteins	

Mouse model	Donor microbiota	Condition	Test substrate	Outcomes	Reference
Swiss-Webster	Various human donors	“humanisation” and diet impact on faecal and urinary metabolome	Polysaccharide-rich diet (PD)	– distinct metabolomes in humanised and conventional mice	Marcobal <i>et al.</i> , 2013
C57BL6/J	<i>B. thetaiotaomicron</i> mono-colonisation		Polysaccharide-deficient diet (PDD)	– distinct clustering of the “humanised” microbiotas (UniFrac distances) and metabolomes based on PD and PDD diet	
GF conventional	<i>B. thetaiotaomicron</i> and <i>B. longum</i> bi-colonisation			– colonisation with simple defined microbiotas model to some extent the “humanisation” metabolic process	
C3H/HeOuJ GF	8 bacterial strains from human gut microbiota	diet-induced obesity	+/- <i>Clostridium ramosum</i> (Erysipelotrichia)	– HFD promoted <i>C. ramosum</i> abundance and obesity	Woting <i>et al.</i> , 2014
			High-fat diet (HFD)	– <i>C. ramosum</i> related transcription upregulation of glucose and fat transport proteins in jejunal and ileum mucosa	
			Low-fat diet (LFD)		
C57BL6/J conventional	-	Frailty and ageing	Standard chow	frailty index correlated to ageing characteristics of the “aged” murine microbiota composition and function : – ↑ <i>Alistipes</i> (Rikenellaceae) – ↓cobalamin, biotin biosynthesis – ↑creatinine degradation related to muscle wasting – ↓di-,oligo- and poly-saccharide utilisation genes	Langille <i>et al.</i> , 2014
C57BL6/J GF	Human	<i>C. difficile</i> infection	Antibiotic treatment <i>C. difficile</i> spores	– “humanised” microbiota transferred to offsprings – establishment of CDI and recurrent CDI in gnotobiotic murine model	Collins <i>et al.</i> , 2015
C57BL6/J /RccHsd conventional GF	Murine	ageing and systemic inflammation	Standard chow	microbiota transfer from conventional old to GF young mice promoted inflammaging : – ↑TM7 and Protoeobacteria taxa – ↓Akkermansia – ↑ T cell activation – ↑inflammation inducing bacterial components in circulation	Fransen <i>et al.</i> , 2017

1844 **1.3 The effect of milk and glycomacropeptide on the gut microbiota**

1845 **1.3.1 The effect of milk on human health and the gut microbiota**

1846 Dairy products and milk have been a component of human nutrition since the
1847 seventh millennium BC (Evershed *et al.*, 2008). According to the Food and
1848 Agriculture Organisation of the United Nations (FAO)
1849 (<http://www.fao.org/home/en/>) in Europe the annual consumption of milk rises to
1850 more than 150 kg per capita providing to consumers 9% of the dietary energy
1851 supply, 19% of the protein supply and the 11%-14% of the dietary fat. Milk
1852 consumption is higher in developed countries but many developing countries are
1853 steadily increasing their per capita milk consumption (FAO; Gateway to dairy
1854 production and products).

1855 Milk is a rich source of nutrients (**Table 4**) composed of lipids including saturated
1856 and unsaturated fatty acids, milk fat globule membranes (MFGM), phospholipids
1857 and glycosphingolipids, proteins such as casein and whey, vitamins such as vitamin
1858 A, E and B12, and minerals including calcium, magnesium, phosphorus and zinc
1859 (Haug *et al.*, 2007). The carbohydrate profile of bovine milk is composed mainly of
1860 the disaccharide lactose, traces of neutral and acidic sialic acid containing
1861 oligosaccharides, and glycoconjugates (Gopal and Gill, 2000; Hsieh *et al.*, 2015).
1862 Glycoproteins like the extensively sialylated mucins, and glycolipids comprise the
1863 glycoconjugate component of bovine milk (O’Riordan *et al.*, 2014).

1864 The beneficial effect of milk and dairy in human diet has been challenged mostly on
1865 the basis of the high saturated fat content in milk but the evidence supporting this
1866 notion is inconclusive. Meta-analysis of various observational and prospective cohort
1867 studies has concluded that no consistent association between milk consumption and

1868 increased all-cause mortality can be established (Guo *et al.*, 2017). Conversely,
 1869 various studies including epidemiological studies indicate the multitude of health
 1870 benefits of moderate milk consumption (Visioli and Strata, 2014).

1871 **Table 4 Bovine milk components.** The data is taken from the Society of Dairy
 1872 Technology (<https://www.sdt.org/pages/>) and O’Riordan *et al.* (2014). The table is
 1873 not exhaustively presenting all milk components, but the main ones.

Component and proportion in bovine milk		Detailed composition
Fat	4%	Small globules covered by the milk fat globule membrane (MFGM), rich in proteins and phospholipids
Proteins	3.3%	- Caseins (76%) in casein micelles structure: α_{s1}, α_{s2}, β, κ - Whey (18%): β-lactoglobulin, α-lactalbumin
Carbohydrates	5%	- Lactose (4.7%) - Free oligosaccharides, glycoconjugates
Minerals	0.7%	Calcium, phosphorus
Organic acids	0.2%	Citric and lactic acid
Vitamins	traces	- Fat-soluble: A, D, E, K - Water-soluble: B₁, B₂, B₆, B₁₂
Water	87%	

1874 1.3.1.1 Cardio-metabolic disease and all-cause mortality

1875 Certain co-occurring metabolic conditions including obesity, insulin resistance and
 1876 high lipidaemia, grouped under the term Metabolic Syndrome (MetS), may be risk
 1877 factors for cardiovascular disease (CVD), diabetes type 2 (T2D) and hepatic steatosis

1878 (reviewed in Lusi *et al.*, 2008; Catrysse and van Loo, 2017). Cardio-metabolic
1879 conditions are prevalent in Western countries; chronic low-grade inflammation is an
1880 underlying factor of these conditions and apart from human genetics, high-fat dietary
1881 choices and sedentary life style are strongly associated with MetS development
1882 (Catrysse and van Loo, 2017).

1883 Evidence from large Western cohorts and meta-analysis of prospective cohort studies
1884 support milk and dairy inclusion in a balanced diet and the positive association of
1885 increased dairy consumption with a reduction in risk of coronary health and MetS
1886 risk (Panagiotakos *et al.*, 2010; Benatar *et al.*, 2014; Crichton and Alkewri, 2014;
1887 Drehmer *et al.*, 2016). In Asian countries like Korea and Japan, where in spite of
1888 Western influence dietary patterns remain different from those in Western countries,
1889 a higher milk and dairy consumption was associated with a lower risk of MetS
1890 development (Jun *et al.*, 2016). In another non-Western cohort of Iranian adults,
1891 regular weekly whole milk consumption but not daily consumption was associated
1892 with reduced CVD risk (Talaie *et al.*, 2017).

1893 Conversely, in two cohorts (n≈100,000 older adults) in Sweden, where consumption
1894 of fluid milk is one of the highest worldwide, a high milk consumption was
1895 associated with adverse health outcomes, i.e. all-cause mortality and hip fracture
1896 (Michaelsson *et al.*, 2014; Tognon *et al.*, 2017). Michaelsson *et al.* (2014) observed
1897 that ≥ 3 glasses of milk per day was associated with increased mortality and hip-
1898 fracture in two Swedish cohorts followed for 11 and 20 years. Tognon *et al.* (2017)
1899 observed that high consumption of non-fermented milk (>2.5 intakes per day)
1900 correlated with an increased risk of all-cause mortality in a dose-response manner in
1901 a mean follow-up time of approximately 14 y. Importantly, Tognon *et al.* (2017)

1902 observed that non-fermented milk consumption was associated with consumers with
1903 poorer educational level compared to cheese consumers who had comparatively
1904 reduced all-cause mortality risk. This observation may indicate that residual
1905 confounding factors associated with life-style may have masked a more relevant to
1906 milk consumption effect on the participants' health.

1907 Milk-based diets were shown to attenuate oxidative stress in obese and overweight
1908 subjects compared to non-dairy intervention control diets (Zemel *et al.*, 2010;
1909 Stancliffe *et al.*, 2011). Milk and dairy consumption may improve blood pressure
1910 and arterial plasticity potentially due to milk proteins and peptides released upon
1911 digestion (Lovegrove and Hobbs, 2016). In spite of the heavy load in whole milk and
1912 dairy products of saturated fatty acids (SFA) (Mansson, 2008) evidence suggests that
1913 whole dairy (excluding butter) does not contribute to significant or detrimental
1914 increase in blood low-density lipoprotein cholesterol (LDL-C) (reviewed in
1915 Lovegrove and Hobbs, 2016). For the milk proteins whey and casein, data from
1916 clinical studies are relatively consistent indicating towards a potential positive effect
1917 on blood glucose regulation (Fekete *et al.*, 2016). Importantly, frequent low-fat milk
1918 and dairy consumption may be associated with a lower risk of T2D (Aune *et al.*,
1919 2013; Díaz-López *et al.*, 2016).

1920 Observational studies have often provoked controversy on the effect of milk
1921 consumption and all-cause mortality (Larsson *et al.*, 2015). Importantly, adjustment
1922 for a wide array of confounding factors such as background dietary patterns, life-
1923 long exposure to dairy, life-style and exercise, smoking, age, sex, education, self-
1924 reported milk and dairy servings could leverage bias in observational study design.
1925 Leverage of the confounding factors effect and clinical trials with well-defined dairy

1926 products (ex. non-fermented milk with variant fat content) could further elucidate
1927 existing controversy.

1928 **1.3.1.2 Frailty**

1929 Frailty is a condition of functional disability observed in older age, that is
1930 characterised by the gradual loss of homeostatic maintenance of the organism even
1931 under minor challenges and it may lead to increased dependency, adverse outcomes
1932 after hospitalization and even death (Clegg *et al.*, 2013). The frailty phenotype is not
1933 restricted to older age but it is also observed in people with advanced HIV and
1934 cancer (Deeks, 2011; Ethun *et al.*, 2017). Sarcopenia which is the state of de-
1935 regulated muscular homeostasis leading to the progressive loss of muscle mass and
1936 functionality decline is widely recognised as a syndrome and it is one of the main
1937 frailty manifestations; metabolic, inflammatory and dietary challenges may
1938 contribute to the development and progression of the syndrome (Fielding *et al.*,
1939 2011). Importantly, frailty assessment models can successfully predict the risk of
1940 osteoporosis-related bone fracture in older age (Li *et al.*, 2017). New drug
1941 development, physical activity and dietary interventions are being employed towards
1942 reversing the frailty scores in sufferers (Keevil and Romero-Ortuno, 2015).

1943 Muscle maintenance results from the balance between muscle protein synthesis
1944 (MPS) triggered by dietary anabolic stimuli like proteins and amino acids and the
1945 naturally occurring process of proteolysis (Mitchell *et al.*, 2016). Glucose regulation
1946 and muscle protein turn-over pass through the homeostatic metabolic junction of the
1947 mechanistic target of rapamycin (mTOR) where the essential amino acids especially
1948 leucine, and insulin are key signalling molecules (Barzilai *et al.*, 2012; Saxton and
1949 Sabatini, 2017). In older age anabolic resistance blunts the sensitivity of the

1950 organism to respond to these signals and consecutively to translate the signals to
1951 muscle accretion eventually leading to wasting; nutritional interventions with
1952 leucine-rich and easily ingested high in protein dietary supplements and physical
1953 activity may reverse the adverse outcome of severe muscle loss (Moore, 2014).

1954 Whey protein is considered “fast” in releasing amino acids in blood after digestion
1955 and may stimulate postprandial MPS whereas casein that is considered “slow”
1956 confers a moderate prolonged aminoacidaemia and supresses muscle protein
1957 breakdown (MPB) (Boirie *et al.*, 1997). Fast postprandial aminoacidaemia was
1958 initially considered as an MPS driver but it seems that the amino acid composition of
1959 the dietary proteins ingested (ex. leucine content) is key to the anabolic activity
1960 (Mitchell *et al.*, 2016). The milk proteins whey and casein that contain all essential
1961 amino acids and are rich in leucine (Hall *et al.*, 2003) have been studied in human
1962 trials in the concept of “fast” and “slow” protein and MPS.

1963 Consumption of fluid skim milk that combines the two types of “slow” and “fast”
1964 proteins after exercise resulted in greater muscle accrual in young adults compared to
1965 isonitrogenous and isoenergetic soy beverage consumption (Hartman *et al.*, 2007;
1966 Wilkinson *et al.*, 2007). Whey supplementation alone was reported efficient to better
1967 sustain protein synthesis and lean mass gain in adults after resistance exercise and
1968 compared to casein or soy protein (Tang *et al.*, 2009; Volek *et al.*, 2013).

1969 For older subjects in whom high quality dietary amino acids intake is essential for
1970 muscle accretion, the rapidly digested rich-in-leucine whey protein may be a suitable
1971 dietary protein source (Landi *et al.*, 2016). Twenty g of whey protein were more
1972 effective in postprandial muscle protein retention in healthy men over 74 yrs without
1973 any exercise intervention compared to the ingestion of the same amount of casein or

1974 casein hydrolysate (Pennings *et al.*, 2011). Mitchell *et al.*, (2015) reported that 20 g
1975 of either milk protein isolate or whey protein dietary supplementation resulted in the
1976 same postprandial muscle protein fractional synthetic rates (FSR) in healthy mostly
1977 sedentary men aged 45-60 yrs. This suggests that milk protein is a widely accessible
1978 food component and is thus adequate source of dietary protein for older adults.

1979 In observational studies of large population cohorts, older people that are regular
1980 consumers of dairy i.e., milk, yoghurt and cheese, have improved measurements for
1981 skeletal muscle and bone mass compared to less regular consumers. Observations
1982 from the Quebec NuAge cohort of people over 67 yrs, showed that community-
1983 residing people consuming ≥ 2 serving of dairy (milk, yoghurt, cheese) per day had
1984 better functionality and mobility measurements and increased lean mass compared to
1985 people consuming less servings per day (Farsijani *et al.*, 2017). In the Quebec
1986 NuAge study the reduced risk of pre-frailty or frailty was more pronounced among
1987 men with high dairy intake compared to low intake than in the respective group of
1988 women. Among 564 community-dwelling women with mean age 85 yrs, high dairy
1989 consumption (≥ 2.2 servings per day) assessed for the previous 12 months associated
1990 with higher bone density and skeletal muscle mass (Radavelli-Bagatini *et al.*, 2014).
1991 Radavelli-Bagatini *et al.* (2013) had previously reported similar positive effects on
1992 muscular and bone health for a larger cohort of women aged 70-85 yrs. In a cohort
1993 (n=1,871) of non-hospitalized non-frail adults over 60 yrs, consumption of ≥ 7
1994 serving per week of low-fat milk and yoghurt was associated with reduced frailty
1995 risk in a 3.5 years follow-up (Lana *et al.*, 2015).

1996 Regarding bone density and risk of fracture in older age, Sahni *et al.* (2014) observed
1997 that for adults over 77 yrs (Farmingham, USA) who regularly consumed milk or

1998 milk and yoghurt, the risk of hip fracture was significantly lower compared to that
1999 low in frequency consumers of dairy products. The group also showed that high milk,
2000 yoghurt and dairy consumption may benefit bone health among elderly under dietary
2001 vitamin D supplementation (Sahni *et al.*, 2017a). Recent studies suggest that diets
2002 rich in dairy may be beneficial for bone mineral density and reduced risk of hip
2003 fracture in elderly population (de Jonge *et al.*, 2017; Durosier-Izart *et al.*, 2017).

2004 Other studies contradict the beneficial effect or report no effect of milk on skeletal
2005 health. For example, a 2011 meta-analysis of cohort studies showed that there was
2006 no association between milk consumption and bone fracture risk for older women
2007 whereas for men there was a marginal inverse association (Bischoff-Ferrari *et al.*,
2008 2011). In two large Swedish cohorts ($\geq 45,000$ subjects each) of adults from 39 to 79
2009 yrs old and for a long follow-up period of over a decade high milk intake was
2010 positively correlated with higher mortality and hip fracture (Michaelsson *et al.*, 2014)
2011 as discussed above in this review. The study of Michaelsson *et al.* (2014) reported
2012 that the female high-milk-consuming participants showed increased health risk in a
2013 20 yrs follow-up period. This prolonged follow-up period potentially increased the
2014 confounding factors; in older adults mortality will increase over the years and older
2015 age co-morbidities may have masked the effect of dairy products on the participants'
2016 health (Sahni *et al.*, 2017b).

2017 **1.3.1.3 Other potential health benefits of milk consumption**

2018 There are conflicting studies on the beneficial effect of milk consumption on
2019 cognition. In older adult populations with Western life-style, total low fat dairy and
2020 cheese consumption but not milk alone was reported beneficial for various cognition
2021 measurements (Crichton *et al.*, 2010; Park and Fulgoni, 2013). Camfield *et al.* (2011)

2022 after a thorough review of existing cross-section studies concluded that low-fat dairy
2023 products including milk incorporated in balanced diet may positively affect cognitive
2024 function of older adults. Similarly, Wu and Sun (2016) after reviewing available
2025 studies on milk and dairy consumption, concluded that there may be a trend
2026 suggesting that regular milk consumption improves cognition. This trend was more
2027 pronounced in Asian cohorts and it was attributed to the fact that total dairy intake is
2028 less common as a background diet in Asia compared to Western countries (Wu and
2029 Sun, 2016) making the effect of milk more detectable. More prospective studies with
2030 standardized measurements and adjustment for a wider range of cofounding factors
2031 (Wu and Sun, 2016) as well as clinical trials are needed in order to draw robust
2032 conclusions on the effect of milk on cognitive function.

2033 Many studies have explored the impact of regular milk consumption on several
2034 cancers but firm conclusion cannot be drawn yet (Visoli and Strata, 2014). An area
2035 of particular interest has been colorectal cancer (CRC) and dairy calcium intake.
2036 According to the WHO, CRC is the second most prevalent cause of cancer death
2037 among Europeans and a major cause of mortality worldwide with life-style factors
2038 such as diet being one of the risk factors ([http://www.euro.who.int/en/health-](http://www.euro.who.int/en/health-topics/noncommunicable-diseases/cancer/news/news/2012/2/early-detection-of-common-cancers/colorectal-cancer)
2039 [topics/noncommunicable-diseases/cancer/news/news/2012/2/early-detection-of-](http://www.euro.who.int/en/health-topics/noncommunicable-diseases/cancer/news/news/2012/2/early-detection-of-common-cancers/colorectal-cancer)
2040 [common-cancers/colorectal-cancer](http://www.euro.who.int/en/health-topics/noncommunicable-diseases/cancer/news/news/2012/2/early-detection-of-common-cancers/colorectal-cancer)).

2041 Park *et al.* (2009) conducted a large prospective study on American older adults ≥ 50
2042 yrs (293,907 men and 198,903 women) and assessed incidents of cancer and dairy
2043 products (milk, yoghurt, cheese) consumption over 7 years. The group found no
2044 adverse outcomes from dairy consumption and cancer incident; high dairy and
2045 supplemental calcium intake was related to 16% and 23% lower risk of GIT cancers

2046 (especially CRC) in men and women respectively. Of note was the observation that
2047 people with higher dairy consumption tended to have a healthier life-style and be
2048 college educated. Aune *et al.* (2012) conducted a meta-analysis on 19 cohort studies
2049 performed until May 2010 and concluded that high milk and total dairy intake (200 g
2050 and 400 g per day respectively) was associated with reduced CRC incident.

2051 More recently, Murphy *et al.* (2013) investigated the effect of dairy product habitual
2052 consumption and colorectal cancer risk in a prospective study on European adults
2053 (EPIC cohort: 10 countries, n=477,122, over 35 yrs old, 11 years follow-up). A
2054 portion of whole-fat or skimmed milk and dairy per day but not supplemental
2055 calcium was inversely associated with CRC occurrence. In a USA cohort of 77,712
2056 older adults, a 7 year old follow-up study showed that milk consumption and dairy
2057 (milk, yoghurt, cheese) may be protective for rectal and CRC; the group suggested
2058 that dietary calcium may be protective against CRC (Tantamango-Bartley *et al.*,
2059 2017).

2060 The existing data do not suggest a significant effect of milk intake on breast cancer
2061 occurrence. A prospective study of 64,904 Norwegian women showed that dairy
2062 consumption had no significant impact on pre- or post-menopausal breast cancer
2063 incidence (Hjartaker *et al.*, 2010). In a meta-analysis of 18 prospective cohort studies
2064 intake level of total dairy products (milk, yoghurt, cheese, butter) but not milk alone
2065 was associated with reduced risk of breast cancer (Dong *et al.*, 2011). Similarly, lung
2066 cancer did not seem to be significantly associated to milk or dairy products
2067 consumption (Yang *et al.*, 2016).

2068 Milk consumption may play a detrimental role in the progression of prostate cancer.
2069 Among 3,918 mostly Caucasian men in U.S.A. (Health Professionals Follow-Up

2070 Study) diagnosed with prostate cancer at baseline those that frequently consumed
2071 whole milk had increased risk of disease progression compared to the less frequent
2072 whole milk consumers or those frequently consuming low-fat dairy products who
2073 demonstrated reduced risk for disease progression after 7.6 years of follow-up
2074 (Pettersson *et al.*, 2013). In another cohort of 926 men diagnosed with non-
2075 metastatic prostate cancer (Physician's Health Study) 3 servings of dairy per day
2076 associated with higher risk of total and prostate cancer-related mortality compared to
2077 men consuming 1 serving per day (Yang *et al.*, 2016). Downer *et al.* (2017) reported
2078 that ≥ 3 servings per day of high-fat milk correlated with higher risk of prostate
2079 cancer mortality compared to 1 serving per day. Both Yang *et al.* and Downer *et al.*
2080 observed a borderline positive association for low-fat dairy and reduced cancer
2081 mortality. Some studies have suggested a link between milk and dairy lactose to risk
2082 for ovarian cancer (Larsson *et al.*, 2006; Qin *et al.*, 2016) whereas dietary calcium
2083 was inversely associated to the disease (Qin *et al.*, 2016; Song *et al.* 2017).

2084 A small number of recent studies suggest a beneficial role of dairy products in renal
2085 health. Regular low fat dairy products and dietary calcium was reported beneficial
2086 for kidney function in a cohort of ≥ 50 yrs old Australians (Gopinath *et al.*, 2016).
2087 Rebholz *et al.* (2016) also observed a protective effect of a healthy diet rich in low-
2088 fat milk and yoghurt against kidney disease in a large cohort of middle-aged African-
2089 American and Caucasians.

2090 **1.3.1.4 Strengths and weaknesses of cohort observation studies assessing the** 2091 **effect of milk on various-cause mortality**

2092 With regards to prospective cohort studies assessing the effect of milk and dairy
2093 products consumption on various causes of mortality in adults, and despite some
2094 conflicting observations, meta-analysis tends to agree that regular milk intake

2095 especially low-fat incorporated in a healthy diet may be beneficial in lowering the
2096 risk of cardiometabolic disease, improving frailty indices in the general adult
2097 population and potentially contributing to cognitive function in elderly (Camfield *et*
2098 *al.*, 2011; Visioli and Strata, 2014).

2099 Authors have suggested, as explanation for conflicting results relating to the effect of
2100 milk and dairy products on human health, the adjustments for various confounding
2101 factors in prospective cohort studies and the consecutive adjustments in meta-
2102 analysis. Background diet, socioeconomic status, smoking and life-style, baseline
2103 measurements of health status, differences in self-reported portion measurement and
2104 lack of discrimination between whole or low-fat dairy product are some of the
2105 possible confounding factors reported in the prospective studies and meta-analysis
2106 studies considered in this review that may have led to conflicting results or masking
2107 potential effects of dairy consumption. Interestingly, some studies report that milk
2108 and dairy products consumers had higher education and socioeconomic status as well
2109 as enjoying a healthier life-style compared to those with lower frequency dairy
2110 product consumption (Wu *et al.*, 2016), whereas in other studies milk consumption
2111 was higher among people of lower socioeconomic status (Talaie *et al.*, 2017).

2112 Milk contains a wealth of nutrients that may synergistically and/or individually
2113 mediate the reported potential effects of milk and dairy products ingestion in human
2114 health. Various peptides such as lactoferrin, α -lactalbumin, β -lactoglobulin, fractions
2115 of α -, β - and κ -casein and peptides released from dairy after digestion, gut
2116 microbiota fermentation and food preparation, have been reported to play a
2117 beneficial role as anti-thrombotic compounds, inhibiting angiotension and potentially
2118 as insulintropics (reviewed in Horner *et al.*, 2016). Calcium, vitamin D and
2119 conjugated linoleic acid (CLA) may be mediators of the chemoprotective activity of

2120 milk (Song *et al.*, 2015). Conversely, D-galactose, lactose, IGF-1, calcium, saturated
2121 fat and hormones contained in milk have been proposed as factors with detrimental
2122 effects in oxidative stress and cancer incidence (Yang *et al.*, 2016(a); Yang *et al.*,
2123 2016(b); Berghlödter *et al.*, 2017). Well-designed prospective studies adjusting for a
2124 wide range of confounding factors and clinical trials on selected cohorts and *in vitro*
2125 */vivo* studies will further elucidate the role of whole dairy and individual milk
2126 components in human health and reveal the mechanism that mediate the effects.

2127 **1.3.1.5 Lactose-intolerance**

2128 Dietary oligosaccharides and disaccharides are hydrolysed by intestinal
2129 disaccharidases in order for the constituent monomers to be absorbed by the
2130 enterocytes in the small intestine (Amiri *et al.*, 2015). Oligosaccharides and
2131 disaccharides that escape hydrolysis and absorption in the small intestine reach the
2132 distal colon where they can cause osmotic flux of water and increased H₂, CO₂ and
2133 fatty acids concentration due to fermentation by colonic bacteria (Amiri *et al.*, 2015).
2134 The disaccharide lactose (consisting of galactose and glucose) is the main
2135 carbohydrate in human and farm animal milk (Silanikove *et al.*, 2015). In humans,
2136 hydrolysis of lactose is highly active during lactation and declines after weaning;
2137 more than half of the human population has low lactase activity as a result of the
2138 genetically programmed decline of lactase synthesis (lactose non-persistent subjects
2139 - LNP) (Deng *et al.*, 2015). Lactase deficiency may also occur after a GIT disease or
2140 as a rare congenital condition in early life; in all cases malabsorbed lactose persists
2141 in the distal GIT and in some cases it may lead to symptoms of lactose intolerance
2142 (Deng *et al.*, 2015). Not all adults lose lactase synthesis activity and the ability to
2143 thoroughly digest lactose (lactose persistent subjects- LP). In modern populations

2144 deriving from ancient pastoral populations, lactase synthesis may remain active
2145 throughout adulthood (Gerbault *et al.*, 2011).

2146 Lactose malabsorption in LNP individuals does not always lead to lactose
2147 intolerance symptoms and to a clinically detectable condition (Misselwitz *et al.*,
2148 2013). Lactose maldigestion can be accompanied by bloating, abdominal pain,
2149 borborygmi, diarrhoea and nausea, and subjects reporting such discomforts may
2150 follow certain diagnostic steps towards the clinical evaluation of their condition
2151 (summarized by Misselwitz *et al.*, 2013). The diagnosis includes tests for
2152 malabsorption such as the lactose H₂-breath test alone or after the ingestion of milk.
2153 Throughout the lactose intolerance diagnosis procedure, other conditions such as IBS
2154 or small intestinal bacterial overgrowth (SIBO) should be considered as potential
2155 contributors to the self-reported discomforts (Misselwitz *et al.*, 2013).

2156 People with self-reported symptoms of lactose malabsorbance or lactose intolerance
2157 tend to avoid milk and dairy products therefore excluding from their daily diet a
2158 valuable source of important nutrients and calcium (Heaney, 2013). Avoidance of
2159 dairy foods is not recommended for the improvement of lactose intolerance (Szilagyi,
2160 2015a) especially when, apart from lactose malabsorption or intolerance, there is
2161 another condition that could be improved by dairy consumption (Szilagyi *et al.*,
2162 2016). Lactase non-persistent individuals can tolerate an average of 12 g of lactose
2163 per day (approximately one glass of milk) without developing symptoms (Szilagyi,
2164 2015b). Lactase non-persistent individuals can develop tolerance to lactose through
2165 adaptation of the colonic microbiota to lactose fermentation, a phenomenon that
2166 indicates that lactose may have a prebiotic effect on the gut microbiota of LNP
2167 subjects (Szilagyi, 2015b).

2168 **1.3.1.6 Milk and the gut microbiota**

2169 Little is known about the effect of non-human fluid non-fermented milk on the
2170 human gut microbiota and the prebiotic potential of its carbohydrate load. The
2171 carbohydrate fraction of milk consists mainly of lactose, oligosaccharides (OS) and
2172 glycoconjugates (**Table 4**). Mature human milk has higher content of lactose (70 g/l)
2173 and OS (5-15 g/l) compared to bovine milk (48 g/l and 0.05 g/l respectively)
2174 (summarized in Bode, 2012). Glucose, galactose, fucose, *N*-acetylneuraminic acid
2175 (NeuAc/sialic acid) and *N*-acetylglucosamine (GlcNAc) are the simple carbohydrates
2176 that compose the milk OS; *N*-glycolylneuraminic acid (NeuGc) and lactose amine
2177 are present only in bovine milk (Tao *et al.*, 2008). The human milk oligosaccharides
2178 (HMOs) are mostly fucosylated whereas a high proportion of the bovine milk
2179 oligosaccharides (BMOs) is sialylated (Bode, 2012).

2180 Fructo-oligosaccharides and GOS that are widely used as prebiotics (see Chapter 1.2)
2181 do not reach the structural and compositional complexity of HMOs or the (similar in
2182 structure) highly complex BMOs (Zivkovic and Barile, 2011). Human milk
2183 oligosaccharides have been extensively studied because of their potent prebiotic
2184 effect on the infant gut microbiota (Smilowitz *et al.*, 2014). If BMO prebiotic
2185 activity is proven, BMOs could then be used as an alternative to HMOs for food
2186 supplementation and the dairy industry could scale-up the relevant BMO isolation
2187 (Zivkovic and Barile, 2011).

2188 Bovine milk OS exist only in trace amounts in mature bovine milk, and whey
2189 concentrate that naturally contains large amounts of OS (approximately 200 mg/L) is
2190 currently used for BMO isolation (Zivkovic and Barile, 2011). There are ongoing
2191 efforts to develop scalable methods to isolate highly pure BMOs and sensitive

2192 analytical tools for the characterisation of the OS isolate profiles in order to further
2193 elucidate the structural complexity and natural variation extend of BMOs
2194 (Sundekilde *et al.*, 2012; Mehra *et al.*, 2014; de Moura Bell *et al.*, 2018). Importantly,
2195 only a few BMOs (approximately 40) have been identified compared to the more
2196 than 100 known HMOs (Bode, 2012).

2197 To my knowledge there are no extensive studies yet exploring the effect of non-
2198 human non-fermented fluid milk on the gut microbiota. Tannock *et al.* (2012)
2199 reported that the faecal microbiota of bovine milk supplemented formula-fed infants
2200 had relatively greater abundance of *Lachnospiraceae* compared to breast-fed. Yin *et al.*
2201 (2014) reported that ingestion of acidified milk-supplemented standard chow
2202 increased the abundance of *Lachnospiraceae* in the murine faecal microbiota
2203 compared to milk supplemented with probiotic *Lactobacillus* or water.

2204 A few recent studies exploring the effect of isolated BMOs on the gut microbiota
2205 gave some insight on the beneficial potential of milk carbohydrates in GIT health.
2206 Charbonneau *et al.* (2016) showed that in both germ-free mice and gnotobiotic
2207 piglets, sialylated BMO (S-BMO) supplemented diet resulted in a faecal microbiota-
2208 mediated attenuation of the effects of malnutrition on lean body mass, muscle and
2209 bone health and cognitive function. Karav *et al.* (2016) demonstrated *in vitro* that OS
2210 derived from bovine milk glycoproteins supported *Bifidobacterium longum* subsp.
2211 *infantis* growth. *Bifidobacterium infantis* is a member of the gut microbiota of
2212 breast-fed infants and selectively grows on HMOs. Bovine milk glycans may
2213 represent a selective substrate for gut microbiota taxa alternative to HMOs (Karav *et al.*,
2214 2016).

2215 Boudry *et al.* (2017) reported that BMOs normalized diet-induced obesity effects on
2216 the gut microbiota composition and gut barrier function in a murine model. Similarly,
2217 Hamilton *et al.* (2017) showed that, in a mouse model, BMO supplemented diet was
2218 more efficient in attenuating the effect of high fat diet on gut barrier function and gut
2219 microbiota compared to prebiotic inulin supplementation. A recent study
2220 investigating the effect of dietary supplementation with BMOs in humans showed
2221 that equivalent to plant-origin prebiotic doses of BMOs were well tolerated by
2222 healthy adults but with no drastic effect on stool consistency and gut microbiota
2223 composition after 11 days of dietary intervention (Smilowitz *et al.*, 2017).

2224 Other components of milk may confer gut microbiota-mediated health benefits.
2225 Norris *et al.* (2016) reported that a bovine milk sphingomyelin-supplemented diet
2226 counteracted the negative effect of high-fat diet in a mouse model by lowering the
2227 serum cholesterol and by reducing the circulating LPS with a concomitant increase
2228 in the relative abundance of faecal Firmicutes and Actinobacteria, predominantly
2229 *Bifidobacterium*. In a neonatal murine model, bovine MFGM-supplemented formula
2230 effectively attenuated the GIT deformities observed under the influence of MFGM-
2231 deprived diet; the gut microbiota composition of the mice fed the MFGM
2232 supplemented formula clustered with the faecal microbiota of mice fed maternal
2233 milk that contains naturally occurring MFGM (Bhinder *et al.*, 2017). Lee *et al.* (2018)
2234 observed improvement of metabolism and changes in microbial metabolites in
2235 malnourished children due to bovine MFGM supplementation. Conversely, in a
2236 murine model, ingestion of a diet high in saturated milk fat resulted in the increased
2237 abundance of the sulphur-reducing bacterium *Bilophila wadsworthia* in the faecal
2238 microbiota, and increased the incidence of colitis in genetically susceptible mice but
2239 not in wild type (Devkota *et al.*, 2012).

2240 Exploring the effect of individual milk components on the gut microbiota and health
2241 is useful on its own merit but it does not allow for a deep comprehension of the
2242 synergistic effect of milk components. Despite some research on the effect of milk
2243 components on the GIT and the gut microbiota, the synergistic effect of all the
2244 constituent components of fluid milk and variations of the standard bovine milk such
2245 as whole and lactose free, remains largely unexplored.

2246 **1.3.1.7 The prebiotic potential of the dairy product Glycomacropeptide (GMP)**

2247 Glycomacropeptide (GMP) is the glycosylated form of the caseinomacropeptide
2248 (CMP) that is released in whey during cheese production when chymosin (rennin)
2249 hydrolyses milk kappa casein (κ -CN) into para- κ -CN and caseinomacropeptide
2250 (CMP) fractions (Neelima, 2013). The genetic variations of the amino acid (AA)
2251 sequence of κ -CN and the variant phosphorylation and glycosylation sites contribute
2252 to the heterogeneity of the GMP peptides (reviewed in Thoma-Worringer *et al.*,
2253 2006). Approximately 50% of the bovine CMP is glycosylated with 5 variations of
2254 O-linked mono- to tetra-saccharide carbohydrate chains containing combinations of
2255 NeuAc, Gal and GalNAc (Thoma-Worringer *et al.*, 2006). Glycomacropeptide has a
2256 high content of essential and branched chain AA (EAA and BCAA respectively)
2257 compared to the average dietary protein and depending on the efficiency of GMP
2258 isolation and purification, none to trace amounts of the aromatic AA phenylalanine
2259 (Phe), tyrosine (Tyr) and tryptophan (Trp) (Etzel, 2004). In whey, GMP makes up
2260 approximately 25% of the protein content, and ultrafiltration of whey is
2261 recommended as the most efficient method for the commercial recovery of GMP
2262 (Neelima, 2013).

2263 Glycomacropeptide has a number of health relevant properties. Based on a number
 2264 of *in vitro* studies it was suggested that GMP may inhibit binding by *Cholera* toxin,
 2265 *Shigella flexneri*, *Salmonella enteritidis* and pathogenic *E. coli* to the mammalian
 2266 cell mostly due to its sialic acid content (Kawasaki *et al.*, 1992; Oh *et al.*, 2000;
 2267 Nakajima *et al.*, 2005; Bruck *et al.*, 2006; Feeney *et al.*, 2017). Glycomacropeptide
 2268 exerts some effect on the immune system as demonstrated in *in vitro* studies
 2269 (Requena *et al.*, 2009; Gong *et al.*, 2014). In murine models of induced colitis and
 2270 ileitis, GMP administration normalized the systemic and local inflammation and
 2271 attenuated the macroscopically observed GIT damage due to the inflammatory
 2272 treatment (Requena *et al.*, 2008; Lopez-Posadas *et al.*, 2010; Xu *et al.*, 2013; Ortega-
 2273 Gonzalez *et al.*, 2014; Cui *et al.*, 2017). Hvas *et al.* (2016) observed the anti-
 2274 inflammatory potential of GMP administration on patients with UC.
 2275 Glycomacropeptide has been successfully used in clinical trials as a naturally
 2276 occurring, low in Phe, dietary alternative to synthetic AA protein for
 2277 phenylketonuria (PKU) dietary management (Ney *et al.*, 2016; Ahring *et al.*, 2017).

 2278 There is inconclusive evidence on the prebiotic potential of GMP. Supplementation
 2279 of growth medium with GMP could sustain the growth of probiotic strains of
 2280 *Lactobacillus* and *Bifidobacterium* in pure culture conditions (Robitaille, 2013). The
 2281 growth promoting effect of GMP on probiotic *Lactobacillus* and *Bifidobacterium*
 2282 taxa was not confirmed when complex faecal microbiota from either infants or adults
 2283 was used for *in vitro* fermentations of GMP supplemented media (Bruck *et al.*, 2006;
 2284 Hernandez-Hernandez *et al.*, 2011; Ntemiri *et al.*, 2017; Chapter 2, this thesis).

 2285 In a piglet model GMP feeding resulted in the increase of *Lactobacillus* counts and
 2286 decrease of pathogenic *E.coli* (Hermes *et al.* 2013). Sawin *et al.* (2015) reported that

2287 wild type (WT) and PKU mice fed GMP-supplemented diet had significantly
2288 reduced relative abundance of the sulphur-reducing taxon *Desulfovibrio* in the faecal
2289 and caecal microbiota compared to mice fed casein and AA supplemented diet
2290 respectively. In the caecum and faecal microbiota of PKU mice fed GMP-
2291 supplemented diet the relative abundance of Bacteroidetes was significantly higher
2292 compared to an AA-based feeding regime; increase in Bacteroidetes was not
2293 observed for WT mice fed GMP (Sawin *et al.*, 2015). Sawin *et al.* (2015) provided
2294 valuable insight on the effect of GMP on the murine faecal and caecal microbiota in
2295 comparison to the AA and casein effect. However, the group did not provide
2296 compositional information of the baseline microbiota of WT and PKU and did not
2297 present microbiota data at a fine taxonomical level which would have put in context
2298 the observed effect of the diets on the microbiota. Recently, Jimenez *et al.* (2017)
2299 using an allergy rat model observed that GMP administration conferred significant
2300 increase in the faecal *Lactobacillus* and *Bifidobacterium* population. *Bacteroides*
2301 population also increased in rats with allergy after GMP administration (Jimenez *et*
2302 *al.*, 2017). These two recent papers report an effect of GMP on the Bacteroidetes
2303 population; baseline microbiota composition information is necessary in order to
2304 clarify what compositional “dysbiosis”, if any, was restored with GMP
2305 administration.

2306 **1.4 Aims and objectives**

2307 In this chapter have reviewed the general aspects of the gut microbiota composition,
2308 how the microbiota is established in the human gut at birth and during the first years
2309 of life, and how the gut microbiota composition and functionality is associated with
2310 health and disease throughout life span, and with ageing. Importantly, changes in the
2311 gut microbiota composition and functionality are associated with age-related frailty.
2312 The gut microbiota is a modifiable factor for maintaining health and ameliorating
2313 disease, and one of the means to modify the microbiota is the use of dietary
2314 components with prebiotic potential. This research thesis focused on exploring the
2315 potential of certain dietary substrates of animal- and plant-origin to modulate the gut
2316 microbiota of older people aiming at identifying changes in the gut microbiota
2317 composition that can be associated to a “healthy” gut microbiota profile. The dietary
2318 substrates of interest were the milk-derived glycomacropeptide (GMP), whole milk
2319 (with or without lactose) and blueberry fruit. Milk is a source of nutrients including
2320 carbohydrates and oligosaccharides but the prebiotic potential of milk and its by-
2321 products remains largely unexplored. Blueberries are rich in polyphenols; these
2322 compounds can modulate the gut microbiota but there are no clear associations
2323 between microbiota members and the bioconversion of phenolic compounds.
2324 Importantly, the potential of “dysbiotic” faecal microbiota to respond to dietary
2325 supplementation was investigated. An *in vitro* colon model was established in order
2326 to evaluate the prebiotic potential of the dietary substrates before proceeding to an *in*
2327 *vivo* model.

2328 1.5 References

- 2329 Adair, K. L., and Douglas, A. E. (2017). Making a microbiome: the many
2330 determinants of host-associated microbial community composition. *Curr Opin*
2331 *Microbiol*, 35, 23–29.
- 2332 Ahring, K. K., Lund, A. M., Jensen, E., Jensen, T. G., Brøndum-nielsen, K.,
2333 Pedersen, M., Bardow, A., Juul, J. *et al.* (2018). Comparison of
2334 Glycomacropeptide with Phenylalanine free synthetic amino acids in test meals
2335 to PKU patients: No significant differences in biomarkers, including plasma Phe
2336 level. *J Nutr Metabol*, 2018, 11.
- 2337 Allison, C., McFarlan, C., MacFarlane, G. T. (1989). Studies on mixed
2338 populations of human intestinal bacteria grown in single-stage and multistage
2339 continuous culture systems. *Appl Environ Microbiol*, 55(3), 672–8.
- 2340 Amiri, M., Diekmann, L., von Köckritz-Blickwede, M., Naim, H. Y. (2015). The
2341 diverse forms of lactose intolerance and the putative linkage to several cancers.
2342 *Nutrients*, 7(9), 7209–7230.
- 2343 Aroniadis, O. C., and Brandt, L. J. (2014). Intestinal microbiota and the efficacy
2344 of fecal microbiota transplantation in gastrointestinal disease. *Gastroenterol*
2345 *Hepatol*, 10(4), 230–237.
- 2346 Arrieta, M. C., Walter, J., Finlay, B. B. (2016). Human microbiota-associated
2347 mice: A model with challenges. *Cell Host Microbe*, 19(5), 575–578.
- 2348 Atarashi, K., Tanoue, T., Oshima, K., Suda, W., Nagano, Y., Nishikawa, H.,
2349 Fukuda, S., Saito, T., Narushima, S., Hase, K., *et al.* (2013). Treg induction by a

2350 rationally selected mixture of Clostridia strains from the human microbiota.
 2351 *Nature*, 500(7461), 232–236.

2352 Aune, D., Lau, R., Chan, D. S. M., Vieira, R., Greenwood, D. C., Kampman, E.,
 2353 Norat, T. (2012). Dairy products and colorectal cancer risk: A systematic review
 2354 and meta-analysis of cohort studies. *Ann Oncol*, 23(1), 37–45.

2355 Aune, D., Norat, T., Romundstad, P., Vatten, L. J. (2013). Dairy products and the
 2356 risk of type 2 diabetes : a systematic review and dose-response meta-analysis of
 2357 cohort studies. *Am J Clin Nutr*, 98(4), 1066–1083.

2358 Bäckhed, F., Ding, H., Wang, T., Hooper, L. V, Koh, G. Y., Nagy, A.,
 2359 Semenkovich, C. F., Gordon, J. I. (2004). The gut microbiota as an
 2360 environmental factor that regulates fat storage. *Proc Natl Acad Sci USA*, 101(44),
 2361 15718–23.

2362 Barzilai, N., Huffman, D. M., Muzumdar, R. H., Bartke, A. (2012). The critical
 2363 role of metabolic pathways in aging. *Diabetes*, 61(6), 1315–22.

2364 Benatar, J. R., Jones, E., White, H. and Stewart, R. A. (2014). A randomized trial
 2365 evaluating the effects of change in dairy food consumption on cardio-metabolic
 2366 risk factors. *Eur J Prev Cardiol*, 21(11), 1376-1386.

2367 Bereswill, S., Fischer, A., Plickert, R., Haag, L. M., Otto, B., Kühl, A. a., Dasti,
 2368 J. I., Zautner, A. E., Loddenkemper, C., Gross, U., Gobel, U. B., Heimesaat, M.
 2369 M. (2011). Novel murine infection models provide deep insights into the
 2370 “Ménage à trois” of campylobacter jejuni, microbiota and host innate immunity.
 2371 *PLoS One*, 6(6), e20953.

2372 Bergholdt, H. K. M., Nordestgaard, B. G., Varbo, A., Ellervik, C. (2017).
 2373 Lactase persistence, milk intake, and mortality in the Danish general population:
 2374 a Mendelian randomization study. *Eur J Epidemiol*, 33(2), 171-181.

2375 Bhinder, G., Allaire, J. M., Garcia, C., Lau, J. T., Chan, J. M., Ryz, N. R.,
 2376 Bosman, E. S., Graef, F. A., Crowley, S. M., *et al.* (2017). Milk fat globule
 2377 membrane supplementation in formula modulates the neonatal gut microbiome
 2378 and normalizes intestinal development. *Sci Rep*, 7, 45274.

2379 Bindels, L. B., Delzenne, N. M., Cani, P. D., Walter, J. (2015). Towards a more
 2380 comprehensive concept for prebiotics. *Nat Rev Gastroenterol Hepatol*, 12(5),
 2381 303–310.

2382 Bischoff-Ferrari, H. A., Dawson-Hughes, B., Baron, J. A., Kanis, J. A., Orav, E.
 2383 J., Staehelin, H. B., Kiel, D. P., Burckhardt, P., *et al.* (2011). Milk intake and risk
 2384 of hip fracture in men and women: A meta-analysis of prospective cohort studies.
 2385 *J Bone Miner Res*, 26(4), 833–839.

2386 Blackwood, B. P., Yuan, C. Y., Wood, D. R., Nicolas, J. D., Grothaus, J. S.,
 2387 Hunter, C. J. (2017). Probiotic *Lactobacillus* species Strengthen intestinal barrier
 2388 function and tight junction integrity in experimental necrotizing enterocolitis. *J*
 2389 *Probiotics Health*, 5(01), 1–20.

2390 Boirie, Y., Dangin, M., Gachon, P., Vasson, M. P., Maubois, J. L., Beaufrere, B.
 2391 (1997). Slow and fast dietary proteins differently modulate postprandial protein
 2392 accretion. *Proc Natl Acad Sci USA*, 94(26), 14930–14935.

2393 Bode, L., Contractor, N., Barile, D., Pohl, N., Prudden, A. R., Boons, G. J., Jin,
 2394 Y. S., Jennewein, S. (2016). Overcoming the limited availability of human milk

2395 oligosaccharides: Challenges and opportunities for research and application. *Nutr*
2396 *Rev*, 74(10), 635–644.

2397 Brück, W. M., Kelleher, S. L., Gibson, G. R., Graverholt, G., Lönnerdal, B. L.
2398 (2006). The effects of α -lactalbumin and glycomacropeptide on the association of
2399 CaCo-2 cells by enteropathogenic *Escherichia coli*, *Salmonella typhimurium* and
2400 *Shigella flexneri*. *FEMS Microbiol Lett*, 259(1), 158–162.

2401 Brück, W. M., Redgrave, M., Tuohy, K. M., Lönnerdal, B., Graverholt, G.,
2402 Hernell, O., Gibson, G. R. (2006). Effects of bovine alpha-lactalbumin and
2403 casein glycomacropeptide-enriched infant formulae on faecal microbiota in
2404 healthy term infants. *J Pediatr Gastroenterol Nutr* 43(5), 673–9.

2405 Buffie, C. G., Bucci, V., Stein, R. R., McKenney, P. T., Ling, L., Gobourne, A.,
2406 No, D., Liu, H., Kinnebrew, M., *et al.* (2015). Precision microbiome
2407 reconstitution restores bile acid mediated resistance to *Clostridium difficile*.
2408 *Nature*, 517(7533), 205–208.

2409 Camfield, D. A., Owen, L., Scholey, A. B., Pipingas, A., Stough, C. (2011).
2410 Dairy constituents and neurocognitive health in ageing. *Br J Nutr*, 106(2), 159–
2411 174.

2412 Cammarota, G., Ianiro, G., Tilg, H., Rajilić-Stojanović, M., Kump, P., Satokari,
2413 R., Sokol, H., Arkkila, P., Pintus, C., Hart, A., *et al.* (2017). European consensus
2414 conference on faecal microbiota transplantation in clinical practice. *Gut*, 66(4),
2415 569-580.

2416 Cardarelli, H. R., Martinez, R. C. R., Albrecht, S., Schols, H., Franco, B. D. G.
2417 M., Saad, S. M. I., Smidt, H. (2016). *In vitro* fermentation of prebiotic

2418 carbohydrates by intestinal microbiota in the presence of *Lactobacillus*
 2419 *amylovorus* DSM 16998. *Benef Microbes*, 7(1), 119–133.

2420 Catrysse, L., and van Loo, G. (2017). Inflammation and the metabolic syndrome:
 2421 The tissue-specific functions of NF-κB. *Trends Cell Biol*, 27(6), 417–429.

2422 Charbonneau, M. R., O'Donnell, D., Blanton, L. V., Totten, S. M., Davis, J. C.
 2423 C., Barratt, M. J., Cheng, J., Guruge, J., Talcott, M., *et al.* (2016). Sialylated milk
 2424 oligosaccharides promote microbiota-dependent growth in models of infant
 2425 undernutrition. *Cell*, 164(5), 859–871.

2426 Chung, H., Pamp, S. J., Hill, J. A., Surana, N. K., Sanna, M., Troy, E. B.,
 2427 Reading, N. C., Villablanca, E. J., Wang, S. *et al.* (2013). Gut immune
 2428 maturation depends on colonization with a host-specific microbiota, 149(7),
 2429 1578–1593.

2430 Chung, W. S. F., Walker, A. W., Louis, P., Parkhill, J., Vermeiren, J., Bosscher,
 2431 D., Duncan, S. H., Flint, H. J. (2016). Modulation of the human gut microbiota
 2432 by dietary fibres occurs at the species level. *BMC Biol*, 14(1), 3.

2433 Cinquin, C., Le Blay, G., Fliss, I., Lacroix, C. (2006). New three-stage *in vitro*
 2434 model for infant colonic fermentation with immobilized fecal microbiota. *FEMS*
 2435 *Microbiol Ecol*, 57(2), 324–36.

2436 Clegg, A., Young, J., Iliffe, S., Rikkert, M. O., Rockwood, K. (2013). Frailty in
 2437 elderly people. *The Lancet*, 381(9868), 752–762.

2438 Clemente, J. C., Pehrsson, E. C., Blaser, M. J., Sandhu, K., Gao, Z., Wang, B.,
 2439 Magris, M., Hidalgo, G., Contreras, M., Noya-Alacron, O., *et al.* (2015). The
 2440 microbiome of uncontacted Amerindians. *Sci Adv*, 1(3), e1500183.

2441 Cockburn, D. W., and Koropatkin, N. M. (2016). Polysaccharide degradation by
 2442 the intestinal microbiota and its influence on human health and disease. *J Mol*
 2443 *Biol*, 428(16), 3230–3252.

2444 Collins, J., Auchtung, J. M., Schaefer, L., Eaton, K. A., Britton, R. A. (2015).
 2445 Humanized microbiota mice as a model of recurrent *Clostridium difficile* disease.
 2446 *Microbiome*, 3, 35.

2447 Crichton, G. E., and Alkerwi, A. (2014). Whole-fat dairy food intake is inversely
 2448 associated with obesity prevalence: Findings from the Observation of
 2449 Cardiovascular Risk Factors in Luxembourg study. *Nutr Res*, 34(11), 936–943.

2450 Crichton, G. E., Murphy, K. J., Bryan, J. (2010). Dairy intake and cognitive
 2451 health in middle-aged South Australians. *Asia Pac J Clin Nutr*, 19(2), 161–171.

2452 Cui, Y., Zhu, C., Ming, Z., Cao, J., Yan, Y., Zhao, P., Pang, G., Deng, Z., Yao,
 2453 Y., Chen, Q. (2017). Molecular mechanisms by which casein glycomacropeptide
 2454 maintains internal homeostasis in mice with experimental ulcerative colitis. *PLoS*
 2455 *One*, 12(7), e0181075.

2456 Deeks, S. G. (2011). HIV infection, inflammation, immunosenescence, and
 2457 aging. *Annu Rev Med*, 62, 141–55.

2458 de Jonge, E. A. L., Kiefte-De Jong, J. C., Hofman, A., Uitterlinden, A. G.,
 2459 Kieboom, B. C. T., Voortman, T., Franco, O. H, Rivadeneira, F. (2017). Dietary

2460 patterns explaining differences in bone mineral density and hip structure in the
 2461 elderly: The Rotterdam study. *Am J Clin Nutr*, 105(1), 203–211.

2462 de Moura Bell, J. M. L. N., Cohen, J. L., de Aquino, L. F. M. C., Lee, H., de
 2463 Melo Silva, V. L., Liu, Y., Domizio, P., Barile, D. (2018). An integrated
 2464 bioprocess to recover bovine milk oligosaccharides from colostrum whey
 2465 permeate. *J Food Eng*, 216, 27–35.

2466 Deng, Y., Misselwitz, B., Dai, N., Fox, M. (2015). Lactose intolerance in adults:
 2467 Biological mechanism and dietary management. *Nutrients*, 7(9), 8020–8035.

2468 Díaz-López, A., Bulló, M., Martínez-González, M. A., Corella, D., Estruch, R.,
 2469 Fitó, M., Gomez-Gracia, E., Fiol, M., Garcia de la Corte, F. J., *et al.* (2016).
 2470 Dairy product consumption and risk of type 2 diabetes in an elderly Spanish
 2471 Mediterranean population at high cardiovascular risk. *Eur J Nutr*, 55(1), 349–360.

2472 Dong, J. Y., Zhang, L., He, K., Qin, L. Q. (2011). Dairy consumption and risk of
 2473 breast cancer: a meta-analysis of prospective cohort studies. *Breast Cancer Res*
 2474 *Treat*, 127(1), 23–31.

2475 Downer, M. K., Batista, J. L., Mucci, L. A., Stampfer, M. J., Epstein, M. M.,
 2476 Håkansson, N., Wolk, A., Johansson, J. E., Andren, O., Fall, K., Andersson, S.
 2477 O. (2017). Dairy intake in relation to prostate cancer survival. *Int J Cancer*,
 2478 140(9), 2060–2069

2479 Drehmer, M., Pereira, M. A., Ine, M., Alvim, S., Lotufo, P. A., Luft, V. C.,
 2480 Duncan, B. B. (2016). Total and full-fat, but not low-fat, dairy product intakes
 2481 are inversely associated with metabolic syndrome in adults. *J Nutr*, 146(1), 81–
 2482 89.

2483 Duncan, S. H., and Flint, H. J. (2013). Probiotics and prebiotics and health in
2484 ageing populations. *Maturitas*, 75(1), 44–50.

2485 Durosier-Izart, C., Biver, E., Merminod, F., Van Rietbergen, B., Chevalley, T.,
2486 Herrmann, F. R., Ferrari, S. L., Rizzoli, R. (2017). Peripheral skeleton bone
2487 strength is positively correlated with total and dairy protein intakes in healthy
2488 postmenopausal women. *Am J Clin Nutr*, 105(2), 513–525.

2489 El Aidy, S., Derrien, M., Aardema, R., Hooiveld, G., Richards, S. E., Dane, A.,
2490 Dekker, J., Vreeken, R., *et al.* (2014). Transient inflammatory-like state and
2491 microbial dysbiosis are pivotal in establishment of mucosal homeostasis during
2492 colonisation of germ-free mice. *Benef Microbes*, 5(1), 67–77.

2493 El Aidy, S., van Baarlen, P., Derrien, M., Lindenbergh-Kortleve, D. J., Hooiveld,
2494 G., Levenez, F., Dore, J., Dekker, J. *et al.* (2012). Temporal and spatial interplay
2495 of microbiota and intestinal mucosa drive establishment of immune homeostasis
2496 in conventionalized mice. *Mucosal Immunol*, 5(5), 567–579.

2497 Ellekilde, M., Selfjord, E., Larsen, C. S., Jakešević, M., Rune, I., Tranberg, B.,
2498 Vogensen, F. K., Nielsen, D. S., Bahl, M. I., Licht, T. R., Hansen, A. K., Hansen,
2499 C. H. (2015). Transfer of gut microbiota from lean and obese mice to antibiotic-
2500 treated mice. *Sci Rep*, 4(1), 5922.

2501 Etzel, M. R. (2004). Manufacture and use of dairy protein fractions. *J. Nutr.*,
2502 134(4), 996S–1002S.

2503 Evershed, R. P., Payne, S., Sherratt, A. G., Copley, M. S., Coolidge, J., Urem-
2504 Kotsu, D., *et al.* (2008). Earliest date for milk use in the Near East and
2505 southeastern Europe linked to cattle herding. *Nature*, 455(7212), 528–531.

2506 Farsijani, S., Payette, H., Morais, J. A., Shatenstein, B., Gaudreau, P., Chevalier,
2507 S. (2017). Dairy consumption is associated with body composition, physical
2508 function and frailty in community-dwelling older adults: The Quebec NuAge
2509 longitudinal study. *FASEB*, 31(1), 139-146.

2510 Feeney, S., Ryan, J., Kilcoyne, M., Joshi, L., Hickey, R. (2017).
2511 Glycomacropeptide reduces intestinal epithelial cell barrier dysfunction and
2512 adhesion of entero-hemorrhagic and entero-pathogenic *Escherichia coli* *in vitro*.
2513 *Foods*, 6(11), 93.

2514 Fehlbaum, S., Chassard, C., Haug, M. C., Fourmestraux, C., Derrien, M.,
2515 Lacroix, C. (2015). Design and investigation of PolyFermS *in vitro* continuous
2516 fermentation models inoculated with immobilized fecal microbiota mimicking
2517 the elderly colon. *PloS One*, 10(11), e0142793.

2518 Fekete, Á. A., Givens, D. I., Lovegrove, J. A. (2016). Can milk proteins be a
2519 useful tool in the management of cardiometabolic health? An updated review of
2520 human intervention trials. *Proc Nutr Soc*, 75(3), 828-41.

2521 Fielding RA, Vellas B, Evans WJ, Bhasin S, Morley JE, Newman AB, Abellan
2522 van Kan G, Andrieu S, Bauer J, Breuille D, Cederholm T, Chandler J, *et al.*
2523 (2011). Sarcopenia: An undiagnosed condition in older adults. Current consensus
2524 definition: Prevalence, etiology, and consequences. *J Am Med Dir Assoc*, 12(4),
2525 249–256.

2526 Flint, H. J., Duncan, S. H., Louis, P. (2017). The impact of nutrition on intestinal
2527 bacterial communities. *Curr Opin Microbiol*, 38, 59–65.

2528 Fransen, F., van Beek, A. A., Borghuis, T., El Aidy, S., Hugenholtz, F., van der
2529 Gaast - de Jongh, C., Savelkoul, H. F. J., *et al.* (2017). Aged gut microbiota
2530 contributes to systemical inflammaging after transfer to germ-free mice. *Front*
2531 *Immunol*, 8, 1385.

2532 Gareau, M. G., Sherman, P. M., Walker, W. A. (2010). Probiotics and the gut
2533 microbiota in intestinal health and disease. *Nat Rev Gastroenterol Hepatol*, 7(9),
2534 503–514.

2535 Gerbault, P., Liebert, A., Itan, Y., Powell, A., Currat, M., Burger, J., Swallow, D.
2536 M., Thomas, M. G. (2011). Evolution of lactase persistence: an example of
2537 human niche construction. *Philos Trans R Soc Lond B Biol Sci*, 366(1566), 863–
2538 77.

2539 Gibson, G. R., Hutkins, R., Sanders, M. E., Prescott, S. L., Reimer, R. A.,
2540 Salminen, S., Scott, K., Stanton, C., *et al.* (2017). Expert consensus document:
2541 The International Scientific Association for Probiotics and Prebiotics (ISAPP)
2542 consensus statement on the definition and scope of prebiotic. *Nat Rev*
2543 *Gastroenterol Hepatol*, 11(8), 506–514.

2544 Gibson, G. R., and Roberfroid, M. B. (1995). Dietary modulation of the human
2545 colonic microbiota : Introducing the concept of prebiotics. *J Nutr*, 125(6), 1401–
2546 12.

2547 Gilbert, J. A., Quinn, R. A., Debelius, J., Xu, Z. Z., Morton, J., Garg, N.,
2548 Jansson, J. K., Dorrestein, P. C., Knight, R. (2016). Microbiome-wide
2549 association studies link dynamic microbial consortia to disease. *Nature*,
2550 535(7610), 94–103.

2551 Goh, Y. J., and Klaenhammer, T. R. (2015). Genetic mechanisms of prebiotic
 2552 oligosaccharide metabolism in probiotic microbes. *Annu Rev Food Sci Technol*,
 2553 6(1), 137–156.

2554 Gong, J., Chen, Q., Yan, Y., Pang, G. (2014). Effect of casein glycomacropeptide
 2555 on subunit p65 of nuclear transcription factor- κ B in lipopolysaccharide-
 2556 stimulated human colorectal tumor HT-29 cells. *Food Science Human Wellness*,
 2557 3(2), 51–55.

2558 Gopal, P. K., and Gill, H. S. (2000). Oligosaccharides and glycoconjugates in
 2559 bovine milk and colostrum. *British J Nutr*, 84 Suppl 1, S69-74.

2560 Guo, J., Astrup, A., Lovegrove, J. A., Gijsbers, L., Givens, D. I., Soedamah-
 2561 Muthu, S. S. (2017). Milk and dairy consumption and risk of cardiovascular
 2562 diseases and all-cause mortality: dose–response meta-analysis of prospective
 2563 cohort studies. *Eur J Epidemiol*, 32(4), 269–287.

2564 Gupta, S., Allen-Vercoe, E., Petrof, E. O. (2016). Fecal microbiota
 2565 transplantation: in perspective. *Therap Adv Gastroenterol*, 9(2), 229–39.

2566 Hall, W. L., Millward, D. J., Long, S. J., Morgan, L. M. (2003). Casein and whey
 2567 exert different effects on plasma amino acid profiles, gastrointestinal hormone
 2568 secretion and appetite. *Br J Nutr*, 89(2), 239.

2569 Hartman, J. W., Tang, J. E., Wilkinson, S. B., Tarnopolsky, M. A., Lawrence, R.
 2570 L., Fullerton, A. V., Phillips, S. M. (2007). Consumption of fat-free fluid milk
 2571 after resistance exercise promotes greater lean mass accretion than does
 2572 consumption of soy or carbohydrate in young, novice, male weightlifters. *Am J*
 2573 *Clin Nutr*, 86(2), 373–381.

2574 Haug, A., Høstmark, A. T., Harstad, O. M. (2007). Bovine milk in human
2575 nutrition – a review. *Lipids Health Dis*, 6(25), 25.

2576 He, Z., Li, P., Zhu, J., Cui, B., Xu, L., Xiang, J., Zhang, T., Long, C., Huang, J.
2577 G., Nie, Y., Wu, K., Fan, D., Zhang, F. (2017). Multiple fresh fecal microbiota
2578 transplants induces and maintains clinical remission in Crohn's disease
2579 complicated with inflammatory mass. *Sci Rep*, 7(1), 4753.

2580 Heaney, R. P. (2013). Dairy intake, dietary adequacy, and lactose intolerance.
2581 *Adv Nutr*, 4(2), 151–156.

2582 Hernandez-Hernandez, O., Sanz, M. L., Kolida, S., Rastall, R. A, Moreno, F. J.
2583 (2011). *In vitro* fermentation by human gut bacteria of proteolytically digested
2584 caseinomacropeptide nonenzymatically glycosylated with prebiotic
2585 carbohydrates. *J Agric Food Chem*, 59(22), 11949–55.

2586 Hintze, K. J., Cox, J. E., Rompato, G., Benninghoff, A. D., Ward, R. E.,
2587 Broadbent, J., Lefevre, M. (2014). Broad scope method for creating humanized
2588 animal models for animal health and disease research through antibiotic
2589 treatment and human fecal transfer. *Gut Microbes*, 5(2), 183–91.

2590 Hjartåker, A., Thoresen, M., Engeset, D., Lund, E. (2010). Dairy consumption
2591 and calcium intake and risk of breast cancer in a prospective cohort: the
2592 Norwegian Women and Cancer study. *Cancer Causes Control*, 21(11), 1875–85.

2593 Hobden, M. R., Martin-Morales, A., Guérin-Deremaux, L., Wils, D., Costabile,
2594 A., Walton, G. E., Rowland, I., Kennedy, O. B., Gibson, G. R. (2013). *In Vitro*
2595 fermentation of NUTRIOSE(®) FB06, a wheat dextrin soluble fibre, in a
2596 continuous culture human colonic model system. *PloS One*, 8(10), e77128.

2597 Horner, K., Drummond, E., Brennan, L. (2016). Bioavailability of milk protein-
 2598 derived bioactive peptides: A glycaemic management perspective. *Nutr Res Rev*,
 2599 29(1), 91–101.

2600 Hryckowian, A. J., Pruss, K. M., Sonnenburg, J. L. (2017). The emerging
 2601 metabolic view of *Clostridium difficile* pathogenesis. *Curr Opin Microbiol*, 35,
 2602 42–47.

2603 Hsieh, C. C., Hernandez-Ledesma, B., Fernandez-Tome, S., Weinborn, V.,
 2604 Barile, D., De Moura Bell, J. M. L. N. (2015). Milk proteins, peptides, and
 2605 oligosaccharides: Effects against the 21st century disorders. *BioMed Res Int*,
 2606 2015, 146840.

2607 Hugenholtz, F., and de Vos, W. M. (2018). Mouse models for human intestinal
 2608 microbiota research: a critical evaluation. *Cell Mol Lif Sci*, 75(1), 149–160.

2609 Hvas, C. L., Dige, A., Bendix, M., Wernlund, P. G., Christensen, L. A., Dahlerup,
 2610 J. F., Agnholt, J. (2016). Casein glycomacropeptide for active distal ulcerative
 2611 colitis: a randomised pilot study. *Eur J Clin Invest*, 46(6), 555–563.

2612 Jackson, M., Jeffery, I. B., Beaumont, M., Bell, J. T., Clark, A. G., Ley, R. E.,
 2613 O'Toole, P. W., Spector, T. D., Steves, C. J. (2016). Signatures of early frailty in
 2614 the gut microbiota. *Genome Med*, 8(1), 8.

2615 Jun, S., Ha, K., Chung, S., Joung, H. (2016). Meat and milk intake in the rice-
 2616 based Korean diet: impact on cancer and metabolic syndrome. *Proc Nutr Soc*,
 2617 75(3), 374-84.

2618 Karav, S., Le Parc, A., Leite Nobrega de Moura Bell, J. M., Frese, S. A., Kirmiz,
 2619 N., Block, D. E., Barile, D., Mills, D. A. (2016). Oligosaccharides released from
 2620 milk glycoproteins are selective growth substrates for infant-associated
 2621 bifidobacteria. *Appl Environ Microbiol*, 82(12), 3622–3630.

2622 Karczewski, J., Troost, F. J., Konings, I., Dekker, J., Kleerebezem, M.,
 2623 Brummer, R. M., Wells, J. M. (2010). Regulation of human epithelial tight
 2624 junction proteins by *Lactobacillus plantarum* in vivo and protective effects on the
 2625 epithelial barrier. *Am J Physiol Gastrointest Liver Physiol.*, 298, G851–G859.

2626 Kawasaki, Y., Isoda, H., Tanimoto, M., Dosako, S., Idota, T., Ahiko, K. (1992).
 2627 Inhibition by lactoferrin and kappa-casein glycomacropeptide of binding of
 2628 cholera toxin to its receptor. *Biosci Biotech Biochem*, 52(2), 195–198.

2629 Keevil, V. L., and Romero-Ortuno, R. (2015). Ageing well: a review of
 2630 sarcopenia and frailty. *Proc Nutr Soc*, 74(4), 337-47.

2631 Kelly, C. J., Zheng, L., Campbell, E. L., Saeedi, B., Scholz, C. C., Bayless, A. J.,
 2632 Wilson, K. E., Glover, L. E., Kominsky, D. J., *et al.* (2015). Crosstalk between
 2633 microbiota-derived short-chain fatty acids and intestinal eEpithelial HIF
 2634 augments tissue barrier function. *Cell Host Microbe*, 17(5), 662–671.

2635 Khanna, S., Pardi, D. S., Kelly, C. R., Kraft, C. S., Dhere, T., Henn, M. R.,
 2636 Lombardo, M. J., Viluc, M., Oshumi, T., *et al.* (2016). A novel microbiome
 2637 therapeutic increases gut microbial diversity and prevents recurrent *Clostridium*
 2638 *difficile* infection. *J Infect Dis*, 214(2), 173–181.

2639 Khanna, S., Vazquez-Baeza, Y., González, A., Weiss, S., Schmidt, B., Muñoz-
 2640 Pedrego, D. A., Rainey, J. F., Kammer, P., *et al.* (2017). Changes in microbial

2641 ecology after fecal microbiota transplantation for recurrent *C. difficile* infection
 2642 affected by underlying inflammatory bowel disease. *Microbiome*, 5(1), 55.

2643 König, J., Wells, J., Cani, P. D., García-Ródenas, C. L., MacDonald, T.,
 2644 Mercenier, A., Whyte, J., Troost, F., Brummer, R. J. (2016). Human intestinal
 2645 barrier function in health and disease. *Clin Transl Gastroenterol*, 7(10), e196.

2646 Koutsos, A., Lima, M., Conterno, L., Gasperotti, M., Bianchi, M., Fava, F.,
 2647 Vrhovsek, U., Lovegrove, J. A., Tuohy, K. M. (2017). Effects of commercial
 2648 apple varieties on human gut microbiota composition and metabolic output using
 2649 an in vitro colonic model. *Nutrients*, 9(6), pii E533.

2650 Kurilshikov, A., Wijmenga, C., Fu, J., Zhernakova, A. (2017). Host genetics and
 2651 gut microbiome: Challenges and perspectives. *Trends Immunol*, 38(9), 633–647.

2652 Lagier, J. C., Hugon, P., Khelaifia, S., Fournier, P. E., La Scola, B., Raoult, D.
 2653 (2015). The rebirth of culture in microbiology through the example of
 2654 culturomics to study human gut microbiota. *Clin Microbiol Rev*, 28(1), 237–264.

2655 Lana, A., Rodriguez-Artalejo, F., Lopez-Garcia, E. (2015). Dairy consumption
 2656 and risk of frailty in older adults: A prospective cohort study. *J Am Geriatr Soc*,
 2657 63(9), 1852–1860.

2658 Larsson, S. C., Crippa, A., Orsini, N., Wolk, A., Michaëlsson, K. (2015). Milk
 2659 consumption and mortality from all causes, cardiovascular disease, and cancer: A
 2660 systematic review and meta-analysis. *Nutrients*, 7(9), 7749–7763.

2661 Landi, F., Calvani, R., Tosato, M., Martone, A. M., Ortolani, E., Saveria, G.,
 2662 D’Angelo, E., Sisto, A., Marzetti, E. (2016). Protein intake and muscle health in

2663 old age: From biological plausibility to clinical evidence. *Nutrients*, 8(5), pii
 2664 E295.

2665 Larsson, S. C., Orsini, N., Wolk, A. (2006). Milk, milk products and lactose
 2666 intake and ovarian cancer risk: A meta-analysis of epidemiological studies. *Int J*
 2667 *Cancer*, 118(2), 431–441.

2668 Lebeer, S., Bron, P. A., Marco, M. L., Van Pijkeren, J. P., O’Connell
 2669 Motherway, M., Hill, C., Pot, B., Roos, S., Klaenhammer, T. (2018).
 2670 Identification of probiotic effector molecules: present state and future
 2671 perspectives. *Curr Opin Biotechnol*, 49, 217–223.

2672 Lee, H., Zavaleta, N., Chen, S., Lönnerdal, B., Slupsky, C. (2018). Effect of
 2673 bovine milk fat globule membranes as a complementary food on the serum
 2674 metabolome and immune markers of 6-11-month-old Peruvian infants. *Npj*
 2675 *Science of Food*, 2(1), 6.

2676 Levy, M., Kolodziejczyk, A. A., Thaïss, C. A., Elinav, E. (2017). Dysbiosis and
 2677 the immune system. *Nat Rev Immunol*, 17(4), 219–232.

2678 Lewis, B. B., Buffie, C. G., Carter, R. A., Leiner, I., Toussaint, N. C., Miller, L.
 2679 C., Gobourne, A., Ling, L., Pamer, E. G. (2015). Loss of microbiota-mediated
 2680 colonisation resistance to *Clostridium difficile* infection with oral vancomycin
 2681 compared with metronidazole. *J Infect Dis*, 212(10), 1656–65.

2682 Ley, R. E., Bäckhed, F., Turnbaugh, P., Lozupone, C. A, Knight, R. D., Gordon,
 2683 J. I. (2005). Obesity alters gut microbial ecology. *Proc Natl Acad Sci USA*,
 2684 102(31), 11070–5.

2685 Li, G., Thabane, L., Papaioannou, A., Ioannidis, G., Levine, M. A. H., Adachi, J.
 2686 D. (2017). An overview of osteoporosis and frailty in the elderly. *BMC*
 2687 *Musculoskelet Disord*, 18(1), 46.

2688 Miyauchi, E., O’Callaghan, J., Buttó, L. F., Hurley, G., Melgar, S., Tanabe, S.,
 2689 Shanahan, F., Nally, K., O’Toole, P.W. (2012). Mechanism of protection of
 2690 transepithelial barrier function by *Lactobacillus salivarius*: strain dependence
 2691 and attenuation by bacteriocin production. *Am J Physiol Gastrointest Liver*
 2692 *Physiol*, 303(9), G1029–G1041.

2693 O’Riordan, N., Kane, M., Joshi, L., Hickey, R. M. (2014). Structural and
 2694 functional characteristics of bovine milk protein glycosylation. *Glycobiology*,
 2695 24(3), 220–236.

2696 Rocio Lopez-Posadas, Pilar Requena, Raquel Gonzalez, Maria Dolores Suarez,
 2697 Antonio Zarzuelo, Fermin Sanchez de Medina, Martinez-Augustin, O. (2010).
 2698 Bovine glycomacropeptide has intestinal antiinflammatory effects in rats with
 2699 dextran sulfate-induced colitis. *J Nutr*, 140(11), 2014–2019.

2700 Lagkouvardos, I., Pukall, R., Abt, B., Foesel, B. U., Meier-Kolthoff, J. P.,
 2701 Kumar, N., *et al.* (2016). The Mouse Intestinal Bacterial Collection (miBC)
 2702 provides host-specific insight into cultured diversity and functional potential of
 2703 the gut microbiota. *Nat Microbiol*, 1(10), 16131.

2704 Langille, M. G. I., Meehan, C. J., Koenig, J. E., Dhanani, A. S., Rose, R. A.,
 2705 Howlett, S. E., Beiko, R. G. (2014). Microbial shifts in the aging mouse gut.
 2706 *Microbiome*, 2(1), 1–12.

2707 Larcombe, S., Hutton, M. L., Lyras, D. (2016). Involvement of bacteria other
 2708 than *Clostridium difficile* in antibiotic-associated diarrhoea. *Trends Microbiol*,
 2709 24(6), 463–476.

2710 Li, H., Qi, Y., and Jasper, H. (2016). Preventing age-related decline of gut
 2711 compartmentalization limits microbiota dysbiosis and extends lifespan. *Cell Host*
 2712 *Microbe*, 19(2), 240–253.

2713 Li, S. S., Zhu, A., Benes, V., Costea, P. I., Hercog, R., Hildebrand, F., Huerta-
 2714 Cepas, J., Nieuwdorp, M., Salojarvi, J., *et al.* (2016). Durable coexistence of
 2715 donor and recipient strains after fecal microbiota transplantation. *Science*,
 2716 352(6285), 586–9.

2717 Likotrafiti, E., Tuohy, K. M., Gibson, G. R., Rastall, R. A. (2014). An *in vitro*
 2718 study of the effect of probiotics, prebiotics and synbiotics on the elderly faecal
 2719 microbiota. *Anaerobe*, 27, 50–55.

2720 Liu, Y., Gibson, G. R., Walton, G. E. (2016). An *in vitro* approach to study
 2721 effects of prebiotics and probiotics on the faecal microbiota and selected immune
 2722 parameters relevant to the elderly. *PLoS One*, 11(9), 1–18.

2723 Lovegrove, J. A., and Hobbs, D. A. (2016). Milk and dairy produce and CVD:
 2724 new perspectives on dairy and cardiovascular health. *Proc Nutr Soc*, 44, 1–12.

2725 Lundberg, R., Toft, M. F., August, B., Hansen, A. K., Hansen, C. H. F. (2016).
 2726 Antibiotic-treated versus germ-free rodents for microbiota transplantation
 2727 studies. *Gut Microbes*, 7(1), 68–74.

2728 Lusi, A. J., Attie, A. D., Reue, K. (2008). Metabolic syndrome: From
 2729 epidemiology to systems biology. *Nat Rev Gen*, 9(11), 819–830.

2730 Macfarlane, G. T., and Macfarlane, S. (2007). Models for intestinal fermentation:
 2731 association between food components, delivery systems, bioavailability and
 2732 functional interactions in the gut. *Curr Opin Biotechnol*, 18(2), 156–62.

2733 Macfarlane, G., Macfarlane, S., Gibson, G. (1998). Validation of a three-stage
 2734 compound continuous culture system for investigating the effect of retention
 2735 time on the ecology and metabolism of bacteria in the human colon. *Microb*
 2736 *Ecol*, 35(2), 180–7.

2737 Macfarlane, S., Woodmansey, E. J., George, T., Macfarlane, G. T. (2005).
 2738 Colonisation of mucin by human intestinal bacteria and establishment of biofilm
 2739 communities in a two-stage continuous culture system. *Appl Environ Microbiol*,
 2740 71(11), 7483–7492.

2741 Mancabelli, L., Milani, C., Lugli, G. A., Turrone, F., Ferrario, C., van Sinderen,
 2742 D., Ventura, M. (2017). Meta-analysis of the human gut microbiome from
 2743 urbanized and pre-agricultural populations. *Environ Microbiol*, 19(4), 1379–
 2744 1390.

2745 Mansson, H. L. (2008). Fatty acids in bovine milk fat. *Food Nutr Res*, 52, 1–3.

2746 Marchesi, J. R., Adams, D. H., Fava, F., Hermes, G. D. A., Hirschfield, G. M.,
 2747 Hold, G., Quraishi, M. N., Kinross, J., *et al.* (2016). The gut microbiota and host
 2748 health: a new clinical frontier. *Gut*, 65(2), 330–390.

2749 Marcobal, A, Kashyap, P. C., Nelson, T. A, Aronov, P. A, Donia, M. S.,
 2750 Spormann, A, *et al.* (2013). A metabolomic view of how the human gut
 2751 microbiota impacts the host metabolome using humanized and gnotobiotic mice.
 2752 *ISME J*, 7(10), 1933–43.

2753 Martín, R., Bermúdez-Humarán, L. G., Langella, P. (2016). Gnotobiotic rodents:
 2754 An *in vivo* model for the study of microbe-microbe interactions. *Front Microbiol*,
 2755 7, 409.

2756 Martinez, R. C. R., Cardarelli, H. R., Borst, W., Albrecht, S., Schols, H.,
 2757 Gutiérrez, O. P., Maathuis, A. J., de Meiro Franco, B. D., *et al.* (2013). Effect of
 2758 galactooligosaccharides and *Bifidobacterium animalis* Bb-12 on growth of
 2759 *Lactobacillus amylovorus* DSM 16698, microbial community structure, and
 2760 metabolite production in an in vitro colonic model set up with human or pig
 2761 microbiota. *FEMS Microbiol Ecol*, 84(1), 110–123.

2762 Martz, S. L. E., McDonald, J. A. K., Sun, J., Zhang, Y., Gloor, G. B., *et al.*
 2763 (2015). Administration of defined microbiota is protective in a murine
 2764 *Salmonella* infection model. *Sci Rep*, 5, 16094.

2765 Marzorati, M., Vanhoecke, B., De Ryck, T., Sadaghian Sadabad, M., Pinheiro, I.,
 2766 Possemiers, S., Van den Abbeele, P. Derycke, M., *et al.* (2014). The HMITM
 2767 module: A new tool to study the Host-Microbiota Interaction in the human
 2768 gastrointestinal tract *in vitro*. *BMC Microbiol*, 14, 133.

2769 McCarville, J. L., Caminero, A., Verdu, E. F. (2016). Novel perspectives on
 2770 therapeutic modulation of the gut microbiota. *Therap Adv Gastroenterol*, 9(4),
 2771 580–593.

2772 Mehra, R., Barile, D., Marotta, M., Lebrilla, C. B., Chu, C., German, J. B.
 2773 (2014). Novel high-molecular weight fucosylated milk oligosaccharides
 2774 identified in dairy streams. *PLoS One*, 9(5), 1–7.

2775 Messer, J. S., Liechty, E. R., Vogel, O. A., Chang, E. B. (2017). Evolutionary
 2776 and ecological forces that shape the bacterial communities of the human gut.
 2777 *Mucosal Immunol*, 10(3), 567–579.

2778 Michaelsson, K., Wolk, A., Langenskiöld, S., Basu, S., Warensjö Lemming, E.,
 2779 Melhus, H., Byberg, L. (2014). Milk intake and risk of mortality and fractures in
 2780 women and men: cohort studies. *BMJ*, 349, g6015.

2781 Misselwitz, B., Pohl, D., Fruhauf, H., Fried, M., Vavricka, S. R., Fox, M. (2013).
 2782 Lactose malabsorption and intolerance: pathogenesis, diagnosis and treatment.
 2783 *United European Gastroenterol J*, 1(3), 151–159.

2784 Mitchell, C. J., McGregor, R. A., D’Souza, R. F., Thorstensen, E. B., Markworth,
 2785 J. F., Fanning, A. C., Poppitt, S. D., Cameron-Smith, D. (2015). Consumption of
 2786 milk protein or whey protein results in a similar increase in muscle protein
 2787 synthesis in middle aged men. *Nutrients*, 7(10), 8685–8699.

2788 Mitchell, W. K., Wilkinson, D. J., Phillips, B. E., Lund, J. N., Smith, K.,
 2789 Atherton, P. J. (2016). Human skeletal muscle protein metabolism responses to
 2790 amino acid nutrition. *Adv Nutr*, 7(4), 828S–38S.

2791 Miquel, S., Martín, R., Rossi, O., Bermúdez-Humarán, L. G., Chatel, J. M.,
 2792 Sokol, H., Thomas, M., Wells, J. M., Langella, P. (2013). *Faecalibacterium*
 2793 *prausnitzii* and human intestinal health. *Curr Opin Microbiol*, 16(3), 255–261.

2794 Moore, D. (2014). Keeping older muscle “young” through dietary protein and
2795 physical activity. *Adv Nutr*, 5(5), 599–607.

2796 Moayyedi, P., Surette, M. G., Kim, P. T., Libertucci, J., Wolfe, M., Onischi, C.,
2797 Armstrong, D., Marshall, J. K., Kassam, Z., Reinisch, W., Lee, C. H. (2015).
2798 Fecal microbiota transplantation induces remission in patients with active
2799 ulcerative colitis in a randomized controlled trial. *Gastroenterology*, 149(1),
2800 102–109.

2801 Murphy, N., Norat, T., Ferrari, P., Jenab, M., Bueno-de-Mesquita, B., Skeie, G.,
2802 Olsen, A., Tjønneland, A., Dahm, C. C., Overvad, K., *et al.* (2013). Consumption
2803 of dairy products and colorectal cancer in the European prospective investigation
2804 into cancer and nutrition (EPIC). *PLoS One*, 8(9), e72715.

2805 Nakajima, K., Tamura, N., Kobayashi-Hattori, K., Yoshida, T., Hara-Kudo, Y.,
2806 Ikedo, M., Sugita-Konishu, Y., Hattori, M. (2005). Prevention of intestinal
2807 infection by glycomacropeptide. *Biosci Biotechnol Biochem*, 69(12), 2294–301.

2808 Neelima, Sharma, R., Rajput, Y.S., Mann, B. (2013). Chemical and functional
2809 properties of glycomacropeptide (GMP) and its role in the detection of cheese
2810 whey adulteration in milk: a review. *Dairy Sci Technol*, 93(1), 21–43.

2811 Ney, D. M., Stroup, B. M., Clayton, M. K., Murali, S. G., Rice, G. M., Rohr, F.,
2812 Levy, H. L. (2016). Glycomacropeptide for nutritional management of
2813 phenylketonuria : a randomized, controlled, crossover trial. *Am J Nutr*, 104(2),
2814 334–345.

2815 Neyrinck, A M., Van Hée, V. F., Piront, N., De Backer, F., Toussaint, O., Cani,
2816 P. D., Delzenne, N. M. (2012). Wheat-derived arabinoxylan oligosaccharides

2817 with prebiotic effect increase satietogenic gut peptides and reduce metabolic
 2818 endotoxemia in diet-induced obese mice. *Nutr Diabetes*, 2, e28.

2819 Neyrinck, A. M., Possemiers, S., Druart, C., Van de Wiele, T., De Backer, F.,
 2820 Cani, P. D., Larondelle, Y., Delzenne, N. M. (2011). Prebiotic effects of wheat
 2821 arabinoxylan related to the increase in bifidobacteria, *Roseburia* and
 2822 *Bacteroides/Prevotella* in diet-induced obese mice. *PloS One*, 6(6), e20944.

2823 Ng, K. M., Ferreyra, J. A., Higginbottom, S. K., Lynch, J. B., Kashyap, P. C.,
 2824 Gopinath, S., Naidu, N., Choudhury, B., *et al.* (2013). Microbiota-liberated host
 2825 sugars facilitate post-antibiotic expansion of enteric pathogens. *Nature*,
 2826 502(7469), 96–99.

2827 Nguyen TL, Vieira-Silva S, Liston A, R. J. (2015). How informative is the
 2828 mouse for human gut microbiota research? *Dis Model Mech*, 8(1), 1–16.

2829 Norris, G. H., Jiang, C., Ryan, J., Porter, C. M., Blesso, C. N. (2016). Milk
 2830 sphingomyelin improves lipid metabolism and alters gut microbiota in high fat
 2831 diet-fed mice. *J Nutr Biochem*, 30, 93–101.

2832 Ntemiri, A., Ni Chonchuir, F., O’Callaghan, T. F., Stanton, C., Ross, R. P.,
 2833 O’Toole, P. W. (2017). Glycomacropeptide sustains microbiota diversity and
 2834 promotes specific taxa in an artificial colon model of elderly gut microbiota. *J*
 2835 *Agric Food Chem*, 65(8), 1836–1846.

2836 Oh, S., Worobo, R. W., Kim, B. C., Rheem, S., Kim, S. (2000). Detection of
 2837 cholera toxin-binding activity of kappa-casein macropeptide and optimization of
 2838 its production by the response surface methodology. *Biosci Biotechnol Biochem*,
 2839 64(3), 516–522.

2840 O’Keefe, S. J. D., Li, J. V., Lahti, L., Ou, J., Carbonero, F., Mohammed, K., ...
 2841 Zoetendal, E. G. (2015). Fat, fibre and cancer risk in African Americans and
 2842 rural Africans. *Nat Commun*, 6(28), 6342.

2843 Oliveira, D. L., Wilbey, R. A., Grandison, A. S., Roseiro, L. B. (2015). Milk
 2844 oligosaccharides: A review. *Int J Dairy Technol*, 68(3), 305–321.

2845 O’Riordan, N., Kane, M., Joshi, L., Hickey, R. M. (2014). Structural and
 2846 functional characteristics of bovine milk protein glycosylation. *Glycobiology*,
 2847 24(3), 220–236.

2848 Ortega-González, M., Capitán-Cañadas, F., Requena, P., Ocón, B., Romero-
 2849 Calvo, I., Aranda, C., Suarez, M. D., *et al.* (2014). Validation of bovine
 2850 glycomacropeptide as an intestinal anti-inflammatory nutraceutical in the
 2851 lymphocyte-transfer model of colitis. *British J Nutr*, 111(7), 1202–12.

2852 O’Toole, P. W., Marchesi, J. R., Hill, C. (2017). Next-generation probiotics: the
 2853 spectrum from probiotics to live biotherapeutics. *Nat Microbiol*, 2(5), 17057.

2854 Panagiotakos, D. B., Pitsavos, C. H., Zampelas, A. D., Chrysoshoou, C. A.,
 2855 Stefanadis, C. I. (2010). Dairy products consumption is associated with
 2856 decreased levels of inflammatory markers related to cardiovascular disease in
 2857 apparently healthy adults: the ATTICA study. *J Am Coll Nutr*, 29(4), 357–364.

2858 Park, Y., Leitzmann, M. F., Subar, A. F., Hollenbeck, A., Schatzkin, A. (2009).
 2859 Dairy food, calcium, and risk of cancer in the NIH-AARP Diet and Health Study.
 2860 *Arch Intern Med*, 169(4), 391-401.

- 2861 Park, K. M., and Fulgoni, V. L. (2013). The association between dairy product
2862 consumption and cognitive function in the National Health and Nutrition
2863 Examination Survey. *Br J Nutr*, 109(6), 1135–1142.
- 2864 Paveljšek, D., Juvan, P., Košir, R., Rozman, D., Hacin, B., Rogelj, I. (2018).
2865 *Lactobacillus fermentum* L930BB and *Bifidobacterium animalis* subsp. *animalis*
2866 IM386 initiate signalling pathways involved in intestinal epithelial barrier
2867 protection. *Benef Microbes*, 9(3), 515-525.
- 2868 Payne, A. N., Zihler, A., Chassard, C., Lacroix, C. (2012). Advances and
2869 perspectives in *in vitro* human gut fermentation modeling. *Trends Biotechnol*,
2870 30(1), 17–25.
- 2871 Pennings, B., Boirie, Y., Senden, J. M. G., Gijsen, A. P., Kuipers, H., Van Loon,
2872 L. J. C. (2011). Whey protein stimulates postprandial muscle protein accretion
2873 more effectively than do casein and casein hydrolysate in older men. *Am J Clin*
2874 *Nutr*, 93(5), 997–1005.
- 2875 Perez, M., Ntemiri, A., Tan H., O’Toole P. (2017). Developing an artificial
2876 bacterial consortium for modulating the microbiota of frail older people. (In
2877 preparation)
- 2878 Pettersson, A., Kasperzyk, J. L., Kenfield, S. A., Richman, E. L., Chan, J. M.,
2879 Willett, W. C., *et al.* (2013). Milk and dairy consumption among men with
2880 prostate cancer and risk of metastases and prostate cancer death. *Cancer*
2881 *Epidemiol Biomarkers*, 21(3), 428–436.
- 2882 Poeker, S. A., Geirnaert, A., Berchtold, L., Greppi, A., Krych, L., Steinert, R. E.,
2883 de Wouters, T., Lacroix, C. (2018). Understanding the prebiotic potential of

2884 different dietary fibers using an in vitro continuous adult fermentation model
 2885 (PolyFermS). *Sci Rep*, 8(1), 1–12.

2886 Qin, B., Moorman, P. G., Alberg, A. J., Barnholtz-Sloan, J. S., Bondy, M., Cote,
 2887 M. L., Funkhouser, E., Peters, E.S., Schwartz, A. G., Terry, P., Schildkraut, J.
 2888 M., Bandera, E. V. (2016). Dairy, calcium, Vitamin D and ovarian cancer risk in
 2889 African-American women. *Br J Cancer*, 115(9), 1122–1130.

2890 Rea, M. C., Dobson, A., O’Sullivan, O., Crispie, F., Fouhy, F., Cotter, P. D.,
 2891 Shanahan, F., Kiely, B., Hill, C., Ross, R. P. (2011). Effect of broad- and narrow-
 2892 spectrum antimicrobials on *Clostridium difficile* and microbial diversity in a
 2893 model of the distal colon. *Proc Natl Acad Sci USA*, 108(Supplement_1), 4639–
 2894 4644.

2895 Requena, P., Daddaoua, A, Martínez-Plata, E., González, M., Zarzuelo, A,
 2896 Suárez, M. D., Sanchez de Medina, F., Martinez-Augustin, O. (2008). Bovine
 2897 glycomacropeptide ameliorates experimental rat ileitis by mechanisms involving
 2898 downregulation of interleukin 17. *Br J Pharmacol*, 154(4), 825–32.

2899 Radavelli-Bagatini, S., Zhu, K., Lewis, J. R., Dhaliwal, S. S., Prince, R. L.
 2900 (2013). Association of dairy intake with body composition and physical function
 2901 in older community-dwelling women. *J Acad Nutr Diet*, 113(12), 1669–1674.

2902 Radavelli-Bagatini, S., Zhu, K., Lewis, J. R., Prince, R. L. (2014). Dairy food
 2903 intake, peripheral bone structure, and muscle mass in elderly ambulatory women.
 2904 *J Bone Miner Res*, 29(7), 1691–1700.

2905 Rebholz, C. M., Crews, D. C., Grams, M. E., Steffen, L. M., Levey, A. S.,
 2906 Miller, E. R., Appel, L. J., Coresh, J. (2016). DASH (Dietary Approaches to Stop

2907 Hypertension) Diet and Risk of Subsequent Kidney Disease. *Am J Kidney Dis*,
2908 68(6), 853–861.

2909 Requena, P., Daddaoua, A., Guadix, E., Zarzuelo, A., Suárez, M. D., Sánchez de
2910 Medina, F., Martínez-Augustin, O. (2009). Bovine glycomacropeptide induces
2911 cytokine production in human monocytes through the stimulation of the MAPK
2912 and the NF-kappa B signal transduction pathways. *Br J Pharmacol*, 157(7),
2913 1232–40.

2914 Ridaura, V. K., Faith, J. J., Rey, F. E., Cheng, J., Duncan, A. E., Kau, A. L.,
2915 Griffin, N. W., Lombard, V., Henrissat, B., *et al.* (2013). Gut microbiota from
2916 twins discordant for obesity modulate metabolism in mice. *Science*, 341(6150),
2917 1241214.

2918 Rivière, A., Selak, M., Lantin, D., Leroy, F., De Vuyst, L. (2016). Bifidobacteria
2919 and butyrate-producing colon bacteria: Importance and strategies for their
2920 stimulation in the human gut. *Front Microbiol*, 7(979).

2921 Robitaille, G. (2013). Growth-promoting effects of caseinomacropeptide from
2922 cow and goat milk on probiotics. *J Dairy Res*, 80(1), 58–63.

2923 Rothschild, D., Weissbrod, O., Barkan, E., Korem, T., Zeevi, D., Costea, P. I.,
2924 Kalka, I. N., Bar, N., Shilo, S., Lador, D., *et al.* (2018). Environmental factors
2925 dominate over host genetics in shaping human gut microbiota composition.
2926 *Nature*, 555(25973), 210–215.

2927 Round, J. L., and Mazmanian, S. K. (2009). The gut microbiome shapes
2928 intestinal immune responses during health and disease. *Nat Rev Immunol*, 9(5),
2929 313–323.

2930 Sahni, S., Mangano, K. M., Kiel, D. P., Tucker, K. L., Hannan, M. T. (2017).
 2931 Dairy intake is protective against bone loss in older vitamin D supplement users:
 2932 The Framingham study. *J Nutr*, 147(4), 645–652. (a)

2933 Sahni, S., Mangano, K. M., Tucker, K. L., Douglas, P. K., Casey, V. A., Hannan,
 2934 M. T. (2015). Protective association of milk intake on the risk of hip fracture:
 2935 Results from the Framingham Original Cohort. *J Bone Miner Res*, 29(8), 37–54.

2936 Sahni, S., Soedamah-Muthu S., C.M.Weaver. (2017). Higher milk intake
 2937 increases fracture risk: confounding or true association? *Osteopor Int*, 28(8),
 2938 2263–2264. (b)

2939 Sánchez-Moya, T., Lopez-Nicolas, R., Planes, D., Gonzales-Bermudez, C. A.,
 2940 Ros-Berruezo, G., Frontela-Saseta, C. (2017). *In vitro* modulation of gut
 2941 microbiota by whey protein to preserve intestinal health. *Food Funct*, 8(9), 3053-
 2942 3063

2943 Sánchez-Patán, F., Barroso, E., Van De Wiele, T., Jiménez-Girón, A., Martín-
 2944 Alvarez, P. J., Moreno-Arribas, M. V., Martinez-Cuesta, M. C., Pelaez, C.,
 2945 Requena, T., Bartolomé, B. (2015). Comparative *in vitro* fermentations of
 2946 cranberry and grape seed polyphenols with colonic microbiota. *Food Chem*, 183,
 2947 273–282.

2948 Sanders, M. E., Benson, A., Lebeer, S., Merenstein, D. J., Klaenhammer, T. R.
 2949 (2018). Shared mechanisms among probiotic taxa: implications for general
 2950 probiotic claims. *Curr Opin Biotechnol*, 49, 207–216.

2951 Sanders, M. E., Guarner, F., Guerrant, R., Holt, P. R., Quigley, E. M. M., Sartor,
 2952 R. B., Sherman, P. M., Mayer, E. A. (2013). An update on the use and
 2953 investigation of probiotics in health and disease. *Gut*, 62(5), 787–796.

2954 Sarbini, S. R., Kolida, S., Gibson, G. R., Rastall, R. A. (2013). *In vitro*
 2955 fermentation of commercial α -gluco-oligosaccharide by faecal microbiota from
 2956 lean and obese human subjects. *Br J Nutr*, 109(11), 1980–1989.

2957 Sarkar, A., Lehto, S. M., Harty, S., Dinan, T. G., Cryan, J. F., Burnet, P. W. J.
 2958 (2016). Psychobiotics and the manipulation of bacteria–gut–brain signals. *Trends*
 2959 *Neurosci*, 39(11), 763–781.

2960 Saxton, R. A., and Sabatini, D. M. (2017). mTOR signaling in growth,
 2961 metabolism, and disease. *Cell*, 168(6), 960–976.

2962 Schubert, A. M., Sinani, H., Schloss, P. D. (2015). Antibiotic-induced alterations
 2963 of the murine gut microbiota and subsequent effects on colonisation resistance
 2964 against *Clostridium difficile*. *MBio*, 6(4), 1–10.

2965 Shin, N. R., Lee, J. C., Lee, H. Y., Kim, M. S., Whon, T. W., Lee, M. S., Bae, J.
 2966 W. (2014). An increase in the *Akkermansia* spp. population induced by
 2967 metformin treatment improves glucose homeostasis in diet-induced obese mice.
 2968 *Gut*, 63(5), 727–735.

2969 Simon, M. C., Strassburger, K., Nowotny, B., Kolb, H., Nowotny, P., Burkart,
 2970 V., Zivehe, F., Hwang, J. H., Stehle, P., *et al.* (2015). Intake of *Lactobacillus*
 2971 *reuteri* improves incretin and insulin secretion in glucose-tolerant humans: A
 2972 proof of concept. *Diabetes Care*, 38(10), 1827–1834.

- 2973 Simrén, M., Barbara, G., Flint, H. J., Spiegel, B. M. R., Spiller, R. C., Vanner, S.,
2974 Verdu, E., Whorwell, P. J., Zoetendal, E. G. (2013). Intestinal microbiota in
2975 functional bowel disorders: a Rome foundation report. *Gut*, 62(1), 159–176.
- 2976 Smilowitz, J. T., Lebrilla, C. B., Mills, D. A., German, J. B., Freeman, S. L.
2977 (2014). Breast milk oligosaccharides: Structure-function relationships in the
2978 neonate. *Annual Rev Nutr*, 34, 143–169.
- 2979 Smilowitz, J. T., Lemay, D. G., Kalanetra, K. M., Chin, E. L., Zivkovic, A. M.,
2980 Breck, M. A., German, J. B., Mills, D. A., Slupsky, C. Barile, D. (2017).
2981 Tolerability and safety of the intake of bovine milk oligosaccharides extracted
2982 from cheese whey in healthy human adults. *J Nutr Sci*, 6, e6.
- 2983 Song, M., Garrett, W. S., Chan, A. T. (2015). Nutrients, foods, and colorectal
2984 cancer prevention. *Gastroenterology*, 148(6), 1244–1260.
- 2985 Song, X., Li, Z., Ji, X., Zhang, D. (2017). Calcium intake and the risk of ovarian
2986 cancer: A meta-analysis. *Nutrients*, 9(7), 679.
- 2987 Staley, C., Kelly, C. R., Brandt, L. J., Khoruts, A., Sadowsky, M. J. (2016).
2988 Complete microbiota engraftment is not essential for recovery from recurrent
2989 *Clostridium difficile* infection following fecal microbiota transplantation. *MBio*,
2990 7(6), e01965-16.
- 2991 Stancliffe, R. A., Thorpe, T., Zemel, M. B. (2011). Dairy attenuates oxidative
2992 and inflammatory stress in metabolic syndrome. *Am J Clin Nutr*, 94(2), 422–430.

- 2993 Szilagyi, A. (2015). Adaptation to lactose in lactase non persistent people:
2994 Effects on intolerance and the relationship between dairy food consumption and
2995 evalution of diseases. *Nutrients*, 7(8), 6751–6779. (b)
- 2996 Szilagyi, A. (2015). Adult lactose digestion status and effects on disease. *Can J*
2997 *Gastroenterol Hepatol*, 29(3), 149–56. (a)
- 2998 Szilagyi, A., Galiatsatos, P., Xue, X. (2016). Systematic review and meta-
2999 analysis of lactose digestion, its impact on intolerance and nutritional effects of
3000 dairy food restriction in inflammatory bowel diseases. *Nutr J*, 15, 67.
- 3001 Ulrik K Sundekilde, Daniela Barile, Mickael Meyrand, Nina A Poulsen, L. B. L.,
3002 Carlito B. Lebrilla, German J. Bruce, and H. C. B. (2012). Natural variability in
3003 bovine milk oligosaccharides from Danish Jersey and Holstein-Friesian breeds. *J*
3004 *Agric Food Chem*, 60(24), 6188-6196.
- 3005 Takagi, R., Sasaki, K., Sasaki, D., Fukuda, I., Tanaka, K., Yoshida, K., Kondo,
3006 A., Osawa, R. (2016). A single-batch fermentation system to simulate human
3007 colonic microbiota for high-throughput evaluation of prebiotics. *Plos One*, 11(8),
3008 e0160533.
- 3009 Talaei, M., Hosseini, N., van Dam, R. M., Sadeghi, M., Oveisgharan, S.,
3010 Dianatkhah, M., Sarrafzadegan, N. (2017). Whole milk consumption and risk of
3011 cardiovascular disease and mortality: Isfahan Cohort Study. *Eur J Nutr*, [Epub
3012 ahead of print].
- 3013 Tang, J. E., Moore, D. R., Kujbida, G. W., Tarnopolsky, M. A., Phillips, S. M.
3014 (2009). Regulation of protein metabolism in exercise and recovery ingestion of
3015 whey hydrolysate, casein, or soy protein isolate : effects on mixed muscle protein

3016 synthesis at rest and following resistance exercise in young men. *J Appl Physiol*,
3017 *107*(3), 987–992.

3018 Tannock, G. W., Lawley, B., Munro, K., Pathmanathan, S. G., Zhou, S. J.,
3019 Makrides, M., Gibson, R. A., Prosser, C. G., Lowry, D., Hodgkinson, A. J.
3020 (2013). Comparison of the compositions of the stool microbiotas of infants fed
3021 goat milk formula, cow milk-based formula, or breast milk. *Appl Environ*
3022 *Microbiol*, *79*(9), 3040–3048.

3023 Tantamango-Bartley, Y., Knutsen, S. F., Jaceldo-Siegl, K., Fan, J., Mashchak,
3024 A., Fraser, G. E. (2017). Independent associations of dairy and calcium intakes
3025 with colorectal cancers in the Adventist Health Study-2 cohort. *Public Health*
3026 *Nutr*, *20*(14), 2577–2586.

3027 Tao, N., DePeters, E. J., Freeman, S., German, J. B., Grimm, R., Lebrilla, C. B.
3028 (2008). Bovine milk glycome. *J Dairy Sci*, *91*(10), 3768–3778.

3029 Terpend, K., Possemiers, S., Daguet, D., Marzorati, M. (2013). Arabinogalactan
3030 and fructo-oligosaccharides have a different fermentation profile in the Simulator
3031 of the Human Intestinal Microbial Ecosystem (SHIME ®). *Environ Microbiol*
3032 *Rep*, *5*(4), 595–603.

3033 Thevaranjan, N., Puchta, A., Schulz, C., Naidoo, A., Szamosi, J. C., Verschoor,
3034 C. P., Loukov, D., Schenck, L. P., Jury, J. *et al.* (2017). Age-associated microbial
3035 dysbiosis promotes intestinal permeability, systemic inflammation, and
3036 macrophage dysfunction. *Cell Host Microbe*, *21*(4), 455–466.

3037 Thomä-Worringer, C., Sørensen, J., López-Fandiño, R. (2006). Health effects
 3038 and technological features of caseinomacropeptide. *Int Dairy J*, 16(11), 1324–
 3039 1333.

3040 Tognon, G., Lena M. Nilsson, Dmitry Shungin, Lauren Lissner, Jan-Hakan
 3041 Jansson, Frida Renstrom, Maria Wennberg, Anna Winkvist, Johansson, I. (2017).
 3042 Nonfermented milk and other dairy products: associations with all-cause
 3043 mortality. *Am J Clin Nutr*, 105(6), 1502–1511.

3044 Turnbaugh, P. J., Backhed, F., Fulton, L., Gordon, J. I. (2008). Diet-induced
 3045 obesity is linked to marked but reversible alterations in the mouse distal gut
 3046 microbiome. *Cell Host Microbe*, 3(4), 213–223.

3047 Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V., Mardis, E. R.,
 3048 Gordon, J. I. (2006). An obesity-associated gut microbiome with increased
 3049 capacity for energy harvest. *Nature*, 444(7122), 1027–1031.

3050 Turnbaugh, P. J., Ridaura, V. K., Faith, J. J., Rey, F. E., Knight, R., Gordon, J. I.
 3051 (2009). The effect of diet on the human gut microbiom: A metagenomic analysis
 3052 in humanized gnotobiotic mice. *Sci Trans Med*, 1(6), 6ra14.

3053 Turrone, F., Ventura, M., Buttó, L. F., Duranti, S., O’Toole, P. W., Motherway,
 3054 M. O. C., Van Sinderen, D. (2014). Molecular dialogue between the human gut
 3055 microbiota and the host: A *Lactobacillus* and *Bifidobacterium* perspective. *Cell*
 3056 *Mol Life Sci*, 71(2), 183–203.

3057 van Baarlen, P., Wells, J. M., Kleerebezem, M. (2013). Regulation of intestinal
 3058 homeostasis and immunity with probiotic lactobacilli. *Trends Immunol*, 34(5),
 3059 208–215.

3060 Van den Abbeele, P., Belzer, C., Goossens, M., Kleerebezem, M., De Vos, W.
3061 M., Thas, O., De Weirdt, R., Kerckhof, F. M., Van de Wiele, T. (2013).
3062 Butyrate-producing *Clostridium* cluster XIVa species specifically colonize
3063 mucins in an in vitro gut model. *ISME J*, 7(5), 949–61.

3064 Van den Abbeele, P., Marzorati, M., Derde, M., De Weirdt, R., Joan, V.,
3065 Possemiers, S., Van de Wiele, T. (2016). Arabinoxylans, inulin and
3066 *Lactobacillus reuteri* 1063 repress the adherent-invasive *Escherichia coli* from
3067 mucus in a mucosa-comprising gut model. *Npj Biofilms Microbiomes*, 2, 16016.

3068 Van den Abbeele, P., Roos, S., Eeckhaut, V., MacKenzie, D. A, Derde, M.,
3069 Verstraete, W., Marzorati, M., Possemiers, S., Vanhoecke, B., Van Immerseel,
3070 F., Van de Wiele, T. (2012). Incorporating a mucosal environment in a dynamic
3071 gut model results in a more representative colonisation by lactobacilli. *Microb*
3072 *Biotechnol*, 5(1), 106–15.

3073 Van Den Abbeele, P., Venema, K., Van De Wiele, T., Verstraete, W.,
3074 Possemiers, S. (2013). Different human gut models reveal the distinct
3075 fermentation patterns of arabinoxylan versus inulin. *J Agric Food Chem*, 61(41),
3076 9819–9827.

3077 Van de Wiele *et al.* in Verhoeckx, K., Cotter, P., López-Expósito, I., Kleiveland,
3078 C., Lea, T., Mackie, A., *et al.* (2015). The impact of food bio-actives on gut
3079 health: *In vitro* and *Ex Vivo* models, Book Chapter 27. Springer editions.

3080 Varankovich, N. V., Nickerson, M. T., Korber, D. R. (2015). Probiotic-based
3081 strategies for therapeutic and prophylactic use against multiple gastrointestinal
3082 diseases. *Front Microbiol*, 6, 685.

3083 Venema, K. in Verhoeckx, K., Cotter, P., López-Expósito, I., Kleiveland, C.,
 3084 Lea, T., Mackie, A., *et al.* (2015). The impact of food bio-actives on gut health:
 3085 *In vitro* and *Ex Vivo* models, Book Chapter 26. Springer editions.

3086 Venema, K., and van den Abbeele, P. (2013). Experimental models of the gut
 3087 microbiome. *Best Pract Res Clin Gastroenterol*, 27(1), 115–26.

3088 Vincent, C., Stephens, D. A, Loo, V. G., Edens, T. J., Behr, M. A, Dewar, K.,
 3089 Manges, A. R. (2013). Reductions in intestinal Clostridiales precede the
 3090 development of nosocomial *Clostridium difficile* infection. *Microbiome*, 1(1), 18.

3091 Visioli, F., and Strata, A. (2014). Milk, dairy products, and their functional
 3092 effects in humans: a narrative review of recent evidence. *Adv Nutr*, 5(2), 131–
 3093 143.

3094 Volek, J. S., Volk, B. M., Kunces. L. J., Kupchak, B. P., Freidenreich, D. J.,
 3095 Aristizabal, J. C., Saenz, C., Dunn-Lewis, C., Ballard, K. D., *et al.* (2013). Whey
 3096 protein supplementation during resistance training augments lean body mass. *J*
 3097 *Am Coll Nutr*, 32(2), 122-135.

3098 Vrieze, A., Van Nood, E., Holleman, F., Salojärvi, J., Kootte, R. S., Bartelsman,
 3099 J. F., Dallinga-Thie, G. M., Ackermans, M. T., *et al.* (2012). Transfer of
 3100 intestinal microbiota from lean donors increases insulin sensitivity in individuals
 3101 with metabolic syndrome. *Gastroenterology*, 143(4), 913–916.e7.

3102 Vulevic, J., Drakoularakou, A., Yaqoob, P., Tzortzis, G., Gibson, G. R. (2008).
 3103 Modulation of the fecal microflora profile and immune function by a novel trans
 3104 -galactooligosaccharide mixture (B-GOS) in healthy elderly volunteers. *Am J*
 3105 *Clin Nutr*, 88, 1438–1446.

3106 Walton, G. E., van den Heuvel, E. G. H. M., Kusters, M. H. W., Rastall, R. A.,
 3107 Tuohy, K. M., Gibson, G. R. (2012). A randomised crossover study investigating
 3108 the effects of galacto-oligosaccharides on the faecal microbiota in men and
 3109 women over 50 years of age. *B J Nutr*, 107(10), 1466–1475.

3110 Wos-Oxley, M. L., Bleich, A., Oxley, A. P. A., Kahl, S., Janus, L. M., Smoczek,
 3111 A., Nahrstedt, H., Pils, M. C., Taudien, S., *et al.* (2012). Comparative evaluation
 3112 of establishing a human gut microbial community within rodent models. *Gut*
 3113 *Microbes*, 3(3), 234–249.

3114 Woting, A., Pfeiffer, N., Loh, G., Klaus, S., Blaut, M. (2014). *Clostridium*
 3115 *ramosum* promotes high-fat diet-induced obesity in gnotobiotic mouse models.
 3116 *MBio*, 5(5), 1–10.

3117 Wu, L., and Sun, D. (2016). Meta-analysis of milk consumption and the risk of
 3118 cognitive disorders. *Nutrients*, 8(12), 1–12.

3119 Xu, S. P., Mao, X. Y., Cheng, X., Chen, B. (2013). Ameliorating effects of
 3120 casein glycomacropeptide on obesity induced by high-fat diet in male Sprague-
 3121 Dawley rats. *Food Chem Toxicol*, 56, 1–7.

3122 Yang, M., Kenfield, S. A., Van Blarigan, E. L., Wilson, K. M., Batista, J. L.,
 3123 Sesso, H. D., Ma, J., Stampfer, M J., Chavarro, J. E. (2016). Dairy intake after
 3124 prostate cancer diagnosis in relation to disease-specific and total mortality. *Int J*
 3125 *Cancer*, 137(10), 2462-2469. (a)

3126 Yang, Y., Wang, X., Yao, Q., Qin, L., Xu, C. (2016). Dairy product, calcium
 3127 intake and lung cancer risk: A systematic review with meta-analysis. *Sci Rep*, 6,
 3128 20624. (b)

- 3129 Yen, C. H., Kuo, Y. W., Tseng, Y. H., Lee, M. C., Chen, H. L. (2011). Beneficial
3130 effects of fructo-oligosaccharides supplementation on fecal bifidobacteria and
3131 index of peroxidation status in constipated nursing-home residents-A placebo-
3132 controlled, diet-controlled trial. *Nutrition*, 27(3), 323–328.
- 3133 Yen, S., McDonald, J. A. K., Schroeter, K., Oliphant, K., Sokolenko, S.,
3134 Blondeel, E. J. M., Allen-Vercoe, E., Aucoin, M. G. (2015). Metabolomic
3135 analysis of human fecal microbiota: A comparison of feces-derived communities
3136 and defined mixed communities. *J Proteome Res*, 14(3), 1472–1482.
- 3137 Yin, X., Yan, Y., Kim, E. B., Lee, B., Marco, M. L. (2014). Short
3138 communication: effect of milk and milk containing *Lactobacillus casei* on the
3139 intestinal microbiota of mice. *J Dairy Sci*, 97(4), 2049–55.
- 3140 Zemel, M. B., Sun, X., Sobhani, T., Wilson, B. (2010). Effects of dairy
3141 compared with soy on oxidative and inflammatory stress in overweight and
3142 obese subjects. *Am J Clin Nutr*, 91(1), 16–22.
- 3143 Zivkovic, A. M., and Barile, D. (2011). Bovine milk as a source of functional
3144 oligosaccharides for improving human health. *Adv Nutr*, 2(3), 284–9.

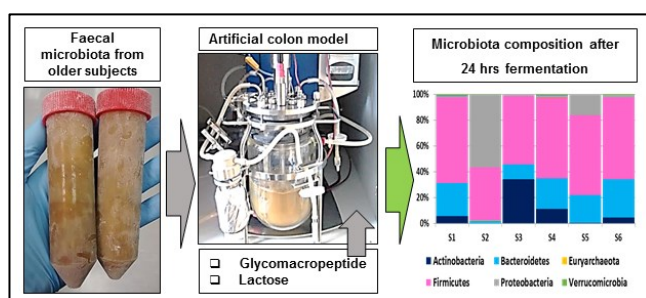
Chapter 2

Glycomacropeptide sustains microbiota diversity and promotes specific taxa in an artificial colon model of elderly gut microbiota

This chapter was published in the Journal of Agricultural Food Chemistry:

Ntemiri, A., Ni Chonchuir, F., O’Callaghan, T. F., Stanton, C., Ross, R. P., O’Toole, P. W. (2017). Glycomacropeptide sustains microbiota diversity and promotes specific taxa in an artificial colon model of elderly gut microbiota. *J Agric Food Chem*, 65(8), 1836–1846.

ORCID: <http://orcid.org/0000-0002-8742-0627>



3157 **2.1 Abstract**

3158 The potential of milk-derived glycomacropeptide (GMP) and lactose for modulating
3159 the human gut microbiota of older people, in whom loss of diversity correlates with
3160 inferior health, was investigated. We used an *in vitro* batch fermentation (artificial
3161 colon model) to simulate colonic fermentation processes of two GMP products, i.e. a
3162 commercially available GMP concentrate and a semi-purified GMP concentrate, and
3163 lactose. Faecal samples were collected from healthy and frail older people. Samples
3164 were analysed by Illumina Miseq sequencing of rRNA gene amplicons. The
3165 commercial GMP preparation had a positive effect on the growth of *Coprococcus*
3166 and *Clostridium* cluster XIVb and sustained a higher faecal microbiota diversity
3167 compared to control substrates or lactose. Lactose fermentation promoted the growth
3168 of Proteobacteria including *Escherichia/Shigella*. This work provides an in-depth
3169 insight on the potential of GMP and lactose for modulating the gut microbiota and
3170 contributes more evidence confirming the prebiotic activity of GMP.

3171 **Keywords:** Glycomacropeptide, lactose, artificial colon model, faecal microbiota,
3172 healthy elderly, frail elderly

3173 **2.2 Introduction**

3174 The human gastrointestinal tract (GIT) harbours a phylogenetically diverse bacterial
3175 community that significantly contributes to host health (Donaldson *et al.*, 2015). The
3176 gut microbiota is determinatively shaped by habitual diet (Claesson *et al.*, 2012;
3177 Jeffery *et al.*, 2016; Sonnenburg *et al.*, 2016). Dysbiosis refers to alterations from
3178 typical proportions of taxa in the gut microbiota composition and altered diversity,
3179 changes often related to non-healthy conditions (Cho *et al.*, 2012). Targeted
3180 modulation of the gut microbiota is emerging as an approach for ameliorating a
3181 series of conditions related to dysbiosis (Foxy-Orenstein *et al.*, 2012).

3182 There is evidence linking the process of ageing-related health loss to the gut
3183 microbiome (Heintz *et al.*, 2014). From adulthood to older age the gut microbiota
3184 undergoes significant compositional and metabolic changes that define the gut
3185 microbiota profile of the elderly (Claesson *et al.*, 2011; O'Tole and Jeffery, 2015).
3186 These shifts in the gut microbiome increase pathobiont infection susceptibility in the
3187 older population (Rea *et al.*, 2012).

3188 Recent studies suggest a link between dairy consumption and a number of health
3189 benefits including muscle maintenance and bone density in older subjects (Sahni *et*
3190 *al.*, 2014; Lana *et al.*, 2015). Little is known about the effect of milk components on
3191 the gut microbiota. The content of lactose in commercial milk is ~4% (Albenzio *et*
3192 *al.*, 2016). Lactose is a candidate prebiotic (Roberfroid, 2007) especially among
3193 lactase non-persistent people (LNP) related to its demonstrated growth promotion of
3194 lactobacilli and bifidobacteria (Szilagyi *et al.*, 2015), with a potential protective role
3195 in colorectal health (Jatvinen *et al.*, 2001).

3196 Caseinomacropeptide (CMP) is produced from milk during cheese making; during
3197 this procedure, chymosin hydrolyses the kappa-casein (κ -CN) of milk resulting in
3198 the para- κ -CN (residues 1-105) and CMP (residues 106-169) (Neelima *et al.*, 2013).
3199 Glycomacropeptide (GMP) is the glycosylated form of CMP; GMP has five mucin-
3200 type glycans namely *N*-acetylneuraminic acid (NeuAc) or sialic acid, galactose
3201 (Gal), and *N*-acetylgalactosamine (Gal-Nac) that anchor to the CMP through *O*-
3202 glycosidic linkages (Kreuss *et al.*, 2008). GMP is rich in branched chain amino acids
3203 (BCAA) and essential amino acids (EAA) and purified GMP concentrate is depleted
3204 of aromatic amino acids (Thoma-Worringer *et al.*, 2006).

3205 The major health-relevant effects of GMP are anti-inflammatory activity, protection
3206 against GIT pathogens and contribution to phenylketonouria (PKU) nutritional
3207 management (Thoma-Worringer *et al.*, 20106; Van Calcar and Ney, 2012). There are
3208 contradictory data on the effects of GMP on the growth promotion of
3209 *Bifidobacterium* and *Lactobacillus* strains (Bruck *et al.*, 2006; Hernandez-Hernandez
3210 *et al.*, 2011; Robitaille, 2013). Recently Sawin *et al.* (2015) reported that GMP
3211 feeding resulted in a significant decrease in the abundance of Proteobacteria and the
3212 genus *Desulfovibrio* in the caecal and faecal microbiota of wild type and
3213 phenylketonouric mice. Although the data were based on murine microbiota and no
3214 extended description of the microbiota was provided, the study offered a more
3215 conclusive view of the effect of GMP on the gut microbiota compared to previous
3216 studies.

3217 The aim of this study was to explore the prebiotic potential of two GMP products, a
3218 commercially available product and a semi-purified preparation, and of lactose, on
3219 the faecal microbiota in an *in vitro* batch fermentation system over 24 hrs. The

3220 responsiveness to these growth substrates of low diversity and higher diversity
3221 microbiota was investigated in faecal samples obtained from healthy and frail elderly
3222 subjects, since the latter are characterised by reduced microbiota diversity.

3223 **2.3 Material and Methods**

3224 **Faecal samples:** Faecal samples were collected from healthy older donors
3225 (community samples) (EM425 male 81 yrs, EM278 female 69 yrs, EM604 female 75
3226 yrs) and older donors that reside in long term residential care units (longstay
3227 samples) (EM703 male 93 yrs, EM297 female 82 yrs, EM704 male 89 yrs). All
3228 practices were approved by the local Clinical Research Ethics Committee. Each stool
3229 sample was placed in a container with a reducing agent (Anaerocult A; Merck
3230 Millipore, Ireland) and transferred to an anaerobic cabinet less than an hour after
3231 defecation. Each stool was thoroughly homogenised (10% w/v) in sterile reduced
3232 phosphate buffer saline (PBS) and 20% glycerol. Faecal slurries from each stool
3233 sample were prepared as inocula for the fermentation vessel and stored frozen in
3234 aliquots at -80 °C before thawing and adding to the fermenter.

3235 **Commercial and semi-purified GMP:** For the *in vitro* tests, Lacprodan CGMP-10
3236 (CGMP-10) was obtained from Arla Foods Ingredients, Viby J, Denmark. The semi-
3237 purified GMP (tGMP) was produced as described below. Sweet whey protein isolate
3238 powder (sWPI) was sourced from Kerry Group plc, Listowel Ireland. A GMP
3239 enriched whey fraction was prepared using a modified method as developed by
3240 Tanimoto *et al* (1991). Three hundred grams of sWPI was reconstituted in 1 L
3241 distilled water, stirred at room temperature for 2 hrs and left to rehydrate at 4 °C
3242 overnight. The sWPI was pH adjusted to 3.8 using 1 M HCl and stirred at room temp
3243 for 30 min. The sWPI was then processed through a 30 kDa molecular weight cut off

(MWCO) membrane (Vivaflow 200 tangential flow units; Sartorius, Göttingen, Germany) at an operating TMP of ~2.25 bar, and 300 mL of permeate (Pr1) was collected. Peristaltic pumps (230V Masterflex L/S Easy-Load peristaltic pumps; Cole Parmer, Niles, IL, USA) and size 16 PVC pump tubing was used. Pr1 was then pH adjusted to pH 7.0 using NaOH and processed using 10 kDa MWCO polyethersulfone membrane following 30 min acclimatisation period in full recirculation mode, and the GMP enriched retentate was collected. Diafiltration was carried out with equivalent volume of distilled water and diluted retentate was concentrated back to its original volume and frozen at -20°C. GMP enriched solutions were freeze dried using Wizard 2.0 freeze drier (Virtis AdVantage, UK). Quantification of the whey proteins α -lactalbumin, β -lactoglobulin and GMP (for both GMP products) was carried out by RP-HPLC using automated Waters 2695 Separation module (Waters Associates, Milford, MA, USA) fitted with a quaternary pump, mobile phase degasser, auto-sampler and a multi-wavelength detector. Calibration curves were obtained for each of the standards i.e. α -lac, β -lg and GMP and Waters Empower data analysis software was used to quantify the α -lac, β -lg and GMP content.

***In vitro* fermentations:** A single stage chemostat system and basal medium (no carbohydrates supplementation) was used as described previously to simulate the colonic microbiota fermentation of the selected substrates (Hernandez-Hernandez *et al.*, 2011). The pH (pH 7.0) and temperature (37°C) controlled batch fermentations were conducted in 1 L working volume, under continuous flow of CO₂ over a period of 24 h. Twenty-four hr fermentation was chosen over a continuous fermentation as a standard approach to study prebiotic effects on the faecal microbiota (Hernandez-Hernandez *et al.*, 2011; Zhang *et al.*, 2016).^{22, 26} Cysteine-HCL was filter sterilised

3269 (0.2 μ m filters) and added to the basal medium after sterilisation. Glucose and
3270 lactose were filtered sterilised and added to the sterilised medium at a final
3271 concentration of 1% w/v. GMP preparations were added to the sterilised medium
3272 after dilution in sterile distilled water. Six substrate conditions were used for each of
3273 the faecal samples: i. basal medium, ii. Glucose (Sigma-Aldrich), iii. lactose (Sigma-
3274 Aldrich), iv. CGMP-10, v. tGMP, vi. CGMP-10 combined with glucose. All
3275 substrates were added to a final concentration of 1%; for substrate condition vi.
3276 CGMP-10 and glucose were added to 0.5% respectively. The faecal samples were
3277 thawed in an anaerobic cabinet and inoculation in the fermenter vessel was
3278 performed anaerobically immediately after thawing at a final concentration of 1%
3279 w/v. At time points zero hours and twenty-four hours 50 mL of fully stirred
3280 fermentation culture was retrieved and immediately centrifuged. Faecal pellet and
3281 supernatant were kept separately in -20 °C for genomic DNA (gDNA) extraction and
3282 SCFA analysis respectively.

3283 **Genomic DNA extraction:** Genomic DNA (gDNA) was extracted using the QIamp
3284 Fast DNA Stool (Qiagen) kit. Faecal pellets were weighed and suspended in 1 ml
3285 sterile PBS. 200 μ L of the suspended slurry was retrieved for gDNA extractions in
3286 order to provide the 200 mg of faeces required for the QIamp Fast DNA Stool
3287 (Qiagen) extraction kit protocol. The faecal slurry was placed in sterile tubes
3288 containing 0.1 mm, 0.5 mm and 1.0 mm zirconia / glass beads (Thistle Scientific,
3289 UK). One ml of InhibitEX buffer was added to the samples and then homogenised
3290 under two pulses of 1 min and a final of 30 sec using a Minibeadbeater (Biospec
3291 Products). In the intervals of the homogenisation steps the samples were placed on
3292 ice for 1 min. The samples were then placed in a 70 °C heat-block for 10 min. The
3293 following steps of the DNA extraction were carried out as described in the Qiagen

3294 protocol using 15 µL of proteinase K with 200 µL of AL buffer, 200 µL of lysate and
3295 200 µL of ethanol at the relevant steps.

3296 **16S rRNA amplicon Illumina library preparation:** The Illumina MiSeq System
3297 (San Diego, California, USA) was used for amplicon sequencing of the 16S rRNA
3298 variable region V3 and V4. The universal 16S ribosomal RNA gene primers forward
3299 primer for V3 region 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCC
3300 TACGGGNGGCWGCAG 3' and reverse primer for V4 region 5' GTCTC GTGG
3301 GCTC GGAG ATGTGTATA AGA GACAGGACTAC HV GGG TATC TA ATCC
3302 3' were used (Klindworth *et al.*, 2013). The library preparation and sequencing was
3303 performed according to the Illumina MiSeq System protocol. The PCR products
3304 were purified and dual-index barcodes attached to the amplicon (Nextera XT V.2
3305 Index Kits sets A and D, Illumina). Amplicons were purified using Agencourt
3306 AMPure XP-PCR Purification system (Beckman Coutler, Inc.) and quantified using
3307 a Qubit dsDNA HS Assay Kit (Thermo Fischer Scientific, MA, USA). Samples were
3308 pooled in order to achieve the same concentration of DNA per amplicon and
3309 sequenced by Eurofins Genetics Services Ltd. (Wolverhampton UK) on a MiSeq
3310 Illumina platform and 2x300 bp Chemistry.

3311 **16S amplicon sequence analysis:** For all demultiplexed samples barcodes were
3312 reattached with relevant custom scripts (Flemer *et al.*, 2016), primers were removed
3313 by the cutadapt tool, forward and reverse reads per sample were joined using the
3314 FLASH computational tool (Magoc *et al.*, 2011). The reads were demultiplexed
3315 using the split_libraries.py script in Qiime. *De novo* operation taxonomic unit (OTU)
3316 table based on the data reads was generated via Usearch; reads were assigned to
3317 OTUs as follows. Based on length size unique sequences were selected using the

derep_fulllength command discarding singletons by `--minuniquesize -2` command. Clustering of the sequences was performed using `cluster_otus` command (Edgar *et al.*, 2013). Sequences were screened for chimeras using the ChimeraSlayer utility (Haas *et al.*, 2011). To map all reads to the representative sequences OTUs database the `Usearch_global` command was used based on 97% identity threshold. The `uc` file generated was converted to an OTU table via `uc2otutab.py` command. Taxonomy was assigned to each OTU (`classify.seqs`) to genus level using Mothur and the RDP 16S rRNA database (Wang *et al.*, 2007).

Microbiota composition statistics: The R software package was used for the statistical analysis of the sequenced data (R Core Team, 2014). From the sequenced samples only those with a number of reads >5000 were kept for further analysis. The OTU counts per sample were rarefied to a common minimum of reads per sample. Metrics of phylogenetic distance between the sequenced samples were calculated using the weighted and unweighted UniFrac method (Lozupone and Knight, 2008). The data was visualised using principal components analysis (PCoA) with functions `s.class` and `dudi.pco` from the `ade4` package. The row counts data table was transformed to relative abundances (0:100) table for obtaining relative abundance of the bacterial populations in the samples to genus level. Relative abundances results were obtained for individual samples and aggregated community-type and longstay-type samples. The Shannon diversity index was used as a metric of within-sample alpha diversity and species evenness (Lozupone and Knight, 2008). A pairwise Wilcoxon rank test was performed to identify significant Shannon diversity changes from 0 hrs to 24 hrs due to medium fermentation. The DESeq2 package (Love *et al.*, 2014) was used for pairwise comparisons of 0hrs and 24hrs for the fold changes ($\log_2$ fold change) in the bacterial populations at genus level based on row read

counts per sample. Normalisation of read counts is embedded in the DESeq2 function. P value for DESeq2 results was adjusted for multiple comparisons using the Benjamini-Hochberg method. Results were considered significant if the p adjusted values were $\text{padj} \leq 0.05$ while $\text{padj} < 0.1$ were regarded as a trend.

Short chain fatty acid analysis and statistics: High performance liquid chromatography (HPLC) was used for the analysis of acetate, propionate and butyrate production (Hernandez-Hernandez *et al.*, 2011). Slurry retrieved from the fermenter vessel was centrifuged for 30 min. The supernatant was filtered through 0.2 μm filter and 1 ml was used for SCFA analysis HPLC. An agilent 1200 HPLC system with a refractive index detector was used. An Agilent Hi-Plex H 300x7.7mm column was used with 0.01N H_2SO_4 elution fluid, at a flow rate of 0.6 mL/min and column temperature at 65°C. Graph Pad Prism 5 Software was used for the analysis and visualisation of the HPLC results. A Kruskal-Wallis non-parametric test with a post hoc Dunn's test was applied using GraphPad Prism 5 in order to compare the SCFA production in the faecal microbiota after 24 hrs fermentation with the supplements against glucose-supplemented medium fermentation.

2.4 Results

Chemical composition of GMP preparations

Two GMP preparations were investigated in this study, a commercial fraction obtained from Arla Foods Ingredients called Lacprodan CGMP-10 (CGMP-10), and a GMP preparation prepared in our laboratories in Teagasc Moorepark Food Research Centre (tGMP). The chemical composition of these two GMP products is shown in **Table 5**. CGMP-10 had a higher content of GMP (70.2%) and lower content of lactose (<2%) compared to tGMP with 51.4% and 4.4% respectively. The

protein content in CGMP-10 and tGMP was 80-84% and 65.6% respectively. The two products varied in their content of the whey proteins beta-lactoglobulin (β -lactoglobulin) and alpha-lactalbumin (α -lac) with 10.6% and 8% respectively for CGMP-10 and 1.5% and 19.3% respectively for tGMP. To investigate the feasibility of using less purified GMP, both preparations were carried forward into the artificial colon model.

Table 5 Chemical composition of the two glycomacropeptide products used in this study, Lacprodan CGMP-10 and semi-purified tGMP.

<i>Component</i>	<i>CGMP-10</i>	<i>tGMP</i>
<i>β-Ig^a</i>	10.63%	1.48%
<i>α-lac^b</i>	8.02%	19.32%
<i>GMP</i>	70.22($\pm$ 4.7)%	51.39($\pm$ 7.1)%
<i>Lactose</i>	<2%	4.4%
<i>Total protein</i>	80-84%	65.61%

^a *β -Ig*, beta lactoglobulin. ^b *α -Lac*, alpha lactalbumin.

Compositional differences between the faecal microbiota from healthy and frail older donors

We have previously reported that elderly subjects living in long-term residential care units have a lower diversity microbiota of a characteristic composition compared to that of community dwelling subjects (Claesson *et al.*, 2012; Jeffery *et al.*, 2016). The compositional differences include significantly reduced Lachnospiraceae abundance in the microbiota of longstay subjects compared to community including genera such as *Coprococcus* and *Roseburia*, and significant enrichment in Proteobacteria abundance including Enterobacteriaceae. We re-enrolled 3 community-dwelling and 3 long-term care unit residing ELDERMET (Claesson *et al.*, 2012) subjects (i.e., a

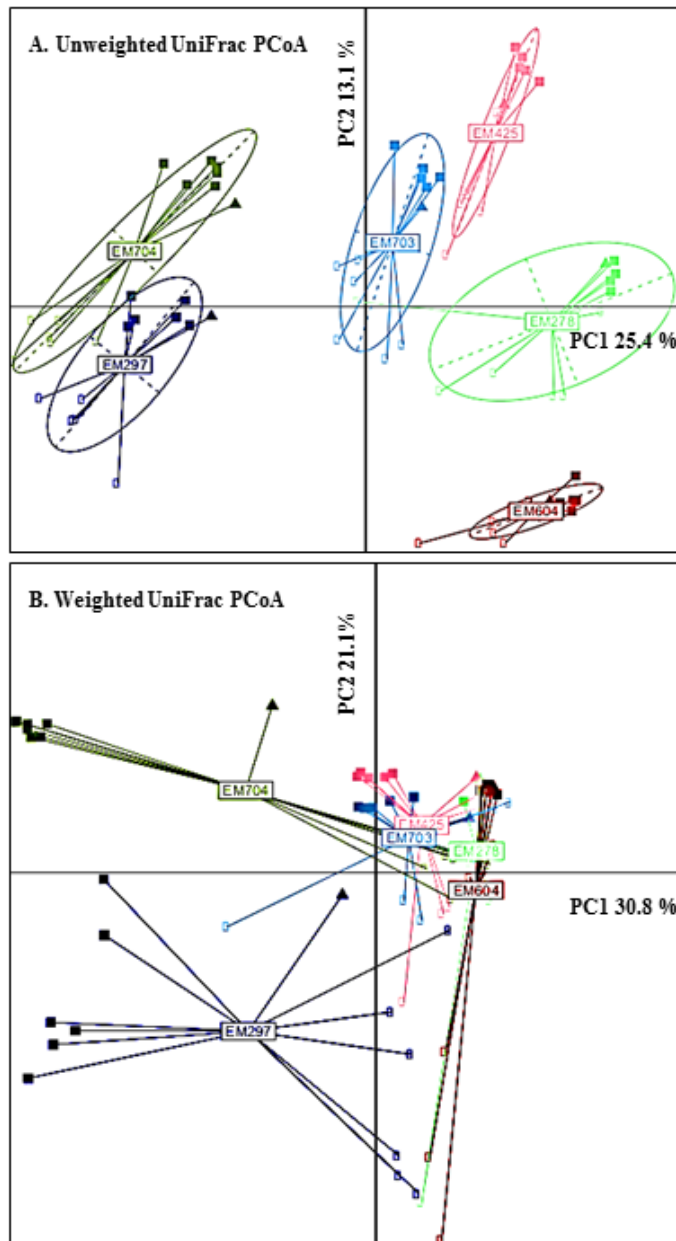
3385 cohort of 500 elderly whose gut microbiota composition was characterised and link
3386 between health and microbiota was investigated) and re-examined their microbiota
3387 several years after initial analysis to ensure that the composition had the expected
3388 profile as described in previous studies (Claesson *et al.*, 2012; Jeffery *et al.*, 2016).

3389 Based on the principal coordinates analysis (PCoA) of the Weighted (**Figure 3 A**)
3390 and Unweighted UniFrac (**Figure 3 B**) distances based on the sequenced 16S rRNA
3391 gene sequences in the faecal samples at baseline (time point 0 hrs), two of the
3392 samples obtained from frail elderly (EM297 and EM704) clustered closer together
3393 and separated from the other four samples (EM703, EM425, EM604, EM278). Thus,
3394 one of the longstay subjects had a community type microbiota. For the downstream
3395 analysis of the samples collected after 24 hrs of *in vitro* fermentation, the samples
3396 were aggregated as follows. Samples EM297 and EM704 were aggregated into
3397 longstay type (LS), and sample EM703 obtained from a frail individual was
3398 considered together with EM425, EM604, EM278 obtained from healthy older
3399 individuals as community type (COM). Across all six medium / supplementation
3400 combinations, all six time point 0 hrs microbiota clustered by donor, indicating an
3401 accurate baseline microbiota / inoculum for each fermenter condition tested.

3402 The composition of the community and longstay type faecal microbiota at phylum
3403 and family level is shown in **Figure 4** and **5** respectively. The two types of
3404 microbiota had distinct differences. Longstay type samples were significantly
3405 enriched for Euryarchaeota (average 27% relative abundance) and Proteobacteria
3406 (average 14% relative abundance) compared to the community type microbiota. The
3407 community type microbiota had higher relative abundance of Firmicutes
3408 (approximately 68%) and Bacteroidetes (approximately 22.5%) compared to an

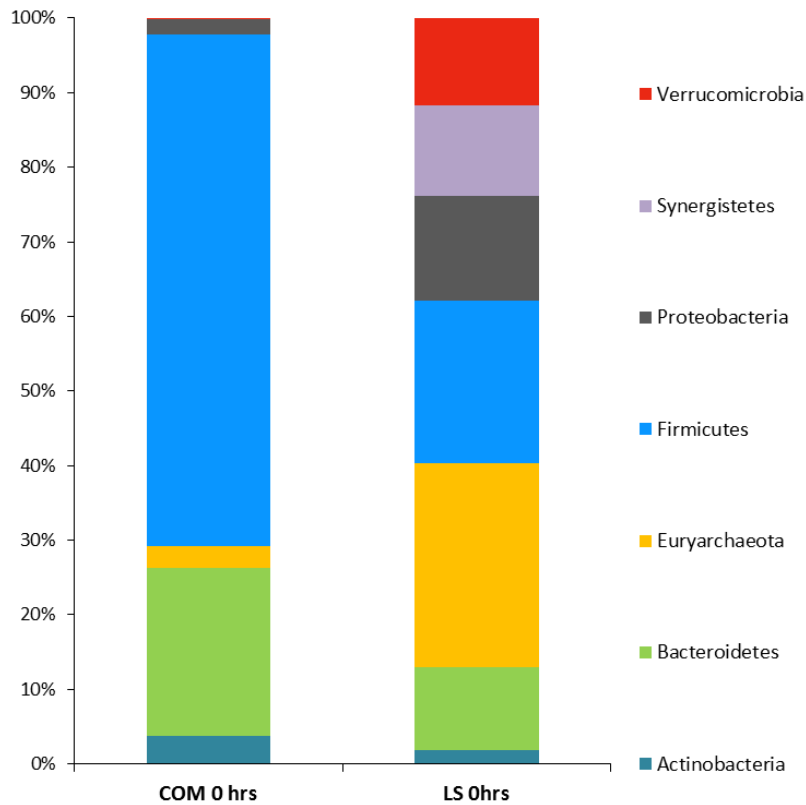
3409 average 22% and 11% respectively in the longstay type microbiota. At family level
3410 Lachnospiraceae comprised up to around 37% of the community type microbiota
3411 whereas in longstay type microbiota, Lachnospiraceae were present with an average
3412 4% relative abundance. Other distinct differences between the two sample types
3413 were the relative abundance of the Ruminococcaceae family, average 14% in
3414 community type and 6% in longstay type, and the enrichment in the longstay type
3415 faecal microbiota of the Verrucomicrobiaceae, Synergistetes and
3416 Methanobacteriaceae compared to community type (**Figure 5 B**).

3417 The observations here are broadly consistent with our previous description of
3418 microbiota composition in frail elderly subjects, allowing us to conclude that the
3419 available samples were suitable representatives to allow for the investigation of the
3420 effect of the selected dairy ingredients on the microbiota of elderly people.



3421

3422 **Figure 3 Principal coordinates analysis of UniFrac Unweighted (A) and**
 3423 **Weighted (B) distances. “■” time points 0 hrs and “□” time points 24 hrs, “▲”**
 3424 **stool microbiota before faecal slurry preparation.**



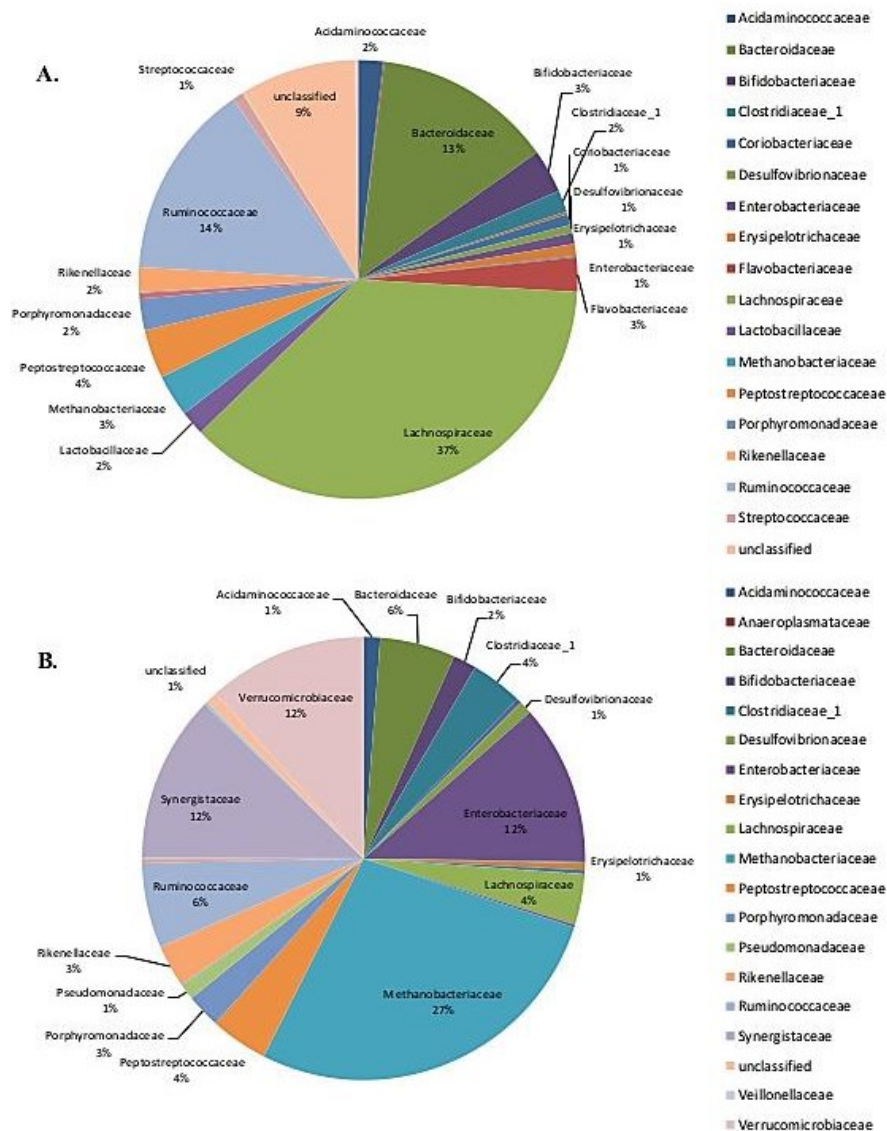
3425

3426 **Figure 4 Composition of the baseline (0 hrs) faecal microbiota at Phylum level.**

3427 The relative abundance of the various phyla is shown for aggregated community

3428 (COM; EM425, EM278, EM604 and EM703) and aggregated longstay (LS; EM297,

3429 EM704) faecal microbiota. Only phyla with relative abundance $\geq 0.5\%$ are shown.



3430

3431 **Figure 5 Composition of the baseline (0hrs) faecal microbiota at Family level.**

3432 The relative abundance of the various families is shown for (A) aggregated
 3433 community (EM425, EM278, EM604 and EM703) and (B) aggregated longstay type
 3434 faecal microbiota (EM297, EM704).

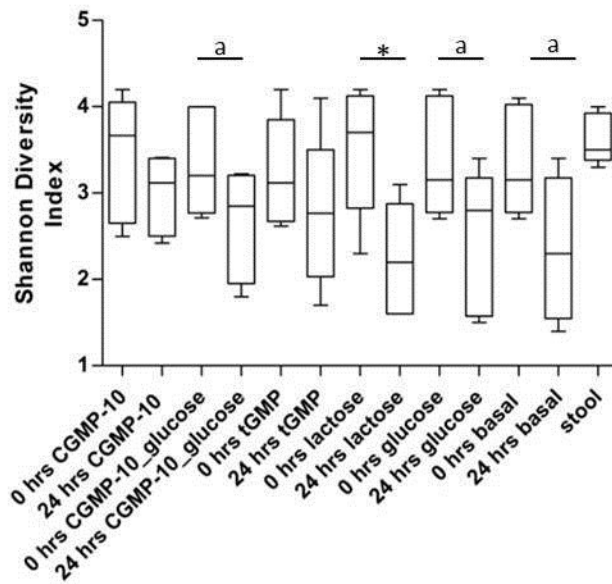
3435 **GMP supplementation sustained a higher diversity faecal microbiota compared**
 3436 **to other media**

3437 We tested the development of the microbiota in the artificial colon in a basal medium
 3438 without carbohydrates supplementation, compared to five supplementation regimes

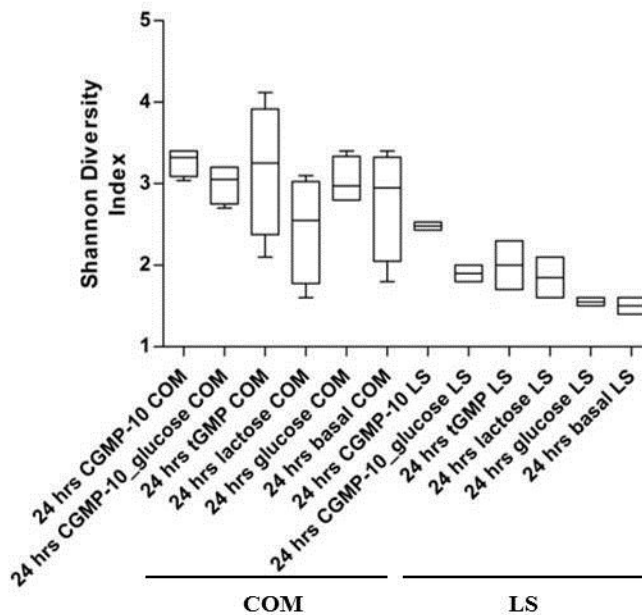
3439 with the two GMP preparations, glucose (a common carbohydrate of typical human
 3440 diet) and lactose. Regardless of any supplementation of the medium, there was an
 3441 overall decrease in the alpha diversity of the faecal microbiota after 24 hrs *in vitro*
 3442 fermentation, measured here by the Shannon diversity index. The decrease in the
 3443 microbiota diversity was expected due to the adaptation to the *in vitro* conditions and
 3444 it was in line with published *in vitro* studies that reported that the microbiota
 3445 diversity decreased over the first fermentation hours and began to increase after 72
 3446 hrs without attaining the diversity level of the initial stool sample (Rajilic-Stojanovic
 3447 *et al.*, 2010). Here, we aimed to observe which of the supplementation regimes
 3448 sustained the highest microbiota diversity. The effect on faecal microbiota by
 3449 medium supplementation is shown in **Figure 6 A** aggregated across all six donors
 3450 for each growth medium; the diversity of the respective 0 hrs sample, and the
 3451 diversity of the initial stool are also shown in **Figure 6 A**. The mean Shannon
 3452 diversity index value at time-point 0 hrs was 3.4 (± 0.6 SD). After 24 hrs
 3453 fermentation with basal medium (no carbohydrate supplementation), supplemented
 3454 with glucose or with lactose, the diversity decreased to a Shannon diversity value of
 3455 ca. 2.4. Fermentation with medium supplemented with GMP resulted in higher
 3456 diversity values. Supplementation with CGMP-10, CGMP-10 combined with
 3457 glucose, and supplementation with tGMP resulted in Shannon diversity scores of 3.0
 3458 (± 0.4 SD), 2.7 (± 0.6 SD) and 2.8 (± 1 SD) respectively. Wilcoxon test of ranking of
 3459 the Shannon diversity index of paired individual samples by medium
 3460 supplementation and time (between 24 hrs and 0 hrs) showed that after lactose
 3461 fermentation, the faecal microbiota alpha diversity decreased significantly ($p=0.03$)
 3462 compared to 0 hrs. There was a stronger trend in the decrease of Shannon diversity
 3463 after fermentation with basal medium or medium supplemented with glucose, or with

3464 CGMP-10 combined with glucose ($p=0.06$) compared to the decrease in Shannon
3465 diversity after fermentation with CGMP-10 supplemented medium ($p=0.16$). Similar
3466 effect of the substrates on the faecal microbiota diversity was observed on
3467 aggregated community samples, and aggregated longstay type samples, where the
3468 CGMP-10 supplemented medium sustained higher microbiota diversity compared to
3469 the other substrates tested (**Figure 6 B**).

A. Aggregated microbiota composition across all six donors



B. Microbiota composition separated by donor residential location



3470

3471 **Figure 6 Effect of the fermentation substrates on the alpha diversity (Shannon**
 3472 **diversity index) of the faecal microbiota.** Effect on diversity of aggregated
 3473 microbiota composition across all donors (**A**) and microbiota separated by donor
 3474 residential location, i.e. community (COM) and longstay (LS) (**B**). Stool sample
 3475 represents the faecal microbiota before the preparation of inoculum faecal slurry.
 3476 Wilcoxon test result: “*” $p < 0.05$; “a” $p < 0.1$.

3477 Detailed microbiota responses to GMP supplementation

3478 **Community type microbiota:** Although retention of microbiota diversity upon
3479 supplementation with GMP is desirable, it was necessary to examine fine-detail
3480 microbiota effects. The microbiota profile at family and genus level for community
3481 aggregated samples is shown in **Figure 7 A** and **B**. The baseline high proportion of
3482 Lachnospiraceae was maintained after fermentation supplemented with all substrates
3483 tested (except after basal medium fermentation) and predominantly with CGMP-10
3484 combined with glucose (average 51% relative abundance). Supplementation of the
3485 fermentation medium with CGMP-10 had a unique effect on the relative abundance
3486 of the families Rickenellaceae and Acidaminococcaceae that increased from an
3487 average 1.8% abundance to 3% and 4.5% respectively. Supplementation of the
3488 fermentation medium with CGMP-10 suppressed the growth of Erysipelotrichaceae
3489 compared to supplementation with the other substrates as shown in **Figure 7 A**. The
3490 lowest proportion of the family Bacteroidaceae was observed after lactose
3491 fermentation (average 4% relative abundance) whereas after fermentation with the
3492 other tested media the relative abundance of the family remained similar to baseline
3493 (approximately 10%). High Enterobacteriaceae relative abundance increase was
3494 observed after fermentation with basal medium from an average 0.8% relative
3495 abundance at baseline to an average 30%. Substantial increase in the relative
3496 abundance of Enterobacteriaceae was also observed after fermentation with lactose
3497 or tGMP supplemented medium. In both supplementation regimes, i.e. lactose or
3498 tGMP, the proportion of the Enterobacteriaceae increased to approximately 17%
3499 relative abundance. The Enterobacteriaceae affected comprised mainly of the genera
3500 *Escherichia/Shigella* (**Figure 7 B**). Clostridiaceae 1 (comprised of the *Clostridium*
3501 sensu stricto cluster) relative abundance demonstrated an increasing trend regardless

3502 of fermentation substrate (**Figure 7 A**). After basal medium fermentation
3503 Clostridiaceae 1 relative abundance had the highest increase compared to the other
3504 substrate fermentations, increasing from approximately 1.6% relative abundance at
3505 baseline to 13%. Fermentation with GMP-supplemented medium resulted in >6.5%
3506 relative abundance of Clostridiaceae 1.

3507 **Longstay type microbiota:** In fermenters seeded with the longstay type microbiota
3508 the increase in relative abundance of the Clostridiaceae 1 and Enterobacteriaceae
3509 families was the predominant observation irrespective of the fermentation medium
3510 suggesting loss of major taxa in the faecal microbiota under 24 hrs *in vitro*
3511 conditions (**Figure 7 A**). The dominant genera were *Clostridium* sensu stricto and
3512 *Escherichia/Shigella* respectively (**Figure 7 B**). The proportion of the relative
3513 abundance occupied by the families Clostridiaceae 1, Enterobacteriaceae and
3514 Peptostreptococcaceae exceeded 60% across the samples. The predominant group in
3515 Peptostreptococcaceae was *Clostridium* cluster XI (**Figure 7 B**). Interestingly,
3516 although fermentation supplemented with CGMP-10 resulted in a high combined
3517 relative abundance of the families Clostridiaceae 1, Enterobacteriaceae and
3518 Peptostreptococcaceae (approximately 70%) there was also an increase in the
3519 relative abundance of Lachnospiraceae from approximately 4% to 11%, and
3520 maintenance of the low relative abundance of Streptococcaceae (<0.5%).
3521 Fermentation supplemented with lactose also resulted in increased Lachnospiraceae
3522 relative abundance (to approximately 7.5%) but lactose also promoted the growth of
3523 Streptococcaceae from <0.5% relative abundance to approximately 20%.

3524 **CGMP-10 positively affected the growth of health-related taxa, predominantly**
3525 **in community type microbiota**

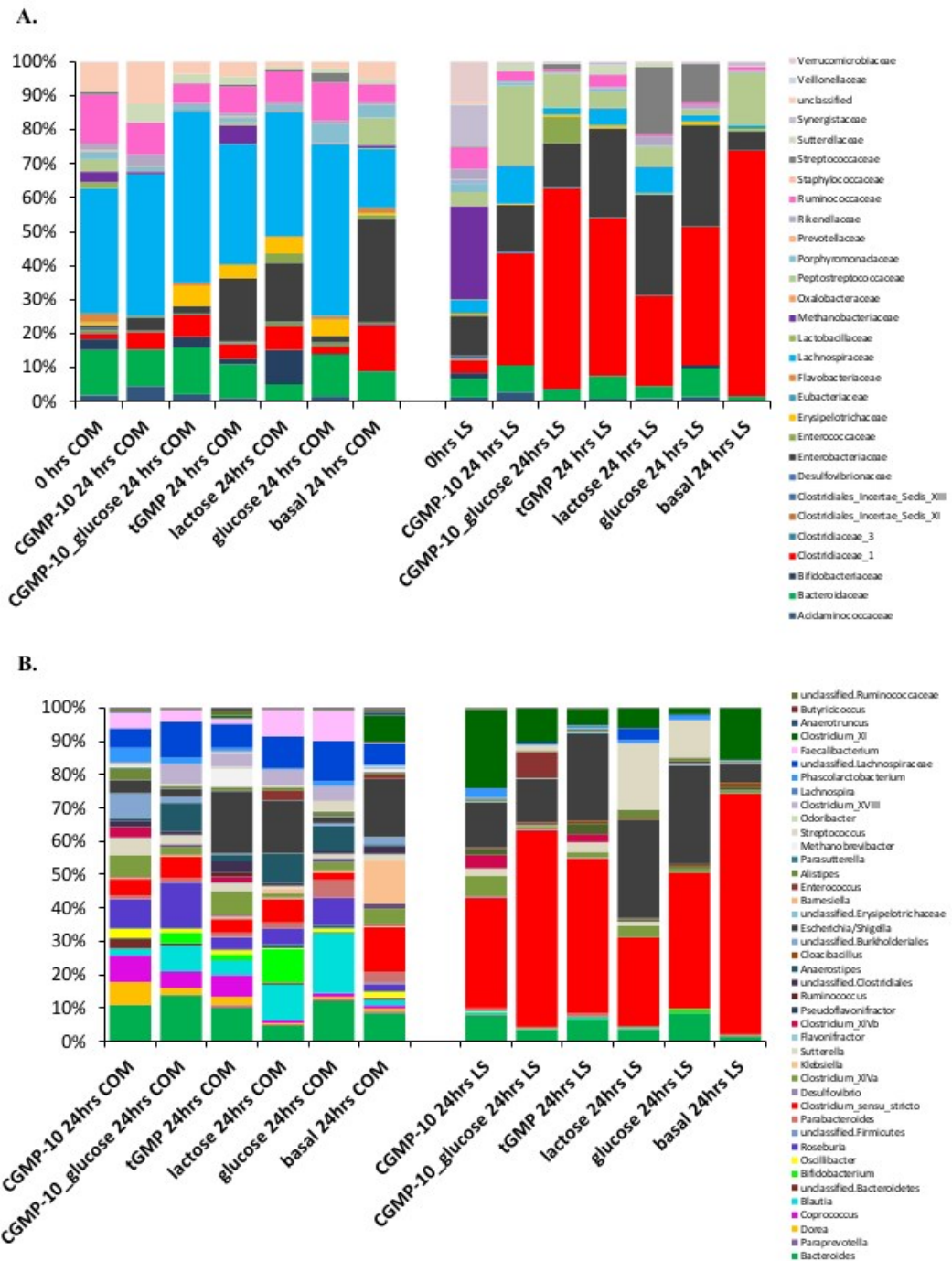
3526 To provide further resolution we used the DESeq2 algorithm to identify bacterial
3527 taxa showing altered abundance after 24 hrs fermentation supplemented with the
3528 selected substrates (**Figure 8**). For certain taxa, change in the abundance after 24 hrs
3529 fermentation was similar irrespective of the fermentation supplement. The
3530 abundance of *Clostridium* cluster IV, *Ruminococcus* and *Lachnospira* decreased in
3531 both faecal microbiota types irrespective of fermentation supplement. With one
3532 exception (fermentation of glucose-supplemented medium by longstay type
3533 microbiota) *Sutterella* abundance increased in both microbiota types after
3534 fermentation with all fermentation supplements. *Clostridium* cluster sensu stricto and
3535 *Escherichia/Shigella* abundance also showed an increasing trend irrespective of
3536 fermentation supplement.

3537 Supplementation with CGMP-10 of the artificial colon medium seeded with either
3538 microbiota type led to an increase of *Clostridium* cluster XIVb and *Coprococcus*
3539 abundance. In the community type microbiota the increase was approximately 6-fold
3540 ($\text{padj} < 0.0005$) and 3-fold ($\text{padj} = 0.05$) respectively. In the longstay type microbiota
3541 the respective fold-changes were approximately 10 ($\text{padj} < 0.0005$) and 1 (not
3542 significant). An increasing trend in the abundance of *Pseudoflavonifractor* was
3543 observed after supplementation with CGMP-10; approximately 2-fold (not
3544 significant) and 16-fold ($\text{padj} = 0.08$) increase in community and longstay microbiota
3545 respectively. After fermentation with medium supplemented with CGMP-10 by the
3546 community type microbiota an increasing trend in the abundance of the genus
3547 *Roseburia* by approximately 6-fold ($\text{padj} = 0.08$) was observed. The abundance of the
3548 genera *Dorea* increased by 8-fold without reaching significance. Importantly,
3549 analysis of the composition of the faecal microbiota per individual sample after
3550 fermentation with medium supplemented with CGMP-10 showed a consistent

3551 increase of the relative abundance of *Dorea* in all samples from healthy donors and
3552 EM703 (data not shown). Fermentations with CGMP-10 combined with glucose
3553 yielded similar changes (but not significant) in the abundance of the aforementioned
3554 taxa in the community samples as with fermentation with CGMP-10 alone. The
3555 tGMP also increased the abundance of *Clostridium* cluster XIVb; approximately 16-
3556 fold (not significant) in community type microbiota and 9 log2fold (padj<0.0005) in
3557 longstay microbiota.

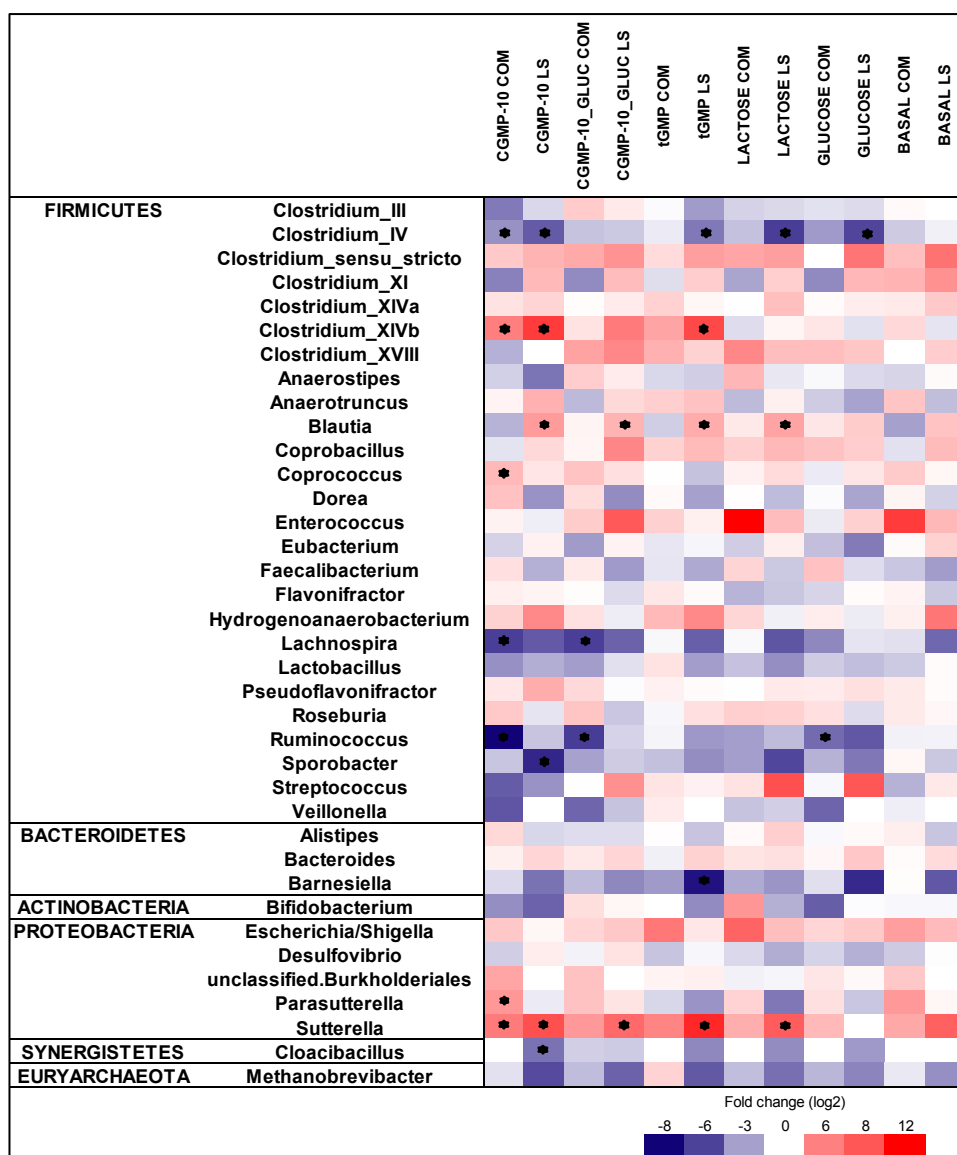
3558 For both microbiota types fermentation of lactose supplemented-medium yielded
3559 (mostly not significant) increase in the abundance of *Blautia* (longstay type;
3560 approximately 16-fold, padj<0.007), *Clostridium* cluster XVIII (community type;
3561 approximately 64-fold, padj<0.06), *Enterococcus*, *Streptococcus*,
3562 *Escherichia/Shigella*. Increase in *Bifidobacterium* abundance was observed only
3563 when baseline abundance was high as in the case of EM278 (data not shown).

3564



3565

3566 **Figure 7 Faecal microbiota profile after 24 hrs fermentation.** Community type
 3567 (COM) and longstay type aggregated (LS) microbiotas are presented at family level
 3568 (A) and genus level (B).

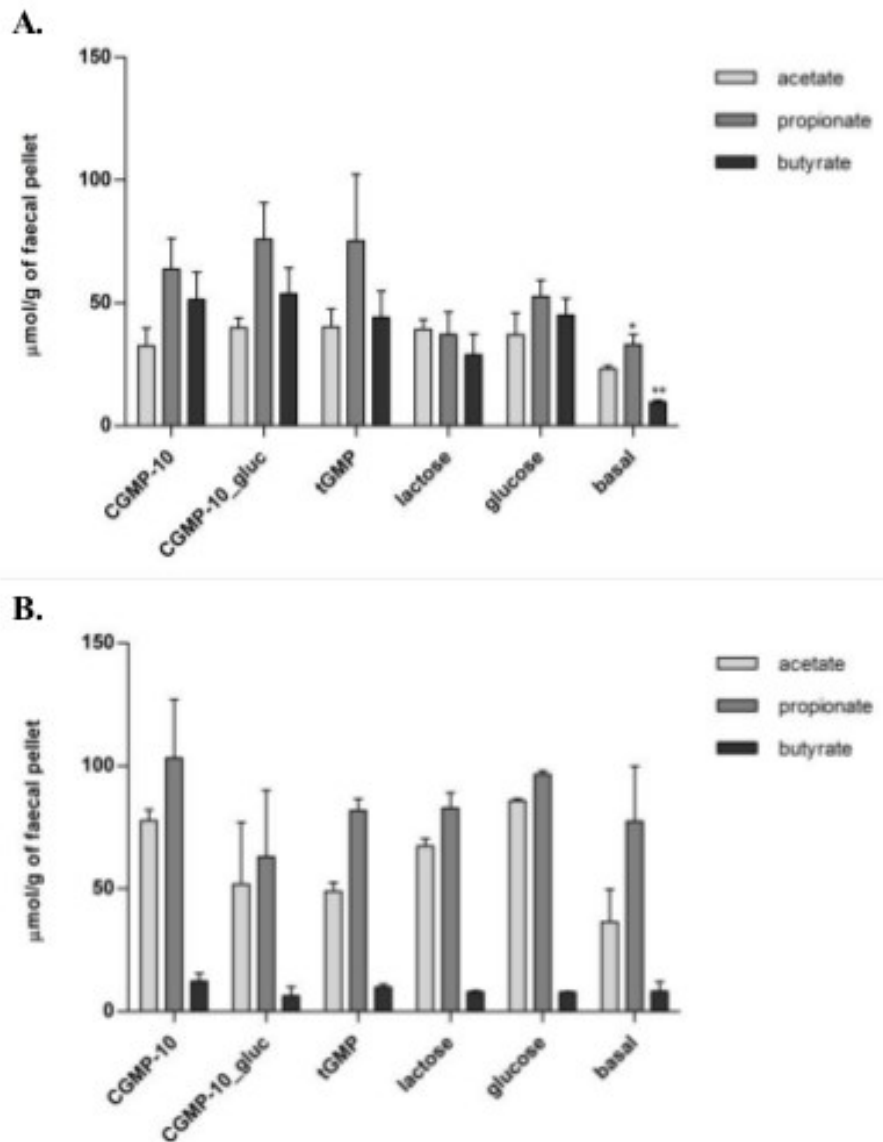


3569

3570 **Figure 8 Heat-map of the changes in bacterial genus abundance in the faecal**
3571 **microbiota after 24 hrs fermentation with selected substrates.** Comparisons were
3572 performed pairwise between 0hrs and 24 hrs for aggregated microbiota types by
3573 residential location as defined in main text. The logarithmic (log2) fold change
3574 results were generated using the DeSeq2 package. COM and LS correspond to
3575 community type and longstay type aggregated faecal microbiotas respectively.
3576 Significance is denoted by “*” for p values adjusted for multiple testing using
3577 Benjamini-Hochberg, padj<0.05.

3578 **GMP supplementation and SCFA production in the faecal microbiota of**
3579 **healthy elderly subjects**

3580 Community type faecal microbiota fermentation with GMP supplementation
3581 (substrate conditions iv, v, vi as shown in Materials and Methods) resulted in higher
3582 SCFA (i.e. propionate, acetate, butyrate) production compared to the other media
3583 tested; GMP-supplemented media: >148 μ mol of propionate, acetate and butyrate
3584 combined per grams of faecal pellet, lactose supplemented medium: mean of 113.3
3585 μ mol combined SCFA per g of faecal pellet, glucose supplemented medium: mean
3586 of 133.7 μ mol combined SCFA per g of faecal pellet, basal medium: mean of 66
3587 μ mol combined SCFA per g of faecal pellet (**Figure 9 A**). No significant differences
3588 were obtained when comparing the SCFA production after supplementation with
3589 either GMP supplementation regime or lactose against the SCFA production after
3590 fermentation with medium supplemented with glucose; only fermentation with basal
3591 medium resulted in significantly lower production of propionate ($p<0.05$) and
3592 butyrate ($p<0.005$). For the longstay type microbiota the profile of the SCFA
3593 production was similar irrespective of the fermentation substrate (**Figure 9 B**)
3594 mirroring the loss of major taxa, among them potentially butyrate producers,
3595 observed for the profile of the microbiota composition (**Figure 7**). No statistical
3596 analysis was performed for longstay microbiota SCFA production due to the small
3597 group size.



3598

3599 **Figure 9 Short chain fatty acid (SCFA) levels in the faecal microbiota after 24**
 3600 **hrs fermentation in media supplemented as indicated.** Concentration ($\mu\text{mol/g}$ of
 3601 faecal pellet) of acetate, propionate and butyrate in aggregated community (A) and
 3602 longstay (B) microbiotas. Kruskal-Wallis test with Dunn's post hoc test was
 3603 performed to compare SCFA production at 24 hrs after supplementation with the
 3604 various substrates against glucose supplementation in the community type
 3605 microbiota; “*” $p < 0.05$, “**” $p < 0.005$.

3606 2.5 Discussion

3607 Prebiotic effect of GMP on the faecal microbiota of healthy older donors

3608 We investigated the *in vitro* effect of GMP supplementation on the gut microbiota
3609 using two GMP preparations and two types of faecal microbiota, i.e. community type
3610 and longstay type. CGMP-10 had a lower concentration of lactose compared to
3611 tGMP. The two microbiota types had major composition differences summarised by
3612 reduced overall diversity and abundance of Firmicutes, enrichment in Euryarchaeota,
3613 Proteobacteria and proteolytic taxa like Synergistetes and Verrucomicrobia in the
3614 longstay compared to the community type. The distinctive longstay microbiota
3615 profile apparently explains the loss of major taxa and the dominance of *Clostridium*
3616 sensu stricto cluster that was observed after fermentations with all tested substrates,
3617 because ecosystem stability and adaptive responsiveness to environmental changes
3618 are known to increase with phylogenetic diversity (Cadotte *et al.*, 2012; Salonene *et*
3619 *al.*, 2014; Alberdi *et al.*, 2016).

3620 For both microbiota types, supplementation of the fermentation medium with
3621 CGMP-10 sustained a higher diversity microbiota compared to the other media
3622 followed in rank order by the other two GMP-containing media, ie. tGMP and
3623 CGMP-10 combined with glucose. GMP containing medium resulted in higher total
3624 SCFA production from community microbiota-seeded fermentations compared to
3625 the other substrates tested. Increased SCFA concentration in the colon is known to
3626 correlate with a wide portfolio of health benefits (Koh *et al.*, 2016).

3627 The Lachnospiraceae member *Coprococcus* spp. is one of the health-relevant taxa
3628 that was positively affected by CGMP-10 supplementation as shown by the increase
3629 in the relative abundance of this group from the starting levels in the faecal

3630 microbiota of mostly healthy donors after 24 hrs fermentation. In our previous
3631 studies, low abundance of coprococci in the faecal microbiota of older donors
3632 correlated with poor health status and a less diverse diet that was low in fibre
3633 (Claesson *et al.*, 2012; Jeffery *et al.*, 2016). Based on studies of the faecal microbiota
3634 from patients with colorectal cancer increased relative abundance of *Coprococcus*
3635 was negatively correlated with the condition (Flemer *et al.*, 2016). CGMP-10 had a
3636 positive effect on the abundance of *Dorea* in the faecal microbiota of community
3637 dwelling older subjects. The increase in abundance of these two taxa indicates
3638 potentially important health benefits deriving from the consumption of GMP because
3639 *Coprococcus* and *Dorea*-enriched gut microbiota was found to be significantly
3640 resistant to pathobiont colonisation (Kampmann *et al.*, 2016). CGMP-10 also
3641 positively affected the abundance of *Clostridium* cluster XIVb in both sample types.
3642 In a murine model of leukaemia and cachexia, cluster XIVb and *Parasutterella*
3643 species were among the first taxa to reduce in abundance in the murine faecal
3644 microbiota in these two diseases (Bindels *et al.*, 2016). In this study the abundance
3645 of *Parasutterella* in the community type microbiota significantly increased after
3646 CGMP-10 supplementation. Importantly, *Clostridium* cluster XIVb is among the
3647 bacterial groups that increase in abundance after restoration of dysbiotic *Clostridium*
3648 *difficile*-associated diarrhoea gut microbiota by faecal microbiota transplantation
3649 (Brown *et al.*, 2016). CGMP-10 had a positive effect on the abundance of *Roseburia*
3650 but only for the community type microbiota. For this taxon it is well-documented
3651 that it contributes to butyrate production which is a major contributor for colonic
3652 health (Koh *et al.*, 2016). The differential response may be due to the low starting
3653 abundance levels of the genus *Roseburia* in the longstay type microbiota.

3654 **Lactose content of supplemented medium increased Proteobacteria abundance**

3655 Lactose-dependent modulation of the gut microbiota resulted in increased relative
3656 abundance of taxa related to inferior health status. Both lactose and tGMP enhanced
3657 the growth of Enterobacteria of the phylum Proteobacteria. This Phylum harbours
3658 pathobionts like the facultatives *Escherichia/Shigella* that can lead to disease
3659 (Kamada *et al.*, 2013). Lactose fermentation promoted the increase in abundance of
3660 the Erysipelotrichaceae *Clostridium* cluster XVIII. This taxon has been associated
3661 with inferior health status in older subjects residing in long-term care units (Jeffery
3662 *et al.*, 2016). In this study we could not conclude on the prebiotic potential of lactose.
3663 Other studies have reported a positive effect of lactose on lactobacilli and
3664 bifidobacteria (Szilagyi, 2015). *In vivo* trials will be conducted to further elucidate
3665 the effect of lactose on the faecal microbiota and to allow for lactose-related dietary
3666 recommendations.

3667 **Effect of the *in vitro* conditions on the faecal microbiota**

3668 In this study faecal samples were treated individually as opposed to samples being
3669 pooled from several donors, an approach used in some other studies (Aguirre *et al.*,
3670 2014). We opted for this because pooling faecal sample produce inocula that would
3671 be non-representative of average “real” microbiota (Payne *et al.*, 2012). Partly in
3672 agreement with previous studies (Rajilic-Stojanovic *et al.*, 2012), here we report
3673 decrease of microbiota diversity and decrease in abundance of *Clostridium* cluster IV
3674 as a result of all of the *in vitro* conditions rather than a result of any individual
3675 fermentation substrate. A decrease in the abundance of *Ruminococcus* was also
3676 observed irrespective of fermentation substrate or microbiota type and potentially
3677 due to the lack of resistant starch in the fermentation media (Ze *et al.*, 2012).

3678 Omitting resistant starch was done to avoid missing the effects conferred by the
3679 carbohydrates attached to the GMP. Interestingly, increase in *Blautia* spp. was
3680 observed in most longstay samples irrespective of fermentation medium suggesting a
3681 potential underlying longstay-type related trend. Bacteria of the genus *Blautia* are a
3682 prominent member of the healthy adult gut microbiota (Touyama *et al.*, 2015) and
3683 are reduced in abundance in older individuals (Claesson *et al.*, 2012). In spite of the
3684 predominant role of this taxon in the “healthy” adult microbiota, increased
3685 abundance of *Blautia* spp. can correlate to colorectal cancer (Flemer *et al.*, 2016).
3686 For this reason the health benefit of promoting their growth by prebiotics is
3687 debatable, at least until species detail is available.

3688 In search for new potential prebiotics we demonstrate here the capacity of GMP, a
3689 milk derivative with documented anti-inflammatory properties, to modulate *in vitro*
3690 the gut microbiota of elderly subjects by promoting the growth of several health-
3691 relevant taxa. GMP also increased SCFA production and sustained the diversity of
3692 the microbiota from healthy elderly inocula and to a lesser extend in inocula from
3693 frail elderly under *in vitro* conditions. *In vivo* trials are necessary to confirm the
3694 microbiota programming by GMP observed here since *in vitro* systems present
3695 certain limitations in simulating the real-life effect of diet compounds on the gut
3696 microbiota.⁴⁹ Importantly, since there are indications that inferior gut microbiota
3697 diversity may be associated with frailty in older age (O’Toole and Jeffery, 2015)
3698 GMP could contribute to amelioration of frailty scores by promoting gut microbiota
3699 diversity.

3700 **2.6 Funding Sources**

3701 This work was financially supported by the Government of Ireland National
3702 Development Plan by way of a Department of Agriculture, Food and the Marine
3703 (DAFM) under a Food Institutional Research Measure (FIRM) award (11/F/053) for
3704 the ELDERFOOD project.

3705 **2.7 Acknowledgements**

3706 We thank Arla Foods Ingredients, Denmark for providing Lacprodan CGMP-10 and
3707 Kerry Group plc, Listowel, Ireland for providing the sweet whey concentrate. We
3708 thank Dr Burkhardt Flemer for his help in the amplicon sequence analysis and Dr
3709 Emilio José Laserna-Mendieta for assistance in the SCFA analysis. Fodhla Ní
3710 Chonchúir and Tom F. O'Callaghan are supported by Teagasc Walsh Fellowships.

3711 2.8 References

- 3712 Aguirre, M., Ramiro-Garcia, J., Koenen, M. E., Venema, K. (2014). To pool or
3713 not to pool? Impact of the use of individual and pooled fecal samples for *in vitro*
3714 fermentation studies. *J. Microbiol Methods*, 107, 1–7.
- 3715 Albenzio, M., Santillo, A., Ciliberti, M. G., Figliola, L., Caroprese, M., Marino,
3716 R., Polito, A. N. (2016). Milk from different species : Relationship between
3717 protein fractions and inflammatory response in infants affected by generalized
3718 epilepsy. *J Dairy Sci*, 99(7), 5032-5038.
- 3719 Alberdi, A., Aizpurua, O., Bohmann, K., Zepeda-Mendoza, M. L., Gilbert, M. T.
3720 P. (2016). Do vertebrate gut metagenomes confer rapid ecological adaptation?
3721 *Trends Ecol Evol*, 31(9), 689–699.
- 3722 Bindels, L. B., Neyrinck, A. M., Claus, S. P., Le Roy, C. I., Grangette, C., Pot, B.,
3723 *et al.* (2016). Synbiotic approach restores intestinal homeostasis and prolongs
3724 survival in leukaemic mice with cachexia. *ISME J*, 10(6), 1456-1470.
- 3725 Brown, J. R. M., Zulquernain, A., Flemer, B., Joyce, S., Sheehan, D., Gahan, C.,
3726 Shanahan, F., O'Toole, P. W. (2016). Microbiota and metabolic changes
3727 associated with successful faecal micorbiota transplantation for *Clostridium*
3728 *difficile*-associated diarrhoea. In preparation.
- 3729 Brück, W. M., Redgrave, M., Tuohy, K., Lönnerdal, B., Graverholt, G., Hernell,
3730 O., Gibson, G. R. (2006). Effects of bovine alpha-lactalbumin and casein
3731 glycomacropeptide-enriched infant formulae on faecal microbiota in healthy term
3732 infants. *J Pediatr Gastroenterol Nutr*, 43(5), 673–679.

3733 Cadotte M. C., Dinnage, R., Tilman, D. (2012). Phylogenetic diversity promotes
3734 ecosystem stability. *Ecology*, 93, S223-S233.

3735 Cho, I. and Blaser, M. J. (2012). The human microbiome: at the interface of
3736 health and disease. *Nat Rev Genet*, 13(4), 260–70.

3737 Claesson, M. J., Cusack, S., O’Sullivan, O., Greene-Diniz, R., de Weerd, H.,
3738 Flannery, E., *et al.* (2011). Composition, variability, and temporal stability of the
3739 intestinal microbiota of the elderly. *Proc Nat Acad Sci USA*, 108 Suppl, 4586–91.

3740 Claesson, M. J., Jeffery, I. B., Conde, S., Power, S. E., O’Connor, E. M., Cusack,
3741 S., *et al.* (2012). Gut microbiota composition correlates with diet and health in
3742 the elderly. *Nature*, 488(7410), 178–184.

3743 Donaldson, G. P., Lee, S. M., Mazmanian, S. K. (2015). Gut biogeography of the
3744 bacterial microbiota. *Nature Rev Microbiol*, 14(1), 20–32.

3745 Edgar, R. C. (2013). UPARSE: highly accurate OTU sequences from microbial
3746 amplicon reads. *Nat. Methods*, 10(10), 996–8.

3747 Flemer, B., Lynch, D. B., Brown, J. M. R., Jeffery, I. B., Ryan, F. J., Claesson,
3748 M. J., O’Riordan, M., Shanahan, F. (2016) Tumour-associated and non-tumour-
3749 associated microbiota in colorectal cancer. *Gut*, 66(4), 633–643.

3750 Foxx-Orenstein, A. E. and Chey, W. D. (2012). Manipulation of the gut
3751 microbiota as a novel treatment strategy for gastrointestinal disorders. *Am J*
3752 *Gastroenterol, Supplements*, 1(1), 41–46.

3753 Guerra, A., Etienne-Mesmin, L., Livrelli, V., Denis, S., Blanquet-Diot, S., Alric,
 3754 M. (2012). Relevance and challenges in modelling human gastric and small
 3755 intestinal digestion. *Trends Biotechnol*, 30, 591–600.

3756 Haas, B. J., Gevers, D., Earl, A. M., Feldgarden, M., Ward, D. V., Giannoukos,
 3757 *et al.* (2011). Chimeric 16S rRNA sequence formation and detection in Sanger
 3758 and 454-pyrosequenced PCR amplicons. *Genome Res*, 21(3), 494–504.

3759 Heintz, C. and Mair, W. (2014). You are what you host: microbiome modulation
 3760 of the aging process. *Cell*, 156(3), 408–11.

3761 Hernandez-Hernandez, O., Sanz, M. L., Kolida, S., Rastall, R. A., Moreno, F. J.
 3762 (2011). *In vitro* fermentation by human gut bacteria of proteolytically digested
 3763 caseinomacropeptide nonenzymatically glycosylated with prebiotic
 3764 carbohydrates. *J Agric Food Chem*, 59(22), 11949–55.

3765 Järvinen, R., Knekt, P., Hakulinen, T., Aromaa, A. (2001). Prospective study on
 3766 milk products, calcium and cancers of the colon and rectum. *Eur J Clin Nutr*, 55,
 3767 1000–1007.

3768 Jeffery, I. B., Lynch, D. B., O’Toole, P. W. (2016). Composition and temporal
 3769 stability of the gut microbiota in older persons. *ISME J*, 10(1), 170–182.

3770 Kamada, N., Chen, G. Y., Inohara, N., Núñez, G. (2013). Control of pathogens
 3771 and pathobionts by the gut microbiota. *Nat Immunol*, 14(7), 685–690.

3772 Kampmann, C., Dicksved, J., Engstrand, L., Rautelin, H. (2016). Composition of
 3773 human faecal microbiota in resistance to *Campylobacter* infection. *Clin*
 3774 *Microbiol Infect*, 22(1), 61.e1–61.e8.

3775 Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M.,
 3776 Glöckner, F. O. (2013). Evaluation of general 16S ribosomal RNA gene PCR
 3777 primers for classical and next-generation sequencing-based diversity studies.
 3778 *Nucleic Acids Res.*, *41*(1), e1.

3779 Koh, A., De Vadder, F., Kovatcheva-Datchary, P., Bäckhed, F. (2016). From
 3780 dietary fiber to host physiology: Short-chain fatty acids as key bacterial
 3781 metabolites. *Cell*, *165*(6), 1332–1345.

3782 Kreuss, M., Krause, I., Kulozik, U. (2008). Separation of a glycosylated and non-
 3783 glycosylated fraction of caseinomacropeptide using different anion-exchange
 3784 stationary phases. *J Chromatogr A*, *1208*(1–2), 126–32.

3785 Lana, A., Rodriguez-Artalejo, F., Lopez-Garcia, E. (2015). Dairy consumption
 3786 and risk of frailty in older adults: A prospective cohort study. *J Am Geriatr Soc*,
 3787 *63*(9), 1852–1860.

3788 Love, M. I., Huber, W., Anders, S. (2014). Moderated estimation of fold change
 3789 and dispersion for RNA-seq data with DESeq2. *Genome Biol*, *15*(12), 550.

3790 Lozupone C. A., Knight R. (2008). Species divergence and measurement of
 3791 microbial diversity. *FEMS Microbiol Rev*, *32*(4), 557–578.

3792 Magoč, T., Salzberg, S. L. (2011). FLASH: Fast length adjustment of short reads
 3793 to improve genome assemblies. *Bioinformatics*, *27*(21), 2957–2963.

3794 Neelima, Sharma, R., Rajput, Y.S., Mann, B. (2013). Chemical and functional
 3795 properties of glycomacropeptide (GMP) and its role in the detection of cheese
 3796 whey adulteration in milk: a review. *Dairy Sci Technol*, *93*(1), 21–43.

3797 O'Toole, P. W., Jeffery, I. B. (2015). Gut microbiota and aging. *Science*,
3798 350(6265), 1214–1216.

3799 Payne, A. N., Zihler, A., Chassard, C., Lacroix, C. (2012). Advances and
3800 perspectives in *in vitro* human gut fermentation modelling. *Trends Biotechnol*,
3801 30(1), 17–25.

3802 R Core Team. (2014). R: A language and environment for statistical computing.
3803 Vienna, Austria: R Foundation for Statistical Computing. [http://www.R-](http://www.R-project.org/)
3804 [project.org/](http://www.R-project.org/)

3805 Rajilić-Stojanović, M., Maathuis, A., Heilig, H. G., Venema, K., De Vos, W. M.,
3806 Smidt, H. (2010). Evaluating the microbial diversity of an *in vitro* model of the
3807 human large intestine by phylogenetic microarray analysis. *Microbiology*,
3808 156(11), 3270–3281.

3809 Rea, M. C., O'Sullivan, O., Shanahan, F., O'Toole, P. W., Stanton, C., Ross, R.
3810 P., Hill, C. (2012). *Clostridium difficile* carriage in elderly subjects and
3811 associated changes in the intestinal microbiota. *J Clin Microbiol*, 50(3), 867–875.

3812 Roberfroid, M. (2007). Prebiotics: the concept revisited. *J Nutr*, 137, 830S–837S.

3813 Robitaille, G. (2013). Growth-promoting effects of caseinomacropeptide from
3814 cow and goat milk on probiotics. *J Dairy Res*, 80, 58–63.

3815 Sahni, S., Mangano, K. M., Tucker, K. L., Douglas, P. K., Casey, V. A., Hannan,
3816 M.T. (2015). Protective association of milk intake on the risk of hip fracture:
3817 Results from the Framingham Original Cohort. *J Bone Miner Res*, 29(8), 37–54.

3818 Salonen, A., Lahti, L., Salojärvi, J., Holtrop, G., Korpela, K., Duncan, S. H., *et*
3819 *al.* (2014). Impact of diet and individual variation on intestinal microbiota
3820 composition and fermentation products in obese men. *ISME J*, 8(11), 2218-2230.

3821 Sawin, E. A., De Wolfe, T. J., Aktas, B., Stroup, B. M., Murali, S. G., Steele, J.
3822 L., Ney, D. M. (2015). Glycomacropeptide is a prebiotic that reduces
3823 *Desulfovibrio* bacteria, increases cecal short chain fatty acids and is anti-
3824 inflammatory in mice. *Am J Physiol Gastrointes Liver Physiol*, 309 (7), G590–
3825 G01.

3826 Sonnenburg, E. D.; Smits, S. A, Tikhonov, M., Higginbottom, S. K., Wingreen,
3827 N. S., Sonnenburg, J. (2016). Diet-induced extinctions in the gut microbiota
3828 compound over generations. *Nature*, 529(7585), 212–215.

3829 Szilagy, A. (2015). Adaptation to lactose in lactase non persistent people:
3830 Effects on intolerance and the relationship between dairy food consumption and
3831 evaluation of diseases. *Nutrients*, 7(8), 6751–6779.

3832 Tanimoto, M., Kawasaki, Y., Shinmoto, H., Dosako, S., Tomizawa A. (1991).
3833 Process of producing κ -casein glycomacropeptides. In Google patents.

3834 Thomä-Worringer, C., Sørensen, J., López-Fandiño, R. (2006). Health effects
3835 and technological features of caseinomacropeptide. *Int Dairy J*, 16(11), 1324–
3836 1333.

3837 Touyama, M., Jin, J. S., Kibe, R., Hayashi, H., Benno, Y. (2015). Quantification
3838 of *Blautia wexlerae* and *Blautia luti* in human faeces by real-time PCR using
3839 specific primers. *Benef Microbes*, 6(4), 583–590.

- 3840 van Calcar, S. C., Ney, D.M. (2012). Food products made with
3841 glycomacropeptide, a low-phenylalanine whey protein, provide a new alternative
3842 to amino Acid-based medical foods for nutrition management of phenylketonuria.
3843 *J Acad Nutr Diet*, 112(8), 1201–10.
- 3844 Wang, Q. Garrity, G. M. Tiedje, J. M. Cole, J. R. (2007). Naive Bayesian
3845 classifier for rapid assignment of rRNA sequences into the new bacterial
3846 taxonomy. *Appl Environ Microbiol*, 73(16), 5261–5267
- 3847 Ze, X., Duncan, S. H., Louis, P., Flint, H. J. (2012). *Ruminococcus bromii* is a
3848 keystone species for the degradation of resistant starch in the human colon. *ISME*
3849 *J*, 6(8), 1535–1543.
- 3850 Zhang, X., Yang, Y., Wu, Z., Weng, P. The modulatory effect of anthocyanins
3851 from purple sweet potato on human intestinal microbiota in vitro. *J Agric Food*
3852 *Chem*, 64 (12), 2582–2590.

3853

Chapter 3

3854

3855

3856

3857

Retention of microbiota diversity by lactose
free milk-supplemented diet was comparable
to soy protein diet in a mouse model of
healthy and frail elderly gut microbiota

3858 3.1 Abstract

3859 Compositional and phylogenetic diversity shifts of the gut microbiota, i.e. dysbiosis,
3860 may lead to disease. Dysbiotic changes in the microbiota are observed during ageing.
3861 Manipulation of the gut microbiota using prebiotics is a therapeutic strategy towards
3862 improving dysbiosis. Milk is a source of nutrients including complex
3863 oligosaccharides (OS), the prebiotic potential of which remains unexplored. We used
3864 a murine model to explore the effect of milk products on high and low diversity
3865 faecal microbiota (from healthy and frail older subjects, respectively) in comparison
3866 to soy protein supplementation, a product known to promote microbiota diversity
3867 and health in mice.

3868 The gut microbiota of conventional mice was eradicated using antibiotics; the mice
3869 were gavaged with microbiota from either a frail or a healthy donor. The mice
3870 received purified diets supplemented with either lactose free or whole milk,
3871 glycomacropeptide GMP, (a potentially prebiotic rich in sialic acid dairy product) or
3872 soy-protein. The gut microbiota was studied after a month of diet.

3873 Lactose-free milk diet was as efficient as the control diet in retaining microbiota
3874 diversity and it retained significantly higher diversity than the other diets in mice
3875 transplanted with microbiota deriving from either human donor type. Whole milk
3876 was not as efficient as lactose-free milk in retaining microbiota diversity but the
3877 relative abundance of health relevant taxa like *Ruminococcus bromii* were increased.
3878 Glycomacropeptide supplementation alone led to significantly decreased diversity
3879 compared to lactose-free milk and previously reported prebiotic activity was not
3880 confirmed *in vivo*. Bacteroidetes and Enterobacteriaceae were highly responsive to
3881 GMP supplemented diet; the sialic acid content of GMP may partly explain this

3882 microbiota response. When lacking lactose, the remaining carbohydrate component
3883 of milk sustained the growth of a wider range of microbiota members and promoted
3884 phylogenetic diversity in this murine model. Our results indicate the prebiotic
3885 potential of lactose-free milk.

3886 **3.2 Introduction**

3887 During the last decade, the bacterial component of the human gut microbiota has
3888 been extensively studied allowing for a more comprehensive understanding of the
3889 compositional and functional profile of the gut microbiota in health and disease
3890 (Marchesi *et al.*, 2015). Predominantly the habitual diet and to a lesser extent host
3891 genetics are two shaping factors of the gut microbiota (Sonnenburg and Bäckhed,
3892 2016; Kurilshikov *et al.*, 2017; Rothschild *et al.*, 2018). The term dysbiosis refers to
3893 shifts in the composition, function and phylogenetic diversity of the gut microbiota
3894 of non-healthy individuals in comparison to healthy controls (Manor *et al.*, 2017).
3895 Metabolic disease, functional GIT disorders and cognitive disorders have been
3896 linked to dysbiosis (reviewed in Levy *et al.*, 2017).

3897 The low-grade chronic inflammation that characterises older age reflects the decline
3898 of immune system fitness to respond to stressors, a phenomenon called
3899 inflammageing (Franceschi and Campisi, 2014). Aberrant intestinal permeability and
3900 dysbiotic gut microbiota are potential inflammageing drivers (Franceschi and
3901 Campisi, 2014; Li *et al.*, 2016; Thevaranjan *et al.*, 2017). Gut microbiota dysbiosis
3902 can be further fuelled by the inflamed GIT environment (Zeng *et al.*, 2016). For
3903 prevention and treatment of dysbiosis and associated conditions, therapeutic
3904 interventions based on probiotics, prebiotics or faecal microbiota transplants (FMT)
3905 are being developed (Scott *et al.*, 2014).

3906 Milk combines many nutrients including calcium, vitamin K, conjugated linoleic
3907 acid, fatty acids, complex sialylated oligosaccharides and glycoproteins (Rozenberg
3908 *et al.*, 2016; O’Riordan *et al.*, 2014). There is accumulating data pointing towards
3909 certain health benefits of milk and dairy consumption (Song *et al.*, 2016; Larsson *et*

3910 *al.*, 2015; Rozenberg *et al.*, 2016). Importantly, milk consumption may contribute to
3911 muscle and bone density maintenance in older consumers (Sahni *et al.*, 2014; Lana *et*
3912 *al.*, 2015). Despite the potential health benefits, studies have shown that elderly
3913 people often fail to meet current recommendations for daily dairy consumption
3914 partly due to misconceptions on lactose mal-absorption and dairy fat content (Power
3915 *et al.*, 2014; Laird *et al.*, 2016).

3916 The effect of milk on the gut microbiota has not been extensively studied. A few
3917 recent studies have focused on the potential prebiotic effect of milk oligosaccharides
3918 on the gut microbiota. Charbonneau *et al.* (2016) using animal models “humanised”
3919 with infant gut microbiota showed that sialylated bovine milk oligosaccharides
3920 (BMO) promoted weight gain associated with *Bacteroides fragilis* and *Escherichia*
3921 *coli* responsiveness to BMOs under malnutrition conditions. Karav *et al.* (2016)
3922 showed that BMO released from glycoproteins could mimic human milk
3923 oligosaccharide (HMO) selectivity on *Bifidobacterium* strains in the infant gut
3924 microbiota. Boudry *et al.* (2017) observed that BMO-supplemented diet enhanced
3925 gut barrier function, increased caecal and colonic microbiota diversity and
3926 *Lactobacillus* relative abundance in a murine model of diet-induced obesity.

3927 Glycomacropeptide (GMP) is a by-product of cheese making and it is released in
3928 whey protein from the kappa-casein fraction of milk by the activity of chymosin
3929 (Neelima *et al.*, 2013). Glycomacropeptide has five mucin-type glycans, high content
3930 of sialic acid, high content of branched chain amino acids (BCAA) and essential
3931 amino acids (EAA) and it is depleted of aromatic amino acids (Neelima *et al.*, 2013).
3932 Glycomacropeptide is associated with a number of health benefits including anti-
3933 inflammatory and anti-GIT pathogen activity, and it is currently being used as an

3934 alternative source of protein for phenylketonuria (PKU) nutritional management (van
3935 Calcar and Ney, 2012; Hvas *et al.*, 2016).

3936 There is inconclusive data on the prebiotic potential of GMP. *Bifidobacterium* and
3937 *Lactobacillus* selective growth promotion by GMP has been reported based on tests
3938 in pure cultures but the selectivity failed to be confirmed when complex faecal
3939 microbiota communities were tested *in vitro* (Bruck *et al.*, 2006; Hernandez-
3940 Hernandez *et al.*, 2011; Robitaille, 2013; Ntemiri *et al.*, 2017). Some *in vivo* studies
3941 showed that GMP administration increased the colonic abundance of *Lactobacillus*
3942 and *Bifidobacterium* (Hermes *et al.*, 2013; Jimenez *et al.*, 2017). Sawin *et al.* (2015)
3943 reported that a GMP-supplemented diet significantly decreased the caecal and
3944 colonic relative abundance of *Desulfovibrio* and Proteobacteria in wild type and
3945 PKU mice. Interestingly, GMP selectivity on promoting growth of Bacteroidetes
3946 taxa has also been reported (Sawin *et al.*, 2015; Jimenez *et al.*, 2017).

3947 In this study, conventional mice were treated with a cocktail of antibiotics before
3948 receiving FMT from either a healthy or a frail older donor. The effect of a diet
3949 enriched in either whole milk, lactose-free milk or GMP on the faecal microbiota of
3950 older humans was then investigated in these “humanised” mice.

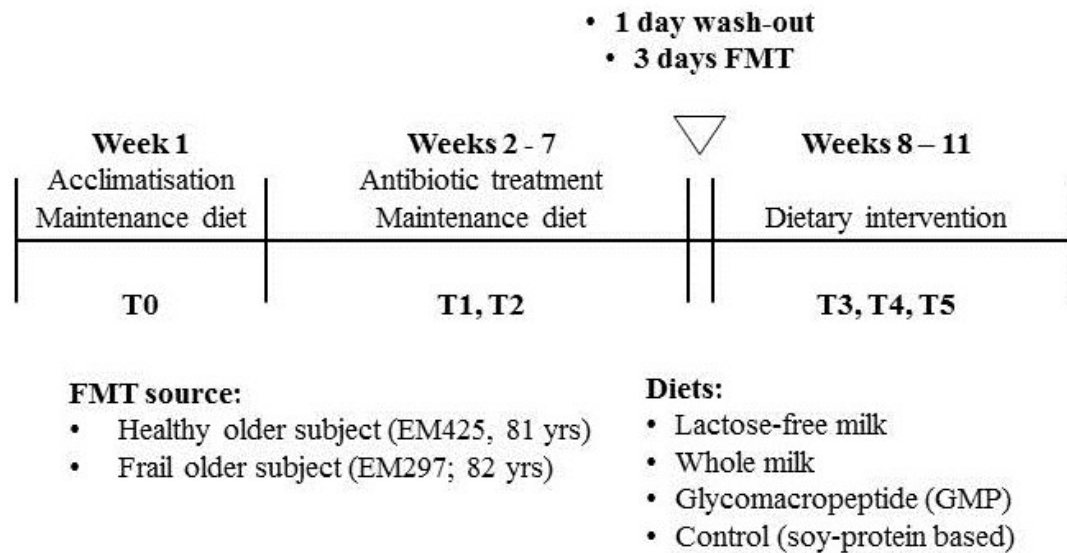
3951 **3.3 Materials and Methods**

3952 **Animals and experimental design:** The Health Products Regulatory Authority
3953 (HPRA) under the European Union Regulations authorised this project with
3954 authorisation number AE19130/P033. Ninety-six 6 week-old female and male
3955 C57BL/6 mice were purchased from Envigo RMS Ltd, Oxon, UK and caged in
3956 groups of six of the same sex. The mice were weighed during the first week and
3957 every subsequent week of the trial.

3958 The timeline of the mouse trial is outlined in **Figure 10 A**. The mice acclimatised to
3959 the animal facility for one week before the commencement of the six weeks
3960 antibiotics (Abx) treatment for the depletion of the indigenous murine gut
3961 microbiota. Ampicillin (1g/L; Sigma Aldrich), Metronidazole (1g/L; Molekula),
3962 Vancomycin (500 mg/L; Molekula), Imipenem (250 mg/L; Molekula) and
3963 Ciprofloxacin-HCl (200 mg/L; Santa Cruz) were diluted in tap water and filtered
3964 sterilised before given to animals as described before (Bereswill *et al.*, 2011).
3965 Animals had *ad libidum* access to Abx-water. Limited administration of HydroGel
3966 (ClearH2O.com) during the Abx treatment was used for the prevention of weight
3967 loss. One day wash-out with filtered tap-water followed the Abx treatment.
3968 Subsequently, the animals were “humanised” with FMT from either a healthy older
3969 donor or a frail older donor.

3970 The mice were introduced to the experimental diets on the wash-out day. The
3971 duration of the dietary intervention was one month. Six male and six female mice
3972 were allocated to each diet **Supplementary S Table 1**. The animals were fed
3973 maintenance diet (ssniff, Spezialdiäten GmbH, German) during the acclimatisation
3974 period and during the Abx treatment. The experimental diets were isoenergetic and
3975 isonitrogenous refined maintenance diets (ssniff, Spezialdiäten GmbH, Germany)
3976 supplemented with one of the following powders: **i. lac-free diet:** 20% lactose-free
3977 whole milk powder (Valio Ltd., Helsinki, Finland), **ii. wmlk diet:** 20% whole milk
3978 powder (Valio Ltd., Helsinki, Finland), **iii. GMP diet:** 20% CGMP-10 (Arla Food
3979 Ingredients, Denmark), **iv. control diet:** 20% soy protein isolate. In order to achieve
3980 the isoenergetic and isonitrogenous profile of the diets, all diets were additionally
3981 supplemented with soy protein isolate that was the basic protein ingredient for the
3982 maintenance diet as shown in **Table 6**. The complete compositional content of the

3983 experimental diets is attached in **Supplementary Material**. All diets were sterilized
 3984 by irradiation.



3985

3986 **Figure 10 Timeline of the 11 weeks mouse trial.** Time-points of samples
 3987 collection: T0 during the acclimatisation, T1 and T2 during the Abx treatment, T3,
 3988 T4 and T5 during the dietary intervention. The FMT source was used for the
 3989 “humanisation” of the Abx- treated animals. The isocaloric and isonitrogenous diets
 3990 were refined diets with 20% supplementation with the shown ingredients.

3991 **Table 6 Composition of the customized experimental diets (ssniff Spezialdiäten**
3992 **GmbH).**

Ingredient %	Control	20 % whole milk powder	20 % lactose free milk powder	20 % CGMP-10 powder
Soy protein isolate	20.0	14.5	12.8	1.8
Whole milk powder	-	20.0	-	-
Lactose-free milk powder	-	-	20.0	-
Glycomacropeptide CGMP-10	-	-	-	20.0
Maltodextrin	33.1	33.2	32.9	32.0
Sucrose	10.5	2.5	4.3	10.3
Cellulose powder	6.0	4.9	5.0	5.2
Soybean oil	7.5	2.3	2.3	7.5
Proximate contents %				
Crude protein	18.1	18.1	18.1	18.1
Crude fat	7.6	7.6	7.6	7.6
Crude ash	4.6	5.4	5.6	5.5
Starch	31.8	31.9	31.6	30.8
Sugar (total)	12.3	12.3	12.3	12.3
Lactose	-	8.0	-	-
Energy (MJ/kg)	16.0	16.0	16.0	16.0
kcal % Protein	19	19	19	19
kcal % Fat	18	18	18	18
kcal %	63	63	63	63
Carbohydrates				

3993 **Faecal slurry preparation and mouse “humanisation”:** One faecal sample
3994 from a healthy 81 yr old subject (EM425; community type microbiota, COM) and
3995 one faecal sample from a frail 82 yrs old subject (EM297; longstay type microbiota,
3996 LS) were collected under the approval of the local Clinical Research Ethics

3997 Committee. The samples were processed under anaerobic conditions for the
3998 preparation of 10 % w/v faecal slurries in PBS and 20 % glycerol. The faecal slurries
3999 were kept in aliquots at -80°C and thawed in the anaerobic cabinet before gavage to
4000 mice. The animals received by oral gavage a total of 300 µl of faecal slurry in three
4001 days.

4002 **Faecal and caecal sample collection:** Faecal samples were collected during
4003 the trial at various time points (T0 to T5) as indicated in **Figure 10**. One to two
4004 faecal pellets were collected at each time point. Caecum content was collected at the
4005 end of the trial by dissecting the caecum and emptying the caecum content in sterile
4006 Eppendorf tubes. All samples were immediately frozen in liquid nitrogen before
4007 being transferred to -80°C.

4008 **Genomic DNA extraction:** Genomic DNA was extracted from murine faecal
4009 pellets and caecum content using the QIamp Fast DNA Stool (Qiagen) kit. Murine
4010 faecal and caecal pellets were weighed before performing the extraction using the
4011 QIamp Fast DNA Stool (Qiagen) extraction kit protocol. The samples were placed in
4012 sterile tubes containing 0.1 mm, 0.5 mm and 1.0 mm zirconia / glass beads (Thistle
4013 Scientific, UK). Eight hundred ml of InhibitEX buffer was added to the samples and
4014 then homogenised under one pulse of 1 min and a final of 30 sec using a
4015 Minibeadbeater (Biospec Products). In the intervals of the homogenisation steps the
4016 samples were placed on ice for 1 min. The samples were then placed in a 95°C heat-
4017 block for 5 min. The subsequent steps of the DNA extraction were carried out as
4018 described in the Qiagen protocol using 15 µl of proteinase K with 200 µl of AL
4019 buffer, 200 µl of lysate and 200 µl of ethanol at the relevant steps. The final elution
4020 was performed at 70 µl of elution buffer.

4021 **Library preparation for 16S rRNA gene amplicon sequencing:** The V3 /
 4022 V4 variable region of the 16S rRNA gene was targeted for Illumina MiSeq System
 4023 sequencing (San Diego, California, USA). The region was targeted and amplified
 4024 using the universal 16S ribosomal RNA gene primers; forward primer for V3 region
 4025 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGC
 4026 CAG 3' and reverse primer for V4 region 5' GTCTCGTGGGCTCGGAGATGTG
 4027 TATAAGAGACAGGACTACHVGGGTATCTAATCC 3' (Klindworth *et al.* 2012).
 4028 The PCR products were purified and the subsequent library preparation steps were
 4029 performed according to the Illumina MiSeq system protocol. Dual-index barcodes
 4030 were attached to the amplicon (Nextera XT V.2 Index Kits sets A and D, Illumina)
 4031 and then purified using the Agencourt AMPure XP-PCR Purification system
 4032 (Beckman Coulter, Inc.). The indexed amplicons were quantified using the Qubit
 4033 dsDNA HS Assay Kit (Thermo Fischer Scientific, MA, U.S.A.). The pooled library
 4034 containing the same concentration of each amplicon was sequenced on a MiSeq
 4035 Illumina platform using a 2 x 250 bp chemistry in the Teagasc Next Generation
 4036 Sequencing Facility (Fermoy, Ireland).

4037 **Microbiota composition analysis and statistics:** FLASH (Magoč *et al.*,
 4038 2011) was used to join the paired reads that were subsequently demultiplexed in
 4039 Qiime with the split_libraries.py script and forward and reverse primers were
 4040 removed from the reads. Usearch was used for the *de novo* generation of an
 4041 operational taxonomic unit (OTU) table. The reads were filtered by read length and
 4042 the number of identical sequences, before chimera filtering was applied.
 4043 Subsequently, all reads were mapped against the representative reads generated by
 4044 the filtering based on 97% identity. The reads per sample were rarefied at a number
 4045 of reads in order to include the majority of the samples collected throughout the

study. The sequences were aligned using the PyNast tool (Caporaso *et al.*, 2010) in Qiime in order to generate alpha diversity indices i.e., Shannon, Simpson, PD whole tree, Chao1 and observed species, and beta diversity indices i.e. Weighted UniFrac and Unweighted UniFrac. The *de novo* OTU table generated based on the filtered reads was taxonomically classified (classify.seqs) down to genus level by using Mothur and the RDP v11.4/trainset 14 database (Wang *et al.*, 2007). The Spingo database was used in order to classify the reads at species level (Allard *et al.*, 2015). The software R (R Core Team) was used in order to convert the reads per sample into relative abundances, to analyse and visualise the UniFrac distances, and to visualise the alpha diversity of the microbiota. A Kruskal-Wallis with Dunn's post hoc test with p adjusted value with the Benjamini-Hochberg method were applied in R in order to detect significant differences in the faecal microbiota diversity due to diet at various time points. A Kruskal-Wallis with Dunn's post hoc test with p adjusted value with the Benjamini-Hochberg method was applied in order to detect the differentially abundant taxa in the aggregated microbiota of female and male mice at the end of the trial as a result of the different diets. Values were considered significant when Kruskal-Wallis p value was ≤ 0.5 , p_{adj} value ≤ 0.05 and Dunn's post hoc test p_{adj} values was ≤ 0.05 .

3.4 Results

Milk supplemented diets resulted in the highest increase in body weight from baseline

Due to severe weight loss during the first 2 weeks of Abx treatment, 6 animals were euthanized (**S Table 1**). Some animals were given HydroGel for up to 9 days (**S Table 1**) in order to prevent further weight loss due to a version of the mice to the

4070 Abx-containing water and subsequent abstaining from food. All animals began to
4071 recover their body weight after the 2nd week of Abx treatment (**Figure 11**) and at the
4072 end of the trial the diets had a differential effect on weight gain compared to the
4073 baseline body weight (**Figure 11**). Lactose-free milk supplemented diet resulted in
4074 the highest increase in body weight in mice humanised with COM type microbiota
4075 (**Figure 11 A**), while whole milk diet resulted in the highest increase in weight in
4076 mice humanised with the LS type microbiota (**Figure 11 B**). Upon dissection all
4077 animals had normal spleen weight (data not shown).

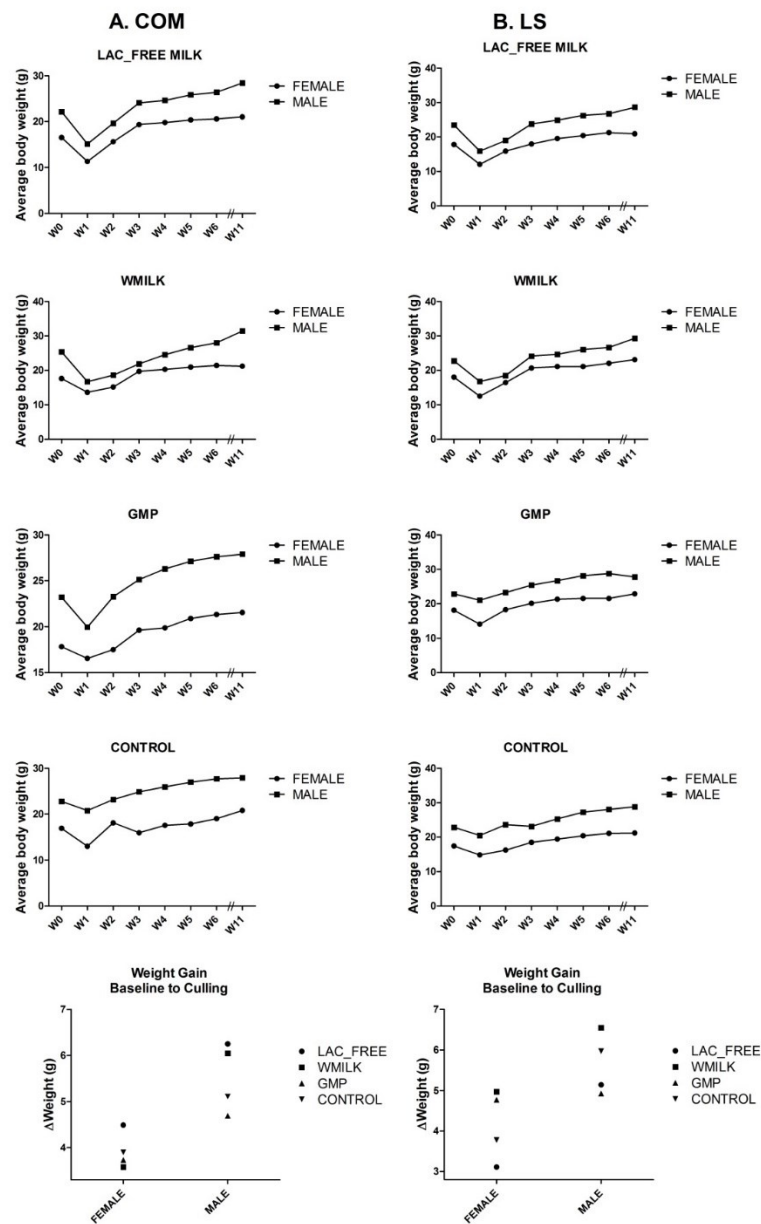
4078 **Differences between the human donors and the murine baseline faecal** 4079 **microbiota composition**

4080 Compositional and phylogenetic diversity differences in the faecal microbiota of frail
4081 long-term care unit-residing elderly subjects and healthy community-living were
4082 described in the ElderMet study (Claesson *et al.*, 2012). The ElderMet donors
4083 EM297 (LS) and EM425 (COM) were re-enrolled and their faecal microbiota was
4084 re-analysed in order to confirm suitability for the mouse trial. The compositional
4085 differences between the faecal microbiotas from subjects EM297 (LS) and EM425
4086 (COM) have been partly described in Chapter 2 (Ntemiri *et al.*, 2017). Compared to
4087 the COM type microbiota, the LS had lower Firmicutes relative abundance, it was
4088 enriched in Proteobacteria, Synergistetes and Bacteroidetes, whereas the alpha
4089 diversity was lower (**Figure 12, S Tables 2 and 3**). Thus, the inocula were
4090 considered suitable for proceeding with mouse humanisation.

4091 The composition of the murine faecal microbiotas is shown in **Figure 12 (S Table**
4092 **2)**. In the murine faecal microbiota, the phylum Bacteroidetes was the prevalent
4093 phylum (56 % average relative abundance) followed by the Firmicutes. Compared to

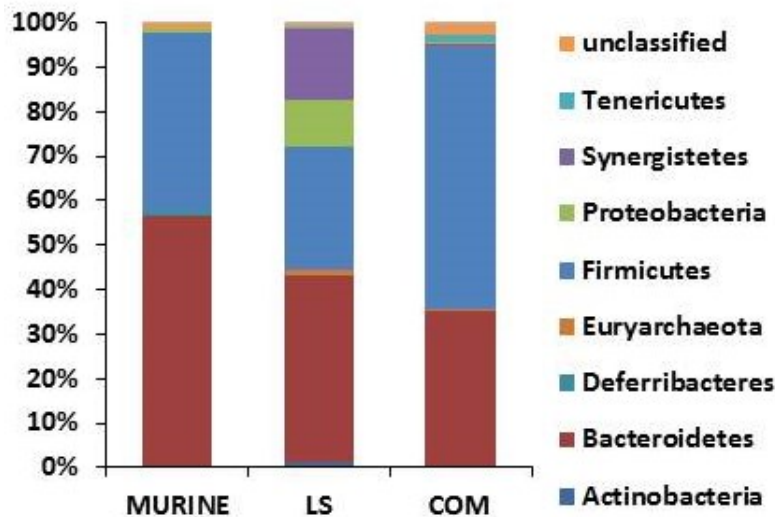
4094 the human microbiota, the murine microbiota was enriched in certain Bacteroidia
4095 including *Barnesiella intestinihominis*, *Alistipes massiliensis*, unclassified
4096 Prevotellaceae and Porphyromonadaceae (**S Table 2**). Among the Firmicutes, taxa
4097 that belonged to *Lactobacillus* and unclassified Lachnospiraceae were dominant in
4098 the murine faecal microbiota. The alpha diversity of the baseline murine microbiota
4099 was comparable to the COM type microbiota alpha diversity (**S Table 3**).

4100



4101

4102 **Figure 11 Animal body weight during the 11 weeks of the trial.** The body weight
4103 from the first week of Abx treatment (W0) till the completion of the trial (W11), is
4104 shown. Body weight change between baseline and end of trial is shown at the
4105 bottom. **A** and **B**: COM: community type humanisation and LS: longstay type
4106 humanisation, respectively.



4107

4108 **Figure 12 Composition of the human and murine baseline faecal microbiota at**
 4109 **phylum level.** Phyla that were present at ≥ 1 % relative abundance are presented. LS:
 4110 longstay type faecal microbiota; COM: community type faecal microbiota; murine:
 4111 aggregated faecal microbiota across all mice at baseline.

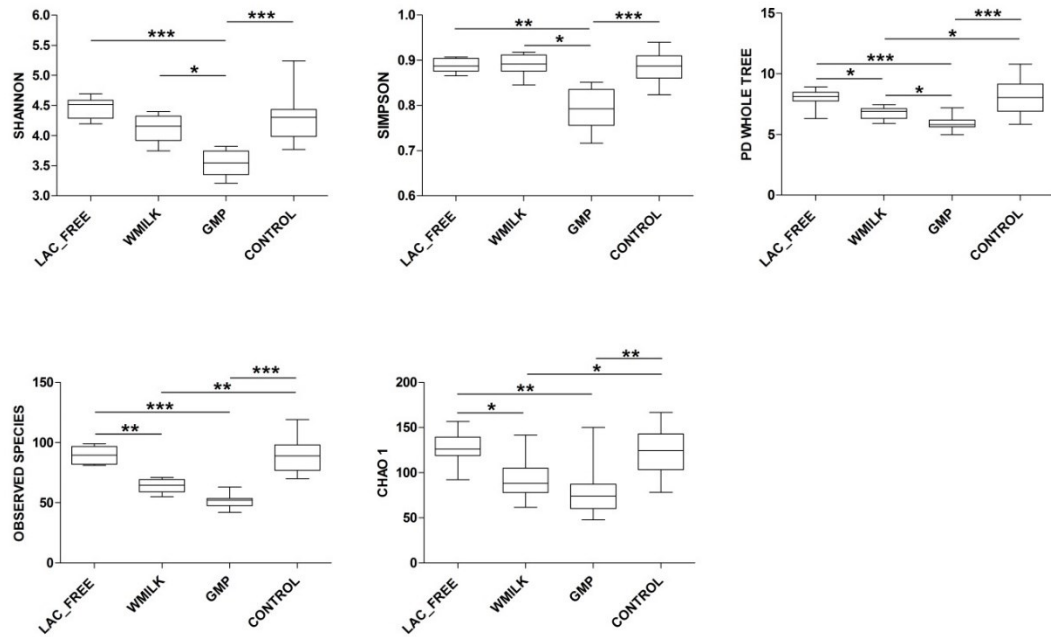
4112 **Efficiency of colonisation of the murine gut by the human faecal**
 4113 **microbiota**

4114 One week after humanisation (T3), the murine faecal microbiota had diverged from
 4115 baseline and did not revert back to the murine phylogenetic profile by the completion
 4116 of the trial as demonstrated by β diversity UniFrac analysis throughout the trial (S
 4117 **Figure 1, 2**). Importantly, the COM or LS phylogenetic profile was retained
 4118 throughout the trial as shown by principal coordinates analysis (PCoA) of the
 4119 Unweighted UniFrac distances at the end of the trial (T5) in spite of the strong effect
 4120 of diet (S **Figure 1 B** and **2 B**).

4121 **Lactose-free milk supplemented diet sustained significantly higher**
4122 **alpha diversity faecal microbiota compared to the other diets in both**
4123 **community and longstay type humanised mice.**

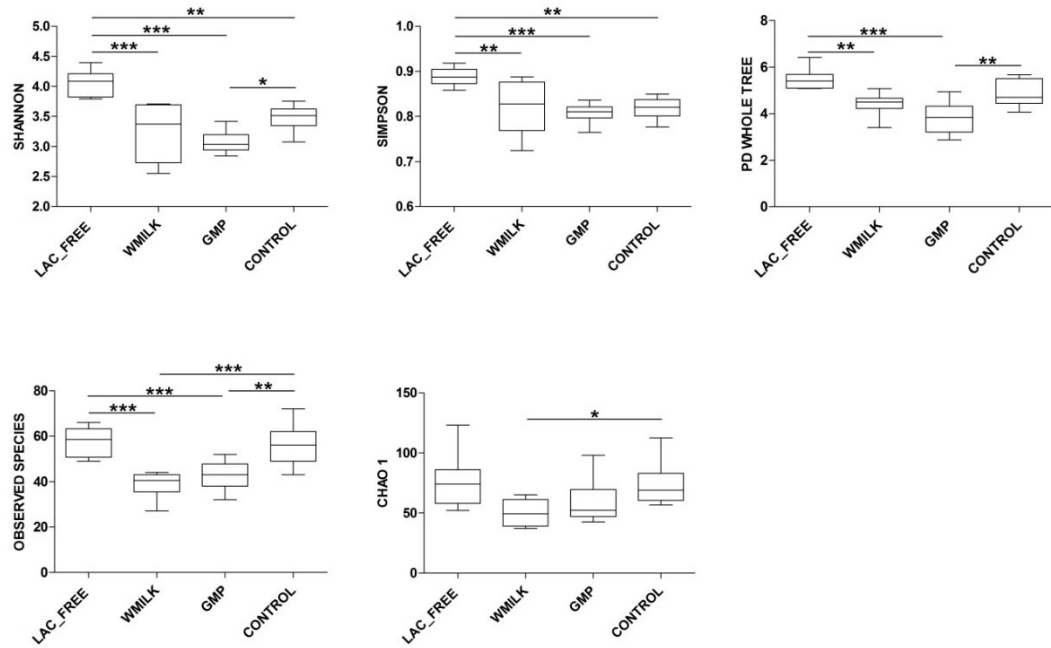
4124 *Community type microbiota:* There was no difference in the effect of lactose-free
4125 diet and control diet on the microbiota diversity. In contrast the GMP diet resulted in
4126 the lowest retention of alpha diversity across all indices (**Figure 13**). Lactose-free
4127 milk, control and whole milk diets resulted in significantly higher Shannon, Simpson
4128 and PD indices compared to GMP diet. For the indices Observed species and Chao1,
4129 lac-free and control diet retained significantly higher faecal microbiota diversity
4130 compared to both wmilk and GMP diet. A detailed summary of the statistical
4131 analysis results is shown **S Table 4**.

4132 *Longstay type microbiota:* The lactose-free milk diet intake resulted in significantly
4133 higher Shannon and Simpson diversity indices compared to all diets including
4134 control (**Figure 14**). The lactose-free diet intake resulted in significantly higher PD
4135 whole tree index compared to the whole milk and GMP diet intake whereas alpha
4136 diversity scores after control diet were significantly higher only compared to GMP
4137 diet. The lactose-free and control diet resulted in significantly higher Observed
4138 Species index compared to the whole milk and GMP diets. A detailed summary of
4139 the statistical analysis results is shown **S Table 5**.



4140

4141 **Figure 13 Alpha diversity indices at the end of the trial (T5) for mice humanised**
 4142 **with community (COM) type faecal microbiota.** LAC_FREE: lactose-free milk
 4143 supplemented diet, WMILK: whole milk supplemented diet, GMP:
 4144 glycomacropeptide supplemented diet, CONTROL: soy-protein based control diet.
 4145 Only tests that passed the $p_{adj} \leq 0.05$ threshold for the Kruskal Wallis test are
 4146 presented. Asterisks refer to the Dunn's post hoc test: * $p_{adj} \leq 0.05$, ** $p_{adj} \leq 0.005$, ***
 4147 $p_{adj} \leq 0.0005$. Details on the values are given in S Table 4.



4148

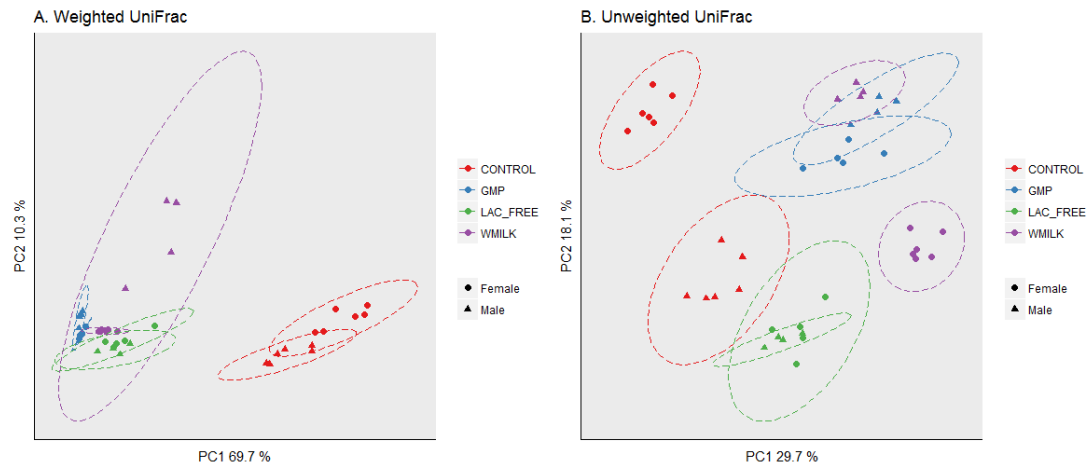
4149 **Figure 14 Alpha diversity indices at the end of the trial (T5) for the faecal**
 4150 **microbiota of mice humanised with longstay (LS) type faecal microbiota.**

4151 LAC_FREE: lactose-free milk supplemented diet, WMILK: whole milk
 4152 supplemented diet, GMP: glycomacropeptide supplemented diet, CONTROL: soy-
 4153 protein based control diet. Only tests that passed the $p_{adj} \leq 0.05$ threshold for the
 4154 Kruskal Wallis test are presented. Asterisks refer to the Dunn's post hoc test: *
 4155 $p_{adj} \leq 0.05$, ** $p_{adj} \leq 0.005$, *** $p_{adj} \leq 0.0005$. Details on the values are given in S Table
 4156 5.

4157 **Lactose-free milk diet sustained higher abundances of health relevant**
 4158 **taxa including Ruminococcaceae compared to the other diets in mice**
 4159 **humanised with community type microbiota.**

4160 At the end of the dietary intervention (T5) the faecal microbiota clustered based on
 4161 diet; microbiota from mice fed all three dairy-supplemented diets clustered closer
 4162 compared to microbiota from mice fed control diet as shown in **Figure 15** (Weighted
 4163 PCoA of the UniFrac distances of the sequenced 16S rRNA gene sequences in the

4164 relevant faecal microbiota samples). In the faecal microbiota of mice fed lac-free diet
4165 the Porphyromonadaceae was the most abundant family (41% average relative
4166 abundance) (**Figure 16**). The families Ruminococcaceae and Lachnospiraceae (17%
4167 and 15% average relative abundance respectively) were the next most abundant
4168 families in the microbiota composition of lactose-free diet fed mice; both families
4169 reached significantly high abundance upon lac-free diet compared to the other diets
4170 (S **Figure 3 A**). Other dominant families were the Bacteroidaceae,
4171 Desulfovibrionaceae and Rikenellaceae. (**Figure 16**). *Parabacteroides goldsteinii*
4172 was the most abundant taxon (28% average relative abundance) of the
4173 Porphyromonadaceae followed by *B. intestinhominis* and *Parabacteroides merdae*
4174 (**Figure 17**). *Anaerotruncus colihominis* and *Oscillibacter* taxa were the most
4175 abundant among the Ruminococcaceae (6% and 5% average relative abundance
4176 respectively) followed by *Flavonifractor plautii* (**Figure 17**). The Lachnospiraceae
4177 representatives belonged to unclassified taxa (**Figure 17**).



4178

4179 **Figure 15 Principal coordinates analysis (PCoA) of the murine faecal**
 4180 **microbiota at the end of the trial (T5).** The mice were humanised with community
 4181 **(COM)** type faecal microbiota. **A:** Weighted UniFrac distances; **B:** Unweighted
 4182 UniFrac distances. LAC_FREE: lactose-free milk supplemented diet, WMILK:
 4183 whole milk supplemented diet, GMP: glycomacropeptide supplemented diet,
 4184 CONTROL: soy-protein based control diet.”●” denotes female mouse microbiota;
 4185 “▲” denotes male mouse microbiota.

4186 **Gender dependent gut microbiota response to whole milk in mice**
4187 **humanised with community type microbiota.**

4188 Based on the UniFrac analysis of the faecal microbiota of mice fed a whole milk
4189 supplemented diet, two sub-clusters were formed separating female and male
4190 microbiota responses (**Figure 15 A, B**). The Bacteroidaceae were more responsive to
4191 the wmlk diet in the female mouse faecal microbiota (23.6% and 6.5% average
4192 relative abundance in female and male mice respectively) whereas the
4193 Erysipelotrichaceae were more abundant in the male mouse microbiota (23% and 2%
4194 average relative abundance in male and female mice respectively) (**S Table 6**).
4195 *Bacteroides thetaiotaomicron* and *B. uniformis* were the predominant representatives
4196 of the Bacteroidaceae, and *A. stercorialis* and *Turicibacter sanguinis* were the major
4197 representative taxa of the Erysipelotrichaceae in male and female mouse microbiota
4198 respectively (**Table 7**). Gender based differences were also observed for the families
4199 Ruminococcaceae (more abundant in male) and Peptostreptococcaceae (more
4200 abundant in female) (**S Table 6**). *Ruminococcus bromii* (Ruminococcaceae) was
4201 sustained only by the whole milk diet in the microbiota of male mice (6% average
4202 relative abundance).

4203 The compositional profile of the faecal microbiota aggregated across both genders is
4204 shown in **Figures 16** and **17**. Bacteroidaceae and Erysipelotrichaceae relative
4205 abundance was significantly increased upon whole milk diet compared to the other
4206 tested diets (**S Figure 3 A**). Compared to the lactose-free diet Porphyromonadaceae
4207 and Ruminococcaceae were less responsive to the whole milk diet whereas
4208 Rikenellaceae and Acidaminococcaceae were more responsive (**Figures 16; S**
4209 **Figure 3 A**). The Lachnospiraceae reached a relatively high abundance (11.7%

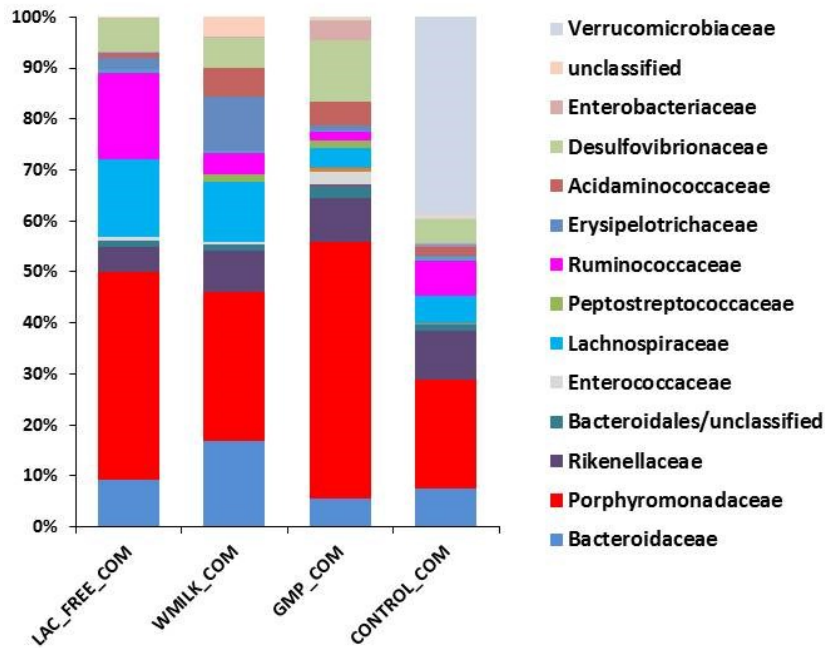
4210 average relative abundance) similar to that after lac-free diet and compared to the
4211 other diets (**S Figure 3 A**).

4212 **Enrichment of the faecal microbiota in Porphyromonadaceae,**
4213 **Desulfovibrionaceae and potential pathobionts due to**
4214 **glycomacropeptide supplementation in community type humanised**
4215 **mice.**

4216 We have previously presented evidence of GMP prebiotic activity in an artificial
4217 colon model of elderly gut microbiota (Chapter 2; Ntemiri *et al.*, 2017) but did not
4218 confirm these findings in our mouse model. The microbiota of both female and male
4219 mice responded similarly to the GMP diet as observed by the clustering on the PCoA
4220 analysis (**Figure 15 A and B**). Compared to the other diets, the GMP diet resulted in
4221 significant enrichment of the faecal microbiota in Porphyromonadaceae and the
4222 Desulfovibrionaceae (50% and 12% average relative abundance respectively)
4223 compared to most of the diets (**Figure 16; S Figure 3 A**). The main representative
4224 taxa of the two aforementioned families were *P. goldsteinii* and *B. wadsworthia*
4225 (46% and 12% average relative abundance respectively) (**Figure 17**). The GMP diet
4226 had, similar to the wmilk diet, an effect on the Acidaminococcaceae (5% average
4227 relative abundance) represented by *P. faecium* (**Figure 17**). Compared to the other
4228 diets, feeding with the GMP diet resulted in significant microbiota enrichment in
4229 Enterobacteriaceae and Enterococcaceae (4% and 2.5% average relative abundance
4230 respectively) (**Figure 16; S Figure 3 A**); *Enterococcus* and *Escherichia/Shigella*
4231 taxa were the dominant representatives (**Figure 17**).

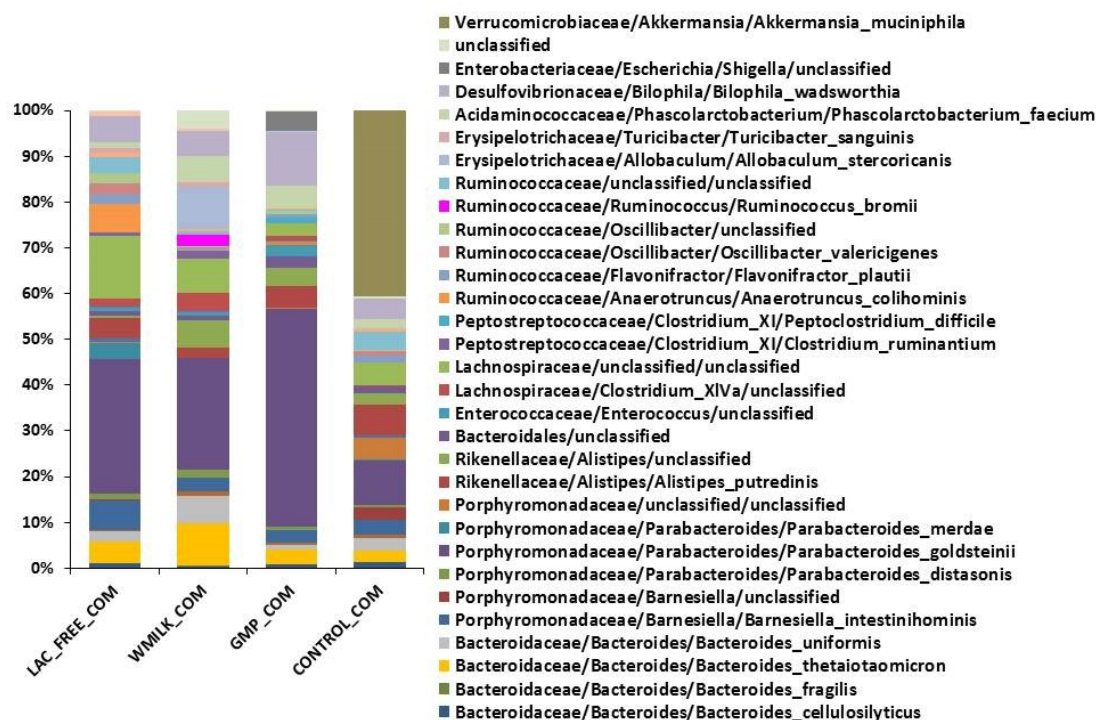
4232 **Gender dependent gut microbiota response to control diet in**
4233 **community type humanised mice.**

4234 The faecal microbiota of mice fed the control diet clustered separately from the
4235 microbiota of mice fed dairy-supplemented diets and gender based sub-clustering
4236 was also observed (**Figure 15**). Differences in the composition between female and
4237 male mice fed the control diet included differences in the abundance of taxa
4238 belonging to the Porphyromonadaceae, Rikenellaceae, Ruminococcaceae and
4239 Acidaminococcaceae (**Table 7**). Porphyromonadaceae was the next most abundant
4240 family (21% average relative abundance) in the microbiota of control fed mice
4241 following the Verrucomicrobiaceae (40% average relative abundance) represented
4242 by *A. muciniphila* (**Figures 16, 17**). The compositional profile of the faecal
4243 microbiota of mice fed the wmlk diet aggregated across both gender is shown in
4244 **Figures 16 and 17**.



4245

4246 **Figure 16 Composition of the murine faecal microbiota at family level at the**
 4247 **end of the trial (T5) in mice humanised with community (COM) type human**
 4248 **faecal microbiota.** LAC_FREE: lactose-free milk supplemented diet, WMILK:
 4249 whole milk supplemented diet, GMP: glycomacropeptide supplemented diet,
 4250 CONTROL: soy protein based control diet. Families present at $\geq 1\%$ are shown.



4251

4252 **Figure 17 Composition of the murine faecal microbiota at species level at the**
 4253 **end of the trial (T5) in mice humanised with community (COM) type human**
 4254 **faecal microbiota. LAC_FREE: lactose-free milk supplemented diet, WMILK:**
 4255 **whole milk supplemented diet, GMP: glycomacropeptide supplemented diet,**
 4256 **CONTROL: soy protein based control diet. Only species present at ≥ 1 % are shown.**

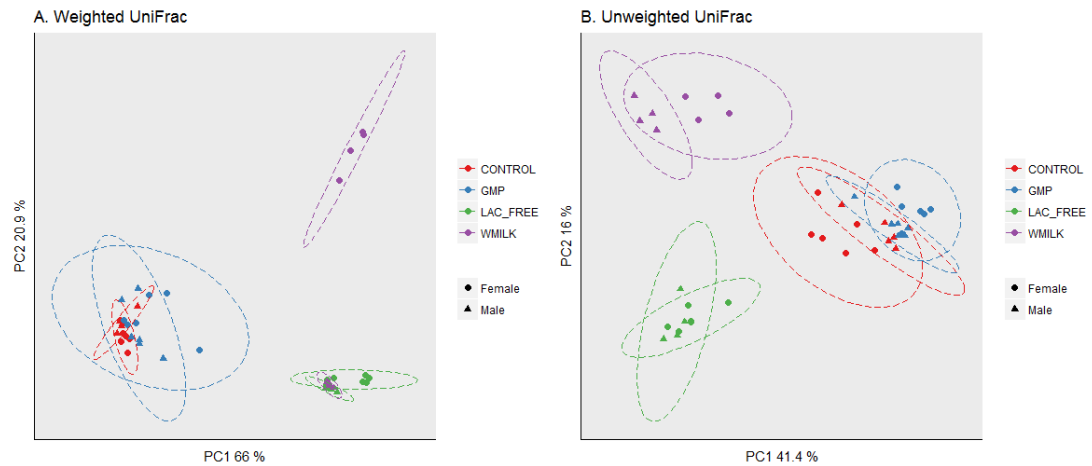
4257 **Table 7 Differences in the female and male murine microbiota composition**
4258 **associated with separation in the PCoA grouping by whole milk (WMILK) and**
4259 **control diet at the end of the trial (T5).** Only species present at ≥ 1 % relative
4260 abundance are presented. The humanisation of the mice was performed with
4261 community (**COM**) type microbiota.

	WMILK FEMALE	WMILK MALE	CONTROL FEMALE	CONTROL MALE
OTU classification	% average relative abundance			
Bacteroidaceae/Bacteroides/Bacteroides cellulosilyticus	0.0	0.6	2.6	0.0
Bacteroidaceae/Bacteroides/Bacteroides thetaiotaomicron	14.1	1.3	2.3	2.3
Bacteroidaceae/Bacteroides/Bacteroides uniformis	7.7	2.7	1.7	4.0
Bacteroidaceae/Bacteroides/unclassified	1.3	0.4	0.2	1.0
Porphyromonadaceae/Barnesiella/Barnesiella intestinihominis	3.1	2.7	2.4	4.1
Porphyromonadaceae/Barnesiella/unclassified	0.1	0.04	4.7	0.2
Porphyromonadaceae/Butyricimonas/Butyricimonas virosa	0.5	1.3	1.0	0.4
Porphyromonadaceae/Parabacteroides/Parabacteroides distasonis	1.8	1.4	1.1	0.3
Porphyromonadaceae/Parabacteroides/Parabacteroides goldsteinii	24.5	22.0	6.0	12.7
Porphyromonadaceae/unclassified/unclassified	0.1	0.1	9.3	0.1
Rikenellaceae/Alistipes/Alistipes putredinis	0.01	5.4	0.01	12.6
Rikenellaceae/Alistipes/unclassified	6.5	4.2	0.4	4.7
Bacteroidales/unclassified/unclassified/unclassified	1.3	1.0	0.8	1.7
Lachnospiraceae/Clostridium XIVa/unclassified	6.4	0.2	0.002	0.6
Lachnospiraceae/unclassified/unclassified	4.1	12.3	5.2	4.5
Peptostreptococcaceae/Clostridium XI/ Clostridium ruminantium	2.7	0.0	0.0	0.0
Ruminococcaceae/Flavonifractor/Flavonifractor plautii	0.8	0.4	2.1	0.7
Ruminococcaceae/Oscillibacter/Oscillibacter valericigenes	0.0	0.02	1.6	0.2
Ruminococcaceae/Ruminococcus/Ruminococcus bromii	0.0	6.0	0.0	0.0
Ruminococcaceae/unclassified/unclassified	0.7	0.7	4.9	2.6
Clostridiales/unclassified/unclassified/unclassified	0.6	0.4	0.5	1.1
Erysipelotrichaceae/Allobaculum/Allobaculum stercoricanis	0.001	22.0	0.0	0.0
Erysipelotrichaceae/Turicibacter/Turicibacter sanguinis	1.6	0.0	0.0	0.001
Acidaminococcaceae/Phascolarctobacterium/ Phascolarctobacterium faecium	5.7	5.5	3.0	0.6
Desulfovibrionaceae/Bilophila/Bilophila wadsworthia	7.5	1.8	3.7	4.9
unclassified	5.3	1.5	0.9	0.2
Verrucomicrobiaceae/Akkermansia/ Akkermansia muciniphila	0.0	0.0	40.6	37.2

4262

4263 **Faecal microbiota compositional profile of mice fed lactose-free milk**
4264 **supplemented diet and humanised with longstay type microbiota**

4265 At the end of the trial (T5) the faecal microbiota from mice humanised with LS
4266 microbiota and fed milk-supplemented diets clustered closer compared to the
4267 microbiota from mice fed GMP or control diet as shown in the PCoA in **Figures 18**
4268 **A and B**. Compared to the other diets, the lactose-free diet resulted in the highest
4269 Porphyromonadaceae abundance increase (29% average relative abundance)
4270 represented predominantly by *Parabacteroides merdae* and to a lesser extent by
4271 *Parabacteroides distasonis* (**Figures 19, 20; S Figure 3 B**). Lachnospiraceae (16%
4272 average relative abundance), Rikenellaceae and Bacteroidaceae (14% average
4273 relative abundance each) were the next most abundant families (**Figure 19**). The
4274 families Desulfovibrionaceae (*Desulfovibrio piger*), Peptostreptococcaceae (*C.*
4275 *ruminantium*), Ruminococcaceae (*F. plautii*) and Enterobacteriaceae (*Shigella* taxa)
4276 reached the highest abundance after the lac-free diet feeding compared to the other
4277 diets (**Figures 19, 20**). Limited differences in the composition of the faecal
4278 microbiota between female and male mice, based on abundances of certain families
4279 such as the Bacteroidaceae (more abundant in male) and Porphyromonadaceae (more
4280 abundant in female) (**Table 8, S Table 7**) may explain the sub-clustering within the
4281 Weighted PCoA (**Figure 18 A**).



4282

4283 **Figure 18 Principal coordinates analysis (PCoA) of the murine faecal**
 4284 **microbiota at the end of the trial (T5).** The mice were humanised with long stay
 4285 **(LS)** type faecal microbiota. **A:** Weighted UniFrac distances; **B:** Unweighted
 4286 UniFrac distances. LAC_FREE: lactose-free milk supplemented diet, WMILK:
 4287 whole milk supplemented diet, GMP: glycomacropeptide supplemented diet,
 4288 CONTROL: soy protein based control diet. "●" denotes female mouse microbiota;
 4289 "▲" denotes male mouse microbiota.

4290 **Gender dependent enrichment of Lachnospiraceae and**
4291 **Erysipelotrichaceae due to whole milk supplemented diet in mice**
4292 **humanised with longstay type microbiota.**

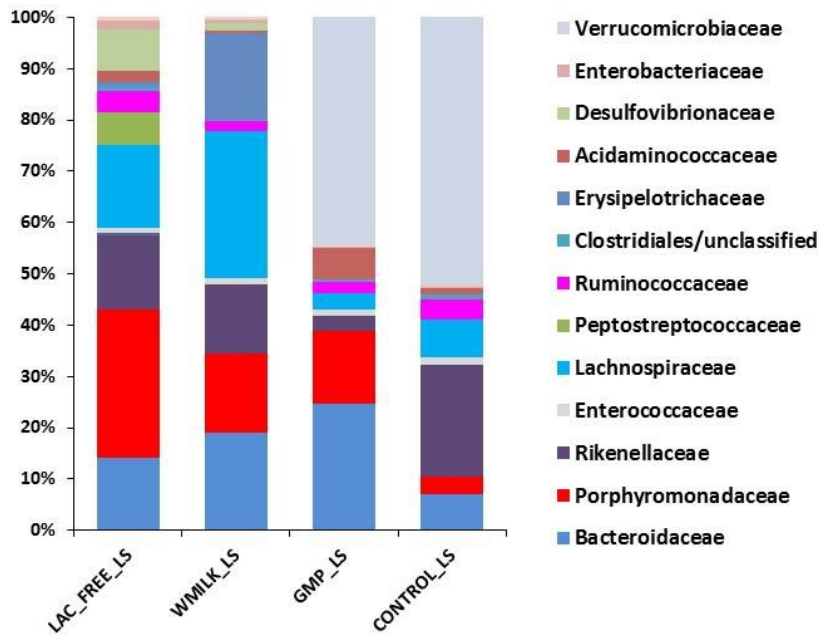
4293 The Weighted UniFrac analysis of the faecal microbiota of mice fed the whole milk
4294 diet, resulted in two sub-clusters based on gender-dependent responses, (**Figures 18**
4295 **A**). Different Lachnospiraceae taxa were responsive in the microbiota of female and
4296 male mice whereas the Erysipelotrichaceae represented by *A. stercorialis* increased
4297 only in the female mice microbiota (**Table 8**). The Bacteroidaceae that were among
4298 the most abundant families in both female and male mouse microbiota (19% average
4299 relative abundance in both) had gender associated differences in the responsive taxa
4300 (**S Table 7, Table 8**). Other dominant taxa with gender associated variations in the
4301 relative abundance belonged to the Porphyromonadaceae and Rikenellaceae (**Table**
4302 **8**). The aggregated across gender mouse microbiota composition is shown in
4303 **Figures 19 and 20**. Compared to the other feeding regimes, Lachnospiraceae and
4304 Erysipelotrichaceae were significantly enriched upon the whole milk diet and
4305 compared to the other feeding regimes (29% and 19% average relative abundance
4306 respectively) (**S Figure 3 B**).

4307 **Effect of GMP and control diet on the longstay type microbiota**

4308 The Bacteroidaceae was the second most abundant family (25% average relative
4309 abundance) in the microbiota of mice fed GMP diet following the
4310 Verrucomicrobiaceae, with *B. dorei* and *B. thetaiotaomicron* as the main
4311 representatives of the Bacteroidaceae family (**Figures 19, 20**). Compared to the other
4312 feeding regimes, GMP diet sustained the highest Bacteroidaceae and

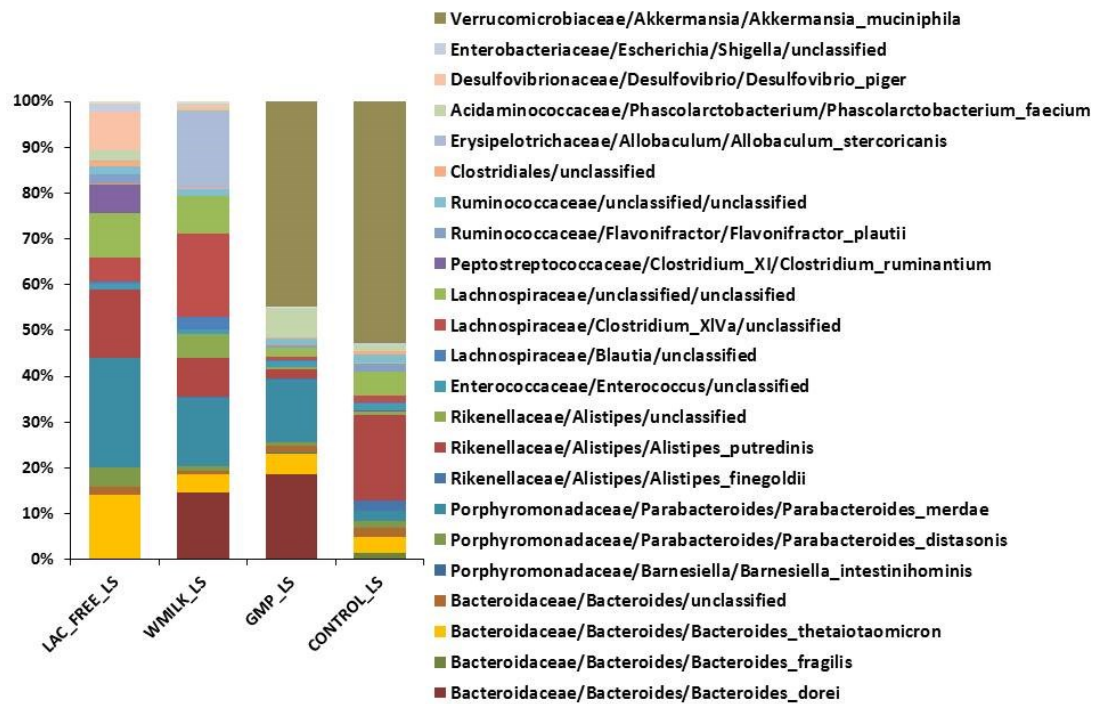
4313 Acidaminococcaceae (represented by *P. faecium*) relative abundances in the faecal
4314 microbiota of mice humanised with LS microbiota (**Figures 19, 20; S Figure 3 B**).

4315 Similar to aforementioned feeding regimes, the Verrucomicrobia were
4316 overrepresented in the faecal microbiota of mice fed the control diet and humanised
4317 with LS microbiota (**Figure 19**). Compared to the other diets, the control diet
4318 resulted in enrichment of the microbiota in the Rikenellaceae family (22% average
4319 relative abundance) (**S Figure 3 B**) and *Alistipes putredinis* was the prevalent
4320 representative taxon (**Figure 20**). The Ruminococcaceae (represented by *F. plautii*
4321 and other unclassified taxa) had a relatively high abundance comparable to that
4322 observed under the lactose-free feeding regime whereas the families
4323 Lachnospiraceae, Bacteroidaceae and Porphyromonadaceae had the lowest
4324 abundance compared to the other diets (**Figure 19**).



4325

4326 **Figure 19 Composition of the murine faecal microbiota at family level at the**
 4327 **end of the trial (T5) in mice humanised with longstay (LS) type human faecal**
 4328 **microbiota.** LAC_FREE: lactose-free milk, WMILK: whole milk, GMP:
 4329 glycomacropeptide, CONTROL: soy-protein based control diet. Only families
 4330 present at ≥ 1 % relative abundance are shown.



4331

4332 **Figure 20 Composition of the murine faecal microbiota at family level at the end**
 4333 **of the trial in mice humanised with longstay (LS) type human faecal microbiota.**
 4334 LAC_FREE: lactose-free milk, WMILK: whole milk, GMP: glycomacropeptide,
 4335 CONTROL: soy-protein based control diet. Only families present at ≥ 1 % relative
 4336 abundance are shown.

4337 **Table 8 Differences in the female and male murine microbiota composition**
 4338 **associated with separation in the PCoA grouping by lactose-free milk and whole**
 4339 **milk diets.** Only species that were present at ≥ 1 % relative abundance are presented.
 4340 The humanisation was performed with longstay (LS) microbiota.

	LAC_FREE FEMALE	LAC_FREE MALE	WMILK FEMALE	WMILK MALE
OTU classification	% average relative abundance			
Bacteroidaceae/Bacteroides/Bacteroides dorei	0.0	0.0	9.9	18.7
Bacteroidaceae/Bacteroides/Bacteroides thetaiotaomicron	8.6	25.0	7.8	0.002
Bacteroidaceae/Bacteroides/unclassified	0.90	3.5	1.4	0.003
Porphyromonadaceae/Parabacteroides/Parabacteroides distasonis	4.3	3.8	0.5	1.5
Porphyromonadaceae/Parabacteroides/Parabacteroides merdae	28.2	14.9	3.1	26.0
Rikenellaceae/Alistipes/Alistipes putredinis	13.5	15.3	5.1	11.8
Enterococcaceae/Enterococcus/unclassified	1.0	1.4	0.002	2.2
Lachnospiraceae/Blautia/unclassified	0.6	0.2	4.6	0.9
Lachnospiraceae/Clostridium XIVa/unclassified	5.8	2.4	29.7	5.6
Lachnospiraceae/unclassified/unclassified	11.6	7.9	0.2	15.4
Peptostreptococcaceae/Clostridium_XI/Clostridium ruminantium	6.5	4.6	0.001	0.0
Ruminococcaceae/Flavonifractor/Flavonifractor plautii	1.7	2.9	0.1	0.10
Ruminococcaceae/unclassified/unclassified	2.1	2.0	0.1	3.0
Clostridiales/unclassified/unclassified/unclassified	1.2	0.8	0.2	0.4
Erysipelotrichaceae/Allobaculum/Allobaculum stercoricanis	0.001	0.001	32.3	0.01
Acidaminococcaceae/Phascolarctobacterium/ Phascolarctobacterium faecium	2.2	1.8	1.1	0.002
Desulfovibrionaceae/Desulfovibrio/Desulfovibrio piger	8.0	8.0	1.4	0.0
Desulfovibrionaceae/unclassified/unclassified	0.1	0.01	0.0	1.2
Enterobacteriales/Enterobacteriaceae/Escherichia/ Shigella/unclassified	1.1	2.7	0.5	0.001

4341 **Enrichment of the microbiota in Verrucomicrobiaceae in some**
 4342 **dietary groups**

4343 In the faecal microbiota of some groups humanised with either microbiota type, an
 4344 enrichment ($\geq 40\%$ average relative abundance) in the Verrucomicrobiaceae family
 4345 represented by *A. muciniphila* was observed at the end of the trial (T5) (**Figures 17,**
 4346 **20**). Alpha diversity scores may have been biased due to the *Akkermansia* bloom
 4347 observed at (T5) and for this reason the microbiota composition and alpha diversity

indices were analysed at an earlier time point, i.e. in the middle of the dietary intervention (T4) (S Figure 4, S Tables 8, 9). The Verrucomicrobiaceae bloom was detected at time point T4 for the mice humanised with COM microbiota but not for the mice humanised with LS type microbiota (S Figure 4). At T4, the lactose-free diet sustained significantly higher alpha diversity in the murine microbiota of COM type humanised mice compared to the other feeding regimes; however, the Verrucomicrobiaceae bloom may have contributed to the alpha diversity observed for control feeding (S Table 8). For the LS type humanised mice at T4, both milk supplemented diets sustained significantly higher alpha diversity indices compared to both GMP and control diet (S Table 8).

3.5 Discussion

Prebiotic effect of the lactose-free milk supplemented diet on the gut microbiota of mice humanised with faecal microbiota from older donors

In mice colonised with either human microbiota type the lactose-free diet sustained significantly higher faecal microbiota diversity compared to the whole milk or GMP diets, performing as efficiently as soy protein supplemented diet. Some animal and human studies indicate to the prebiotic potential of soy and its products (reviewed in Huang *et al.*, 2016). Decreased gut microbiota phylogenetic diversity is indication biomarker of ageing and frailty, and dietary supplements contributing in sustaining diversity may improve the ageing process (Claesson *et al.*, 2012; Jackson *et al.*, 2016). The efficiency of the lactose-free milk diet in retaining microbiota diversity can be attributed partly to the BMOs available for microbiota fermentation. Bovine milk oligosaccharides have attracted scientific interest as potential prebiotics due to

4372 their structural complexity and similarity to HMOs (Zivkovic and Barile, 2011;
4373 Smilowitz *et al.*, 2014).

4374 The Ruminococcaceae enrichment of the microbiota associated with the lactose-free
4375 diet associated observed mostly in COM type humanised animals could indicate that
4376 such a dietary supplementation might contribute to colonic health and healthy ageing
4377 as reported before (Kong *et al.*, 2016; Mottawea *et al.*, 2016). In the current study the
4378 dominant Ruminococcaceae taxon was *Anaerotruncus*. Others have reported
4379 probiotic-associated *Anaerotruncus* enrichment of dysbiotic murine microbiota after
4380 Abx treatment (Grazul *et al.*, 2016). Other dominant Ruminococcaceae taxa reported
4381 here i.e., *Oscillibacter* and *Flavonifractor*, have been associated with resistant starch
4382 degradation and identified in the gut microbiota of lean individuals (Walker *et al.*,
4383 2011; Kasai *et al.*, 2015). However, caution is needed before extrapolating these
4384 findings to human dietary interventions because some studies report enrichment of
4385 *Oscillibacter* in cancer associated microbiota and *Anaerotruncus* enrichment in the
4386 microbiota of frail elderly (Jeffery *et al.*, 2016; Flemer *et al.*, 2017). In the LS
4387 humanised mice the Ruminococcaceae reached the highest relative abundance after
4388 lactose-free (and control) diet but not as high as in COM type humanised mice,
4389 potentially due to low starting abundance of this family in the LS microbiota.
4390 Importantly, in LS humanised mice, lac-free diet resulted in increased *D. piger*
4391 relative abundance; a taxon negatively correlated to health in elderly people (Jeffery
4392 *et al.*, 2016). The balance of these effects needs to be measured and considered in
4393 human studies.

4394 **Prebiotic potential of whole milk**

4395 The whole milk supplemented diet was less efficient than the lactose-free diet in
 4396 sustaining microbiota diversity. However, the comparative increase in the relative
 4397 abundance of certain taxa after whole milk diet warrants further investigation.
 4398 *Ruminococcus bromii* (Ruminococcaceae) was detected at high abundance
 4399 exclusively in (male) mice humanised with COM type microbiota fed whole milk
 4400 diet. *Ruminococcus bromii* is a dominant gut microbiota taxon highly responsive to
 4401 resistant starch, essential in nutrient degradation and as such it can be a prebiotic
 4402 target leading to substrate release in the colon and increase in cross-feeding mediated
 4403 by SCFA production (Ze *et al.*, 2012; Scott *et al.*, 2015). The (gender dependent)
 4404 Erysipelotrichaceae/*A. stercorialis* enrichment of the faecal microbiota of whole
 4405 milk fed mice may be a positive effect on the microbiota. In murine trials, high-fat
 4406 diet-associated dysbiosis and impaired gut barrier function were restored after BMOs
 4407 supplementation correlating to *Allobaculum* (and Ruminococcaceae) enrichment of
 4408 the microbiota (Boudry *et al.*, 2017; Hamilton *et al.*, 2017). Inulin supplementation
 4409 had similar effect on *Allobaculum* taxa associated with improved vascular function
 4410 (Catry *et al.* 2018). However, careful extrapolation to human diet is needed due to
 4411 *Allobaculum* association with thrombosis risk through TMAO metabolism and to
 4412 other erysipelotrichi associations with the obesity phenotype in murine models
 4413 (Woting *et al.*, 2014; Zhu *et al.*, 2016). Importantly the whole milk diet, similarly to
 4414 the lactose-free diet, sustained the highest Lachnospiraceae abundance in the murine
 4415 microbiota compared to the control and GMP diets, and especially in LS humanised
 4416 mice the whole milk diet resulted in a favourable ratio of Lachnospiraceae and
 4417 Bacteroidaceae. In aged humans with frailty indications, Bacteroidaceae were found
 4418 to dominate in abundance over Lachnospiraceae (Jeffery *et al.*, 2016). Interestingly,
 4419 the increased relative ratio of Lachnospiraceae to Bacteroidaceae may explain the

4420 fact that milk-supplemented diets resulted in the highest weight gain from baseline
4421 (**Figure 11**) (Turnbaugh *et al.*, 2006; Jumpertz *et al.*, 2011).

4422 **GMP was not associated with prebiotic activity**

4423 The Bacteroidetes (Bacteroidaceae, Bacteroidales unclassified and
4424 Porphyromonadaceae), Enterobacteriaceae and Desulfovibrionaceae were responsive
4425 to GMP and the milk diets depending on humanisation type (**S Figure 3**). In this
4426 study we did not observe *in vivo* beneficial effects of the GMP supplementation on
4427 the gut microbiota in contrast to previous studies in a conventional mouse model
4428 (Sawin *et al.*, 2015). In our study increased Desulfovibrionaceae/*B. wadsworthia*
4429 abundance due to GMP supplementation in COM humanised mice was observed,
4430 contrary to previous studies reporting decreased Desulfovibrionaceae/*D. piger*
4431 relative abundance associated with 20% GMP-enriched diet (Sawin *et al.*, 2015).
4432 Intake of high-fat diet (milk-derived fat) resulted in *B. wadsworthia* associated
4433 dysbiosis and inflammation in a conventional mouse model (Devkota *et al.*, 2012).
4434 Colonic health is promoted by reduced *Bilophila* relative abundances in human gut
4435 microbiota (Vandeputte *et al.*, 2017). The sialic acid content of the GMP may have
4436 contributed to the comparative increase in Enterobacteriaceae/*Escherichia/Shigella*
4437 taxa relative abundance in COM type humanised mice microbiota (Huang *et al.*,
4438 2015). A similar response was not observed in LS humanised mice; the
4439 Enterobacteriaceae were more responsive to the lactose-free milk diet. In the
4440 microbiota of LS colonised mice the Bacteroidaceae were highly responsive to
4441 GMP-supplemented diet (and the other two dairy diets). Increase in *Bacteroides*
4442 population due to GMP-supplementation has been reported in mouse models (Sawin
4443 *et al.*, 2015; Jimenez *et al.*, 2017). Interestingly, different Bacteroidaceae taxa have

4444 been associated with sialidase activity with or without sialic acid utilisation ability
4445 (Huang *et al.*, 2015; reviewed in Juge *et al.*, 2016). Charbonneau *et al.* (2016)
4446 reported growth improvement in a gnotobiotic mouse and piglet model through
4447 cross-feeding between *Bacteroides* taxa that could degrade sialic acid-containing
4448 carbohydrates from sialylated BMO and secondary utilisers of sialic acid-containing
4449 compounds like *E. coli*.

4450 In Chapter 2, I reported the increased relative abundance of Lachnospiraceae taxa
4451 like *Coprococcus* and *Roseburia* due to GMP *in vitro* supplementation; in this *in*
4452 *vivo* model we did not observe similar microbiota responsiveness. As discussed in
4453 the following section (and in details in Chapter 1.2.4 of this thesis), the mouse gut
4454 may favour the colonisation of xenomicrobiota Bacteroidetes taxa and may be
4455 refractory to Lachnospiraceae and other Firmicutes taxa colonisation. This could
4456 partly explain why, in this *in vivo* model, Lachnospiraceae taxa were not observed to
4457 be major responders to GMP supplementation.**Establishment of the human**
4458 **faecal microbiota in the murine GIT tract**

4459 Although the faecal microbiota across groups at the end of the trial strongly
4460 separated from that at baseline grouping by humanisation type and diet (**S Figures 2,**
4461 **3**), it is considered that the murine host played a role in re-shaping the
4462 xenomicrobiota. For example, at the end of the trial, the Bacteroidetes and especially
4463 Porphyromonadaceae enrichment across all dietary groups and the compositional
4464 profile of the Lachnospiraceae can be viewed as the result of selective pressure
4465 applied by the murine host to reshape the relative abundances of shared humans
4466 xenomicrobiota taxa to best fit murine host physiology and immunity (Nguyen *et al.*,
4467 2015; Messer *et al.*, 2016). In this study, similarly to previous observations, we

4468 observed gender dependent differential responses to diet partly attributed to
4469 hormonal effects (Org *et al.*, 2016; Wang *et al.*, 2016). The *Akkermansia* bloom
4470 could be attributed to the HydroGel treatment that interrupted the Abx treatment in
4471 some dietary groups, to coprophagy and to cross-contamination between cages.
4472 Interestingly, Dubourg *et al.* (2012) reported increased *A. muciniphila* colonisation
4473 in gut microbiota after broad-spectrum Abx treatment.

4474 **3.6 Conclusions**

4475 Milk is a widely accessible dietary product, it combines an array of valuable
4476 nutrients and many recent human cohort studies have demonstrated the health
4477 benefits of milk consumption (Haug *et al.*, 2007; Visioli and Strata, 2014; Guo *et al.*,
4478 2017). Importantly, BMOs may be an efficient prebiotic substrate similarly to HMOs
4479 (Zivkovic and Barile, 2011; Smilowitz *et al.*, 2014). We have discussed the gut
4480 microbiota modulatory potential of (lactose free and whole) milk in a murine model.
4481 Lactose free milk was found a better prebiotic candidate compared to whole milk as
4482 it retained higher microbiota diversity. However, whole milk diet affected the
4483 abundance of significant health-relevant microbiota taxa. When translating the
4484 findings of the current study to humans, the fact that mice may not fully tolerate
4485 lactose (van de Heijning *et al.*, 2015), and that the lactose fermentation in wmlk diet
4486 fed mice may have prevented BMOs utilisation by the gut microbiota, must be taken
4487 into consideration. If the two milk types, i.e., whole milk and lactose-free milk, have
4488 a differential effect on the gut microbiota of lactase persistent subjects remains to be
4489 clarified in a clinical trial.

4490 **3.7 Funding Sources**

4491 This work was financially supported by the Government of Ireland National
4492 Development Plan by way of a Department of Agriculture, Food and the Marine
4493 (DAFM) under a Food Institutional Research Measure (FIRM) award (11/F/053) for
4494 the ELDERFOOD project.

4495 **3.8 Acknowledgements**

4496 We thank Arla Foods Ingredients, Denmark and Valio Ltd Finland for providing
4497 Lacprodan CGMP-10 and milk powders, respectively. We thank Dr Celine Ribiere
4498 for her help in the amplicon sequence analysis.

4499 **3.9 References**

- 4500 Allard, G., Ryan, F. J., Jeffery, I. B., Claesson, M. J. (2015). SPINGO: A rapid
4501 species-classifier for microbial amplicon sequences. *BMC Bioinformatics*, 16(1),
4502 1–8.
- 4503 Bereswill, S., Fischer, A., Plickert, R., Haag, L. M., Otto, B., Kühl, A. A., Dasti,
4504 J. I., Zautner, A. E., Munoz, M., Loddenkemper, C., Gross, U., *et al.* (2011).
4505 Novel murine infection models provide deep insights into the “Ménage à Trois”
4506 of *Campylobacter jejuni*, microbiota and host innate immunity. *PLoS One*, 6(6),
4507 e20953.
- 4508 Boudry, G., Hamilton, M. K., Chichlowski, M., Wickramasinghe, S., Barile, D.,
4509 Kalanetra, K. M., Mils, D. A., Raybould, H. E. (2017). Bovine milk
4510 oligosaccharides decrease gut permeability and improve inflammation and
4511 microbial dysbiosis in diet-induced obese mice. *J Dairy Sci*, 100(4), 2471–2481.
- 4512 Brück, W. M., Redgrave, M., Tuohy, K. M., Lönnerdal, B., Graverholt, G.,
4513 Hernell, O., Gibson, G. R. (2006). Effects of bovine alpha-lactalbumin and
4514 casein glycomacropeptide-enriched infant formulae on faecal microbiota in
4515 healthy term infants. *J Pediatr Gastroenterol Nutr*, 43(5), 673–9.
- 4516 Caporaso, J. G., Bittinger, K., Bushman, F. D., Desantis, T. Z., Andersen, G. L.,
4517 Knight, R. (2010). PyNAST: A flexible tool for aligning sequences to a template
4518 alignment. *Bioinformatics*, 26(2), 266–267.
- 4519 Catry, E., Bindels, L. B., Tailleux, A., Lestavel, S., Neyrinck, A. M., Goossens,
4520 J.-F., Lobysheval, I., Plovier, H., *et al.* (2018). Targeting the gut microbiota with

4521 inulin-type fructans: preclinical demonstration of a novel approach in the
 4522 management of endothelial dysfunction. *Gut*, 67(2), 271–283.

4523 Charbonneau, M. R., O'Donnell, D., Blanton, L. V., Totten, S. M., Davis, J. C.
 4524 C., Barratt, M. J., Cheng, J., Guruge, J., *et al.* (2016). Sialylated milk
 4525 oligosaccharides promote microbiota-dependent growth in models of infant
 4526 undernutrition. *Cell*, 164(5), 859–871.

4527 Claesson, M. J., Jeffery, I. B., Conde, S., Power, S. E., O'Connor, E. M., Cusack,
 4528 S., Harris, H. M., Coakley, M., *et al.* (2012). Gut microbiota composition
 4529 correlates with diet and health in the elderly. *Nature*, 488(7410), 178–184.

4530 Devkota, S., Wang, Y., Musch, M. W., Leone, V., Fehlner-Peach, H., Nadimpalli,
 4531 A., Antonopoulos, D. A., Jabri, B., Chang, E. B. (2012). Dietary-fat-induced
 4532 taurocholic acid promotes pathobiont expansion and colitis in Il10 $-/-$ mice.
 4533 *Nature*, 487(7405), 104–108.

4534 Dubourg, G., Lagier, J. C., Armougom, F., Robert, C., Audoly, G., Papazian, L.,
 4535 Raoult, D. (2013). High-level colonisation of the human gut by Verrucomicrobia
 4536 following broad-spectrum antibiotic treatment. *Int J Antimicrob Agents*, 41(2),
 4537 149–155.

4538 Flemer, B., Lynch, D. B., Brown, J. M. R., Jeffery, I. B., Ryan, F. J., Claesson,
 4539 M. J., O'Riordan M., Shanahan, F., O'Toole, P. W. (2017). Tumour-associated
 4540 and non-tumour-associated microbiota in colorectal cancer. *Gut*, 66(4), 633–643.

4541 Franceschi, C., and Campisi, J. (2014). Chronic inflammation (inflammaging)
 4542 and its potential contribution to age-associated diseases. *J Gerontol A Biol Sci*
 4543 *Med Sci*, 69(Suppl 1), S4–S9.

4544 Grazul, H., Kanda, L. L., Gondek, D. (2016). Impact of probiotic supplements on
 4545 microbiome diversity following antibiotic treatment of mice. *Gut Microbes*, 7(2),
 4546 101–114.

4547 Guo, J., Astrup, A., Lovegrove, J. A., Gijsbers, L., Givens, D. I., Soedamah-
 4548 Muthu, S. S. (2017). Milk and dairy consumption and risk of cardiovascular
 4549 diseases and all-cause mortality: dose–response meta-analysis of prospective
 4550 cohort studies. *Eur J Epidemiol*, 32(4), 269–287.

4551 Gustavo Hermes, R., Molist, F., Francisco Pérez, J., Gómez de Segura, A.,
 4552 Ywazaki, M., Davin, R., *et al.* (2013). Casein glycomacropeptide in the diet may
 4553 reduce *Escherichia coli* attachment to the intestinal mucosa and increase the
 4554 intestinal lactobacilli of early weaned piglets after an enterotoxigenic *E. coli* K88
 4555 challenge. *Br J Nutr*, 109(6), 1001–12.

4556 Hamilton, M. K., Ronveaux, C. C., Rust, B. M., Newman, J. W., Hawley, M.,
 4557 Barile, D., Mills, D. A., Raybould, H. E. (2017). Prebiotic milk oligosaccharides
 4558 prevent development of obese phenotype, impairment of gut permeability, and
 4559 microbial dysbiosis in high fat-fed mice. *Am J Physiol Gastrointest Liver*
 4560 *Physiol*, 312(5), G474–G487.

4561 Haug, A., Høstmark, A. T., Harstad, O. M. (2007). Bovine milk in human
 4562 nutrition – a review. *Lipids Health Dis*, 6(1), 25.

4563 Hernandez-Hernandez, O., Sanz, M. L., Kolida, S., Rastall, R. A, Moreno, F. J.
 4564 (2011). *In vitro* fermentation by human gut bacteria of proteolytically digested
 4565 caseinomacropeptide nonenzymatically glycosylated with prebiotic
 4566 carbohydrates. *J Agric Food Chem*, 59(22), 11949–55.

- 4567 Huang, Y.-L., Chassard, C., Hausmann, M., von Itzstein, M., Hennet, T. (2015).
4568 Sialic acid catabolism drives intestinal inflammation and microbial dysbiosis in
4569 mice. *Nature Commun*, 6(1), 8141.
- 4570 Huang, H., Krishnan, H. B., Pham, Q., Yu, L. L., Wang, T. T. Y. (2016). Soy and
4571 gut microbiota: Interaction and implication for human health. *J Agric Food*
4572 *Chem*, 64(46), 8695–8709.
- 4573 Hvas, C. L., Dige, A., Bendix, M., Wernlund, P. G., Christensen, L. A., Dahlerup,
4574 J. F., Agnholt, J. (2016). Casein glycomacropeptide for active distal ulcerative
4575 colitis: a randomised pilot study. *Eur J Clin Invest*, 46(6), 555–563.
- 4576 Jackson, M., Jeffery, I. B., Beaumont, M., Bell, J. T., Clark, A. G., Ley, R. E.,
4577 O'Toole, P. W., Spector, T. D., Steves, C. J. (2016). Signatures of early frailty in
4578 the gut microbiota. *Genome Med*, 8(1), 8.
- 4579 Jeffery, I. B., Lynch, D. B., O'Toole, P. W. (2016). Composition and temporal
4580 stability of the gut microbiota in older persons. *ISME J*, 10(1), 170–182.
- 4581 Jimenez, M., Cervantes-Garcia, D., Munoz, Y. H., Garcia, A., Haro, L. M.,
4582 Salinas, E. (2017). Novel mechanisms underlying the therapeutic effect of
4583 glycomacropeptide on allergy: Change in gut Microbiota, upregulation of TGF- β ,
4584 and inhibition of mast cells. *Int Arch Allergy Immunol*, 171(3–4), 217–226.
- 4585 Juge, N., Tailford, L., Owen, C. D. (2016). Sialidases from gut bacteria: a mini-
4586 review. *Biochem Soc Trans*, 44(1), 166–175.
- 4587 Jumpertz, R., Le, D. S., Turnbaugh, P. J., Trinidad, C., Bogardus, C., Gordon, J.
4588 I., Krakoff, J. (2011). Energy-balance studies reveal associations between gut

4589 microbes, caloric load, and nutrient absorption in humans. *Am J Clin Nutr*, 94(1),
 4590 58–65.

4591 Kasai, C., Sugimoto, K., Moritani, I., Tanaka, J., Oya, Y., Inoue, H., Tameda,
 4592 M., Shiraki, K., Ito, M., Takei, Y., Takase, K. (2015). Comparison of the gut
 4593 microbiota composition between obese and non-obese individuals in a Japanese
 4594 population, as analyzed by terminal restriction fragment length polymorphism
 4595 and next-generation sequencing. *BMC Gastroenterol*, 15, 100.

4596 Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M.,
 4597 Glöckner, F. O. (2012). Evaluation of general 16S ribosomal RNA gene PCR
 4598 primers for classical and next-generation sequencing-based diversity studies.
 4599 *Nucleic Acids Res*, 41(1), e1.

4600 Kurilshikov, A., Wijmenga, C., Fu, J., Zhernakova, A. (2017). Host genetics and
 4601 gut microbiome: Challenges and perspectives. *Trends Immunol*, 38(9), 633–647.

4602 Laird, E., Casey, M. C., Ward, M., Hoey, L., Hughes, C. F., McCarroll, K.,
 4603 Cunningham, C., Strain, J. J., McNulty, Molloy, A. M. (2016). Dairy intakes in
 4604 older Irish adults and effects on vitamin micronutrient status: Data from the
 4605 TUDA study. *J Nutr Health Aging*, 21 (9), 954-961.

4606 Lana, A., Rodriguez-Artalejo, F., Lopez-Garcia, E. (2015). Dairy consumption
 4607 and risk of frailty in older adults: A prospective cohort study. *J Am Geriatr Soc*,
 4608 63(9), 1852–1860.

4609 Larsson, S. C., Crippa, A., Orsini, N., Wolk, A., Michaëlsson, K. (2015). Milk
 4610 consumption and mortality from all causes, cardiovascular disease, and cancer: A
 4611 systematic review and meta-analysis. *Nutrients*, 7(9), 7749–7763.

4612 Levy, M., Kolodziejczyk, A. A., Thaiss, C. A., Elinav, E. (2017). Dysbiosis and
4613 the immune system. *Nat Rev Immunol*, 17(4), 219–232.

4614 Li, H., Qi, Y., Jasper, H. (2016). Preventing age-related decline of gut
4615 compartmentalization limits microbiota dysbiosis and extends lifespan. *Cell Host*
4616 *Microbe*, 19(2), 240–253.

4617 Lundberg, R., Toft, M. F., August, B., Hansen, A. K., Hansen, C. H. F. (2016).
4618 Antibiotic-treated versus germ-free rodents for microbiota transplantation
4619 studies. *Gut Microbes*, 7(1), 68–74.

4620 Magoc, T., and Salzberg, S. L. (2011). FLASH: Fast length adjustment of short
4621 reads to improve genome assemblies. *Bioinformatics*, 27(21), 2957–2963.

4622 Manor, O., and Borenstein, E. (2017). Systematic characterization and analysis
4623 of the taxonomic drivers of functional shifts in the human Microbiome. *Cell Host*
4624 *Microbe*, 21(2), 254–267.

4625 Marchesi, J. R., Adams, D. H., Fava, F., Hermes, G. D. A., Hirschfield, G. M.,
4626 Hold, G., Quraishi, M. N., Kinross J., *et al.* (2016). The gut microbiota and host
4627 health: a new clinical frontier. *Gut*, 65, 330–390.

4628 Messer, J. S., Liechty, E. R., Vogel, O. A., Chang, E. B. (2017). Evolutionary
4629 and ecological forces that shape the bacterial communities of the human gut.
4630 *Mucosal Immunol*, 10(3), 567–579.

4631 Mottawea, W., Chiang, C.-K., Mühlbauer, M., Starr, A. E., Butcher, J.,
4632 Abujamel, T., Deeke, S. A., Brandel, A., *et al.* (2016). Altered intestinal

4633 microbiota–host mitochondria crosstalk in new onset Crohn’s disease. *Nat*
4634 *Commun*, 7, 13419.

4635 Neelima, Sharma, R., Rajput, Y.S., Mann, B. (2013). Chemical and functional
4636 properties of glycomacropeptide (GMP) and its role in the detection of cheese
4637 whey adulteration in milk: a review. *Dairy Sci Technol*, 93(1), 21–43.

4638 Nguyen, T. L. A., Vieira-Silva, S., Liston, A., Raes, J. (2015). How informative
4639 is the mouse for human gut microbiota research? *Dis Model Mech*, 8(1), 1–16.

4640 Ntemiri, A., Ni Chonchuir, F., O’Callaghan, T. F., Stanton, C., Ross, R. P.,
4641 O’Toole, P. W. (2017). Glycomacropeptide sustains microbiota diversity and
4642 promotes specific taxa in an artificial colon model of elderly gut microbiota. *J*
4643 *Agric Food Chem*, 65(8), 1836–1846.

4644 Org, E., Mehrabian, M., Parks, B. W., Shipkova, P., Liu, X., Drake, T. A., Lusi,
4645 A. J. (2016). Sex differences and hormonal effects on gut microbiota
4646 composition in mice. *Gut Microbes*, 7(4), 313–322.

4647 O’Riordan, N., Kane, M., Joshi, L., Hickey, R. M. (2014). Structural and
4648 functional characteristics of bovine milk protein glycosylation. *Glycobiology*,
4649 24(3), 220–236.

4650 Power, S. E., Jeffery, I. B., Ross, R. P., Stanton, C., O’Toole, P. W., O’Connor,
4651 E. M., Fitzgerald, G. F. (2014). Food and nutrient intake of Irish community-
4652 dwelling elderly subjects: who is at nutritional risk? *J Nutr Health Aging*, 18(6),
4653 561–572.

4654 Robitaille, G. (2013). Growth-promoting effects of caseinomacropeptide from
 4655 cow and goat milk on probiotics. *J Dairy Res*, 80(1), 58–63.

4656 Rothschild, D., Weissbrod, O., Barkan, E., Korem, T., Zeevi, D., Costea, P. I.,
 4657 Godneva, A., Kalka, I. N., Bar, N., Shilo, S., Lador, D., Vila, A. V., *et al.* (2018).
 4658 Environmental factors dominate over host genetics in shaping human gut
 4659 microbiota composition. *Nature*, 555(25973), 210–215.

4660 Rozenberg, S., Body, J. J., Bruyère, O., Bergmann, P., Brandi, M. L., Cooper, C.,
 4661 Devogelaer, J. P., Gielen, E., *et al.* (2016). Effects of dairy products consumption
 4662 on health: Benefits and beliefs—A commentary from the Belgian Bone Club and
 4663 the European Society for Clinical and Economic Aspects of Osteoporosis,
 4664 Osteoarthritis and Musculoskeletal Diseases. *Calcif Tissue Int*, 98(1), 1–17.

4665 Sahni, S., Mangano, K. M., Tucker, K. L., Douglas, P. K., Casey, V. A., Hannan,
 4666 M. T. (2015). Protective association of milk intake on the risk of hip fracture:
 4667 Results from the Framingham Original Cohort. *J Bone Miner Res*, 29(8), 37–54.

4668 Sawin, E. a, De Wolfe, T. J., Aktas, B., Stroup, B. M., Murali, S. G., Steele, J. L.,
 4669 Ney, D. M. (2015). Glycomacropeptide is a prebiotic that reduces *Desulfovibrio*
 4670 bacteria, increases cecal short chain fatty acids and is anti-inflammatory in mice.
 4671 *Am J Physiol Gastrointest Liver Physiol*, 309(7), G590-601.

4672 Scott, K. P., Antoine, J.-M., Midtvedt, T., van Hemert, S. (2015). Manipulating
 4673 the gut microbiota to maintain health and treat disease. *Microb Ecol Health Dis*,
 4674 26, 25877.

4675 Smilowitz, J. T., Lebrilla, C. B., Mills, D. A., German, J. B., Freeman, S. L.
 4676 (2014). Breast milk oligosaccharides: Structure-function relationships in the
 4677 neonate. *Annu Rev Nutr*, 34(1), 143–169.

4678 Song, M., Garrett, W. S., and Chan, A. T. (2015). Nutrients, foods, and
 4679 colorectal cancer prevention. *Gastroenterology*, 148(6), 1244–1260.

4680 Sonnenburg, J. L., and Bäckhed, F. (2016). Diet–microbiota interactions as
 4681 moderators of human metabolism. *Nature*, 535(7610), 56–64.

4682 Thevaranjan, N., Puchta, A., Schulz, C., Naidoo, A., Szamosi, J. C., Verschoor,
 4683 C. P., Loukov, D., Schenck, L. P., *et al.* (2017). Age-associated microbial
 4684 dysbiosis promotes intestinal permeability, systemic inflammation, and
 4685 macrophage dysfunction. *Cell Host Microbe*, 21(4), 455–466.e4.

4686 Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V., Mardis, E. R.,
 4687 Gordon, J. I. (2006). An obesity-associated gut microbiome with increased
 4688 capacity for energy harvest. *Nature*, 444(7122), 1027–31.

4689 van Calcar, S. C., and Ney, D. M. (2012). Food products made with
 4690 glycomacropeptide, a low-phenylalanine whey protein, provide a new alternative
 4691 to amino Acid-based medical foods for nutrition management of
 4692 phenylketonuria. *J Acad Nutr Diet*, 112(8), 1201–10.

4693 Van de Heijning, B., Kegler, D., Schipper, L., Voogd, E., Oosting, A., Beek, E.
 4694 (2015). Acute and chronic effects of dietary lactose in adult rats are not explained
 4695 by residual intestinal lactase activity. *Nutrients*, 7(7), 5542–5555.

4696 Vandeputte, D., Falony, G., Vieira-Silva, S., Wang, J., Sailer, M., Theis, S.,
 4697 Verbeke, K., Raes, J. (2017). Prebiotic inulin-type fructans induce specific
 4698 changes in the human gut microbiota. *Gut*, 66(11), 1968–1974.

4699 Visioli, F., and Strata, A. (2014). Milk, dairy products, and their functional
 4700 effects in humans: a narrative review of recent evidence. *Adv Nutr*, 5(2), 131–
 4701 143.

4702 Walker, A. W., Ince, J., Duncan, S. H., Webster, L. M., Holtrop, G., Ze, X.,
 4703 Brown, D., Stares, M. D., Scott, P., *et al.* (2011). Dominant and diet-responsive
 4704 groups of bacteria within the human colonic microbiota. *ISME J*, 5(2), 220–30.

4705 Wang, J., Wang, J., Pang, X., Zhao, L., Tian, L., Wang, X. (2016). Sex
 4706 differences in colonization of gut microbiota from a man with short-term
 4707 vegetarian and inulin-supplemented diet in germ-free mice. *Sci Rep*, 6(1), 36137.

4708 Woting, A., Pfeiffer, N., Loh, G., Klaus, S., Blaut, M. (2014). *Clostridium*
 4709 *ramosum* promotes high-fat diet-induced obesity in gnotobiotic mouse models.
 4710 *MBio*, 5(5), e01530-14-e01530-14.

4711 Ze, X., Duncan, S. H., Louis, P., Flint, H. J. (2012). *Ruminococcus bromii* is a
 4712 keystone species for the degradation of resistant starch in the human colon. *ISME*
 4713 *J*, 6(8), 1535–1543.

4714 Zivkovic, A. M., and Barile, D. (2011). Bovine milk as a source of functional
 4715 oligosaccharides for improving human health. *Adv Nutr (Bethesda, Md.)*, 2(3),
 4716 284–9.

4717 Zhu, W., Gregory, J. C., Org, E., Buffa, J. A., Gupta, N., Wang, Z., Li, L., Wu,
4718 Y., Mehrabian, M., *et al.* (2016). Gut microbial metabolite TMAO enhances
4719 platelet hyperreactivity and thrombosis risk. *Cell*, 165(1), 111–124.

4720 Zeng, M. Y., Inohara, N., Nuñez, G. (2016). Mechanisms of inflammation-driven
4721 bacterial dysbiosis in the gut. *Mucosal Immunol*, 10(1), 18–26.

4722 3.10 Supplementary Chapter 3

4723 **S Table 1 Mouse treatment groups tested by diet, gender and the human**
 4724 **microbiota type.** The number of mice at the end of the trial per group is indicated.
 4725 The experimental arms of groups A, B, C and D were performed simultaneously
 4726 followed by the experimental arms of groups E, F, G and H. The animals that
 4727 received HydroGel treatment during Abx period, is indicated.

	Community type human microbiota (COM)		Longstay type human microbiota (LS)	
	female	male	female	male
Lactose free milk	A (n=5)	A (n=5)	B (n=6)	B (n=6)
Whole milk	C (n=6)	C (n=6)	D (n=4)	D (n=5)
GMP	E (n=6)#	E (n=6)#	G (n=6)	G (n=6)
Control	H (n=6)###	H (n=6)	F (n=6)##	F (n=5)###

“#”, “##”, “###” three, six and nine days, respectively, of HydroGel intake during Abx treatment

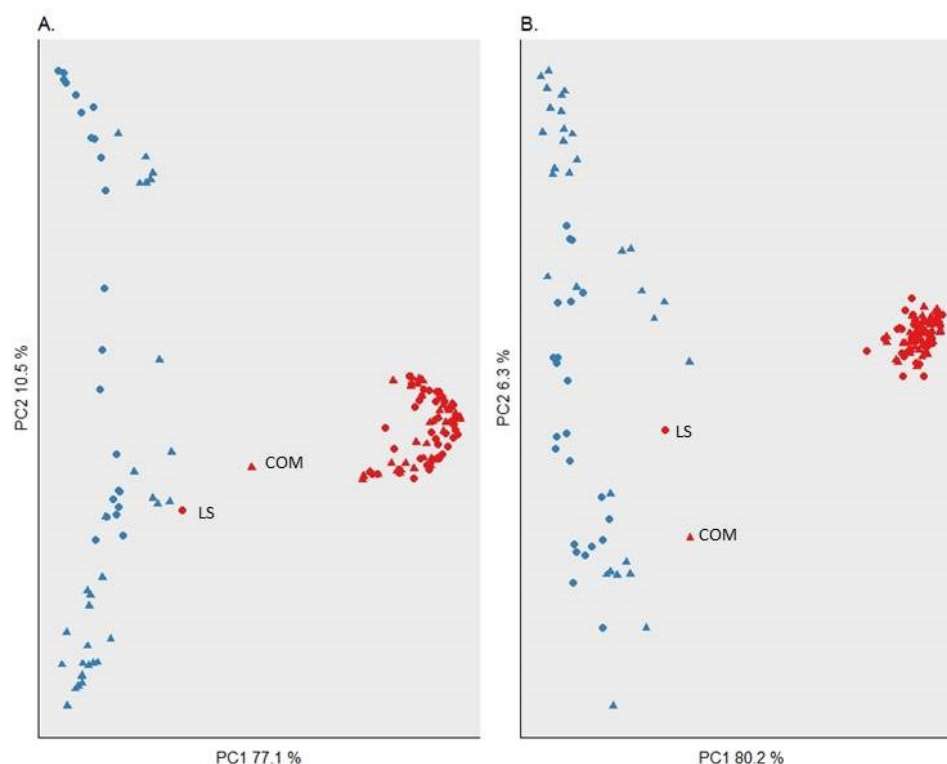
4728

4729 **S Table 2 Composition of the baseline murine and the human faecal microbiota.**
 4730 EM297; longstay type faecal microbiota (LS); EM425 community type faecal
 4731 microbiota (COM). Only species present at ≥ 1 % relative abundance in at least one
 4732 group are presented.

	MURINE	EM297 LS	EM425 COM
OTU classification	% average relative abundance		
Bifidobacteriaceae/Bifidobacterium/unclassified	0.0	1.1	0.0
Bacteroidaceae/Bacteroides/Bacteroides acidifaciens	1.5	0.01	0.7
Bacteroidaceae/Bacteroides/Bacteroides dorei	0.2	9.5	0.0
Bacteroidaceae/Bacteroides/Bacteroides fragilis	0.0001	15.4	1.4
Bacteroidaceae/Bacteroides/unclassified	0.1	8.0	0.6
Porphyromonadaceae/Barnesiella/Barnesiella intestinihominis	7.9	0.7	2.6
Porphyromonadaceae/Barnesiella/unclassified	1.1	0.02	0.2
Porphyromonadaceae/Odoribacter/Odoribacter splanchnicus	0.4	1.7	0.3
Porphyromonadaceae/Parabacteroides/Parabacteroides merdae	0.2	3.0	1.3
Porphyromonadaceae/unclassified/unclassified	34.6	0.02	0.03
Prevotellaceae/unclassified/unclassified	4.1	0.0	0.0
Rikenellaceae/Alistipes/Alistipes massiliensis	3.2	0.0	0.0
Rikenellaceae/Alistipes/Alistipes putredinis	0.04	0.7	1.3
Rikenellaceae/Alistipes/unclassified	0.8	2.5	0.8
Deferribacteraceae/Mucispirillum/Mucispirillum schaedleri	1.1	0.0	0.0
Methanobacteriaceae/Methanobrevibacter/ Methanobrevibacter smithii	0.0	1.6	0.3
Lactobacillaceae/Lactobacillus/Lactobacillus reuteri	0.8	0.04	0.0
Lactobacillaceae/Lactobacillus/unclassified	1.9	0.3	0.0
Clostridiaceae_1/Clostridium sensu stricto/Clostridium disporicum	0.0	0.0	1.5
Lachnospiraceae/Clostridium XIVa/unclassified	1.7	2.6	0.04
Lachnospiraceae/Ruminococcus2/Ruminococcus torques	0.003	2.2	0.1
Lachnospiraceae/unclassified/unclassified	24.6	0.8	18.3
Peptostreptococcaceae/Clostridium_XI/Clostridium ruminantium	0.0001	0.0	1.5
Ruminococcaceae/Clostridium IV/Clostridium leptum	0.02	3.9	0.004
Ruminococcaceae/unclassified/unclassified	1.9	4.2	3.8
Clostridiales/unclassified/unclassified/unclassified	5.2	0.8	10.2
Acidaminococcaceae/Succiniclasticum/Succiniclasticum ruminis	0.0	0.0	1.3
Firmicutes/unclassified/unclassified/unclassified/ unclassified/unclassified	0.6	0.01	4.7
Pseudomonadaceae/Pseudomonas/Pseudomonas aeruginosa	0.0001	4.2	0.003
Synergistaceae/Cloacibacillus/Cloacibacillus evryensis	0.0001	21.3	0.0
Anaeroplasmataceae/Anaeroplasma/unclassified	0.004	0.0	1.7
unclassified/unclassified/unclassified/unclassified/ unclassified/unclassified	1.1	0.5	2.5
Verrucomicrobiaceae/Akkermansia/Akkermansia muciniphila	0.8	0.0	0.0

4733 **S Table 3 Alpha diversity indices of the faecal microbiotas of the human donors**
4734 **and the murine baseline faecal microbiota.** EM297: longstay type faecal
4735 microbiota (LS); EM425: community type faecal microbiota (COM); murine:
4736 aggregated faecal microbiota across all mice at baseline.

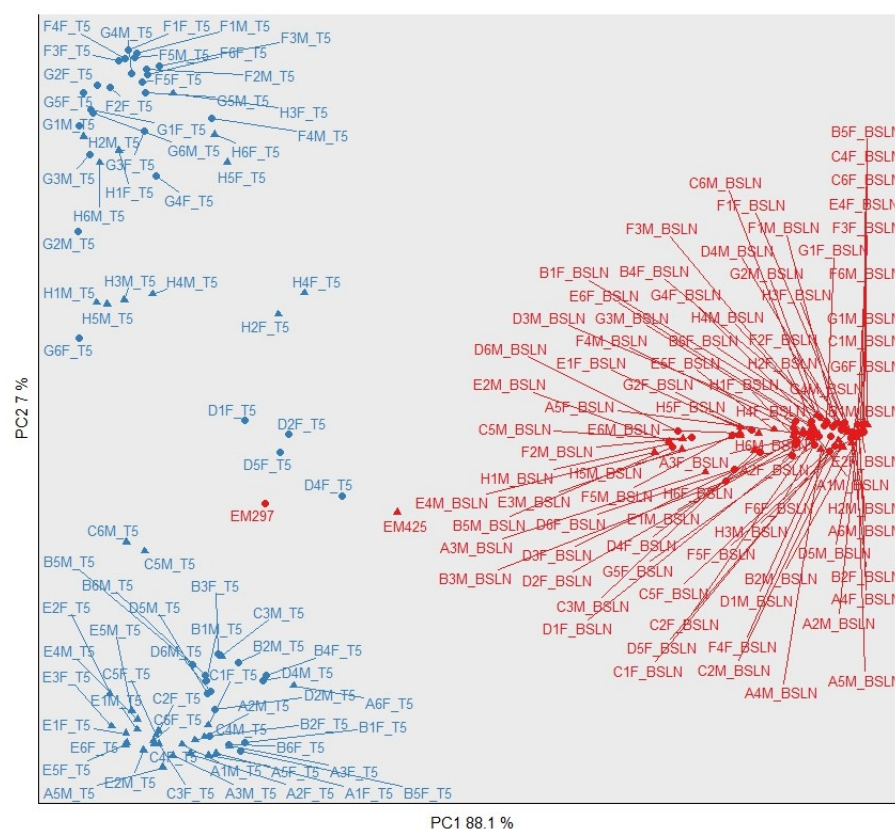
Alpha Diversity Index	MURINE	EM297 LS	EM425 COM
Shannon	6.0	4.0	6.0
Simpson	1.0	1.0	1.0
PD Whole tree	15.0	9.0	17.0
Observed species	207.0	73.0	169.0
Chao 1	346.0	104.0	264.0



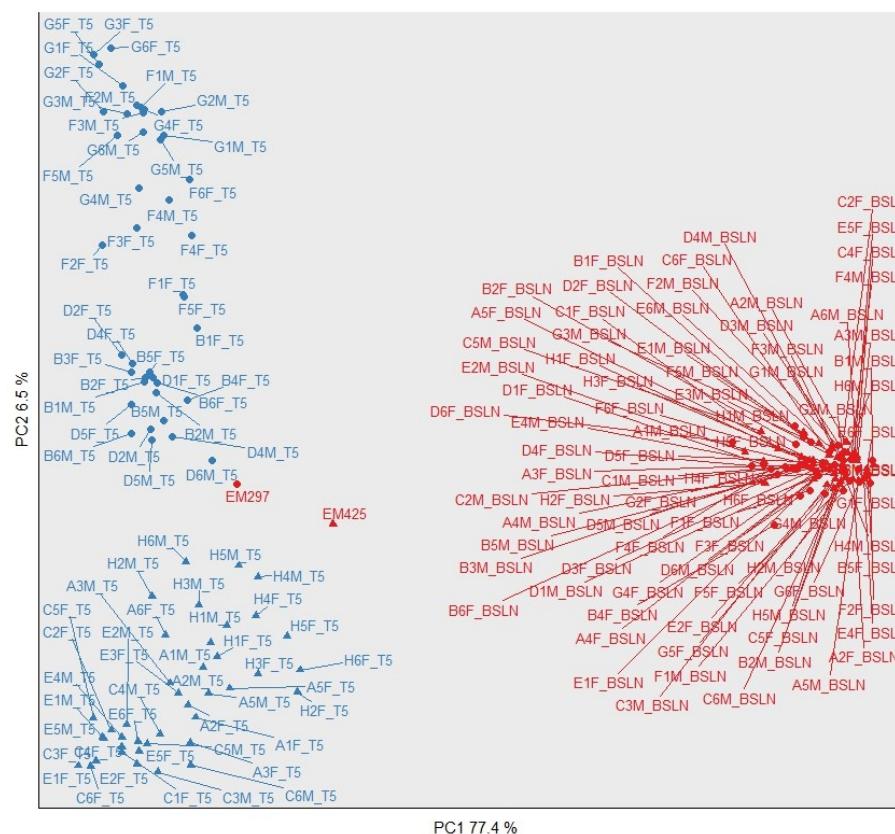
4737

4738 **S Figure 1 Principal coordinates analysis (PCoA) of the UniFrac distances one**
4739 **week after “humanisation” (T3).** A. Weighted UniFrac distances; B. Unweighted
4740 UniFrac distances; “▲” community (COM) type faecal microbiota; “●” longstay
4741 (LS) type faecal microbiota; **red**: baseline murine faecal microbiota; **blue**: T3 time
4742 point. The inocula of human faecal microbiota are denoted as COM and LS.

A.



B.



4743

4744 **S Figure 2 Principal coordinates analysis (PCoA) of the UniFrac distances at**
 4745 **end of the trial (T5). A.** Weighted UniFrac distances; **B.** Unweighted UniFrac
 4746 distances; “▲” community (COM) type faecal microbiota; “●” longstay (LS) type
 4747 faecal microbiota; **red:** baseline murine faecal microbiota; **blue:** T5 time point. The
 4748 inocula of human faecal microbiota are denoted as COM and LS.

4749 **S Table 4 Alpha diversity statistical analysis for community (COM) type**
 4750 **microbiota at the end of the trial (T5)** LAC_FREE: lactose free diet; WMILK:
 4751 whole milk diet; GMP: GMP diet; CONTROL: control diet.

Diversity indices	Kruskal-Wallis P _{adj} -value	Dunn's Post Hoc test p _{adj} values					
		CONTROL VS GMP	CONTROL VS LAC-FREE	GMP VS LAC-FREE	CONTROL VS WMILK	GMP VS WMILK	LAC-FREE VS WMILK
Shannon	3.30E-05	0.0004	0.1	1.17E-05	0.2	0.01	0.03
Simpson	0.0002	0.0004	0.4	0.001	0.4	0.0003	0.4
PD	0.0001	0.0002	0.4	0.0003	0.02	0.06	0.02
Observed species	1.41E-06	1.20E-05	0.3	1.40E-05	0.004	0.06	0.003
Chao1	0.001	0.002	0.4	0.004	0.02	0.23	0.02

4752

4753 **S Table 5 Alpha diversity statistical analysis for longstay (LS) type microbiota**
 4754 **at the end of the trial (T5)** LAC_FREE: lactose free diet; WMILK: whole milk
 4755 diet; GMP: GMP diet; CONTROL: control diet.

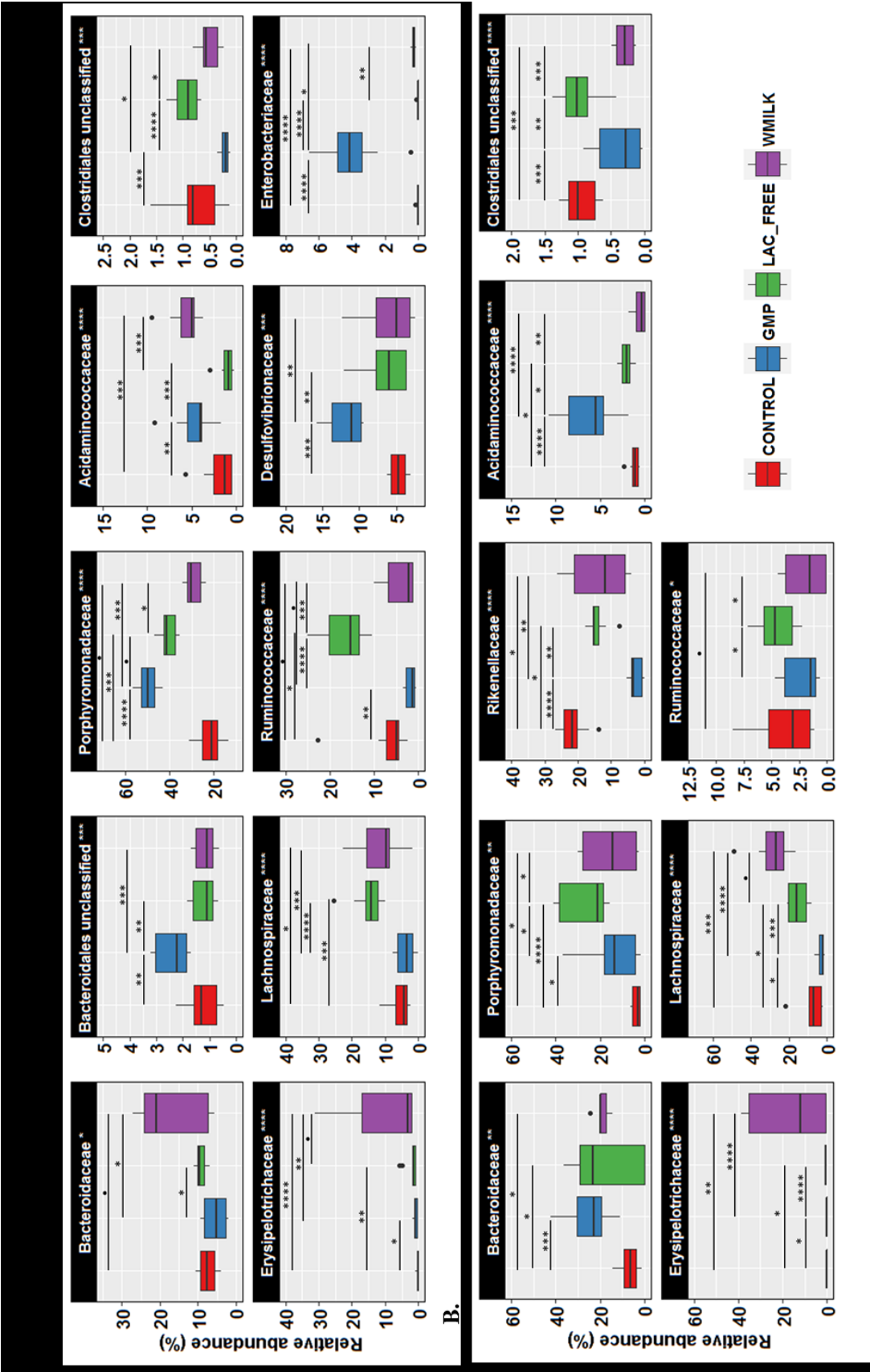
		Dunn's Post Hoc test p _{adj} values					
Diversity indices	Kruskal-Wallis P _{adj} -value	CONTROL VS GMP	CONTROL VS LAC-FREE	GMP VS LAC-FREE	CONTROL VS WMILK	GMP VS WMILK	LAC-FREE VS WMILK
Shannon	3.83E-06	0.01	0.007	1.20E-06	0.1	0.2	0.0003
Simpson	0.0002	0.30	0.001	7.73E-05	0.4	0.3	0.003
PD	1.85E-05	0.004	0.1	5.01E-06	0.1	0.1	0.004
Observed species	8.68E-06	0.001	0.3	0.0004	0.0004	0.2	0.0002
Chao1	0.05	0.09	0.4	0.09	0.05	0.3	0.066

4756

4757 **S Table 6 Differences in the female and male murine microbiota composition**
 4758 **associated with separation in the PCoA grouping by whole milk and control**
 4759 **diet.** Only families that were present at ≥ 1 % relative abundance in at least one group
 4760 are presented. WMILK: whole milk diet; CONROL: control diet; community (COM)
 4761 type microbiota.

	WMILK FEMALE	WMILK MALE	CONTROL FEMALE	CONTROL MALE
OTU assignment	% average relative abundance			
Bacteroidaceae	23.6	6.6	7.3	7.7
Porphyromonadaceae	30.3	28.0	25.0	17.9
Rikenellaceae	6.7	9.9	0.8	18.1
Bacteroidales/unclassified	1.3	1.0	0.8	1.7
Lachnospiraceae	10.9	12.8	5.4	5.4
Peptostreptococcaceae	2.7	0.001	0.0	0.0
Ruminococcaceae	1.7	7.7	9.2	4.6
Clostridiales/unclassified	0.6	0.4	0.5	1.1
Erysipelotrichaceae	2.3	23.1	0.6	0.004
Acidaminococcaceae	5.7	5.5	3.0	0.6
Desulfovibrionaceae	7.7	3.0	4.5	5.0
unclassified	5.3	1.5	0.9	0.2
Verrucomicrobiaceae	0.002	0.0	40.6	37.2

4762



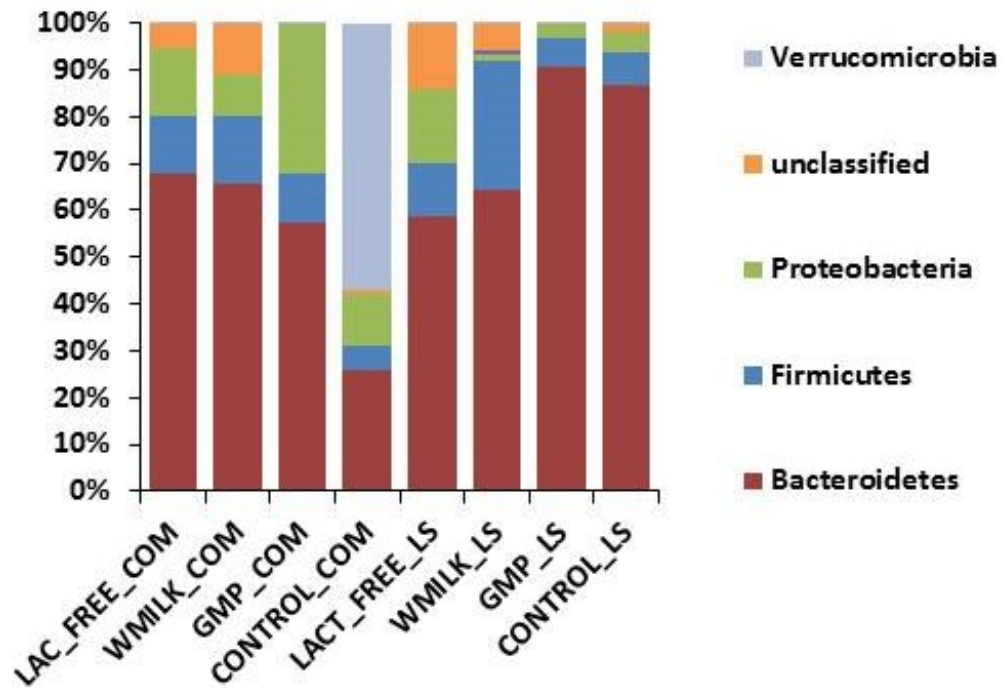
4764 **S Figure 3 Differentially abundant taxa at family level at the end of the trial**
4765 **(T5) for aggregated female and male mouse faecal microbiota.** A: community
4766 (COM) type colonisation; B: longstay (LS) type colonisation. Asterisks next to taxa
4767 denote the result of Kruskal-Wallis comparison; markings above boxplots denote the
4768 Dunn's post hoc result. Significant values: “●” padj<0.1; “*” padj<0.05; “**”
4769 padj<0.01; “***” padj<0.001; “****” padj<0.0001. COM type humanisation:

4770

4771 **S Table 7 Differences in the female and male murine microbiota composition**
4772 **associated with separation in the PCoA grouping by the lac-free and whole milk**
4773 **diet.** Only families that were present at ≥ 1 % relative abundance in at least one group
4774 are presented. LAC_FREE: lactose free diet; WMILK: whole milk diet; longstay
4775 (LS) type microbiota.

	LAC-FREE FEMALE	LAC-FREE MALE	WMILK FEMALE	WMILK MALE
	% average relative abundance			
Bacteroidaceae	9.5	28.5	19.1	18.7
Porphyromonadaceae	32.6	19.0	3.6	27.4
Rikenellaceae	13.5	15.4	5.4	21.6
Enterococcaceae	1.0	1.4	0.002	2.2
Lachnospiraceae	18.5	10.8	34.6	22.8
Peptostreptococcaceae	6.5	4.6	0.001	0.0
Ruminococcaceae	4.2	5.2	0.1	3.7
Clostridiales/unclassified	1.2	0.8	0.2	0.4
Erysipelotrichaceae	0.8	0.6	33.1	0.6
Acidaminococcaceae	2.2	1.8	1.1	0.002
Desulfovibrionaceae	8.1	8.0	1.4	1.2
Enterobacteriaceae	1.1	2.8	0.6	0.7

4776



4777

4778 **S Figure 4 Composition of the murine faecal microbiota at phylum level at the**
 4779 **mid part of the dietary intervention (T4).** COM: community type microbiota, LS:
 4780 longstay type microbiota. LAC_FREE: lactose free milk, WMILK: whole milk,
 4781 GMP: glycomacropeptide, CONTROL: soy protein based control diet. Only phyla
 4782 present at ≥ 1 % relative abundance are presented.

4783 **S Table 8 Alpha diversity analysis for community (COM) type microbiota at the**
 4784 **mid part of the trial (T4)** LAC_FREE: lactose free diet; WMILK: whole milk diet;
 4785 GMP: GMP diet; CONTROL: control diet.

		Dunn's Post Hoc test p _{adj} values					
Index	Kruskal-Wallis P _{adj} -value	CONTROL VS GMP	CONTROL VS LAC-FREE	GMP VS LAC-FREE	CONTROL VS WMILK	GMP VS WMILK	LAC-FREE VS WMILK
Shannon	1E-05	0.3	8E-05	7E-06	0.1	0.02	0.01
Simpson	1E-04	0.3	6E-05	0.0003	0.2	0.4	0.001
Observed species	0.0002	0.01	0.04	4E-05	0.4	0.01	0.05
PD whole tree	0.0002	0.003	0.21	0.0003	0.3	0.001	0.3
Chao1	0.001	0.02	0.05	0.0002	0.3	0.01	0.1
Alpha diversity average values							
Index		CONTROL	GMP	LAC-FREE	WMILK		
Shannon		3	3	4	3		
Simpson		1	1	1	1		
Observed species		46	28	57	46		
PD whole tree		5	3	6	6		
Chao1		66	33	71	57		

4786

4787 **S Table 9 Alpha diversity analysis for longstay (LS) type microbiota at the mid**
 4788 **part of the trial (T4)** LAC-FREE: lactose free diet; WMILK: whole milk diet;
 4789 GMP: GMP diet; CONTROL: control diet.

		Dunn's Post Hoc test p_{adj} values					
Index	Kruskal-Wallis P_{adj} -value	CONTROL VS GMP	CONTROL L VS LAC-FREE	GMP VS LAC-FREE	CONTROL VS WMILK	GMP VS WMILK	LAC-FREE VS WMILK
Shannon	1E-06	0.4	0.0001	0.00004	0.0008	0.0002	0.4
Simpson	2E-06	0.5	0.0001	0.0001	0.0003	0.0002	0.5
PD whole tree	1E-06	0.2	0.0007	1E-05	0.002	6E-05	0.4
Observed species	1E-06	0.4	0.0001	4E-05	0.001	0.0002	0.4
Chao1	1E-06	0.4	0.0004	9E-05	0.0003	5E-05	0.4
Alpha diversity average values							
Index		CONTROL L	GMP	LAC-FREE	WMILK		
Shannon		2	2	4	4		
Simpson		1	1	1	1		
PD whole tree		2	1	3	3		
Observed species		14	12	31	31		
Chao1		16	14	39	42		

4790

4791

Chapter 4

4792

Effect of blueberry on the composition and

4793

metabolism activity of gut microbiota and

4794

artificial microbiota community from the

4795

elderly

4796 **4.1 Abstract**

4797 We tested the modulatory effect of freeze-dried blueberry powder on the human gut
4798 microbiota of older subjects using an *in vitro* batch fermentation system. The
4799 blueberry powder was rich in polyphenols and fibre. In older people, lower gut
4800 microbiota diversity is associated with poor health and mortality. Therefore, two
4801 types of faecal microbiota were used, i.e. low diversity longstay type (LS)
4802 microbiota from older subjects residing in long-term care units, and higher diversity
4803 community type (COM) microbiota from community-dwelling subjects. An artificial
4804 gut microbiota community (MCC100) of 100 strains designed to emulate the
4805 compositional profile of the gut microbiota of a healthy adult, was also used. The
4806 fermentation medium was supplemented with either blueberry powder or prebiotic
4807 mix alone, or a combination of the two supplementation regimes. The microbiota
4808 was analysed at baseline and at time points 16 h and 24 h of fermentation by
4809 Illumina MiSeq amplicon sequencing of the 16S rRNA gene. The differential
4810 microbiota responsiveness to the blueberry supplementation was associated with the
4811 baseline microbiota composition. Blueberry supplementation resulted in increased
4812 Lactobacillales/*Streptococcus* relative abundance most likely due to the high sugar
4813 content of the supplement. The blueberry supplementation increased the relative
4814 abundance of Bifidobacteriales/*Bifidobacterium* when the taxon was abundant at
4815 baseline. The increase in the relative abundance of the Proteobacteria across all
4816 microbiotas tested could be the result of simple sugar fermentation. In the lower
4817 complexity MCC100 artificial microbiota the combined supplementation resulted in
4818 comparatively increased alpha diversity; a similar result was not observed in the
4819 tested faecal microbiotas. The combined supplementation resulted in increased short
4820 chain fatty acid production in MCC100 and the faecal microbiota. The high sugar

4821 content of the whole blueberry powder had a strong effect on the observed microbiota
4822 responses to blueberry supplementation. Follow-up *in vitro* trials with isolated
4823 phenolic compounds such as anthocyanins will elucidate the role of the gut
4824 microbiota in the bioconversion of blueberry polyphenol extracts.

4825 **4.2 Introduction**

4826 The gut microbiota is a recognised component of human health and it is shaped by
4827 host genetics, environment, life-style and diet (Sonneburg and Backhed, 2016;
4828 Messer *et al.*, 2017). Based mostly on cohorts representing western populations and
4829 life-style, and despite significant inter-individual variations in gut microbiota
4830 composition, the existing data has enabled a general description of the healthy adult
4831 gut microbiota (Marchesi *et al.*, 2015). Various events throughout the lifespan such
4832 as excessive antibiotic use, changes in dietary habits, and infections may ultimately
4833 cause dysbiosis, that is, perturbations in the composition and reduction in the
4834 phylogenetic diversity of the gut microbiota that is associated with disease (Petersen
4835 and Round, 2014).

4836 Immuno-senescence, hospitalization and changes in dietary habits may collectively
4837 contribute to age-related microbiota alterations observed in the gut microbiota of
4838 older individuals and to the increase of the inflammatory status in the elderly, a
4839 known risk factor for mortality in humans and animal models (Jeffery *et al.*, 2015;
4840 Theveranjan *et al.*, 2017). In conditions where dysbiosis is a disease feature,
4841 manipulation of the gut microbiota can be a tool for prevention, improvement or
4842 even therapy (Salazar *et al.*, 2014). Probiotics, faecal microbiota transplants (FMT),
4843 live bacteriotherapy, prebiotics and supplementation such as with polyphenols are
4844 potential mechanisms for gut microbiota modulation (Petrof *et al.*, 2013; Marchesi *et*
4845 *al.*, 2015).

4846 Flavonoids are polyphenolic compounds that naturally occur in fruits, vegetables and
4847 other plant-derived dietary components and can be sub-divided in to seven sub-
4848 classes including flavones, and anthocyanins (reviewed in Panche *et al.*, 2016).

4849 Anthocyanins are present in plants as glycosylated anthocyanidins conjugated with
4850 sugars including glucose, galactose, arabinose, rhamnose and xylose (reviewed in
4851 Pojer *et al.*, 2013). Dietary intake of these compounds has been associated with
4852 health benefits based on *in vitro* and *in vivo* experimental models and human studies
4853 (reviewed in Li *et al.*, 2017). Ivey *et al.* (2017) reported that flavonoid-rich diet was
4854 associated with improved health and reduced mortality in a follow-up cohort study of
4855 young and middle-aged adults.

4856 The reported flavonoid consumption in adult populations varies due to differences in
4857 the analytical methods used to assess the flavonoid content in food products, and
4858 because of widely varying dietary habits (reviewed in Chun *et al.*, 2007; Chun *et al.*,
4859 2012; Pojer *et al.*, 2013). Adults in the US, Europe and the UK have a daily
4860 consumption of flavonoids that ranges from 177 mg/d up to 428 mg/d and
4861 consumption of anthocyanidins that ranges from 4.2 mg/d up to 19 mg/d (Chun *et al.*
4862 2007; Beking and Vieira 2011; Vogiatzoglou *et al.* 2015).

4863 Unabsorbed phenolic compounds reach the colon where they may serve as substrates
4864 for faecal microbiota fermentation (reviewed in Selma *et al.* 2009). Several *in vitro*
4865 studies (Sánchez-Patán *et al.*, 2012; Hidalgo *et al.*, 2012; Cueva *et al.*, 2013; Sánchez-Patán
4866 *et al.*, 2015; Zhang *et al.*, 2016) and *in vivo* studies (Del Bo *et al.*, 2010; Lacombe *et*
4867 *al.*, 2013; Fotschki *et al.*, 2016; Collins *et al.*, 2016) indicate towards the potential of
4868 polyphenols to modulate the gut microbiota. There is also scientific interest in the
4869 combined effect of dietary polyphenols and fibre on the gut microbiota and health
4870 (Edwards *et al.*, 2017).

4871 In this study, we applied 16S rRNA gene amplicon sequencing in order to describe
4872 the compositional changes that occurred in the faecal microbiota of older (>65 yrs)

4873 individuals after 24 h of pH and temperature controlled *in vitro* fermentations
4874 supplemented with whole blueberry powder. Faecal samples collected from frail and
4875 healthy older people were used to differentiate the responsiveness of low and higher
4876 phylogenetic diversity faecal microbiota respectively to the selected substrates. To
4877 move beyond whole community analysis, identifying the functional properties of
4878 isolated gut bacteria will further enable preclinical studies towards targeted
4879 restoration of dysbiosis based on artificial bacterial consortia (Clavel *et al.*, 2017).
4880 We therefore also tested an artificial bacterial consortium consisting of isolated
4881 faecal bacteria from healthy donors and representing the faecal microbiota of a
4882 healthy adult in the 24 h *in vitro* batch fermentation system for its fermentative
4883 potential on the blueberry powder.

4884 **4.3 Materials and Methods**

4885 **Faecal Samples:** Faecal samples were collected from two community-dwelling
4886 (COM) healthy older people (EM425 male 81 yrs, EM278 female 69 yrs) and two
4887 frail long-term care unit-residing (LS) older people (EM297 female 82 yrs, EM704
4888 male 89 yrs). The local Clinical Research Ethics Committee approved all practices.
4889 The faecal samples were transferred to an anaerobic cabinet within an hour after
4890 passing. Several 10% w/v slurries were prepared from each homogenised faecal
4891 sample using sterile reduced phosphate buffer saline (PBS) with 20% v/v glycerol.
4892 All faecal slurries were aliquoted and stored frozen at -80°C.

4893 **Artificial gut microbiota MCC100:** This artificial bacterial consortium consisted of
4894 100 strains of strict anaerobic and facultative anaerobic bacteria isolated from faeces
4895 of healthy individuals and it was assembled based on the composition of the gut
4896 microbiota profile of healthy western adult (Perez *et al.*, in preparation). The

4897 bacterial consortium was premixed from individual purified cultures, concentrated
4898 and stored in -80°C.

4899 **Single stage chemostat:** Batch fermentations were used in order to simulate colonic
4900 bacterial fermentation of the selected substrates (Hidalgo *et al.*, 2012). The
4901 fermentation conditions were as described before with some variations (Ntemiri *et*
4902 *al.*, 2017). The temperature and pH were kept stable at 37°C and 7.0 respectively.
4903 The duration of the fermentation was 24 h. The fermentation vessel was purged
4904 continuously with NO₂ and the working volume used was 400 ml. The basal medium
4905 was supplemented with 3 g/l of casein and antifoam A emulsion (1 ml/l) (Sigma
4906 Aldrich). The vessels were inoculated in triplicate at 1% w/v faecal sample; faecal
4907 samples were treated individually. Ten ml of thoroughly stirred fermentation liquid
4908 was retrieved from the fermenter vessel for analysis at time points 0 h and 16 h. This
4909 volume (10 ml) was selected in order not to reduce significantly the total volume of
4910 the fermentation. At time point 24 h, 50 ml faecal slurry was retrieved. The
4911 fermentation samples were immediately centrifuged and both supernatant and pellet
4912 were kept at -20°C for future analysis.

4913 **Fermentation Substrates:** The basal medium was supplemented in three different
4914 ways: A: 1% w/v a mix of complex carbohydrates (MIX) consisting of xylan from
4915 corncob (0.6 g/l), pectin from apple (0.6 g/l), amylopectin (0.6 g/l), arabinogalactan
4916 (0.6 g/l), soluble starch (4.0 g/l), β-glucan (0.1 g/l), mucin type II (1.0 gr/l), glucose
4917 (2.0 g/l); B: 1% w/v MIX supplemented with 1.5 % w/v blueberry powder
4918 (BB.MIX); C: 1.5 % blueberry powder (BB) alone. The blueberry powder was
4919 freeze-dried whole fruit powder, 50:50 tifblue:rubel blend; courtesy of the U.S.
4920 Highbush Blueberry Council, California) containing 21.4 g/100g of dietary fibre

4921 (16.8 g insoluble fibre, 4.6 g soluble fibre). The BB dose was calculated to approach
4922 that of a high total flavonoid and anthocyanins daily dietary intake; approximately
4923 200 mg per day and 70 mg per day respectively (Beking and Vieira, 2011) in an
4924 approximately 400 ml of simulated wet colon content (Sender *et al.*, 2016). The BB
4925 powder contained 33 mg/g phenolics and 10.2 mg/g anthocyanins.

4926 **Bacterial DNA Extraction:** Faecal pellets were weighted and resuspended in sterile
4927 PBS in order to provide approximately 200 mg of pellet required for the QIamp Fast
4928 DNA Stool (Qiagen) extraction kit protocol. The samples were homogenised
4929 mechanically in sterile tubes containing InhibitEX solution and zirconia glass beads
4930 of three sizes 0.1, 0.5 and 1.0 mm (Thistle Scientific, UK) using a Minibeadbeater
4931 (Biospec Products). Subsequent steps of gDNA extraction were performed as
4932 described before (Ntemiri *et al.*, 2017).

4933 **Preparation of Illumina Library for 16S rRNA Amplicon Sequencing:** The
4934 variable region V3/V4 of the 16S rRNA gene was targeted for amplicon sequencing
4935 using the Illumina MiSeq System (San Diego, California, USA). The universal 16S
4936 rRNA gene primers were forward primer for V3 region 5' TCGTCGGC
4937 AGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG 3' and
4938 reverse primer for V4 region 5' GTCTCGTGGGCTCGGAGATGTGTATAAG
4939 AGACAGGACTACHVGGGTATCTAATCC 3' (Klindworth *et al.* 2012). The
4940 amplicons were purified using the Agencourt AMPure XP-PCR Purification system
4941 (Beckman Coulter, Inc.). Dual index barcoding was performed to the purified
4942 products following the Illumina MiSeq System Protocol (Nextera XT V.2 Index Kits
4943 sets A and D, Illumina). The barcoded amplicons were purified (Agencourt AMPure
4944 XP-PCR Purification system; Beckman Coulter, Inc.) and quantified using the Qubit

4945 dsDNA HS Assay Kit (Thermo Fischer Scientific, MA, U.S.A.). Each sample was
4946 pooled at the same concentration and sequenced in the Teagasc Next Generation
4947 Sequencing Facility (Fermoy, Ireland) on a MiSeq Illumina platform using 2x250 bp
4948 chemistry.

4949 **Analysis of 16S rRNA Amplicon Sequencing:** Paired reads were joined using
4950 FLASH (Magoč *et al.*, 2011) and demultiplexed in Qiime using the split_libraries.py
4951 script. The forward and reverse primers are removed and operational taxonomic unit
4952 (OTU) table was generated *de novo* with Usearch. Filtering of the reads based on the
4953 read length and the number of identical sequences was performed. Subsequently,
4954 chimera filtering was performed and mapping of all the reads against the
4955 representative reads generated by the filtering, based on 97% identity. The reads per
4956 sample were rarefied and the sequences were aligned using the Qiime PyNast tool
4957 (Caporaso *et al.*, 2010). Alpha diversity indices i.e., Shannon, Simpson, PD whole
4958 tree, Chao1 and observed species, and beta diversity indices i.e. Weighted UniFrac,
4959 Unweighted UniFrac and Bray-Curtis, were generated in Qiime. The OTU table was
4960 taxonomically classified (classify.seqs) to genus level by using Mothur and the RDP
4961 v11.4/trainset 14 database (Wang *et al.*, 2007). In order to further classify the OTUs
4962 to species level Spingo database was used (Allard *et al.*, 2015).

4963 **Statistical Analysis of microbiota composition:** The R package (R Core Team)
4964 was used for the generating taxa relative abundance from the rarified reads, for the
4965 further analysis and visualisation of the UniFrac distances, for the visualisation of
4966 the alpha diversity and for statistical analysis. A Kruskal-Wallis with Dunn's post
4967 hoc test and p adjusted value with the Benjamini-Hochberg method was applied in R
4968 in order to detect significant differences in the microbiota composition due to

4969 substrate fermentation after 16 h and 24 h at Order, Family, Genus and Species level
4970 and for the alpha diversity indices. The Kruskal-Wallis test was applied in order to
4971 detect significant differences due to the effect of the substrate per donor, across all
4972 donors and MCC100 per time point and per substrate. Values were considered
4973 significant when Kruskal-Wallis p value was ≤ 0.05 , p_{adj} value ≤ 0.05 and Dunn's
4974 post hoc test p_{adj} value (for readability denoted as q_{adj}) were ≤ 0.05 . Significant
4975 trends in data were considered when Kruskal-Wallis p value was ≤ 0.5 , p_{adj} value $\leq$
4976 0.1 and Dunn's post hoc test value p_{adj} (for readability denoted as q_{adj}) was ≤ 0.1 .

4977 **Short Chain Fatty Acid Analysis:** High performance liquid chromatography
4978 (HPLC) was used in order to detect acetate, propionate and butyrate production, as
4979 described before (Chapter 2; Ntemiri *et al.*, 2017). Slurry retrieved from the
4980 fermenter vessels was centrifuged for 30 min and the supernatant was filtered
4981 through 0.2 μ m filter. For the SCFA analysis 1 ml was loaded in an Agilent 1200
4982 HPLC system with a refractive index detector. An Agilent Hi-Plex H 300x7.7mm
4983 column was used with 0.01N H₂SO₄ elution fluid, at a flow rate of 0.6 mL/min and
4984 column temperature at 65°C. For the visualization and analysis of SCFA levels,
4985 Graph Pad Prism 5 Software was used. A Kruskal-Wallis non-parametric test and
4986 Dunn's post hoc test were applied in order to detect significant differences in SCFA
4987 production per donor per substrate at 24 h. The same test was applied in order to
4988 detect significant differences in combined SCFA production in the faecal microbiota
4989 of all donors aggregated at 24 h.

4990 **4.4 Results**

4991 **Blueberry responses of Bifidobacteriaceae, Bacteroidaceae and Lactobacillales**
4992 **depend on baseline abundance**

4993 We have previously described the compositional and phylogenetic differences in the
4994 faecal microbiota of healthy or frail older subjects (COM type microbiota EM425
4995 and EM278, and LS type microbiota EM297 and EM704, respectively) (Jeffery *et*
4996 *al.*, 2015; Ntemiri *et al.*, 2017; Chapters 2 and 3 this thesis). Significant differences
4997 between the two types of microbiota include the enrichment in Lachnospiraceae and
4998 Ruminococcaceae in the COM type faecal microbiota composition and the
4999 enrichment in Proteobacteria observed in LS type microbiota. The LS type
5000 microbiota is also characterised by lower phylogenetic diversity compared to the
5001 community type (**S Table 10**). Principal coordinates analysis (PCoA) of the
5002 microbiotas at 0 h showed the differential clustering between COM and LS type (**S**
5003 **Figure 8**).

5004 Supplementation with the prebiotic MIX sustained the highest microbiota diversity
5005 across all faecal microbiotas (**S Figure 9 and 10**). Principal coordinates analysis
5006 (PCoA) of the microbiotas at the end of the fermentation (24 h) showed that the
5007 microbiotas clustered predominantly by donor and microbiota type and to a lesser
5008 extent by supplementation (**S Figure 11 A and B**).

5009 At baseline, the COM type microbiota of donor EM278 was naturally abundant in
5010 Bifidobacteriales/Bifidobacteriaceae and Bacteroidales/Bacteroidaceae;
5011 approximately 25% combined relative abundance (**Figures 21 and 22**). Blueberry
5012 supplementation (BB) resulted in increased Bifidobacteriaceae relative abundance in
5013 the composition of the EM278 COM faecal microbiota whereas prebiotic MIX
5014 supplementation increased the Bacteroidaceae abundance after 24 h fermentation.
5015 Supplementation with combined blueberry and prebiotic mix (BB.MIX) resulted in
5016 the concomitant increase of both Bifidobacteriaceae and Bacteroidaceae relative

5017 abundance; the combined relative abundance increased to approximately 67%
 5018 (**Figure 22** and **Table 9**). The Bifidobacteriaceae were represented by
 5019 *Bifidobacterium bifidum*, *Bifidobacterium longum* and other unclassified
 5020 *Bifidobacterium* taxa whereas the major Bacteroidaceae representative taxa after 24
 5021 h MIX or BB.MIX supplementation were *Bacteroides uniformis* and *Bacteroides*
 5022 *caccae* (Species level information for COM type microbiota in **S Table 13**). Another
 5023 faecal microbiota enriched in Bifidobacteriaceae and Bacteroidaceae was the LS
 5024 faecal microbiota from donor EM297 (approximately 20% combined abundance).
 5025 Bifidobacteriaceae in the EM297 LS type microbiota were not responsive to
 5026 blueberry supplementation and the baseline abundance (approximately 2%)
 5027 decreased across all supplementation regimes (**Figure 22**). Bacteroidaceae in the LS
 5028 microbiota EM297 were predominantly responsive to BB.MIX supplementation
 5029 whereas across the COM type microbiota EM425 and LS type microbiota EM704
 5030 the Bacteroidaceae were predominantly responsive to the prebiotic MIX
 5031 supplementation (**Figure 22**).

5032 Lactobacillales were highly responsive to blueberry supplementation (BB or
 5033 BB.MIX) predominantly in the EM425 COM type faecal microbiota and in the
 5034 EM704 LS type microbiota whereas inclusion of the prebiotic MIX in the blueberry
 5035 supplemented medium (BB.MIX) promoted the growth of a wider array of taxa
 5036 resulting in a lower scale relative abundance of Lactobacillales after 24 h
 5037 fermentation (**Figure 21**). In the EM425 COM microbiota, BB supplementation
 5038 increased the Lactobacillales relative abundance from approximately 0.5% (0 h) to
 5039 33% ($p_{\text{adj}} \& q_{\text{adj}} < 0.1$) at 24 h (comparison data at Order level for COM and LS
 5040 microbiota in **S Table 11** and **12**, respectively). In the EM704 LS microbiota the
 5041 Lactobacillales relative abundance increased after BB supplementation from 0.1 % at

0 h to 72 % at 24 h (p_{adj} & $q_{adj} < 0.1$) (**Figure 21**). In both EM425 and EM704 the taxon *Streptococcus* (unclassified) was the dominant representative of the Lactobacillales (**S Tables 13 and 14**).

In the EM297 LS microbiota the Lactobacillales was represented by the taxon *Enterococcus* (unclassified) (**S Table 14**). The taxon relative abundance was significantly (trend) increased by both BB supplementation (0 h: 2.5%, 24 h: 9% p_{adj} & $q_{adj} < 0.1$) and BB.MIX supplementation (0 h: 1.5%, 24 h: 9.6%, p_{adj} & $q_{adj} \leq 0.1$). The responsiveness of enterococci to blueberry supplementation was of a lower scale compared to the streptococci responsiveness, and prebiotic MIX supplementation promoted a higher increase in the relative abundance of enterococci in the EM297 faecal microbiota (**Figures 22**).

Blueberry supplementation increased the abundance of Enterobacteriaceae across all donors faecal microbiota

Blueberry supplementation (BB) increased the relative abundance of the Proteobacteria/Enterobacteriaceae represented by *Escherichia/Shigella* taxa across all donors faecal microbiotas whereas supplementation of fermentation medium with prebiotic MIX (MIX or BB.MIX) balanced the overrepresentation of the Enterobacteriaceae family observed with BB supplementation (**Figure 22**). The increase in the enterobacteria was partly independent the baseline Proteobacteria abundance. For example, in the COM type EM425 microbiota the low in abundance Enterobacteriaceae increased from $< 0.05\%$ to 40% at 24 h (**Table 9**) whereas in the EM297 LS type faecal microbiota that was significantly enriched in Proteobacteria, BB supplementation resulted in the highest Enterobacteriaceae relative abundance increase from approximately 9% to 69% at 24 h (p_{adj} & $q_{adj} < 0.1$) (**Table 10**). A

comparatively lower scale increase in the enterobacteria relative abundance was observed in the EM704 LS type microbiota; combined BB and MIX supplementation resulted in the highest abundance increase of the enterobacteria (**Table 10**).

The prebiotic MIX retained relatively higher Lachnospiraceae and Ruminococcaceae combined abundance whereas *Faecalibacterium prausnitzii* showed some responsiveness to BB supplementation

The high Lachnospiraceae and Ruminococcaceae relative abundance observed in the COM type microbiotas (approximately 66% combined abundance in both EM278 and EM425) was not retained across any of the three supplementation regimes (**Figure 22**). Supplementation with prebiotic MIX alone retained the highest portion of the Lachnospiraceae abundance in COM type microbiotas EM278 and EM425 and LS type EM704, whereas supplementation with BB and MIX combined retained the highest Lachnospiraceae abundance in the LS type microbiota EM297 (**Tables 9 and 10**). The taxon *Anaerostipes hadrus* was highly responsive to co-supplementation in both COM microbiotas (**S Table 13**).

Similarly, Ruminococcaceae abundance was partially retained with MIX and BB.MIX supplementation in COM type EM425 and LS type EM704 microbiotas (**Tables 9 and 10**), whereas the Ruminococcaceae in the COM microbiota EM278 were highly responsive to BB supplementation; the same responsiveness was not observed after MIX or BB.MIX supplementation (**Figure 22, TABLE 9**).

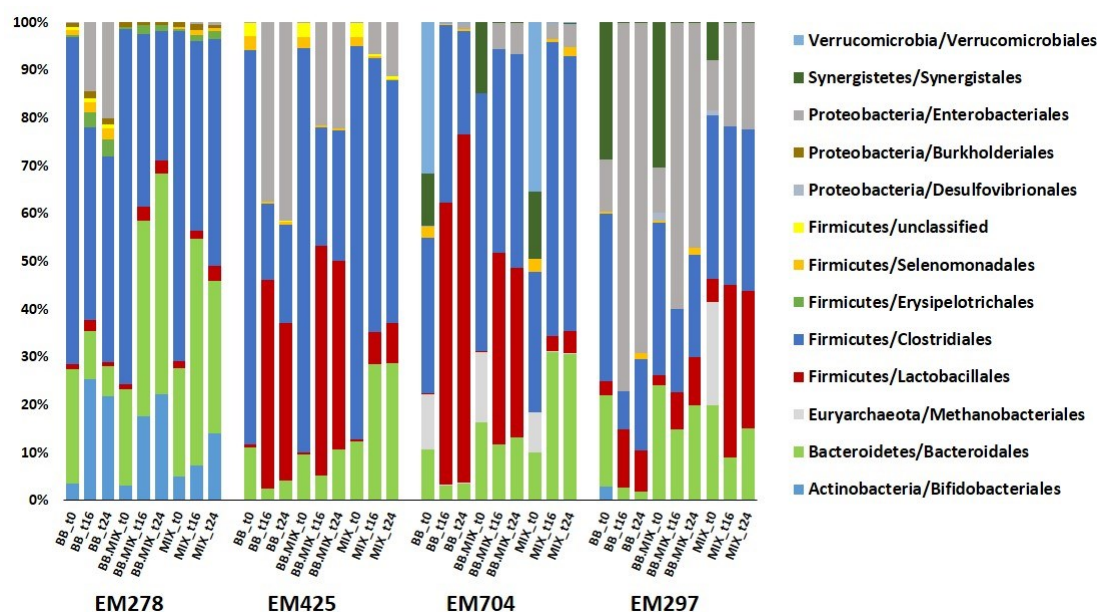
Faecalibacterium prausnitzii was the predominant taxon of the Ruminococcaceae family (approximately 13% relative abundance) in the EM278 microbiota after BB supplementation (**S Table 13**). In EM425 microbiota, the taxon *F. prausnitzii* was the predominant member of the Ruminococcaceae family after BB or MIX

5090 supplementation (2.5% and 5% relative abundance respectively), whereas the taxon
5091 *Gemmiger formicilis* of the Ruminococcaceae was dominant after BB.MIX or MIX
5092 supplementation (approximately 7% and 2% relative abundance respectively) (S
5093 **Table 13**). In EM704 LS faecal microbiota, of unclassified taxa were the dominant
5094 representatives of the Ruminococcaceae family (S **Table 14**).

5095 **Blueberry supplemented prebiotic medium promoted the abundance of**
5096 **Peptostreptococcaceae and Clostridiaceae 1 when abundant at baseline and**
5097 **irrespective of microbiota type.**

5098 In the EM425 COM type faecal microbiota where the Peptostreptococcaceae family
5099 was present with approximately 3% relative abundance at baseline, BB and BB.MIX
5100 but not MIX supplementation alone resulted in increased relative abundance during
5101 the first 16 h of BB (4.5% relative abundance) and BB.MIX (3% relative
5102 abundance); however, the abundance of the taxon was not retained until the
5103 fermentation end (**Figure 22, Table 9**). In the LS type faecal microbiota EM704
5104 where the Peptostreptococcaceae was present with approximately 11% relative
5105 abundance at baseline, BB or MIX supplementation alone could not retain
5106 Peptostreptococcaceae relative abundance whereas BB.MIX supplementation
5107 resulted in increased relative abundance at 24 h (**Figure 22, Table 10**). *Clostridium*
5108 *glycolicum* and other unclassified taxa were the dominant *Clostridium* cluster XI
5109 representatives of the Peptostreptococcaceae at 24 h (S **Table 14**). Abundant
5110 baseline Clostridiaceae 1 (represented by *Clostridium* sensu stricto taxa) increased in
5111 abundance with BB.MIX and MIX supplementation in both the COM type
5112 microbiota EM425 and the LS type EM704 microbiota whereas when non-abundant

5113 at baseline as in LS type EM297 microbiota, the abundance increased after MIX
5114 supplementation (Figure 22, Tables 9 and 10).



5115 **Figure 21 Order level development of the faecal microbiota from COM donors**
5116 **(EM278, EM425) and LS donors (EM704, EM297).** Orders present with relative
5117 abundance $\geq 1\%$ are shown. Substrate conditions are basal medium supplemented
5118 with: BB: blueberry powder; BB.MIX: blueberry powder and prebiotic mix of
5119 carbohydrates; MIX: mix of prebiotic carbohydrates. Time points: 0 h, 16 h and 24 h
5120 of fermentation.

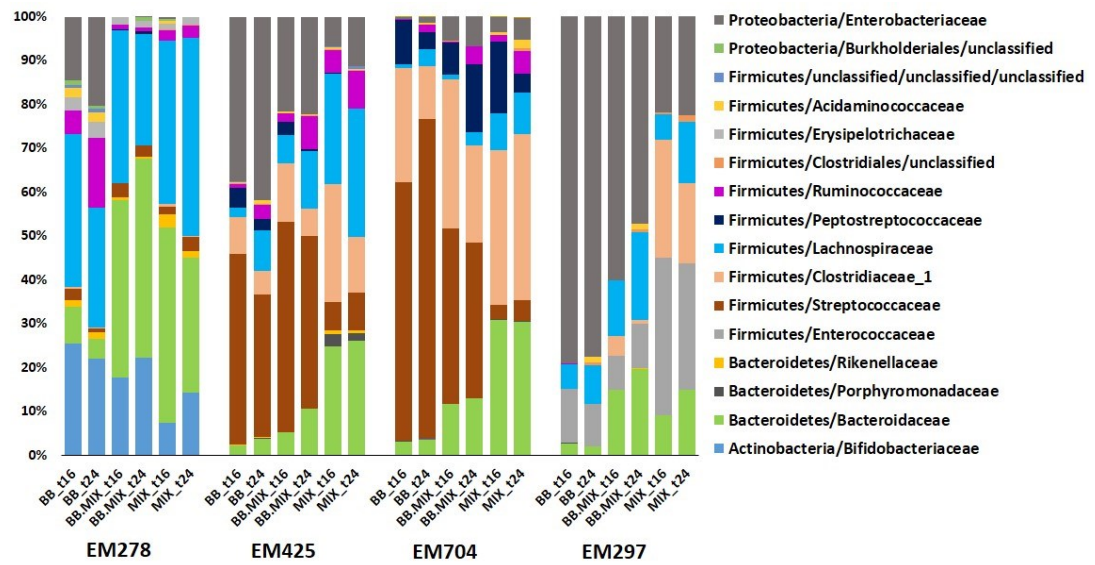


Figure 22 Family level development of the faecal microbiota from COM donors (EM278, EM425) and LS donors (EM704, EM297). Families present at $\geq 1\%$ are shown. Substrate conditions are basal medium supplemented with: BB: blueberry powder; BB.MIX: blueberry and prebiotic mix of carbohydrates; MIX: mix of prebiotic carbohydrates. Time points: 16 h and 24 h of fermentation.

5128 **Table 9 Development of the composition and comparison between time points of**
5129 **the relative abundance and comparison between time points of the relative**
5130 **abundance of dominant families after 24 h fermentation with COM faecal**
5131 **microbiota (EM278, EM425). BB: blueberry powder supplementation; BB.MIX:**
5132 **blueberry powder and prebiotic carbohydrate mix supplementation; MIX: prebiotic**
5133 **carbohydrate mix supplementation. Missing values indicate that the relevant taxon**
5134 **had <1% abundance in all vessels (two or three) summed for mean abundance. The**
5135 **mean value of the relative abundance per time point is shown.**

	EM278						EM425					
	BB		BB.MIX		MIX		BB		BB.MIX		MIX	
	t0	t24	t0	t24	t0	t24	t0	t24	t0	t24	t0	t24
Bifidobacteriaceae	3.5	21.6	3.0	21.9	4.9	13.9 ^a	-	-	-	-	-	-
Bacteroidaceae	20.0	4.5	16.9	44.8	19.1	29.9 ^a	5.8	3.7	4.6	10.6	6.8	26.1
Porphyromonadaceae	-	-	-	-	-	-	2.7	0.3	-	-	2.7	1.7
Rikenellaceae	1.7	1.5	1.3	0.4	1.8	1.5	1.6	0.1	-	-	1.6	0.6 ^a
Streptococcaceae	1.1	0.9	1.0	2.6	1.3	3.1	0.5	32.1 ^a	0.4	39.1	0.4	8.5
Clostridiaceae_1	0.1	0.2	-	-	0.1	0.1	3.1	5.3	3.9	6.2	2.7	12.6 ^a
Lachnospiraceae	43.7	26.7	49.5	25.0	43.4	43.8	44.4	9.3 ^a	42.8	13.0	46.4	29.0 ^a
Peptostreptococcaceae	-	-	0.3	0.6	-	-	3.2	2.5	3.5	0.4	2.9	0.2
Ruminococcaceae	20.1	15.7	19.2	0.8	21.0	2.9	21.2	3.2 ^a	22.8	7.6	19.4	8.5 ^a
Clostridiales unclas.	2.7	0.1	2.5	0.1	2.5	0.2 ^a	3.8	0.2 ^a	4.0	0.1	3.6	0.2
Erysipelotrichaceae	0.3	3.5	0.4	1.4	0.5	1.7 ^a	-	-	-	-	-	-
Acidaminococcaceae	0.4	2	-	-	0.2	0.6 ^a	2.6	0.7 ^a	2.0	0.4	1.8	0.3 ^a
Firmicutes/unclassified	0.8	1	-	-	-	-	2.6	0.2 ^a	2.7	0.02	2.7	0.6 ^a
Enterobacteriaceae	0.01	20	-	-	0.01	0.5	0.04	41.3	0.03	22.0	0.03	11.1 ^a

* Statistically significant result, Kruskal-Wallis test result $p \leq 0.05$, $\text{padj} \leq 0.05$ with Dunn's test $\text{qadj} \leq 0.05$
a Statistical trend, Kruskal-Wallis test result $p \leq 0.05$, $\text{padj} \leq 0.1$, with Dunn's test $\text{qadj} \leq 0.1$.

5136

5137 **Table 10 Development of the composition and comparison between time points**
5138 **of the relative abundance of dominant families after 24 h fermentation with LS**
5139 **faecal microbiota (EM704, EM297).** BB: blueberry powder supplementation;
5140 BB.MIX: blueberry powder and prebiotic carbohydrate mix supplementation; MIX:
5141 prebiotic carbohydrate mix supplementation. Missing values indicate that the
5142 relevant taxon had <1% abundance in all vessels (two or three) summed for mean
5143 abundance. The mean value of the relative abundance per time point is shown.

OTUU Classification	EM704						EM297					
	BB		BB.MIX		MIX		BB		BB.MIX		MIX	
	t0	t24	t0	t24	t0	t24	t0	t24	t0	t24	t0	t24
Bifidobacteriaceae	-	-	-	-	-	-	2.3	0.02	-	-	-	-
Bacteroidaceae	6.0	3.5	6.4	12.9	5.4	29.9	12.9	1.8	13.9	18.5	15.1	14.6
Porphyromonadaceae	3.2	0.1	-	-	3.5	0.3	1.4	0.04	1.5	0.1 ^a	2.7	0.02
Rikenellaceae	-	-	-	-	1.0	0.04	1.2	0.02	1.3	0.02	1.5	0.01
Enterococcaceae	-	-	-	-	-	-	1.1	8.6 ^a	0.5	9.6 ^a	1.6	28.1
Streptococcaceae	0.1	72.0 ^a	0.1	35.0	0.1	4.5 ^a	-	-	-	-	-	-
Clostridiaceae_1	10.1	11.8	11.1	21.7	9.2	37.3	-	-	0.01	0.9 ^a	0.01	17.9
Lachnospiraceae	3.3	3.7	3.2	3.1	3.2	9.3	15.3	7.7	11.9	18.7	17.0	13.6
Peptostreptococcaceae	11.7	4.0	13.4	15.1	10.3	4.3 ^a	-	-	-	-	-	-
Ruminococcaceae	3.6	1.6 ^a	3.2	4.0	3.7	5.2a ^a	11.2	0.1	8.7	0.1	13.7	0.1
Clostridiales unclas.	1.8	0.1	2.1	0.1	1.9	0.6 ^a	2.0	0.5 ^a	1.5	0.5 ^a	2.3	1.4
Acidaminococcaceae	2.3	0.3 ^a	-	-	2.6	1.9 ^a	0.3	1.1	0.2	1.3	-	-
Enterobacteriaceae	0.1	1.2	0.04	6.6	0.1	4.9	8.8	68.5 ^a	6.6	44.3 ^a	10.2	21.9

* Statistically significant result, Kruskal-Wallis test result $p \leq 0.05$, $p_{adj} \leq 0.05$ with Dunn's test $q_{adj} \leq 0.05$

^a Statistical trend, Kruskal-Wallis test result $p \leq 0.05$, $p_{adj} \leq 0.1$, with Dunn's test $q_{adj} \leq 0.1$

5144

5145 **Blueberry supplemented prebiotic medium sustained high microbiota diversity**
5146 **and *Bacteroides* taxa abundance in the artificial consortium MCC100 compared**
5147 **to the other supplementation regimes**

5148 The artificial consortium MCC100 composition was designed in order to mimic the
5149 faecal microbiota composition of a healthy adult. The analysis of the relatedness by
5150 Weighted Principal Coordinates Analysis (PCoA) of the microbiota composition in
5151 the bacterial consortium and faecal microbiotas showed that at 0 h the MCC100
5152 grouped with the community type faecal microbiotas separating from the LS type
5153 microbiotas (PCoA information in **S Figure 8 A and B**), as expected from its design.
5154 The distance between PCoA positions of some MCC100 microbiota (**S Figure 8 A**)
5155 was associated with the presence of OTUs assigned to chloroplasts deriving from the
5156 blueberry supplementation. Therefore, it was not considered a true compositional
5157 difference between the MCC100 preparations inoculated at 0 h.

5158 Supplementation of fermentation medium with BB.MIX sustained similar or higher
5159 levels of alpha diversity to the prebiotic MIX supplementation in the MCC100
5160 microbiota (**Table 11**). The Shannon and Simpson indices were similar after
5161 BB.MIX or MIX supplementation, whereas species richness indices (Chao1,
5162 Observed species and PD whole tree) were higher after BB.MIX supplementation.
5163 BB supplementation alone sustained the lowest diversity among the supplementation
5164 regimes.

5165 Supplementation of the MCC100 fermentation with BB.MIX was more effective in
5166 sustaining the abundance of Bacteroidetes compared to prebiotic MIX
5167 supplementation alone (**Figure 23 A**). Bacteroidetes relative abundance increased
5168 from approximately 12% to 52% over the 24 h of BB.MIX fermentation ($p_{\text{adj}}=0.05$,

5169 $q_{adj}=0.02$) whereas MIX supplementation resulted in a decrease in the relative
 5170 abundance of Bacteroidetes during the first 16 h; the relative abundance of the
 5171 phylum subsequently increased to an average of 45% (24 h, $p_{adj}=0.04$, $q_{adj}=0.1$). The
 5172 Bacteroidaceae family were the main Bacteroidetes representatives (**Figure 23 B**).
 5173 The average relative abundance of the Bacteroidaceae increased from approximately
 5174 6% (16 h) to 46% (24 h) and from 6% (0 h) to 52% (24 h) after MIX and BB.MIX
 5175 supplementation respectively (**Figure 23 B**). Supplementation with BB had a
 5176 relatively lower scale impact on the Bacteroidaceae (**Figure 23 B**). *Bacteroides*
 5177 *fragilis* and *Bacteroides thetaiotaomicron* were the two most responsive taxa of the
 5178 Bacteroidaceae family across supplementation regimes (**Table 12**). After 24 h
 5179 BB.MIX supplementation *B. fragilis* relative abundance increased from 0.5% to
 5180 24%, ($p_{adj}=0.04$, $q_{adj}=0.01$) whereas after MIX supplementation the abundance was
 5181 approximately 38%, ($p_{adj}=0.05$ $q_{adj}=0.01$). The highest relative abundance increase
 5182 for *B. thetaiotaomicron* was observed after BB.MIX supplementation from
 5183 approximately 1% to 26% ($p_{adj}=0.04$, $q_{adj}=0.01$). The taxon *Bacteroides caccae* was
 5184 responsive to BB.MIX supplementation and its average relative abundance had a
 5185 significant increasing trend from 0.4% to 1% after 24 h fermentation, ($p_{adj}=0.04$,
 5186 $q_{adj}=0.1$).

5187 Firmicutes tended to decrease across the three supplementation regimes whereas
 5188 MIX supplementation sustained a relatively higher portion of the Firmicutes
 5189 abundance (**Figure 23 A**). The differences in the relative abundance of Firmicutes
 5190 taxa observed at baseline (**Table 12**) between the three supplementation conditions
 5191 can be attributed to some variation introduced during the preparation of the
 5192 consortium; gDNA extraction and sequencing bias may have amplified this variation

5193 resulting in increased relative representation of the phylum at baseline of the MIX
5194 supplementation regime.

5195 Lachnospiraceae relative abundance showed a decreasing trend across all
5196 supplementation regimes during the first 16 h of fermentations; the abundance of the
5197 Lachnospiraceae tended to increase thereafter (**Figure 23 B**). Twenty-four h
5198 BB.MIX supplementation retained a comparatively high Lachnospiraceae relative
5199 abundance (14%) compared to MIX (11%) and BB supplementation (6%). The
5200 relative abundance of Lachnospiraceae taxon *Clostridium symbosium* reached its
5201 highest abundance after BB supplementation followed by BB.MIX supplementation
5202 whereas *Coprococcus comes* retained the highest abundance after BB.MIX
5203 supplementation (approximately 8 %) (**Table 12**). Other dominant Firmicutes taxa
5204 responsive to blueberry supplementation belonged to the *Clostridium* cluster XI of
5205 the Peptostreptococcaceae family (**Table 12**). The Lactobacillales were more
5206 responsive to the MIX supplementation. The taxon *Enterococcus* was the main
5207 representative of the Lactobacillales and increased in relative abundance from <0.1%
5208 (0 h) to approximately 21% ($p_{adj}=0.05$, $q_{adj}=0.01$) during the first 16 h of MIX
5209 supplementation; a similar but to a lesser scale response was observed with BB and
5210 BB.MIX supplementation (**Table 12**).

5211 The Proteobacteria relative abundance increased over the first 16 h across all
5212 supplementation regimes and tended to decrease thereafter; the highest relative
5213 abundance was observed after BB supplementation (60% average relative abundance
5214 at 24 h; $p_{adj}=0.09$, $q_{adj}=0.08$) (**Figure 23 A**). Supplementation with prebiotic MIX or
5215 BB.MIX had a similar effect on the abundance of the main Proteobacteria taxa that

5216 belonged to the *Escherichia/Shigella* group, i.e., approximately 20% relative
 5217 abundance after 24 h fermentation (**Table 12**).

5218 **Table 11 MCC100 alpha diversity indices after 24 h fermentation with the**
 5219 **selected substrates.** BB: blueberry powder supplementation; BB.MIX: blueberry
 5220 powder and prebiotic carbohydrate mix supplementation; MIX: prebiotic
 5221 carbohydrate mix supplementation.

Mean alpha diversity value at 24 h with indicated substrate			
Alpha Diversity Indices	BB	BB.MIX	MIX
Shannon	2.0	2.9	2.7
Simpson	0.6	0.8	0.8
PD whole tree	3.3	4.4	3.7
Chao1	34.8	68.2	45.4
Observed species	32.0	43.0	35.0

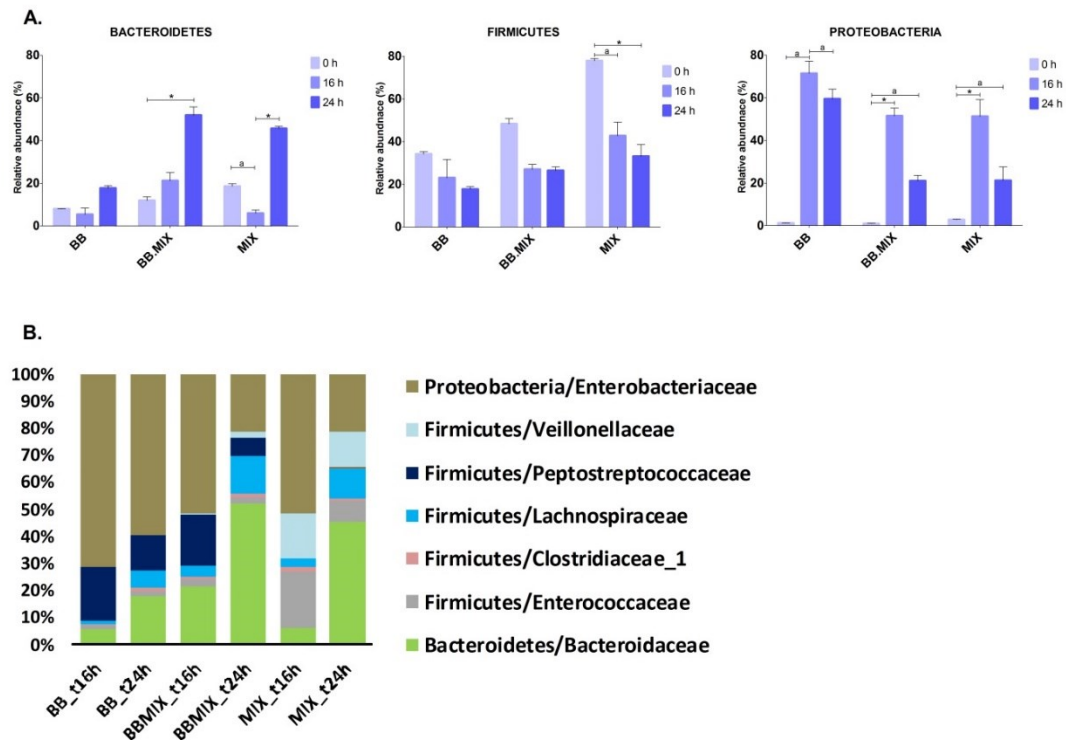
5222

5223 **Table 12 Development of the relative abundance at species level in the MCC100**
5224 **after 24 h fermentation and comparison by time point.** BB: blueberry powder
5225 supplementation; BB.MIX: blueberry powder and prebiotic carbohydrate mix
5226 supplementation; MIX: prebiotic carbohydrate mix supplementation. Missing values
5227 indicate that the relevant taxon had <1% abundance in all vessels (two or three)
5228 summed for mean abundance.

OTU Classification	MIX			BB. MIX			BB		
	t0	t16	t24	t0	t16	t24	t0	t16	t24
Bacteroides/Bacteroides caccae	-	-	-	0.4	0.1	1.0*	-	-	-
Bacteroides/Bacteroides dorei	4.1	0.01*	0.1	-	-	-	-	-	-
Bacteroides/Bacteroides fragilis	1.0	5.9	37.8*	0.5	14.7	24.3*	0.5	0.9	2.1
Bacteroides/Bacteroides thetaiotaomicron	1.81	0.1	7.5*	1.0	6.4	26.3*	1.0	4.5	15.4
Odoribacter/Odoribacter splanchnicus	-	-	-	2.9	0.004 ^a	0.01	-	-	-
Enterococcus/unclassified	0.1	20.8*	7.6	0.01	2.8	2.2	0.02	1.7	1.9
Clostridium sensu stricto/ Clostridium baratii	0.03	1.9	1.0	0.04	1.2	1.3	0.01	0.1	1.3 ^a
Clostridium XIVa/ Clostridium hathewayi	0.3	0.2	2.1	-	-	-	-	-	-
Anaerostipes/Anaerostipes hadrus	-	-	-	-	-	-	6.5	0.02	0.2
Clostridium XIVa/Clostridium symbiosum	0.1	1.0	2.4*	0.04	0.8	3.4*	0.04	1.5	5.0 ^a
Clostridium XIVa/unclassified	0.1	0.03	0.6*	-	-	-	-	-	-
Coprococcus/Coprococcus comes	25.5	0.5*	1.2 ^a	13.5	3.0*	7.8 ^a	9.6	0.1	0.1
Roseburia/Eubacterium rectale	-	-	-	2.7	0.02	0.1	1.9	0.0	0.01
Lachnospiraceae/ unclassified	5.3	1.6	4.9	3.1	0.1*	0.7	2.6	0.02*	0.1
Clostridium XI/unclassified	-	-	-	0.01	18.2*	6.2	0.0	16.8	11.2
Veillonella/Veillonella dispar	0.03	16.0	12.7	0.01	0.1	2.2	-	-	-
Escherichia/Shigella/ unclassified	2.6	51.3*	21.2	0.8	51.5*	21.0	1.1	71.4 ^a	59.5

*Statistically significant result, Kruskal-Wallis test result $p \leq 0.05$, $p_{adj} \leq 0.05$ with Dunn's test $q_{adj} \leq 0.05$.

^a Statistical trend, Kruskal-Wallis test result $p \leq 0.05$, $p_{adj} \leq 0.1$, with Dunn's test $q_{adj} \leq 0.1$.



5229

5230 **Figure 23 Development of the MCC100 composition after 24 h fermentation.**

5231 The relative abundance of the dominant phyla Bacteroidetes, Firmicutes and
 5232 Proteobacteria (A) and the relevant families (B) is shown for time points 0 h, 16 h
 5233 and 24 h. BB: blueberry powder supplementation; BB.MIX: blueberry powder and
 5234 prebiotic carbohydrate mix supplementation; MIX: prebiotic carbohydrate mix
 5235 supplementation. Only families present with $\geq 1\%$ relative abundance are shown. “*”
 5236 statistically significant: Kruskal-Wallis test result $p \leq 0.05$, $p_{adj} \leq 0.05$, Dunn’s test
 5237 $q_{adj} \leq 0.05$. “a” statistical trend: Kruskal-Wallis test result $p \leq 0.05$, $p_{adj} \leq 0.1$, Dunn’s
 5238 test $q_{adj} \leq 0.1$.

5239 **Blueberry supplemented prebiotic medium resulted in increased short chain**
 5240 **fatty acid production across the four faecal microbiotas and MCC100.**

5241 The production levels of the SCFAs acetate, propionate and butyrate levels in the
 5242 community type faecal microbiotas (EM278, EM425) and the longstay type

5243 (EM297, EM704) after 24 h fermentation are shown in **Figure 25 A and B**
5244 respectively. Combined supplementation of fermentation medium with blueberry
5245 powder and prebiotic carbohydrates (BB.MIX) resulted in increased SCFA
5246 production across all donors faecal microbiota irrespective of community or longstay
5247 type and in comparison to prebiotic (MIX) or blueberry (BB) supplementation alone.
5248 The effect of substrate supplementation on combined SCFA levels in the aggregated
5249 across the four donors faecal microbiota is shown in **Figure 25 C**. BB.MIX
5250 supplementation resulted in significantly higher SCFA levels (mean 210 μmol mean
5251 SCFA per g of faecal pellet) compared to BB (mean 124 μmol per g of faecal pellet,
5252 $p<0.005$) and MIX (mean 150 μmol per g of faecal pellet, $p<0.0005$). Similarly, in
5253 the MCC100 microbiota BB.MIX supplementation resulted in higher SCFA levels
5254 after 24 h fermentation compared to the other supplementation regimes (**Figure 25**
5255 **D**). Acetate and butyrate production were significantly higher after BB.MIX
5256 supplementation (mean 66 μmol per g of bacterial pellet, $p<0.05$ and 14 μmol per g
5257 of bacterial pellet, $p<0.05$ respectively) compared to MIX supplementation (5.5 and
5258 7 μmol per g of bacterial pellet respectively).

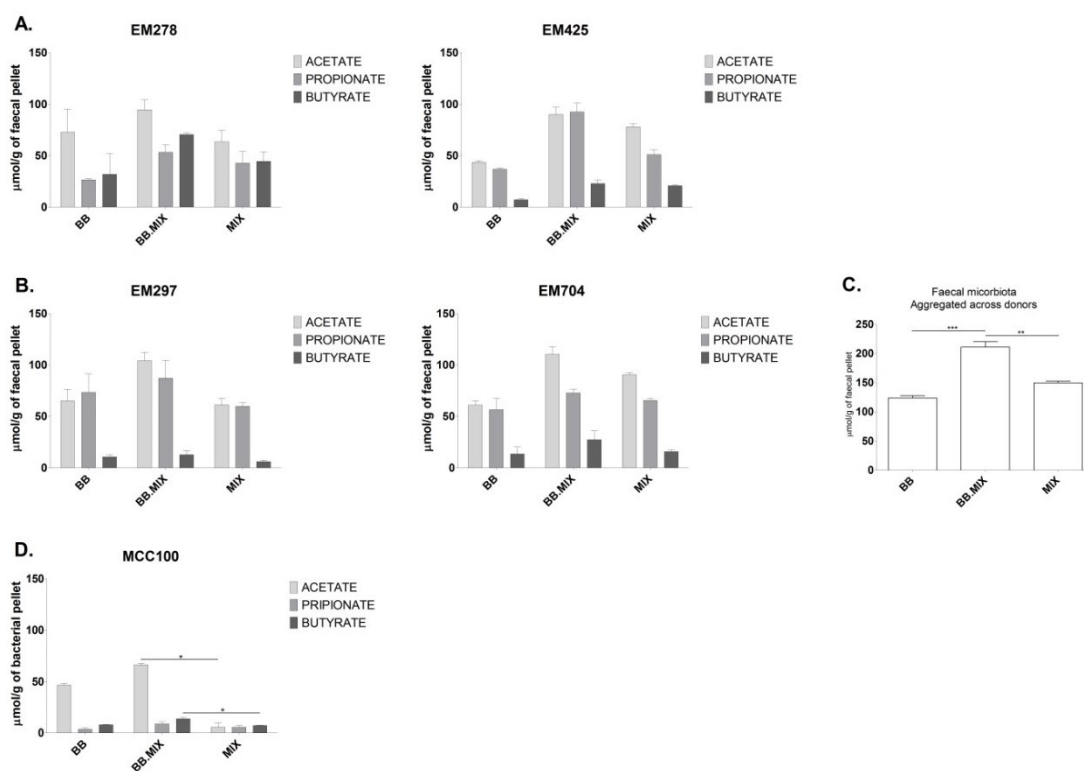


Figure 24 Short chain fatty acid (SCFA) production by the faecal microbiota and artificial consortium MCC100 after 24 h fermentation with selected substrates. The concentration (μmol/g of faecal pellet) of acetate, propionate and butyrate in the fermentation fluid from the COM type (EM278 and EM425) and LS type (EM297, EM704) faecal microbiotas and consortium MCC100 is shown in **A**, **B** and **D** respectively. The combined SCFA production in the aggregated (i.e. across all donors faecal microbiotas) is shown in panel **C**. Kruskal-Wallis test with Dunn's post hoc test was applied to compare the SCFA levels at 24 h. *** $p \leq 0.0005$, ** $p \leq 0.005$, * $p \leq 0.05$. BB: blueberry powder supplementation; BB.MIX: blueberry powder and prebiotic supplementation; MIX: prebiotic carbohydrate mix supplementation.

5271 4.5 Discussion

5272 Baseline-dependent prebiotic effect of whole blueberry extract on the gut 5273 microbiota of older donors

5274 In this study we explored the modulatory effect of the rich in polyphenols and
5275 dietary fibre whole blueberry powder *in vitro* on the faecal microbiota of older
5276 subjects. Blueberry powder was used either as the sole fermentation substrate or in
5277 combination with prebiotics. By testing the supplementation regimes and analysing
5278 its effect on individual faecal microbiotas we observed the differential prebiotic
5279 potential of blueberries that was broadly determined by the baseline microbiota
5280 composition. Differential responsiveness to prebiotic treatment due to inter-
5281 individual compositional differences in the gut microbiota has been repeatedly
5282 reported calling for personalized strategies towards restoring the dysbiotic gut
5283 microbiota and retaining health (Duncan and Flint, 2013). Notwithstanding, due to
5284 the increasing understanding of the ecological forces that assemble the gut
5285 microbiota communities and the role of certain health-related taxa in disease,
5286 prebiotics remain a promising generic therapeutic approach (Scott *et al.*, 2015;
5287 Bashan *et al.*, 2016).

5288 The presence of a dominant *Bifidobacterium* and *Bacteroides* population responsive
5289 to blueberry supplementation differentiated the EM278 community type microbiota
5290 from the other community type and the longstay type microbiotas. In the case of the
5291 community type EM278 bifidobacteria and *F. prausnitzii* relative abundance
5292 increased when blueberries were the sole fermentation substrate whereas
5293 bifidobacteria and *Bacteroides* increased after co-supplementation with blueberries
5294 and prebiotics suggesting extensive cross-feeding between bifidobacteria and either

5295 butyrate-producers like *F. prausnitzii* or *Bacteroides* taxa. Cross-feeding between
 5296 butyrate-producing bacteria like *F. prausnitzii* and acetate-producing bifidobacteria
 5297 have been extensively described before (Moens *et al.*, 2016). Strain-dependent cross-
 5298 feeding phenomena between *Bifidobacterium* and *Bacteroides* taxa have also been
 5299 described (Rios-Covian *et al.*, 2013). *In vitro* responsiveness of bifidobacteria
 5300 predominantly and to a lesser extent of *F. prausnitzii* to polyphenols-rich extracts
 5301 from fruits like blueberries, pomegranate and apple, has been reported before (Molan
 5302 *et al.*, 2009; Balionska *et al.*, 2010; Guergoletto *et al.*, 2016; Koutsos *et al.*, 2017).
 5303 There are well-documented health-claims for *bifidobacteria*, *F. prausnitzii* and
 5304 *Bacteroides* taxa. Decreased relative abundance of *F. prausnitzii* population
 5305 associates to IBD whereas bifidobacteria abundance decreases with ageing (Miquel
 5306 *et al.*, 2013; Arbolea *et al.*, 2016). Various *Bacteroides* are next generation
 5307 probiotic candidates (El Hage *et al.*, 2017). Interestingly, although the combined
 5308 supplementation favoured other dominant bacterial groups and inhibited *F.*
 5309 *prausnitzii* from accessing the blueberries substrate, the resulting SCFA production
 5310 was the highest among supplementation regimes, including the highest production of
 5311 butyrate indicating that although *F. prausnitzii* population was not sustained other
 5312 butyrate producing Lachnospiraceae like *A. hadrus* may have benefitted from cross-
 5313 feeding phenomena with bifidobacteria as reported before (Belenguer *et al.*, 2006).

 5314 Increased SCFA production was observed across all faecal microbiotas and MC100
 5315 after co-supplementation compared to prebiotics supplementation alone. Although
 5316 acetate and propionate were the major SCFAs, butyrate production profile was
 5317 promoted by the co-supplementation. In animal trials diet combining strawberry
 5318 polyphenols-rich extracts with fructooligosaccharides (FOS) resulted in increased
 5319 caecal SCFA production especially butyrate suggesting a synergistic beneficial effect

5320 of the two substrates on the GIT health (Singh *et al.*, 2017). Others have reported
5321 beneficial metabolic effects of similar co-supplementation in murine models but not
5322 increased SCFA production (Fotschki *et al.*, 2016). The SCFA propionate and
5323 butyrate are strongly associated to host health produced mostly by Bacteroidetes and
5324 certain Clostridiales respectively, whereas acetate is an abundant SCFA by-product
5325 of the colonic bacteria fermentation activity (Louis and Flint, 2017).

5326 Co-supplementation supported a greater production of SCFA which was
5327 accompanied by a concomitant increase in alpha diversity indices only in MCC100.
5328 Due to its wide repertoire of carbohydrate utilization (Flint *et al.*, 2012) *Bacteroides*
5329 were among the taxa that increased in abundance after co-supplementation across the
5330 faecal microbiotas and especially in MCC100. The *Clostridium* cluster XIVa taxa
5331 *Clostridium symbiosum* and *Coprococcus comes* were identified as highly responsive
5332 to co-supplementation in MCC100. Members of the *Clostridium* cluster XIVa are
5333 major butyrate producers and potential candidates for probiotic consortium design
5334 (El Hage *et al.*, 2017). The synthetic community MCC100 has allowed monitoring
5335 the responsive behaviour of selected dominant faecal bacteria to supplementation
5336 potentially through what Clavel *et al.* (2017) has described as “functional enrichment
5337 via community reduction”.

5338 Importantly, Lactobacillales represented here by *Streptococcus* taxa were highly
5339 responsive to blueberry supplementation whereas *Enterococcus* taxa demonstrated
5340 lower responsiveness. Others have reported *in vitro* responsiveness of
5341 Lactobacillales (measured with *Lactobacillus/Enterococcus* FISH and qPCR probes)
5342 to supplementation with various polyphenols rich extracts (Balionska *et al.*, 2010;
5343 Cueva *et al.*, 2013; Zhang *et al.*, 2016; Gil-Sanchez *et al.*, 2017). Importantly, Lee *et*

5344 *al.* (2018) reported that in mice fed high-fat diet blueberry powder administration
5345 resulted in amelioration of the GIT physiology and inflammation that correlated to
5346 an increase in Lactobacillales and Proteobacteria abundance. The Lactobacillales is a
5347 prominent member of the human gut microbiota; the order harbours probiotic strains
5348 but also potential pathobionts (Rajilic-Stojanovic and de Vos, 2014). Enterococci
5349 and streptococci have been identified to be involved in polyphenols metabolism
5350 (Rowland *et al.*, 2018). Streptococci may have readily responded to both the
5351 blueberry fruit sugars and the polyphenol content.

5352 **Effect of whole blueberry extract on the abundance of Enterobacteriaceae**

5353 We observed a relatively high increase in the Gammaproteobacteria with the
5354 blueberry supplementation. It has been reported before that Enterobacteriaceae may
5355 play a role in polyphenols metabolism (Couteau *et al.*, 2001) and increase in
5356 Enterobacteriaceae population with a concomitant increase in *Bacteroides* was
5357 observed due to tannin-rich diets in murine models (Smith and Mackie, 2004).
5358 Recently, Lee *et al.* (2018) reported that in the faecal microbiota of rats fed high-fat
5359 diet supplemented with whole blueberry extract the abundance of
5360 Gammaproteobacteria, mainly Pasteurellaceae, Fusobacteriaceae and
5361 Porphyromonadaceae was increased compared to feeding with high-fat diet or low-
5362 fat diet. Proliferation of Proteobacteria and Fusobacteria in the gut microbiota is
5363 associated with dysbiosis (Gevers *et al.*, 2014; Eklof *et al.*, 2017; Litvak *et al.*,
5364 2017). In the Lee *et al.* (2018) study the seemingly dysbiotic modulation of the
5365 murine faecal microbiota after blueberry supplementation was accompanied with
5366 improved GIT function and lowered inflammation markers. Increase in
5367 Proteobacteria abundance was observed in the faecal microbiota of rats after Roux-

5368 en Y gastric bypasses and was correlated to postoperative weight loss (Shao *et al.*,
5369 2017).

5370 **4.6 Conclusion**

5371 Importantly, dietary polyphenolic compounds may be bound to dietary fibre
5372 impacting the bioavailability and the metabolism of the substrates by the colonic
5373 microbiota (Edwards *et al.*, 2017). Dietary fibre in polyphenols rich substrates may
5374 better support the growth of various taxa such as *Bacteroides* taxa as observed in *in*
5375 *vitro* and in human trials (Eid *et al.*, 2014; Henning *et al.*, 2017) and is more relevant
5376 to human diet. Here, we reported that rich in polyphenols and dietary fibre whole
5377 blueberry supplementation can affect certain health-relevant taxa and drive an
5378 increased SCFA production in the faecal microbiota of elderly. Blueberries
5379 potentially promote elderly health by contributing fibre to the fibrolytic colonic
5380 bacterial community thus, increasing the SCFA production that is known to decline
5381 with ageing (Duncan and Flint, 2013) and also by contributing phenolic metabolites.
5382 The lack of compartmentalisation and the reduction in diversity of the faecal
5383 microbiota under *in vitro* conditions significantly impact on microbiota
5384 responsiveness to supplementation tests (Guerra *et al.*, 2012). The awaited phenolic
5385 compound analysis will reveal bioconversion of these compounds by the microbiota
5386 during the 24 h fermentation. Follow-up *in vitro* trials with isolated phenolic
5387 compounds will elucidate the role of blueberry extracts on gut microbiota
5388 modulation. *In vivo* trials will leverage the *in vitro* bias and further elucidate the
5389 microbiota modulatory potential of whole blueberries allowing for safe dietary
5390 recommendations for the elderly.

5391 **4.7 Funding Sources**

5392 This work was financially supported by the Government of Ireland National
5393 Development Plan by way of a Department of Agriculture, Food and the Marine
5394 (DAFM) under a Food Institutional Research Measure (FIRM) award (11/F/053) for
5395 the ELDERFOOD project.

5396 **4.8 Acknowledgements**

5397 We thank U.S. Highbush Blueberry Council, California for providing the blueberry
5398 powder. We thank Dr Celine Ribiere for her help in the amplicon sequence analysis.

5399 **4.9 References**

- 5400 Allard, G., Ryan, F. J., Jeffery, I. B., Claesson, M. J. (2015). SPINGO: A rapid
5401 species-classifier for microbial amplicon sequences. *BMC Bioinformatics*, 16(1),
5402 1–8.
- 5403 Arboleya, S., Watkins, C., Stanton, C., Ross, R. P. (2016). Gut bifidobacteria
5404 populations in human health and aging. *Front Microbiol*, 7, 1204.
- 5405 Bialonska, D., Ramnani, P., Kasimsetty, S. G., Muntha, K. R., Gibson, G. R.,
5406 Ferreira, D. (2010). The influence of pomegranate by-product and punicalagins
5407 on selected groups of human intestinal microbiota. *Int J Food Microbiol*, 140(2–
5408 3), 175–182.
- 5409 Bashan, A., Gibson, T. E., Friedman, J., Carey, V. J., Weiss, S. T., Hohmann, E.
5410 L., Liu, Y. Y. (2016). Universality of human microbial dynamics. *Nature*,
5411 534(7606), 259–262.
- 5412 Beking, K., and Vieira, A. (2011). An assessment of dietary flavonoid intake in
5413 the UK and Ireland. *Int J Food Sci Nutr*, 62(1), 17–19.
- 5414 Belenguer, A., Duncan, S. H., Calder, A. G., Holtrop, G., Louis, P., Lobley, G.
5415 E., Flint, H. J. (2006). Two routes of metabolic cross-feeding between
5416 *Bifidobacterium adolescentis* and butyrate-producing anaerobes from the human
5417 gut. *Appl Environ Microbiol*, 72(5), 3593-9.
- 5418 Caporaso, J. G., Bittinger, K., Bushman, F. D., Desantis, T. Z., Andersen, G. L.,
5419 Knight, R. (2010). PyNAST: A flexible tool for aligning sequences to a template
5420 alignment. *Bioinformatics*, 26(2), 266–267.

- 5421 Chun, O. K., Chung, S. J., Song, W. O. (2007). Estimated dietary flavonoid
5422 intake and major food sources of U.S. adults. *J Nutr*, 137(5), 1244–1252.
- 5423 Chun, O. K., Lee, S. G., Wang, Y., Vance, T., Song, W. O. (2012). Estimated
5424 flavonoid intake of the elderly in the United States and around the world. *J Nutr*
5425 *Gerontol Geriatr*, 31(3), 190–205.
- 5426 Clavel, T., Lagkouvardos, I., Stecher, B. (2017). From complex gut communities
5427 to minimal microbiomes via cultivation. *Curr Opin Microbiol*, 38, 148–155.
- 5428 Collins, B., Hoffman, J., Martinez, K., Grace, M., Lila, M. A., Cockrell, C.,
5429 Nadimpalli, A., Chang, E., Chuang, C. C., *et al.* (2016). A polyphenol-rich
5430 fraction obtained from table grapes decreases adiposity, insulin resistance and
5431 markers of inflammation and impacts gut microbiota in high-fat-fed mice. *J Nutr*
5432 *Biochem*, 31, 150–165.
- 5433 Couteau, D., McCartney, A. L., Gibson, G. R., Williamson, G. (2001). Isolation
5434 and characterization of human colonic bacteria able to hydrolyse chlorogenic
5435 acid. *J Appl Microbiol*, 90(6), 873–881.
- 5436 Cueva, C., Sánchez-Patán, F., Monagas, M., Walton, G. E., Gibson, G. R.,
5437 Martín-Álvarez, P. J., Bartolome, B., Moreno-Arribas, M. V. (2013). *In vitro*
5438 fermentation of grape seed flavan-3-ol fractions by human faecal microbiota:
5439 Changes in microbial groups and phenolic metabolites. *FEMS Microbiol Ecol*,
5440 83(3), 792–805.
- 5441 Del Bò, C., Ciappellano, S., Klimis-Zacas, D., Daniela, M., Claudio, G., Patrizia,
5442 R., Marisa, P. (2010). Anthocyanin absorption, metabolism, and distribution

5443 from a wild blueberry-enriched diet (*Vaccinium angustifolium*) is affected by diet
5444 duration in the sprague-dawley rat. *J Agric Food Chem*, 58(4), 2491–2497.

5445 Donaldson, G. P., Lee, S. M., Mazmanian, S. K. (2015). Gut biogeography of the
5446 bacterial microbiota. *Nat Rev Microbiol*, 14(1), 20–32.

5447 Duncan, S. H., and Flint, H. J. (2013). Probiotics and prebiotics and health in
5448 ageing populations. *Maturitas*, 75(1), 44–50.

5449 Eid, N., Enani, S., Walton, G., Corona, G., Costabile, A., Gibson, G., Rowland,
5450 I., Spencer, J. P. E. (2014). The impact of date palm fruits and their component
5451 polyphenols, on gut microbial ecology, bacterial metabolites and colon cancer
5452 cell proliferation. *J Nutr Sci*, 3(22), e46.

5453 Edwards, C. A., Havlik, J., Cong, W., Mullen, W., Preston, T., Morrison, D. J.,
5454 Combet, E. (2017). Polyphenols and health: Interactions between fibre, plant
5455 polyphenols and the gut microbiota. *Nutr Bull*, 42(4), 356–360.

5456 Eklöf, V., Löfgren-Burström, A., Zingmark, C., Edin, S., Larsson, P., Karling, P.,
5457 Alexeyev, O., Rutegard, J., Wikberg, M. L., Palmqvist, R. (2017). Cancer-
5458 associated fecal microbial markers in colorectal cancer detection. *Int J of Cancer*,
5459 141(12), 2528–2536.

5460 El Hage, R., Hernandez-Sanabria, E., Van de Wiele, T. (2017). Emerging trends
5461 in “smart probiotics”: Functional consideration for the development of novel
5462 health and industrial applications. *Front Microbiol*, 8, 1889.

5463 Flint, H. J., Scott, K. P., Duncan, S. H., Louis, P., Forano, E. (2012). Microbial
5464 degradation of complex carbohydrates in the gut. *Gut Microbes*, 3(4), 289–306.

- 5465 Fotschki, B., Juśkiewicz, J., Jurgoński, A., Kołodziejczyk, K., Milala, J.,
5466 Kosmala, M., Zduńczyk, Z. (2016). Anthocyanins in strawberry polyphenolic
5467 extract enhance the beneficial effects of diets with fructooligosaccharides in the
5468 rat cecal environment. *PLoS One*, *11*(2), e0149081.
- 5469 Gevers, D., Kugathasan, S., Denson, L. A., Vázquez-Baeza, Y., Van Treuren,
5470 W., Ren, B., Schwager, E., Knights, D., Song, S. J., *et al.* (2014). The treatment-
5471 naive microbiome in new-onset Crohn's disease. *Cell Host Microbe*, *15*(3), 382–
5472 392.
- 5473 Gil-Sánchez, I., Ayuda-Durán, B., González-Manzano, S., Santos-Buelga, C.,
5474 Cueva, C., Martín-Cabrejas, M. A., Sanz-Buenhombre, M., *et al.* (2017).
5475 Chemical characterization and in vitro colonic fermentation of grape pomace
5476 extracts. *J Sci Food Agric*, *97*(10), 3433–3444.
- 5477 Guerra, A., Etienne-Mesmin, L., Livrelli, V., Denis, S., Blanquet-Diot, S., Alric,
5478 M. (2012). Relevance and challenges in modeling human gastric and small
5479 intestinal digestion. *Trends Biotechnol*, *30*, 591–600.
- 5480 Guergoletto, K. B., Costabile, A., Flores, G., Garcia, S., Gibson, G. R. (2016). *In*
5481 *vitro* fermentation of jucara pulp (*Euterpe edulis*) by human colonic microbiota.
5482 *Food Chem*, *196*, 251–258.
- 5483 Henning, S. M., Yang, J., Shao, P., Lee, R. P., Huang, J., Ly, A., Ly, A., Hsu,
5484 M., Lu, Q. Y., Thames, G., Heber, D., Li, Z. (2017). Health benefit of
5485 vegetable/fruit juice-based diet: Role of microbiome. *Sci Rep*, *7*(1), 2167.
- 5486 Hidalgo, M., Oruna-Concha, M. J., Kolida, S., Walton, G. E., Kallithraka, S.,
5487 Spencer, J. P. E., de Pascual-Teresa, S. (2012). Metabolism of anthocyanins by

5488 human gut microflora and their influence on gut bacterial growth. *J Agric Food*
5489 *Chem*, 60(15), 3882–3890.

5490 Ivey, K. L., Jensen, M. K., Hodgson, J. M., Eliassen, A. H., Cassidy, A., Rimm,
5491 E. B. (2017). Association of flavonoid-rich foods and flavonoids with risk of all-
5492 cause mortality. *Br J Nutr*, 117(10), 1470–1477.

5493 Jeffery, I. B., Lynch, D. B., O'Toole, P. W. (2015). Composition and temporal
5494 stability of the gut microbiota in older persons. *ISME J*, 10(1), 1002-14.

5495 Jeyabalan, J., Aqil, F., Munagala, R., Gupta, R. (2013). Chemopreventive and
5496 therapeutic activity of high anthocyanin-content blueberry against estrogen-
5497 mediated breast cancer. *Cancer Res*, 73(8 Suppl).

5498 Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M.,
5499 Glöckner, F. O. (2012). Evaluation of general 16S ribosomal RNA gene PCR
5500 primers for classical and next-generation sequencing-based diversity studies.
5501 *Nucleic Acids Res*, 41(1), e1.

5502 Koutsos, A., Lima, M., Conterno, L., Gasperotti, M., Bianchi, M., Fava, F.,
5503 Vrhovsek, U., Lovegrove, J. A., Tuohy, K. M. (2017). Effects of commercial
5504 apple varieties on human gut microbiota composition and metabolic output using
5505 an in vitro colonic model. *Nutrients*, 9(6), piiE533.

5506 Lacombe, A., Li, R. W., Klimis-Zacas, D., Kristo, A. S., Tadepalli, S., Krauss,
5507 E., Young, R., Wu, V. C. H. (2013). Lowbush wild blueberries have the potential
5508 to modify gut microbiota and xenobiotic metabolism in the rat colon. *PLoS One*,
5509 8(6), e67497.

- 5510 Lee, S., Keirsey, K. I., Kirkland, R., Grunewald, Z. I., Fischer, J. G., de La Serre,
5511 C. B. (2018). Blueberry supplementation influences the gut microbiota,
5512 inflammation, and insulin resistance in high-fat-diet-fed rats. *J Nutr*, 148(2),
5513 209–219.
- 5514 Li, D., Wang, P., Luo, Y., Zhao, M., Chen, F. (2017). Health benefits of
5515 anthocyanins and molecular mechanisms: Update from recent decade. *Crit Rev*
5516 *Food Sci Nutr*, 57(8), 1729–1741.
- 5517 Litvak, Y., Byndloss, M. X., Tsois, R. M., Bäumler, A. J. (2017). Dysbiotic
5518 Proteobacteria expansion: a microbial signature of epithelial dysfunction. *Curr*
5519 *Opin Microbiol*, 39, 1–6.
- 5520 Louis, P., and Flint, H. J. (2017). Formation of propionate and butyrate by the
5521 human colonic microbiota. *Environ Microbiol*, 19(1), 29–41.
- 5522 Magoč, T.; Salzberg, S. L. (2011). FLASH: Fast length adjustment of short reads
5523 to improve genome assemblies. *Bioinformatics*. 27, 2957–2963.
- 5524 Marchesi, J. R., Adams, D. H., Fava, F., Hermes, G. D. A., Hirschfield, G. M.,
5525 Hold, G., Quraishi, M. N., Kinross, J., Smidt, H., *et al.* (2015). The gut
5526 microbiota and host health: a new clinical frontier. *Gut*, 65(2), 330-9.
- 5527 Messer, J. S., Liechty, E. R., Vogel, O. A., Chang, E. B. (2017). Evolutionary
5528 and ecological forces that shape the bacterial communities of the human gut.
5529 *Mucosal Immunol*, 10(3), 567–579.

- 5530 Miquel, S., Martín, R., Rossi, O., Bermúdez-Humarán, L. G., Chatel, J. M.,
5531 Sokol, H., Thomas, M., Wells, J. M., Langella, P. (2013). *Faecalibacterium*
5532 *prausnitzii* and human intestinal health. *Curr Opin Microbiol*, 16(3), 255–261.
- 5533 Moens, F., Weckx, S., and De Vuyst, L. (2016). Bifidobacterial inulin-type
5534 fructan degradation capacity determines cross-feeding interactions between
5535 bifidobacteria and *Faecalibacterium prausnitzii*. *Intl J Food Microbiol*, 231, 76–
5536 85.
- 5537 Molan, A. L., Lila, M. A., Mawson, J., De, S. (2009). *In vitro* and *in vivo*
5538 evaluation of the prebiotic activity of water-soluble blueberry extracts. *World J*
5539 *Microbiol Biotechnol*, 25(7), 1243–1249.
- 5540 Ntemiri, A., Ni Chonchuir, F., O’Callaghan, T. F., Stanton, C., Ross, R. P.,
5541 O’Toole, P. W. (2017). Glycomacropeptide sustains microbiota diversity and
5542 promotes specific taxa in an artificial colon model of elderly gut microbiota. *J*
5543 *Agric Food Chem*, 65(8), 1836–1846.
- 5544 Ogawa, K., Sakakibara, H., Iwata, R., Ishii, T., Sato, T., Goda, T., Shimol, K.,
5545 Kumazawa, S. (2008). Anthocyanin composition and antioxidant activity of the
5546 crowberry (*Empetrum nigrum*) and other berries. *J Agric Food Chem*, 56(12),
5547 4457–4462.
- 5548 Panche, A. N., Diwan, A. D., Chandra, S. R. (2016). Flavonoids: an overview. *J*
5549 *Nutr Sci*, 5, e47.
- 5550 Perez, M., Ntemiri, A., Tan, H. and O’Toole, P. (in preparation). Developing an
5551 artificial bacterial consortium for modulating the microbiota of frail older people.

5552 Petersen, C., and Round, J. L. (2014). Defining dysbiosis and its influence on
5553 host immunity and disease. *Cell Microbiol*, 16(7), 1024–1033.

5554 Petrof, E. O., Gloor, G. B., Vanner, S. J., Weese, S. J., Carter, D., Daigneault, M.
5555 C., Brown, E. M., Schroeter, K., Allen-Vercoe, E. (2013). Stool substitute
5556 transplant therapy for the eradication of *Clostridium difficile* infection:
5557 “RePOOPulating” the gut. *Microbiome*, 1(1), 3.

5558 Pojer, E., Mattivi, F., Johnson, D., Stockley, C. S. (2013). The case for
5559 anthocyanin consumption to promote human health: A review. *Comp Rev Food*
5560 *Sci Food Safety*, 12(5), 483–508.

5561 Rajilić-Stojanović, M., and de Vos, W. M. (2014). The first 1000 cultured
5562 species of the human gastrointestinal microbiota. *FEMS Microbiol Rev*, 38, 996–
5563 1047.

5564 R Core Team. R: A language and environment for statistical computing. Vienna,
5565 Austria: R Foundation for Statistical Computing. **2014** <http://www.R-project.org/>

5566 Rios-Covian, D., Arboleya, S., Hernandez-Barranco, A. M., Alvarez-Buylla, J.
5567 R., Ruas-Madiedo, P., Gueimonde, M., De Los Reyes-Gavilan, C. G. (2013).
5568 Interactions between *Bifidobacterium* and *Bacteroides* species in cofermentations
5569 are affected by carbon sources, including exopolysaccharides produced by
5570 bifidobacteria. *Appl Environ Microbiol*, 79(23), 7518–7524.

5571 Routray, W., and Orsat, V. (2011). Blueberries and their anthocyanins: Factors
5572 affecting biosynthesis and properties. *Comp Rev Food Sci Food Safety*, 10(6),
5573 303–320.

5574 Rowland, I., Gibson, G., Heinken, A., Scott, K., Swann, J., Thiele, I., Tuohy, K.
5575 (2018). Gut microbiota functions: metabolism of nutrients and other food
5576 components. *Eur J Nutr*, 57(1), 1-24.

5577 Salazar, N., Arbolea, S., Valdés, L., Stanton, C., Ross, P., Ruiz, L., Gueimonde,
5578 M., de los Reyes-Gavilán, C. G. (2014). The human intestinal microbiome at
5579 extreme ages of life. Dietary intervention as a way to counteract alterations.
5580 *Front Genet*, 5, 406.

5581 Sánchez-Patán, F., Barroso, E., van de Wiele, T., Jiménez-Girón, A., Martín-
5582 Alvarez, P. J., Moreno-Arribas, M. V., Martinez-Cuesta, M. C., Pelaez, C.,
5583 Requena, T., Bartolomé, B. (2015). Comparative *in vitro* fermentations of
5584 cranberry and grape seed polyphenols with colonic microbiota. *Food Chem*, 183,
5585 273–82.

5586 Sánchez-Patán, F., Cueva, C., Monagas, M., Walton, G. E., Gibson, G. R.,
5587 Quintanilla-López, J. E., Lebron-Aquilar, R., *et al.* (2012). *In vitro* fermentation
5588 of a red wine extract by human gut microbiota: Changes in microbial groups and
5589 formation of phenolic metabolites. *J Agric Food Chem*, 60(9), 2136–2147.

5590 Scott, K. P., Antoine, J. M., Midtvedt, T., van Hemert, S. (2015). Manipulating
5591 the gut microbiota to maintain health and treat disease. *Microb Ecol Health Dis*,
5592 26, 25877.

5593 Selma, M. V., Espín, J. C., Tomás-Barberán, F. A. (2009). Interaction between
5594 phenolics and gut microbiota: Role in human health. *J Agric Food Chem*, 57(15),
5595 6485–6501.

5596 Sender, R., Fuchs, S., and Milo, R. (2016). Revised estimates for the number of
5597 human and bacteria cells in the body. *PLoS Biol*, 14(8), e1002533.

5598 Shao, Y., Ding, R., Xu, B., Hua, R., Shen, Q., He, K., Yao, Q. (2017).
5599 Alterations of gut microbiota after Roux-en-Y gastric bypass and sleeve
5600 gastrectomy in Sprague-Dawley rats. *Obes Surg*, 27(2), 295–302.

5601 Singh, D. P., Singh, S., Bijalwan, V., Kumar, V., Khare, P., Baboota, R. K.,
5602 Singh, P., Boparai, R. K., *et al.* (2017). Co-supplementation of isomalto-
5603 oligosaccharides potentiates metabolic health benefits of polyphenol-rich
5604 cranberry extract in high fat diet-fed mice via enhanced gut butyrate production.
5605 *Eur J Nutr*, [Epub ahead of print].

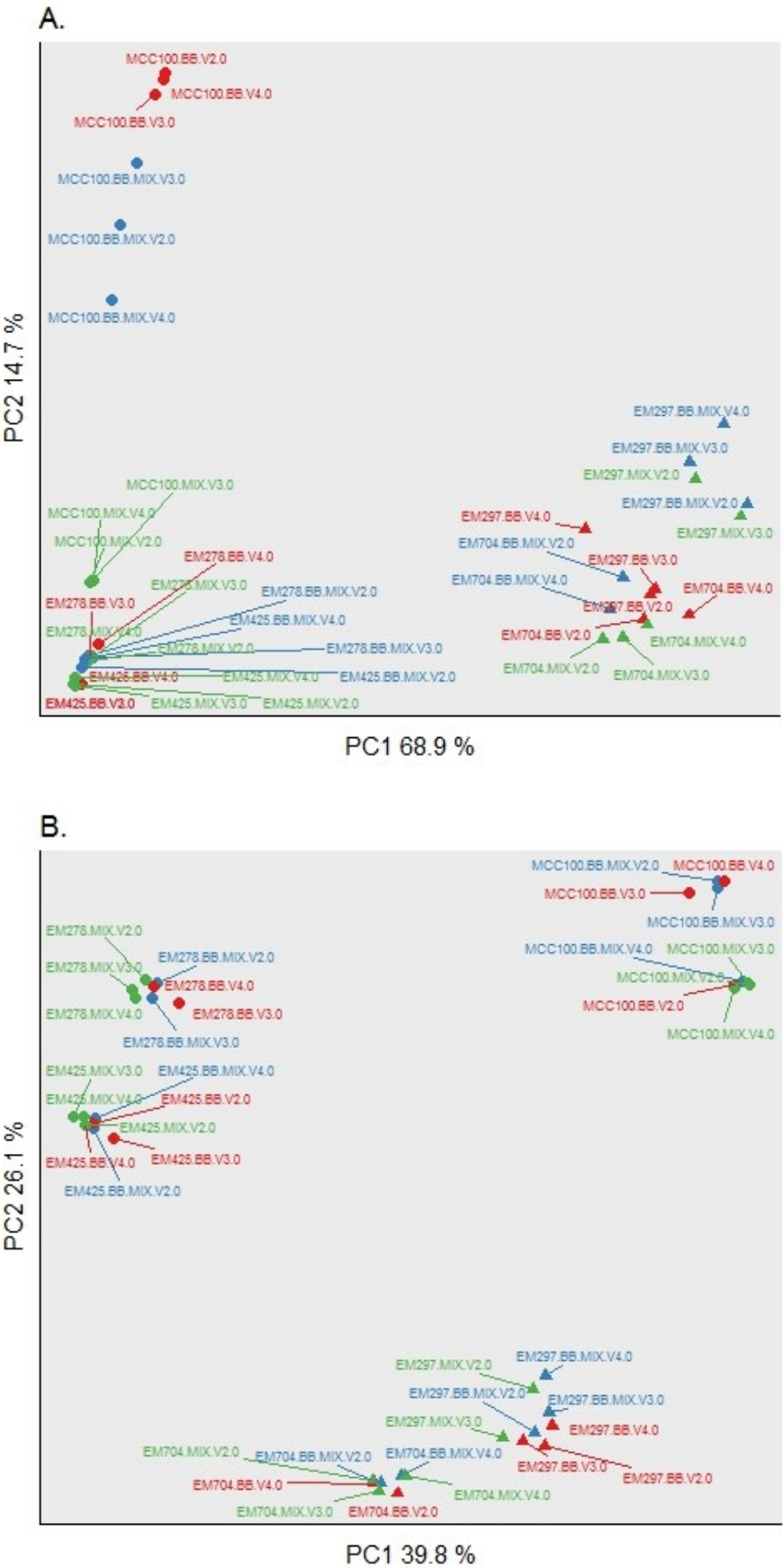
5606 Smith, A. H., and Mackie, R. I. (2004). Effect of condensed tannins on bacterial
5607 diversity and metabolic activity in the rat gastrointestinal tract. *Appl Environ*
5608 *Microbiol*, 70(2), 1104–1115.

5609 Sonnenburg, J. L., and Bäckhed, F. (2016). Diet–microbiota interactions as
5610 moderators of human metabolism. *Nature*, 535(7610), 56–64.

5611 Thevaranjan, N., Puchta, A., Schulz, C., Naidoo, A., Szamosi, J. C., Verschoor,
5612 C. P., Loukov, D., Schenck, L. P., *et al.* (2017). Age-associated microbial
5613 dysbiosis promotes intestinal permeability, systemic inflammation, and
5614 macrophage dysfunction. *Cell Host Microbe*, 21(4), 455–466.e4.

5615 Vogiatzoglou, A., Mulligan, A. A., Lentjes, M. A. H., Luben, R. N., Spencer, J.
5616 P. E., Schroeter, H., Khaw, K. T., Kuhnle, G. G. C. (2015). Flavonoid intake in
5617 European adults (18 to 64 Years). *PLoS One*, 10(5), e0128132.

- 5618 Wang, Q.; Garrity, G. M.; Tiedje, J. M.; Cole, J. R. (2007). Naive Bayesian
5619 classifier for rapid assignment of rRNA sequences into the new bacterial
5620 taxonomy. *Appl Environ Microbiol*, 73, 5261–5267
- 5621 Zhang, X., Yang, Y., Wu, Z., Weng, P. (2016). The modulatory effect of
5622 anthocyanins from purple sweet potato on human intestinal microbiota *in vitro*. *J*
5623 *Agric Food Chem*, 64(12), 2582–2590.

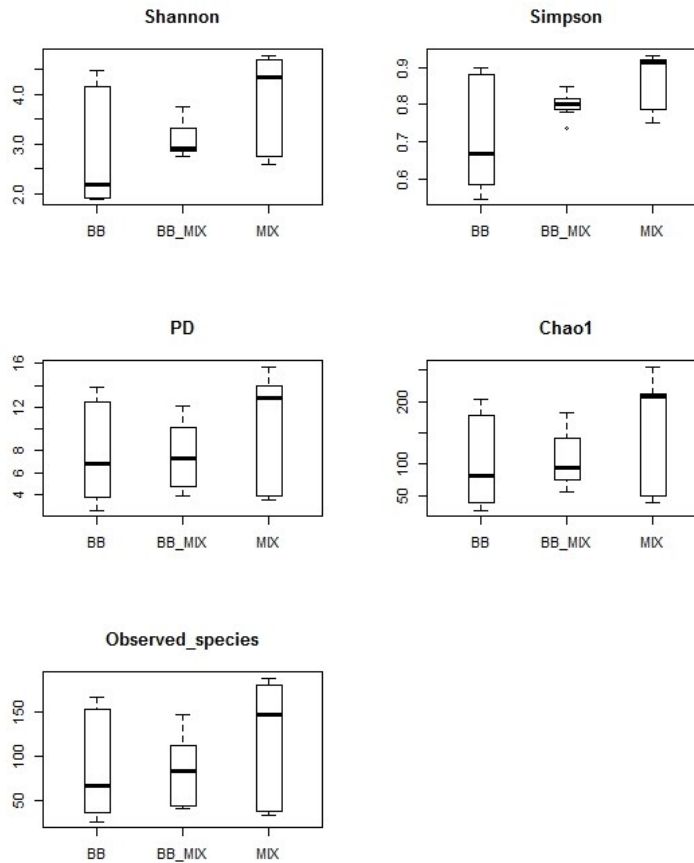


5626 **S Figure 5 Principal Coordinates Analysis (PCoA) of the faecal microbiota and**
5627 **MCC100 at baseline (t0 h). A:** Weighted UniFrac distances; **B:** Unweighted
5628 UniFrac distances. “●” Community (COM) type microbiota; “▲” Longstay (LS)
5629 type microbiota; **red:** BB supplementation; **blue:** BB.MIX supplementation; **green:**
5630 MIX supplementation.

5631 **S Table 10 Alpha diversity indices for the faecal microbiotas and MCC100 at**
5632 **baseline (0 h).**

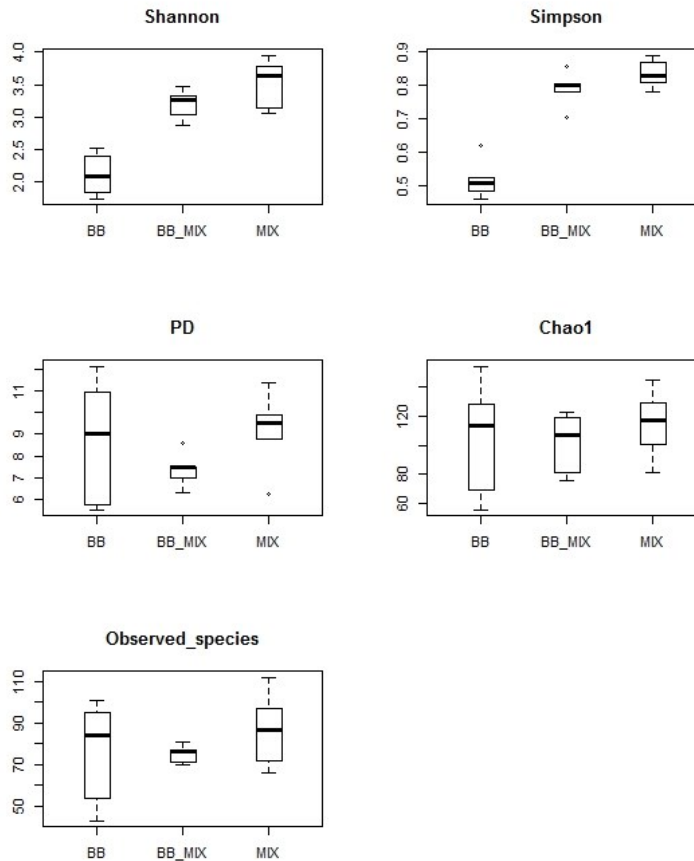
Alpha Diversity Index					
Microbiota	Shannon	Simpson	PD whole tree	Observed species	Chao1
EM278	5.5	0.9	25.3	298	387
EM425	5.5	0.9	29.6	332	404
MCC100	3.8	0.8	10.2	90	109
EM297	4.4	0.9	16.0	165	242
EM704	3.9	0.9	19.0	169	220

5633



5634

5635 **S Figure 6 Alpha diversity of the community (COM) type faecal microbiota at**
5636 **24 h.** The results refer to the aggregated faecal microbiotas EM278 and EM425. BB:
5637 blueberry supplementation; BB_MIX: blueberry and prebiotic carbohydrate mix
5638 supplementation; MIX: carbohydrate mix supplementation.



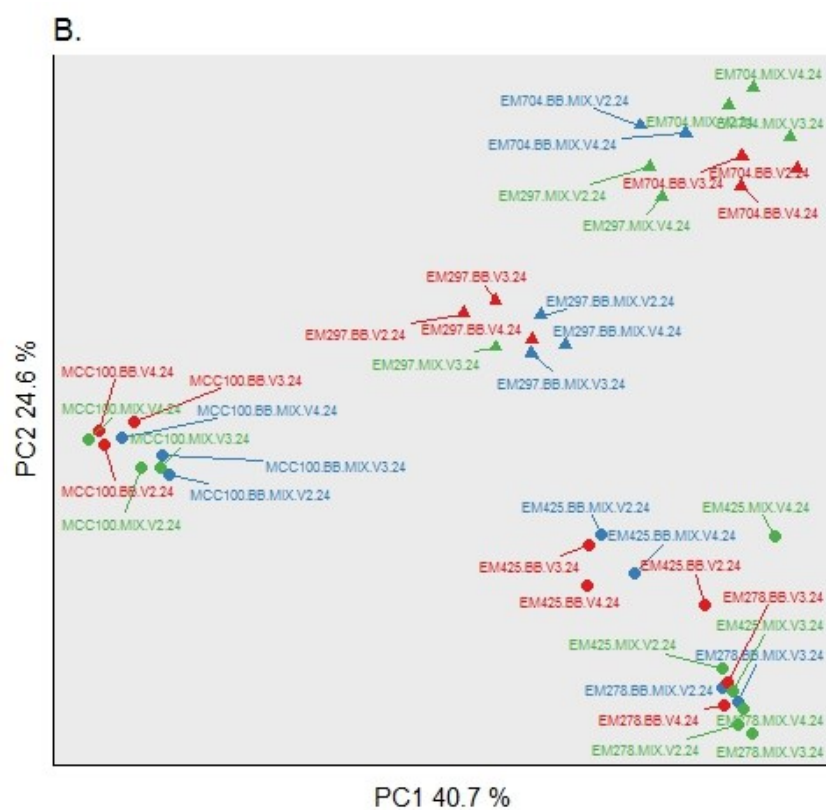
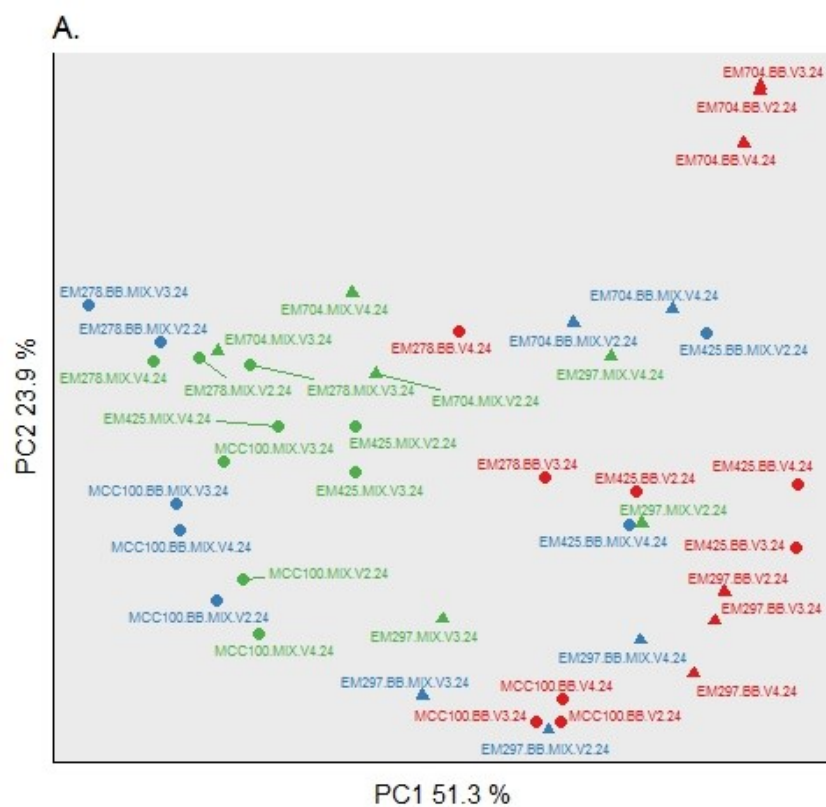
5639

5640 **S Figure 7 Alpha diversity of the longstay (LS) type faecal microbiota at 24 h.**

5641 The results refer to the aggregated faecal microbiotas EM704 and EM297. BB:

5642 blueberry supplementation; BB_MIX: blueberry and prebiotic carbohydrate mix

5643 supplementation; MIX: carbohydrate mix supplementation.



5644

5645 **S Figure 8 Principal Coordinates Analysis (PCoA) of the faecal microbiota and**
5646 **MCC100 at 24 h. A. Weighted UniFrac distances; B. Unweighted UniFrac distances.**
5647 “●” Community (COM) type microbiota and MCC100; “▲” Longstay (LS) type
5648 microbiota; **red**: BB supplementation; **blue**: BB.MIX supplementation; **green**: MIX
5649 supplementation.

5650 **S Table 11 Development of the composition and comparison between time**
5651 **points of the relative abundance of orders after 24 h fermentation with**
5652 **community (COM) type faecal microbiota (EM278, EM425). BB:** blueberry
5653 **powder supplementation; BB.MIX:** blueberry powder and prebiotic carbohydrate
5654 **mix supplementation; MIX:** prebiotic carbohydrate mix supplementation. Missing
5655 values indicate that the relevant taxon had <1% abundance in all vessels (two or
5656 three) summed for mean abundance. The mean value of the relative abundance per
5657 time point is shown.

OTU Classification	EM278						EM425					
	BB		BB.MIX		MIX		BB		BB.MIX		MIX	
	t0	t24	t0	t24	t0	t24	t0	t24	t0	t24	t0	t24
Bifidobacteriales	3.5	21.6	3.0	21.9	4.9	13.9 ^a	-	-	-	-	-	-
Bacteroidales	23.3	6.3	19.4	45.5	22.3	31.8 ^a	10.1	4.1	8.7	10.6	11.2	28.6
Lactobacillales	1.1	0.9	1.0	2.6	1.4	3.1 ^a	0.5	32.8 ^a	0.4	39.2	0.4	8.5
Clostridiales	67.0	42.9	71.5	26.8	67.6	47.1	75.6	20.5	76.9	27.3	75.0	50.5 [*]
Erysipelotrichales	0.3	3.5	0.4	1.4	0.5	1.7 ^a	-	-	-	-	-	-
Selenomonadales	1.0	2.2	-	-	0.4	0.6 ^a	2.7	0.7 ^a	2.1	0.4	1.8	0.3 ^a
Firmicutes/unclassified	0.8	1.0	-	-	-	-	2.6	0.2 ^a	2.7	0.02	2.7	0.6
Burkholderiales	0.8	1.1	0.9	0.5	1.0	0.7	-	-	-	-	-	-
Enterobacteriales	0.0	20.0	-	-	0.01	0.5	0.04	41.3	0.03	22.0	0.03	11.1 [*]

^{*} Statistically significant result, Kruskal Wallis test result $p \leq 0.05$, $p_{adj} \leq 0.05$ with Dunn's test $q_{adj} \leq 0.05$.
^a Statistical trend, Kruskal Wallis test result $p \leq 0.05$, $p_{adj} \leq 0.1$, with Dunn's test $q_{adj} \leq 0.1$.

5658

5659 **S Table 12 Development of the composition and comparison between time**
5660 **points of the relative abundance of orders after 24 h fermentation with longstay**
5661 **(LS) type faecal microbiota (EM704, EM297). BB: blueberry powder**
5662 **supplementation; BB.MIX: blueberry powder and prebiotic carbohydrate mix**
5663 **supplementation; MIX: prebiotic carbohydrate mix supplementation. Missing values**
5664 **indicate that the relevant taxon had <1% abundance in all vessels (two or three)**
5665 **summed for mean abundance. The mean value of the relative abundance per time**
5666 **point is shown.**

OTU Classification	EM704						EM297					
	BB		BB.MIX		MIX		BB		BB.MIX		MIX	
	t0	t24	t0	t24	t0	t24	t0	t24	t0	t24	t0	t24
Bifidobacteriales	-	-	-	-	-	-	2.3	0.02	-	-	-	-
Bacteroidales	10.2	3.6	10.1	13.0	10.0	30.3	15.5	1.9	16.7	18.7	19.3	14.8
Lactobacillales	0.1	72.1 ^a	0.1	35.1	0.1	4.5 ^a	2.5	8.6 ^a	1.5	9.6 ^a	4.7	28.2
Clostridiales	31.1	21.4	33.6	44.2	28.8	56.7	28.5	18.9 ^a	22.1	20.2	33.1	33.2
Selenomonadales	2.3	0.3 ^a	-	-	2.6	1.9 ^a	0.3	1.1	0.2	1.3	-	-
Enterobacteriales	0.1	1.2	0.04	6.6	0.1	4.9	8.8	68.5 ^a	6.6	44.3 ^a	10.2	21.9
Synergistales	10.5	0.1	9.2	0.02	13.8	0.1 ^a	23.3	0.1	21.0	0.04	7.6	0.09

^aStatistically significant result, Kruskal-Wallis test result $p \leq 0.05$, $p_{adj} \leq 0.05$ with Dunn's test $q_{adj} \leq 0.05$.
^a Statistical trend, Kruskal-Wallis test result $p \leq 0.05$, $p_{adj} \leq 0.1$, with Dunn's test $q_{adj} \leq 0.1$.

5667 **S Table 13 Development of the composition and comparison between time points of the relative abundance of species after 24 h**
5668 **fermentation with community (COM) type faecal microbiota (EM278, EM425). BB:** blueberry powder supplementation; BB.MIX:
5669 blueberry powder and prebiotic carbohydrate mix supplementation; MIX: prebiotic carbohydrate mix supplementation. Missing values indicate
5670 that the relevant taxon had <1% abundance in all vessels (two or three) summed for mean abundance. The mean value of the relative abundance
5671 per time point is shown.

OTU Classification	EM278						EM425					
	BB		BB.MIX		MIX		BB		BB.MIX		MIX	
	t0	t24	t0	t24	t0	t24	t0	t24	t0	t24	t0	t24
<i>Bifidobacterium bifidum</i>	0.05	1.8	0.03	0.8	0.1	0.6 ^a	-	-	-	-	-	-
<i>Bifidobacterium longum</i>	0.2	2.3	0.1	2.5	0.4	1.2	-	-	-	-	-	-
<i>Bifidobacterium</i> "	2.7	15.5	2.2	16.3	3.6	9.6 ^a	-	-	-	-	-	-
<i>Bifidobacte-riaceae</i> "	0.6	2.0	0.6	2.3	0.7	2.5 ^a	-	-	-	-	-	-
<i>Bacteroides caccae</i>	0.9	0.4	0.7	3.2	0.8	5.7 ^a	-	-	-	-	-	-
<i>Bacteroides dorei</i>	7.5	0.7	6.4	0.4	7.2	1.4 ^a	-	-	-	-	-	-
<i>Bacteroides eggerthii</i>	1.9	0.2	1.7	0.8	2.0	0.5 ^a	-	-	-	-	-	-
<i>Bacteroides fragilis</i>	-	-	-	-	-	-	0.8	0.6	0.6	10.1	0.8	15.2
<i>Bacteroides thetaiotaomicron</i>	0.6	0.3	-	-	0.4	0.4	-	-	-	-	0.1	1.1 ^a
<i>Bacteroides uniformis</i>	7.8	2.1	6.7	38.3	7.4	19.2 ^a	4.5	2.5	3.6	0.1	5.5	8.2
<i>Bacteroides</i> "	-	-	-	-	0.5	1.8	-	-	-	-	0.1	1.1 ^a
<i>Alistipes</i> "	1.1	1.3	-	-	1.2	1.4	-	-	-	-	-	-

OTU Classification	EM278						EM425					
	BB		BB.MIX		MIX		BB		BB.MIX		MIX	
	t0	t24	t0	t24	t0	t24	t0	t24	t0	t24	t0	t24
Streptococcus"	1.1	0.9	1.0	2.6	1.3	3.1	0.4	32.1 ^a	0.2	39.1	0.2	8.5
Clostridium sensu stricto"	-	-	-	-	-	0.5	7.8	5.0 ^a	0.6	5.7	0.4	12.3 ^a
Anaerostipes hadrus	2.3	11.2	2.6	11.3	2.5	7.8	-	-	0.7	6.7	0.8	8.1
Blautia faecis	2.3	0.5	2.7	2.0	2.0	4.3	-	-	-	-	-	-
Blautia luti	2.0	3.0	2.4	0.7	2.2	2.3	-	-	-	-	-	-
Blautia"	4.5	1.9	5.9	0.9	4.2	2.1	2.6	0.1	2.8	1.6	2.4	3.4
Clostridium XIVa"	-	-	-	-	0.1	0.8 ^a	0.1	0.5	-	-	-	-
Coprococcus catus	-	-	0.3	1.2	-	-	0.2	1.1 ^a	0.2	0.8	-	-
Coprococcus comes	0.6	1.3	-	-	0.8	4.3 ^a	0.2	0.6	0.2	0.9	-	-
Eubacterium rectale	15.1	0.5	16.3	2.7	16.6	10.2	-	-	-	-	-	-
Lachnospiraceae"	13.6	5.8	15.4	5.2	11.9	9.8 ^a	35.4	3.7 ^a	34.3	1.4	38.3	12.0 ^a
Clostridium XI"	-	-	-	-	-	-	0.2	1.4	0.1	0.1	-	-
Faecalibacterium prausnitzii	9.9	12.7	8.8	0.2	10.1	1.4	7.9	2.5	8.8	0.1	7.9	5.3 ^a
Gemmiger formicilis	-	-	-	-	1.4	0.1	2.0	0.1	2.7	7.4	1.8	2.0
Ruminococcaceae"	2.2	1.7	2.3	0.2	2.1	0.5 ^a	7.6	0.2	7.8	0.1	6.2	0.7 ^a
Clostridiales"	2.7	0.1	2.5	0.1	2.5	0.2 ^a	3.8	0.2 ^a	4.0	0.1	3.6	0.2
Clostridium XVIII"	0.1	3.2	0.2	1.3	0.3	1.4	-	-	-	-	-	-

OTU Classification	EM278						EM425					
	BB		BB.MIX		MIX		BB		BB.MIX		MIX	
	t0	t24	t0	t24	t0	t24	t0	t24	t0	t24	t0	t24
Phascolarctobacterium faecium	0.4	2.0	-	-	0.2	0.6 ^a	-	-	-	-	-	-
Firmicutes ^u	0.8	1.0	-	-	-	-	2.6	0.2 ^a	2.7	0.02	2.7	0.6 ^a
Shigella ^u	0.02	9.3	-	-	-	-	0.04	40.8	0.03	21.8	0.03	11.1 ^a

^aStatistically significant result, Kruskal-Wallis test result $p \leq 0.05$, $p_{adj} \leq 0.05$ with Dunn's test $q_{adj} \leq 0.05$

^aStatistical trend, Kruskal-Wallis test result $p \leq 0.05$, $p_{adj} \leq 0.1$, with Dunn's test $q_{adj} \leq 0.1$

^uUnclassified: not classified at a finer phylogenetic level than the indicated

5673 **S Table 14 Development of the composition and comparison between time points of the relative abundance of species after 24 h**
5674 **fermentation with longstay (LS) type faecal microbiota (EM704, EM297). BB:** blueberry powder supplementation; BB.MIX: blueberry
5675 powder and prebiotic carbohydrate mix supplementation; MIX: prebiotic carbohydrate mix supplementation. Missing values indicate that the
5676 relevant taxon had <1% abundance in all vessels (two or three) summed for mean abundance. The mean value of the relative abundance per time
5677 point is shown.

OTU Classification	EM704						EM297					
	BB		BB.MIX		MIX		BB		BB.MIX		MIX	
	t0	t24	t0	t24	t0	t24	t0	t24	t0	t24	t0	t24
<i>Bifidobacterium</i> "	-	-	-	-	-	-	1.6	0.01	-	-	-	-
<i>Bacteroides caccae</i>	-	-	-	-	0.4	1.4	-	-	-	-	-	-
<i>Bacteroides cellulosilyticus</i>	-	-	-	-	0.6	1.3	-	-	-	-	-	-
<i>Bacteroides dorei</i>	1.5	0.4 ^a	1.0	0.7	1.1	0.9	4.0	0.2 ^a	4.8	0.7 ^a	4.1	1.1
<i>Bacteroides fragilis</i>							7.5	0.3 ^a	7.4	8.8	8.9	8.5
<i>Bacteroides thetaiotaomicron</i>	3.3	2.8	4.4	11.9	3.3	25.8	0.3	1.1	0.4	5.1	0.5	2.7
<i>Bacteroides</i> "	-	-	-	-	-	-	0.8	0.2	0.9	3.9	1.2	2.4
<i>Methanobrevibacter smithii</i>	10.4	0.04	-	-	7.5	0.03 ^a	-	-	-	-	20.7	0.003
<i>Enterococcus</i> "	-	-	-	-	-	-	1.1	8.6 ^a	0.5	9.6 ^a	1.6	28.1
<i>Streptococcus</i> "	0.1	72.0 ^a	0.1	35.0	0.1	4.5 ^a	-	-	-	-	-	-
<i>Clostridium baratii</i>	-	-	0.7	2.8	0.7	1.7 ^a	-	-	-	-	-	-
<i>Clostridium disporicum</i>	9.4	0.8	10.3	4.6	8.4	4.4	-	-	-	-	-	-

OTU Classification	EM704						EM297					
	BB		BB.MIX		MIX		BB		BB.MIX		MIX	
	t0	t24	t0	t24	t0	t24	t0	t24	t0	t24	t0	t24
Clostridium sensu stricto^u	0.1	6.9	0.1	10.3	0.1	25.5	-	-	0.003	0.8 ^a	-	-
Blautia^u	0.2	1.7 ^a	0.1	2.1	0.1	5.0 ^a	0.2	1.0	0.2	1.1	0.2	2.7
Clostridium hathewayi	-	-	-	-	-	-	1.6	2.4	1.2	7.0	1.9	4.7
Clostridium XIVa^u	-	-	-	-	0.2	1.4 ^a	2.7	0.8	2.3	0.9 ^a	3.4	1.6
Ruminococcus torques	1.4	0.1	1.4	0.05	1.2	0.3 ^a	6.1	0.1 ^a	4.7	2.4	7.0	0.03
Eubacterium eligens	-	-	-	-	-	-	0.1	0.5	0.1	3.0	-	-
Lachnospiraceae^u	1.4	0.1 ^a	1.4	0.03	-	-	2.5	2.3	2.0	3.6 ^a	3.5	3.1
Clostridium glycolicum	0.1	1.4	0.1	3.5	-	-	-	-	-	-	-	-
Clostridium XI^u	0.2	1.9 ^a	0.2	11.0	0.2	3.9 ^a	-	-	-	-	-	-
Ruminococcaceae^u	2.7	1.5 ^a	2.5	4.0	2.9	5.1 ^a	5.4	0.02	4.7	0.03	5.4	0.02
Clostridiales^u	1.8	0.1	2.1	0.1	1.9	0.6 ^a	2.0	0.5 ^a	1.5	0.5 ^a	2.3	1.4
Phascolarctobacterium faecium	2.3	0.3 ^a	-	-	2.6	1.9 ^a	0.3	1.1	0.2	1.3	-	-
Escherichia/Shigella^u	0.01	0.9	0.01	6.5	0.02	4.8	7.0	66.8 ^a	5.4	44.3 ^a	9.0	20.3
Klebsiella^u	-	-	-	-	-	-	1.8	1.6	-	-	1.2	1.1
Cloacibacillus evryensis	10.3	0.1	9.0	0.02	13.7	0.1 ^a	23.2	0.1	20.9	0.04	7.6	0.1
Akkermansia muciniphila	30.2	0.03	-	-	34.7	0.2	-	-	-	-	-	-

^aStatistically significant result, Kruskal-Wallis test result $p \leq 0.05$, $p_{adj} \leq 0.05$ with Dunn's test $q_{adj} \leq 0.05$
^aStatistical trend, Kruskal-Wallis test result $p \leq 0.05$, $p_{adj} \leq 0.1$, with Dunn's test $q_{adj} \leq 0.1$
^uUnclassified: not classified at a finer phylogenetic level than the indicated

5678

Chapter 5

5679

General Discussion

5680 **Considerations for advancing gut microbiota modulation research for older**
5681 **people**

5682 The population over 60 yrs old is growing fast and people over 80 yrs are expected
5683 to comprise 13% of the European population by 2080 ([http://ec.europa.eu/eurostat/](http://ec.europa.eu/eurostat/statistics-explained/index.php/Population_structure_and_ageing)
5684 [statistics-explained/index.php/Population_structure_and_ageing](http://ec.europa.eu/eurostat/statistics-explained/index.php/Population_structure_and_ageing)). The extension of
5685 human life expectancy comes with increased risk of age-related conditions such as
5686 cognitive decline and non-communicable disease (metabolic disease, CVD, cancer),
5687 which places national economies and health care systems under significant stress
5688 (Divo *et al.*, 2016). While habitual diet can be the major source of key nutrients that
5689 could prevent or at least, ameliorate age-related conditions, the high prevalence of
5690 malnutrition in both community- and long term care unit- dwelling older people has
5691 increased the need for innovative nutritional recommendations and strategies, and
5692 the design of novel functional food products targeting the specific nutritional needs
5693 of the elderly population (de Groot *et al.*, 2016; Hill *et al.*, 2016; Moore *et al.*, 2018).

5694 There is an increasing effort to develop general or targeted gut microbiota
5695 therapeutics for the older population, similar to the efforts aimed at diseases in which
5696 the associated microbiota dysbiosis could be treated with prebiotics, probiotics or
5697 bacteriotherapy (reviewed in Chapter 1; Salazar *et al.*, 2017). While Salazar *et al.*
5698 (2017) in their recent review pointed out that more intervention studies on older
5699 population using a wide range of probiotics and prebiotics are needed, I have also
5700 emphasised in Chapter 1.2 the fact that there is a scarcity of studies using faecal
5701 microbiota from older subjects (≥ 65 yrs) in *in vitro* and also in *in vivo* preclinical
5702 experiments. Furthermore, there is a lack of preclinical trials using microbiota from
5703 non-healthy older subjects (reviewed in Chapter 1.2).

5704 *In vitro* screening of the “elderly” gut microbiota against an array of potential
5705 microbiota modulators (e.g. prebiotics, probiotics, LBT), could be an important
5706 stepping stone to advance the preclinical research of microbiota therapeutics
5707 targeting the older population. Outcomes of *in vitro* screens can lead to the design of
5708 *in vivo* preclinical trials and subsequently, to human interventions (O’Toole *et al.*,
5709 2017; Schmidt *et al.*, 2018). In this research thesis, faecal microbiota from healthy
5710 and non-healthy (frail) older subjects were used in the preclinical trials described in
5711 Chapters 2, 3 and 4. The use of both “healthy” and “non-healthy” microbiota from
5712 elderly people reflects the current scientific interest to develop both preventive and
5713 therapeutic approaches for gut microbiota modulation (Derrien and Veiga, 2016).

5714 The choice between aiming at a general or a more precise gut microbiota modulation
5715 depends on the context of what is considered the healthy or normal state of the
5716 microbiota, and whether whole community or specific taxon perturbations are
5717 associated with the non-healthy state of interest. Targeted and untargeted microbiota
5718 modulations can be achieved through the use of pre/pro-biotics and LBT, or through
5719 FMT (reviewed in Chapter 1.2; Schmidt *et al.*, 2018). Importantly, under the current
5720 updated concept of prebiotics, (potential) prebiotics can serve in untargeted
5721 microbiota modulation approaches because retention of the phylogenetic diversity of
5722 the whole microbiota community in combination with increased SCFA production
5723 especially butyrate, is emerging as a desirable outcome of prebiotic administration
5724 (reviewed in Chapter 1.2; Gibson *et al.*, 2017).

5725 Examples of precision approaches targeting a certain microbiota component include
5726 the following: *F. prausnitzii* is proposed as a therapeutic target for Crohn’s disease
5727 (Sokol *et al.*, 2008; Pascal *et al.*, 2017), therefore, the taxon could be supplemented

5728 in patients or targeted with prebiotics. The discovery of CRC biomarker bacterial
5729 clusters (Flemer *et al.*, 2017) may call for a targeted approach that will aim at a wide
5730 but defined portion of the microbiota community of patients. Importantly, for the
5731 rational design of precision gut microbiota therapeutics, some degree of causation
5732 beyond mere associations, between biomarker taxa and disease needs to be
5733 established before aiming at specific microbiota interventions (Schmidt *et al.*, 2018).

5734 It is also important not to overstate the beneficial effects of certain gut bacteria or
5735 “incriminate” others for deleterious effects based solely on association studies and
5736 without placing the effect in an ecological context (e.g. diet, immunity, cross-talk
5737 with other microbiota members), as the examples of the LBT based on the
5738 “beneficial” taxon *A. muciniphila* and *P. copri* have shown (Cani, 2018; Cirstea *et*
5739 *al.*, 2018). Another example is the normalisation of dysbiosis and inflammation by
5740 precision tailoring of the expanded Enterobacteriaceae population during the
5741 inflamed stage of colitis in mice (Zhu *et al.*, 2018). This potentially pathobiont taxon
5742 which is a common member of the gut microbiota was not eradicated but its
5743 abundance was restored to pre-inflammation levels.

5744 While some conditions may require the targeting of certain taxa or clusters, others
5745 will require a general effect on the whole microbiota community. For example, the
5746 association of metabolic disease with low diversity and low gene counts in the gut
5747 microbiota (Le Chatelier *et al.*, 2013) indicates the need for general modulations, i.e.
5748 to target of the whole community for the increase of the phylogenetic diversity, in
5749 order to decrease the risk for metabolic conditions. Interestingly, boost of the
5750 phylogenetic diversity of the gut microbiota of “westernised/industrialised” cohorts
5751 in which decreased phylogenetic diversity is a potential risk factor for various

5752 “modern” diseases such as metabolic disease and IBD, could be key for general
5753 approaches towards maintaining health and lowering the risk of disease in the
5754 general population (Moeller *et al.*, 2017).

5755 Identifying the “healthy adult” microbiota has been challenging (Lloyd-Price *et al.*,
5756 2016). A “functional healthy core” in which different microbial lineages confer a
5757 similar metabolic activity due to metabolic redundancy in the gut microbiota has
5758 been proposed as a more relevant notion compared to a core of taxa for which
5759 consensus across cohorts is often prevented by inter-individual compositional
5760 variations (Lloyd-Price *et al.*, 2016). Compositional patterns, i.e. enterotypes
5761 (discussed in Chapter 1.1), in spite of the controversy with which the concept was
5762 initially met, may offer a useful way to stratify “healthy” microbiota compositional
5763 profiles across subjects (Costea *et al.*, 2018).

5764 In depth understanding of microbiota assembly dynamics can be key to general
5765 microbiota modulatory strategies. In spite of inter-individual species/strain level
5766 differences, and especially for “healthy” microbiomes, these dynamics are most
5767 likely governed by “universality” reflected in the pathways of inter-species cross-talk,
5768 resilience to stressors and in the combination of compositional adaptiveness and
5769 longitudinal robustness (Bashan *et al.*, 2016; Gilbert *et al.*, 2018). Dysbiotic
5770 microbiota dynamics are more difficult to predict because this “universality” may be
5771 interrupted and some authors argue for “stochastic” changes in the dysbiotic
5772 microbiota (Bashan *et al.*, 2016; Zaneveld *et al.*, 2017).

5773 The wide distance observed in β -diversity analysis among patients (e.g. Crohn’s
5774 disease) compared to the close clustering of healthy controls, indicates that the
5775 underlying dysbiosis may in some conditions, lead to an unstable microbiota state

5776 and unpredictable (stochastic) shifts from the healthy microbiota profile (Zaneveld *et*
5777 *al.*, 2017). The deregulated dynamics of the “dysbiotic microbiota” which is most
5778 likely characterised by unpredictable responsiveness to stressors, may also be
5779 hampering responsiveness to therapeutics. In depth profiling of the gut microbiota
5780 compositional changes in “perturb-to-predict” experiments (e.g. in *in vitro* and *in*
5781 *vivo* colon models), offer valuable insight to microbiota dynamics because they can
5782 help typify shifts of the microbiota from baseline as being stochastic or reproducible
5783 as a response to the experimentally imposed challenge (e.g. response to diet)
5784 (Zaneveld *et al.*, 2017; Schmidt *et al.*, 2018).

5785 The re-evaluated concept of the enterotypes in the analysis of microbiota
5786 composition could further refine the identification of predictable and stochastic
5787 microbiota responses (Costea *et al.*, 2108). Furthermore, many studies have reported
5788 responders and non-responders to prebiotic intervention, underlying the importance
5789 of the baseline microbiota composition in “perturb-to-predict” approaches (Duncan
5790 and Flint, 2013; Zhao *et al.*, 2018). To date, *Bacteroides* and *Prevotella* have been
5791 identified as key taxa that define major enterotype profiles among individuals, and
5792 responsiveness variations, for example as demonstrated in an *in vitro* colon system
5793 after dietary fibre supplementation (Chen *et al.*, 2017; Costea *et al.*, 2018).

5794 Taken together, efforts to categorise microbiota responses to extrinsic challenges (e.g.
5795 diet, antibiotics, LBT, pathogens) in predictable or stochastic manners, overcoming
5796 the inter-individual variations by stratifying responses according to enterotypes and
5797 baseline microbiota characteristics, may facilitate generalised and at the same time
5798 precision approaches in health-maintenance and therapeutic strategies, overcoming
5799 the need for personalised therapeutics (Sonnenburg and Backhed, 2016).

5800 In order to extend our definition of the “healthy” microbiota, an interesting question
5801 to ask is how different is the responsiveness of the “healthy older” from the “healthy
5802 younger” gut microbiota to perturbations. The “healthy older” may not significantly
5803 differ in composition from the “healthy younger adult” microbiota as observed in
5804 principal coordinates (PCoA) analysis in various studies (Claesson *et al.*, 2012; Bian
5805 *et al.*, 2017). In the *in vitro* experiments described in Chapters 2 and 4, microbiota
5806 from younger adults was not used. However, it would be interesting to examine in
5807 future preclinical *in vitro* studies the comparative responsiveness of healthy “young”
5808 and “old” microbiota to substrate supplementation/perturbation, both in taxa and
5809 SCFA production.

5810 *In vitro* microbiota perturbation tests (e.g. challenge of the microbiota with candidate
5811 pre/pro-biotic substrate, LBT, pathobionts), in which simulation of the host factors
5812 that affect the microbiota composition (e.g. lower immune regulation in the elderly;
5813 Kundu *et al.*, 2017) cannot be achieved, apart perhaps from the slower retention time
5814 in the elderly colon (e.g. in Fehlaub *et al.*, 2015), may allow for a better
5815 understanding of the “intrinsic” potential of the “young” and “old” healthy
5816 microbiota to respond to challenges. The potential differences could be associated to
5817 different strains being differentially enriched in the two types of microbiota (Zhu *et*
5818 *al.*, 2015). Strain level differences between microbiotas are often underestimated
5819 because it is difficult to discern them based on phylogenetic gene markers such as
5820 16S rRNA gene (Zhu *et al.*, 2015); however, strain level analysis will refine our
5821 understanding of the microbiota dynamics and functionality. Identification of such
5822 fine strain-level differences may improve the outcome of gut microbiota modulation
5823 strategies (De Filippis *et al.*, 2016) and open a window for preventive modulations
5824 targeting the progression of ageing in the microbiota.

5825 The use of synthetic minimal microbial communities (reviewed in Chapter 1.2,;
5826 experiments in Chapter 4), can be a valuable tool for studying how key microbiota
5827 members respond to dietary substrates, and especially in *in vitro* systems, and they
5828 offer the potential to study the microbiota down to strain level properties. Rationally
5829 assembled artificial microbiota communities that are designed to resemble "healthy"
5830 or "dysbiotic" gut microbiota or microbiotas of certain enterotypes, will allow for
5831 controlled and reproducible experiments through which associations, function,
5832 metabolism, cross-feeding and other parameters can be studied in a reduced in
5833 complexity ecosystem (de Roy *et al.*, 2013). Importantly, *in vitro* studies employing
5834 artificial microbiota communities can be the basis for the design of LBT intervention
5835 in which microbiota consortia and dietary context (i.e. substrates that can sustain the
5836 viability and diversity of the consortium) can be matched in order to achieve the
5837 desired response (e.g. increased butyrate production by increasing the fibrolytic
5838 bacteria in the colon) in microbiotas that otherwise may fail to respond due to
5839 missing responsive members (Makki *et al.*, 2018).

5840 The reproducibility within studies and the comparability across studies that can now
5841 be achieved by thorough documentation of protocols from the wet lab to the data
5842 analysis, can maximise the information extracted from microbiome studies, leading
5843 to improved translatability and rationally designed clinical applications (Knight *et*
5844 *al.*, 2018). While next generation 16S rRNA amplicon sequencing has been a major
5845 analysis method in the microbiota studies to date, it is apparent that in order to refine
5846 the microbiota taxonomic and functionality analysis, a combination of -omics
5847 methods should be employed (e.g. metagenomics, metatranscriptomics) when
5848 economic resources, sample availability and time constraints allow for it (Knight *et*
5849 *al.*, 2018). Especially the metagenomics approach, allows for high taxonomic

5850 resolution of both the bacterial component of the microbiota community and the
5851 whole microbial component, i.e. fungi, yeasts, archaea and viruses and phages,
5852 which although underrepresented in most microbiota studies, still impacts health and
5853 disease (Cani, 2018). Furthermore, accurate profiling of the microbiota composition
5854 could benefit from a combination of qualitative (16S rRNA gene amplicon
5855 sequencing) and quantitative methods (e.g. quantitative PCR) in order to detect true
5856 changes in cell counts in combination to relative abundance changes (Vandeputte *et*
5857 *al.*, 2017; Knight *et al.*, 2018).

5858 Any prediction that is generated in *in vitro* studies requires validation in *in vivo*
5859 systems and ultimately in “real life” clinical trials, before scientific advances in the
5860 field of gut microbiota research can be responsibly disseminated to the public (Cani,
5861 2018; Schmidt *et al.*, 2018). Importantly, some advances in gut microbiota research
5862 are already reaching the public and health authorities are promoting science-backed
5863 dietary recommendations (Derrien and Veiga, 2016). Dietary recommendations
5864 represent low-cost health-improving strategies but require careful dissemination
5865 strategies from the laboratories to society and adherence by the public. Cohort
5866 studies show that adherence to international and national dietary guidelines may
5867 significantly improve public health (Batis *et al.*, 2016; Biesbroek *et al.*, 2017) and
5868 even increase the life expectancy of the older population (Jankovic *et al.*, 2014;
5869 Jankovic *et al.*, 2015).

5870 In spite of the importance of dietary guidelines, adherence is often challenging.
5871 Functional foods supplemented with next generation prebiotics and/or probiotics or
5872 microbial products potentially regulated as food products (Derrien and Veiga, 2016;

5873 Brodman *et al.*, 2017) are expected to play a significant role in the amelioration of
5874 health across all ages and in particularly in the elderly population.

5875 **Widely used plant- and animal- derived dietary substrates as a source for novel**
5876 **prebiotics**

5877 Plants (fruits, vegetables, legumes) are the main source of substrates with potential
5878 prebiotic activity, e.g. dietary fibre (reviewed in. Makki *et al.*, 2018) and in the
5879 search for new prebiotics, dietary substrates of plant origin are being investigated.
5880 There is increasing interest in the study of the effect of dietary polyphenols in the gut
5881 microbiota, found in fruits, vegetables and legumes (Derrien and Veiga, 2016). The
5882 health relevance of polyphenol consumption is associated with the gut microbiota
5883 metabolism of unabsorbed polyphenols and the subsequent release of bioactive
5884 phenolic compounds (Marchesi *et al.*, 2015). Furthermore, some recent studies have
5885 focused on the cumulative prebiotic effect of polyphenols and dietary fibre, because
5886 apart from the documented health benefits of these two food components, the
5887 binding of polyphenols to dietary fibre (e.g. the case of plant cell wall) may increase
5888 the bioavailability of the polyphenols in the distal colon (discussed in Chapter 4).

5889 Another less investigated source of potentially prebiotic substrates is milk. As
5890 reviewed in Chapter 1.3, milk and its carbohydrate components are drawing
5891 scientific attention due to BMOs having complex structures similar to HMOs. The
5892 structural complexity of HMOs is associated to the HMOs prebiotic activity
5893 (reviewed in Chapter 1.2). A similar to HMOs structural complexity has not been
5894 identified in the plant derived prebiotic candidates investigated to date (Chapter 1.2).
5895 The large-scale isolation (or production) of HMOs has been challenging and
5896 therefore, there are limitations in the use of HMOs for study purposes or for food

5897 supplementation (Bode *et al.*, 2016). Should prebiotic activity of BMOs be
5898 documented (as recent studies are indicating; see Chapters 1.3 and 3), they could
5899 replace HMOs in various large-scale *in vitro*, *in vivo* and importantly, in clinical
5900 studies, thus, extending our knowledge on the effect of these non-plant derived OS
5901 on the gut microbiota.

5902 The studies on the effect of isolated milk components such as BMOs or GMP
5903 (Chapter 2) on the gut microbiota can reveal new opportunities for functional foods
5904 design ([https://www.nutrition.org.uk/nutritionscience/foodfacts/functional-](https://www.nutrition.org.uk/nutritionscience/foodfacts/functional-foods.html?limitstart=0)
5905 [foods.html?limitstart=0](https://www.nutrition.org.uk/nutritionscience/foodfacts/functional-foods.html?limitstart=0)) and give new perspectives for the industrial and commercial
5906 exploitation of dairy industry residual by-products. Isolation of BMOs, and GMP,
5907 from whey concentrates may present a productive way for the dairy industry to
5908 resolve the challenging whey surplus problem while also extracting valuable
5909 compounds for foods supplementation (Oliveira *et al.*, 2015; Bode *et al.*, 2016).

5910 To my knowledge (reviewed in Chapter 1.3), research has yet to thoroughly assess
5911 the effect of regular milk consumption on the microbiota composition, an area of
5912 study which could complement the array of health benefits known for milk
5913 consumption. Evidence on the prebiotic potential of (whole, unfermented) milk, such
5914 as those presented in Chapter 3, in combination with large cohort data and meta-
5915 analysis (reviewed in Chapter 1) showing that milk consumption does not correlate
5916 with detrimental health effects, would add compelling scientific evidence towards
5917 milk inclusion in a balanced diet. The inclusion of milk in the diet of the elderly
5918 population is of particular relevance because milk is a source of the vitamins B and
5919 D among others, and of protein and micronutrients such as calcium, the dietary

5920 deficiency of which is associated with degenerative disease in older age (de Groot,
5921 2016).

5922 **Research thesis contribution to current literature and future work**

5923 In this research thesis I investigated the modulatory effect on the human gut
5924 microbiota of selected food ingredients, i.e. milk and milk-derived GMP, and berry
5925 fruit, focusing on older subjects. The aim was to provide scientific evidence for the
5926 prebiotic potential of these products and therefore, evidence to warrant the design of
5927 informed future clinical trials in order to establish the effect of the selected products
5928 on GIT health.

5929 In Chapter 1.3, I have reviewed the existing evidence on the potential of the selected
5930 dairy food ingredients to be used in approaches to modulate the gut microbiota; I
5931 have also reviewed the existing knowledge on the health benefits associated with the
5932 consumption of these products. In Chapter 1.2, I reviewed the major experimental
5933 models that can be used in the study of gut microbiota modulation. Chapter 2
5934 provides experimental evidence for the potential of GMP to modulate *in vitro* the gut
5935 microbiota of older subjects by promoting the growth of several health-relevant taxa
5936 and by sustaining the microbiota diversity close to baseline starting point. As I
5937 reviewed in Chapter 1.3, previous *in vitro* studies yielded controversial evidence on
5938 the prebiotic potential of GMP. One possible reason for this is the fact that they did
5939 not examine the whole microbiota community changes that GMP can promote, but
5940 rather they focused on identifying changes in specific taxa such as *Lactobacillus* and
5941 *Bifidobacterium*, measured by quantitative molecular methods such as FISH (Bruck
5942 *et al.*, 2002; Hernandez-Hernandez *et al.*, 2011). In Chapter 2, I profiled the changes

5943 in the whole community and identified affected taxa beyond the “traditional” targets
5944 of prebiotics.

5945 Importantly, in the *in vitro* experiments I used faecal samples from subjects in which
5946 low phylogenetic diversity correlated with poor health and frailty. As reviewed in
5947 Chapter 1, frailty is associated to dysbiosis, it is prevalent in the elderly population
5948 and it is a risk factor for morbidity. Apart from examining the potential of GMP to
5949 sustain the phylogenetic diversity of the “frail” microbiota, we exposed a major
5950 limitation in the use of low-diversity faecal samples in *in vitro* models. Reduced
5951 diversity can lead to reduced responsiveness of the microbiota, translating into fewer
5952 competent taxa being overrepresented in the compositional profile after fermentation,
5953 as seen in Chapter 2 figures.

5954 This observation could be reflecting “real life” observations from other studies: low
5955 richness faecal microbiota (measured by alpha diversity, species diversity indexes
5956 and gene counts) in obese subjects, was not only predictive of disease but also of low
5957 responsiveness to dietary intervention (Cotillard *et al.*, 2013; Le Chatelier *et al.*,
5958 2013). Some restoration of microbiota dysbiosis was achieved by dietary
5959 intervention but the underlying low-grade inflammation was not significantly
5960 ameliorated (Cotillard *et al.*, 2013), implying that low species richness leads to low
5961 metabolic potential (e.g. SCFA production) through limited functional redundancy in
5962 the microbiota.

5963 In spite of the limitations, *in vitro* colon models can be a valuable tool for the study
5964 of the modulation of the “frail” microbiota. One improvement for future studies is
5965 the use of a rich basal medium which can be supplemented with the test substrate
5966 (similar to the one used in Chapter 4). The adaptation of the faecal microbiota in *in*

5967 *vitro* systems comes with the reduction of the alpha diversity (discussed in Chapters
5968 1.2 and 2); a rich basal medium may be necessary when using microbiota with
5969 reduced diversity in order to sustain the maximum portion of the baseline diversity
5970 and therefore, its dynamics and functionality.

5971 In Chapter 2, I discussed the choice not to pool the faecal samples that were used. An
5972 interesting adjustment in the study design for future experiments, would be the
5973 pooling of “frail” microbiotas following the rationale of Zaneveld *et al.* (2017). The
5974 comparative analysis of the responsiveness of the pooled “frail” faecal microbiota
5975 against single-donor “frail” microbiotas could reveal whether the observed changes
5976 are reproducible or stochastic because in the pooled microbiota the stochastic
5977 responses may be minimised (Zaneveld *et al.* 2017).

5978 In Chapter 3, I described the preclinical trial where the potential of milk and GMP to
5979 modulate the gut microbiota was examined in an *in vivo* colon model of humanised
5980 mice. With the preclinical trial we aimed at evaluating the findings of the *in vitro*
5981 trial with regard to GMP prebiotic activity, and at performing an in-depth profiling
5982 of the changes that milk confers on the gut microbiota and thus, to provide evidence
5983 for health benefits of milk consumption through its potential prebiotic activity.

5984 In the mouse model used here, we found that lactose free milk was the most efficient
5985 in retaining gut microbiota diversity equally to or followed by soy-protein control
5986 diet. Although whole milk retained higher microbiota diversity compared to GMP,
5987 no significant differences were detected between the two feeding regimes.
5988 Importantly, both milk diets positively affected the relative abundance of health-
5989 relevant taxa such as *R. bromii*, in a gender-dependent manner. Same gender mice
5990 are often used in microbiome studies in order to obtain more homogenous results.

5991 However, it is important to address gender variations not only in murine studies
5992 (Wang *et al.*, 2016; Fransen *et al.*, 2017) but also in human studies in order to
5993 identify the extent of the impact of the endocrine system on the gut microbiota inter-
5994 individual variation and therefore, illuminate another aspect of the gut microbiota
5995 dynamics (Neuman *et al.*, 2015).

5996 The inherent limitations of the mouse model used in Chapter 3 experiments
5997 (analysed in Chapters 1.2, 3), generated some questions that may be reducing the
5998 translatability of the results. For example, the murine gut favours the establishment
5999 and prevalence of xenomicrobiota Bacteroidetes including Bacteroidaceae and
6000 Porphyromonadaceae, whereas it is refractory to Firmicutes / Lachnospiraceae
6001 xenomicrobiota colonisation (reviewed in Chapter 1.2). Glycomacropeptide contains
6002 a high portion of sialic acid that, as discussed in Chapter 3, may promote the growth
6003 of Bacteroidetes taxa. Therefore, testing a potentially Bacteroidetes promoting
6004 substrate in a gut microbiota model that is “biased” towards Bacteroidetes
6005 enrichment, may not have been the most useful approach for identifying the prebiotic
6006 potential of GMP. Importantly, it was the milk diets that led to the smallest
6007 Bacteroidetes/Firmicutes ratio, tipping the scale towards an increased Firmicutes
6008 relative abundance. However, because of the poor Lachnospiraceae colonisation (e.g.
6009 Figures 18 and 21) we could not identify effects on a wide range of Lachnospiraceae
6010 taxa.

6011 It has been proposed that for the investigation of the effect of diet on the gut
6012 microbiota, the use of conventional mice with their intact indigenous gut microbiota
6013 and full-range taxonomic representation may be a more informative approach
6014 compared to conventionalisation or humanisation (Arrieta *et al.*, 2016). In spite of

6015 limitations of the study model, the promising findings of our pre-clinical trial
6016 coupled with the fact that most of the cohort studies indicate the health benefits of
6017 moderate milk consumption, warrant a human trial searching for microbiota effects.

6018 Importantly, mice like other rodents, may not fully tolerate lactose (de Heijning *et al.*,
6019 2015) and it can be inferred that the differential effect of the two types of milk on the
6020 gut microbiota described in Chapter 3, is due to the presence or absence of residual
6021 lactose reaching the colon. In LP humans, lactose is fully digested and absorbed in
6022 the small intestine. Whether or not this means that lactose free milk and whole milk
6023 have similar gut microbiota modulatory effect in LP humans, remains to be
6024 investigated in a clinical trial. Furthermore, because LNP subjects can tolerate an
6025 average of 1 glass of whole milk per day, it would be also interesting to further
6026 investigate the prebiotic potential of whole milk in LNP subjects.

6027 Another interesting question in the search of the effect of milk on the gut microbiota
6028 is if different lactose free milk preparations have the same effect on the human gut
6029 microbiota composition. The lactose-free milk used in Chapter 3 was prepared after
6030 lactose was filtered and the remaining lactose was hydrolysed
6031 (<https://www.valio.com/articles/lactose-intolerance-is-real-and-common/>). Other
6032 preparations involve only lactose hydrolysis to glucose and galactose. Potentially,
6033 low load of simple sugar (e.g. the case of filtered lactose free milk preparations)
6034 reaching the small intestine microbiota that competes with the human host for the
6035 uptake of these compounds, can affect the downstream microbiota dynamics
6036 differentially to higher simple sugar load (Zoetendal *et al.*, 2012; Marinez-Guryn *et*
6037 *al.*, 2018).

6038 In Chapter 4, I described the exploratory experiments towards identifying the *in vitro*
6039 effect of the blueberry powder on the gut microbiota of elderly subjects and the
6040 bacterial consortium MCC100. We hypothesised that the high sugar content (e.g.
6041 fructose) of the blueberry powder, resulted in enrichment of the microbiota in
6042 streptococci as these organisms are known to be fast utilisers of simple sugars
6043 (Zoetendal *et al.*, 2018). Only the presence of bifidobacteria outcompeted
6044 streptococci in the utilisation of the provided substrates, showing the importance of
6045 the baseline microbiota in the responsiveness to the dietary substrates. Interestingly,
6046 bifidobacteria taxa were present at baseline in one of the two LS microbiotas used;
6047 however, they failed to respond to supplementation similarly to what observed when
6048 present in the COM type microbiota. If this is an indication of deregulated
6049 microbiota “universality” remains to be further examined in future experiments *in*
6050 *vitro* experiments using both faecal and artificial microbiotas.

6051 A multi-stage fermenter involving compartmentalisation of interconnected vessels
6052 with different pH mimicking parts of the GIT might have allowed for fastidiously
6053 growing fibre utilisers to grow; under *in vivo* conditions, simple sugars would have
6054 been digested or absorbed in the upper parts of the GIT. Results of the phenolic
6055 compound chemical analysis are awaited. In future experiments that will complete
6056 this exploratory work, isolated phenolic compounds will be used in *in vitro*
6057 fermentation experiments in order to identify specific microbiota changes caused by
6058 selected phenolic compound supplementation free of complicating sugars being
6059 present.

6060 The artificial consortium was assembled by our laboratory in order to mimic the
6061 faecal microbiota of a healthy adult. The 100 selected strains belong to the laboratory

6062 microbiome collection of faecal bacteria isolated from healthy donors; the isolation
6063 work was part of this PhD research. A manuscript with details on consortium
6064 assembly and relevant further work is in preparation as mentioned in Chapter 4
6065 (Materials and Methods). As discussed before (Chapters 1, 5), the use of minimal
6066 synthetic microbiotas is expected to facilitate diet-microbiota research. To my
6067 knowledge, this is a first attempt to use a minimal “healthy” faecal microbiota in the
6068 study of the effect of diet on the microbiota. Future experiments with isolated
6069 phenolic compounds and potentially with consortia that will mimic microbiotas of
6070 different enterotypes are expected to elucidate the role and associations of various
6071 gut microbiota members in the metabolism of dietary polyphenols and other dietary
6072 components with potential prebiotic activity.

6073 **5.1 References**

- 6074 Arrieta, M. C., Walter, J., Finlay, B. B. (2016). Human Microbiota-Associated
6075 Mice: A model with challenges. *Cell Host Microbe*, 19(5), 575–578.
- 6076 Bashan, A., Gibson, T. E., Friedman, J., Carey, V. J., Weiss, S. T., Hohmann, E.
6077 L., Liu, Y.-Y. (2016). Universality of human microbial dynamics. *Nature*,
6078 534(7606), 259–262.
- 6079 Batis, C., Aburto, T. C., Sánchez-Pimienta, T. G., Pedraza, L. S., Rivera, J. A.
6080 (2016). Adherence to dietary recommendations for food group intakes is low in
6081 the Mexican population. *J Nutr*, 146(9), 1897S–1906S.
- 6082 Bian, G., Gloor, G. B., Gong, A., Jia, C., Zhang, W., Hu, J., Zhang, H., Zhang,
6083 Y., Zhou, Z., Zhang, J., Burton, J. P., *et al.* (2017). The gut microbiota of healthy
6084 aged Chinese is similar to that of the healthy young. *MSphere*, 2(5), e00327-17.
- 6085 Biesbroek, S., Verschuren, W. M. M., Boer, J. M. A., Van De Kamp, M. E., Van
6086 Der Schouw, Y. T., Geelen, A., Looman, M., Temme, E. H. M. (2017). Does a
6087 better adherence to dietary guidelines reduce mortality risk and environmental
6088 impact in the Dutch sub-cohort of the European Prospective Investigation into
6089 Cancer and Nutrition? *Br J Nutr*, 118(1), 69–80.
- 6090 Bode, L., Contractor, N., Barile, D., Pohl, N., Prudden, A. R., Boons, G. J., Jin,
6091 Y. S., Jennewein, S. (2016). Overcoming the limited availability of human milk
6092 oligosaccharides: Challenges and opportunities for research and application. *Nutr*
6093 *Rev*, 74(10), 635–644.

6094 Brodmann, T., Endo, A., Gueimonde, M., Vinderola, G., Kneifel, W., de Vos, W.
6095 M., Salminen, S., Gómez-Gallego, C. (2017). Safety of novel microbes for
6096 human consumption: Practical examples of assessment in the European Union.
6097 *Front Microbiol*, 8(12), 1–15.

6098 Brück, W. M., Graverholt, G., Gibson, G. R. (2002). Use of batch culture and a
6099 two-stage continuous culture system to study the effect of supplemental alpha-
6100 lactalbumin and glycomacropeptide on mixed populations of human gut bacteria.
6101 *FEMS Microbiol Ecol*, 41(3), 231–7.

6102 Cani, P. D. (2018). Human gut microbiome: hopes, threats and promises. *Gut*,
6103 [Epub ahead of print].

6104 Chen, T., Long, W., Zhang, C., Liu, S., Zhao, L., Hamaker, B. R. (2017). Fiber-
6105 utilizing capacity varies in *Prevotella*- versus *Bacteroides*-dominated gut
6106 microbiota. *Sci Rep*, 7(1), 2594.

6107 Cirstea, M., Radisavljevic, N., Finlay, B. B. (2018). Good Bug, Bad Bug:
6108 Breaking through Microbial Stereotypes. *Cell Host Microbe*, 23(1), 10–13.

6109 Claesson, M. J., Jeffery, I. B., Conde, S., Power, S. E., O'Connor, E. M., Cusack,
6110 S, Harris, H.M.B., Coakley, M., *et al.* (2012). Gut microbiota composition
6111 correlates with diet and health in the elderly. *Nature*, 488(7410), 178–184.

6112 Costea, P. I., Hildebrand, F., Manimozhiyan, A., Bäckhed, F., Blaser, M. J.,
6113 Bushman, F. D., de Vos, W. M., Ehrlich, S. D., Fraser, C. M., Hattori, M., *et al.*
6114 (2018). Enterotypes in the landscape of gut microbial community composition.
6115 *Nat. Microbiol*, 3(1), 8–16.

6116 Cotillard, A., Kennedy, S. P., Kong, L. C., Prifti, E., Pons, N., Le Chatelier, E.,
6117 Almeida, M., Quiquis, B., Levenez, F., *et al.* (2013). Dietary intervention impact
6118 on gut microbial gene richness. *Nature*, 500(7464), 585–588.

6119 de Groot, L. C. P. G. M. (2016). Nutritional issues for older adults: addressing
6120 degenerative ageing with long-term studies. *Proc Nutr Soc*, 75(02), 169–173.

6121 de Heijning, B., Kegler, D., Schipper, L., Voogd, E., Oosting, A., Beek, E.
6122 (2015). Acute and chronic effects of dietary lactose in adult rats are not explained
6123 by residual intestinal lactase activity. *Nutrients*, 7(7), 5542–5555.

6124 De Filippis, F., Pellegrini, N., Laghi, L., Gobetti, M., and Ercolini, D. (2016).
6125 Unusual sub-genus associations of faecal *Prevotella* and *Bacteroides* with
6126 specific dietary patterns. *Microbiome*, 4:57.

6127 De Roy, K., Marzorati, M., Van den Abbeele, P., Van de Wiele, T., Boon, N.
6128 (2014). Synthetic microbial ecosystems: an exciting tool to understand and apply
6129 microbial communities. *Environ Microbiol*, 16(6), 1472–81.

6130 Divo, M. J., Martinez, C. H., Mannino, D. M. (2016). Ageing and the
6131 epidemiology of multimorbidity. *Eur Respir*, 44(4), 1055–1068.

6132 Duncan, S. H., and Flint, H. J. (2013). Probiotics and prebiotics and health in
6133 ageing populations. *Maturitas*, 75(1), 44–50.

6134 Fehlbaum, S., Chassard, C., Haug, M. C., Fourmestraux, C., Derrien, M., Lacroix,
6135 C. (2015). Design and investigation of PolyFermS *in vitro* continuous
6136 fermentation models inoculated with immobilized fecal microbiota mimicking
6137 the elderly colon. *PloS One*, 10(11), e0142793.

6138 Flemer, B., Lynch, D. B., Brown, J. M. R., Jeffery, I. B., Ryan, F. J., Claesson,
6139 M. J., O’Riordan, M., Shanahan, F., O’Toole, P.W. (2017). Tumour-associated
6140 and non-tumour-associated microbiota in colorectal cancer. *Gut*, 66(4), 633–643.

6141 Fransen, F., van Beek, A. A., Borghuis, T., Meijer, B., Hugenholtz, F., van der
6142 Gaast-de Jongh, Sevelkoul, H. F., de Jonge, M. I., Faas, M. M., *et al.* (2017). The
6143 impact of gut microbiota on gender-specific differences in immunity. *Front*
6144 *Immunol*, 8(30), 754.

6145 Gibson, G. R., Hutkins, R., Sanders, M. E., Prescott, S. L., Reimer, R. A.,
6146 Salminen, S., Scott, K., Stanton, C., *et al.* (2017). Expert consensus document:
6147 The International Scientific Association for Probiotics and Prebiotics (ISAPP)
6148 consensus statement on the definition and scope of prebiotic. *Nat Rev*
6149 *Gastroenterol Hepatol*, 11(8), 506–514.

6150 Gilbert, J. A., Blaser, M. J., Caporaso, J. G., Jansson, J. K., Lynch, S. V., Knight,
6151 R. (2018). Current understanding of the human microbiome. *Nat Med*, 24(4),
6152 392–400.

6153 Hernandez-Hernandez, O., Sanz, M. L., Kolida, S., Rastall, R. A, Moreno, F. J.
6154 (2011). *In vitro* fermentation by human gut bacteria of proteolytically digested
6155 caseinomacropeptide nonenzymatically glycosylated with prebiotic
6156 carbohydrates. *J Agric Food Chem*, 59(22), 11949–11955.

6157 Hill, T. R., Mendonça, N., Granic, A., Siervo, M., Jagger, C., Seal, C. J., Kerse,
6158 N., Wham, C., Adamson, A.J., Mathers, J. C. (2016). What do we know about
6159 the nutritional status of the very old? Insights from three cohorts of advanced age
6160 from the UK and New Zealand. *Proc Nutr Soc*, 75(03), 420–430.

6161 Jankovic, N., Geelen, A., Streppel, M. T., de Groot, L. C., Kiefte-de Jong, J. C.,
6162 Orfanos, P., Bamia, C., Trichopoulou, A., Boffetta, P., *et al.* (2015). WHO
6163 guidelines for a healthy diet and mortality from cardiovascular disease in
6164 European and American elderly: the CHANCES project. *Am J Clin Nutr*, 102(4),
6165 745–756.

6166 Jankovic, N., Geelen, A., Streppel, M. T., de Groot, L. C. P. G. M., Orfanos, P.,
6167 van den Hooven, E. H., Pikhart, H., Boffetta, P., Trichopoulou, A., *et al.* (2014).
6168 Adherence to a healthy diet according to the World Health Organization
6169 guidelines and all-cause mortality in elderly adults from Europe and the United
6170 States. *Am J Epidemiol*, 180(10), 978–988.

6171 Knight, R., Vrbanc, A., Taylor, B. C., Aksenov, A., Callewaert, C., Debelius, J.,
6172 Gonzalez, A., Kosciulek, T., McCall, L. I., *et al.* (2018). Best practices for
6173 analysing microbiomes. *Nat Rev Microbiol*, 16(7), 410–422.

6174 Kundu, P., Blacher, E., Elinav, E., Pettersson, S. (2017). Our gut microbiome:
6175 The evolving inner self. *Cell*, 171(7), 1481–1493.

6176 Le Chatelier, E., Nielsen, T., Qin, J., Prifti, E., Hildebrand, F., Falony, G.,
6177 Almeida, M., Arumugam, M., Batto, J.-M., Kennedy, S., *et al.* (2013). Richness
6178 of human gut microbiome correlates with metabolic markers. *Nature*, 500(7464),
6179 541–546.

6180 Lloyd-price, J., Abu-ali, G., Huttenhower, C. (2016). The healthy human
6181 microbiome. *Genome Med*, 8:51.

6182 Makki, K., Deehan, E. C., Walter, J., Bäckhed, F. (2018). The impact of dietary
6183 fiber on gut microbiota in host health and disease. *Cell Host Microbe*, 23(6),
6184 705–715.

6185 Marchesi, J. R., Adams, D. H., Fava, F., Hermes, G. D. A., Hirschfield, G. M.,
6186 Hold, G., Quraishi, M.N., Kinross, J., *et al.* (2016). The gut microbiota and host
6187 health: a new clinical frontier. *Gut*, 65(2), 330–9.

6188 Martinez-Guryn, K., Hubert, N., Frazier, K., Urlass, S., Musch, M. W., Ojeda, P.,
6189 Pierre, J.F., Miyoshi, J., Sontag, T. *et al.* (2018). Small intestine microbiota
6190 regulate host digestive and absorptive adaptive responses to dietary lipids. *Cell*
6191 *Host Microbe*, 23(4), 458–469.e5.

6192 Moeller, A. H. (2017). The shrinking human gut microbiome. *Curr Opin*
6193 *Microbiol*, 38, 30–35.

6194 Moore, K., Hughes, C. F., Ward, M., Hoey, L., McNulty, H. (2018). Diet,
6195 nutrition and the ageing brain: current evidence and new directions. *Proc Nutr*
6196 *Soc*, 77(02), 152–163.

6197 Neuman, H., Debelius, J. W., Knight, R., Koren, O. (2015). Microbial
6198 endocrinology : the interplay between the microbiota and the endocrine system.
6199 *FEMS Microbiol Rev*, 39(4), 509–521.

6200 Oliveira, D. L., Wilbey, R. A., Grandison, A. S., Roseiro, L. B. (2015). Milk
6201 oligosaccharides: A review. *Int J Dairy Technol*, 68(3), 305–321.

6202 O’Toole, P. W., Marchesi, J. R., Hill, C. (2017). Next-generation probiotics: the
6203 spectrum from probiotics to live biotherapeutics. *Nat Microbiol*, 2(5), 17057.

6204 Pascal, V., Pozuelo, M., Borruel, N., Casellas, F., Campos, D., Santiago, A.,
 6205 Martinez, X., Varela, E., Sarrabayrouse, G. *et al.* C. (2017). A microbial
 6206 signature for Crohn's disease. *Gut*, 66(5), 813-822.

6207 Salazar, N., Valdés-Varela, L., González, S., Gueimonde, M., de los Reyes-
 6208 Gavilán, C. G. (2017). Nutrition and the gut microbiome in the elderly. *Gut*
 6209 *Microbes*, 8(2), 82–97.

6210 Schmidt, T. S. B., Raes, J., Bork, P. (2018). The Human Gut Microbiome: From
 6211 Association to Modulation. *Cell*, 172(6), 1198–1215.

6212 Sokol, H., Pigneur, B., Watterlot, L., Lakhdari, O., Bermúdez-Humarán, L. G.,
 6213 Gratadoux, J.-J., Blugeon, S., Bridonneau, C., *et al.* (2008). *Faecalibacterium*
 6214 *prausnitzii* is an anti-inflammatory commensal bacterium identified by gut
 6215 microbiota analysis of Crohn disease patients. *Proc Nat Acad Sci USA*, 105(43),
 6216 16731–6.

6217 Sonnenburg, J. L., and Bäckhed, F. (2016). Diet-microbiota interactions as
 6218 moderators of human metabolism. *Nature*, 535(7610), 56–64.

6219 Vandeputte, D., Kathagen, G., D'hoë, K., Vieira-Silva, S., Valles-Colomer, M.,
 6220 Sabino, J., Wang, L., Tito, R.Y., *et al.* (2017). Quantitative microbiome profiling
 6221 links gut community variation to microbial load. *Nature*, 551(7681), 507–511.

6222 Wang, J., Wang, J., Pang, X., Zhao, L., Tian, L., Wang, X. (2016). Sex
 6223 differences in colonization of gut microbiota from a man with short-term
 6224 vegetarian and inulin-supplemented diet in germ-free mice. *Sci Rep*, 6(1), 36137.

6225 Zaneveld, J. R., McMinds, R., Vega Thurber, R. (2017). Stress and stability:
 6226 applying the Anna Karenina principle to animal microbiomes. *Nat Microbiol*,
 6227 2(9), 17121.

6228 Zhao, L., Zhang, F., Ding, X., Wu, G., Lam, Y. Y., Wang, X., FU, H., Xue, X.,
 6229 Lu, C., Ma, J., Xu, C., Ren, Z., Xu, Y., *et al.* (2018). Gut bacteria selectively
 6230 promoted by dietary fibres alleviate type 2 diabetes. *Science*, 359(6380), 1151–
 6231 1156.

6232 Zhu, A., Sunagawa, S., Mende, D. R., Bork, P. (2015). Inter-individual
 6233 differences in the gene content of human gut bacterial species. *Genome Biol*,
 6234 16(1), 82.

6235 Zhu, W., Winter, M. G., Byndloss, M. X., Spiga, L., Duerkop, B. A., Hughes, E.
 6236 R., Buttner, L., de Lima Romao, E., Behrendt, C. L., Lopez, C. A., *et al.* (2018).
 6237 Precision editing of the gut microbiota ameliorates colitis. *Nature*, 553(7687),
 6238 208–211.

6239 Zoetendal, E. G., Raes, J., van den Bogert, B., Arumugam, M., Booiijink, C. C.,
 6240 Troost, F.J., Bork, P., Wels, M., de Vos, W. M., Kleerebezem, M. (2012). The
 6241 human small intestinal microbiota is driven by rapid uptake and conversion of
 6242 simple carbohydrates. *ISME J*, 6(7), 1415–1426.

Acknowledgements

Miracles happen and I finally hand in my PhD thesis manuscript. I would like to thank the people that helped me make this happen. First, I thank my supervisor Paul O'Toole for giving me the opportunity of this PhD. I would like to sincerely thank him for the challenge, the straightforward no-beating-around-the-bush corrections and critic on my work that I deeply appreciated (although shocked at first), and the support and the trust all these years that I have been a member of his lab. I would also like to thank my co-supervisors Catherine Stanton and Paul Ross who have always been supportive and willing to assist for the successful completion of this work.

I would like to thank my friends and all current and former members of Lab 438. Lab 438 people, the things we saw (and smelled) under those hoods will haunt us ... eh, sorry, connect us for ever. Special thanks to dr Celine Ribiere that has been a supportive friend and a generous colleague always offering scientific expertise. Many special thanks to dr Jillian Brown who welcomed me when I first came to the lab and has always been there for me. Many thanks to “the girls”, Marta Neto and dr Marta Perez and dr Ana Almeida for being there making the PhD life so much nicer. Another big thanks to Valentina Ambrogi for the long chats and the heart-warming coffee.

I would also like to thank the former lab members Huizi Tan who first showed me what a “great fun” it is to work in the anaerobic cabinet, dr Fabien Cousin for all the protocols he provided, and dr Burkhardt Flemer. Many thanks to the Micro department technical stuff and especially Maurice O'Donoghue and Paddy O'Reilly, not only for solving almost any micro/technical issue I had, but also for our chats.

Thanks to the nurses Patricia Egan and Katie Power that worked with me during my PhD and of course, the donors who generously spared no gram in their donations and saved my PhD.

I would also like to thank my loving family and friends back home, Constantina and Stefanos who I have turned into “gut microbiota lay experts”, Iris who awaits for me enthusiastically in Greece, Maria, Stelios and Marilia who have been great company those last five years. Last but not least, I want to thank my dearest people. My partner for so many years (and now my husband) Panagiotis Mentzelidis for taking this trip with me, always supporting me with smart advice and believing in me. My parents Vasiliki Rachouti and Constantinos Ntemiris that taught me the value of education and I can’t thank them enough for this empowering gift.