Insight into the microbial diversity of healthy human milk by metagenomic approach

By
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Topics to be covered

- ✓ Introduction
- ✓ Origin of bacteria in breast milk
- ✓ International & National status
- ✓ Significance of the study
- ✓ Methodology
- ✓ Results and discussion
- ✓ Conclusion


Introduction
MILK

Diversity of Life on Earth

- ✓ Bacterial spp. ~10,000
- ✓ Fungi ~ 60,000
- ✓ Animals and plants ~ 1.3 million

- **Described species: ~1.5 millions**
- **Predicted to exist: >30 millions**
- **Cultivate in the lab: ~thousands**
- **How uncultivable bacterial species can be identified?**
- **How the expression of genes occures in vivo and in vitro?**

Metagenomics

Metagenomics (also Environmental Genomics, Ecogenomics or Community Genomics) is the study of genetic material recovered directly from environmental samples.

Library Preparation



Deep Sequencing



Advanced Data Analysis



- **The ongoing revolution in metagenomic sequencing technology has led to the production of sequencing machines with dramatically lower costs and higher throughput.**

- **Sequencing and analysis can provide relatively rapid and cost-effective methods for assessing bacterial diversity and abundance and may be useful for pathogen discovery and identification.**

Human milk Metagenomics

- **Human milk contains fat, protein, carbohydrate, minerals and bacteria..**

- **Breast milk → Source of bacteria**

  initiation and development of the neonatal gut microflora.

  Infant Health

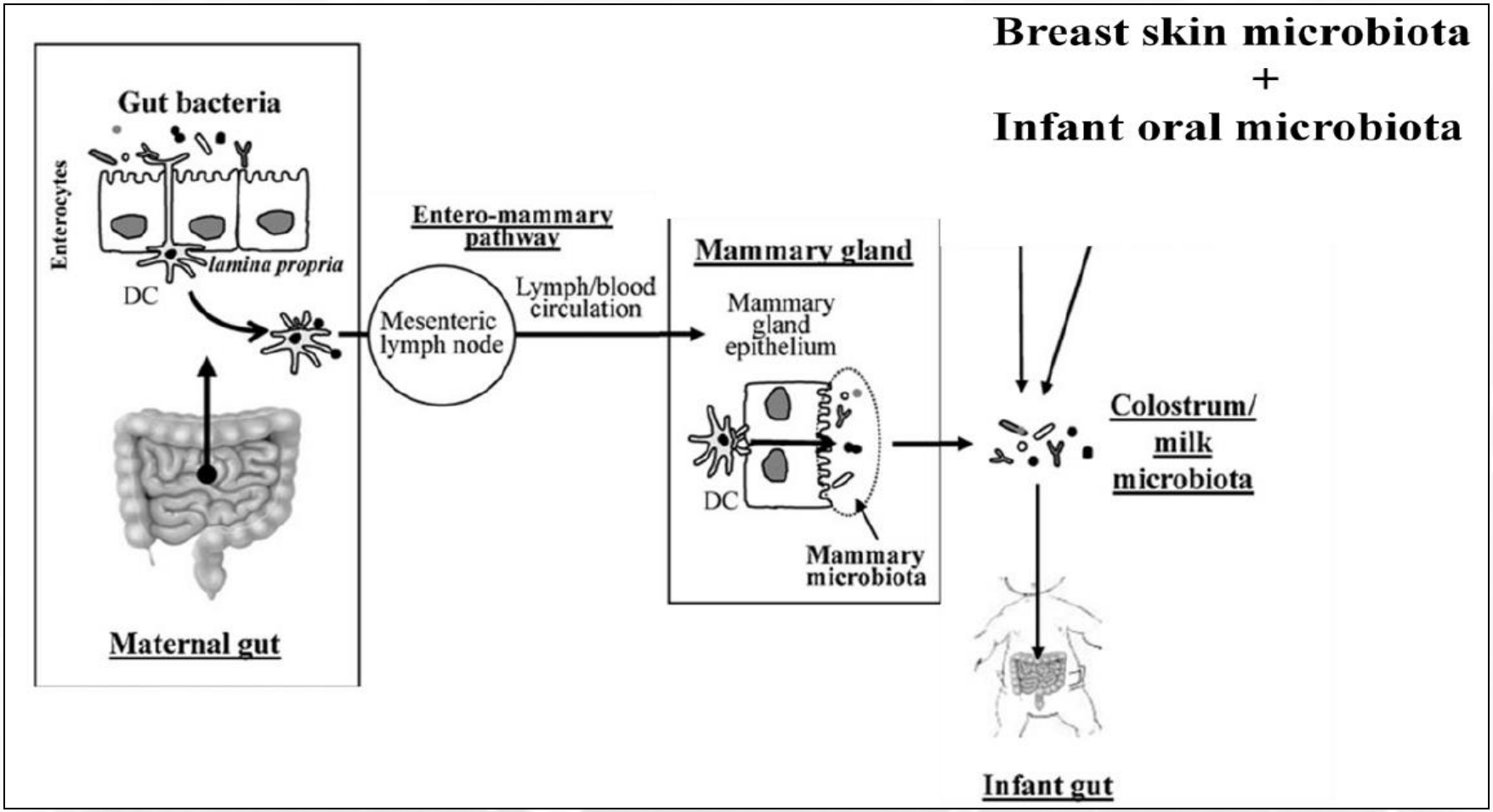
- **Bacteria that are commonly found in human milk**
  - ✓ ***Staphylococci***
  - ✓ ***Streptococci***
  - ✓ ***Lactobacilli***
  - ✓ ***Lactococci***
  - ✓ ***Enterococci and***
  - ✓ ***Bifidobacteria***

FROM WHERE THESE BACTERIA CAME TO THE MAMMARY GLAND



- **Studies articulate transmission of bacterial strains from mother-to-infant through breastfeeding** (*Albesharat et al., 2011; Jimenez et al., 2008c; Makino et al., 2011; Martin, 2012; Martín et al., 2006; Matsumiya et al., 2002).*
- **However, the exact mechanisms by which bacteria reach the mammary gland have been the subject of much debate over the years.**
- **Conventionally, it is believed that the presence of bacteria in human milk is a result of a simple contamination with bacteria from the mother's skin or infant's oral cavity.**
- **Occurrence of Streptococcus in infant saliva, colostrum and human milk is been established by culture independent and culture dependent method** *(Hunt et al., 2011; Jimenez et al., 2008a,b).*

The revolutionary hypothesis: 'active migration'



(A) Hormonal changes occurring in this period may have an influence on gut permeability, which could facilitate bacterial uptake. (B) The mother's skin microbiota and infant's oral microbiota may contribute to the establishment of the human milk microbiome. (C) Bacteria from the maternal intestinal tract may be taken up by different immune cells. The massive migration of immune cells to the mammary glands could provide another possible route to alter the human milk microbiome.

Fernández et al. 2013

- **Despite these interesting findings, several questions arise and need to be answered before the 'migration hypothesis' could be accepted.**
- **It remains unclear how a bacterium interacts with the immune cell and is actually transported to the mammary gland.**

Human Immunodeficiency Virus Load in Breast Milk, Mastitis, and Mother-to-Child Transmission of Human Immunodeficiency Virus Type 1

Richard D. Semba,[1] Newton Kumwenda,[2] Donald R. Hoover,[2] Taha E. Taha,[2] Thomas C. Quinn,[3,6] Laban Mtimavalye,[7] Robert J. Biggar,[5] Robin Broadhead,[8] Paolo G. Miotti,[6] Lori J. Sokoll,[4] Len van der Hoeven,[2] and John D. Chiphangwi[7]

Departments of [1]Ophthalmology, [2]Epidemiology, [3]Medicine, and [4]Pathology, Johns Hopkins University Schools of Medicine and Hygiene and Public Health, Baltimore, and [5]Viral Epidemiology Branch, National Cancer Institute, and [6]National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland; Departments of [7]Obstetrics and Gynaecology and [8]Paediatrics and Child Health, College of Medicine, University of Malawi, Blantyre, Malawi

Human immunodeficiency virus (HIV) type 1 load in breast milk and mastitis were examined as risk factors for vertical transmission of HIV-1. Six weeks after delivery, HIV-1 load and sodium (an indicator of mastitis) were measured in breast milk from 334 HIV-1–infected women in Malawi. Median breast milk HIV-1 load was 700 copies/mL among women with HIV-1–infected infants versus undetectable (<200 copies/mL) among those with uninfected

Downloaded

Can bacteria in human milk influence maternal and infants' health…?

- **Bacteria present in human milk may be crucial in programming the immune system to respond to antigens, pathogens and commensal bacteria** *(Donnet-Hughes et al., 2010).*

- **It has been demonstrate that *L. fermentum* and *L. salivarius*, isolated from breast milk are able to activate NK cells, CD4+ T cells, CD8+ T cells and regulatory T cells** *(Perez-Cano et al. 2011)*

- ***L. fermentum* has been accounted to reduce incidence of gastrointestinal and upper respiratory tract infections in infants (** *Maldonado et al., 2012).*

Journal of Dairy Research (2008) **75** 365–373. © *Proprietors of Journal of Dairy Research 2008*
doi:10.1017/S0022029908003373 *Printed in the United Kingdom*

Intramammary infusion of a live culture of *Lactococcus lactis* for treatment of bovine mastitis: comparison with antibiotic treatment in field trials

Katja Klostermann[1,2], Fiona Crispie[1], James Flynn[3], R Paul Ross[1]*, Colin Hill[2] and William Meaney[3]

[1] Moorepark Food Research Centre, Teagasc, Moorepark, Fermoy, Co. Cork, Ireland
[2] Department of Microbiology, University College Cork, Cork, Ireland
[3] Dairy Production Research Centre, Teagasc, Moorepark, Fermoy, Co. Cork, Ireland

Received 8 November 2007; accepted for publication 4 April 2008

A treatment containing a live food-grade organism, *Lactococcus lactis* DPC3147, was compared with conventional antibiotic therapy for its potential to treat bovine chronic subclinical or clinical mastitis in two separate field trials. Effects on disease symptoms and bacteriology were

Journal of Dairy Research (2008) **75** 374–384. © *Proprietors of Journal of Dairy Research 2008*
doi:10.1017/S0022029908003385 *Printed in the United Kingdom*

Intramammary infusion of a live culture for treatment of bovine mastitis: effect of live lactococci on the mammary immune response

Fiona Crispie[1,3], Mercedes Alonso-Gómez[2], Collette O'Loughlin[2], Katja Klostermann[1,3], James Flynn[3], Seán Arkins[2], William Meaney[3], R Paul Ross[1]* and Colin Hill[4]

[1] Biotechnology Department, Moorepark Food Research Centre, Fermoy, Co. Cork, Ireland
[2] Department of Life Sciences, University of Limerick, Limerick, Ireland
[3] Dairy Production Research Centre, Moorepark, Fermoy, Co. Cork, Ireland
[4] Department of Microbiology, University College Cork, Cork, Ireland

Received 8 November 2007; accepted for publication 4 April 2008

- **The use of more sophisticated culture-dependent and independent techniques to study the Milk Microbiome has revealed a complex ecosystem with a much greater diversity than previously anticipated.**
- **Comparative metagenomic analyses identified unique and/or over-abundant taxonomic and functional elements within milk microbiomes.**
- **These genetic attributes may help in better understanding of microbial genetic factors that are relevant to human health.**

International Status

- **First study on bacterial diversity of human milk from healthy women was reported in 2003, based on in vitro culturing methods** ***(Heikkila and Saris, 2003; Martín et al., 2003).***

- **During 2007 to 2010, it was sophisticated -based on 16s rRNA gene sequencing, real time PCR and Quantitative PCR.**

- **Till 2010 bacterial diversity based on culture dependent methods were investigate.**

- **It included bacterial like *Lactobacillus, Bifidobacterium, Staphylococcus, Streptococcus, Enterococcus, Weisella.***

- **In 2011, Hunt et al. used new approach i.e. 454-pyrosequencing**
- **They characterized the microbial diversity and temporal stability of bacterial in American lactating mother.**

- **Using similar approach, researcher of different country (Finland, Mozambique, Switzerland, Canada, Slovenia Germany and Austria) carried out similar study**

- **All the studied showed abundance of genera *Pseudomonas, Staphylococcus, Serratia, Corynebacterium, Ralstonia, Streptococcus, Sphingomonas, Bradyrhizobium* and *Propionibacterium* in human milk.**

National status:

- **There are no significant reports demonstrating milk microbiome except few.**
- **Presence of *Lactobacillus fermentum, Enterococcus mudtii, Enterococcus faecium, lactobacillus reuteri* i.e. beneficial bacteria in breast milk *(Vaibhav et al., 2012 and Anandharaj & Sivasankari, 2013).***
- **Vaidya et. al 2015 explore the microbial community present in human milk using culture dependent method**

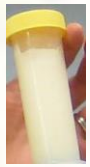
Significance of the study

- **Role of microbes on infant health can be studied.**
- **Unexplored microbial community can be studied using culture-independent techniques.**
- **Information gained can help in formulating maternal diet and baby food.**


METHODOLOGY
SYSTEM
CONSTRUCTION
PROPER
GENERIC
CONSTRUCTIVE
RULES
WORD
QUALITATIVE
ARRAY
TECHNIQUE
RECENT
PARTS
PROCESSES
ANALYSIS
DEVELOPMENT
ATTAINING
THEORIES
FRAMEWORK
INCREASE
SPECIFIC
OBJECTIVE
METHOD
READING
DISCIPLINE
SYSTEMATIC
COMBINED
BUZZWORD
EMPLOYED
LOGICAL
PAST
CRITICISM
CHANGED
ALGORITHM
COMPONENTS
SUBSTITUTE
QUANTITATIVE
METHODOLOGY
TASKS
GUIDELINE
RESEARCH
PROBLEM
DEFINED
STUDY
PHASES
PARADIGM
FREQUENCY
ATTENTION

1. Amplicon preparation

DNA isolation from healthy breast milk



Human milk



DNA

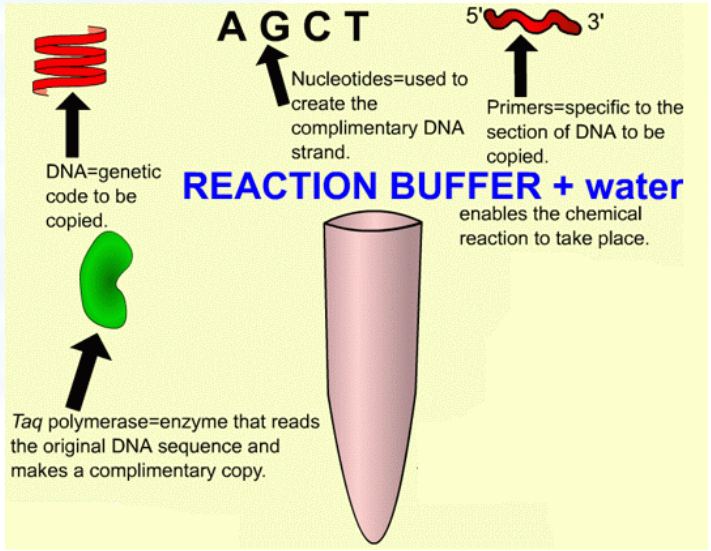
The primers (Forward, 100F: 5'-**CCA TCT CAT CCC TGC GTG TCT CCG ACT** *CAG* XXX XXX XXX XAC TGG CGG ACG GGT GAG TAA';
Reverse 517R, 1: 5'- **CCT CTC TAT GGG CAG TCG GTG GAT** CGT ATT ACC GCG GCT GCT GG-3';
Red font: Ion Torrent specific adapters; **Blue font**: sequencing key for calibrating signal intensity at the beginning of the sequencing run; **Purple font:** X…X: 10 bp barcode; **orange font**: template-specific primers;



Primer

PCR reaction mixture:

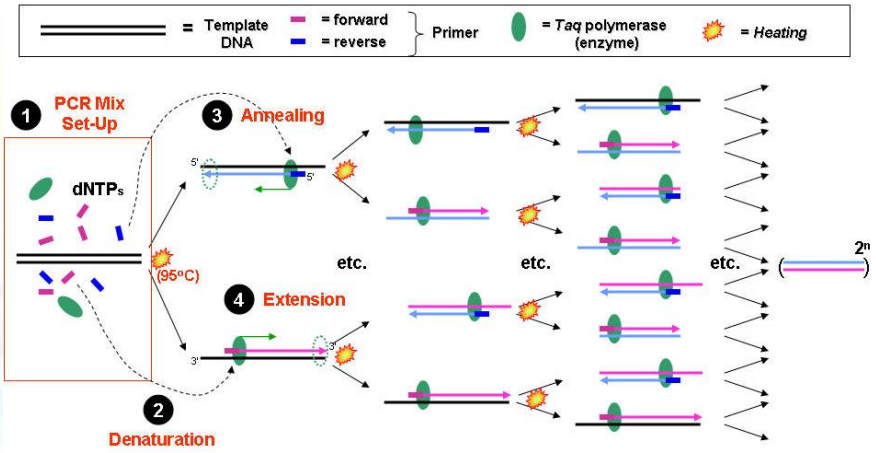
Reagents	Volume (µl)
Buffer	**5**
$MgCl_2$	**0.5**
dNTPs	**1**
Taq DNA polymerase	**1**
Forward primer	**1**
Reverse primer	**1**
Template	**1**
Miliq water	**24.5**



PCR reaction Mixture

PCR condition

Condition	Temperature	Time	
Initial Denaturation	95°C	5min	30 Cycle
Denaturation	95°C	30 sec	
Annealing	60°C	45 sec	
Extension	72°C	60 sec	
Final Extension	72°C	10 min	



Amplification of DNA

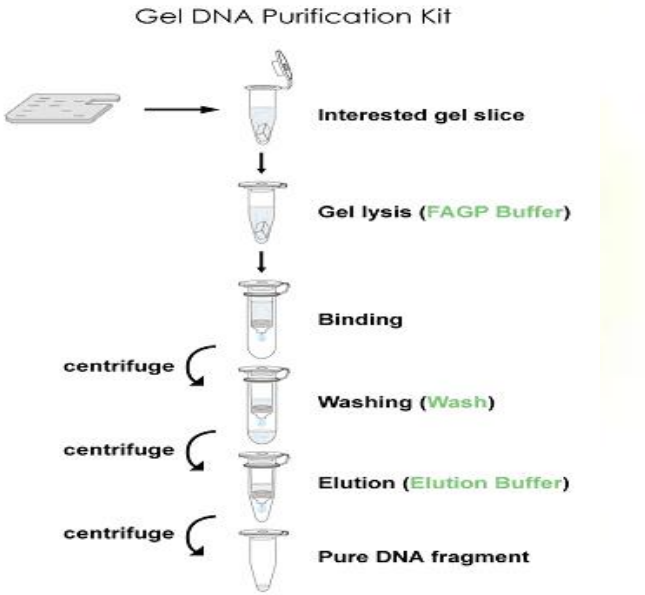
2. Amplicon Purification, Quantification and Pooling

Run the amplicon in 2% agarose gel and Approx 500 bp size band was selected and cut using a clean scalpel

An excised band was weighted and purified by Gel Extraction kit

All the amplions were pooled in equimolar concentration

Quantity of amplions was determined by Qurbitflurometer (Invitrogen. USA).





Qurbitflurometer

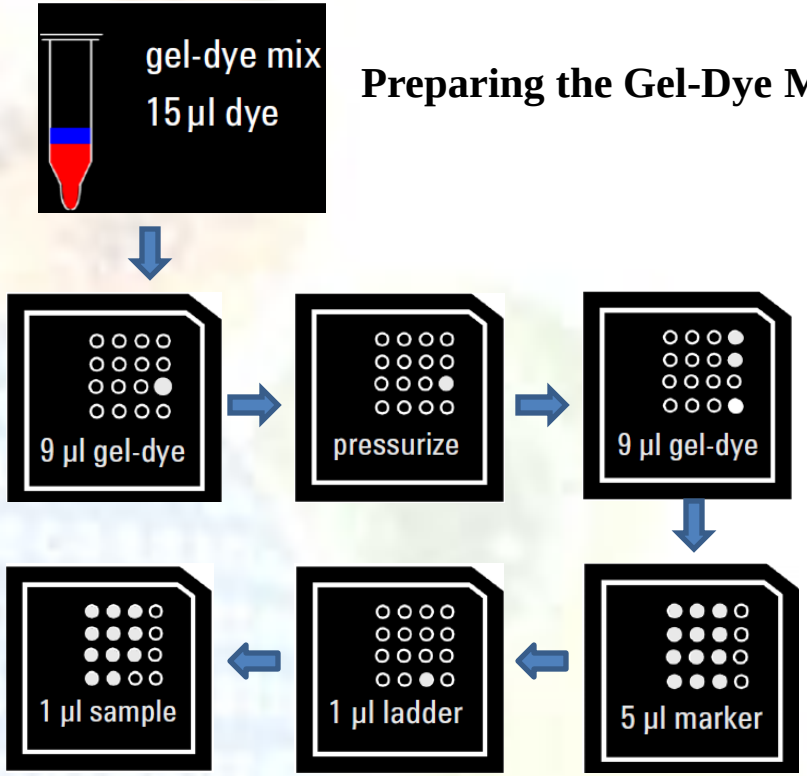
Quality was checked by Bioanalyzer High sensitivity chip (Agilent. USA)



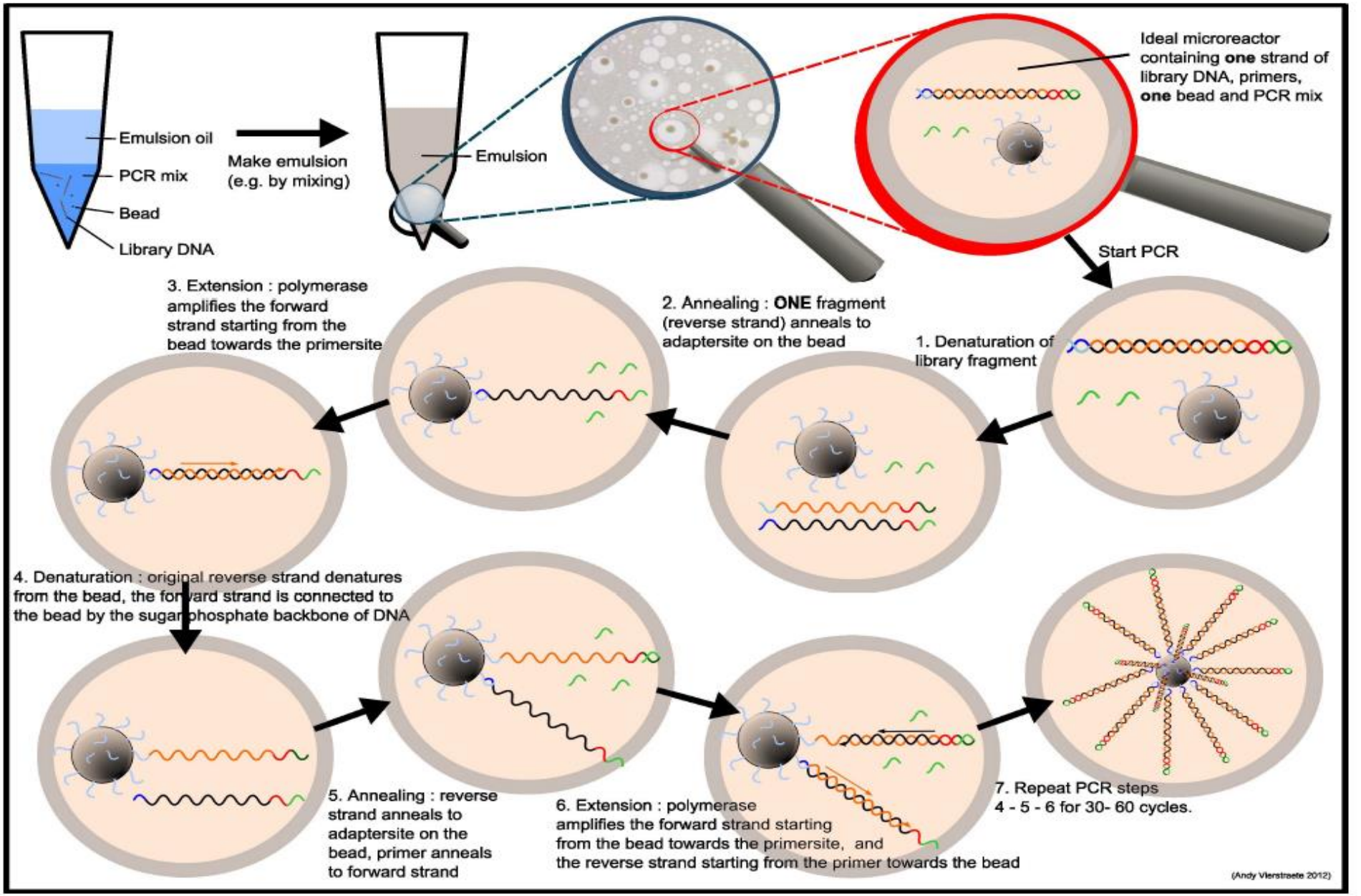


Final concentration of amplicons was made to 26 pM by diluting the purified amplicon.

Preparing the Gel-Dye Mi

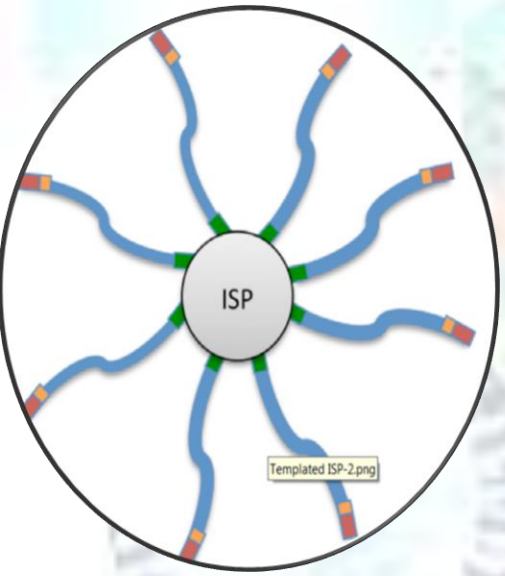


3. Emulsion PCR

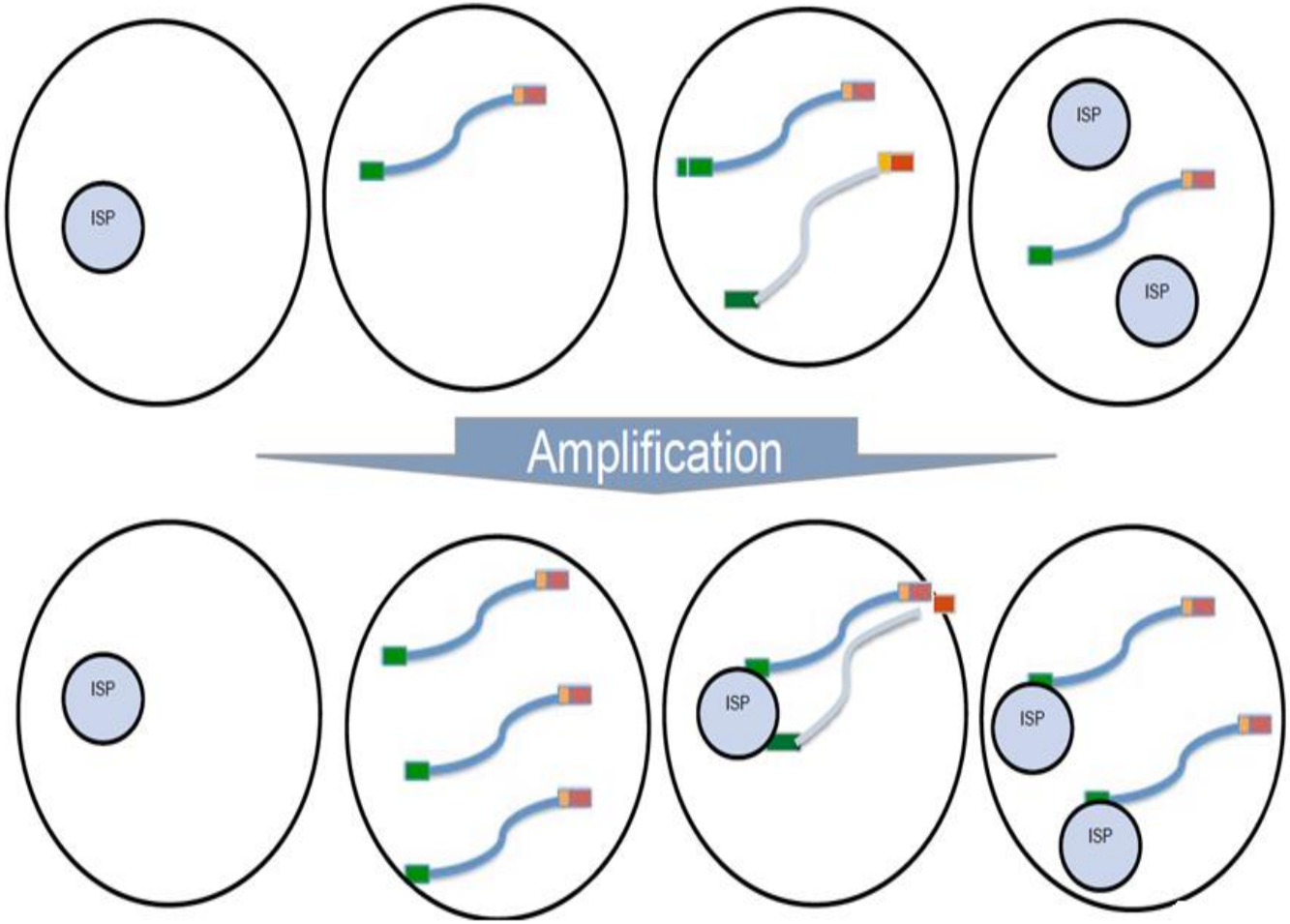


Andy Vlestraete , 2012

Ideal Outcome



Non- Ideal Outcome



No template | No ISP | Polyclonal “mixed” reads | Duplicates reads

Andy Vlestraete , 2012

Quantification of the library is important!

Too much library = lots of polyclonal "mixed" reads



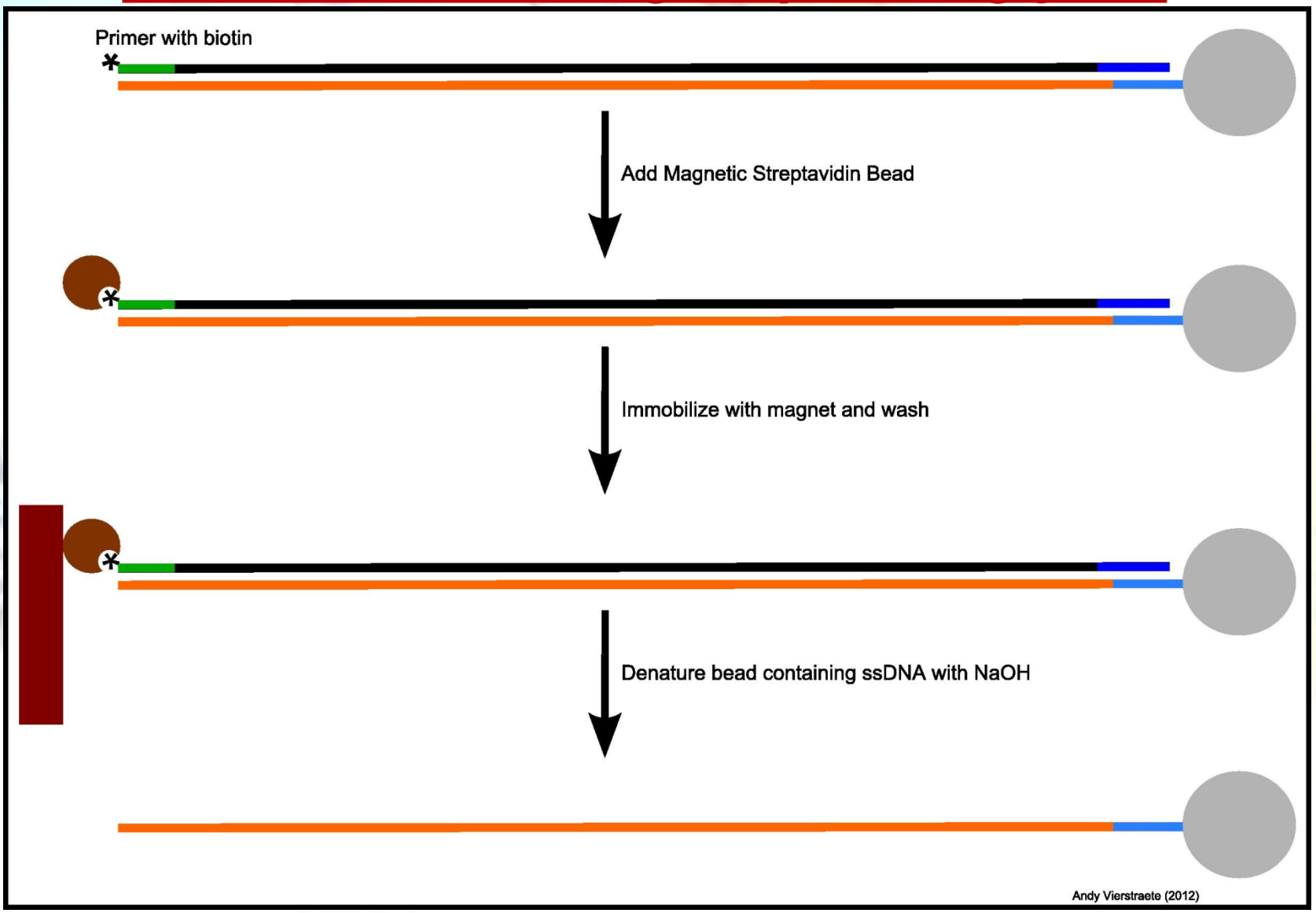
Not enough library = lots of empty ISPs

It's all about balance

$$\mu l \text{ of Library per tube} = \frac{\text{Desired molecule per bead} \times 35 \times 10^6 \text{ Beads per tube}}{\text{Library concentration (in molecules/}\mu l)}$$

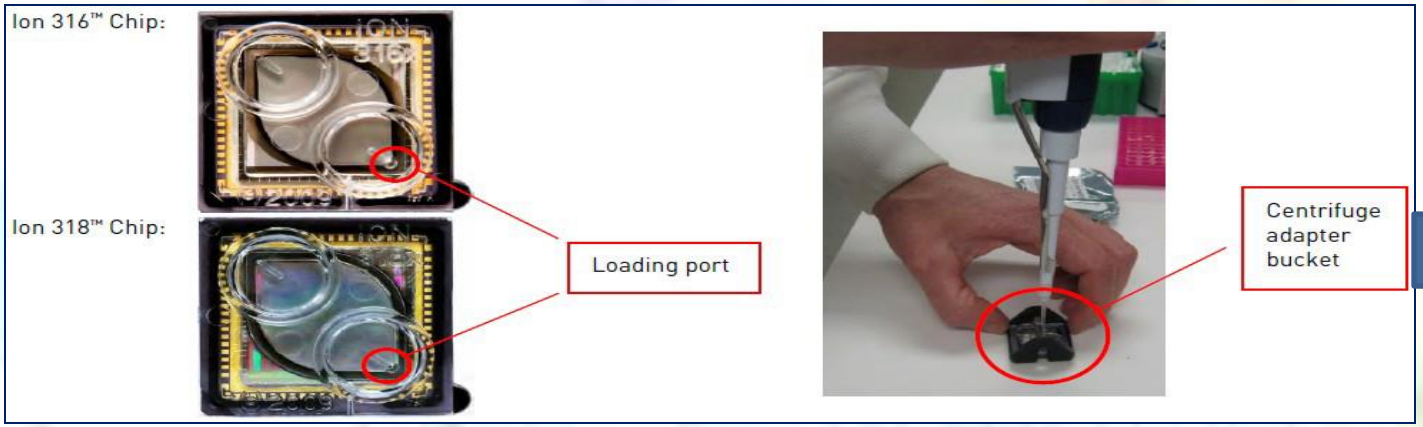
Andy Vlestraete , 2012

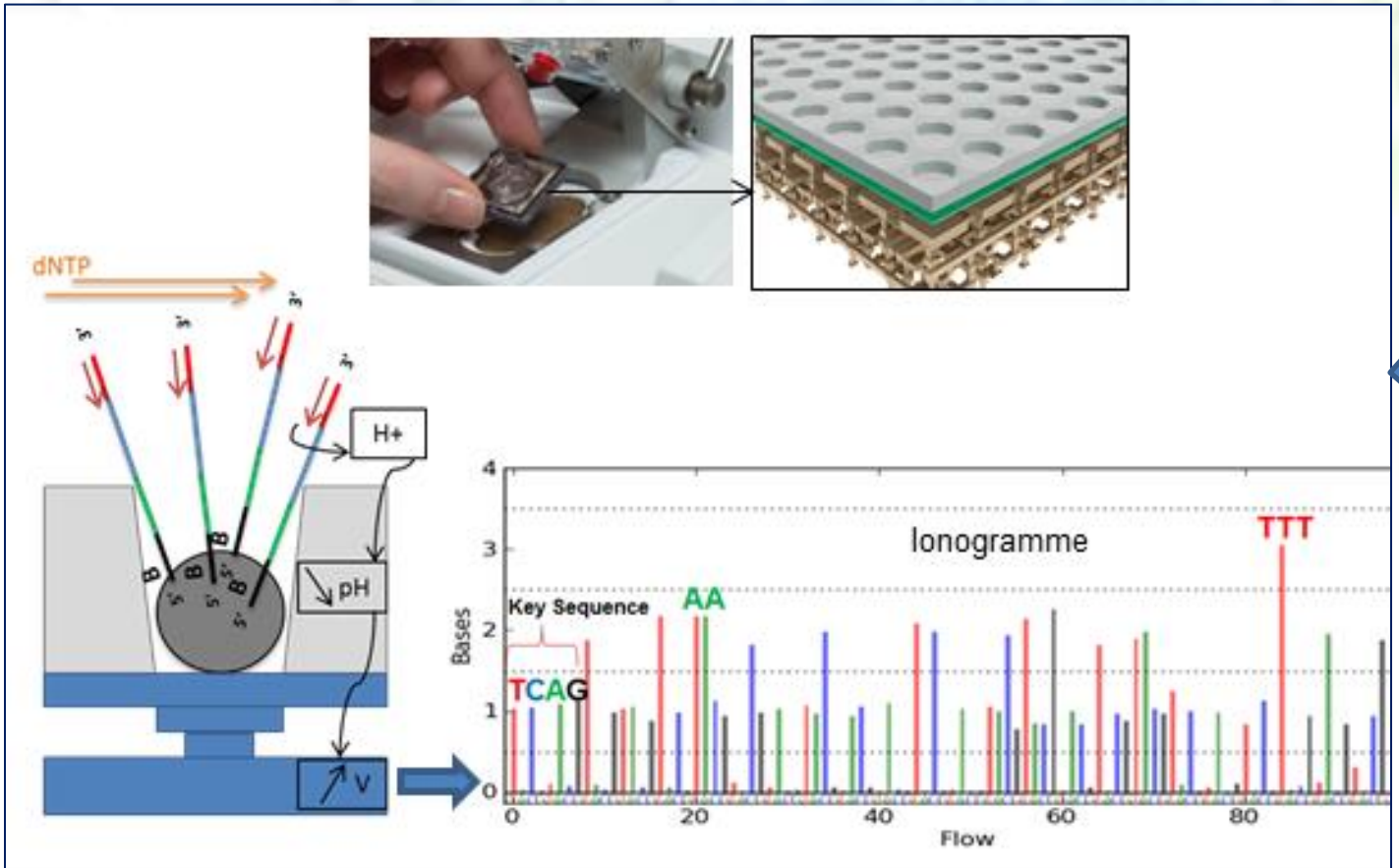
4. Enrichment : select only the beads that contain DNA & maximizing sequencing yield



Andy Vlestraete , 2012

5. Sequencing using Ion Torrent PGM Platform





Andy Vlestraete , 2012

6. Data quality filtration using PRINSEQ





7. Data Analysis using MGRAST

MG-RAST
metagenomics analysis server

YATI DAVE

Metagenome Analysis

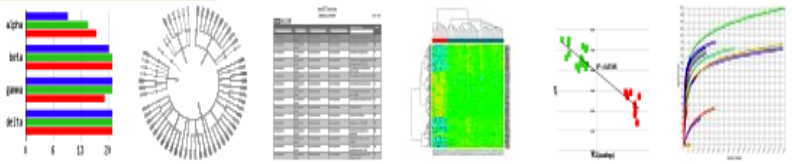
1 Data Type

- ORGANISM ABUNDANCE
  - Representative Hit Classification
  - »Best Hit Classification
  - Lowest Common Ancestor
- FUNCTIONAL ABUNDANCE
  - Hierarchical Classification
  - All Annotations

2 Data Selection

Metagenomes	4559452.3, 4559464.3, 4559463.3, 4559462.3, 4559461.3, 4559460.3, 4559459.3, 4559458.3, 4559457.3, 4559456.3, 4559455.3, 4559454.3, 4559453.3, 4559451.3, 4559450.3
Annotation Sources	RDP
Max. e-Value Cutoff	1e-5
Min. % Identity Cutoff	80 %
Min. Alignment Length Cutoff	20
Workbench	use features from workbench

3 Data Visualization



barchart | tree | table | heatmap | PCoA | rarefaction

generate

Workbench (0 Features) | Getting Started | Organism PCoA 1

This data was calculated for metagenomes 4559452.3, 4559464.3, 4559463.3, 4559462.3, 4559461.3, 4559460.3, 4559459.3, 4559458.3, 4559457.3, 4559456.3, 4559455.3, 4559454.3, 4559453.3, 4559451.3 and 4559450.3. The data was compared to RDP using a maximum e-value of 1e-5, a minimum identity of 80 %, and a minimum alignment length of 20 measured in aa for protein and bp for RNA databases.
The data has been normalized to values between 0 and 1. If you would like to view raw values, redraw using the form below.

redraw using normalized values and bray-curtis distance draw

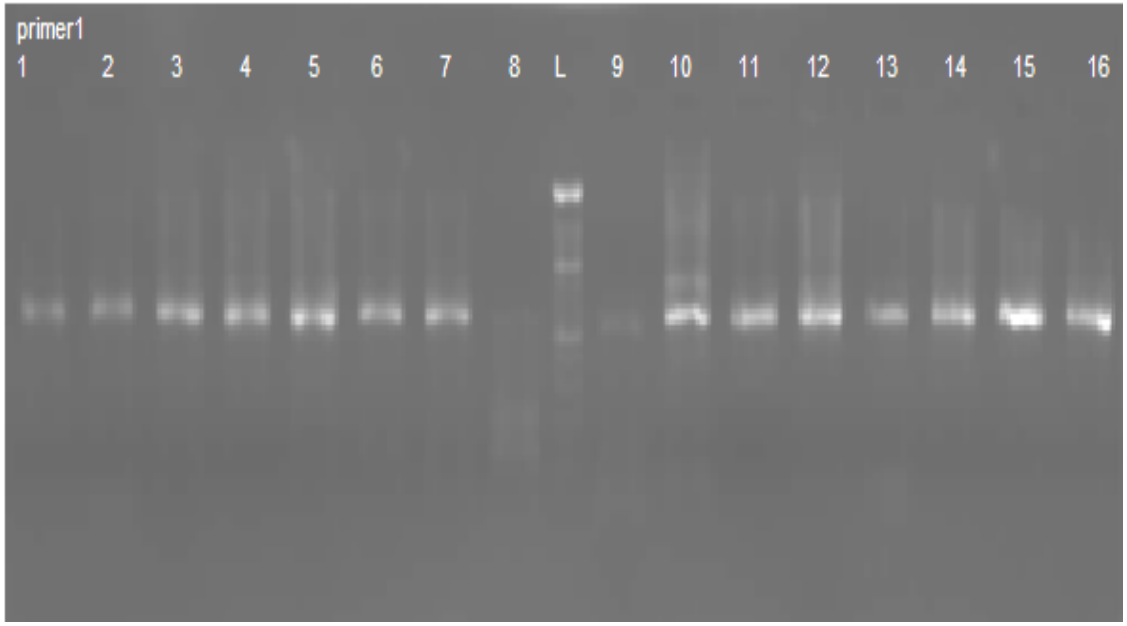
The image is currently dynamic. To be able to right-click/save the image, please click the static button static

PCoA grouping control

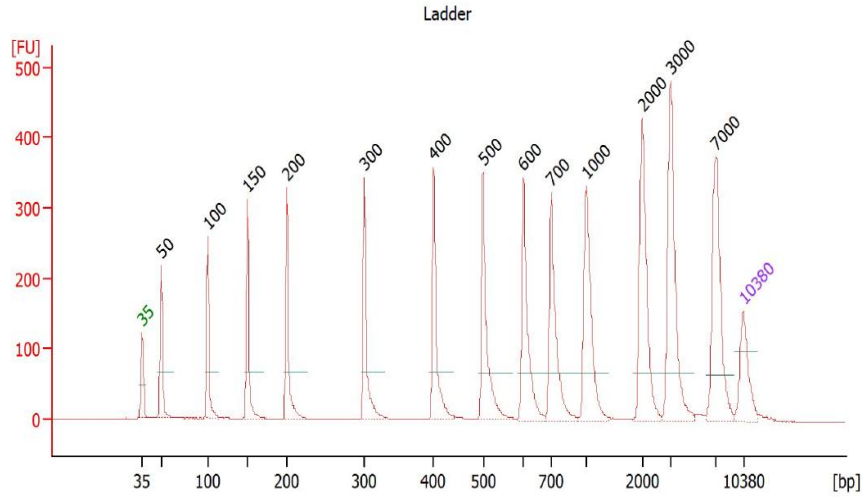
Safely Remove Hardware and Eject Media

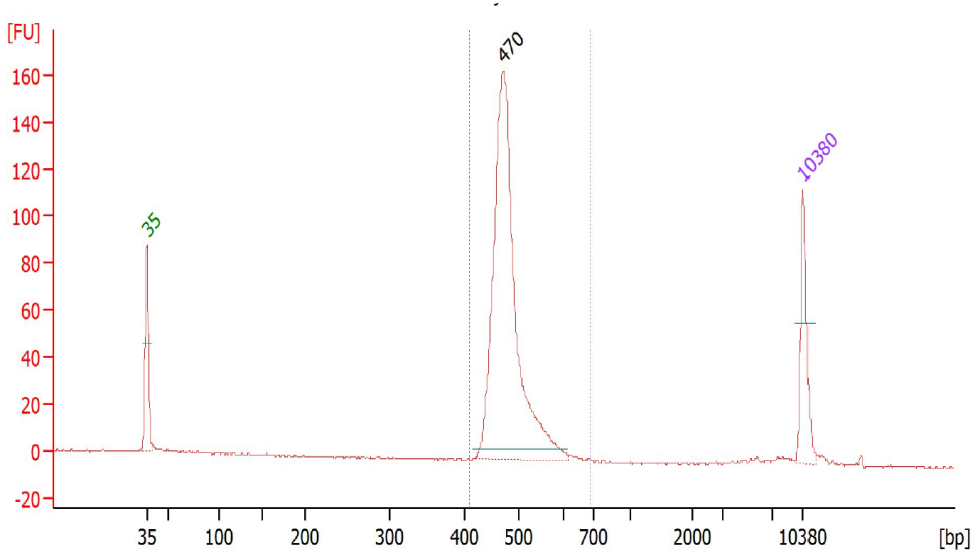

Results

1. Metagenomic library preparation & Quality of amplicon:



The total DNA from 16 milk samples of healthy volunteer women was subjected to 16S rRNA gene amplification by 100R and 517F primer



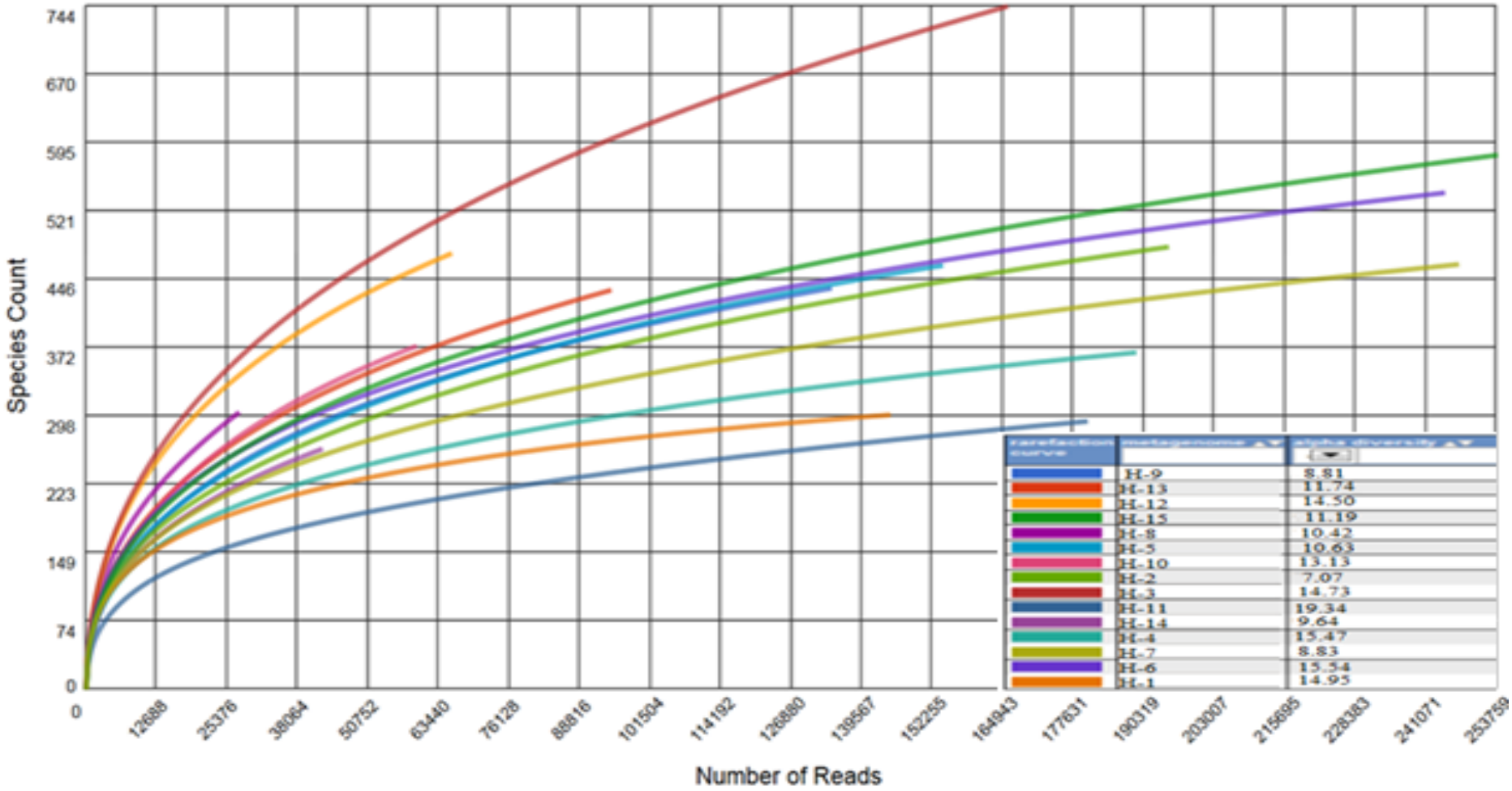


2. Data generated after quality filtration using PRINSEQ

Sample ID	No. of Sequences	No. of Sequences after QC	bp count after QC	Average length of Sequences (bp)	Mean GC% after QC
H1	154309	144633(93.73%)	47427437	328	54.91±2.11%
H2	190510	180060 (94.51%)	61967846	344	54.41±2.02%
H3	209893	194660(92.74%)	6643494	341	54.44±2.25%
H4	261099	246814 (94.53%)	73764368	299	55.60±1.78%
H5	164639	154147 (93.63%)	51250545	314	54.73±2.15%
H6	261066	244391 (93.61%)	728769	435	55.51±1.99%
H7	199861	188814 (94.47%)	56551411	286	55.81±1.79%
H8	266239	253776 (95.32%)	74191890	281	55.72±1.69%
H9	141563	134043 (94.69%)	37357392	266	55.66±1.85%
H10	63464	59422 (93.63%)	21029859	334	54.54±2.16%
H11	178099	165698 (93.04%)	55840524	317	54.26±2.70%
H12	99999	94422 (94.42%)	28185925	284	55.24±2.10%
H13	69988	65729 (93.31%)	18679335	270	55.31±2.29%
H14	45143	42466 (94.07%)	12209569	273	55.70±1.82%
H15	29103	27531 (94.60%)	7898606	274	55.78±1.72%

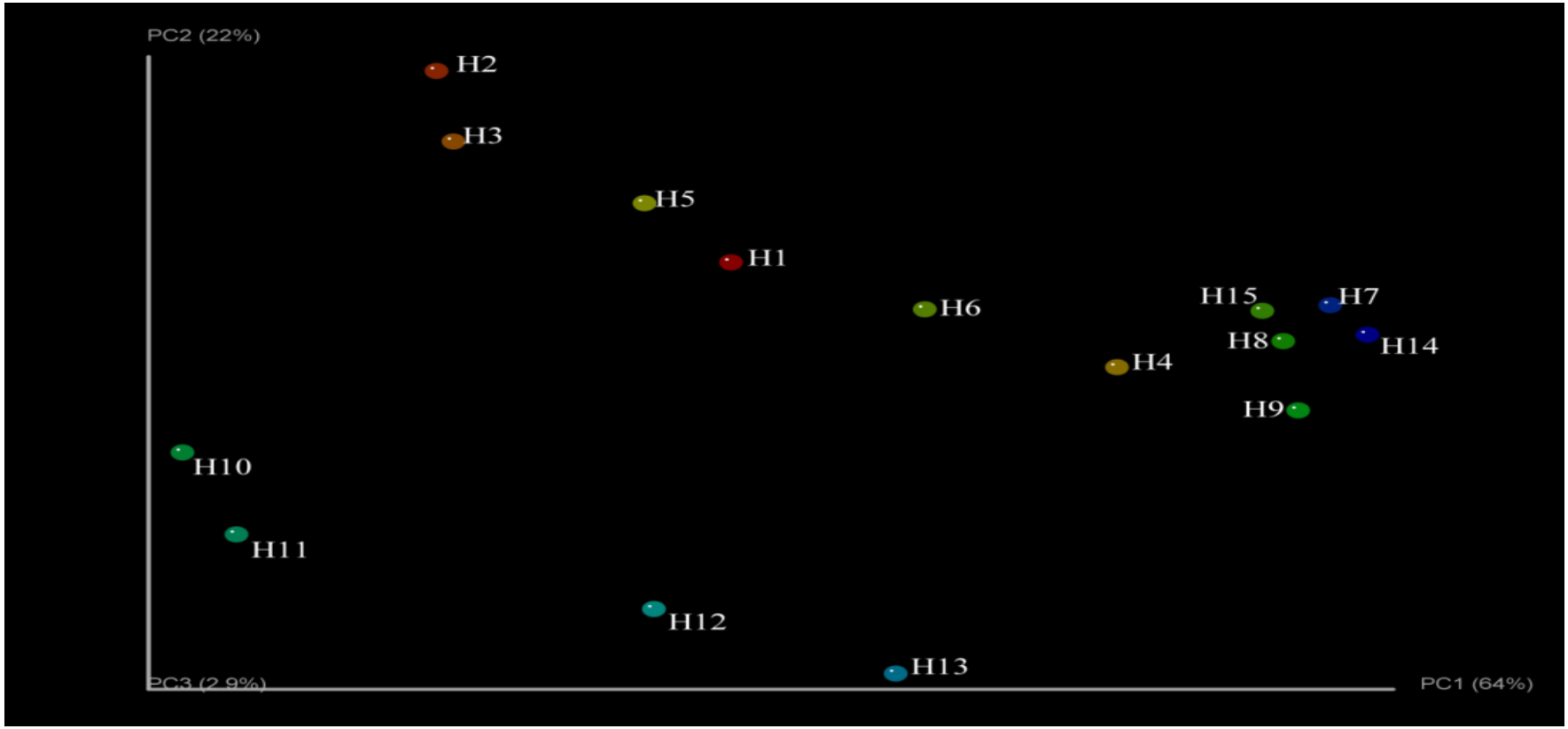
3. Alpha diversity

- Using alpha diversity, the number (richness) and distribution (evenness) of taxa expected within a single population can be identified.
- These give rise to figures known as collector‘s or rarefaction curves, since increasing numbers of sequenced taxa allow increasingly precise estimates of total population diversity.
- An alpha diversity measure thus acts like a summary statistic of a single population.

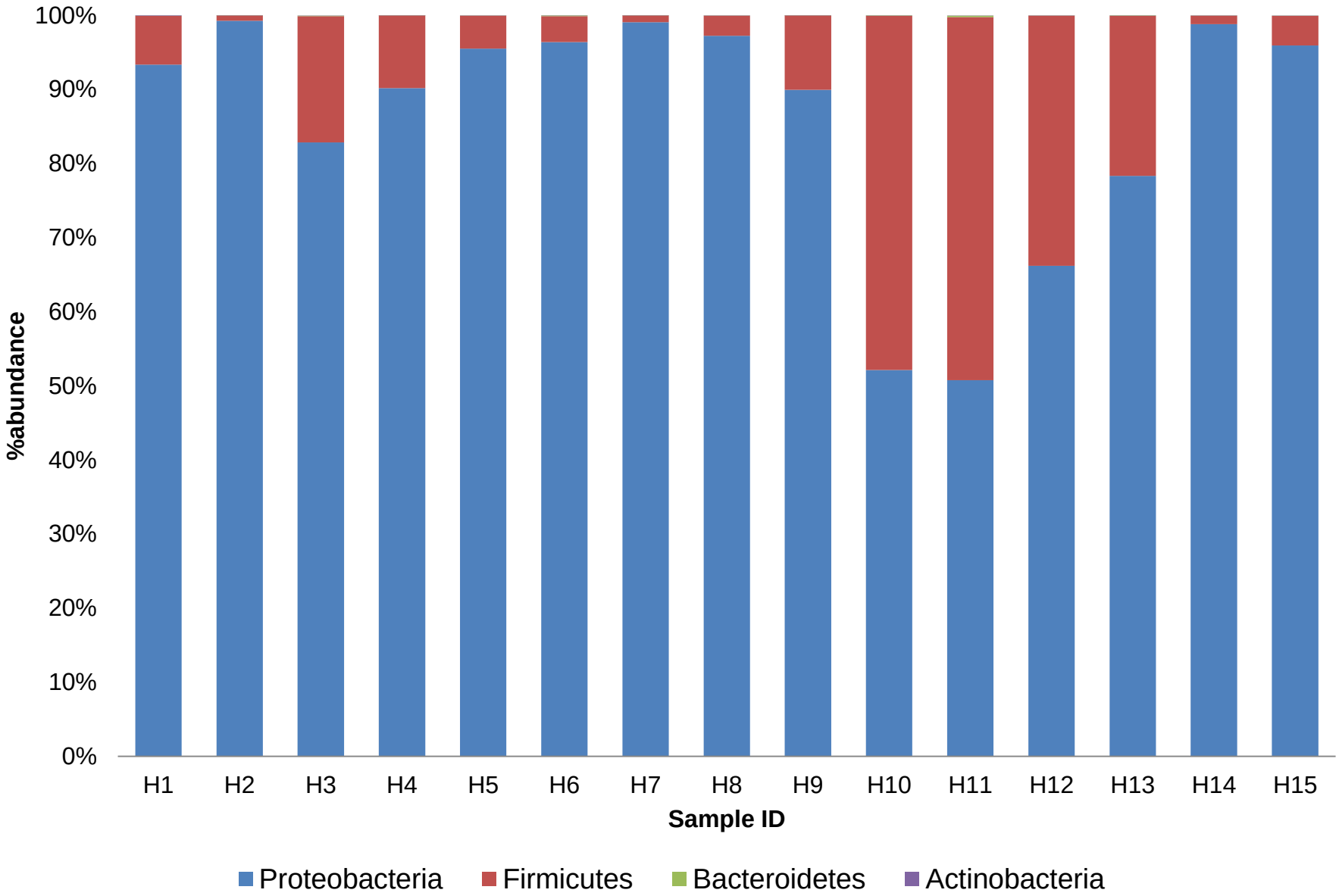


4. Beta diversity

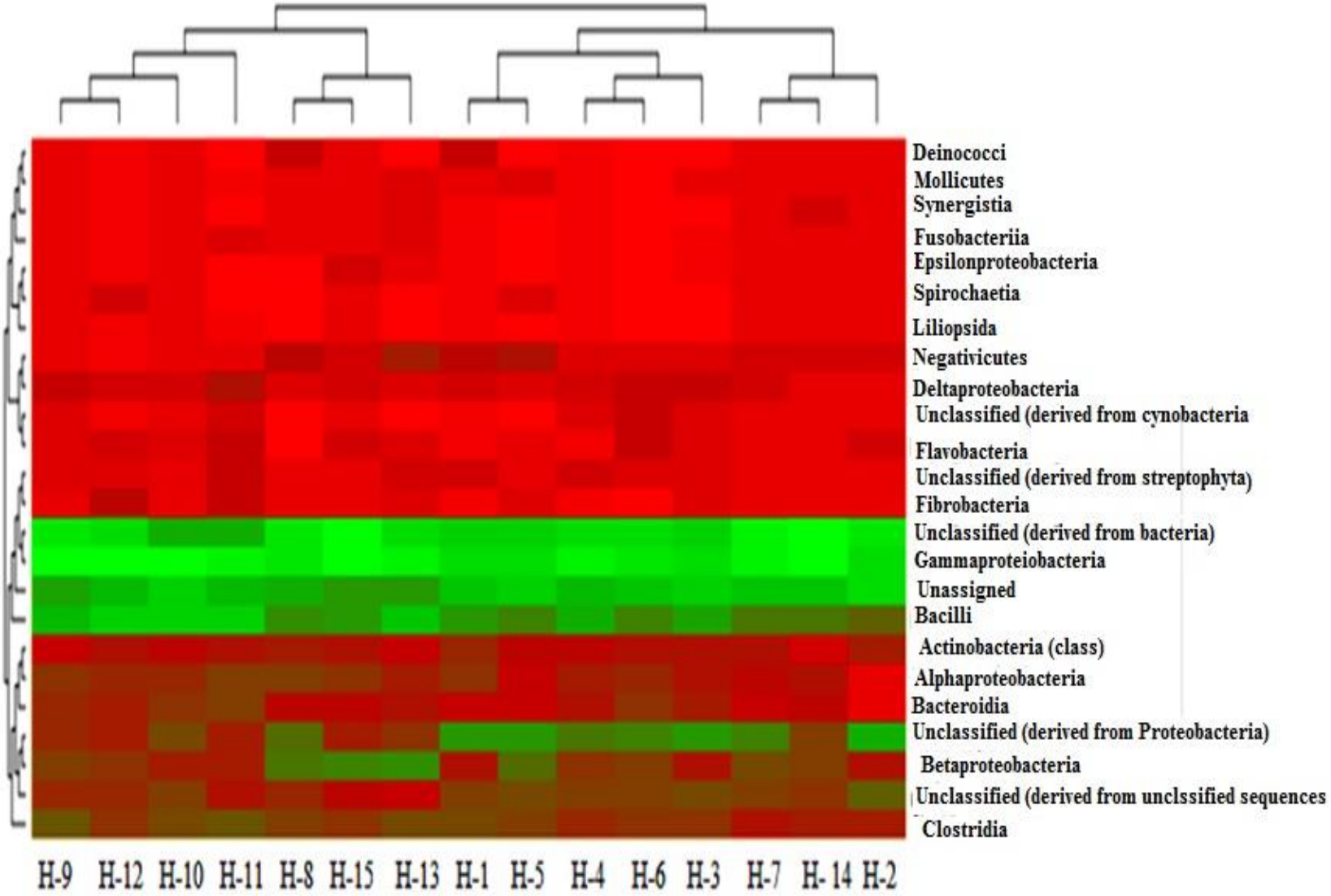
- In order to view relationships among samples based on differences in phylogenetic diversity, principle coordinates (PC) were calculated from jackknifed-UniFrac distances between samples and used to construct three-dimensional principal coordinate analysis (PCoA) plots
- Also changes of the bacterial community in response to different environmental conditions were examined
- However, the PCoA analysis revealed that all samples were assigned to one site of the plot in relation to their geographical region



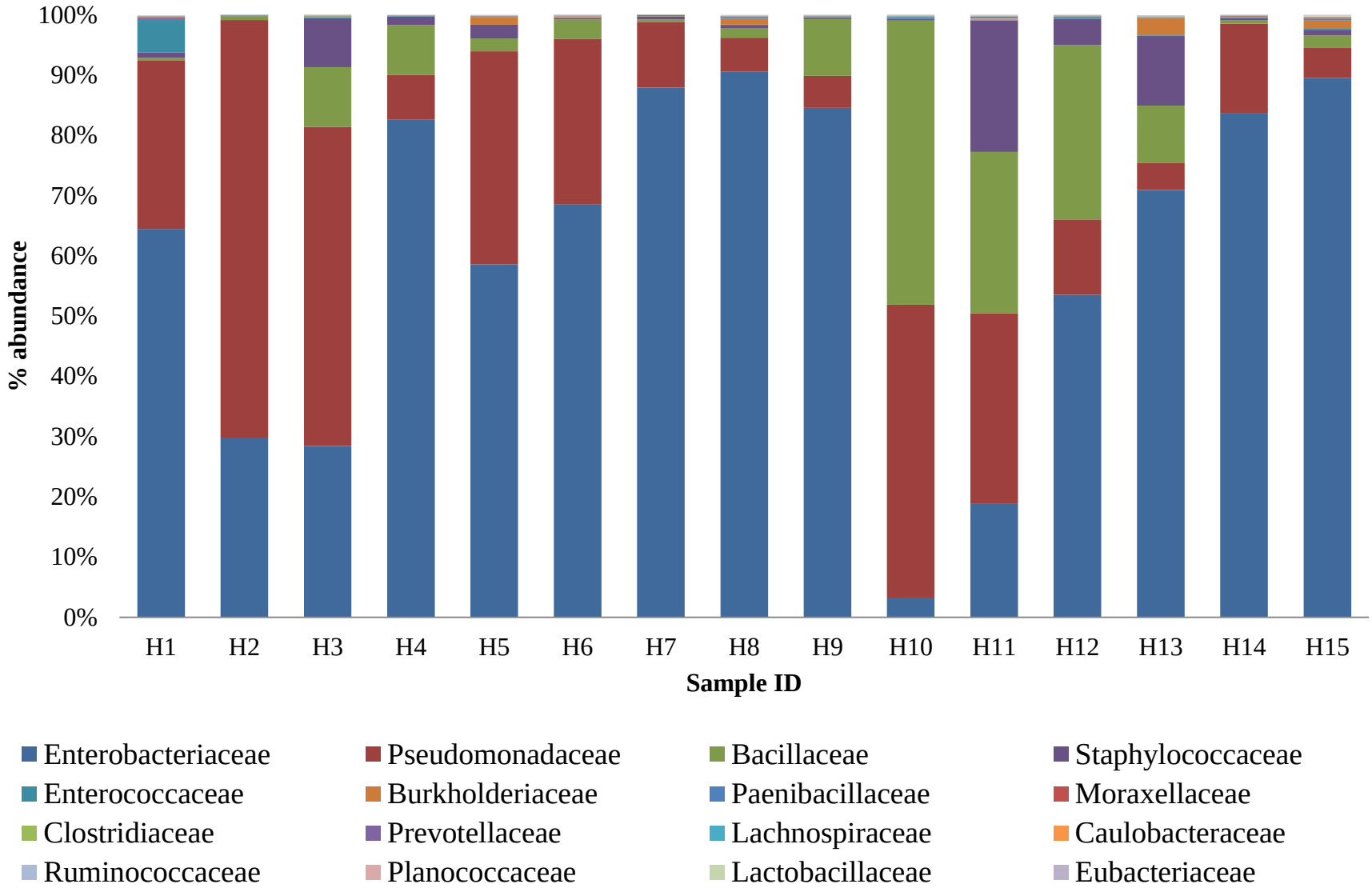
5. Phylum level classification



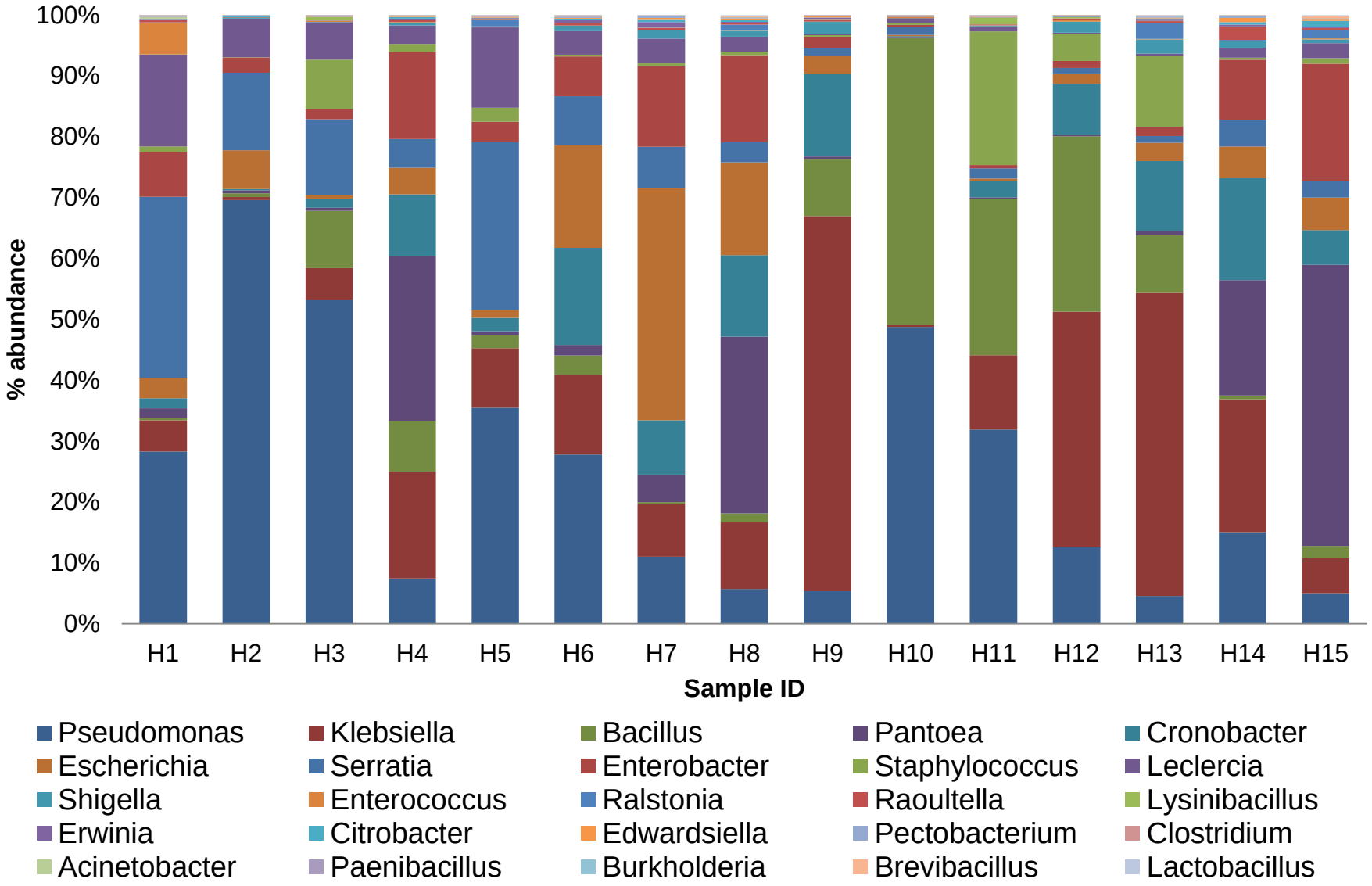
6. Class level classification



7. Family level classification



8. Genus level classification



Ward et al. BMC Microbiology 2013, 13:116
http://www.biomedcentral.com/1471-2180/13/116

BMC Microbiology

RESEARCH ARTICLE Open Access

Human milk metagenome: a functional capacity analysis

Tonya L Ward[1], Sergey Hosid[1,2], Ilya Ioshikhes[1,2*] and Illimar Altosaar[1,2*]

Abstract

Background: Human milk contains a diverse population of bacteria that likely influences colonization of the infant gastrointestinal tract. Recent studies, however, have been limited to characterization of this microbial community by 16S rRNA analysis. In the present study, a metagenomic approach using Illumina sequencing of a pooled milk sample (ten donors) was employed to determine the genera of bacteria and the types of bacterial open reading frames in human milk that may influence bacterial establishment and stability in this primal food matrix. The human milk metagenome was also compared to that of breast-fed and formula-fed infants' feces (n = 5, each) and mothers' feces (n = 3) at the phylum level and at a functional level using open reading frame abundance. Additionally, immune-modulatory bacterial-DNA motifs were also searched for within human milk.

Results: The bacterial community in human milk contained over 360 prokaryotic genera, with sequences aligning predominantly to the phyla of Proteobacteria (65%) and Firmicutes (34%), and the genera of Pseudomonas (61.1%), Staphylococcus (33.4%) and Streptococcus (0.5%). From assembled human milk-derived contigs, 30,128 open reading frames were annotated and assigned to functional categories. When compared to the metagenome of infants' and mothers' feces, the human milk metagenome was less diverse at the phylum level, and contained more open reading frames associated with nitrogen metabolism, membrane transport and stress response ($P < 0.05$). The human milk metagenome also contained a similar occurrence of immune-modulatory DNA motifs to that of infants' and mothers' fecal metagenomes.

Conclusions: Our results further expand the complexity of the human milk metagenome and enforce the benefits of human milk ingestion on the microbial colonization of the infant gut and immunity. Discovery of immune-modulatory motifs in the metagenome of human milk indicates more exhaustive analyses of the functionality of the human milk metagenome are warranted.

Keywords: Human milk, Microbiome, Metagenome, Bacteria, Illumina, DNA, Open reading frames, Immune-modulatory motifs, Infant feces

OPEN ACCESS Freely available online

PLoS one

Characterization of the Diversity and Temporal Stability of Bacterial Communities in Human Milk

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Abstract

Recent investigations have demonstrated that human milk contains a variety of bacterial genera; however, as of yet very little work has been done to characterize the full diversity of these milk bacterial communities and their relative stability over time. To more thoroughly investigate the human milk microbiome, we utilized microbial identification techniques based on pyrosequencing of the 16S ribosomal RNA gene. Specifically, we characterized the bacterial communities present in milk samples collected from 16 women at three time-points over four weeks. Results indicated that milk bacterial communities were generally complex; several genera represented greater than 5% of the relative community abundance, and the community was often, yet not always, stable over time within an individual. These results support the conclusion that human milk, which is recommended as the optimal nutrition source for almost all healthy infants, contains a collection of bacteria more diverse than previously reported. This finding begs the question as to what role this community plays in colonization of the infant gastrointestinal tract and maintaining mammary health.

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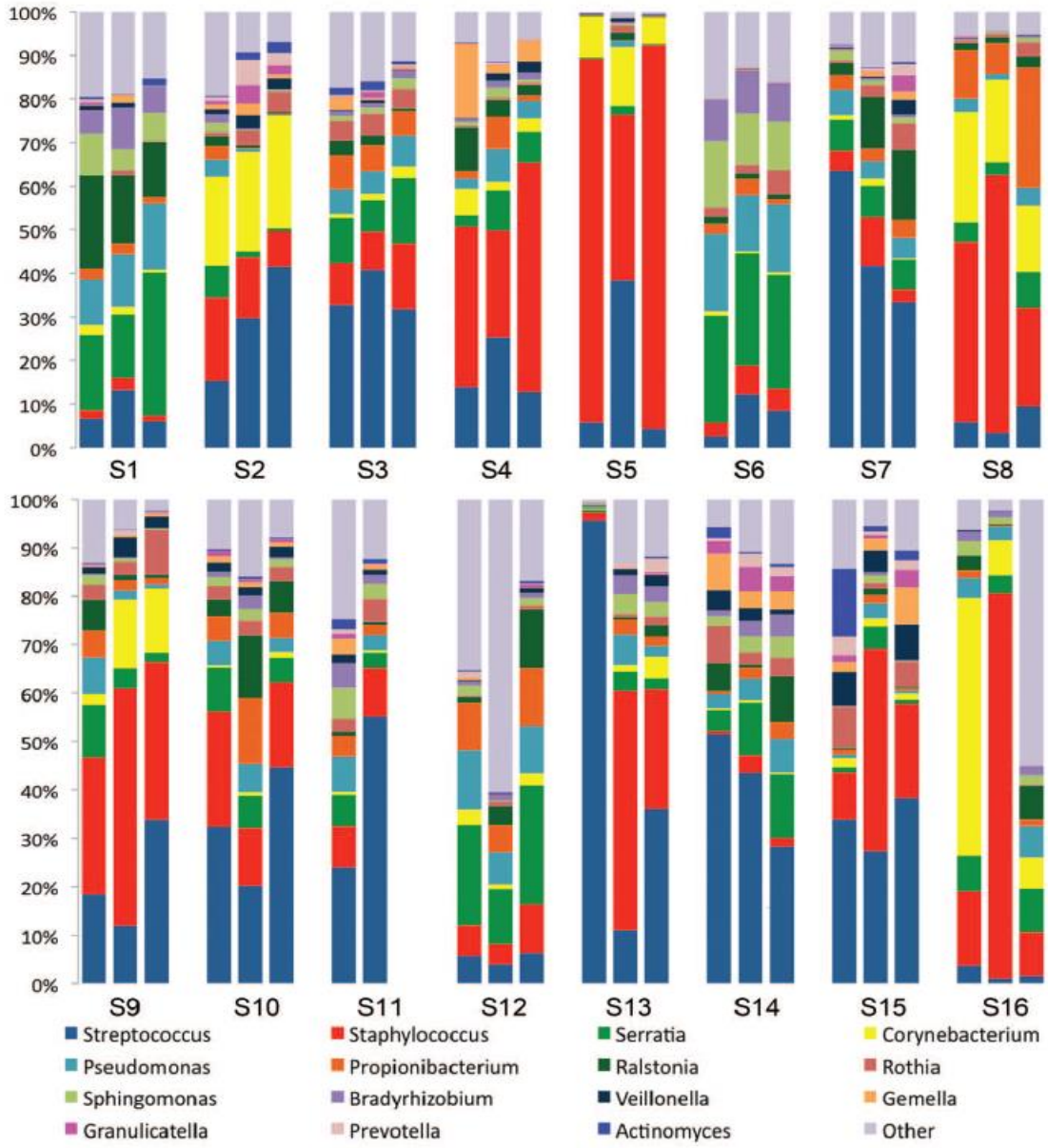


Table 1. Genus assignments of the 9 OTUs identified in every sample ($n = 47$) and their relative abundance (%).

Core OTU Genera	Relative abundance of OTU in total community (%)
Staphylococcus	15.8
Streptococcus	8.2
Serratia	7.6
Pseudomonas	4.5
Corynebacterium	3.8
Ralstonia	3.7
Propionibacterium	3.6
Sphingomonas	2.4
Bradyrhizobiaceae	1.9
Sum of all "core" OTUs	51.5

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When?
Where?
Conclusion
Who?
Who?

- **Using 16s rRNA gene amplicon sequencing were performed using 16 healthy breast milk to explored bacterial diversity**

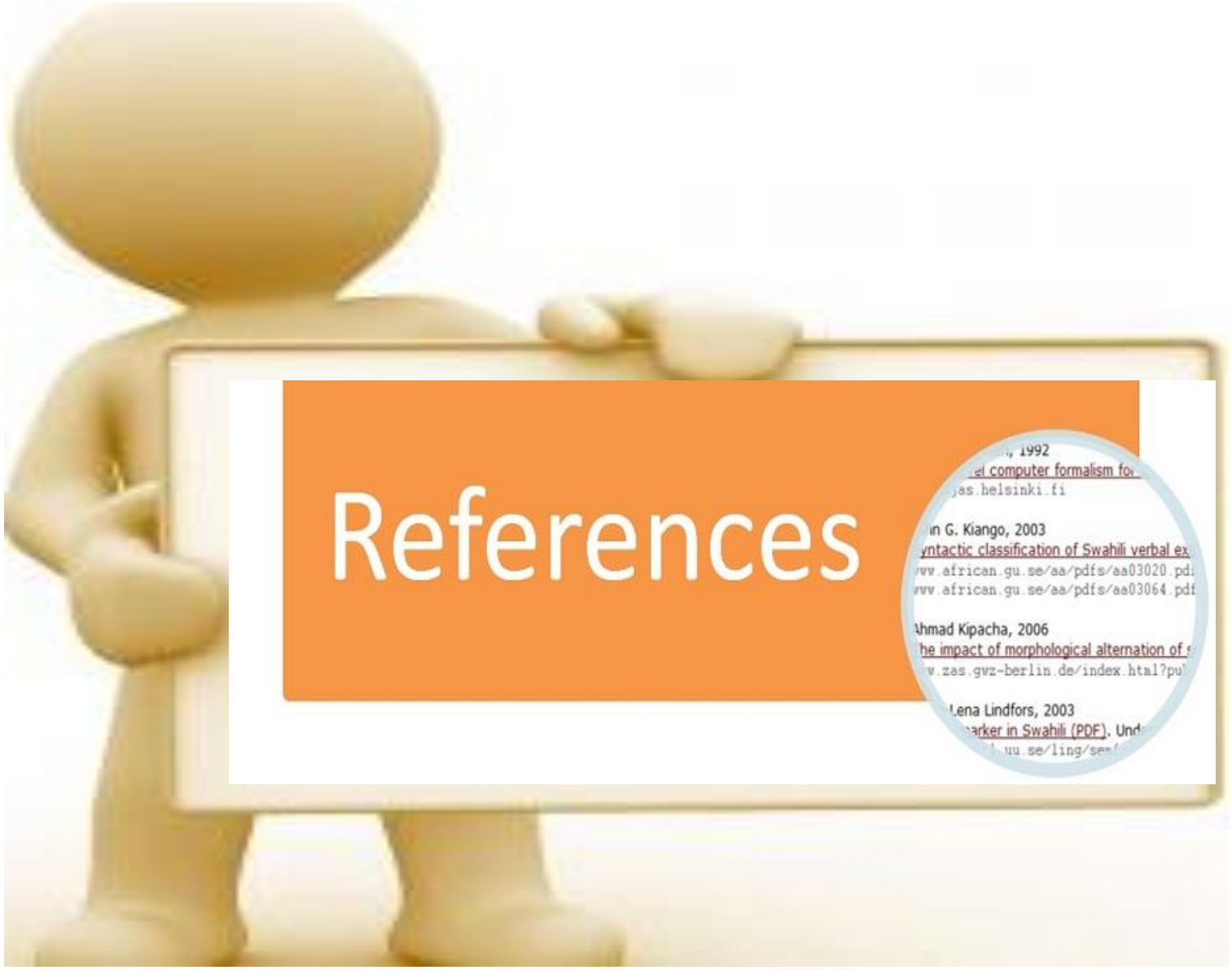
- **Health of breast was interpreted base on somatic cell count.**

- **The data were analyzed in MG-RAST using best hit approach with 80% identity.**

- **86% sequences are predominantly aligned with the phyla *Proteobacteria* followed by *Firmicutes.***

- **Genus like, *pseudomonas* (11.94%), *bacillus* (5.81%), *pantoea* (4.24%), *escherichia* (3.88%), *serratia* (3.74%), *staphylococcus* (2.46%), *enterococcus* (0.19%), *lysinibacillus* (0.12%), *erwinia* (0.10%), *brevibacillus* (0.02%), *paenibacillus* (0.02%), *lactobacillus* (0.02%), *anoxybacillus* (0.01%), *brevundimonas* (0.01%) and *yersinia* (0.01%) were detected in all samples.**

- **Our results indicate that breast milk has a dynamic microbial ecology with a microbiota composed of skin- and enteric-associated bacteria and pathogens. With improved**


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OUR STRENGTH

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Dr. Anju Kunjadia
Research Guide





Charutar Vidya Mandal's ARIBAS

ANY QUESTION



THANK YOU FOR LISTENING