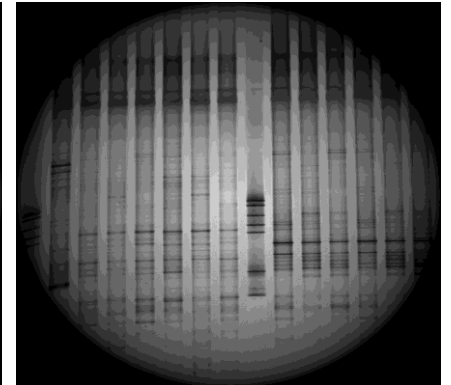
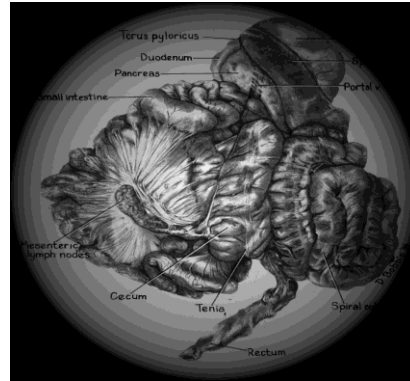


Microbiomics meets Animal Production



Odette Pérez
Hauke Smidt

Wageningen University & Research Centre

- **Mission:** "To explore the potential of nature to improve the quality of life"



WAGENINGEN UNIVERSITY
WAGENINGEN UR

- **Wageningen UR** is collaboration between University, VHL School of Higher Education & Research Institutes (DLO)



- 6,500 staff & 10,000 students from > 100 countries



Laboratory of Microbiology ± 80 co-workers

Head: Prof. Willem M. de Vos in 3 groups

Prof. John van der Oost

Bacterial Genetics
extremophile engineering

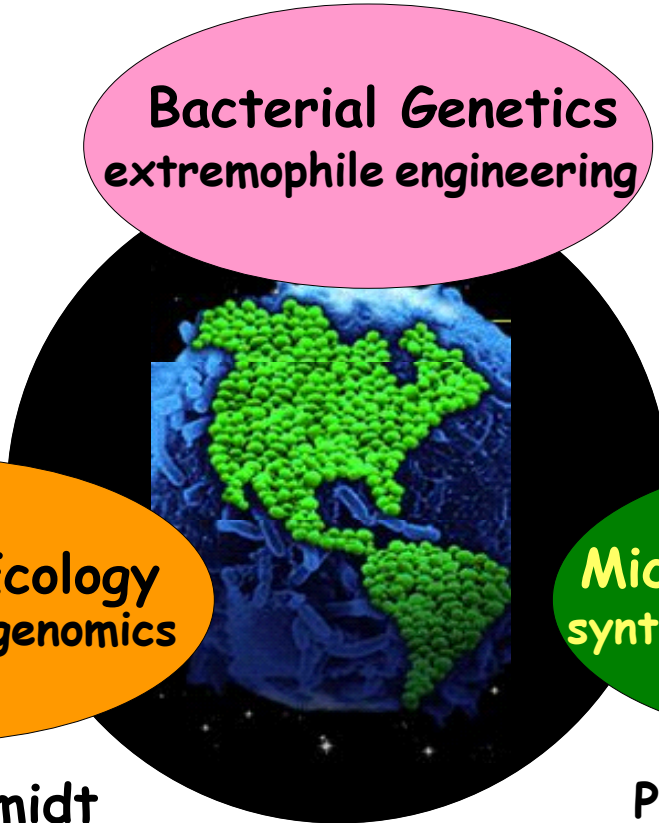
Molecular Ecology
gut & soil ecogenomics

Microbial Physiology
syntrophic bioconversions

Prof. H. Smidt

Dr. E. Zoetendal
Prof. M. Kleerebezem

Prof. Fons Stams



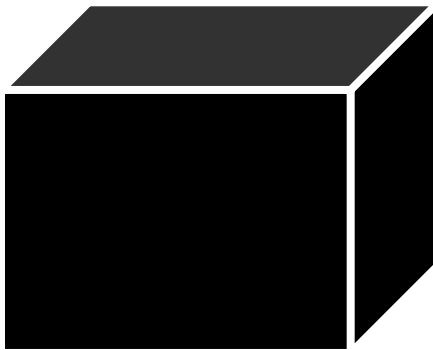
Why are We Studying the GI Tract Microbiota?

GI Tract Microbiota plays a key role in host health

Which microbes are in the GI tract?

What are the microbes doing in the GI tract?

How can we influence the microbiota?

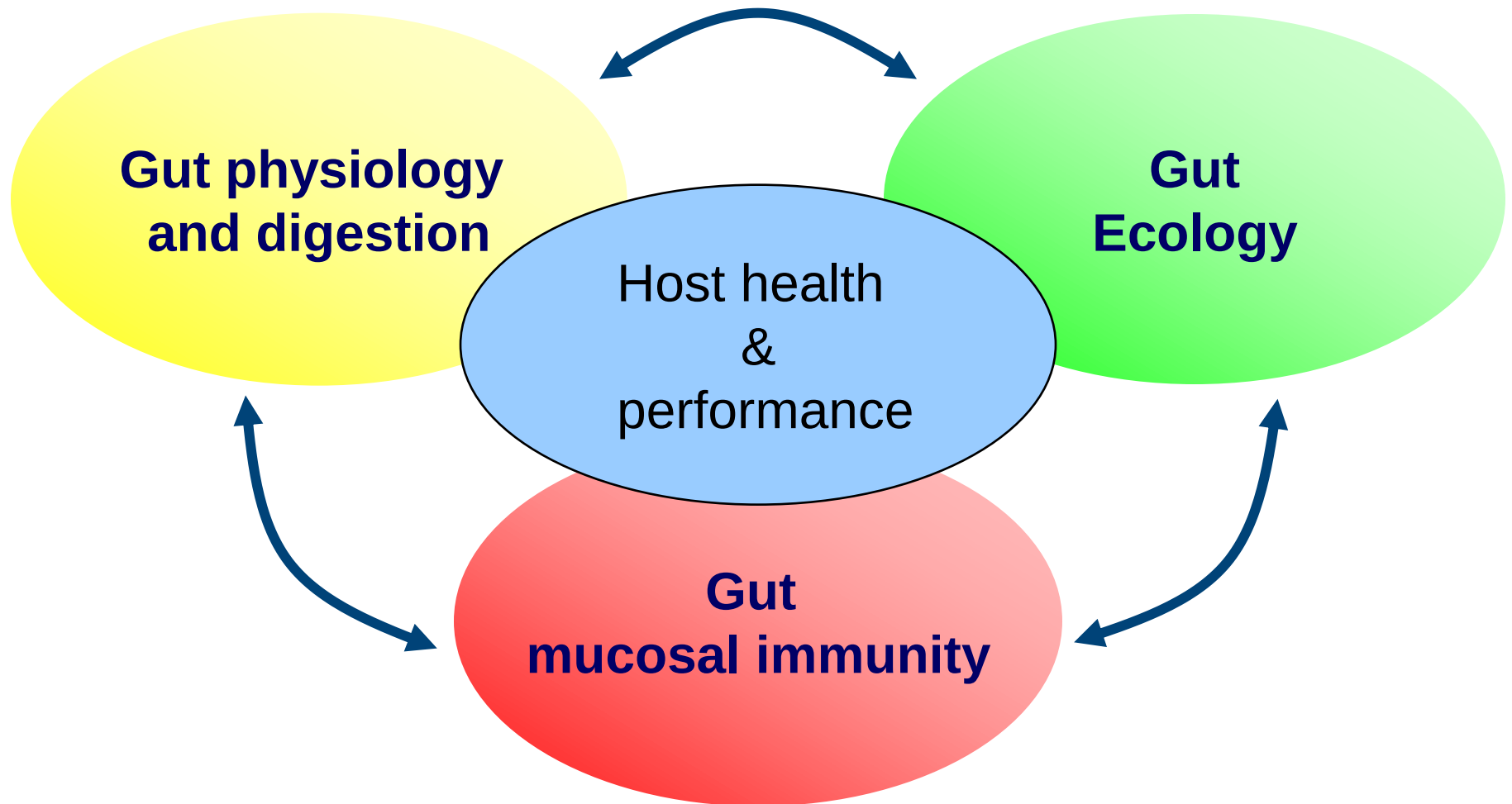


Black box

Understanding

Predictions

Interactions in the GI tract



The Gut and Gut Bacteria



- Most metabolically active organ
 - Dominated by a diverse microbiota
 - Can be modulated by diet
 - Supplies micronutrients
 - Polysaccharides fermentations, SCFA...
-
- Epithelial cells proliferation, gut barrier integrity
 - Direct immune defenses (against pathogens)
 - Non-immune defenses (gut barrier integrity)
 - Energy homeostasis



Gut Bacteria in numbers

Stomach and Duodenum: 10^0 - 10^4 CFU/g

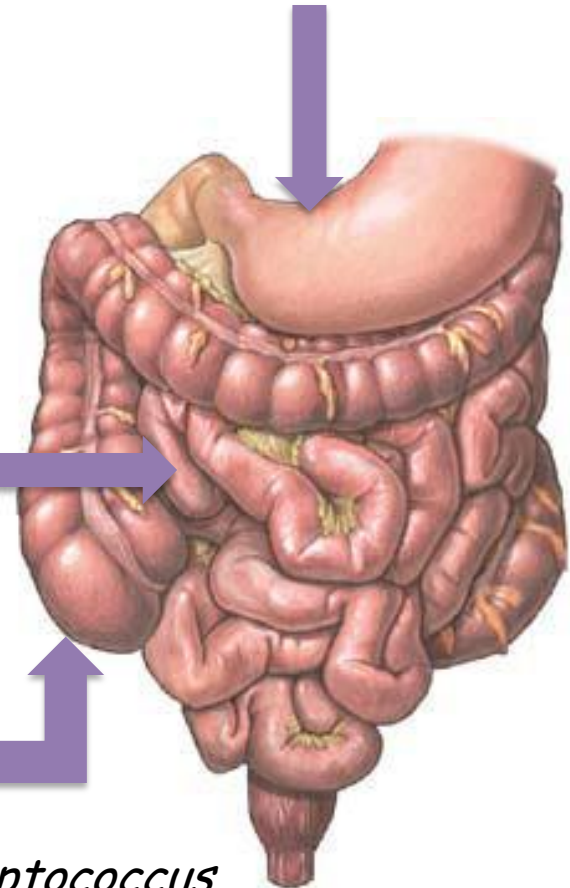
Lactobacillus
Streptococcus
Enterobacteria
Clostridium
Eubacterium
Bifidobacterium

Ileum and Jejunum: 10^7 - 10^9 CFU/g

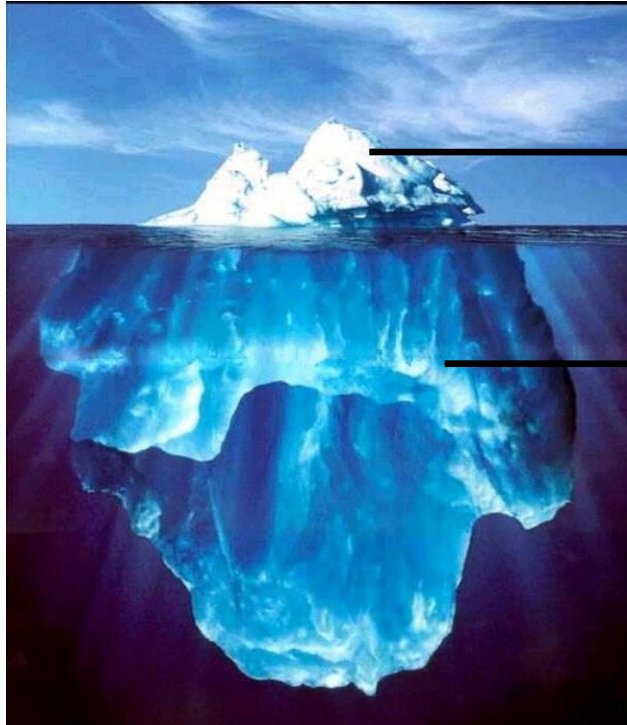
Bacillus
Bacteroides spp.

Caecum and Colon: 10^{11} - 10^{12} CFU/g

Lactobacillus *Peptostreptococcus*
Streptococcus *Bacteroides*
Eubacterium *Prevotella*
Clostridium



The Microbial Iceberg

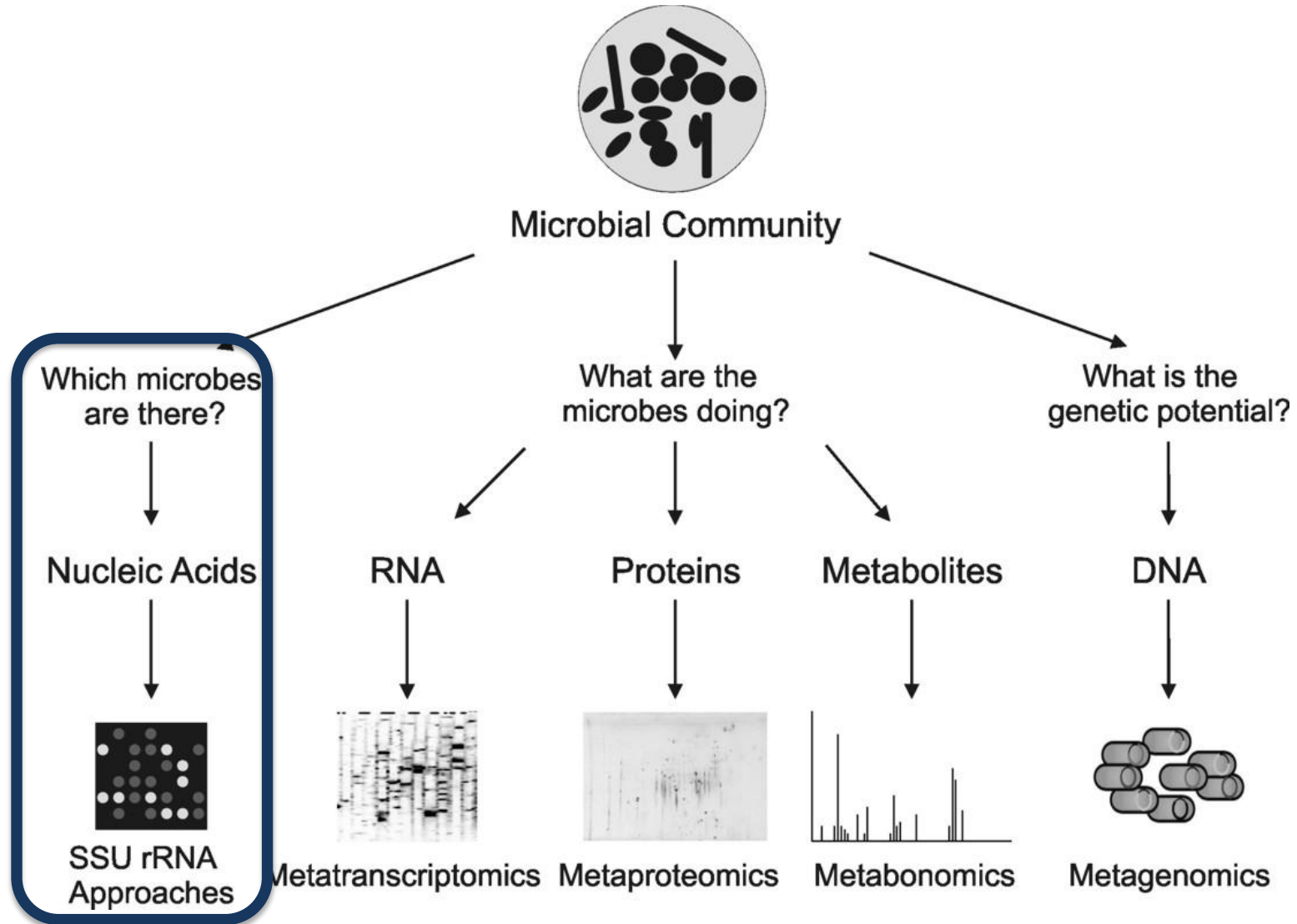


Cultured fraction (~20%):
Some physiological data
available

Uncultured fraction (~80%):
Only (partial) 16S rRNA
sequence known

- Microbial cells outnumber host cells by 10-fold
- > 1000 species of microbes in the gut ecosystem
- Predominance of anaerobic bacteria

Community-based Approaches



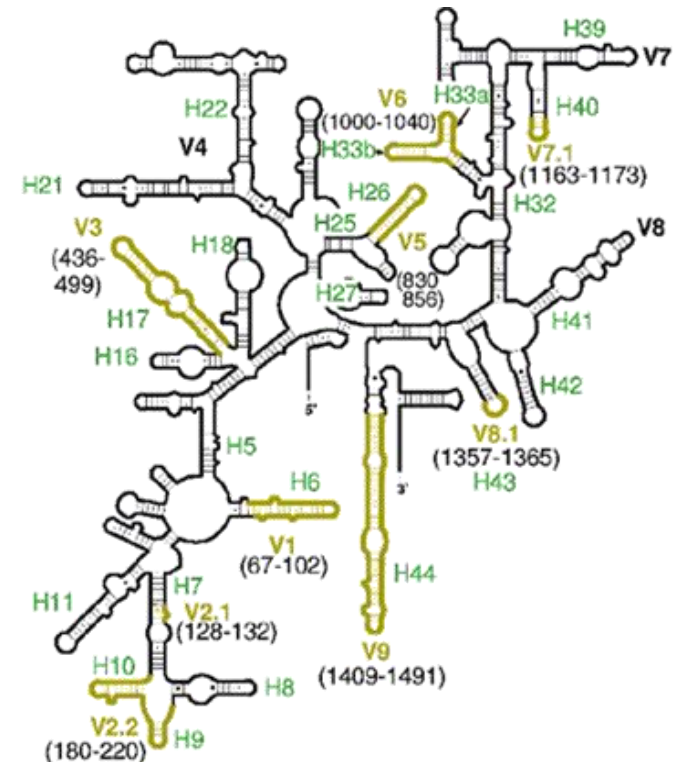
Studying the diversity of the microbiota

MOLECULAR TAXONOMY

rRNA based phylogeny

- Ideal phylogenetic marker
- Domains of different degrees of conservation
- Size: 1500 nucleotides
 - Small enough to sequence easily
 - but still large enough to contain information
- Naturally amplified
- Present in every (microbial) cell
- More than 1,000,000 sequences in databases

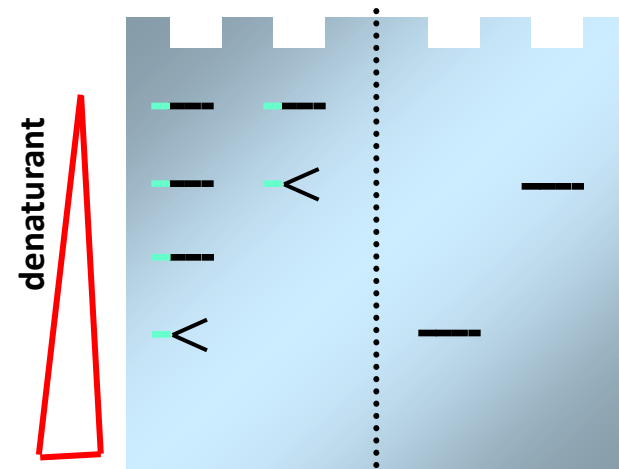
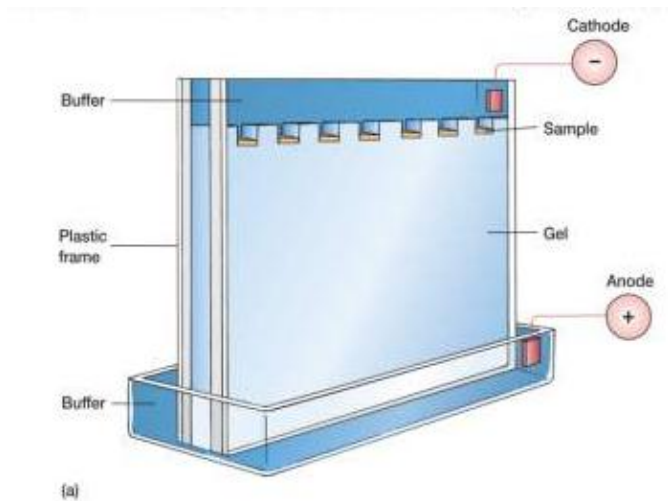
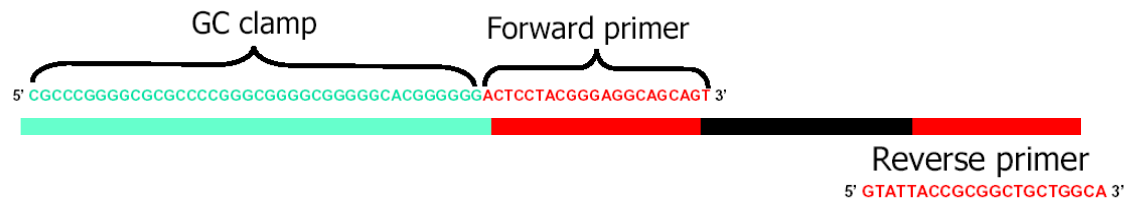
Small subunit rRNA
(16S/18S)



Denaturant Gradient Gel Electrophoresis (DGGE)

DGGE

PCR product: Separation of bands depending on sequence (GC content).
High GC bacteria lowest part of the gel (different gradients used)

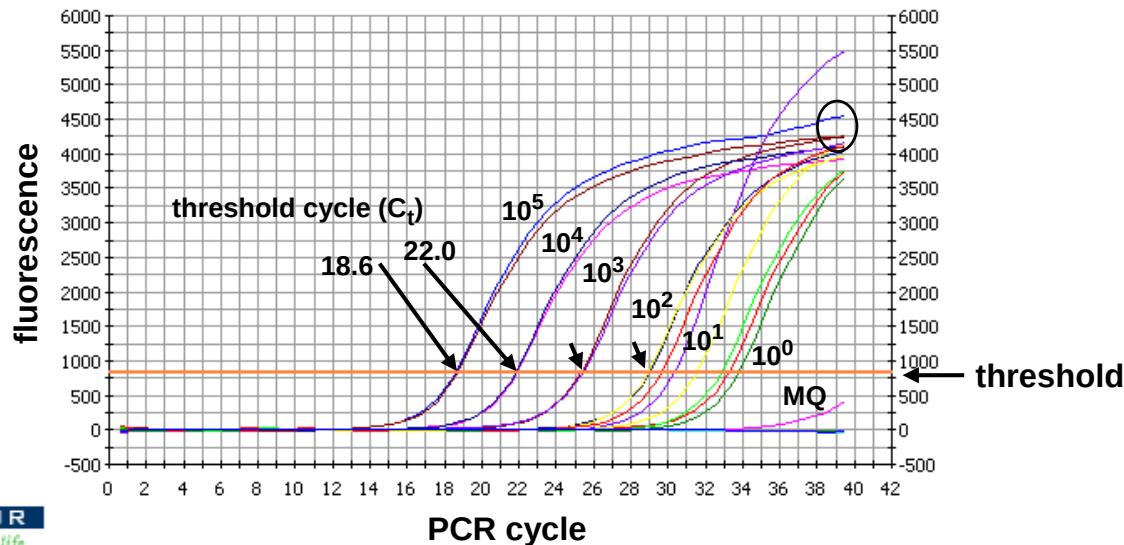


What happens · What is visible

Quantitative PCR (qPCR)

qPCR

- DNA quantification of a gene-of-interest
- SYBR-Green (fluorescent when bound to double-stranded DNA)
- Onset of the logarithmic phase (threshold)
- Standard curve (dilution series) with gene-of-interest
- C_t -values relate to known number of DNA copies initially present



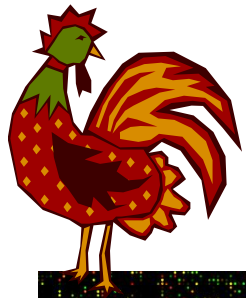
Novel HTP Tools for GIT diversity

The Intestinal Tract Chip Suite



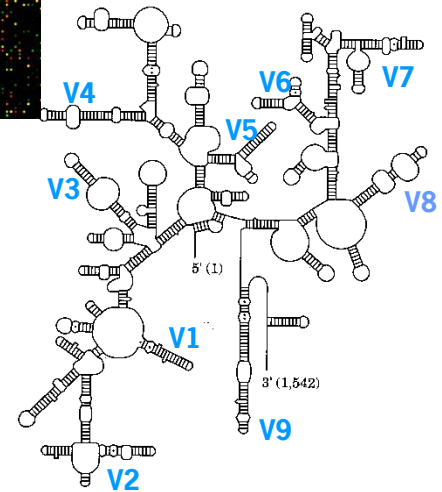
HITChip

MITChip



PITChip

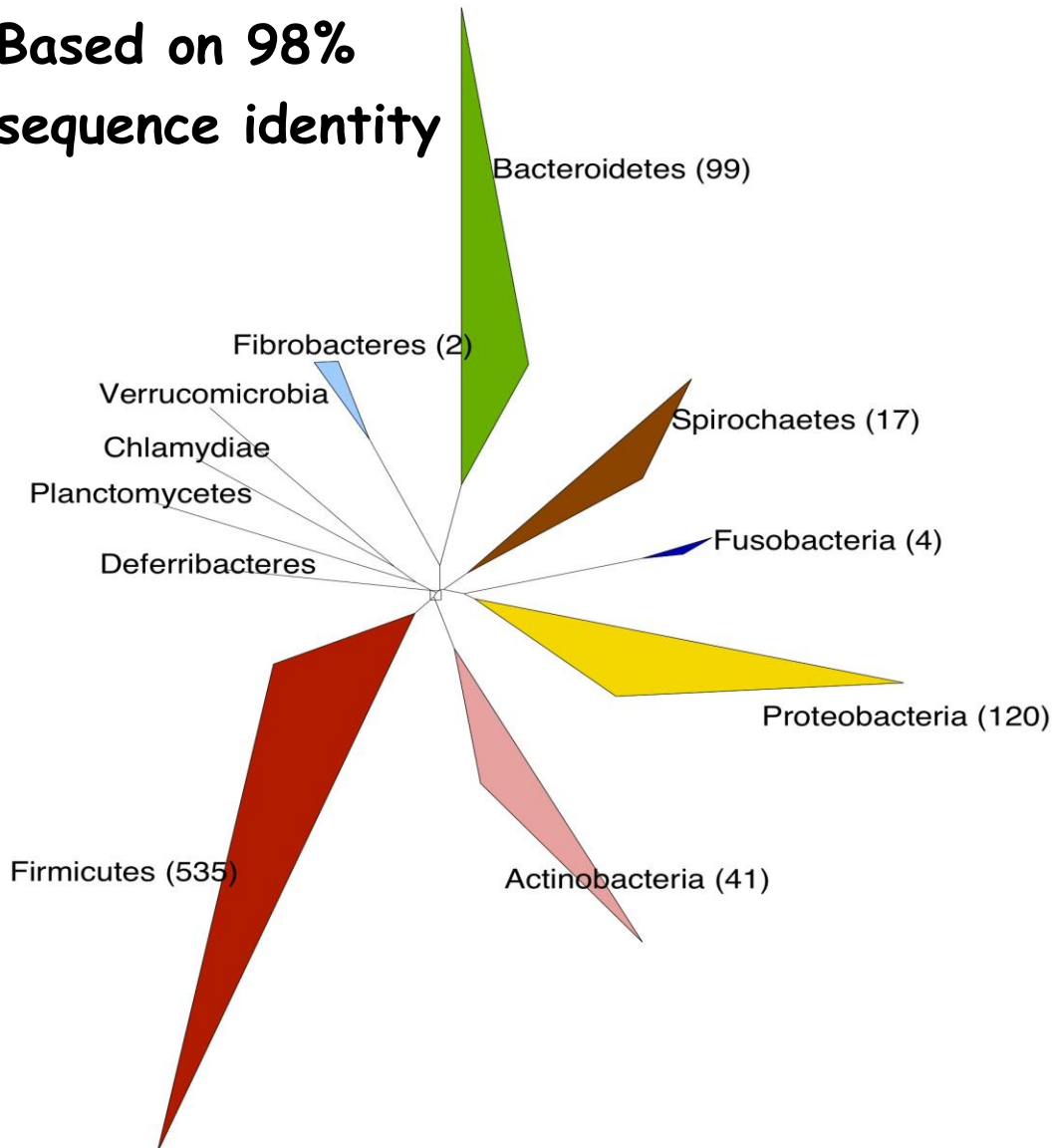
CHICKChip



Agilent Technologies

Pig Intestinal Tract Chip (PITChip)

Based on 98%
sequence identity



Currently > 800 OTU

mostly *Firmicutes*

&

Bacteroides/Prevotella

& *Proteobacteria*

*Mostly uncultured
(53%)*

(Perez *et al.*, in preparation)

PITChip probe design

Export V1 and V6 region (41-68bp)

```
AACGAGAGACTCCTTCGGGACGATCTAGTGGCGAACGGGTGAGTAAC
```

Sequence from the left side

```
AACGAGAGACTCCTTCGGGACGAT
```

Sequence around middle nucleotide

```
TCCTTCGGGACGATCTAGTGGCGA
```

Sequence from the right side

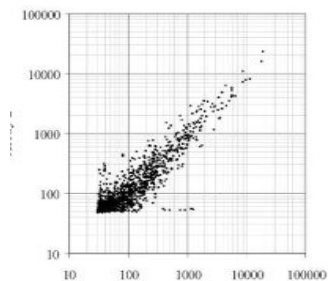
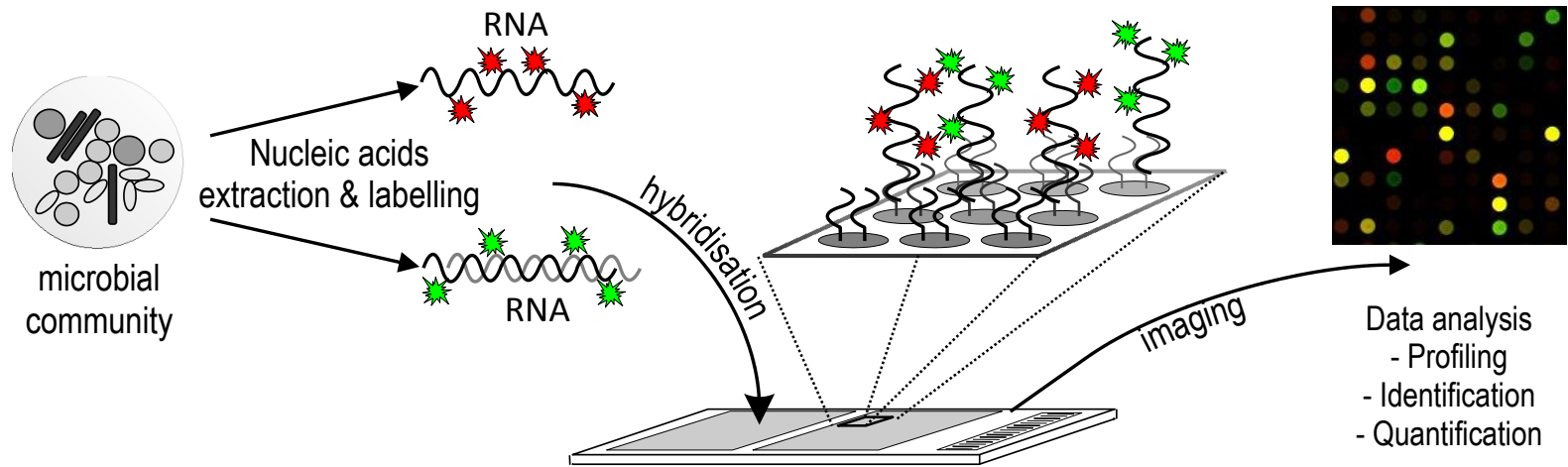
```
TCTAGTGGCGAACGGGTGAGTAAC
```

Optimization of T_m (narrow range of 58.4-63.4°C)

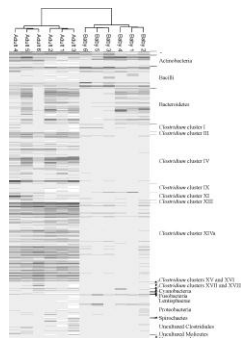
Reverse complement

Aprox. 3000 probes

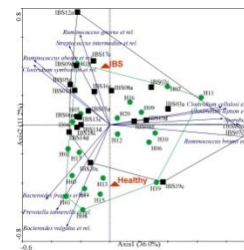
Principle of the phylogenetic microarrays



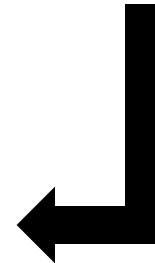
Intensity plots



Phylotype clustering



PCA/RDA



Why the current interest in production animal microbiota?

- What is the role of microbes in the triangle with animal nutrition, health and performance?
 - **AWAY FROM BLACK BOX APPROACHES**
- For many years, antibiotics used as growth promoters
YET:
 - How do they work?
 - Banned since 2006 (EU)
- Quest for alternative additives

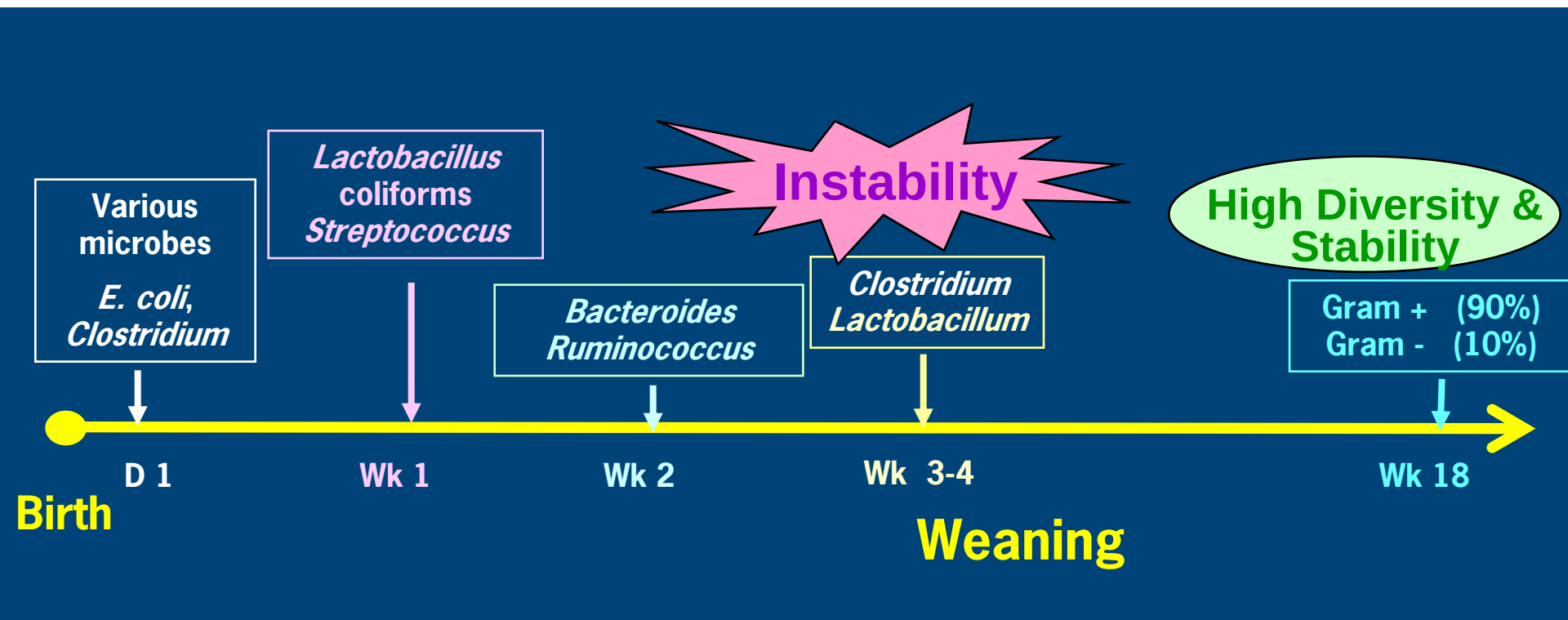
Production Animal Research @ MIB

Focus on Pigs

- Which are the first bacterial colonizers in the pig GIT?
- Which bacteria are part of the indigenous microbiota in the neonate pig GIT?
- Effect of environment factors, diet and host on bacterial colonization?
- Alternatives to antibiotics: Improved dietary intervention strategies possible (pre/probiotics)?

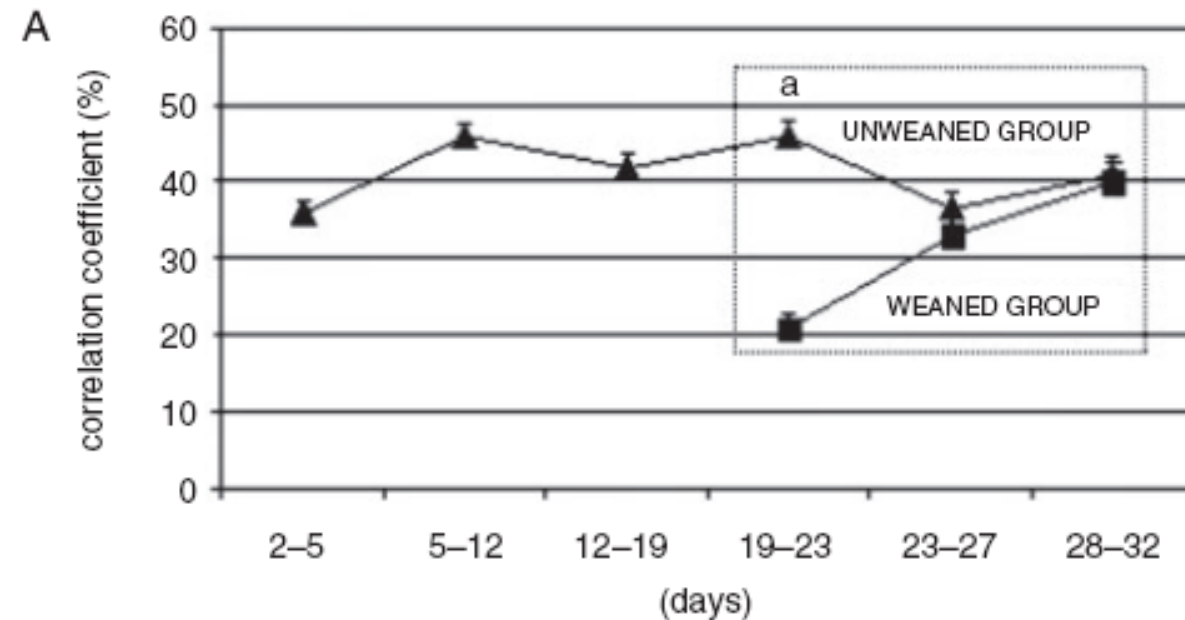


Microbial succession in Piglet feces



- Largely based on cultivation-dependent studies

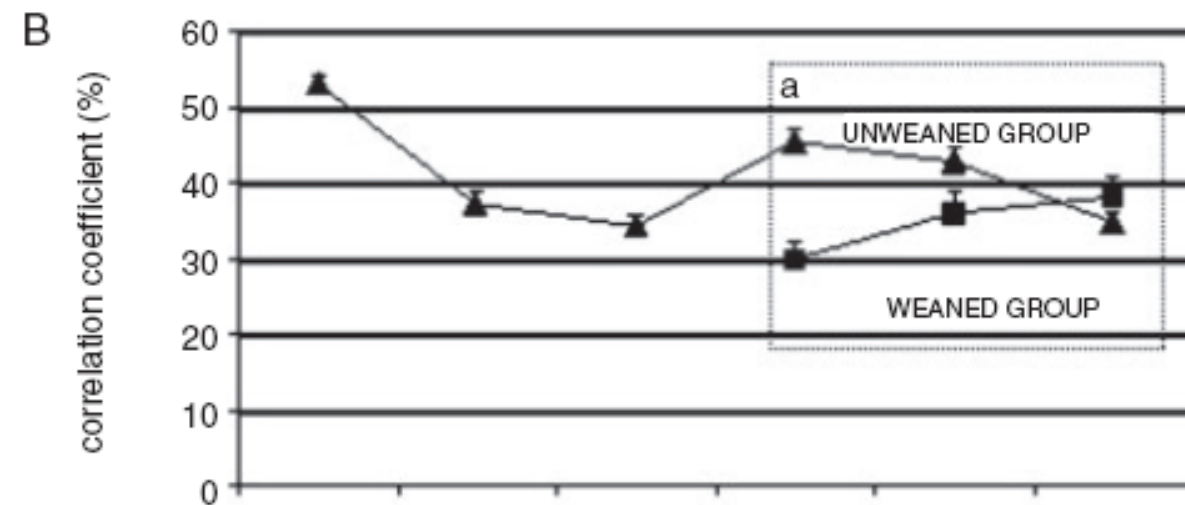
Microbiota instability after weaning



Ileum

16S rRNA gene-targeted
DGGE fingerprinting

Moving Window
Correlation



Colon

Changes in microbiota after weaning

- more *E. coli*, *Shigella*, etc.
- fewer lactobacilli; **especially *L.sobrius* (*L.amylovorus*)**

Day-treatment	<i>L. sobrius</i>	<i>L. reuteri</i>	LAB
2	$4.72 \pm 3.9 \times 10^7$	$1.34 \pm 0.5 \times 10^7$	$7 \pm 0.1 \times 10^7$
5	$9.63 \pm 1.2 \times 10^8$	$2.13 \pm 2.17 \times 10^7$	$9.7 \pm 0.5 \times 10^8$
12	$1.01 \pm 1.05 \times 10^8$	$3.08 \pm 3.97 \times 10^7$	$1.4 \pm 0.9 \times 10^8$
19	$6.85 \pm 0.1 \times 10^8$	$1.48 \pm 1.3 \times 10^7$	$7.1 \pm 2.6 \times 10^8$
23-UW	$4.72 \pm 3.9 \times 10^7$	$1.34 \pm 0.5 \times 10^7$	$7.1 \pm 1.3 \times 10^7$
23-W	$< 10^{3*}$	$< 10^{3*}$	$4.4 \pm 0.5 \times 10^{5*}$
27-UW	$2.46 \pm 0.9 \times 10^9$	$1.07 \pm 0.8 \times 10^7$	$8.2 \pm 0.8 \times 10^9$
27-W	$< 10^{3*}$	$< 10^{3*}$	$5.1 \pm 0.1 \times 10^{6*}$
32-UW	$1.18 \pm 0.9 \times 10^7$	$6.13 \pm 1.3 \times 10^7$	$6.1 \pm 0.5 \times 10^7$
32-W	$< 10^{3*}$	$< 10^{3*}$	$2.1 \pm 0.1 \times 10^{6*}$

- UW: unweaned W: weaned

Changes in *Lactobacillus* spp. carbohydrate utilization capacity after weaning

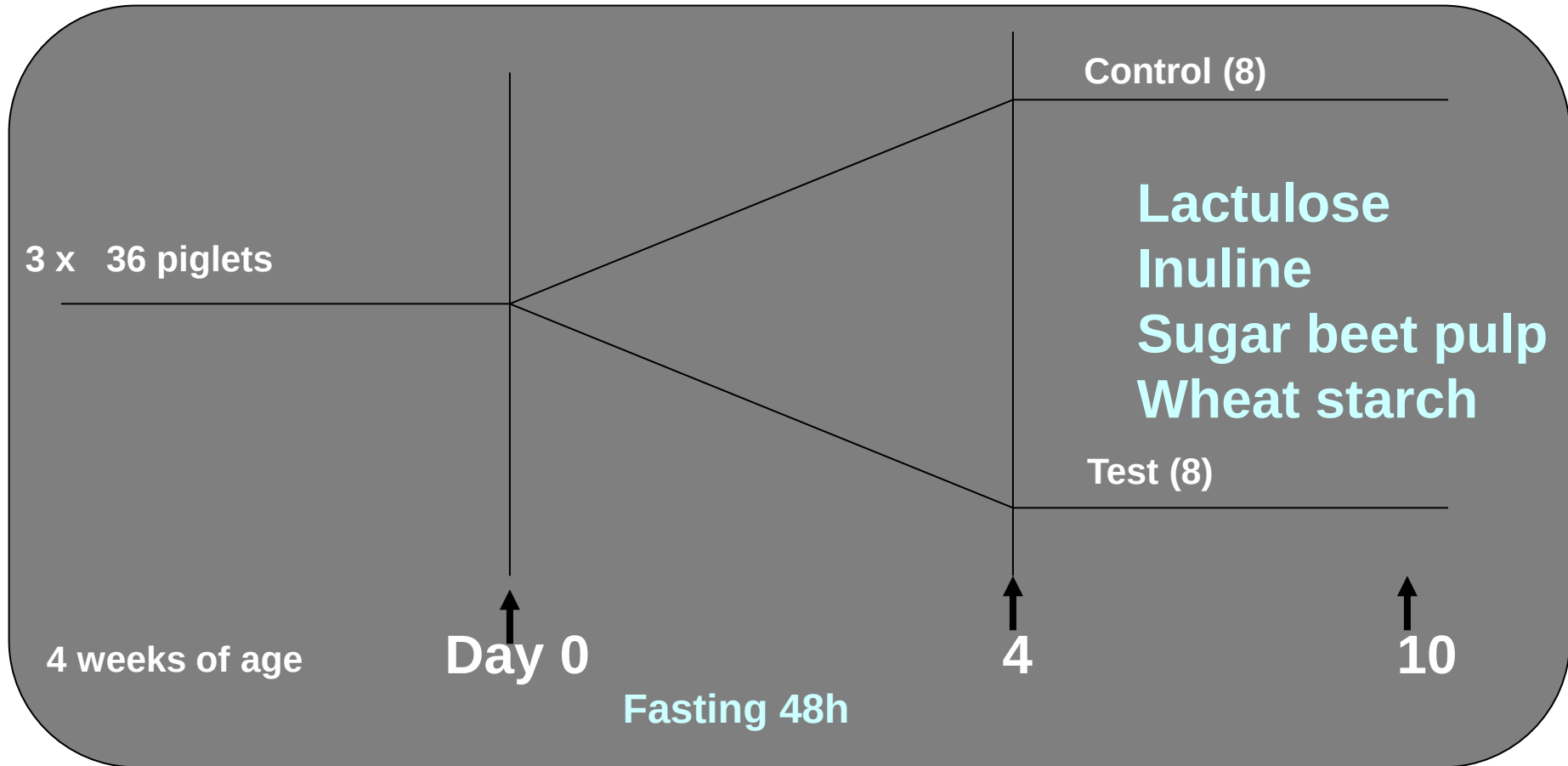
	Days after the weaning (number of isolates)				
	0 (25)	1 (5)	2 (15)	5 (11)	11 (20)
Glucose	100	100	100	100	100
Galactose	100	100	93	91	59
Fructose	56	80	67	27	88
Lactose	100	100	100	91	77
Cellobiose	36	27	20	80	77
Arabinose	0	0	0	55	12
Ribose	16	0	0	36	12
Xylose	0	0	0	55	6
Raffinose	100	100	87	100	53
Starch	8	29	20	36	60

Our main pig research questions during the past 8 years

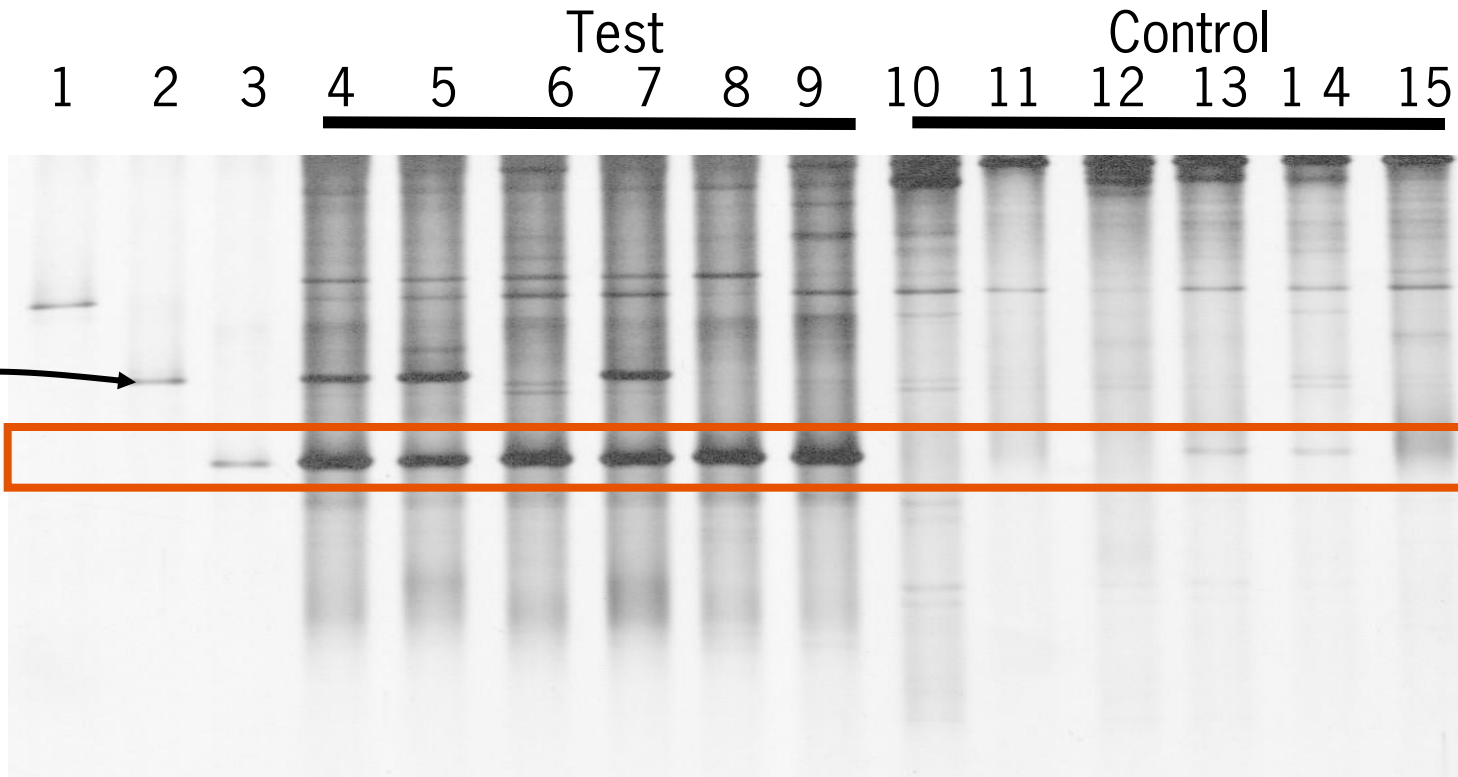
- Which are the first bacterial colonizers in the pig GIT?
- Which bacteria are part of the indigenous microbiota in the neonate pig GIT?
- Effect of environment factors, diet and host on bacterial colonization?
- Alternatives to antibiotics: Improved dietary intervention strategies possible (pre/probiotics)?

Case study 1:

Fermentable carbohydrates affect microbiota especially in the small intestine



Maintenance of *Lactobacillus* spp. populations with mix of fermentable carbs



1- *Lactobacillus acidophilus*, 2 *L. reuteri*, 3 *L. sobrius*,
4-9 Small intestinal samples of piglets on Test diet
10-15 Small intestinal samples of piglets on Control diet

Increased production of lactic acid in test animals

Lactobacillus sobrius/amylovorus

In vitro and in vivo functionality

- *Lactobacillus sobrius* represents new isolate within *Lactobacillus amylovorus* group
- Abundant in piglet small intestine
- Globally distributed
 - (NL, D, UK, China, FIN, IT, US...)
- *Genome sequencing ongoing* (Univ. Helsinki)



Conclusions

- ✓ Composition of gut microbiota changes after birth from a simple to a complex and stable community influenced by the environment

Weaning Piglets:

- ✓ Diet and host are major factors influencing microbiota
- ✓ dietary impact on *Lactobacillus* spp. population
- ✓ *L. sobrius* universal & abundant colonizer pre-weaning
 - ✓ Potential marker for stable community after weaning

Case study 2

Na-butyrate: background

- Product of fermentation (SCFA) in the large intestine
- Energy substrate for the colonocyte
 - trophic effect + colonic barrier integrity
 - immuno-modulating properties
- Pre-gastric hydrolysis of milk fats in suckling mammal
- Anti-bacterial properties

Earlier studies in farm animals (baby calves & pigs):

- Improved growth performance and feed efficiency
- Decreased frequency of diarrhea
- Increased enzyme activities (pancreas, intestine)

Oral Na-butyrate trial

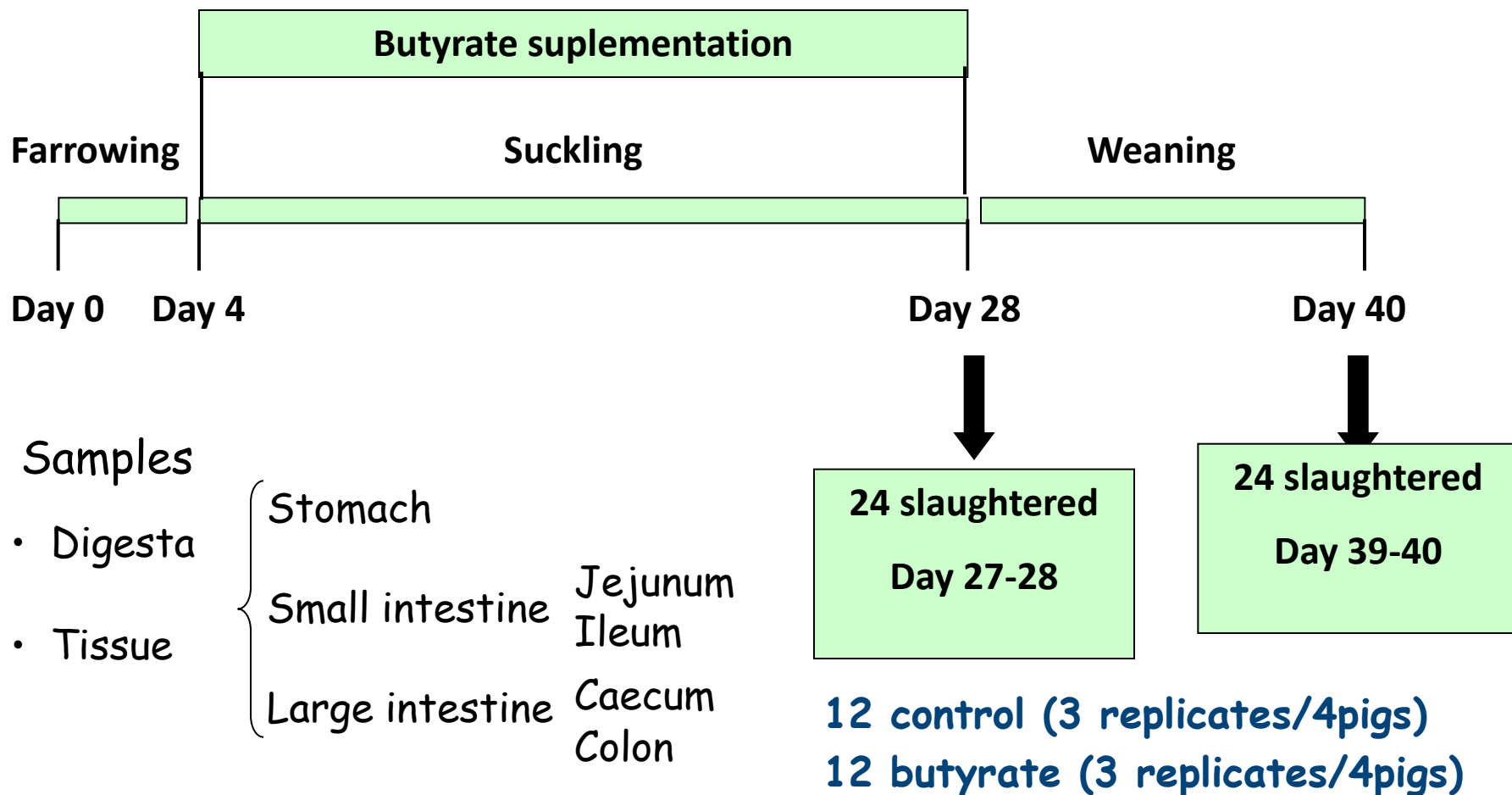
INRA St. Gilles

Hypothesis:

Na-butyrate provided early after birth is able to speed up the maturation of the GIT and help overcome the critical period of weaning

Experimental design

48 piglets



Experimental design

Supplementation with Na-butyrate during suckling period:

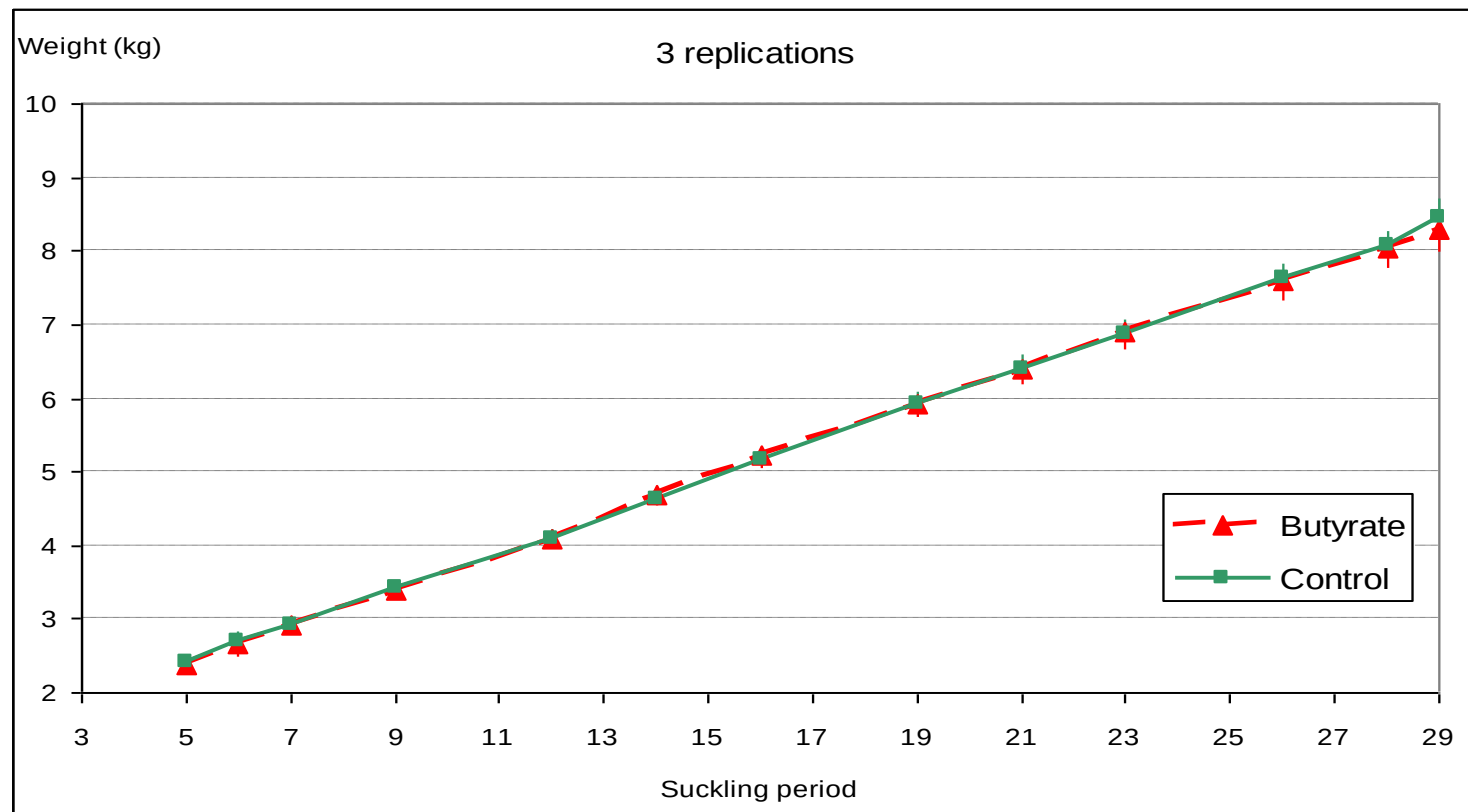
- Dose: 0.3% of the dry matter intake
- Administration: oesophageal tube, twice a day (9:00 am, 3:00 pm)

Post weaning period, administration of reference weaner diet:

- D 0 to D 4: 10 to 80% of energy needs
- D 5 to D 11/12: 100% of energy needs

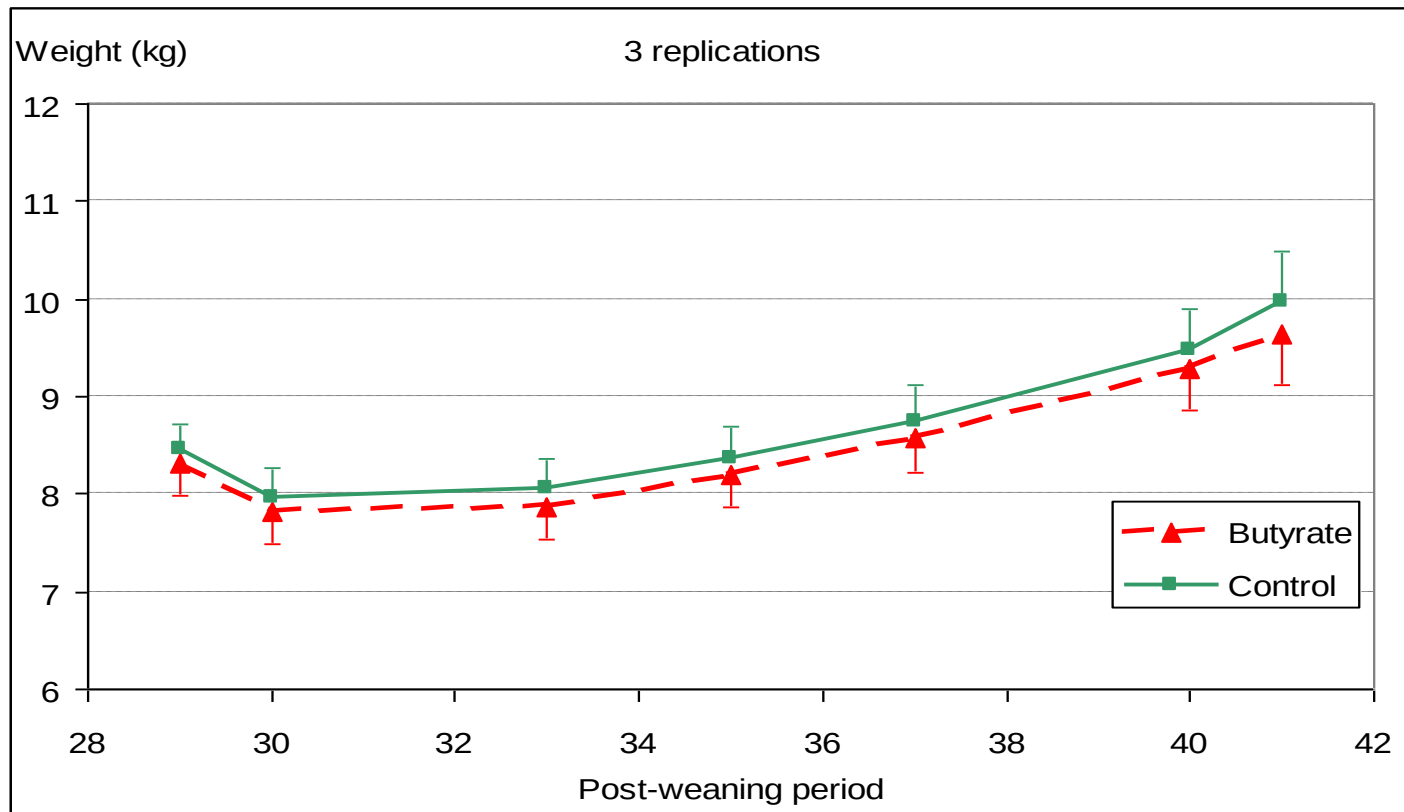
Results: body weight

Growth of piglets during suckling period (3 replicates)



Results: body weight

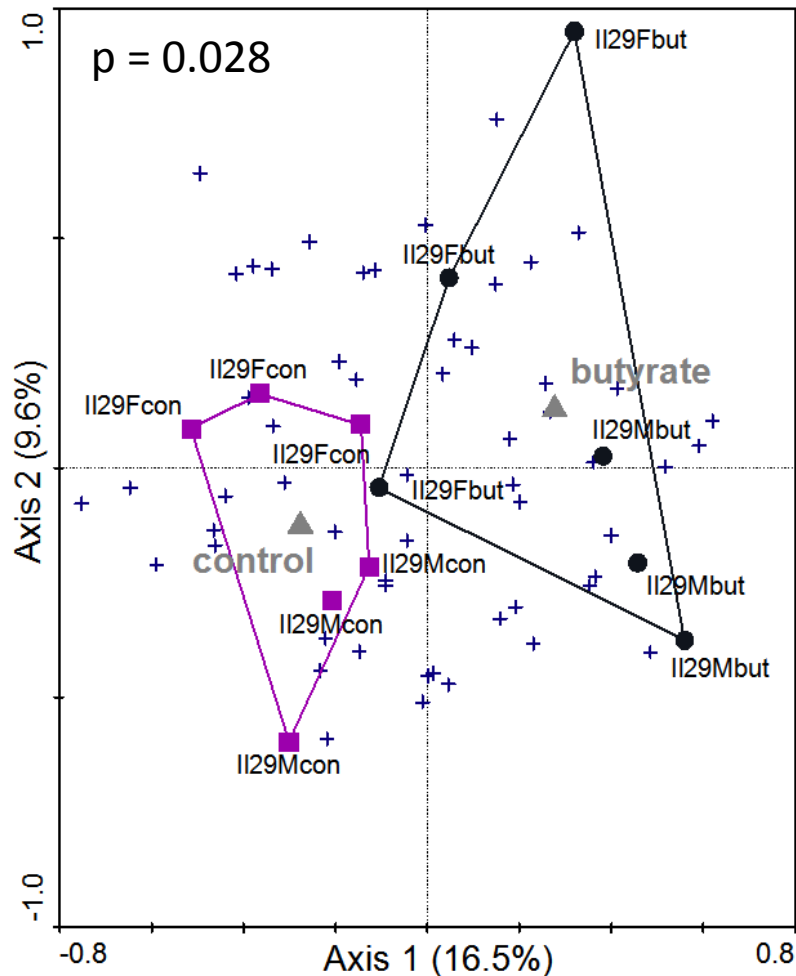
Growth of piglets during post-weaning period



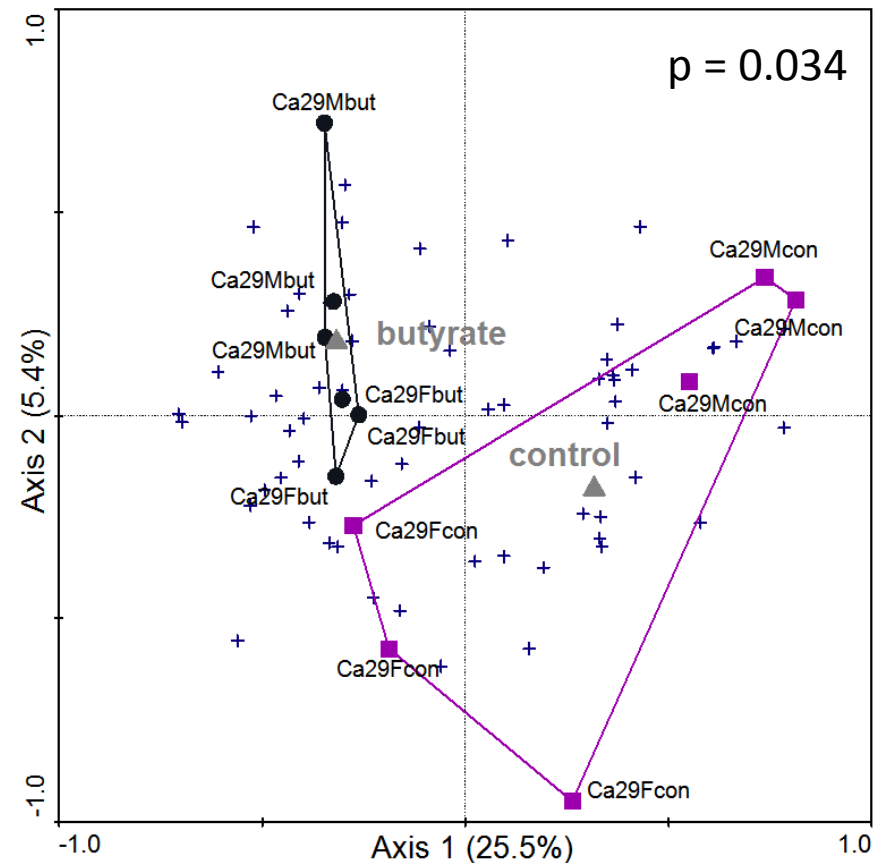
Multivariate analysis of DGGE data

Significant effect on total bacteria in ileum and caecum

Ileum

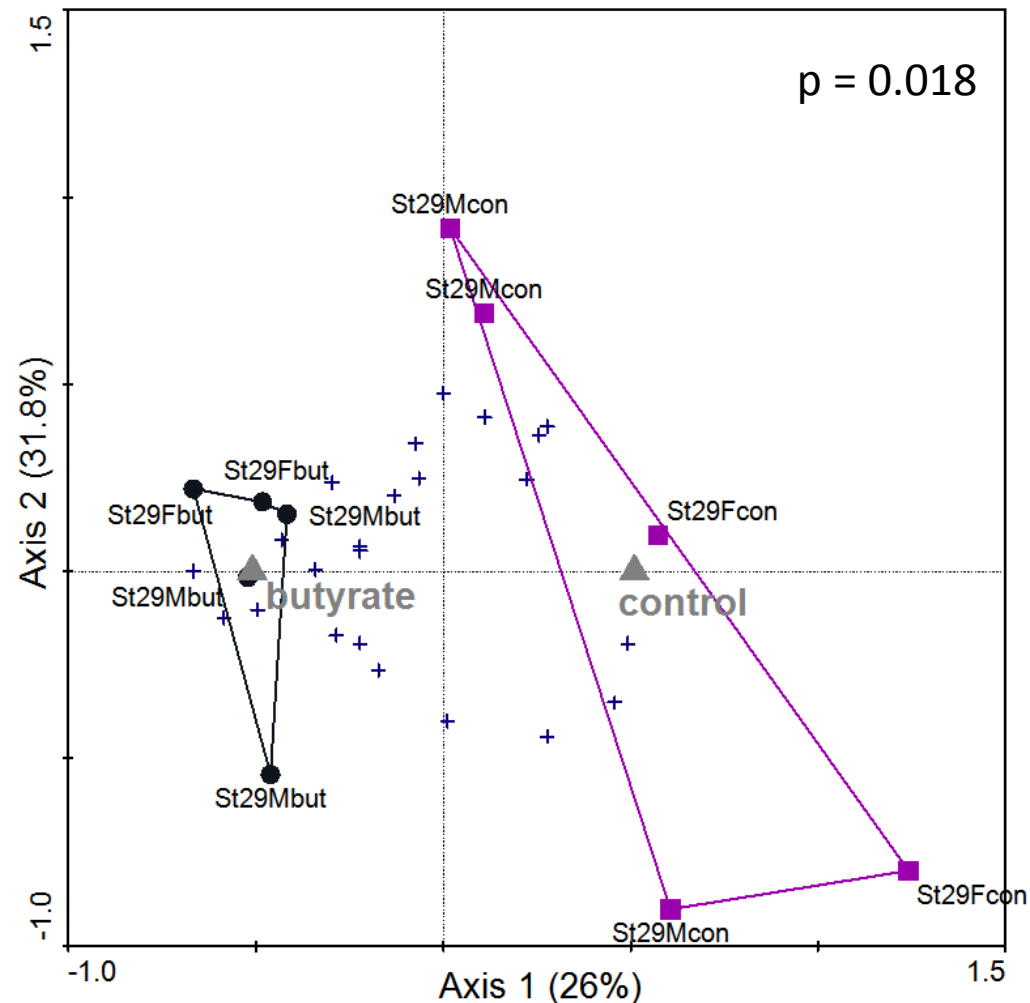


Caecum



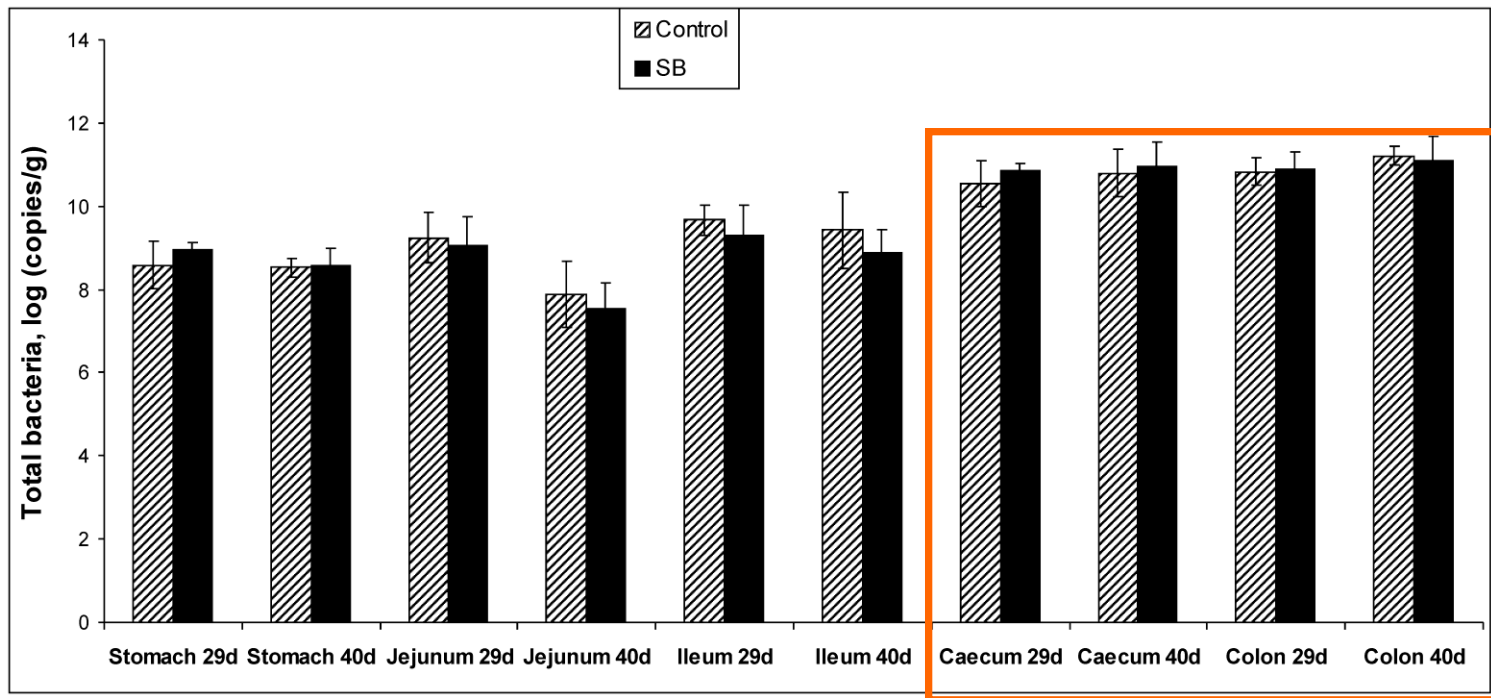
Multivariate analysis of DGGE data

Significant effect on Lactobacillus groups in the stomach

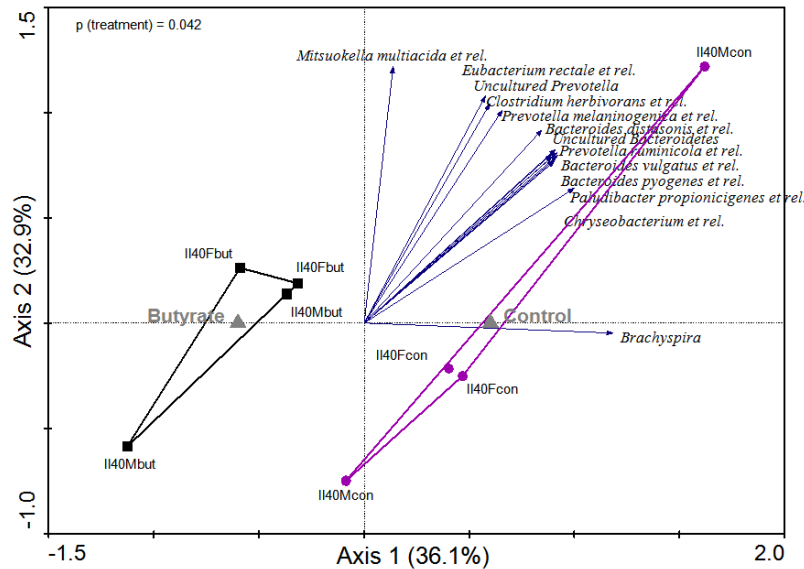


Quantification of total bacteria

qPCR: No effect of SB on bacterial numbers



PITChip data: antimicrobial effect



RDA

SB changes microbial composition

More homogeneous group with SB

SB shows antimicrobial effect after weaning

Site	Higher taxonomic group	Group	Effect	p-value	Relative abundance (%)
Caecum 29d	Alphaproteobacteria	Spingomonas-like	down	0.017	0.08
		Treponema-like	up	0.028	0.04
		Bacteroides pyogenes-like	down	0.039	0.80
Colon 29d	Bacteroidetes	Prevotella melaninogenica-like	down	0.025	0.48
		Uncultured Bacteroidetes	down	0.02	1.0
		Uncultured Porphyromonadaceae	down	0.042	0.74
		Uncultured Prevotella	down	0.013	3.07
		Flavobacteria	down	0.038	0.49
Ileum 29d	Clostridium cluster I	Clostridium perfringens-like	up	0.006	2.13
		Alphaproteobacteria	down	0.037	0.45
Ileum 40d	Clostridium cluster XIVa	Dorea-like	down	0.03	0.83
		Eubacterium plexicaudatum-like	down	0.041	0.37
		Eubacterium ventriosum-like	down	0.043	0.37
		Roseburia intestinalis-like	down	0.037	1.21
	Deltaproteobacteria	Uncultured Deltaproteobacteria	down	0.006	0.10
	Fibrobacteres	Fibrobacter succinogenes-like	down	0.013	0.66
	Spirochaetes	Treponema-like	down	0.025	0.05

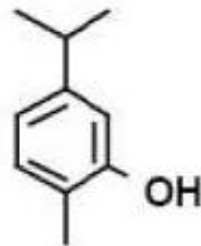
Conclusions

- No difference in BW between the 2 groups (Control / Butyrate)
- SB did not affect total bacterial counts but relative abundance of individual groups
- Pre-weaning effect of SB in the ileum, caecum and stomach
- Post-weaning antimicrobial effect of Na-butyrate in the ileum
- SB homogenizes microbial profiles

Case study 3

Carvacrol: experimental setup

Origanum vulgare



Carvacrol

Identified as the active constituent of Oregano Oil
Main antibiotic molecule in Oreganos

Case study 3

Carvacrol: experimental setup

Animals

- 7 suckling piglets from 1 litter
- T-cannulation at the terminal ileum: 17 - 19 days of age

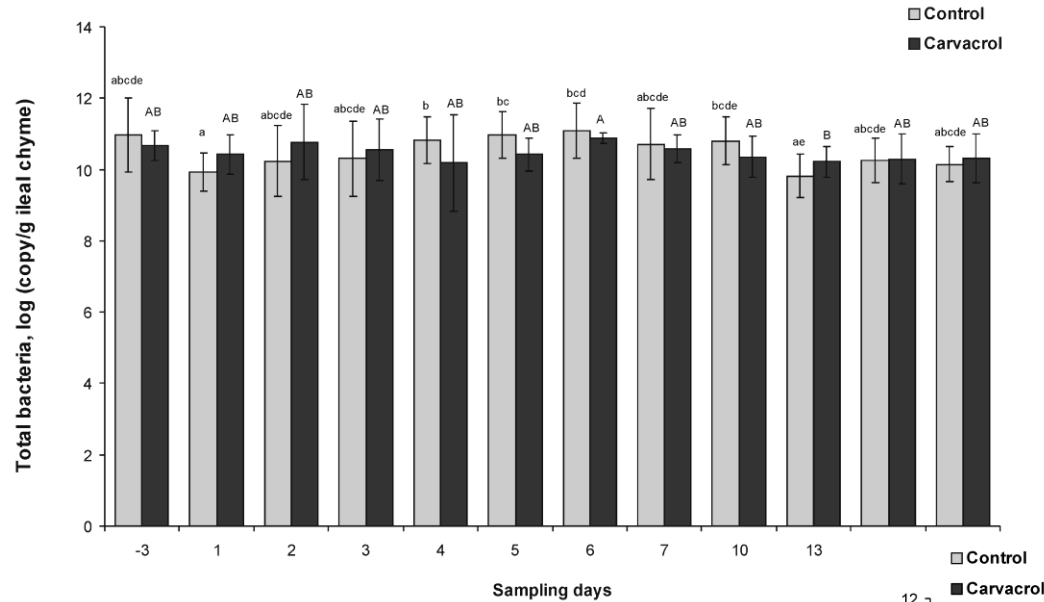
Diets

- before weaning piglets suckled by the sow
- post weaning 2 different starter diets applied
- ❖ **control** = reference diet from FFPH (starter A)
- ❖ **treatment** = carvacrol (150 g/ton)

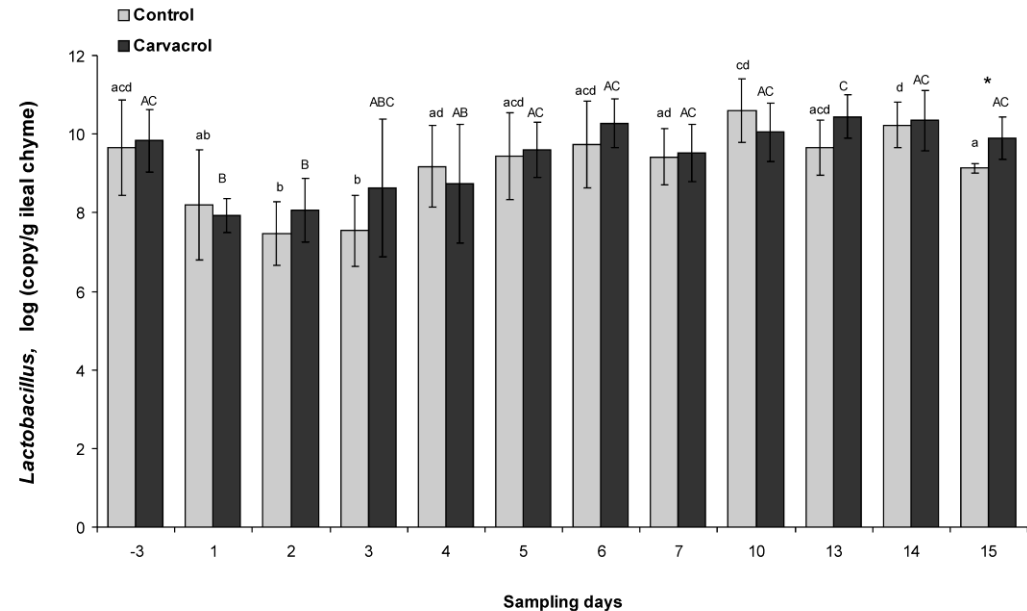
Collection of samples

Day	Day	Day	Day	Day	Day	Day	Day	Day	Day	Day	Day
-3	1	2	3	4	5	6	7	10	13	14	15

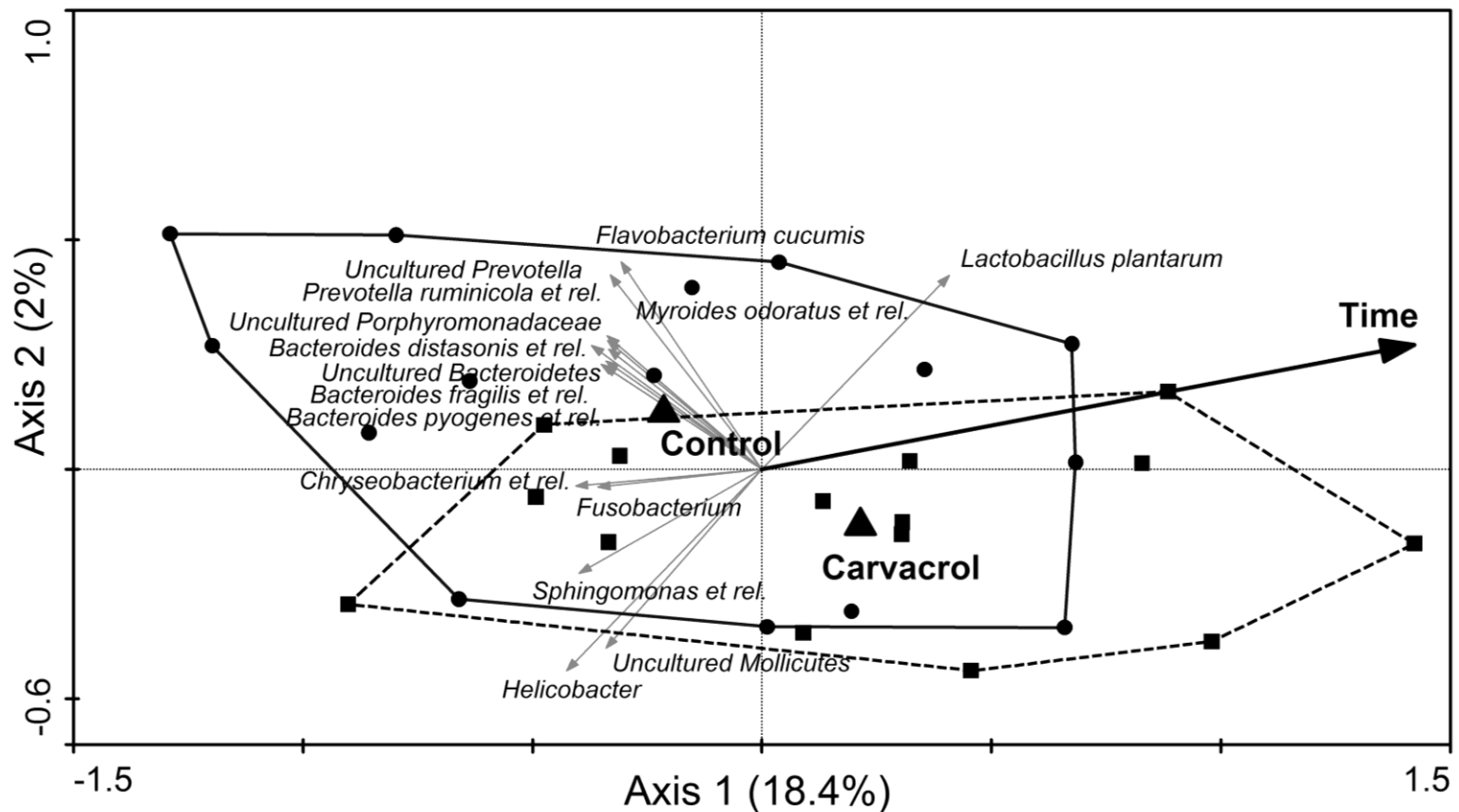
Quantification of total bacteria and *Lactobacillus* by qPCR



dynamics in time of bacterial numbers present in control and treatment piglets



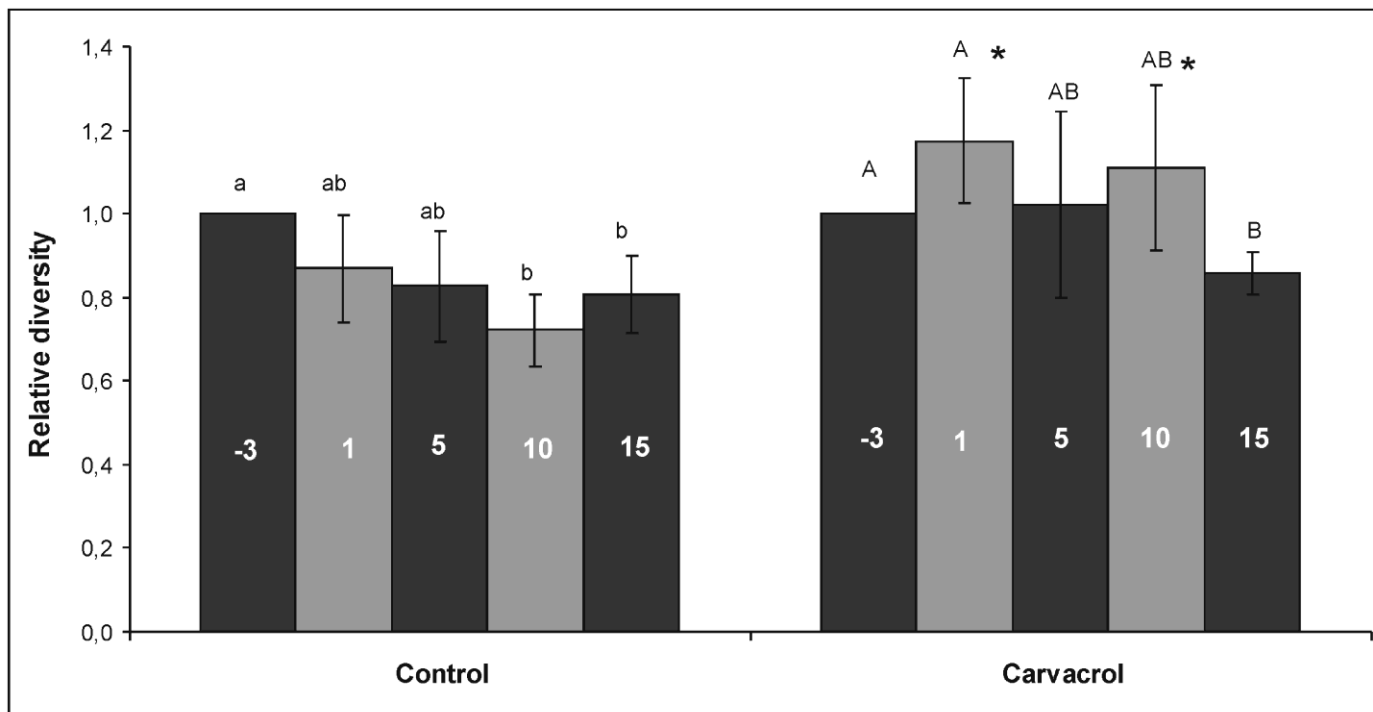
Multivariate analysis: of PITChip data



$p(\text{time}) < 0.01$

PITChip: Diversity per treatment

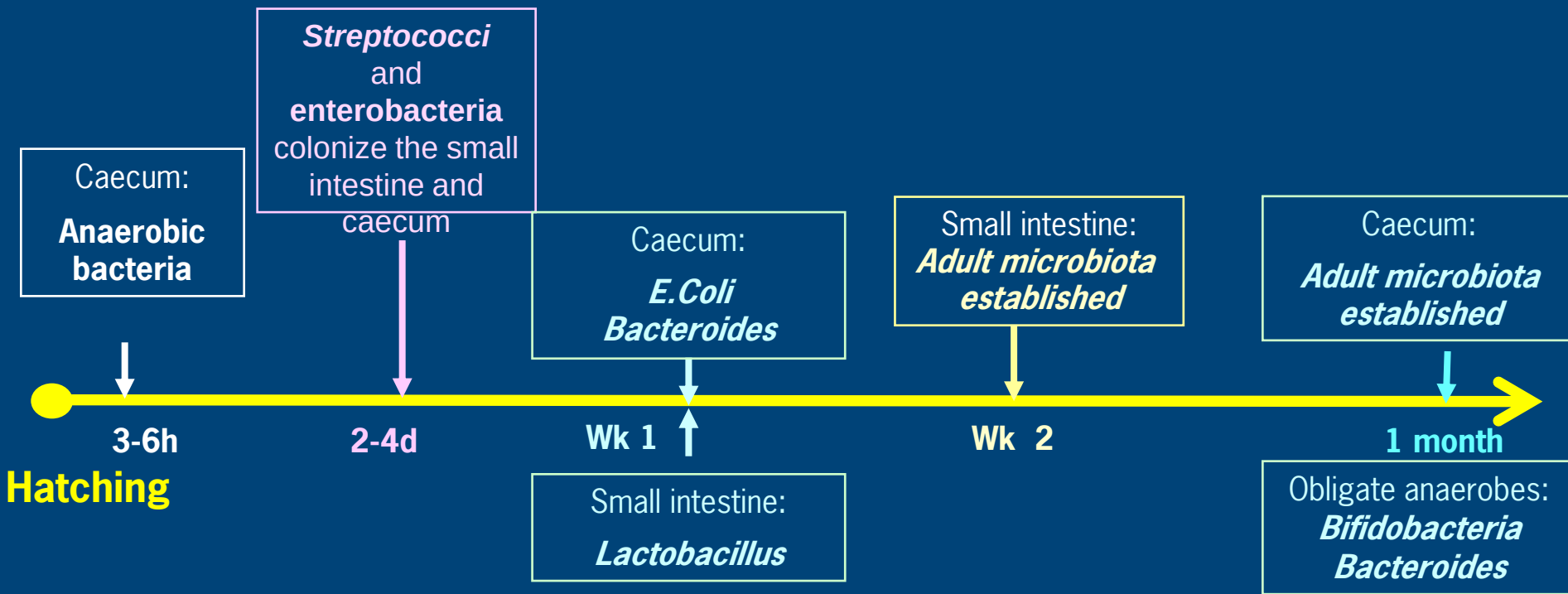
Relative diversity in time



Conclusions

- No effect of carvacrol on performance
- Significant effect of sampling time
- Fermentation-end products analysis reflected an altered microbiota. No effect of carvacrol
- Total bacterial counts stable: decrease in beneficial bacteria → increase in pathogens
- Antimicrobial effect (Gram-negative bacteria)
- Data suggests an increased stability when carvacrol is added

Chicken gut microbiota: microbial succession



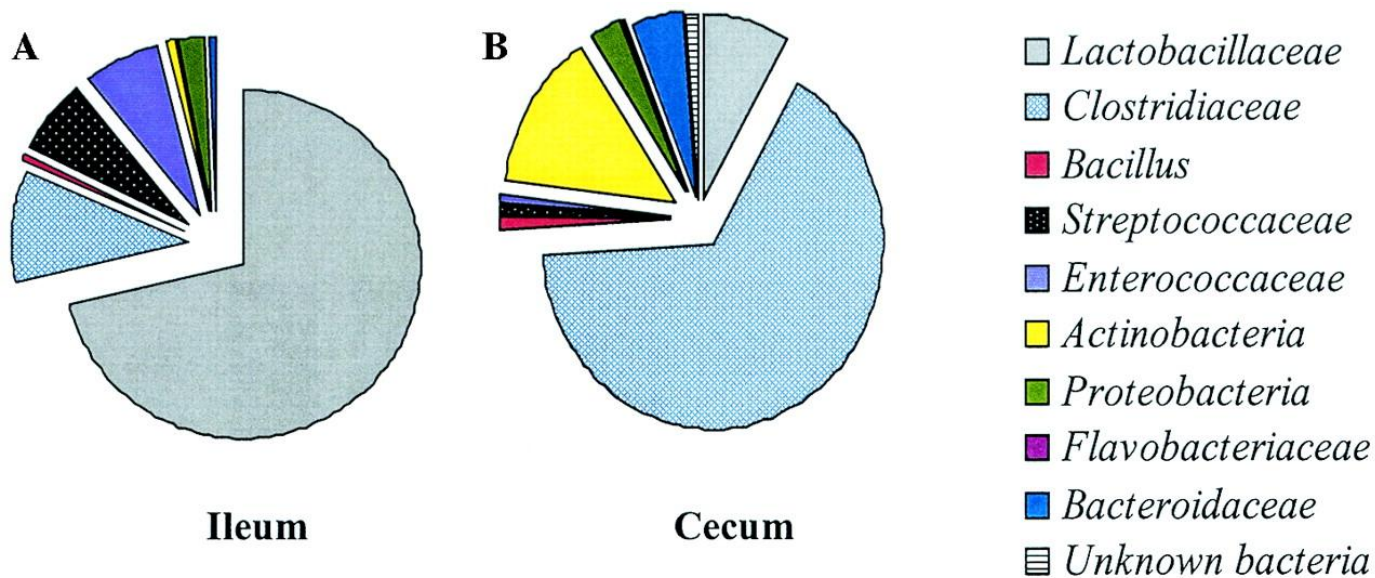
Barnes et al., 1972

Mead and Adams, 1975

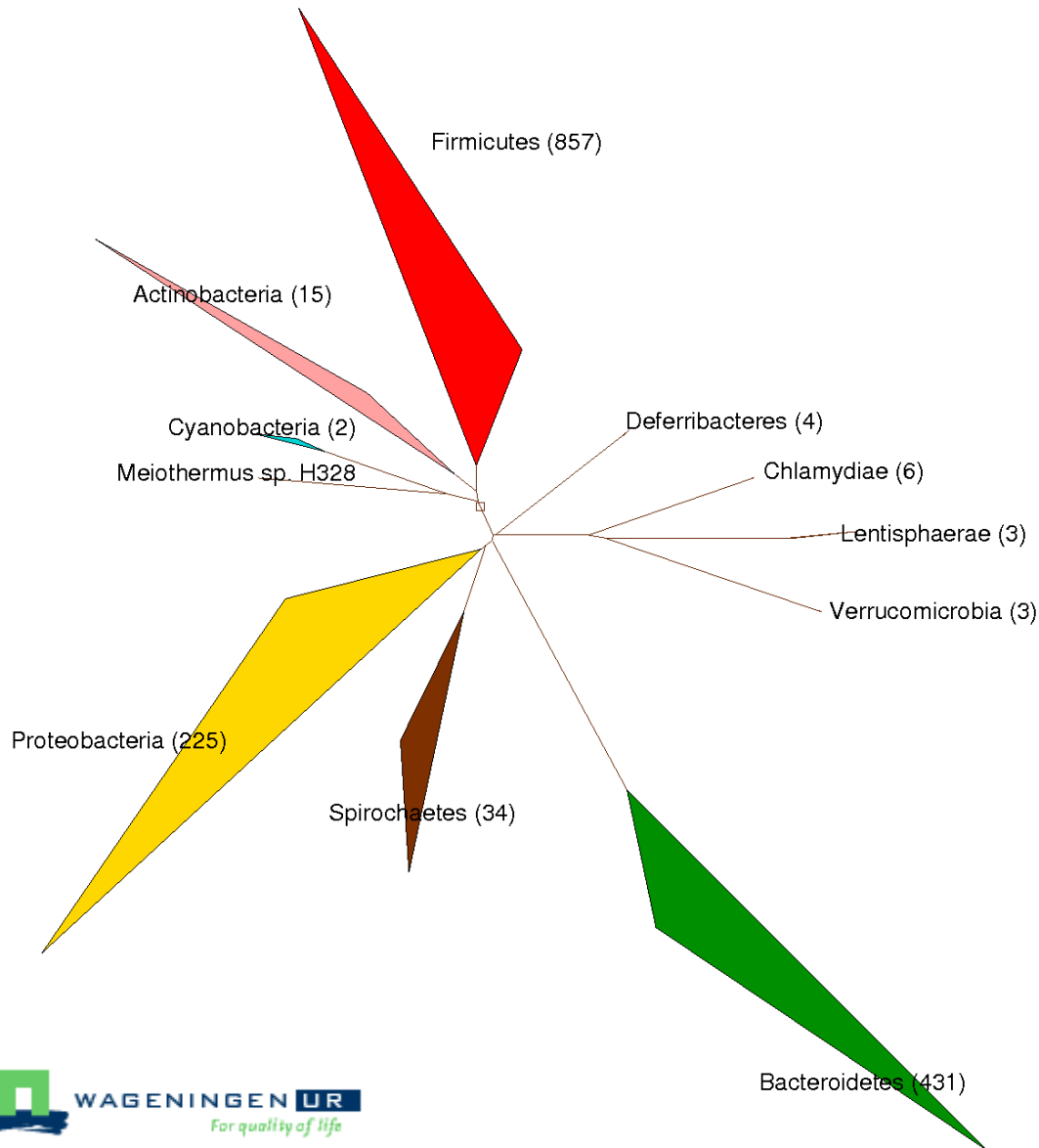
Lev and Briggs, 1956

Chicken gut microbiota

Microbial composition of the ileum and caecum of broiler chickens, determined by sequencing of 1,230 clones from a 16S rDNA community DNA library



Chicken Intestinal Tract Chip (CHICKChip)

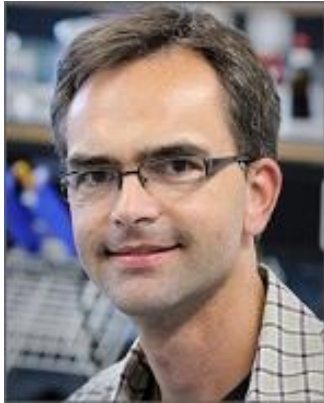


Currently > 1500 seq
mostly *Firmicutes*
&
Bacteroides/Prevotella
& *Proteobacteria*

Mostly uncultured
(>70%)

Still:
Find unique OTUs
Design probes

WU-MIB Animal GIT Research



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Acknowledgements

Wageningen University

Microbiology

Sara de Filippi
Paula Fajardo
Hans Heilig
Sebastian Tims
Tom van den Bogert
Sergey Konstantinov
Hauke Smidt
Willem M. de Vos

Animal Nutrition

Martin W.A. Verstegen
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Schothorst Lelystad

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Jeannette Kluess
Jan Fledderus

INRA

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Terence Pellet