Part of FOOD WORLD





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

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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

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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

TNO innovation for life



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TNO innovation for life

Factory sampling and enrichment for thermophiles

Sample	1 (standard milk)	2 (standard milk)	1 (Pipeline 1)	2 (Pipeline 2)	3 (pipeline 3)	5 (Pipeline 4)	6 (Evaporator. 1)	7 (Evaporator. 2)	8 (Pasteur)	9 (Tank)	0 (Tank filter)	1 (Drying tower)	1 (Dairy concentrates)	2 (Dairy concentrates)	1 (standard milk)	2 (standard milk)	1 (Pipeline 1)	2 (Pipeline 2)	3 (pipeline 3)	5 (Pipeline 4)	6 (Evaporator. 1)	7 (Evaporator. 2)	8 (Pasteur)	9 (Tank)	0 (Tank filter)	1 (Drying tower)	1 (Dairy concentrates)	2 (Dairy concentrates)	1 (standard milk)	2 (standard milk)	1 (Pipeline 1)	2 (Pipeline 2)	3 (pipeline 3)	5 (Pipetine 4)	6 (Evanorator, 1)
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Selection of thermophilic strains with a strong capacity to form biofilms

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	Industrial	16S		С	ulture OD			Biof	ilm stain OD	
Strain Id	sample	type	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								





Our champion: Geobacillus



8

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 tem	perature range		tD (Topt) hr
		Tmin	Tmax	Topt	
TNO-09.006	Anoxybacillus flavithermus LMG [⊤] 18397 (99,60%)	43,0∘C	61,8∘C	57∘C	0,86
TNO-09.008	Geobacillus stearothermophilus LMG [⊤] 6939 (99,80%)	48,6∘C	67,1∘C	61∘C	0,58
TNO-09.020	Geobacillus themoglucosidasius LMG [⊤] 7317 (99,80%)	50,8∘C	69,0∘C	60∘C	0,53
Strain id.	Sporulat	ion (on NA++ ag	gar plate)	hea	t resistance of spores
	sporulation efficiency 3 d	ays		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



12 31-5-2017 14:49 Titel van de presentatie

Compartimentalized growth of thermophilic factory isolates





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2 compartments separated by a 0.4 u membrane



13 31-5-2017 14:49 Titel van de presentatie

innovation for life

Factory isolate GT20 is dependent for growth in UHT milk on the presence of isolate AF06 or GS08





innovation

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) \rightarrow 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

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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



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* (logCRU = 1) indicates the detection limit: *bars* below this limit represent samples where no growth was observed



RELATION BETWEEN SPIKED AND DETECTED SPORES BY NGS



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

A.																														
Treatment:			_											untre	eate	d												_		
Time (days):	t00					t02 t04								ti)6		t08				t10					t12				
Taxon																														
Streptophyta	1734	1721	5379	4357	1509	1231	2658	1745	4204	3770	3709	4095	928	1014	1991	2240	2220	1015	1455	2195	619	1399	191	276	71	103	58			
Pseudomonas	6725	5531	3276	3583	6404	6295	5245	6101	4895	4719	4430	4440	6888	5997	5761	5134	3514	3963	3553	3678	923	787	1694	2747	3688	478	437	4		
Leuconostoc	80	76	54	100	184	223	107	70	38	76	38	69	177	337	133	80	1628	1246	1730	1379	27	7	13	8	2	0	2190	2		
Bacillus	23	0	- 14	10	15	23	13	5	16	17	57	13	16	24	67	76	901	1369	1176	896	2935	1283	1662	1107	1290	991	1100	1		
Paenibacillus	11	4	- 5	0	8	. 8	3	9	-5	12	5	1	35	73	36	34	888	846	944	841	4705	5354	3834	4253	4655	383	5745	6		
3																														
Treatment:	u	untreated sorbate															-													
Time (days):	t00			t02					t(04		t06					t0	8			t1	0	t12							
Taxon																														
Streptophyta	1734	1721	5379	4857	1824	1777	3062	2797	1645	5437	2618	2320	3002	3044	556	656	1275	1785	1001	994	469	544	911	871	643	1363	1837	5		
Pseudomonas	5725	6651	3276	3583	6257	6255	5335	5829	6169	5871	5724	5560	5418	5440	6759	5453	4305	6094	6183	6362	6445	6433	4483	4824	5581	3440	4760	41		
Leuconostoc	80	76	54	100	101	70	55	67	82	70	51	49	123	74	120	100	155	175	103	77	1147	1110	1920	1974	1671	1196	1260	58		
Bacillus	23	0	14	10	0	0	0	4	2	3	2	4	0	a	4	8	5	0	0	5	6	7	28	7	3	з	10			
Paenibacillus	-11	4	-6	0	0		- 16	0	4	0	- 6	4	4	8	٥	4	0		6	5	3	0	7	- 6	0	2	•			
Streptococcus	0	4	27	- 16	8	37	- 16	60	35	118	32	194	2	13	52	112	44	29	83	64	17	28	92	45	33	331	75	1		
C																														
Treatment:	u	ntre	ate	d			pr	ropi	onat	te						lact	ate		Т			ac	te		Т					
Time (days):	t00				tC	2		t12				t02					- t1:	2		t02					2					
Taxon																											┓			
Streptophyta	1734	1721	6379	4857	2187	1801	4475	3990	1832	2101	1096	1321	2998	2428	1689	1424	2429	1742	1762 2	316	4163	1333	1367	3858	3923	2811	3212			
Pseudomonas	6725	6691	3276	3583	5674	5661	4108	4107	8082	6019	6665	6317	5813	5496	5675	6033	5234	4926	4378 2	967	4406	i130 (5873	4404	4094	4893	3632			
Leuconostoc	80	76	54	100	100	133	56	71	63	57	103	78	224	352	153	162	67	204	93	198	155	169	322	171	262	226	135			
Bacillus	23	0	14	10	9	а	18	13	-14	2	3	2	4	0	5	0	8	0	18	3	0	0	0	0	0	8	з			
Paenibacillus	11	4	- 5	0	9	5	0	10	2	6	6	7	4	8	7	17	0	11	9	3	0	31	2	18		11	з			
						40	- 20	40	44	36		32		1.74	104		100	202	374	182	40	192	482	7	44	11	450			



SUMMARY

- Bacterial spores can be identified in soup down to a detection limit of 10² spores/ml
- The detection limit is dependent on the extraction efficiency of DNA from spores, which is straindependent.
- 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:

de Boer et al. Microbiome (2015) 3:30 DOI 10.1186/s40168-015-0096-3

METHODOLOGY







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

Paulo de Boer^{1,2†}, Martien Caspers^{1,2†}, Jan-Willem Sanders³, Robèr Kemperman³, Janneke Wijman⁴, Gijs Lommerse⁴, Guus Roeselers^{1,2}, Roy Montijn¹, Tjakko Abee^{1,5} and Remco Kort^{1,2,6*}

