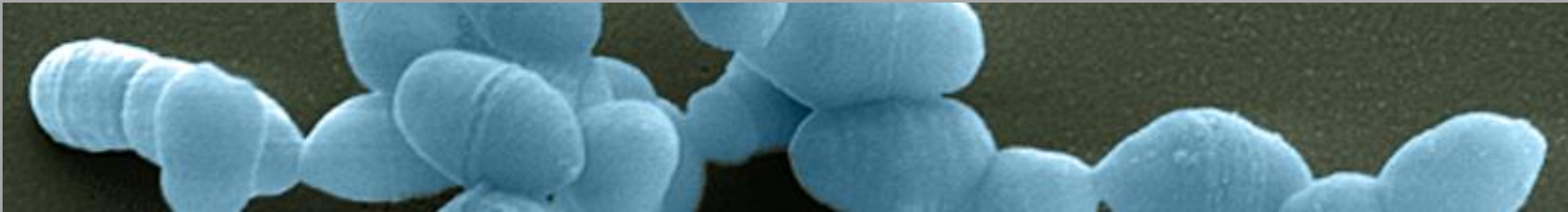〉 **Next Generation Sequencing Techniques in Food Microbiology**
Remco Kort, Professor,

Microbiology & Systems Biology,
Netherlands Institute for Applied Scientific Research (TNO)

Microbe-Host Interactions
VU University Amsterdam (VUA)

TNO innovation for life

NEXT GENERATION SEQUENCING TECHNIQUES IN FOOD MICROBIOLOGY

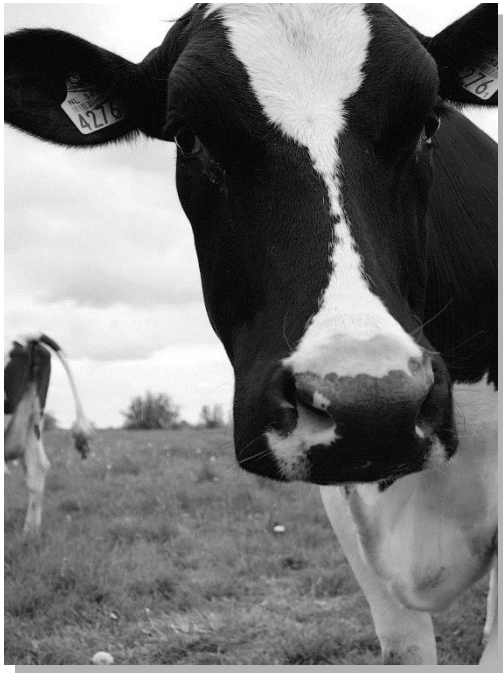› **Spoilage cases**

› A dairy factory environment

› Complex foods

  › Ready-to-eat food matrix
  › Canned food matrix (including bacterial spores)

BIOFILMS OF THERMOPHILIC SPOREFORMERS IN THE DAIRY PROCESSING ENVIRONMENT

>

>

Problem: dairy products contaminated with spores from thermophilic sporeformers

Hypothesis

In factory environments members of spoilage flora become specialists and show metabolic (inter)dependence
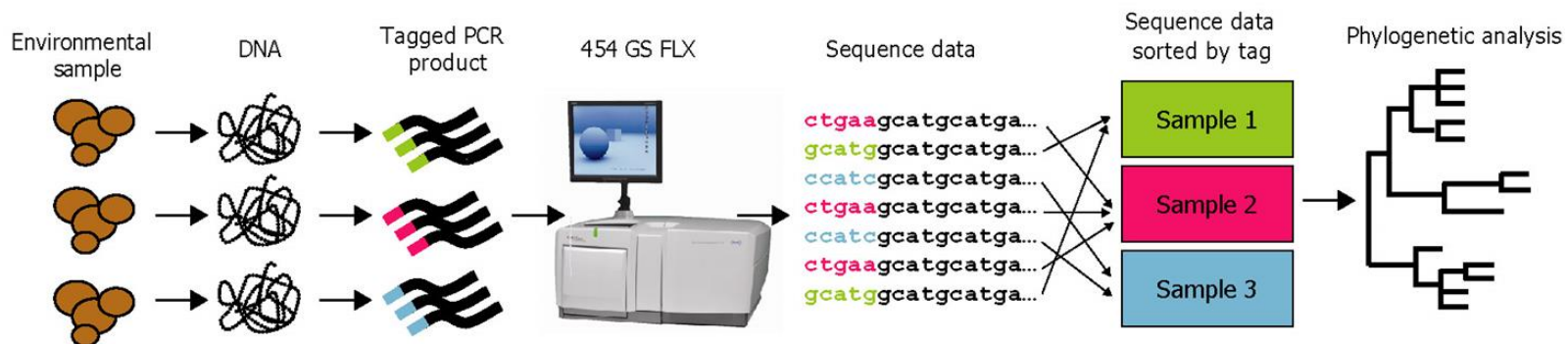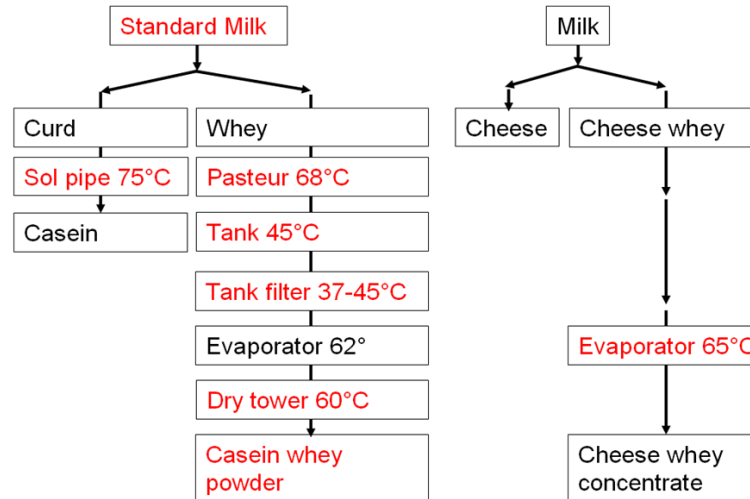
Aim

New strategies for combatting spoilage microbiota

Approach

1. Cultivation-independent population analysis by mass sequencing

2. Analysis of co-occurrence and enrichments

3. Elucidation of metabolic dependencies

# Dairy factory ecology: identification of the bacterial spoilage by mass sequencing

Factory sampling and enrichment for thermophiles

Selection of thermophilic strains with a strong capacity to form biofilms
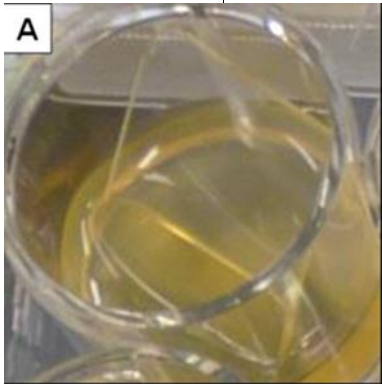
Strain Id	Industrial sample	16S type	Culture OD				Biofilm stain OD			
			30°	60°	65°	70°	30°	60°	65°	70°
TNO-09.001	Milk 4	B. lich								
TNO-09.002	Milk 4	B. lich								
TNO-09.004	Milk 3	A.flav								
TNO-09.006	Milk 3	A.flav								
TNO-09.009	Milk 1	A.flav								
TNO-09.010	Milk 1	A.flav								
TNO-09.011	Milk 1	A.flav								
TNO-09.012	Milk 1	A.flav								
TNO-09.014	Milk 1	A.flav								
TNO-09.015	Milk 1	A.flav								
TNO-09.016	i07	A.flav								
TNO-09.007	i13	G.stea								
TNO-09.008	i13	G.stea								
TNO-09.018	i08	G.stea								
TNO-09.019	i08	G.stea								
TNO-09.026	i05	G.stea								
TNO-09.027	i05	G.stea								
TNO-09.028	i05	G.stea								
TNO-09.020	i02	G.th.gl								
TNO-09.023	i02	G.th.gl								

Culture OD: 0.0 – 1.2   Biofilm stain OD: 0.0 – 1.2

Our champion: Geobacillus
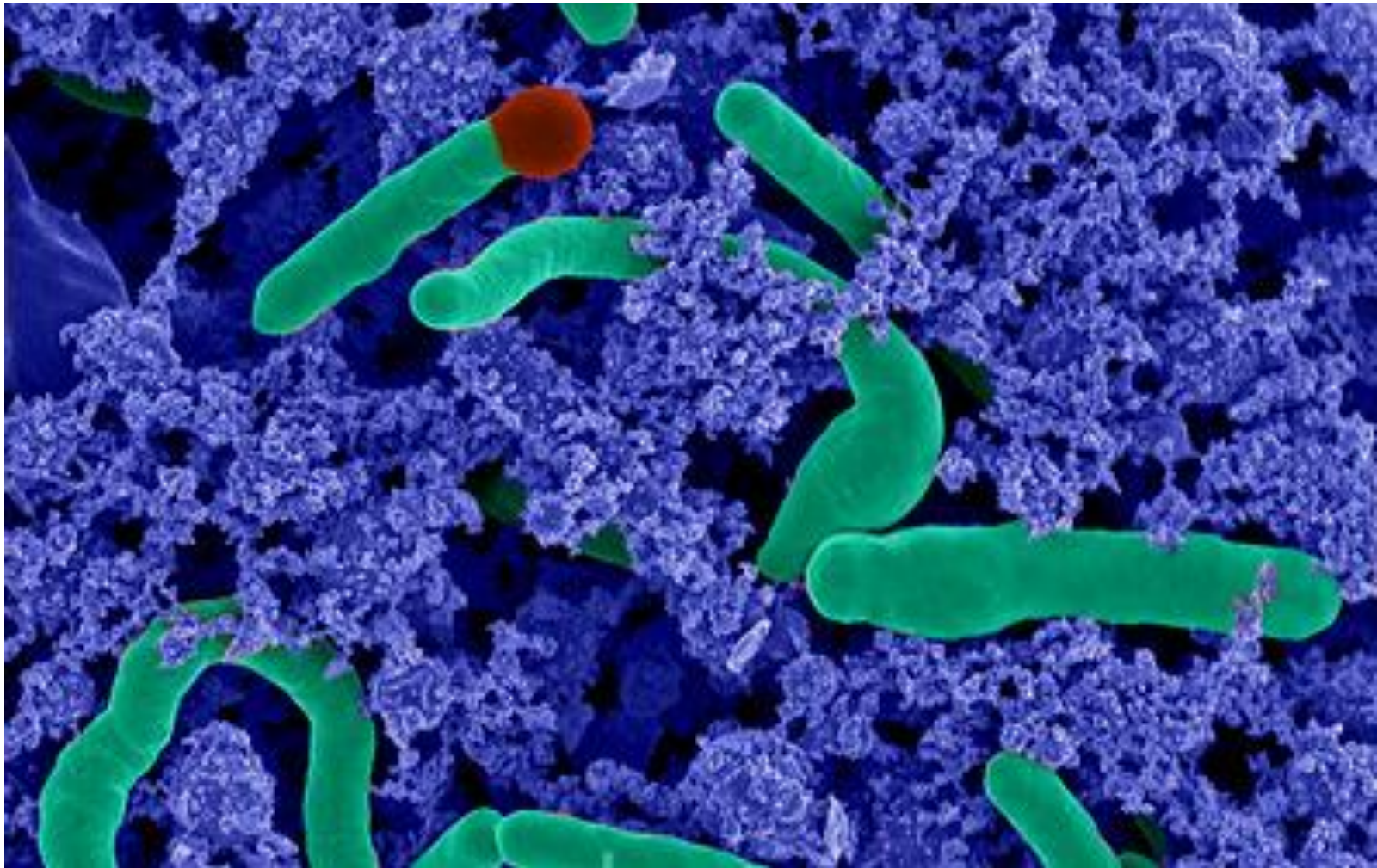
SEM of *Geobacillus* source: science photo library

Thermophilic spore forming bacteria preferentially grow on the air-liquid interface
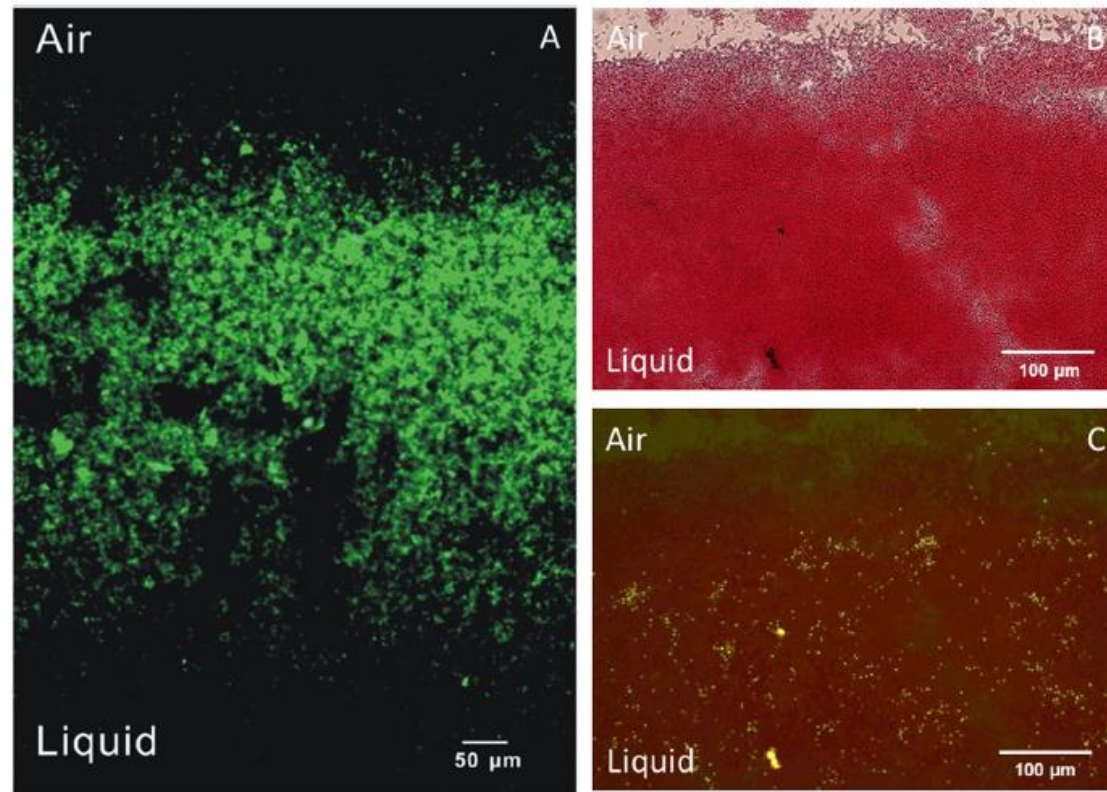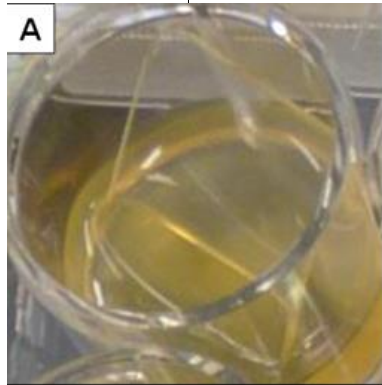

FIG 3 *Geobacillus thermoglucosidans* TNO-09.020 biofilms at the air-liquid interface. (A) Fluorescence microscopy image of Auramine-stained biofilm on a standing stainless steel coupon after 10 h of batch cultivation at 65°C. (B and C) Bright-field (B) and fluorescence (C) microscopy images of Auramine- and Safranine-stained biofilm on a standing glass coupon after 16 h of batch cultivation at 65°C.

Characterization of thermophilic factory isolates

Strain id.	LMG-Typing (% relatedness)	Growth temperature range			tD (Topt) hr
		Tmin	Tmax	Topt	
TNO-09.006	*Anoxybacillus flavithermus* LMG^T 18397 (99,60%)	43,0◦C	61,8◦C	57◦C	0,86
TNO-09.008	*Geobacillus stearothermophilus* LMG^T 6939 (99,80%)	48,6◦C	67,1◦C	61◦C	0,58
TNO-09.020	*Geobacillus themoglucosidasius* LMG^T 7317 (99,80%)	50,8◦C	69,0◦C	60◦C	0,53

Strain id.	Sporulation (on NA++ agar plate)	heat resistance of spores	
	sporulation efficiency 3 days	Z value (°C)	D$_{110}$ (min)
TNO-09.006	77%	13,3	1,9
TNO-09.008	37,5%	11,1	17,7
TNO-09.020	91,25%	8,36	19,7

Growth of factory isolates in milk

- *Anoxybacillus flavithermus* strain 09-006 grows well in milk with a little acidification

- *Geobacillus staerothermophilus* strain 09-008 grows well in milk strong acidification

- *Geobacillus thermoglucosidasius* strain 09-020 does not grow in milk

Compartimentalized growth of thermophilic factory isolates
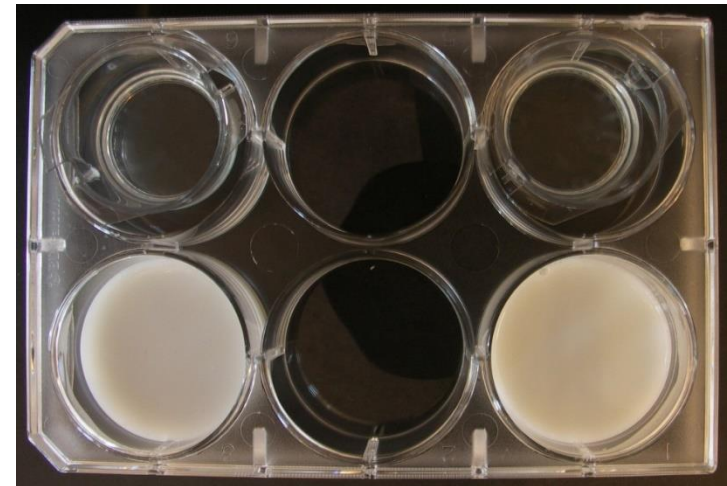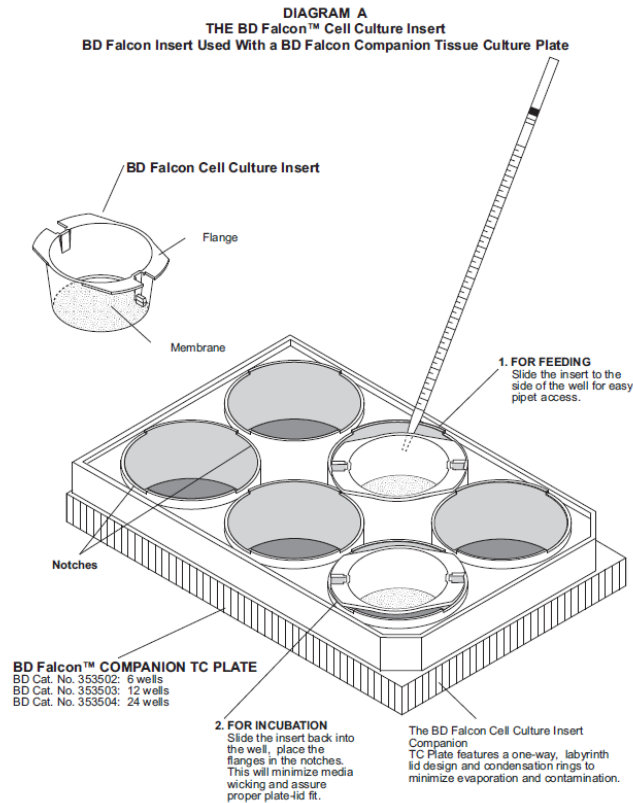
2 compartments separated by a 0.4 u membrane

Factory isolate GT20 is dependent for growth in UHT milk on the presence of isolate AF06 or GS08
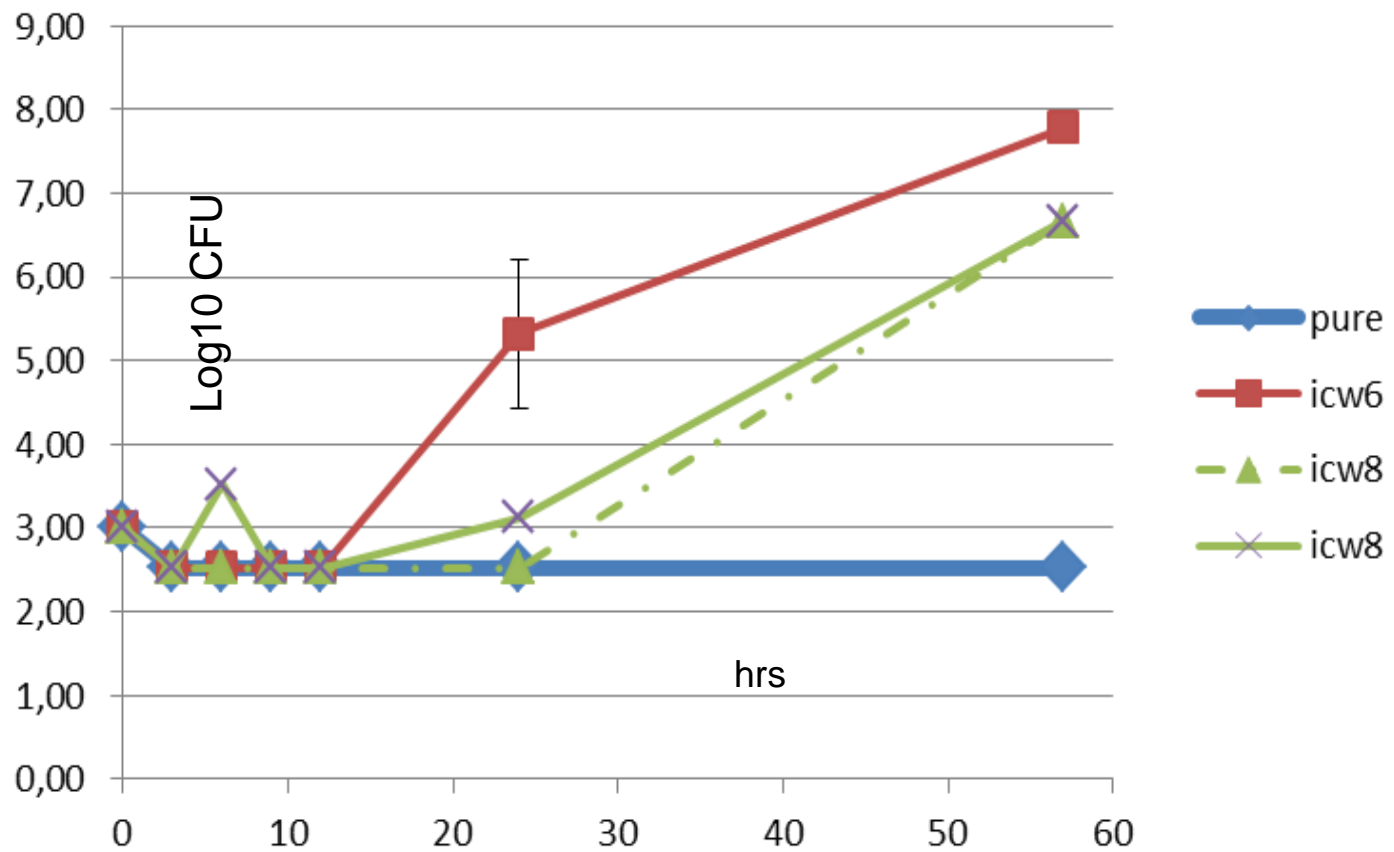
Why does GT020 needs help to grow on milk?

Whole milk:

88% water

3.5 % protein (casein)

4.8 % carbohydrates (lactose)

3.9 % fat

Growth experiments:

GT020 grows very well on trypton (tryptic digest of casein)
→ The strain is dependent on the proteolytic activity of other strains

Summary

› **Spoilage bacteria in factories occur in complex populations**

› **Species that co-occur may gain a growth benefit from sharing the costs of catabolic enzyme production**

› **We identified the champion GT020: extremely heat resistant spores, high growth rate and ability to form biofilms**

› **Our champion is dependent on others for degradation of milk proteins**

› **Design the factory process for an 'early' intervention by heat-inactivation: Combat the protease producers, which are not as difficult to eradicate**

Further reading:

Zhao Y, Caspers MP, Metselaar KI, de Boer P, Roeselers G, Moezelaar R, Nierop Groot M, Montijn RC, Abee T, Kort R. Abiotic and microbiotic factors controlling biofilm formation by thermophilic sporeformers. *Appl Environ Microbiol.* 2013 Sep;79(18):5652-60.

Caspers MP, Boekhorst J, Abee T, Siezen RJ, Kort R. Complete Genome Sequence of *Anoxybacillus flavithermus* TNO-09.006, a Thermophilic Sporeformer Associated with a Dairy-Processing Environment. *Genome Announc.* 2013 Jan;1(1). pii: e00010-13

Zhao Y, Caspers MP, Abee T, Siezen RJ, Kort R. Complete genome sequence of *Geobacillus thermoglucosidans* TNO-09.020, a thermophilic sporeformer associated with a dairy-processing environment. *J Bacteriol.* 2012 Aug;194(15):4118.

EVALUATION OF NGS FOR THE CHARACTERIZATION OF SPOILAGE MICROBIOTA IN FOOD PRODUCTS

Next generation sequencing

- Information about the entire bacterial population in one experiment
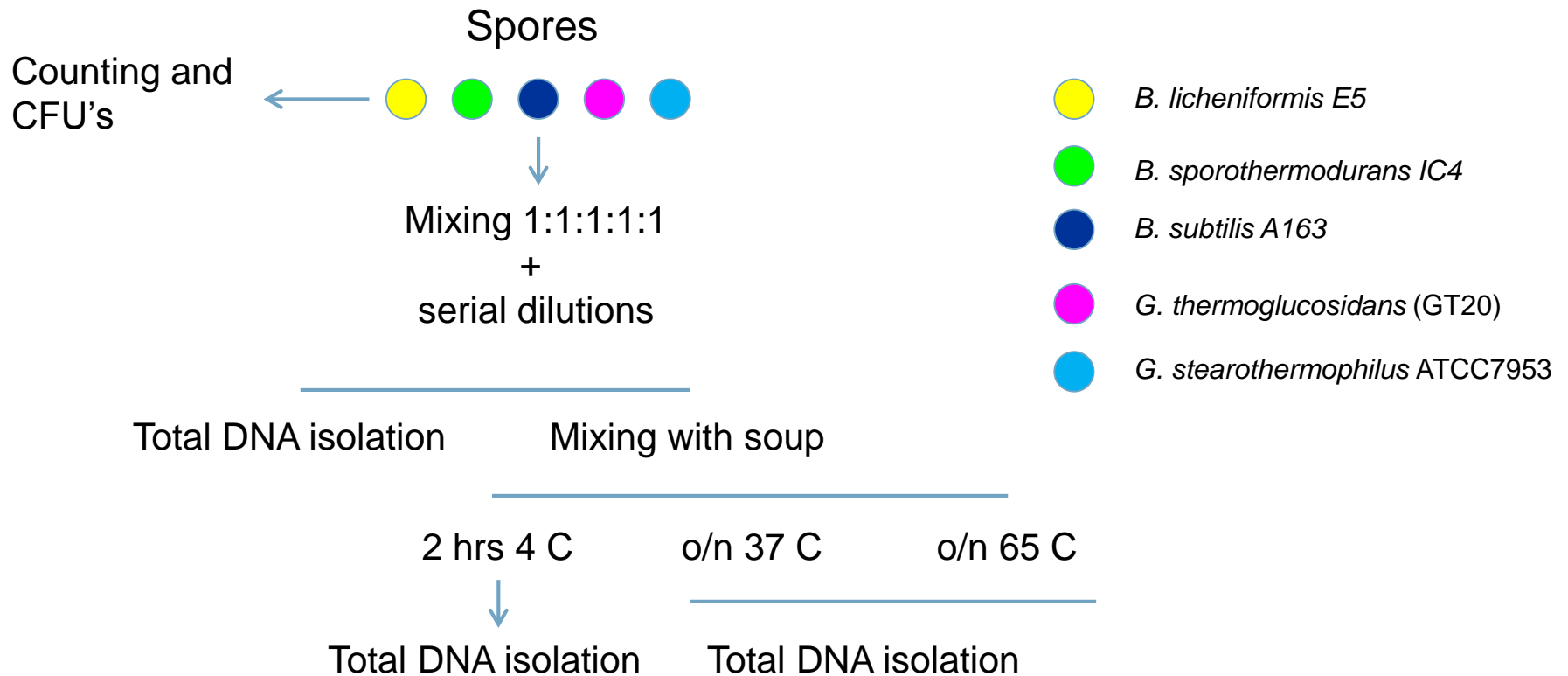- No cultivation bias
- But: not quantitative

Questions about applicability of NGS:

To what extent can we carry out a quantitative analysis (of spores)?
Feasible to monitor effects of preservatives on the spoilage microbiota?

- Ready-to-eat food matrix
- Canned food matrix (including bacterial spores)

EXPERIMENTAL DESIGN CANNED FOOD

Spores

Counting and CFU's

B. licheniformis E5

B. sporothermodurans IC4

B. subtilis A163

G. thermoglucosidans (GT20)

G. stearothermophilus ATCC7953

Mixing 1:1:1:1:1
+
serial dilutions

Total DNA isolation   Mixing with soup

2 hrs 4 C   o/n 37 C   o/n 65 C

Total DNA isolation   Total DNA isolation

VIABILITY COUNTS OF SPORES IN CANNED FOOD

Fig. 1 Viable counts of a spore spoilage model for a canned food matrix. Colony-forming units (CFUs) of pure canned food (soup), the pure spore mixture of five species diluted in physiological salt (spike mixture), canned food inoculated with the spike mixture in diluted series (soup + spike mixture), and canned food spiked with 1 × 10⁶ spores and incubated at 37 or 55 °C (o/n incubation) are shown. CFU counts were determined on TSA plates aerobically incubated at 37 °C (*hatched bars*) or 55 °C (*black bars*). Error bars represent duplicates of separate canned food, spore, or canned food + spore batches. The *dashed black line* (logCFU = 1) indicates the detection limit: *bars* below this limit represent samples where no growth was observed

RELATION BETWEEN SPIKED AND DETECTED SPORES BY NGS
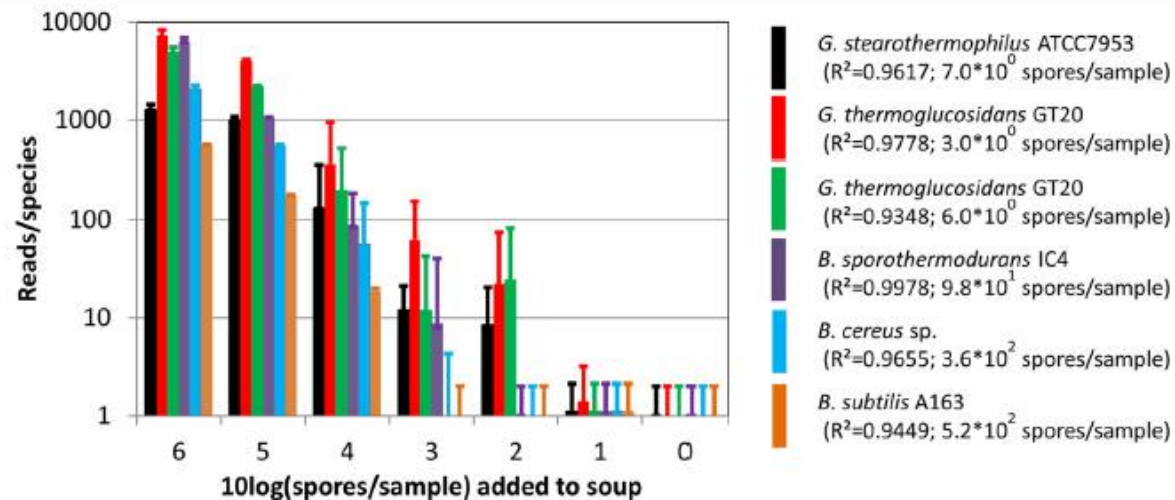

Fig. 3 Linear relation between spiked and detected spores and the detection limit (spores/bacterial species/sample giving one read/OTU/sample). Normalization of the "canned food specific" OTU 8 (*Aeromonas encheleia*) to 340 reads/sample was applied on all other OTUs. Lines were fitted through points with >5 reads/sample) for 6 spiked OTUs not detected in pure canned food (R^2 and detection limit indicated behind strain names). Additional file 2 shows normalized (and raw) frequencies of all 2037 OTUs

EXPERIMENTAL DESIGN RTE FOOD

› 5 batches of fried rice prepared:

1. Untreated

2. Propionate (0.3%)

3. Sorbate (0.1%)

4. Acetate (2.5%)

5. Lactate (2.5%)

› pH of all samples adjusted to 5.5

› Two aliquots prepared for each time point

› Incubation at 7°C, aliquots processed at 0, 2, 4, 6, 8, 10, 12 days

› Samples homogenized in a stomacher and split for CFU counts and DNA extractions

› Duplicate DNA extractions and CFU counts on TSA (aerobic) and MRS (micro-aerophilic) for all samples

MASS SEQUENCE-BASED ANALYSIS OF THE MICROBIAL COMPOSITION DURING SPOILAGE OF RTE MEALS
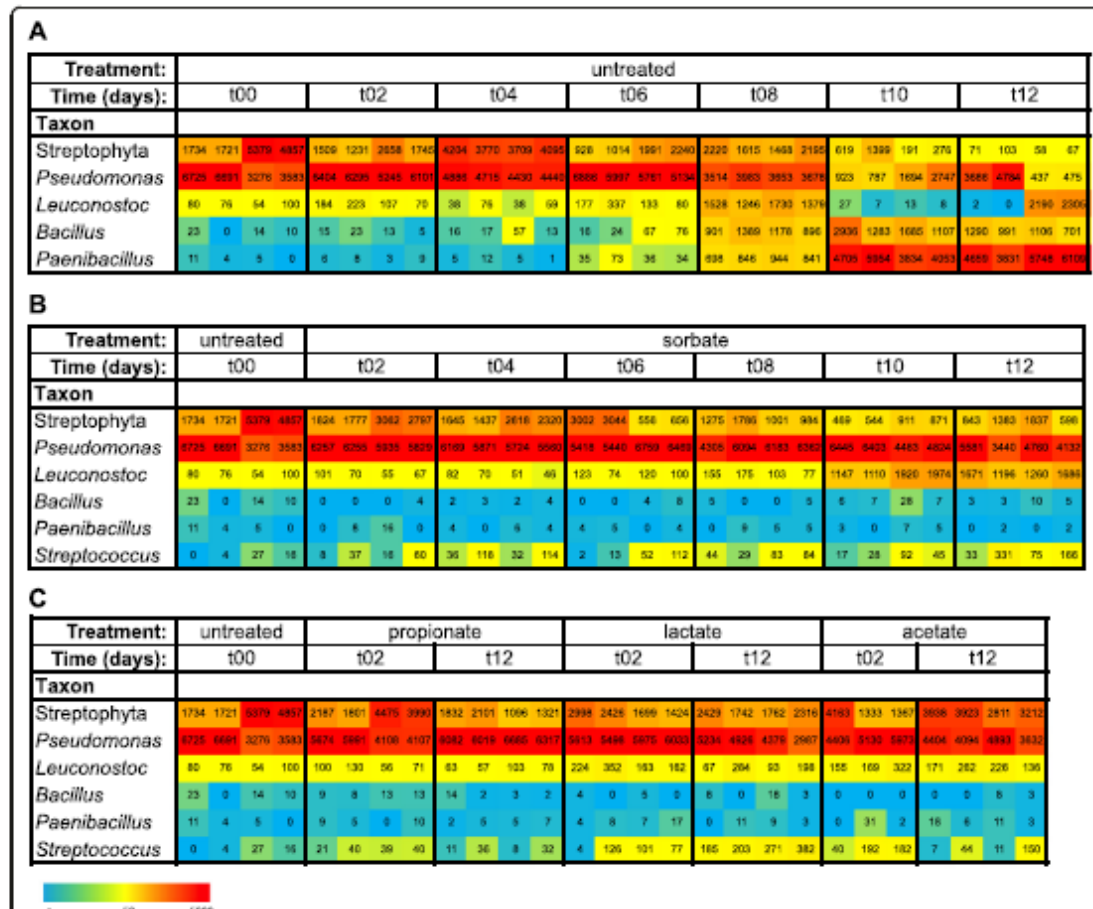
SUMMARY

› Bacterial spores can be identified in soup down to a detection limit of 10^2 spores/ml

› The detection limit is dependent on the extraction efficiency of DNA from spores, which is strain-dependent.

› Normalization of data can be performed by use of DNA present background flora

› A substantial number of sequences can be attributed to chloroplasts (20-50%)

› Novel oligonucleotide design led to 5 – 15 x reduction of the percentage of chloroplasts

› A rise in *Paenibacillus* and *Bacillus* from 8 days.

› Overall growth was suppressed by sorbate, specifically (paeni)bacilli, but not *Leuconostoc*

› Almost no growth in other weak acid/rice samples, except for slight outgrowth of *Streptococcus* (lactate)

FURTHER READING:

Microbiome

METHODOLOGY — **Open Access**

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Amplicon sequencing for the quantification of spoilage microbiota in complex foods including bacterial spores

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