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Interfacing Food & Medicine

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Agriculture and Food Development Authority

‘Health-Promoting Properties Encoded within Fermented Food Microbiota’

Paul Cotter

Head of Food Bioscience

paul.cotter@teagasc.ie

UCC University College Cork, Ireland Coláiste na hOllscoile Corcaigh

teagasc Agriculture and Food Development Authority

CIT Institiúid Teicneolaíochta Chorcaí Cork Institute of Technology

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sfi Fondúireacht Eolaíochta Éireann Science Foundation Ireland

Culture independent approaches to study microbial communities

Traditional, culture-based, approaches to microbiology only reveal the 'tip of the iceberg'

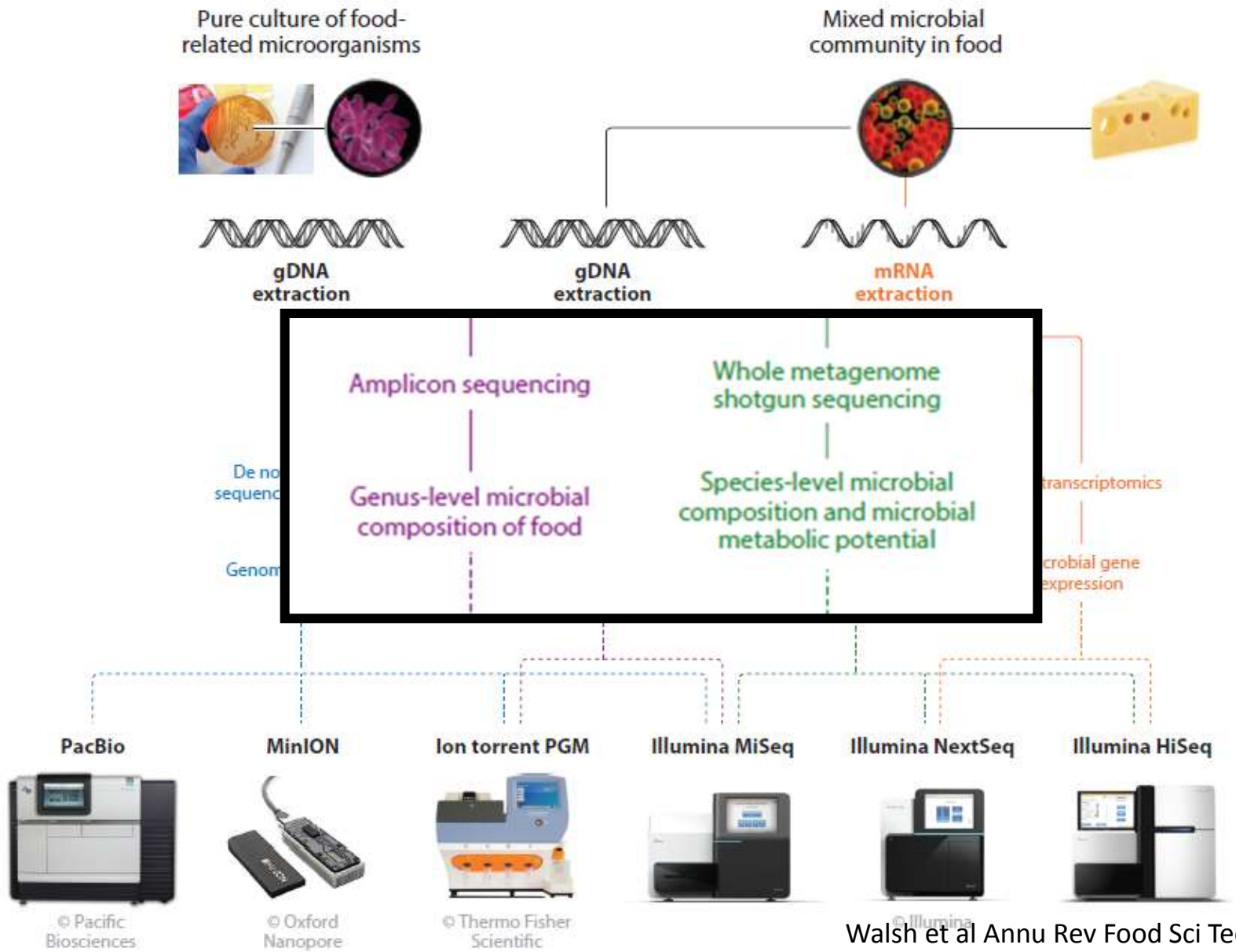
The success of culture based approaches is dependent on having types of agar/culture media/growth conditions that allow all microbes to grow

Such an agar does not exist!!

Indeed only a small % of microbes are easily cultured in the laboratory.

DNA sequencing-based approaches can allow an analysis to the entire population (and the genes present therein) regardless of whether it can be grown or not

Sequencing based approaches to study food microbes

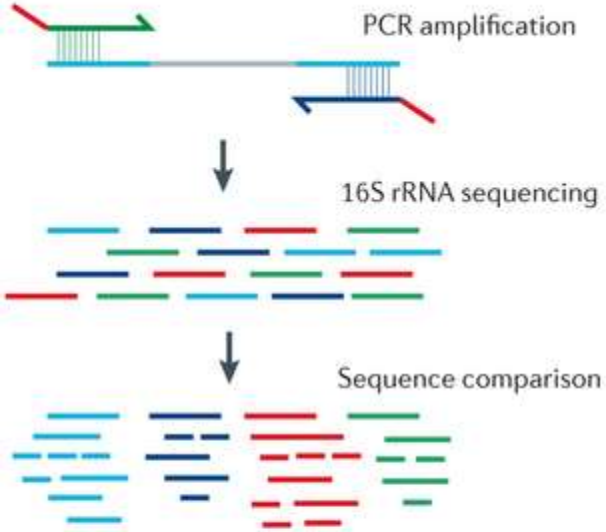


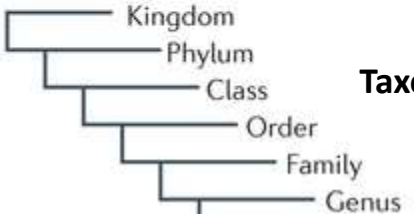
Walsh et al Annu Rev Food Sci Technol 2017

DNA-based community analysis

Community DNA

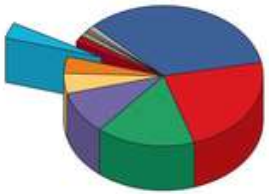
Amplicon sequencing



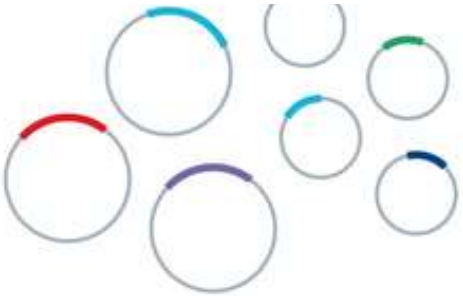


Taxonomy

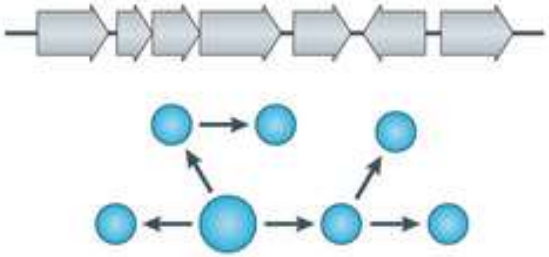
Community Composition



Whole metagenome Shotgun sequencing



Genes or Pathways



Structure of presentation

1. **Health-promoting fermented foods – Introduction**
2. **Microbial composition of selected foods**
3. **Particular focus on kefir**
4. **Feeding studies**
5. **Conclusion**

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Fact vs Fiction!

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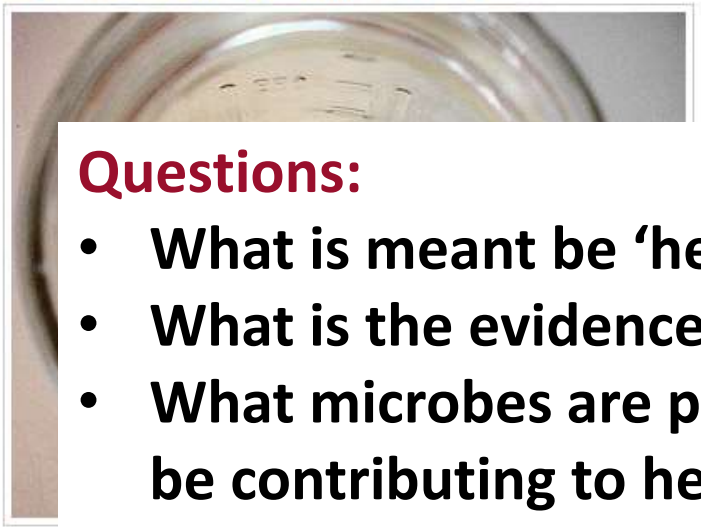
FRIDAY, SEPTEMBER 25, 2009

My Anti-Cancer Treatments: Kefir



Questions:

- **What is meant be 'health-promoting' fermented foods?**
- **What is the evidence that they are health-promoting?**
- **What microbes are present in these foods and how might they be contributing to health benefits?**

Long-term Goal:

- **Harnessing the microbes in these foods to generate fermented foods with genuine health-promoting features.**

by KATIE on MARCH 6, 2013

Everyone knows that drinking tea is good for your health but wouldn't it be great if there were a way to make it *even* healthier? Well actually, there is! (Spoiler alert, it's a *fungus!*)

Fermented beverages

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Table 1. A compilation of various milk, cereal and other fermented beverages popular around the world, with their corresponding microbial populations and substrates.

Product	Substrates	Region	Microflora
Amasi	Milk (cow, various)	Africa (Zimbabwe)	*Lactococcus* (*L. lactis*), *Lactobacillus*, *Leuconostoc*, *Enterococcus*. Uncharacterised fungal component
Aryan	Milk (cow, various)	Turkey	LAB: *Lactobacillus bulgaricus*, *Streptococcus thermophilus*
Garris	Milk (camel)	Africa (Sudan)	Bacteria: *Lactobacillus* (*Lb. paracasei*, *Lb. fermentum* and *Lb. plantarum*), *Lactococcus*, *Enterococcus*, *Leuconostoc*. Uncharacterised fungal component
Kefir	Milk (cow, various)	Eastern Europe (Caucasian region)	Bacteria: *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Acetobacter*; Yeast: *Naumovozyma*, *Kluyveromyces*, *Kazachstania*
Kivuguto	Milk (cow)	Africa (Rwanda)	LAB: *Leuconostoc* (*Leu. mesenteroides*, *Leu. pseudomesenteroides*) and *L. lactis*. Uncharacterised fungal component
Koumiss/Airag	Milk (horse)	Asia/Russia	LAB: *Lactobacillus*; Yeast: *Kluyveromyces*, *Saccharomyces* and *Kazachstania*
Kumis	Milk (cow)	South America (Columbia)	Bacteria: *Lb. cremoris*, *L. lactis*, *Enterococcus* (*E. faecalis*, *E. faecium*); Yeast: *Galactomyces geotrichum*, *Pichia kudriavzevii*, *Clavispora lusitaniae*, *Candida tropicalis*
Nyarmie	Milk (camel)	Africa (Ghana)	LAB: *Leu. mesenteroides*, *Lb. bulgaricus*, *Lb. helveticus*, *Lb. lactis*, *Lactococcus lactis*; Yeast: *Saccharomyces cerevisiae*
Rob	Milk (unspecified)	Africa (Sudan)	LAB: *Lb. fermentum*, *Lb. acidophilus*, *L. lactis*, *Streptococcus salivarius*; Yeast: *Saccharomyces cerevisiae*, *Candida kefyr*
Suusac	Milk (unspecified)	Africa (Kenya)	LAB: *Leu. mesenteroides*, *Lactobacillus* (*Lb. plantarum*, *Lb. cruvatus*, *Lb. salivarius*, *Lb. Raffinolactis*); Yeast: *Candida krusei*, *Geotrichum penicillatum*, *Rhodotorula mucilaginosa*
Shubat	Milk (camel)	China	Bacteria: *Lactobacillus* (*Lb. sakei*, *Lb. Helveticus*, *Lb. brevis*) *Enterococcus* (*E. faecium*, *E. faecalis*), *Leu. lactis* and *Weissella hellenica*; Yeast: *Kluyveromyces marxianus*, *Kazachstania unisporus*, and *Candida ethanolica*
Amazake	Rice	Japan	Fungi: *Aspergillus* spp
Boza	Various (barley, oats, rye, millet, maize, wheat or rice)	Balkans (Turkey, Bulgaria)	LAB: *Leuconostoc* (*Leu. paramesenteroides*, *Leu. sanfranciscensis*, *Leu. mesenteroides*), *Lactobacillus* (*Lb. plantarum*, *Lb. acidophilus*, *Lb. fermentum*); Yeast: *Saccharomyces* (*S. uvarum*, *S. cerevisiae*), *Pichia fermentans*, *Candida* spp.
Bushera	Sorghum, millet flour,	Africa (Uganda)	Bacteria: *Lactobacillus*, *Streptococcus*, *Enterococcus*. Uncharacterised fungal component
Koko	Cereal (pearl millet)	Africa (Ghana)	Bacteria: *Weissella confusa*, *Lb. fermentum*, *Lb. salivarius*, *Pediococcus* spp. Uncharacterised fungal component
Sour Water Kvass	Rye bread, rye and barley malt/flour,	Russia	LAB: *Lb. casei*, *Leu. mesenteroides*; Yeast: *Saccharomyces cerevisiae*
Mahewu	Maize, sorghum/millet	Africa (Zimbabwe)	Unknown
Pozol	Maize	Mexico (Southeast)	Bacteria: *L. lactis*, *Streptococcus suis*, *Lactobacillus* (*Lb. plantarum*, *Lb. casei*, *Lb. alimentarium*, *Lb. delbruekii*), *Bifidobacterium*, *Enterococcus*. Uncharacterised fungal component
Togwa	Maize flour, finger millet malt,	Africa (Tanzania)	LAB: *Lactobacillus* spp.; Yeast: *Saccharomyces cerevisiea*, *Candida* spp.
Hardaliye	Grapes/mustard seeds/cherry leaf	Turkey	LAB: *Lactobacillus* spp. Uncharacterised fungal component
Kombucha	Tea	China, Worldwide	Bacteria: *Gluconacetobacter* (*G. xylinus*), *Acetobacter*, *Lactobacillus*; Yeast: *Zygosaccharomyces*, *Candida*, *Hanseniaspora*, *Torulaspora*, *Pichia*, *Dekkera*, *Saccharomyces*
Water Kefir	Water/sucrose	Mexico, Worldwide	Bacteria: *Lactobacillus* (*Lb. casei*, *Lb. hilgardii*, *Lb. brevis*, *Lb. plantarum*), *L. lactis*, *Leu. mesenteroides*, *Zymomonas*; Yeast: *Dekkera* (*D. anomola*, *D. bruxellensis*), *Hanseniaspora* (*H. valbyensis*, *H. vineae*) *Saccharomyces cerevisiae*, *Lachancea fermentati*, *Zygosaccharomyces* (*Z. lentus*, *Z. florentina*)

Amasi, Aryan, Garris, Kefir, Kivuguto, Koumiss/Arag, Kumis, Nyarmie, Rob, Suusac, Shubat, Amazake, Boza, Bushera, Koko, Sour water, Kvass, Mahewu, Pozol, Togwa, Hardaliye, Kombucha, Water Kefir

CrossMark

Fermented beverages with health-promoting potential: Past and future perspectives

Alan J. Marsh[a,b,c], Colin Hill[b,c], R. Paul Ross[a,b] and Paul D. Cotter[a,b,*]

Trends in Food Science & Technology 38 (2014) 113–124

Health benefits of fermented foods

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Recent human studies involving fermented foods

Type 2 diabetes,
Coronary heart disease,
mortaility,
Cardiovascular disease,
Obesity,
Impaired glucose metabolism,
Hyperlipidemia,
Hypertension,
Osteoporosis,
Muscle soreness,
Depression,
Brain activity,
Infection control,
Bowel movement,
Irritable bowel syndrome

		consumption for 6 months	(1.1 days vs. 2.5 days, $P < 0.05$) and improved bowel movements (constipation: 0.8 times vs. 1 time, $P < 0.05$) (diarrhea: 0.3 days vs. 0.7 days, $P < 0.05$)	
IBS	Rye bread	Adult, PC, DB, crossover RCT, $N = 87$, 7-8 pieces per day, 4 week diet, 4 week washout, 4 week switch	Signs of IBS (flatulence, abdominal pain, cramps and stomach rumbling) were milder on the low-FODMAP rye bread (P values: 0.04; 0.048; 0.01 and 0.001)	Laatikainen *et al.* [27]

T2D, type 2 diabetes; CHD, coronary heart disease; IGM, impaired glucose metabolism; CVD, cardiovascular disease; IBS, irritable bowel syndrome; PC, placebo controlled; DB, double blinded; RCT, randomized clinical trial; HR, hazard ratio; FDP, fermented dairy products; FM, fermented milk; SBP, systolic blood pressure; Hg, mercury; DBP, diastolic blood pressure; BMD, bone mineral density; FODMAP, fermentable oligosaccharides, disaccharides, monosaccharides, polyols.

ELSEVIER
Available online at www.sciencedirect.com
ScienceDirect

ISAPP
International Scientific Association for Probiotics and Prebiotics

Health benefits of fermented foods: microbiota and beyond

Maria L Marco[1], Dustin Heeney[1], Sylvie Binda[2], Christopher J Cifelli[3], Paul D Cotter[4], Benoit Foligné[5], Michael Gänzle[6], Remco Kort[7], Gonca Pasin[8], Anne Pihlanto[9], Eddy J Smid[10] and Robert Hutkins[11]

Traditional or novel food fermentations with probiotic organisms

Species with recognized probiotic activity[a]	Fermented foods that reproducibly contain high cell counts of strains of the species
Lactobacillus acidophilus	NSLAB long ripened cheese
Lactobacillus johnsonii	Kefir
Lactobacillus fermentum	Bushera, ting and other African cereal porridges and beverages
Lactobacillus plantarum	Salami[b], sauerkraut/kimchi, olives, others
Lactobacillus paracasei	Salami, kvass, NSLAB in long-ripened cheese
Lactobacillus rhamnosus	'Villi', fermented oatmeal
Lactobacillus casei	NSLAB long-ripened cheese

NSLAB, non-starter lactic acid bacteria.
[a] According to Health Canada.
[b] Fermented foods are printed in bold if presence of the probiotic organism depends on its addition as competitive starter culture.

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Water kefir

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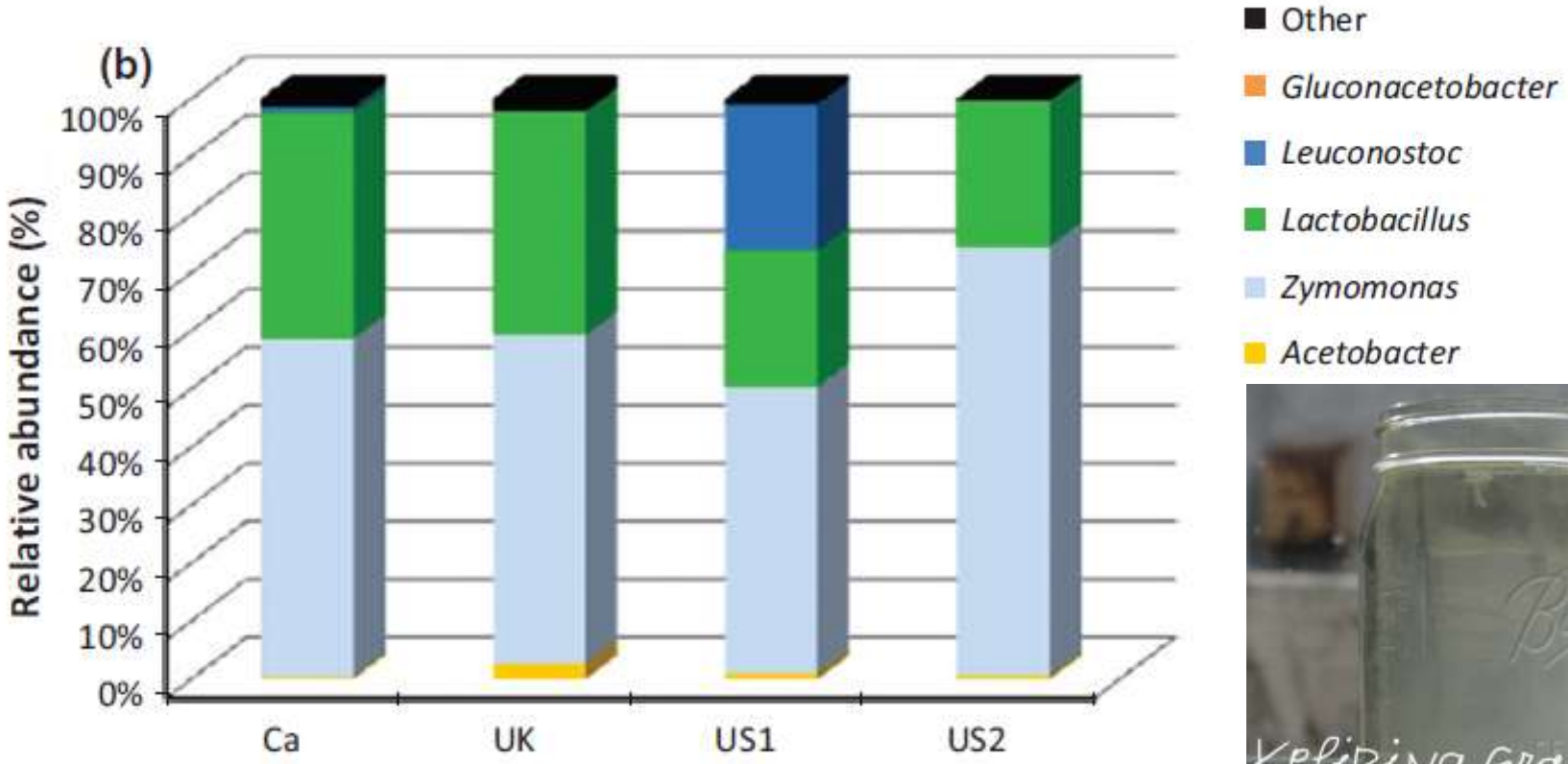
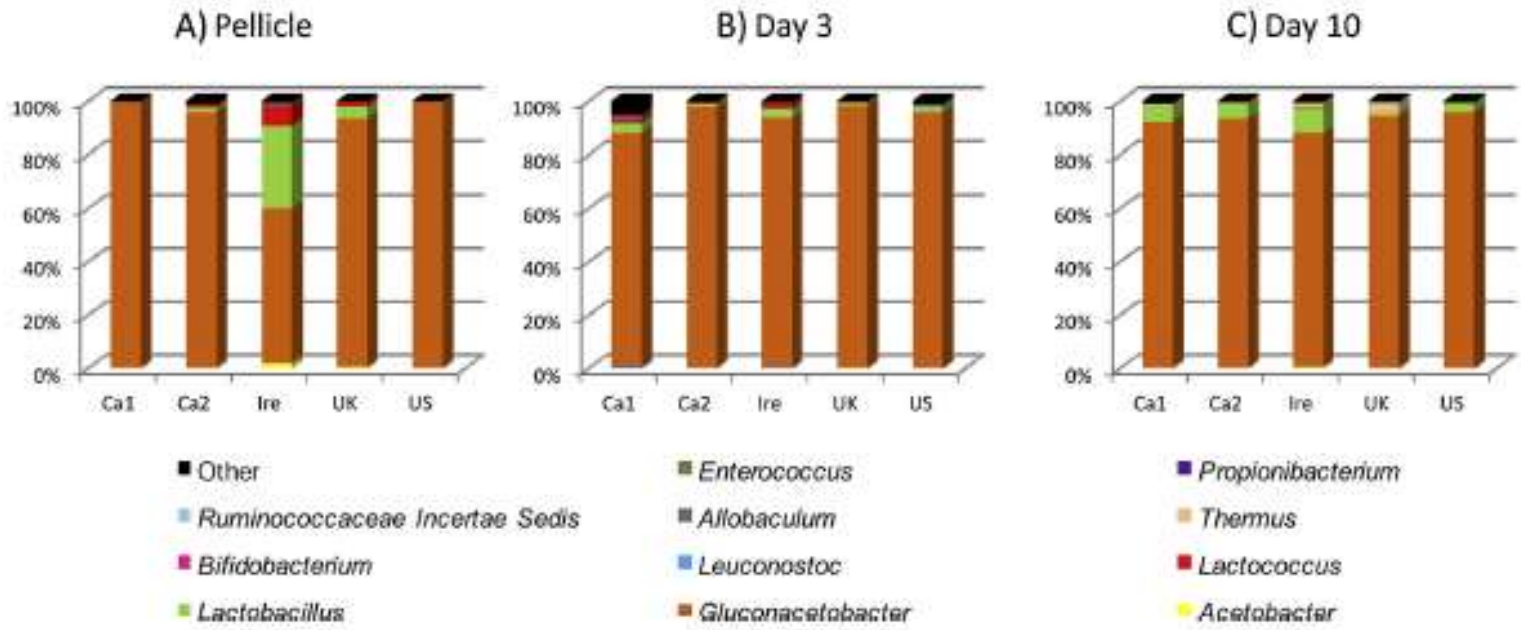


RESEARCH LETTER

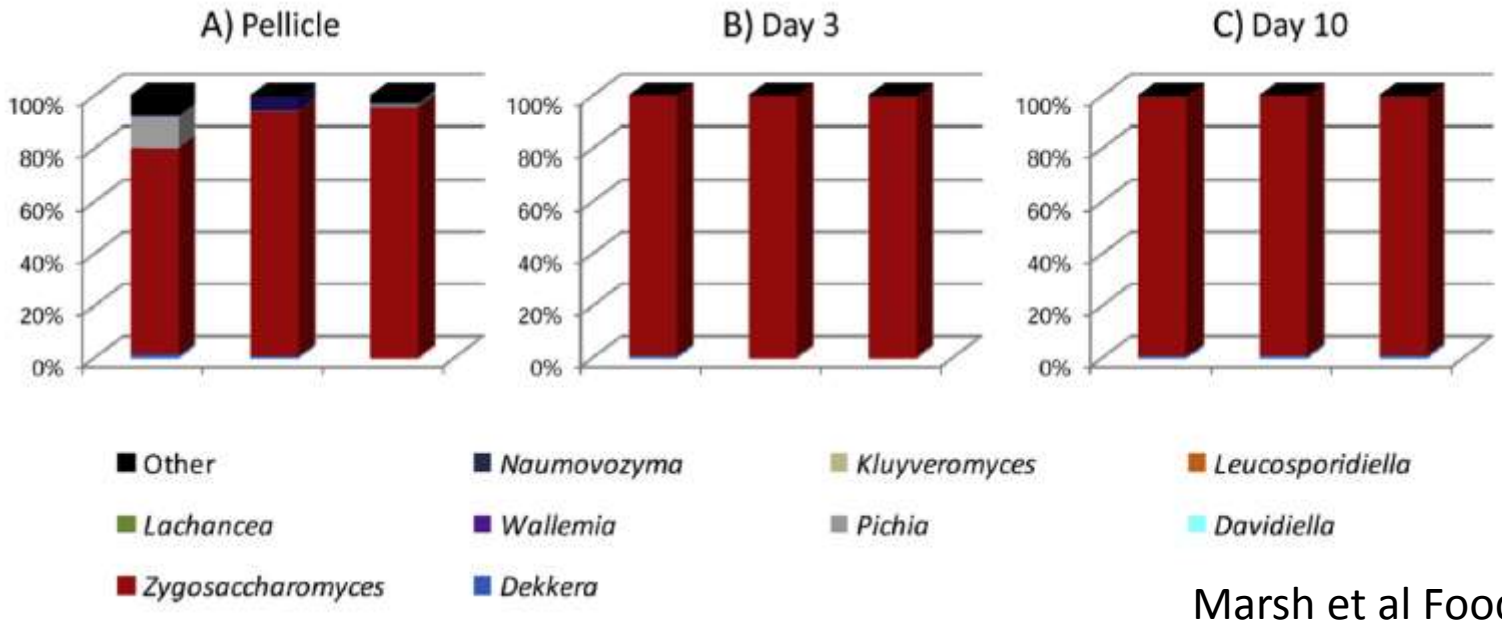
Sequence-based analysis of the microbial composition of water kefir from multiple sources

Alan J. Marsh[1,2,3], Orla O'Sullivan[1], Colin Hill[2,3], R. Paul Ross[1,2] & Paul D. Cotter[1,2]

Kombucha



Bacteria



Fungi

Marsh et al Food Microbiol 2014

Nunu

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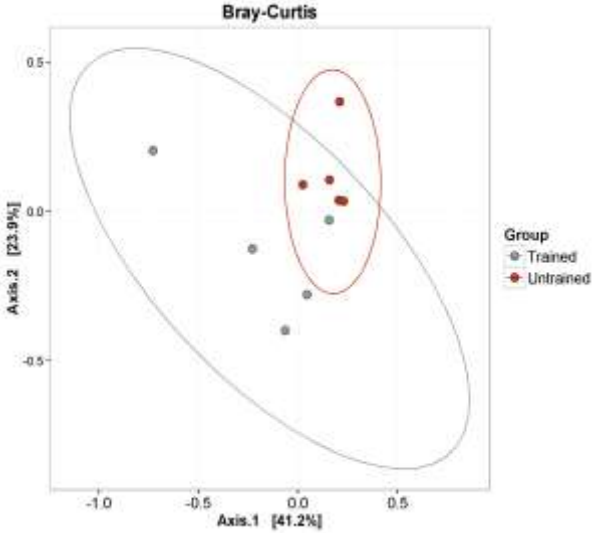
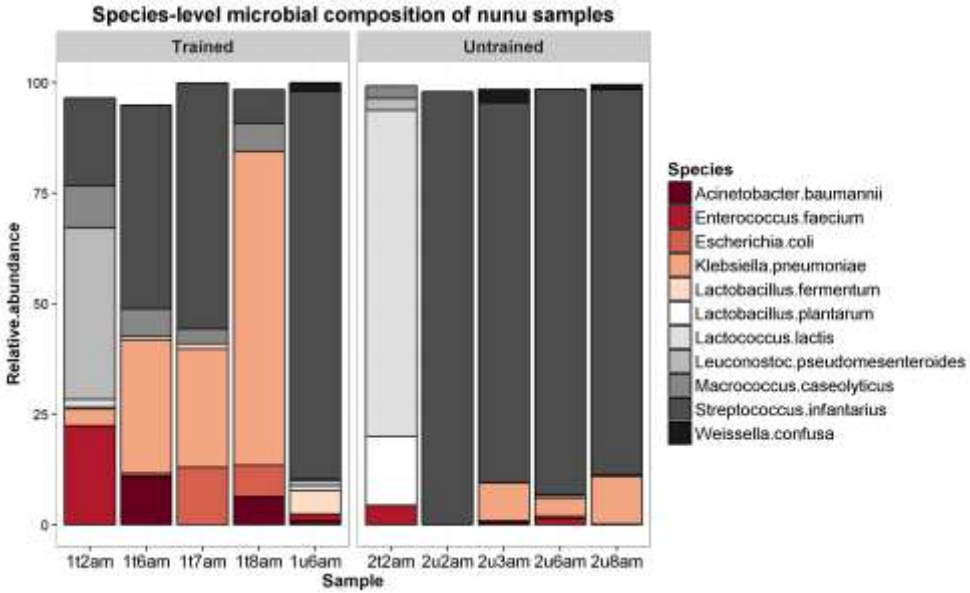
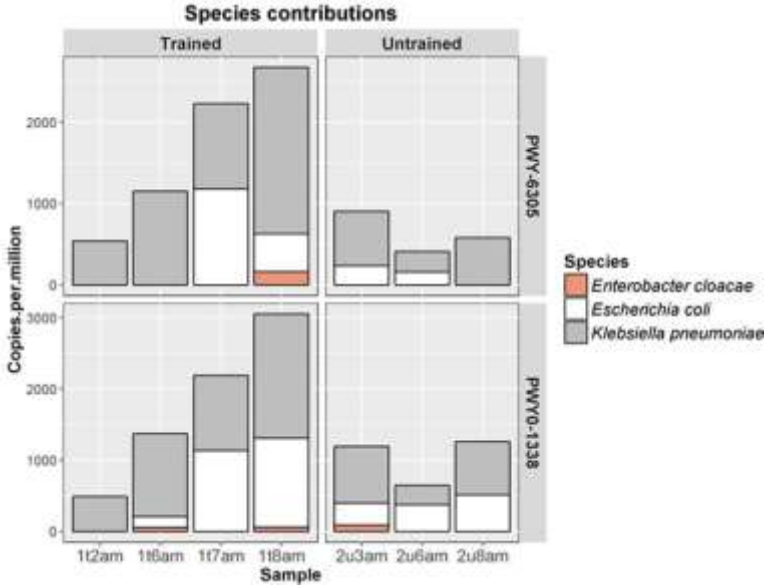
Spontaneously fermented yoghurt-like milk product from Africa….short shelf-life
Trained vs Untrained producers……







Walsh, Scott et al. AEM 2017

Fermented Food Research

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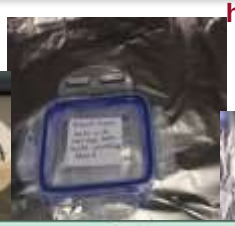
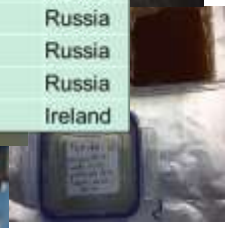



id	Sample N	Name	Type	Origin
1	FS00a_2016	Wagashi	Cheeses, Dairy product	Benin
2	FS01_2016	Bread Kvass	Beverage	Russia
3	FS02_2016	Carrot Kimchi	Fermented Vegetable	UK
4	FS03_2016	Boza	Beverage	UK
5	FS04_2016	Lemon	Preserve	UK
6	FS05_2016	Turnip	Fermented Vegetable	UK
7	FS06_2016	Orange	Preserve	UK
8	FS07_2016	Krauthehi	Fermented Vegetable	Germany
9	FS08_2016	Tepache	Beverage	Mexico
10	FS09_2016	Ginger Beer	Beverage	UK
11	FS10_2016	Tempeh	Fermented Grain	UK
12	FS11_2016	Cucumber	Fermented Vegetable	UK
13	FS12_2016	Milk Kefir	Beverage	UK
14	FS13_2016	Water Kefir	Beverage	UK
15	FS14_2017	Sushi Ginger	Condiment, Pickled vegetables	China
16	FS15_2017	Fukujin Zuke	Condiment, Pickled radish & vegetables	China
17	FS16_2017	Tofu Chilli	Fermented condiment	China
18	FS17_2017	Daikon	Fermented condiment	China
19	FS18_2017	Pickled Bamboo	Condiment, Pickeld vegetables	China
20	FS19_2017	Pickled vegetables	Condiment	China
21	FS20_2017	Black Garlic	Condiment, Pickeld vegetables	England
22	FS21_2017	Papouis Halloumi Cheese	Cheeses, Dairy product	Cyprus
23	FS22_2017	Raw Sauerkraut & Juniper berries	Cabbage	Ireland
24	FS23_2017	Brown rice amazake	Brown Rice	Japan
25	FS24_2017	Beetroot Kvass	Beetroot	Ireland
26	FS25_2017	Kefir and fennel soup	Dairy Soup	Ireland
27	FS26_2017	Mead	Fermented honey	Ireland
28	FS27_2017	Saurkraut	Cabbage	Ireland
29	FS28_2017	Dill dearg	saurkraut	Ireland
30	FS29_2017	Kimchi	cabbage	Ireland
31	FS30_2017	Golden child	Saurkraut	Ireland
32	FS31_2017	Water Kefir Hibiscus	Beverage	Ireland
33	FS32_2017	Water Kefir lemon	Beverage	Ireland
34	FS33_2017	Water Kefir Ginger	Beverage	Ireland
35	FS34_2017	Kombucha Vinegar	Liquid condiment	Ireland
36	FS35_2017	RYAZHENKA	beverage	Russia
37	FS36_2017	Agousha	beverage	Russia
38	FS37_2017	ROSTAGROÈKPORT VOROŽNyJ	Snack	Russia
39	FS38_2017	RUŽ'A	Cheeses, Dairy product	Russia
40	FS39_2017	Raw Milk Kefir	Beverage	Ireland







Grouped by Fermented Food type

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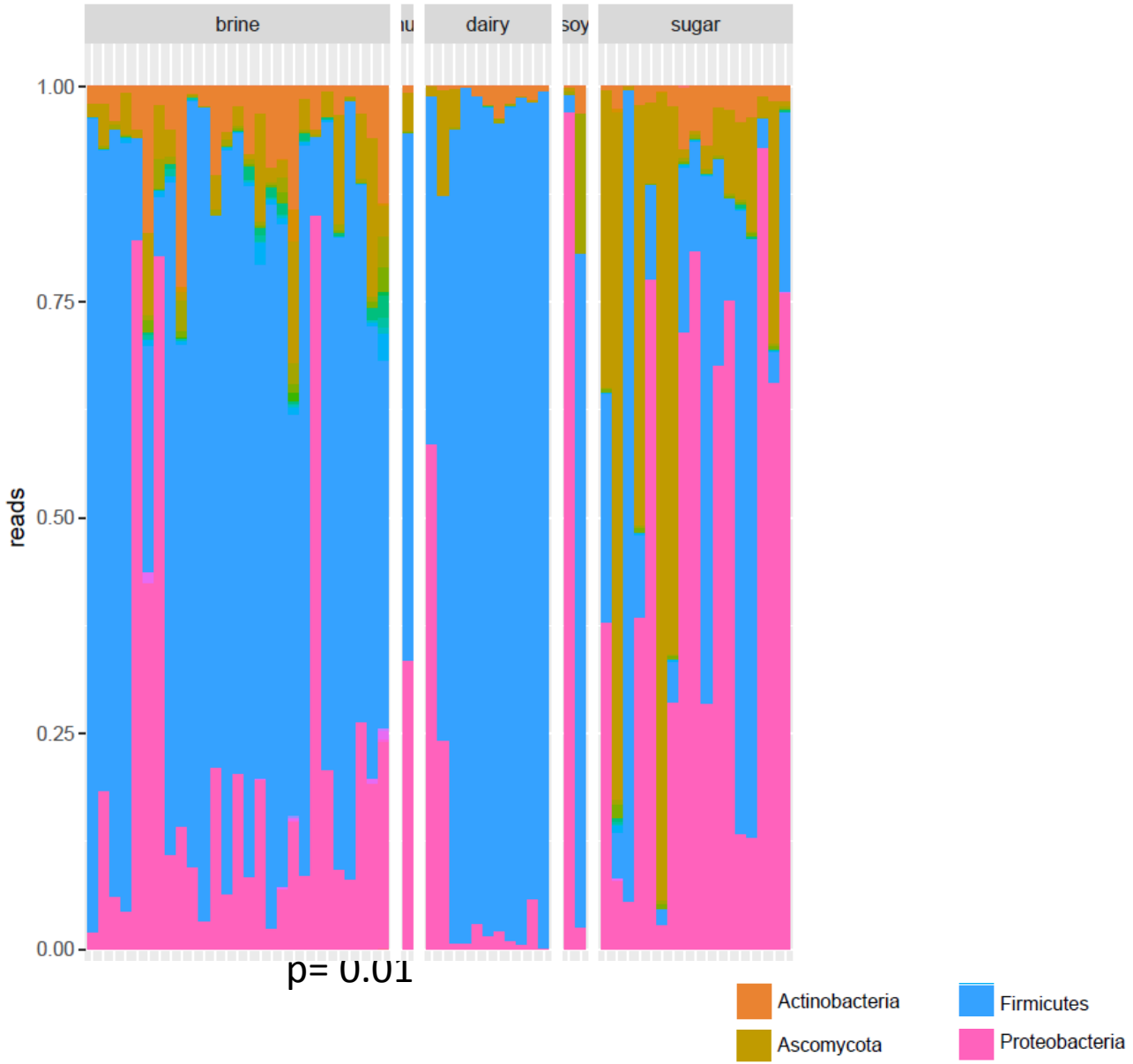
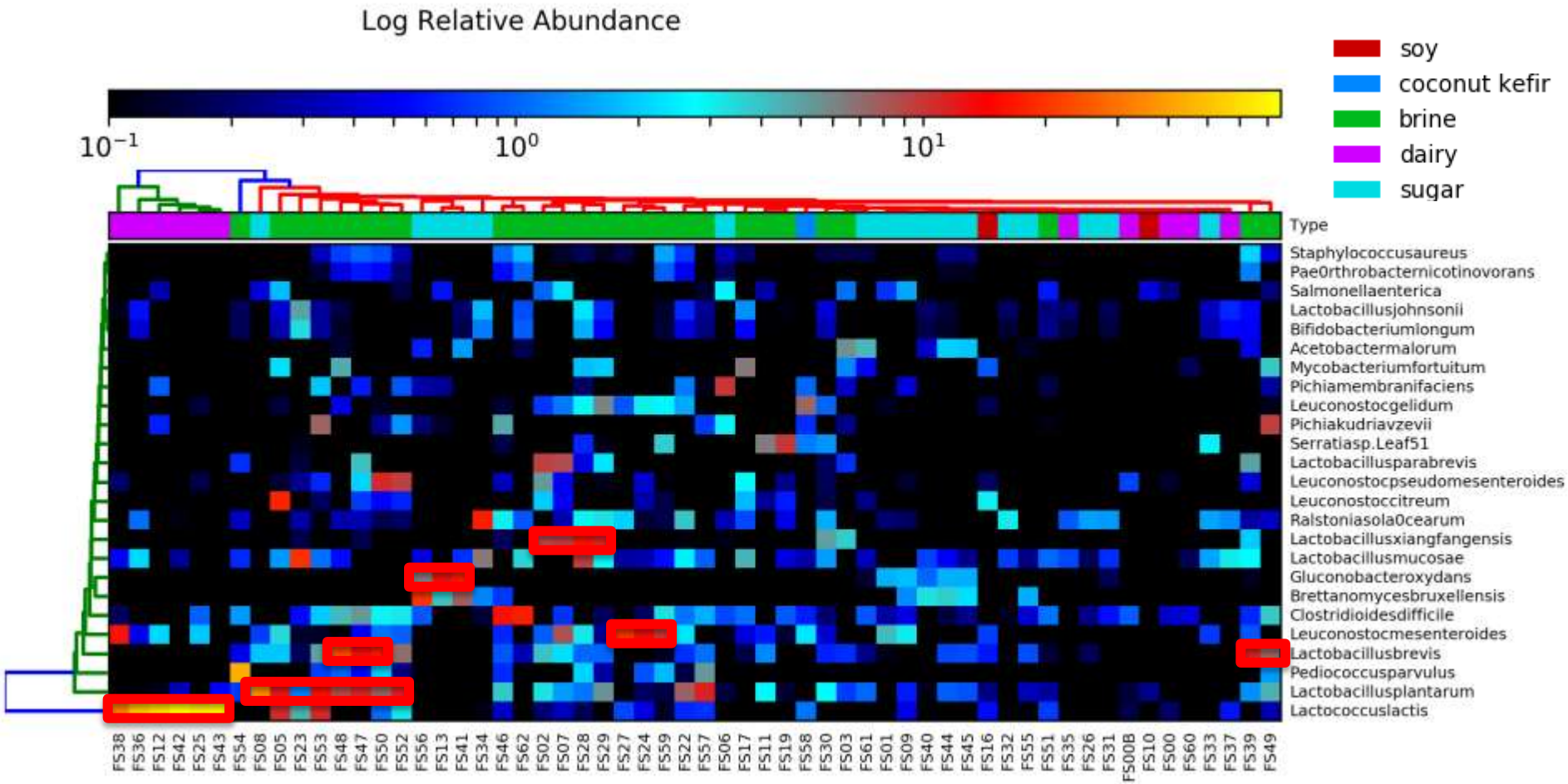


Species level assignment

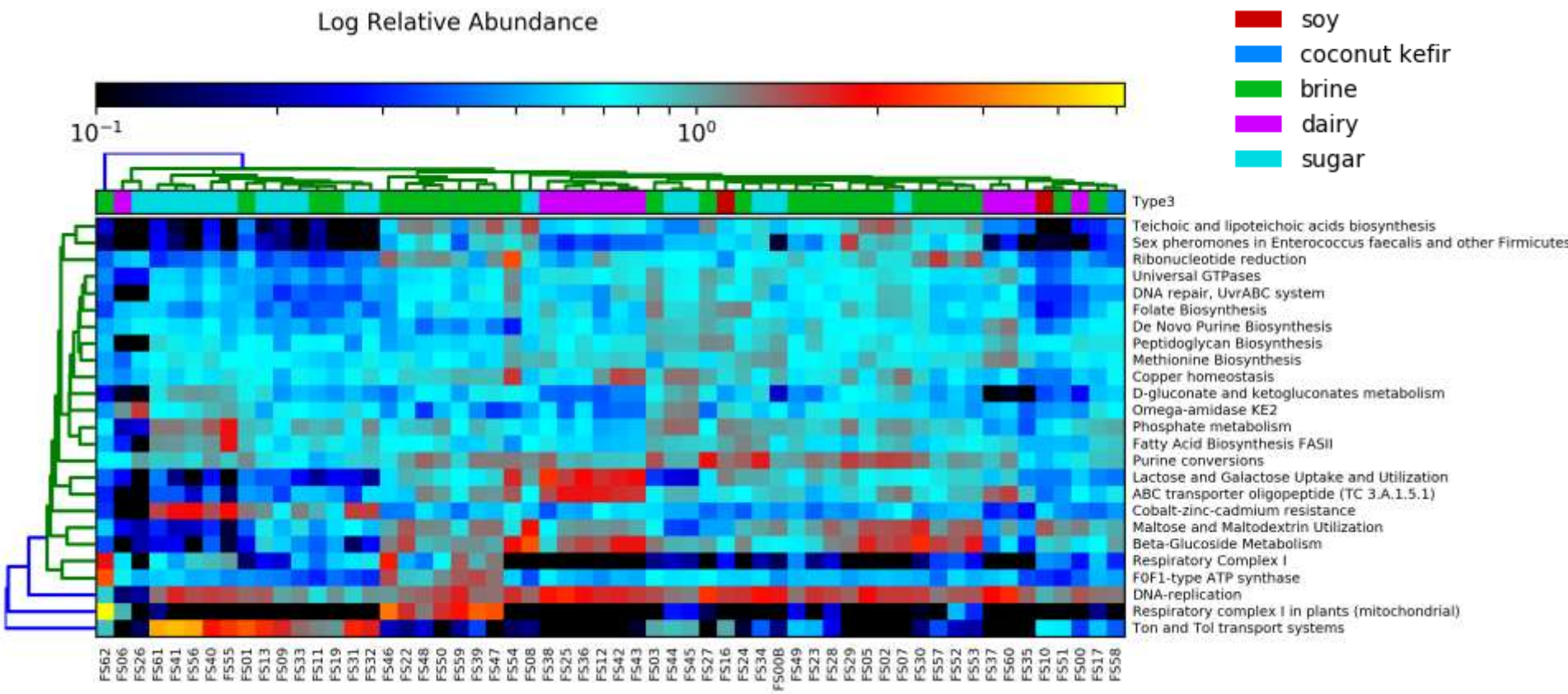


Lactococcus lactis
Lactobacillus plantarum
Lactobacillus brevis

Leuconostoc mesenteroides
Gluconobacter oxydans
Lactobacillus mucosae

Functionalities associated with fermented food types

Superfocus



Bacteriocin production – putative probiotic trait

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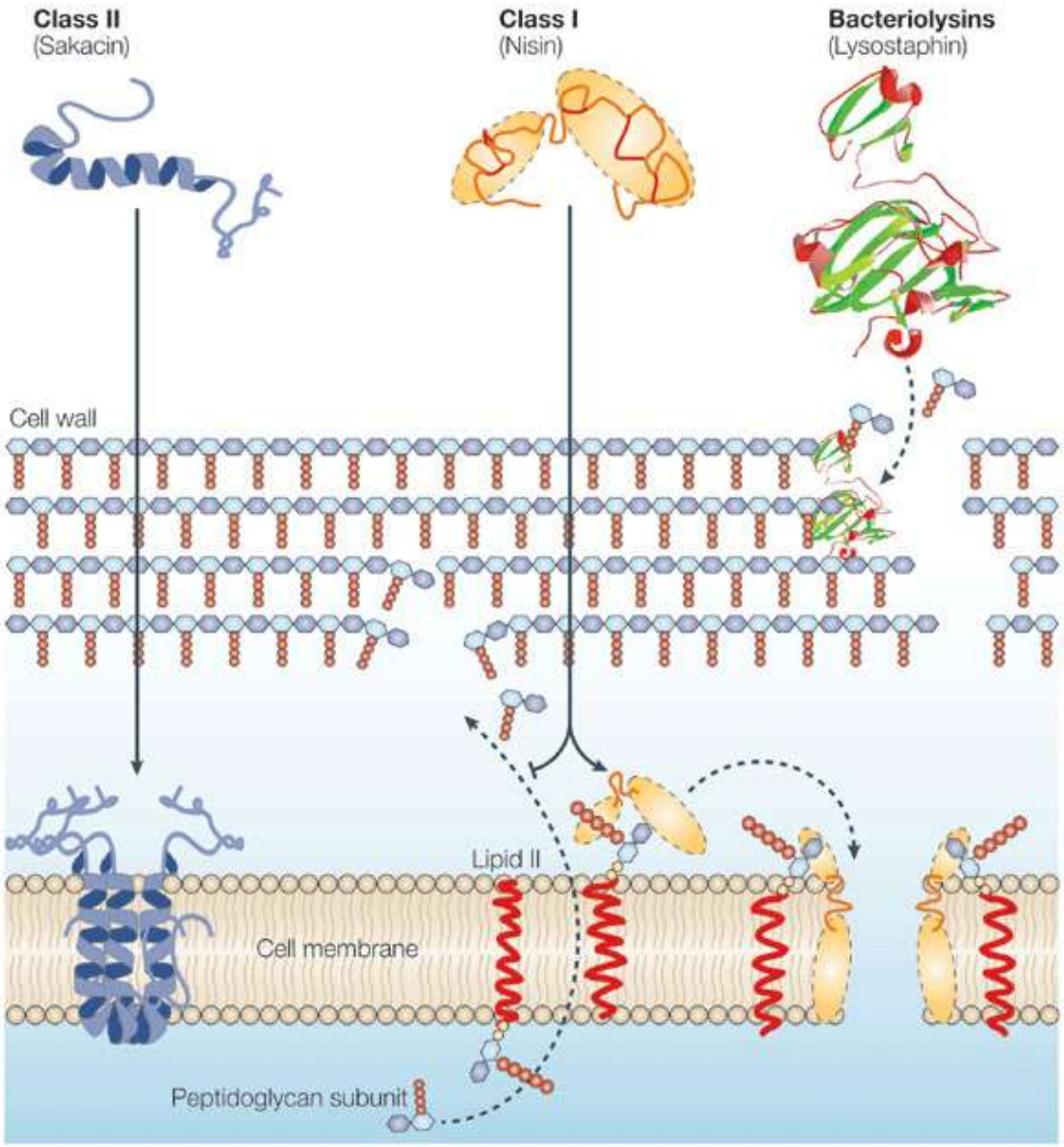
Cotter et al Nature Reviews Microbiol 2005



Distribution of bacteriocin genes in cheeses

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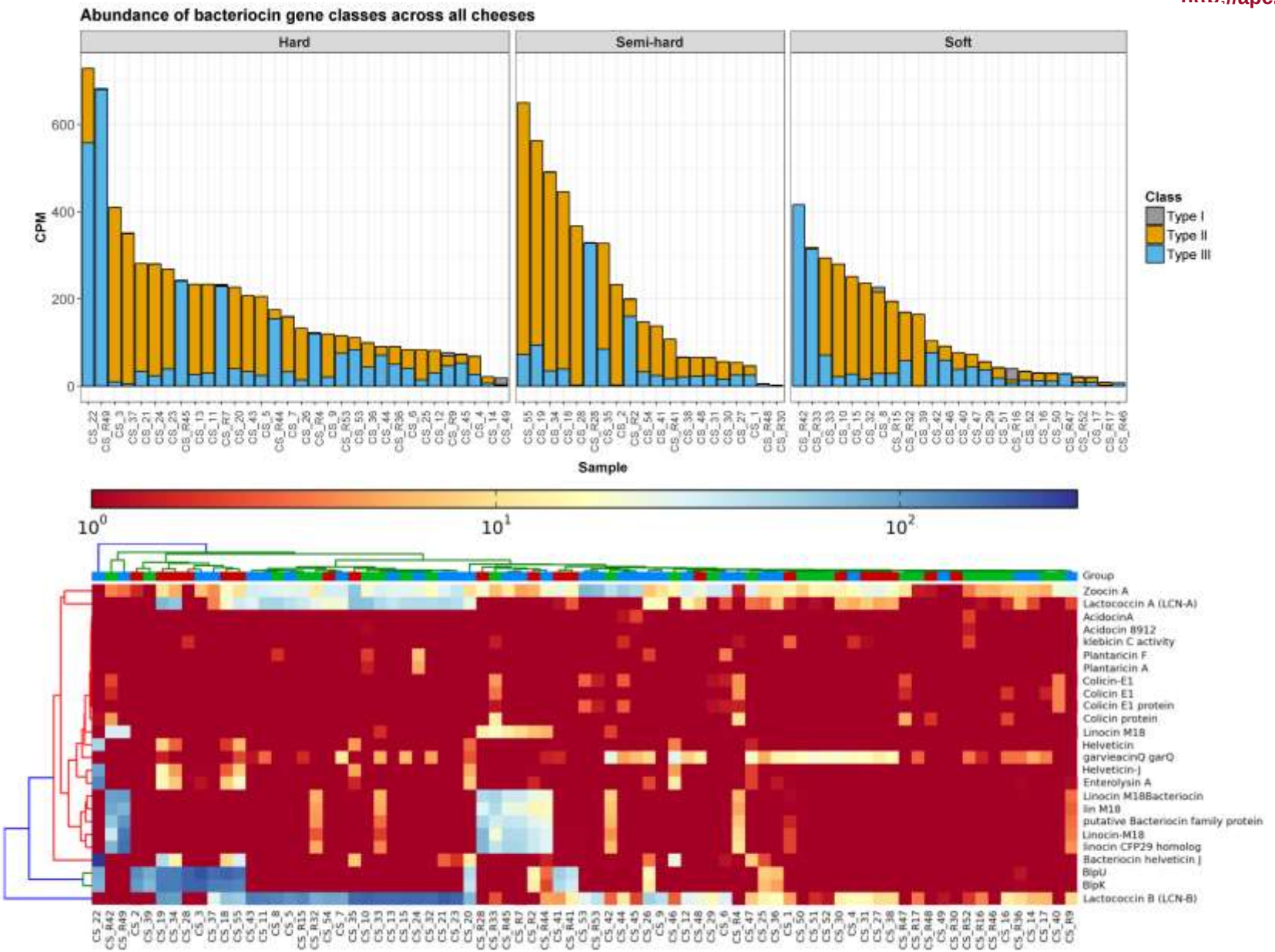

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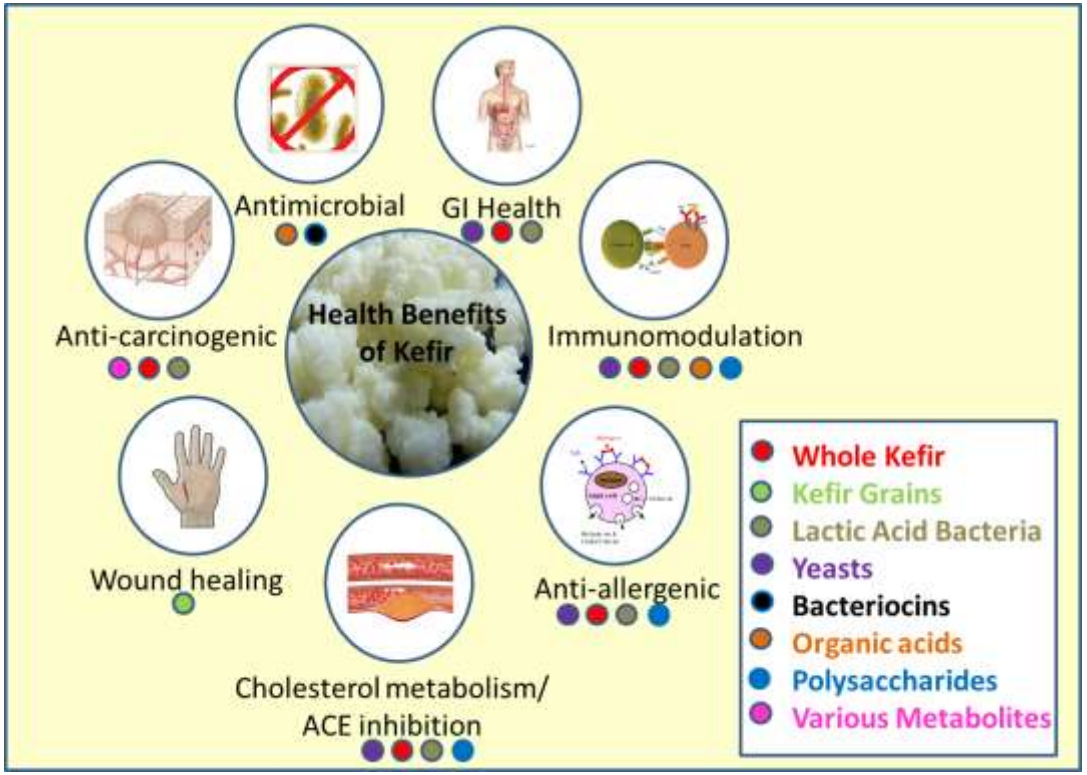
Health Benefits of Kefir

Kefir = milk fermented with kefir grain (containing a consortium of bacteria and yeasts)

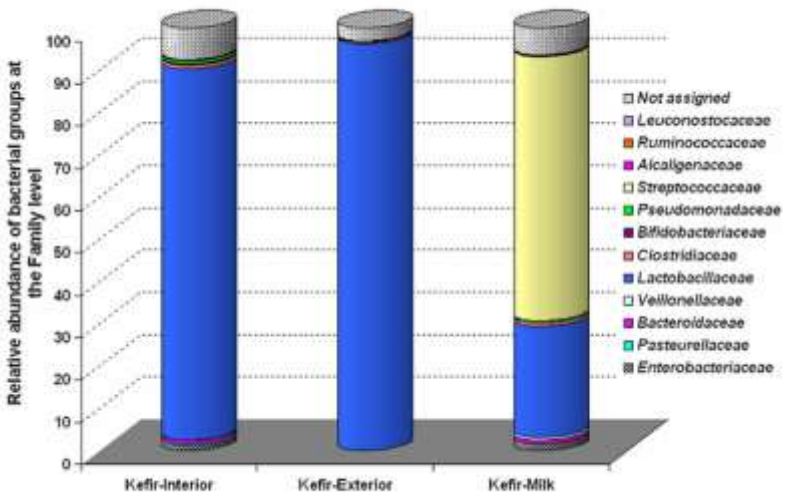


Some of the products on the market that are called 'kefir' are not really kefir

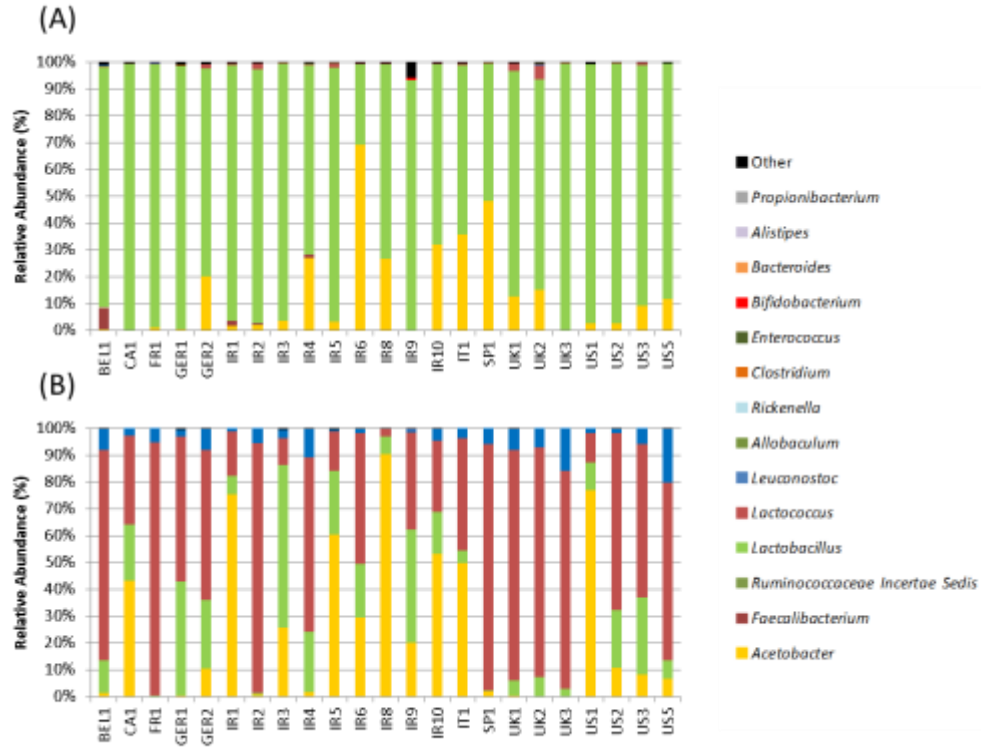
Many putative health benefits but quality of many of the associated publications leaves a lot to be desired

Bourrie et al. Front. Microbiol 2016.

Previous microbiota studies



1 kefir grain and milk (16S)
Dobson et al. 2011



16S phylogenetic composition of the bacterial component of the kefir grain (A) and kefir fermented milk (B) at genus level.

23 kefir milk (16S and ITS)
Marsh et al. 2013

Microbiota – genotype screening

Collected samples at 0, 8 and 24 h of kefir fermentation (x 3 grains)

- Extracted DNA at each time-point for sequencing
- GCMS on milk samples to determine the flavour profile

Amplicon sequencing (16S and ITS) – Changes in microbial composition

Whole metagenome shotgun sequencing – Changes in gene content

Aaron M. Walsh et al. mSystems 2016;1:e00052-16

Bacterial composition (16S)

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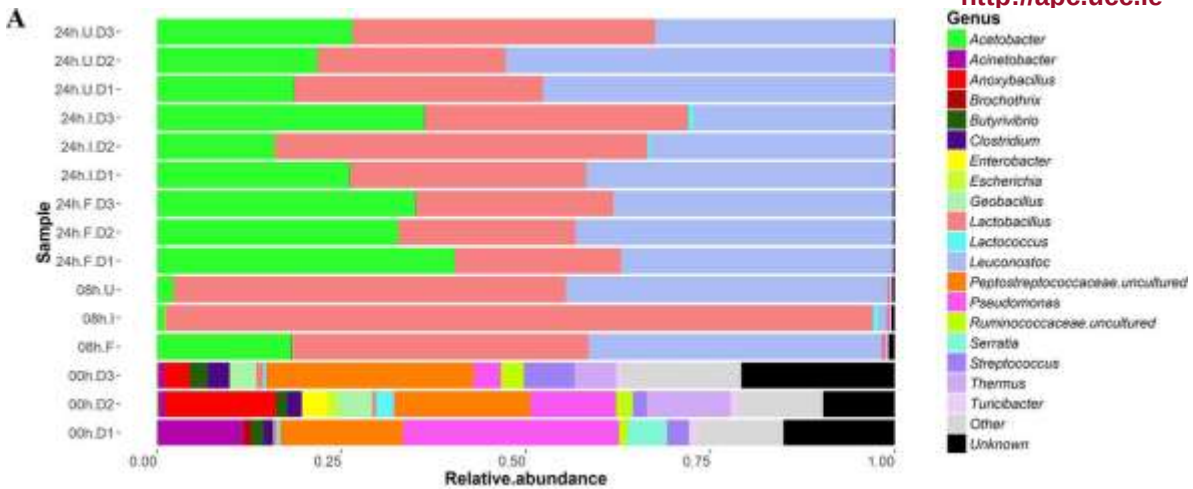
16S rRNA

24 Hr (x3)

8 Hr

0 Hr

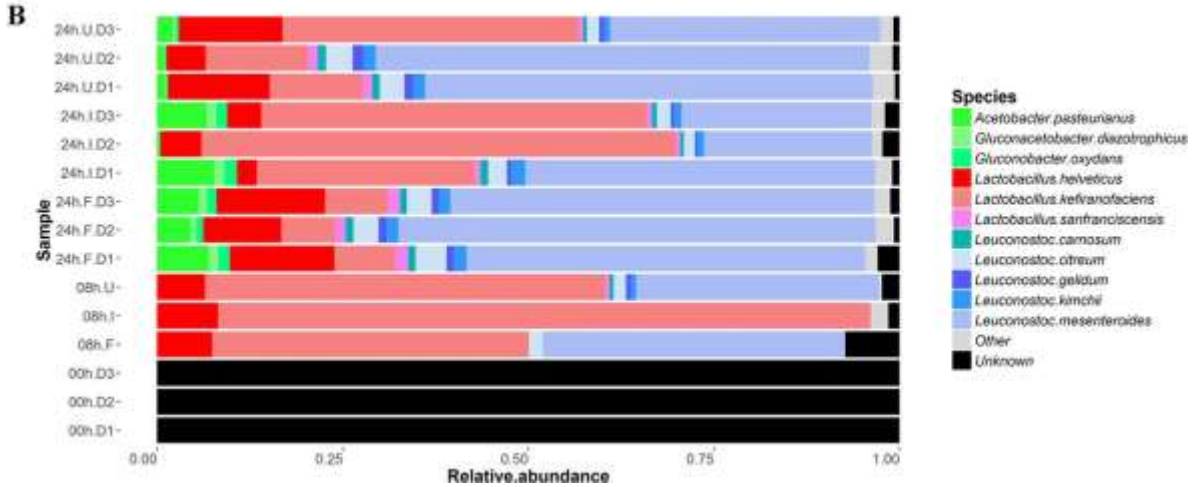


Shotgun
(Kraken)

24 Hr (x3)

8 Hr

0 Hr



- **High bacterial diversity at 0 hours…reflects the milk microbiota**
- **Three genera dominated at 8 hours: *Lactobacillus, Leuconostoc* and *Acetobacter***
- ***Lactobacillus* decreased whereas *Leuconostoc* and *Acetobacter* increased between 8 and 24 hours**

Aaron M. Walsh et al. mSystems 2016;1:e00052-16

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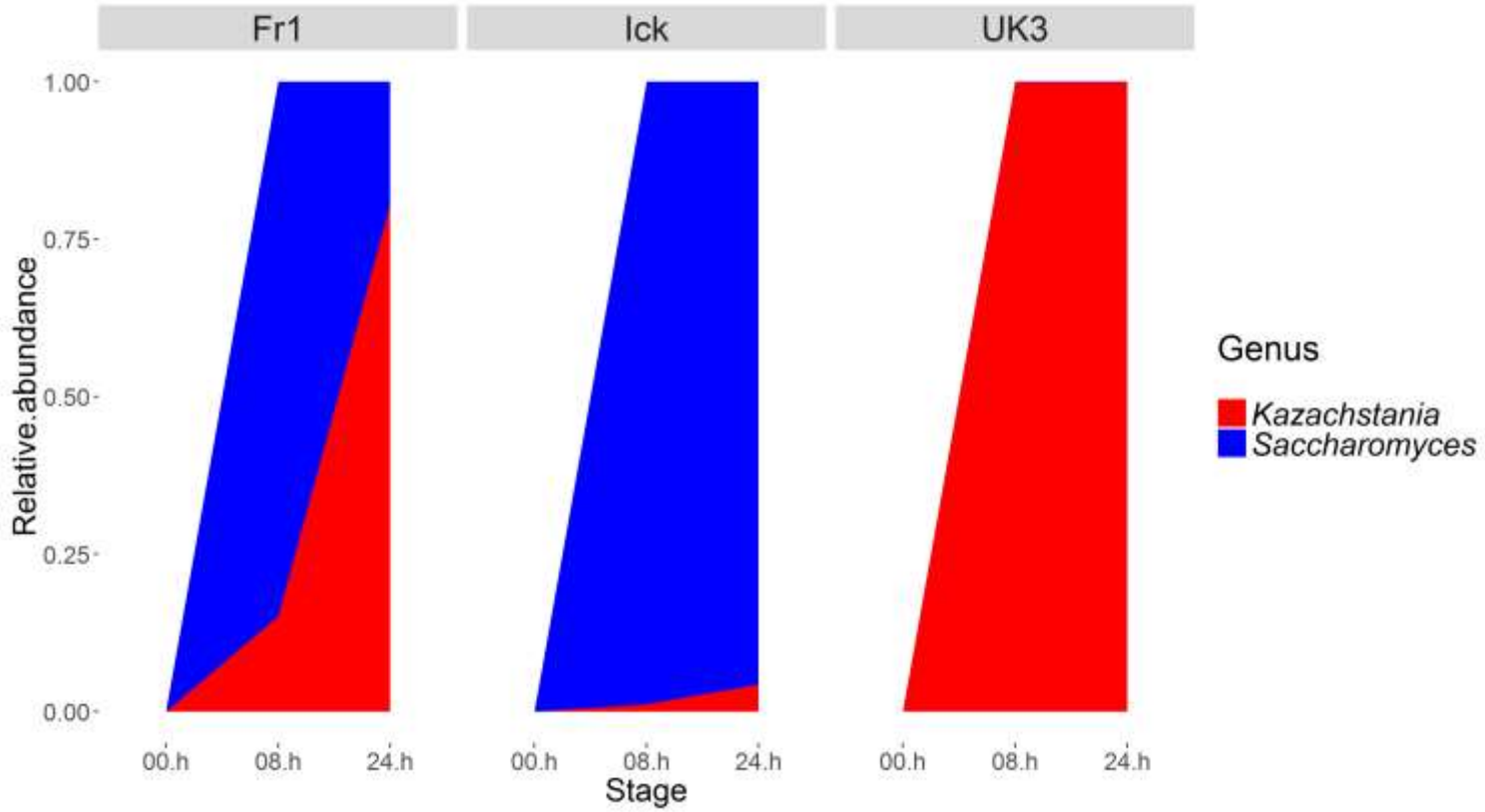
Fungal composition (ITS)



- In contrast to the bacterial communities, there was considerable differences between the fungal communities (and low diversity)

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Cladogram presenting a hierarchical overview of pathways detected

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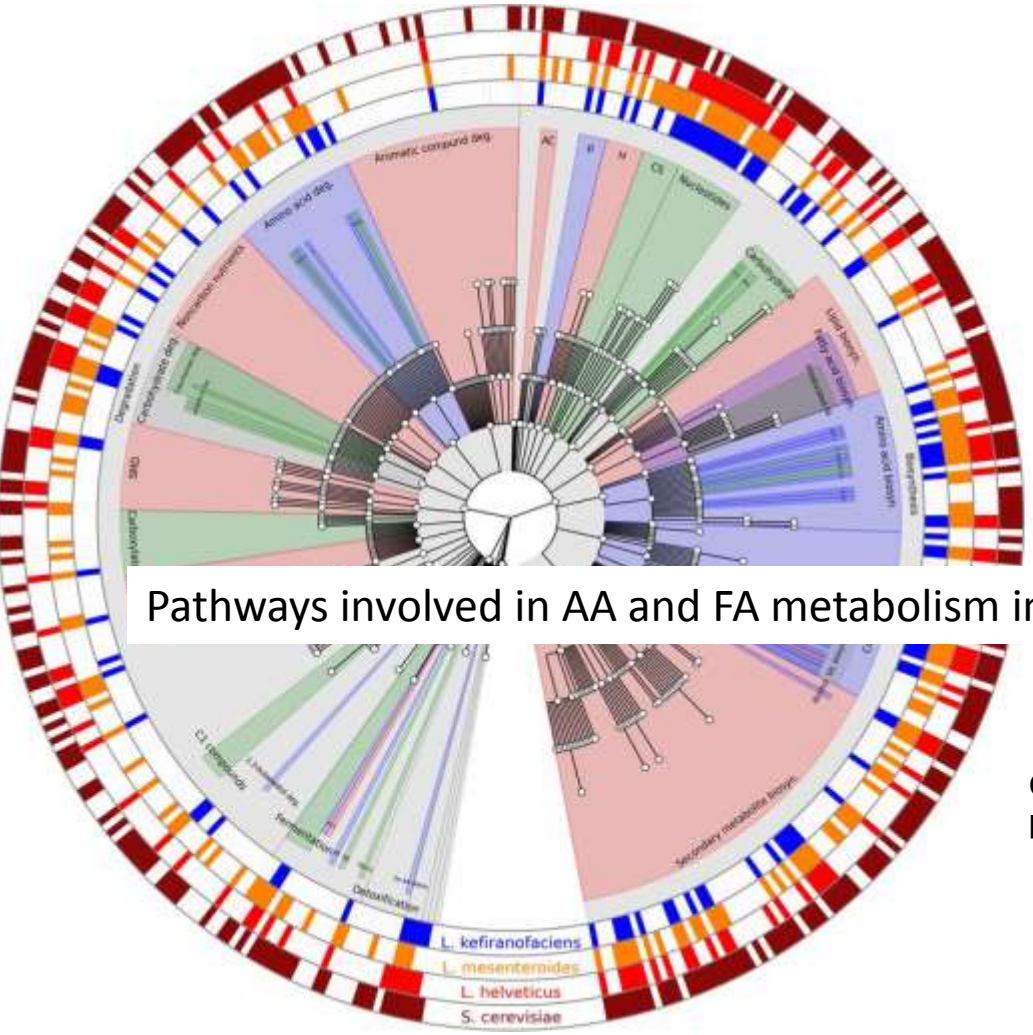

- 00 hours
- 08 hours
- 24 hours

A: Aerobic respiration
AC: Aromatic compounds biosynthesis
Alco.: Alcohol biosynthesis
An: Anaerobic respiration
CS: Cell structure biosynthesis
ED: Enter-Duodoroff pathways
Ga: Galactose degradation
Glyco. deg.: Glycogen degradation
Glyco.: Glycolysis variants
H: Hormone synthesis
La: Lactose degradation
P.saccharides deg.: Polysaccharides degradation
PP: Pentose phosphate cycle
SMD: Secondary metabolite degradation
Se AA detox.: Seleno amino acid detoxification
Su: Sucrose degradation
Unsaturated FA: Unsaturated fatty acid biosynthesis
Vit. biosyn.: Vitamin Biosynthesis
g: Gluconeogenesis
p: Polyamine biosynthesis
s: Sucrose biosynthesis

Pathways involved in AA and FA metabolism increase as the fermentation proceeds

Cladogram presenting a hierarchical overview of the MetaCyc pathways detected in the kefir microbiome with HUMAnN2.

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Correlations between the relative abundances of microbial species and the levels of volatile compounds in kefir samples.

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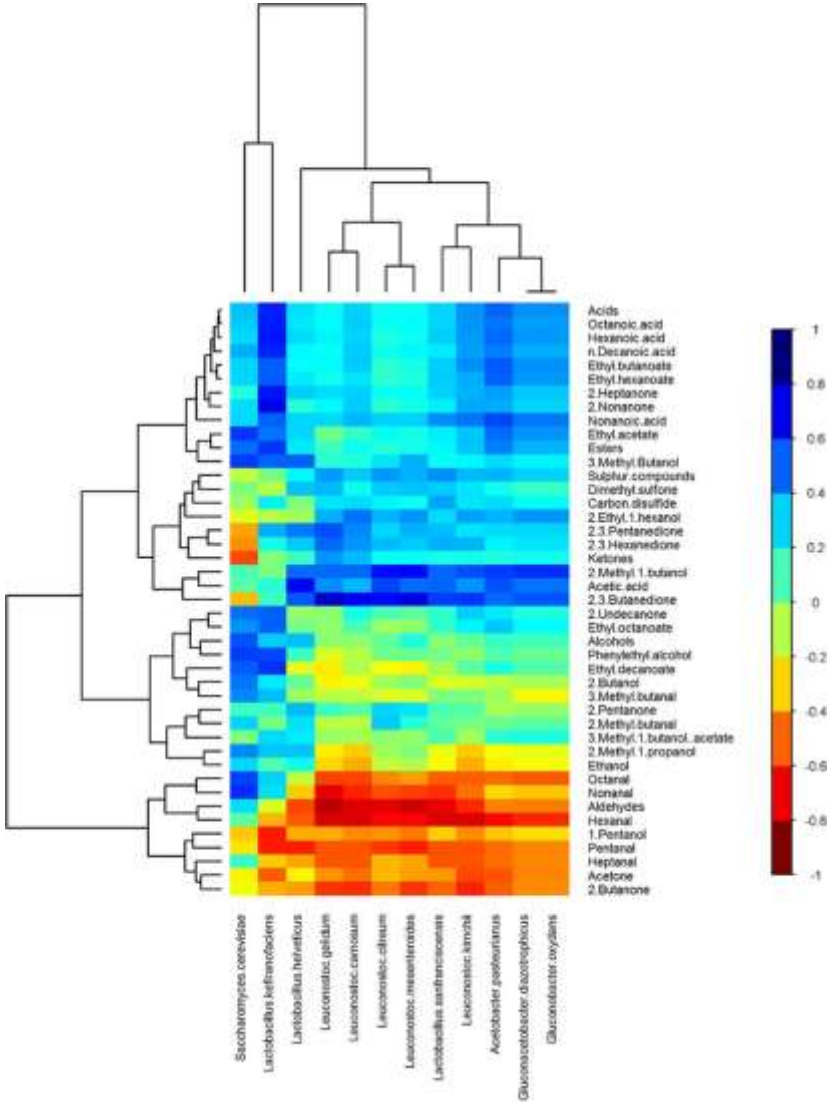

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Identified strong correlations between:

- *Acetobacter pasteurianus* and acetic acid - associated with vinegary flavours
- *Lactobacillus kefiranofaciens* and carboxylic acids associated with cheesy flavours
- *Leuconostoc mesenteroides* and 2,3-Butanedione (associated with buttery flavours)
- *Saccharomyces cerevisiae* and esters associated with fruity flavours

Adding *L. kefiranofaciens* NCFB 2797 to Fr1:

increases in the levels of the esters ethenyl acetate (by 59.15%), ethyl acetate (100%), methyl-3-butyrate (26.83%), and 2-methylbutyl-acetate (11.44%) and the ketone 2-heptanone (65.86%).

Adding *L. mesenteroides* 213M0 to Ick:

increases in the levels of acetic acid (168.28%) and 2,3 butanediol (14.91%), a precursor to 2,3-butanedione.

Health promoting features?

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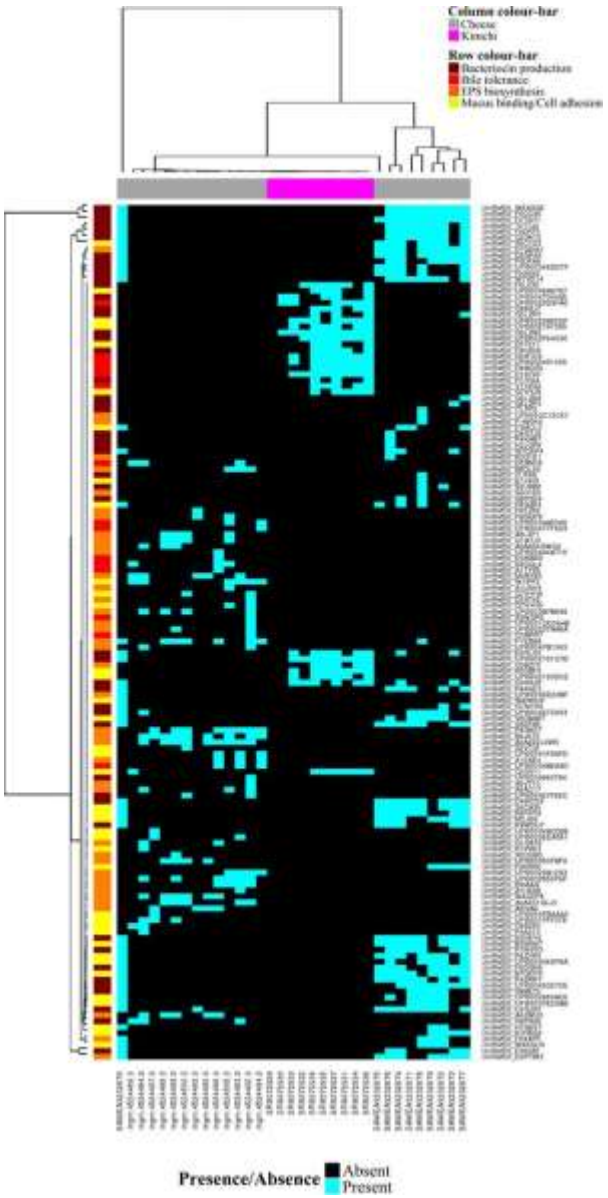
HUMANnN2 gene family table was inspected for genes associated with probiotic functionalities to better understand the basis of the health benefits of kefir.

We observed genes encoding

- **EPS synthesis proteins**
- **Bile salt transporter proteins**
- **Adhesion proteins**
- **Mucus binding proteins**
- **Type III bacteriocins/bacteriolysins**



Aaron M. Walsh et al. mSystems 2016;1:e00052-16

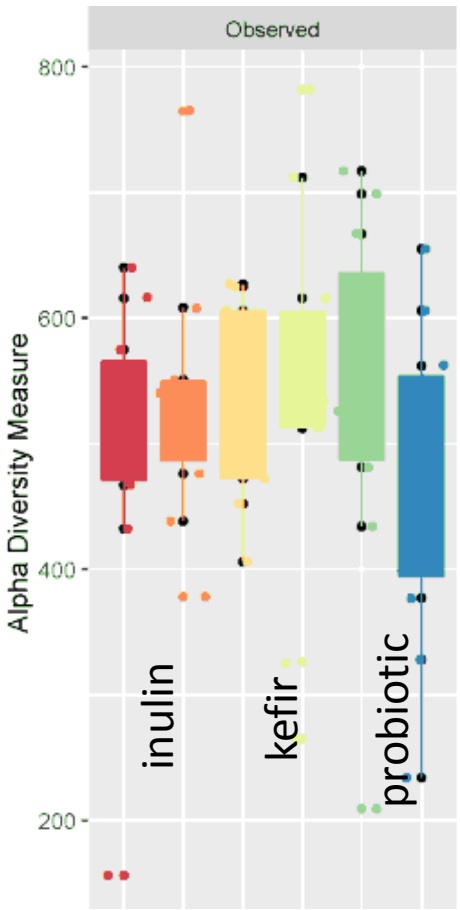
Structure of presentation

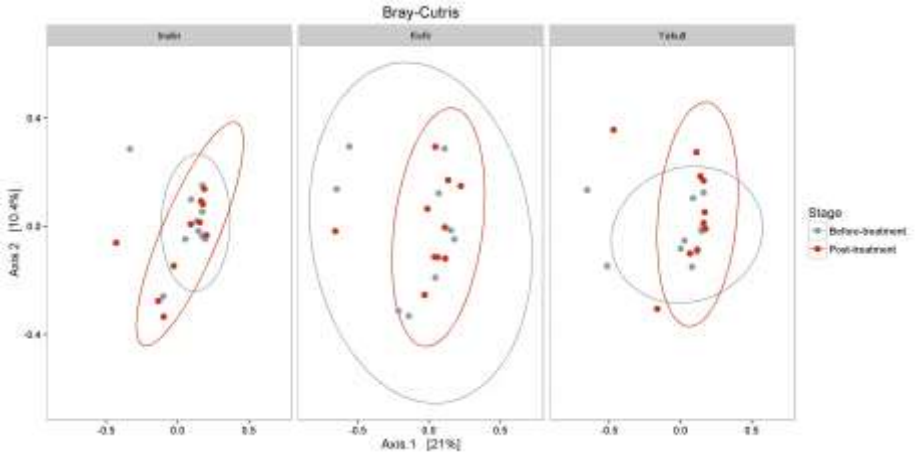
1. Health-promoting fermented foods – Introduction
2. Microbial composition of selected foods
3. Particular focus on kefir
4. **Feeding studies**
5. Conclusion

Human feeding study

- Off the shelf probiotic drink - once daily
- Kefir - once daily
- Attempting to achieve 7g of inulin / day

28 day intervention





None of the treatments had large-scale effects on the overall bacterial diversity of the gut microbiota (This isn't a bad thing!)

Walsh et al Unpub

Family-level bacterial composition of the gut microbiota before/after each treatment

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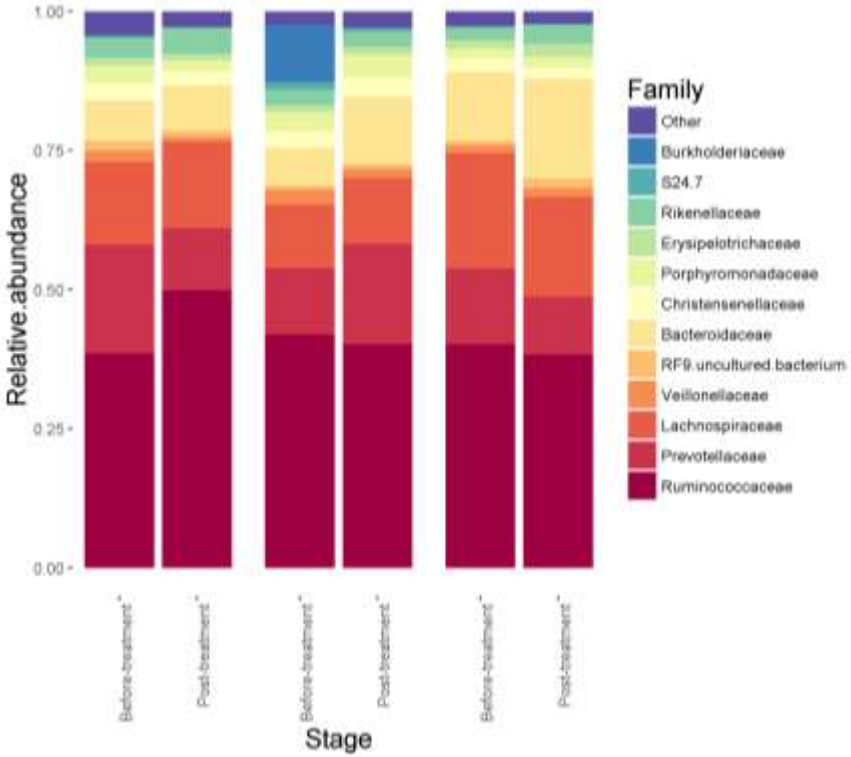
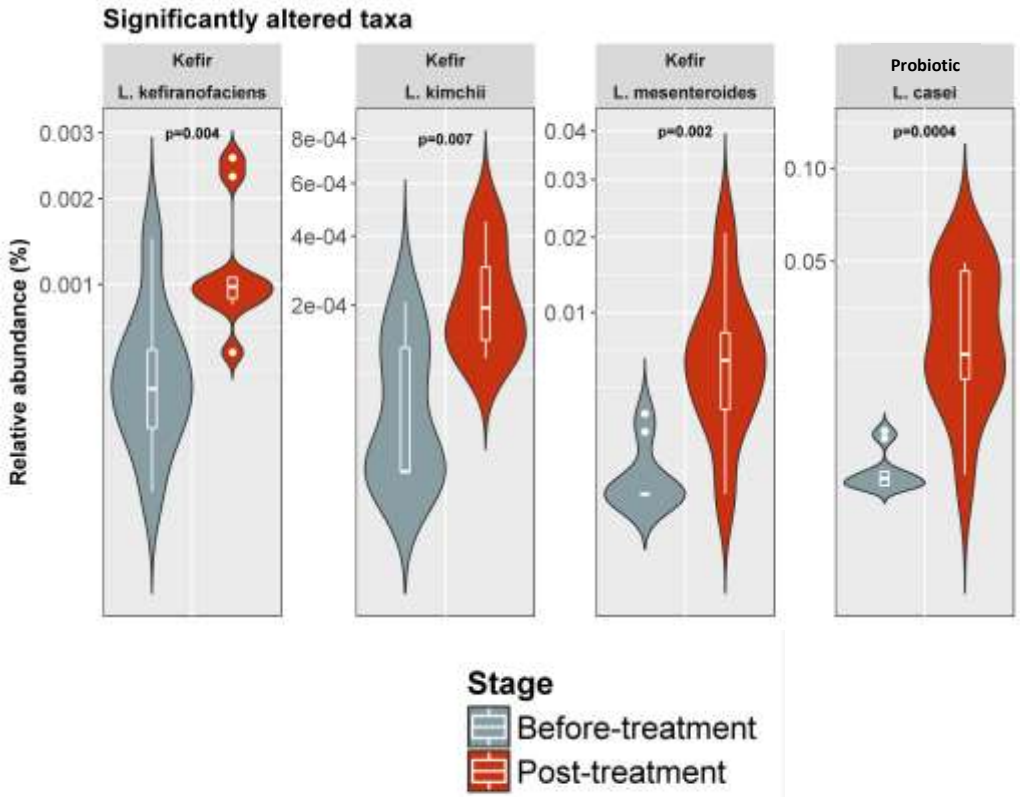
Inulin | Kefir | Probiotic





This graph indicates the average bacterial composition of the gut microbiota in each group/stage

Walsh et al Unpub

Harnessing the potential of kefir

Cultures from 16 kefir grains (and milk) from 6 countries

Phenotype - screening

- 2000 Bacterial and 2000 Fungal isolates
- pH tolerance (pH2) 75% of isolates resistant
- Bile tolerance (3% Oxgall) 75% of isolates resistant
- Antimicrobial production
- Cholesterol assimilation assays
- Adherence and anti-cytotoxicity

Bourrie, Willing et al Unpub

Cholesterol assimilation in milk

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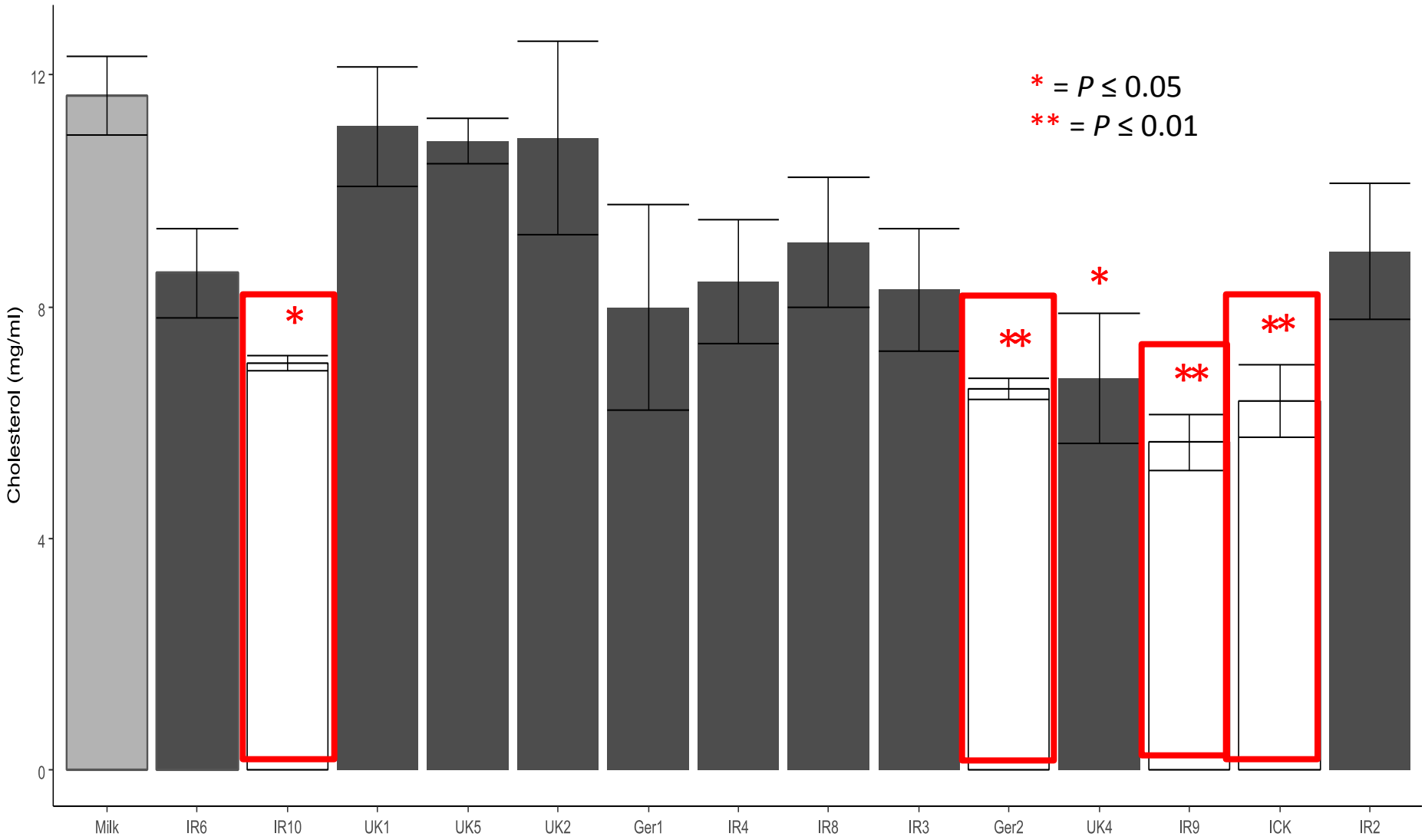
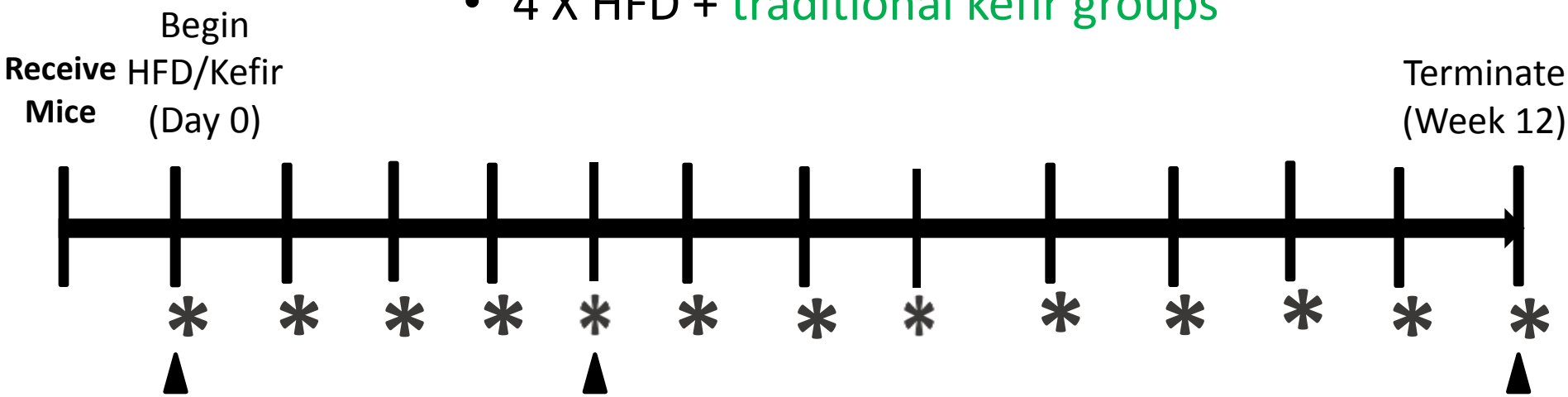


Bourrie, Willing et al Unpub

High fat fed mice

Groups:

- LFD control
- HFD control
- HFD + Commercial
- 4 X HFD + traditional kefir groups



- HFD (40% calories + 1.25% cholesterol) or LFD
- Daily gavage of 100 µl milk (controls) or kefir

✱ = Body weight taken

▲ = Fecal sample taken

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Mouse (DIO) feeding studies

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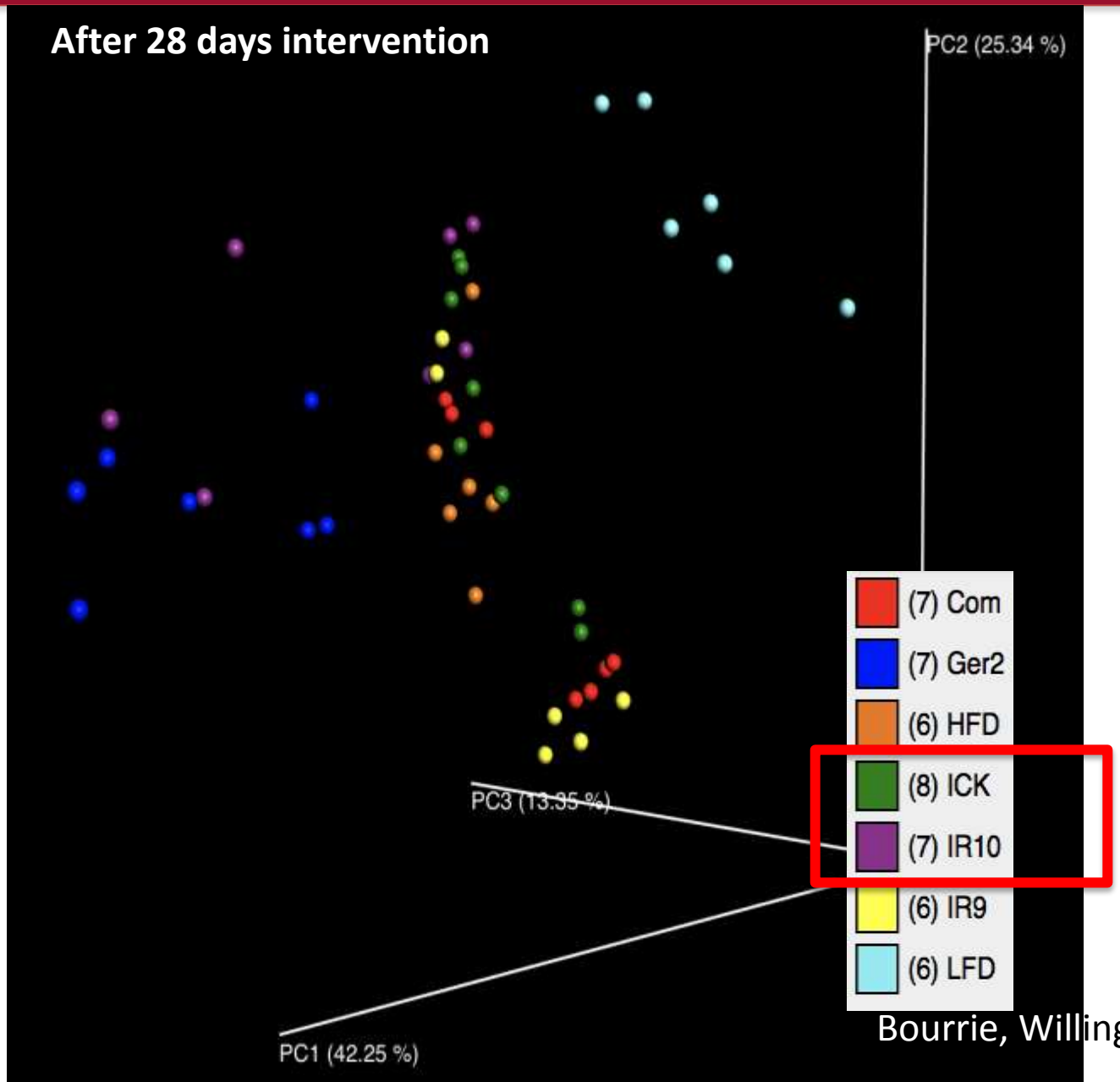

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Mouse feeding studies

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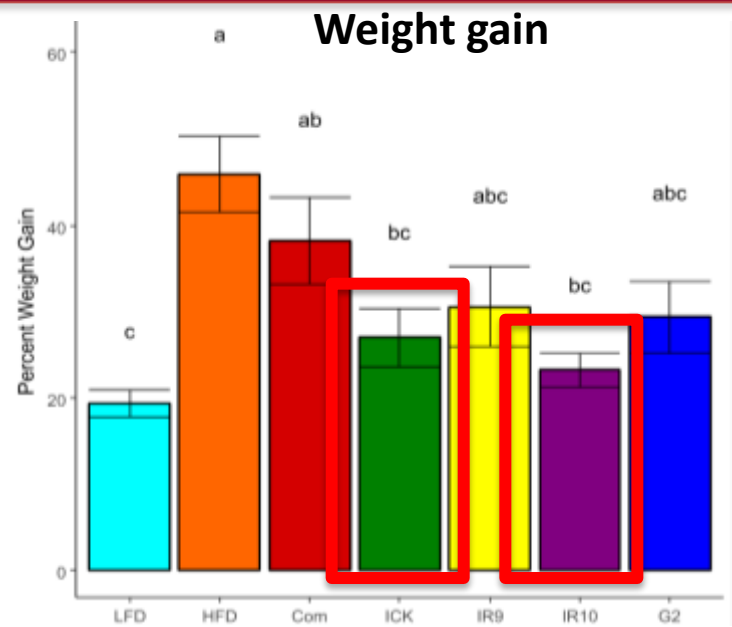

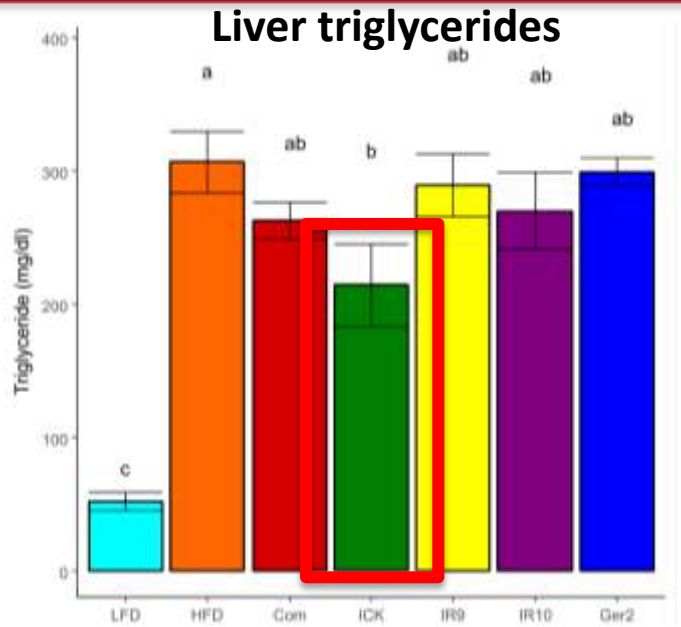

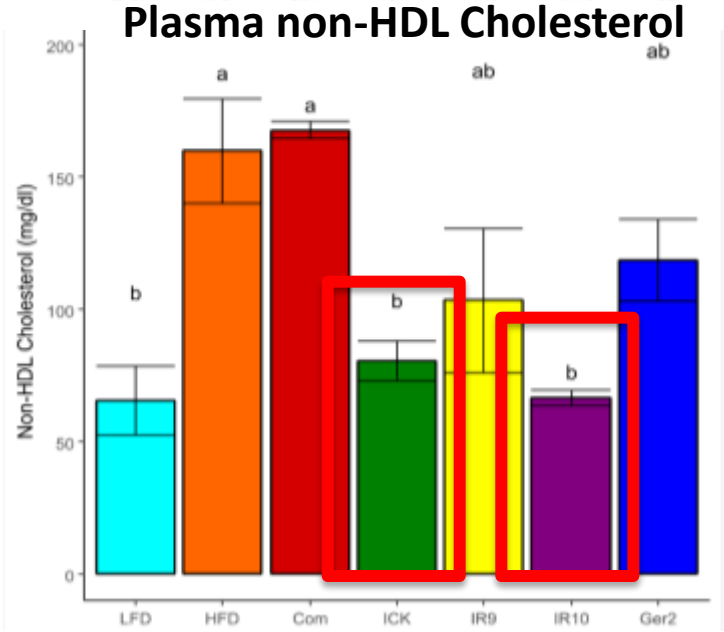

Some traditional, but not commercial kefir, reduced weight gain, levels of plasma LDL Cholesterol and liver TGs

Bourrie, Willing et al Unpub

Van de Wouw, Cryan et al Unpub

Structure of presentation

1. Health-promoting fermented foods – Introduction
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Conclusion

- **There are quite a number of putatively health-promoting fermented beverages (and foods) on the market but the science underlying the associated claims can often be poor**

- **We are trying to address this through a more rigorous examination of the microorganisms present and the health benefits they can confer**

- **These microorganisms can be used for the generation of fermented beverages that closely reflect existing artisanal products but for which health benefits have been clearly determined, and optimised**

- **The availability of strains to facilitate this will also ensure that the quality, flavour and shelf-life of such products can be ensured**

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