Bacterial Spores as Probiotics: Mode of Action

Professor Simon Cutting
School of Biological Sciences
Royal Holloway University of London
Egham, Surrey
TW20 0EX, UK
s.cutting@rhul.ac.uk

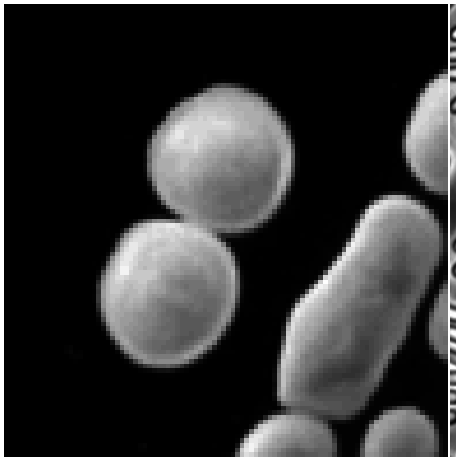
Royal Holloway
University of London

1. Sporulation in *Bacilli*

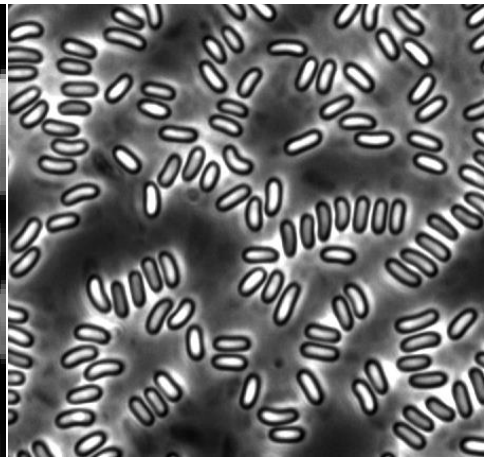
2. Use of Bacillus spores as probiotics

3. How do they work?

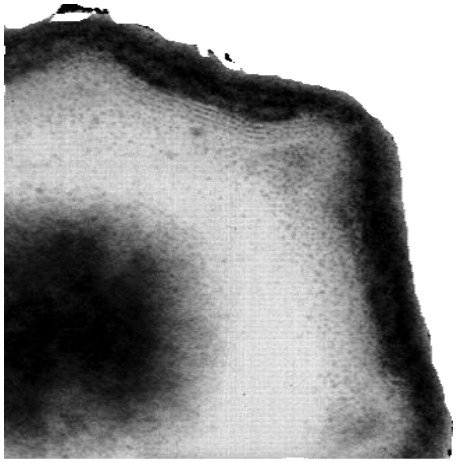
Plate colony



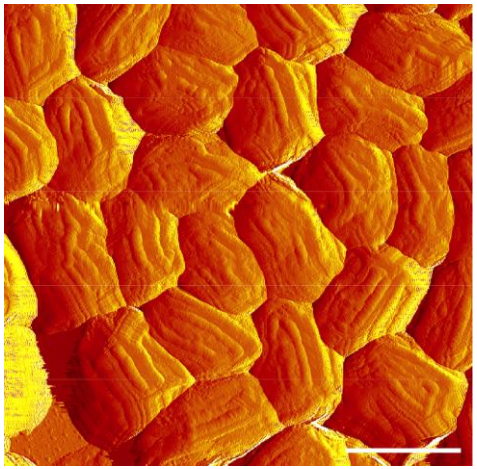
Phase Microscopy



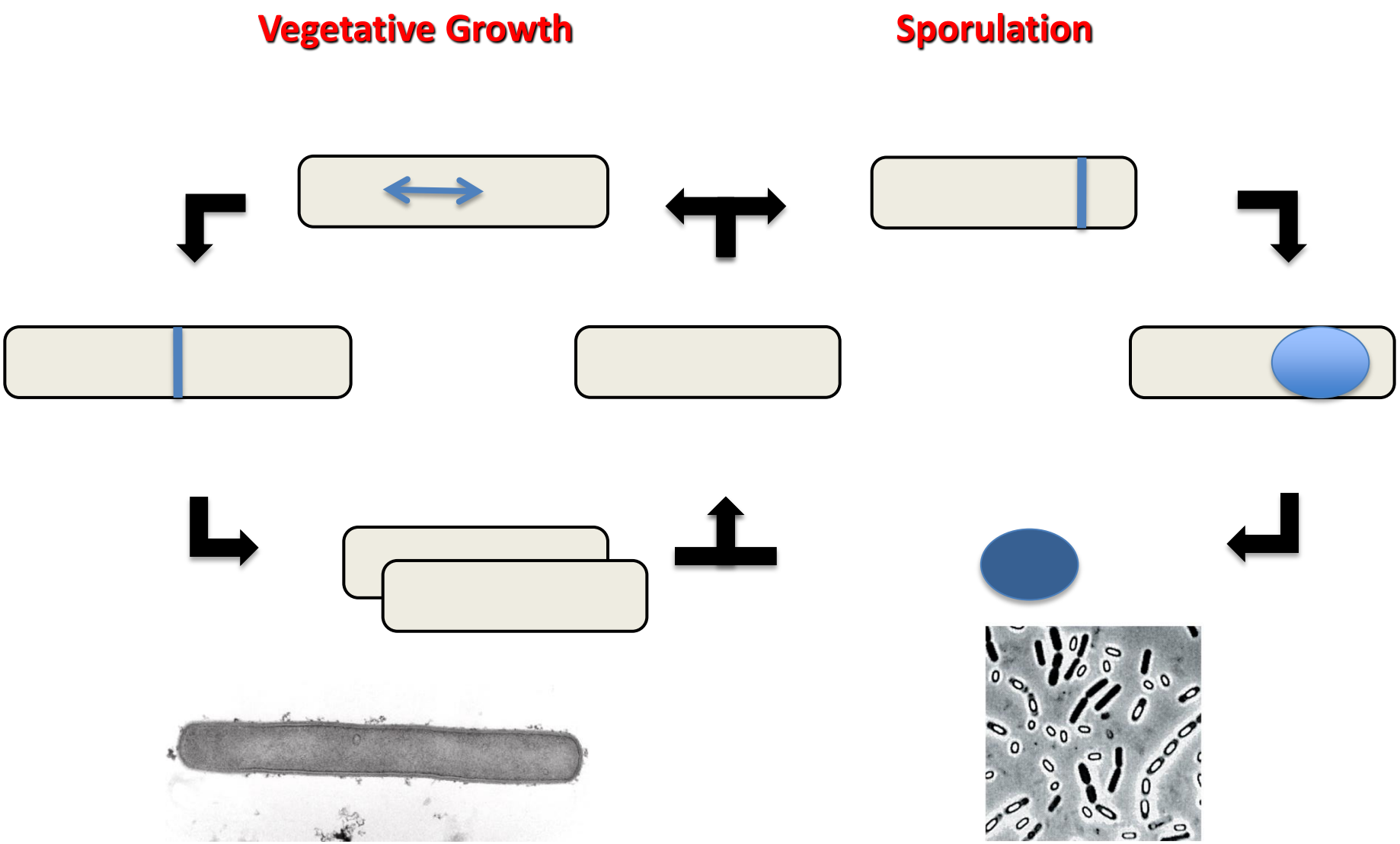
Electron microscopy



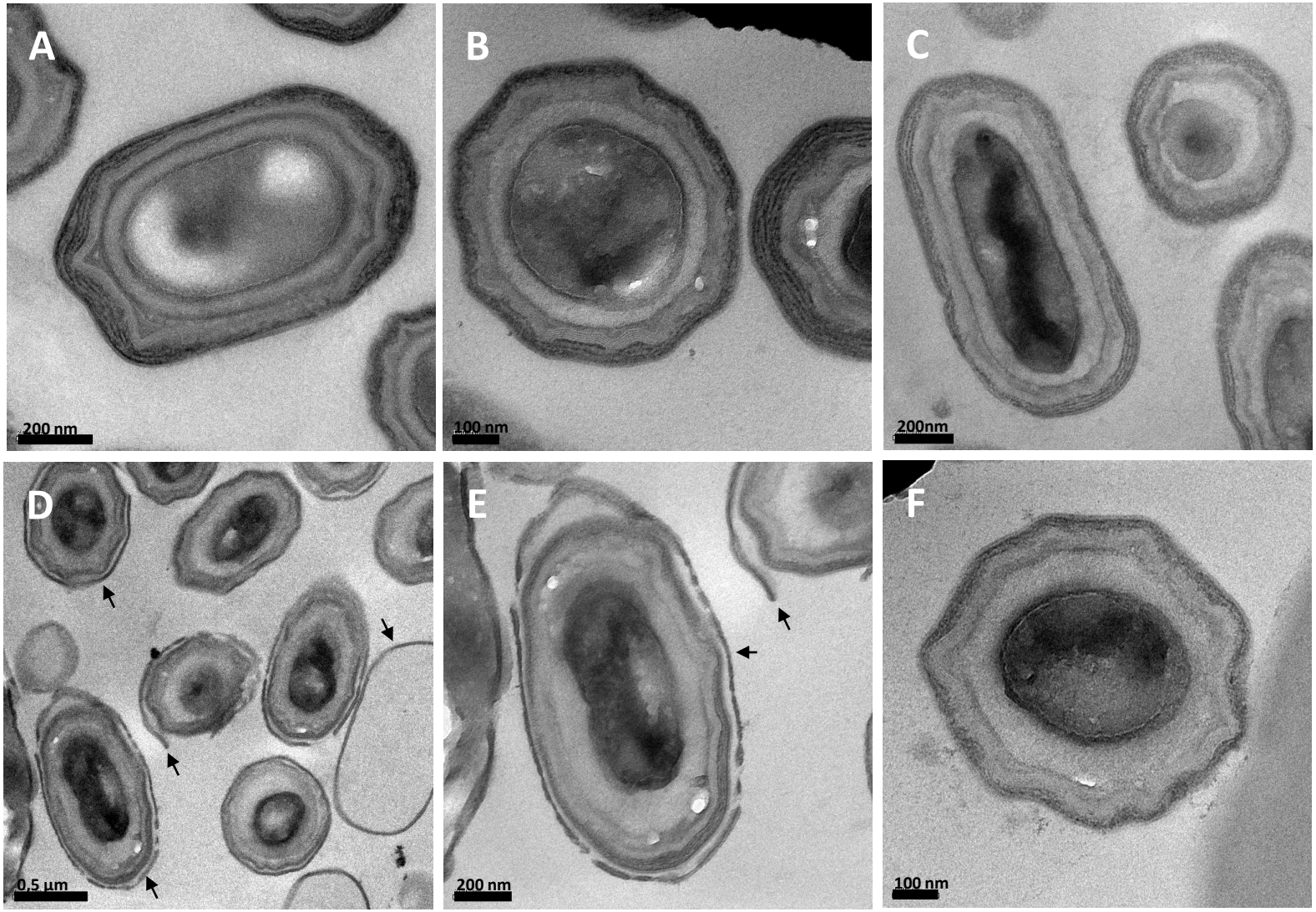
Atomic Force microscopy



Life cycle of *Bacillus* species



Spores of different isolates of *B. subtilis*



Spores are very robust

Revival and Identification of Bacterial Spores in 25- to 40-Million-Year-Old Dominican Amber

Raúl J. Cano* and Monica K. Borucki

A bacterial spore was revived, cultured, and identified from the abdominal contents of extinct bees preserved for 25 to 40 million years in buried Dominican amber. Rigorous surface decontamination of the amber and aseptic procedures were used during the recovery of the bacterium. Several lines of evidence indicated that the isolated bacterium was of ancient origin and not an extant contaminant. The characteristic enzymatic, biochemical, and 16S ribosomal DNA profiles indicated that the ancient bacterium is most closely related to extant *Bacillus sphaericus*.

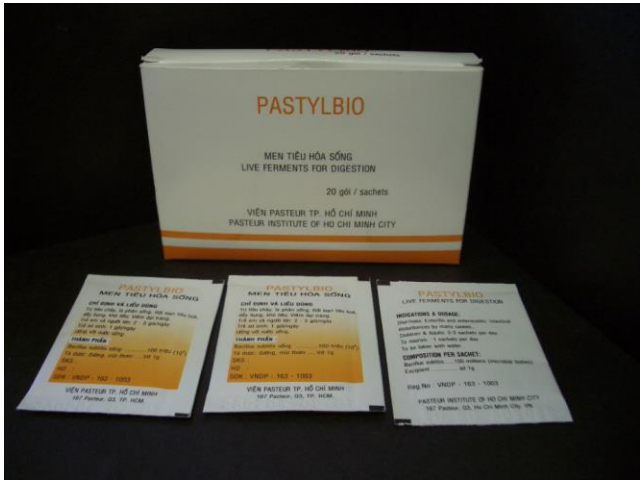
Science (1995). 268: p1060-4

Resistance to UV irradiation
Temp 65°C-80°C
Extreme heat-short term (<8 min) to 235°C
Resistance to solvents and noxious chemicals

Use As Probiotics, Dietary Supplements and Nutrafoods

Dietary supplements, food supplements

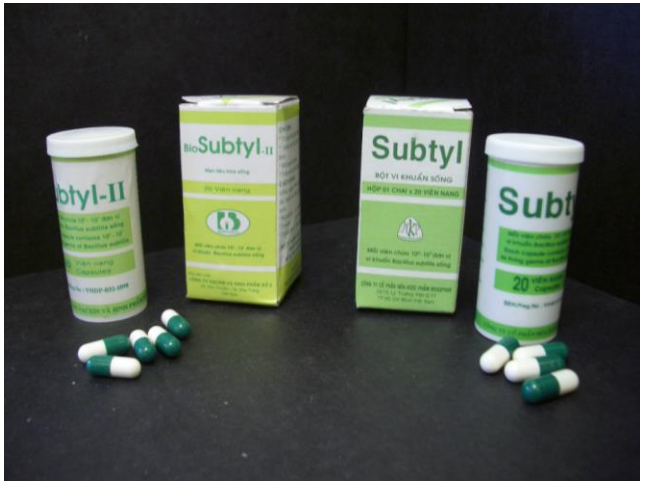
B. subtilis, B. cereus, B. coagulans (aka Lactobacillus sporogenes*), B. clausii*



•GanedenBC^{30}. Spores of *B. coagulans* added to muffins and baked foods, alcohol, chocolate in the USA etc.

•*B. subtilis* has registration for food use in the UK (2007) and Italy (<1991)

•*B. coagulans* has GRAS status in the USA (2008)




For Digestive Fitness!™
Powered by GanedenBC30™ Probiotic
Digestive Advantage
Irritable Bowel Syndrome
Bowel Fitness Formula
Medical Food for Daily Dietary Management of Irritable Bowel Syndrome
Digestive Advantage
FOR DAILY DIGESTIVE FITNESS™
Irritable Bowel Syndrome
Bowel Fitness Formula™
TAKE EVERY DAY
For the Daily Dietary Management of IBS Symptoms
Medical Food
(See Side Panel)
Ganeden BC 30 PROBIOTIC
32 Capsules
The PROBIOTIC That SURVIVES**
For Every Body Every Day
Sustenex
FOR DIGESTIVE & IMMUNE FITNESS
Sustenex®
FOR DIGESTIVE & IMMUNE FITNESS
Daily Probiotic
Delivers 10x More Cells Than Yogurt**
Maintains The Immune System*
Supports Digestive Health*
Dietary Supplement
30 Capsules With 2 Billion Cells Of GanedenBC30
Ganeden BC 30 PROBIOTIC

Natto

B. subtilis var. Natto

Japanese staple

BioPlus 2B

Spores of *B. subtilis* & *B. licheniformis*

Animal feed

Christian Hansen (Denmark)

Main Street Gourmet (USA)
obtained from Isabella's Healthy Bakery

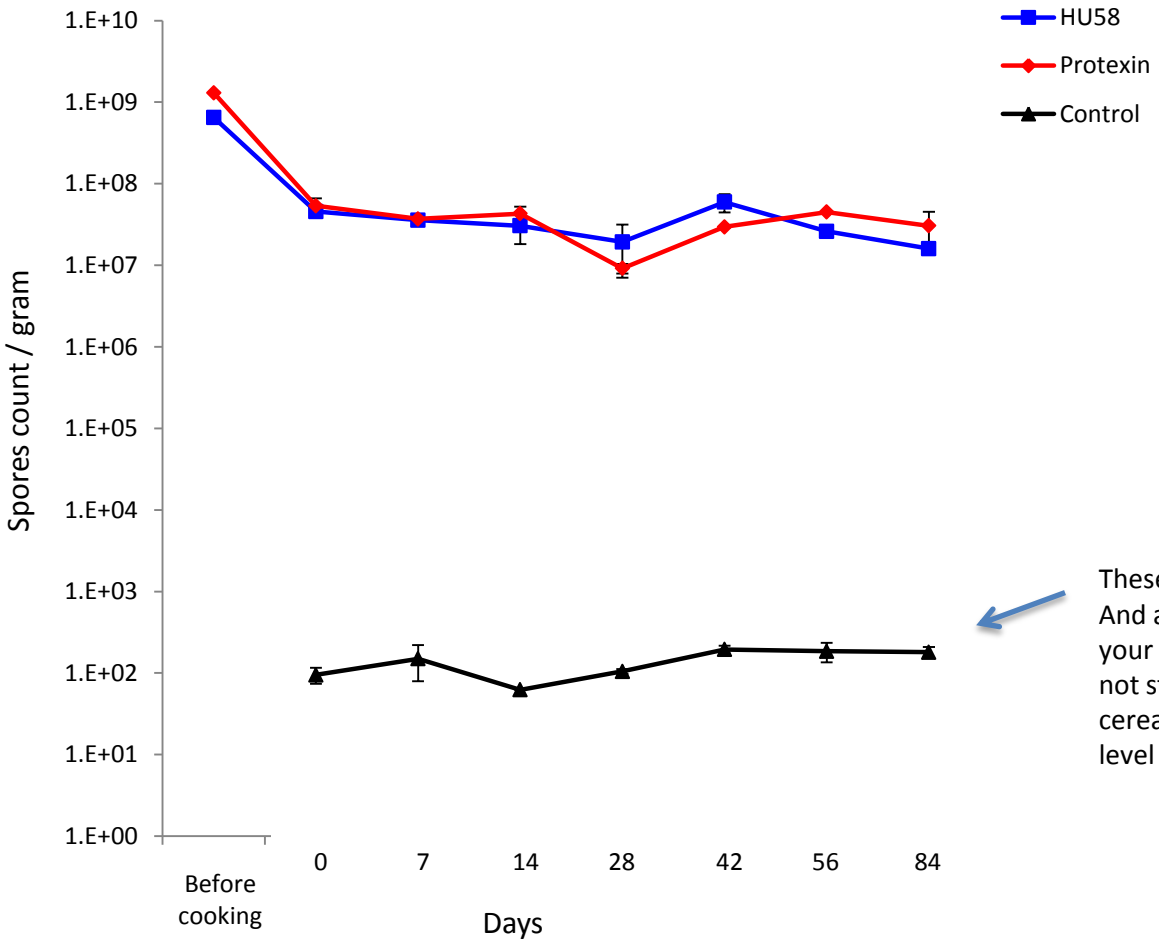
"Activate"
Probiotic muffins impregnated with **spores**

Turtle Island Soup (USA)

"Souper Food"

Soups fortified with **spores**

Survival of spores incorporated in biscuits (McVities) after 84 days



These are background counts And are *Bacillus* spores present in your biscuit mix. The biscuit mix is not sterile and data shows that cereals, wheat etc have a basal level of spore counts.

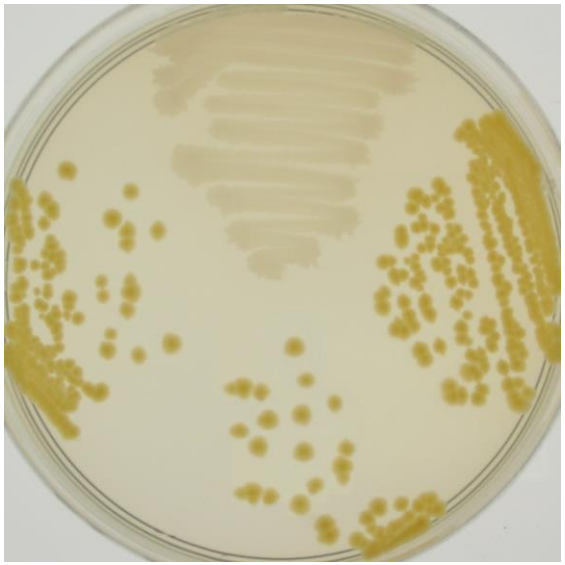
Starting CFU

HU58 Tube 1 + 1kg mix = 6.57 X 10^8 spores/g

PXN21 Tube 1 + 1kg mix = 1.33 X 10^9 spores/g

Pigmented GIT isolates of *B. indicus*

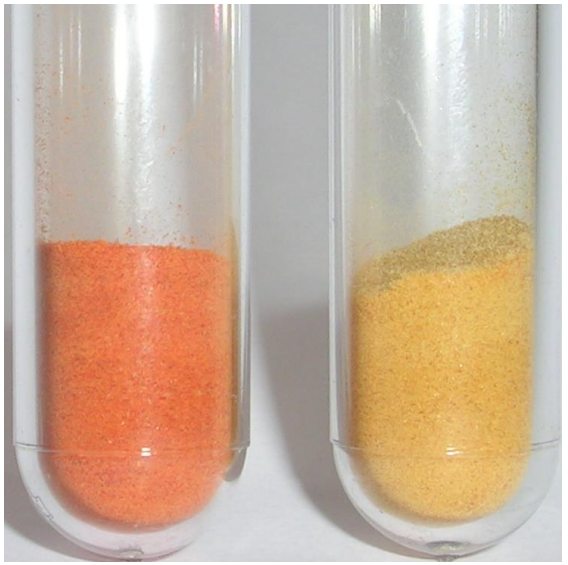
Vegetative Growth



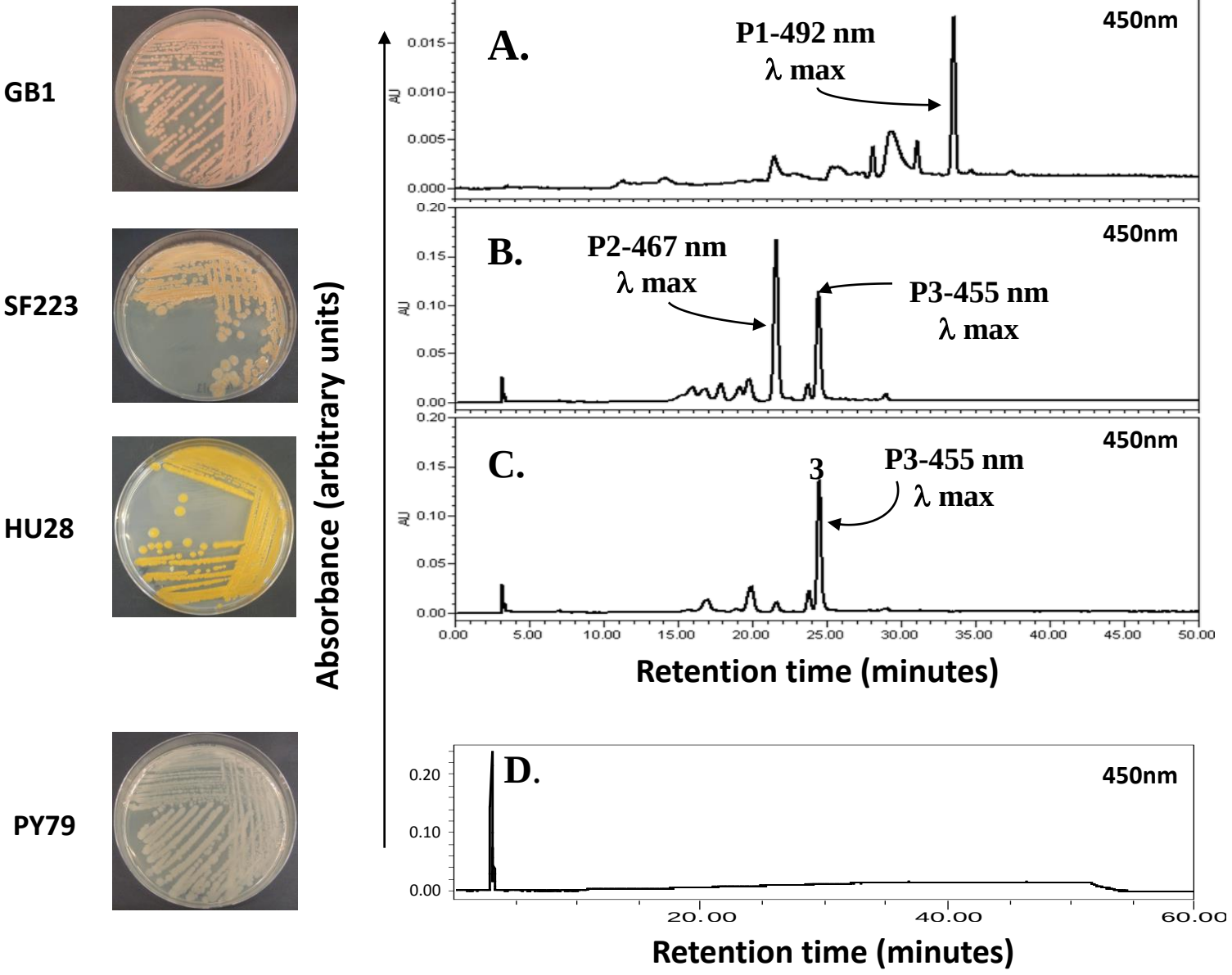
Sporulation

Spores

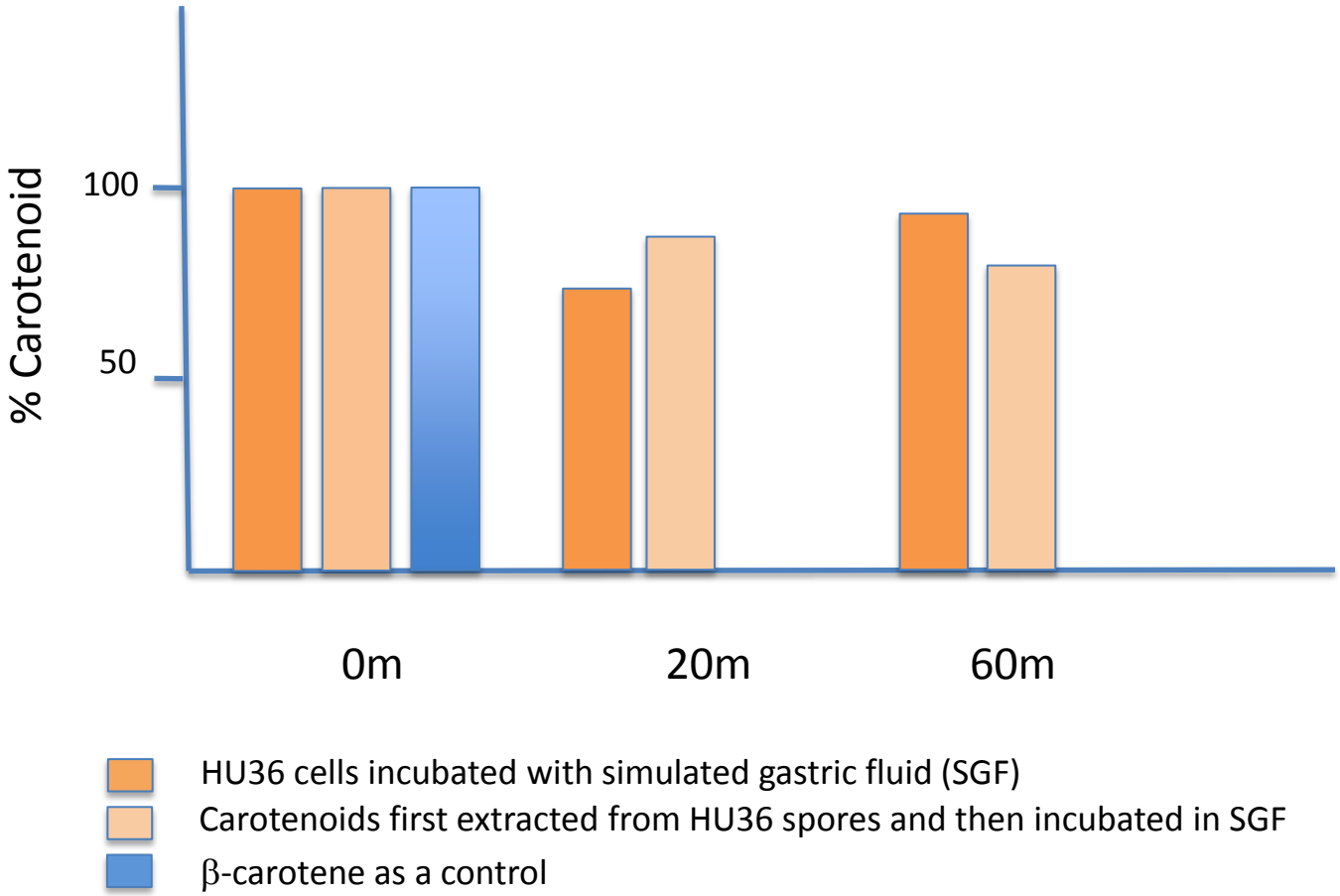
Vegetative Cells



Initial Carotenoid Characterisation

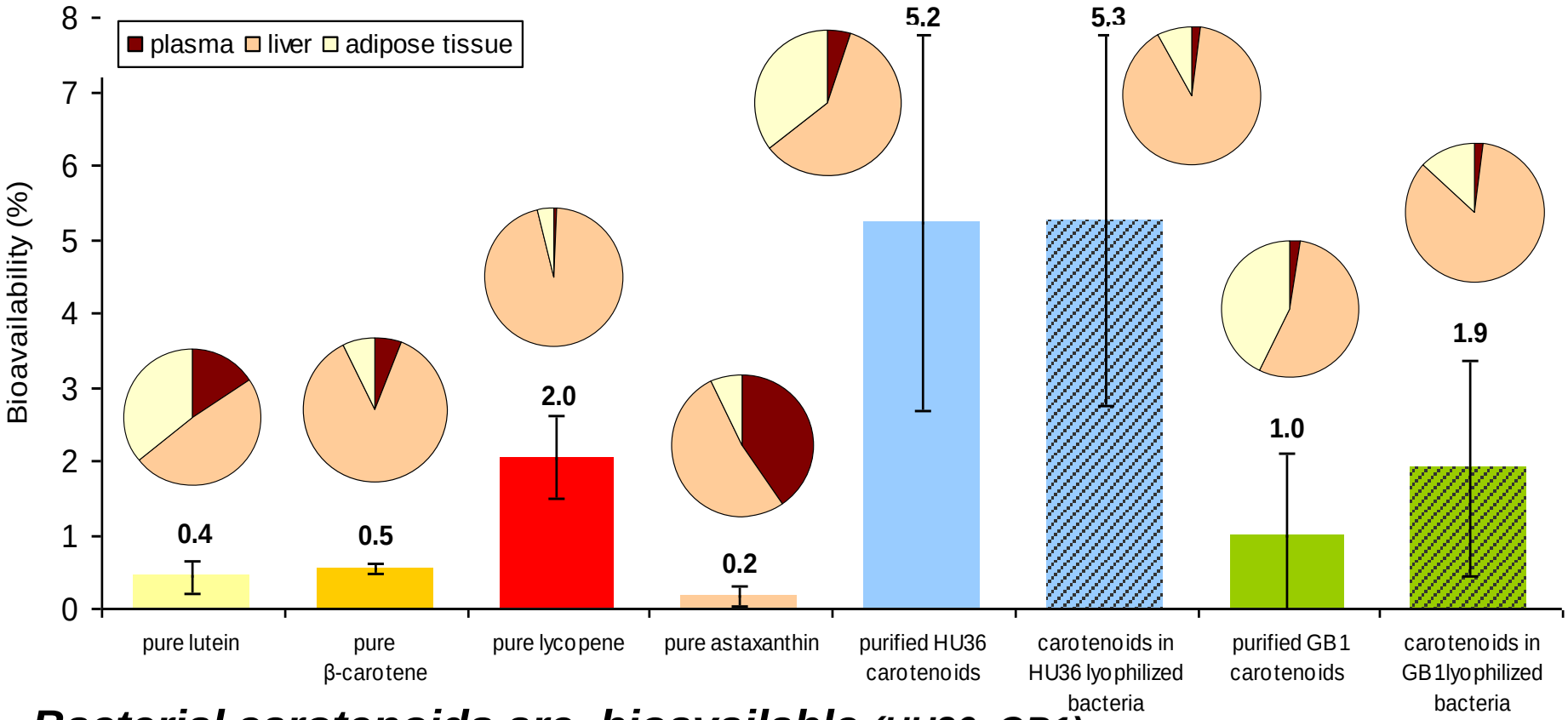


Bacillus Carotenoids are Gastric Stable



IN VIVO BIOAVAILIBILITY RESULTS

- **Bioavailability** = % of carotenoids recovered in plasma + liver + adipose tissue in rats



Bacterial carotenoids are bioavailable (HU36>GB1)
mainly recovered in liver and adipose tissue

CONCLUSIONS

Bacterial carotenoids are

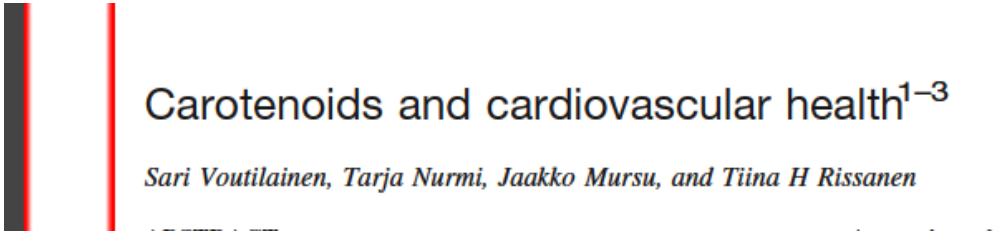
- more stable
than standard carotenoids
(towards dietary iron in micellar system)

- better antioxidants
than standard carotenoids

QPS Species of *Bacillus* that carry Carotenoids

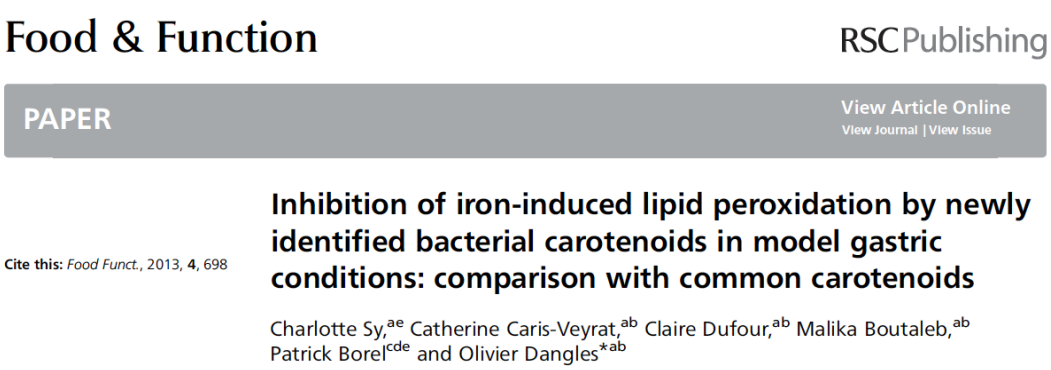
Bacillus megaterium (Yellow/Orange) and *Bacillus pumilus (red)*

- Use for prevention of CardioVascular Disease (CVD)
- Potential anti-cancer agent
- Iron deficiency since carotenoids enhance iron uptake



Carotenoids and cardiovascular health[1–3]

Sari Voutilainen, Tarja Nurmi, Jaakko Mursu, and Tiina H Rissanen



Food & Function

RSC Publishing

PAPER

View Article Online
View Journal | View Issue

Cite this: *Food Funct.*, 2013, **4**, 698

Inhibition of iron-induced lipid peroxidation by newly identified bacterial carotenoids in model gastric conditions: comparison with common carotenoids

Charlotte Sy,[ae] Catherine Caris-Veyrat,[ab] Claire Dufour,[ab] Malika Boutaleb,[ab] Patrick Borel[cde] and Olivier Dangles*[ab]

Vitamin A and β-Carotene Can Improve Nonheme Iron Absorption from Rice, Wheat and Corn by Humans[1,2]

María Nieves García-Casal,* Miguel Layrisse,*‡[3] Liseti Solano,† María Adela Barón,† Franklin Arguello,† Daisy Llovera,† José Ramírez,* Irene Leets* and Eleonora Tropper*

**Centro de Medicina Experimental, Laboratorio de Fisiopatología, Instituto Venezolano de Investigaciones Científicas (IVIC), Caracas 1020A, Venezuela; †Unidad de Investigaciones en Nutrición, Universidad de Carabobo, Valencia, Venezuela; and ‡Universidad Central de Venezuela, Caracas, Venezuela*

Bacillus species are gut commensals

Found in the human gut at ~10^4 spores/g of feces

Recovered from ileal biopsies

30% of the gut flora are spore formers

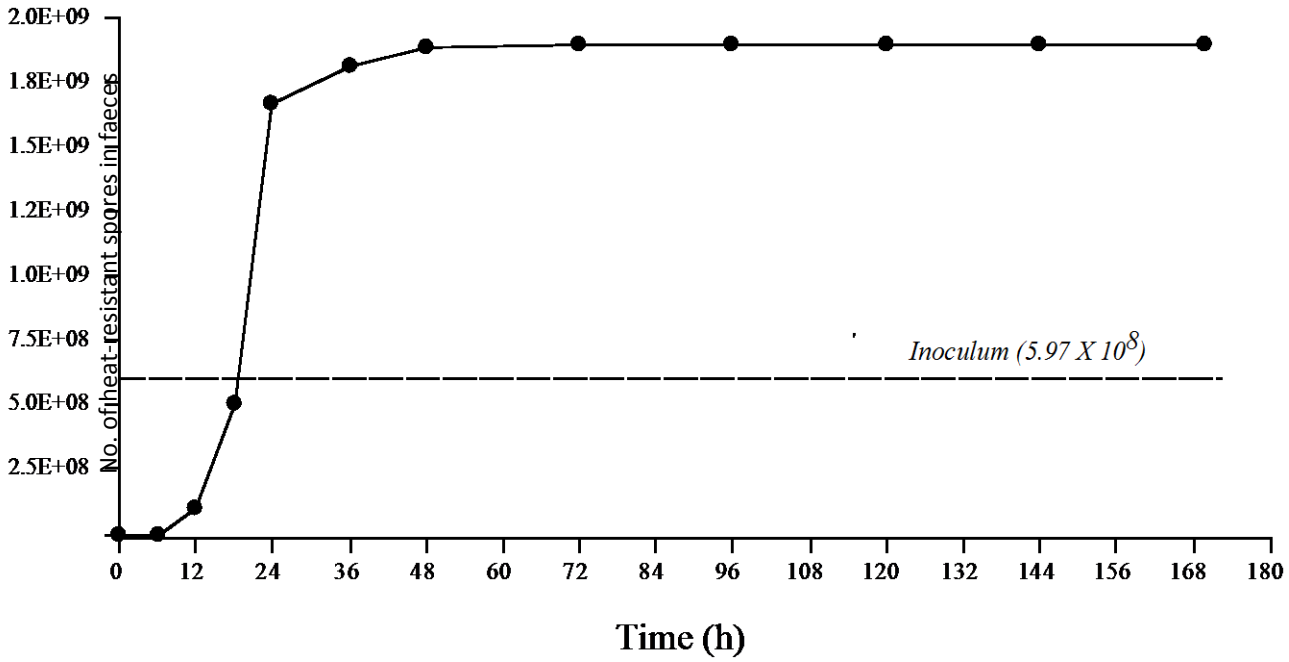
Most Bacilli produce bile salt hydrolase

Some species have evolved into pathogens (*B. anthracis* and *B. cere*us)

Can persist for up to 1 month in the gut of the mouse

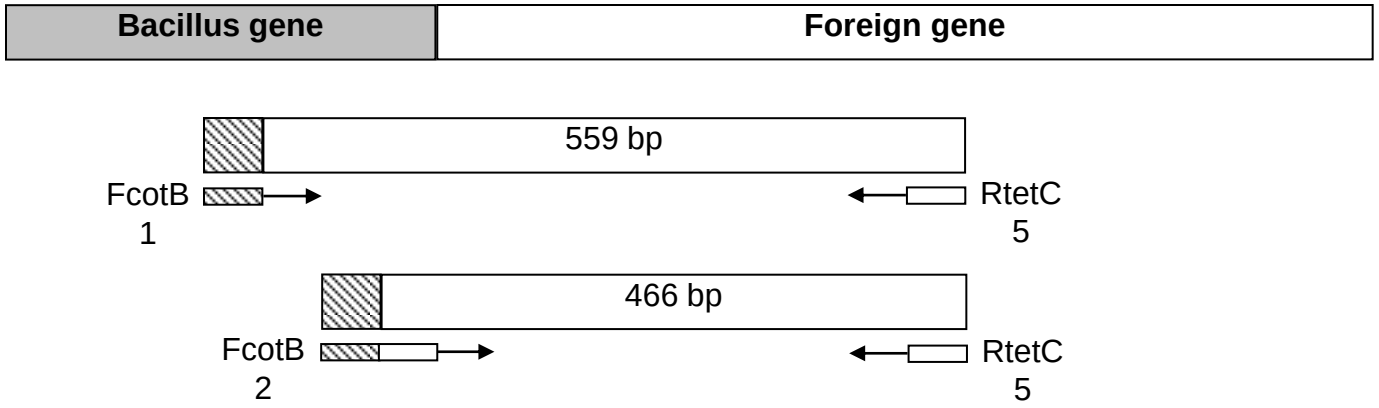
Analysis of Spore Counts in Faeces Shows More Spores Excreted than Inoculated by a Factor of 3-6

Individual mice given single dose of spores and shed heat-resistant cfu determined in faeces

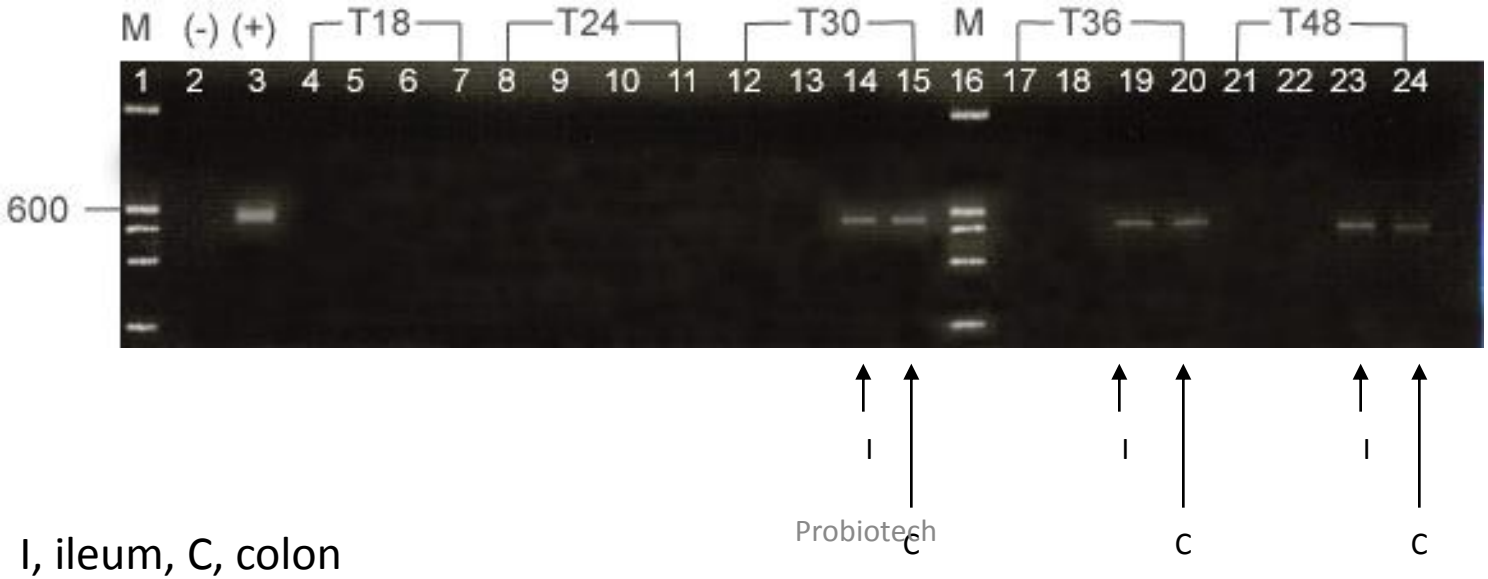


Germination, proliferation and sporulation in the GI-tract

1) Construction of chimeric gene, expressed during germination/growth or during sporulation



2) Dose mice with spores, extract mRNA and quantify expression



I, ileum, C, colon

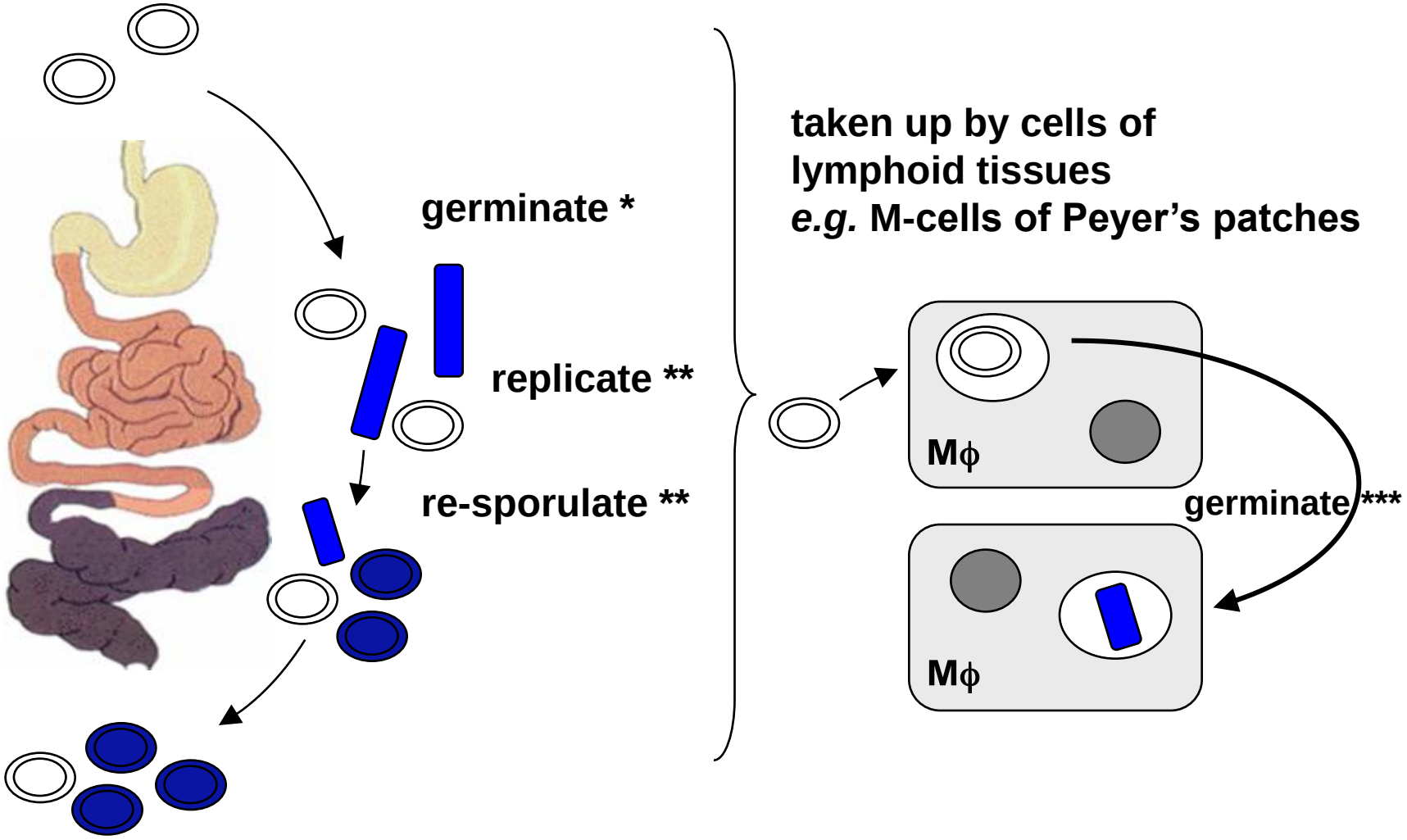
Approx. 10-16% of spores germinate and then re-sporulate in the GI-tract

		18h				24h				30h			
	Mouse	Duodenum	Jejunum	Ileum	Large int.	Duodenum	Jejunum	Ileum	Large int.	Duodenum	Jejunum	Ileum	Large int.
Germination	1	-	++	-	-	-	++	-	-	-	+	-	-
	2	-	++	+	-	-	++	-	-	-	+	-	-
	3	-	++	-	-	-	++	+	-	-	+	-	-
	4	-	++	-	-	-	++		-	-	+	-	-
	Naïve	-	-	-	-	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Sporulation	1	-	-	-	-	-	-	-	-	-	-	+	+
	2	-	-	-	-	-	-	-	-	-	-	+	+
	3	-	-	-	-	-	-	-	-	-	-	+	+
	4	-	-	-	-	-	-	-	-	-	-	+	+
	Naïve	-	-	-	-	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a

		36h				48h			
	Mouse	Duodenum	Jejunum	Ileum	Large int.	Duodenum	Jejunum	Ileum	Large int.
Germination	1	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-
	Naïve	n/a	n/a	n/a	n/a	-	-	-	-
Sporulation	1	-	-	+	+	-	-	+	±
	2	-	-	+	+	-	-	+	±
	3	-	-	+	+	-	-	+	±
	4	-	-	+	+	-	-	+	±
	Naïve	n/a	n/a	n/a	n/a	-	-	-	-

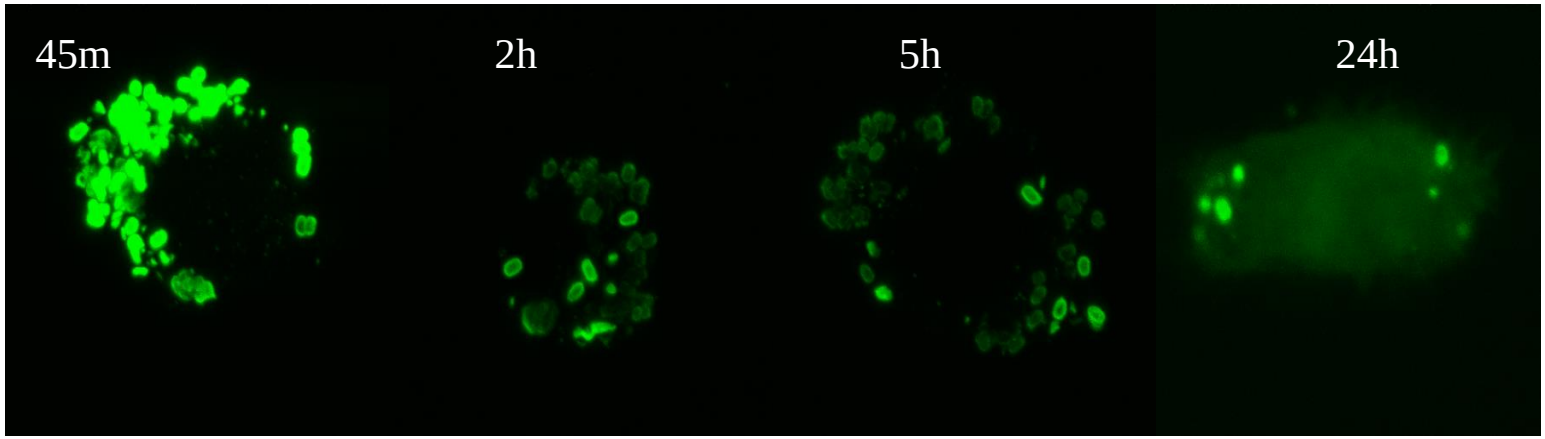
Measure germination by using a vegetative *Bacillus* gene chimera
Measure re-sporulation by using a sporulation *Bacillus* gene chimera

Fate of Spores

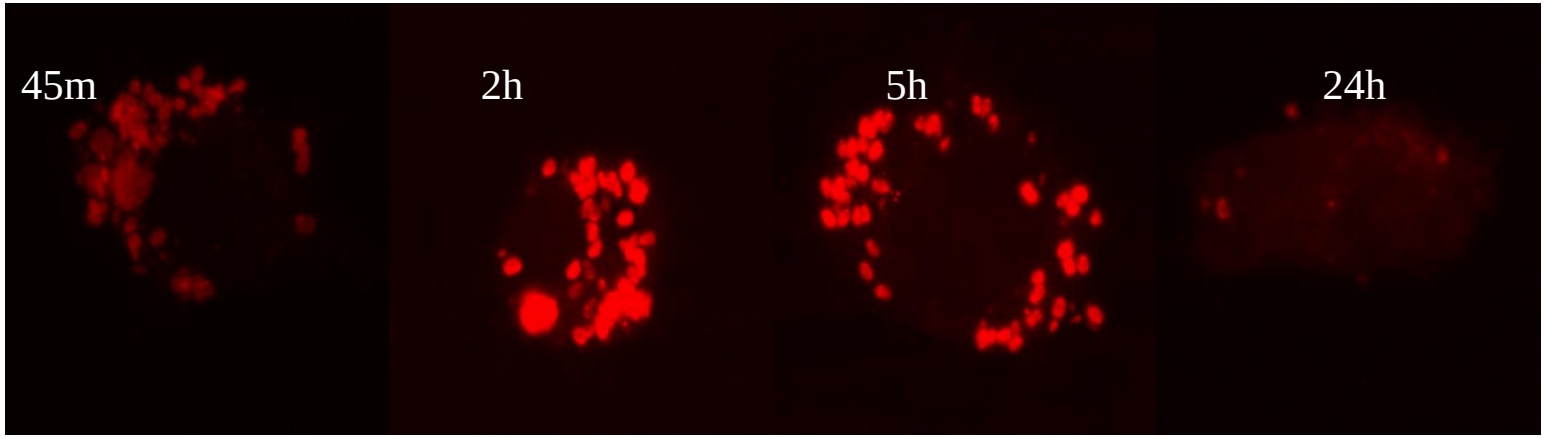


* Casula & Cutting. 2002. *Appl Environ Microbiol* 68:2344-52
** Hoa *et al*. 2001. *Appl Environ Microbiol* 67:3819-23
*** Duc *et al*. 2004. *Vaccine* 22:1873-85

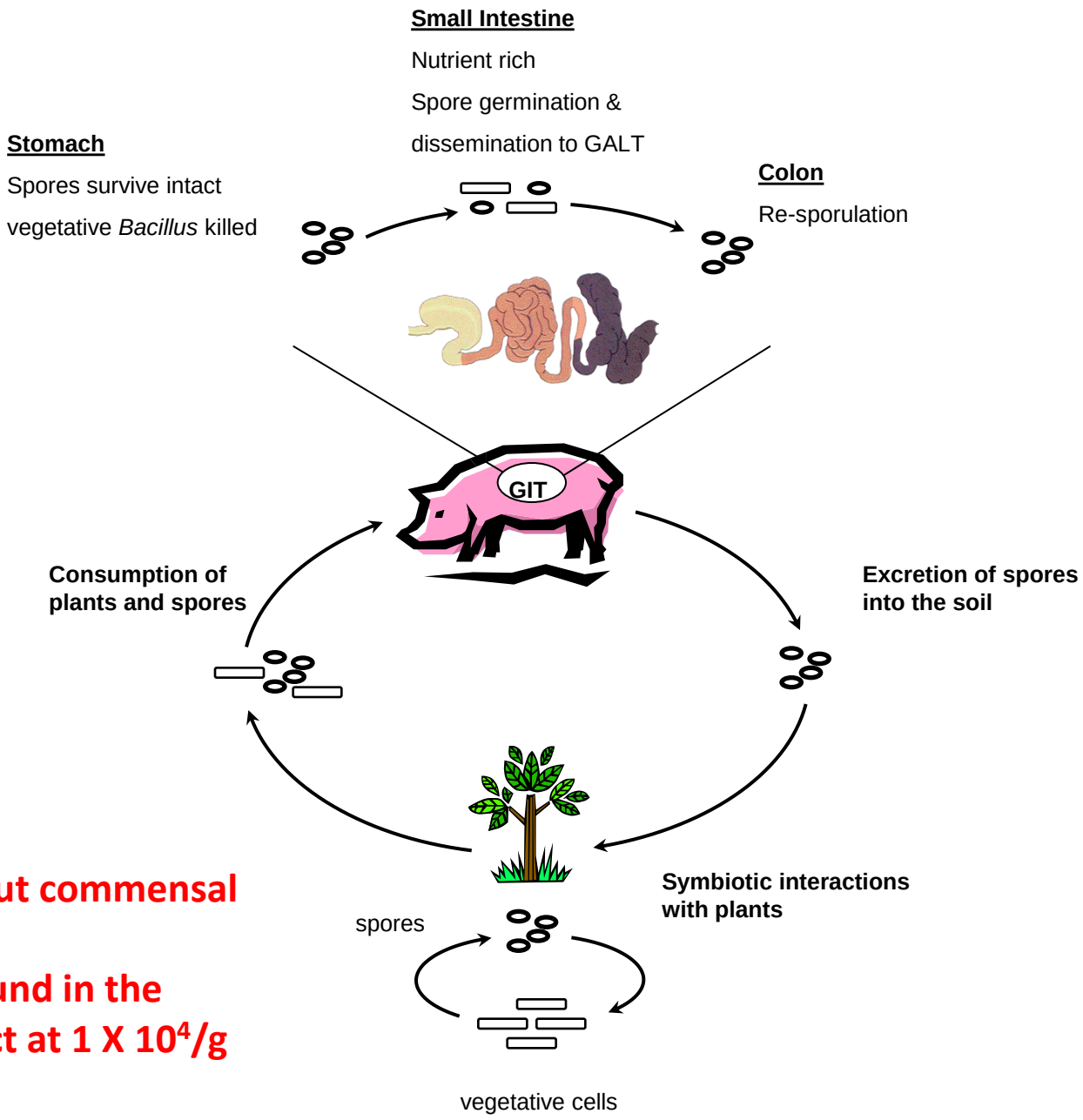
Phagocytosis of *rrn0-lacZ* spores by macrophage-like cell line, RAW264.7
Ingestion and prolonged survival could induce CTL responses



Anti-spore labelling



Anti-β-Gal labelling (vegetative gene expression*)



Bacillus as a gut commensal

Commonly found in the human GI-tract at 1 X 10^4/g

Mechanisms for Probiosis

1) Stimulation of innate immunity

2) Synthesis of antimicrobials that kill pathogens

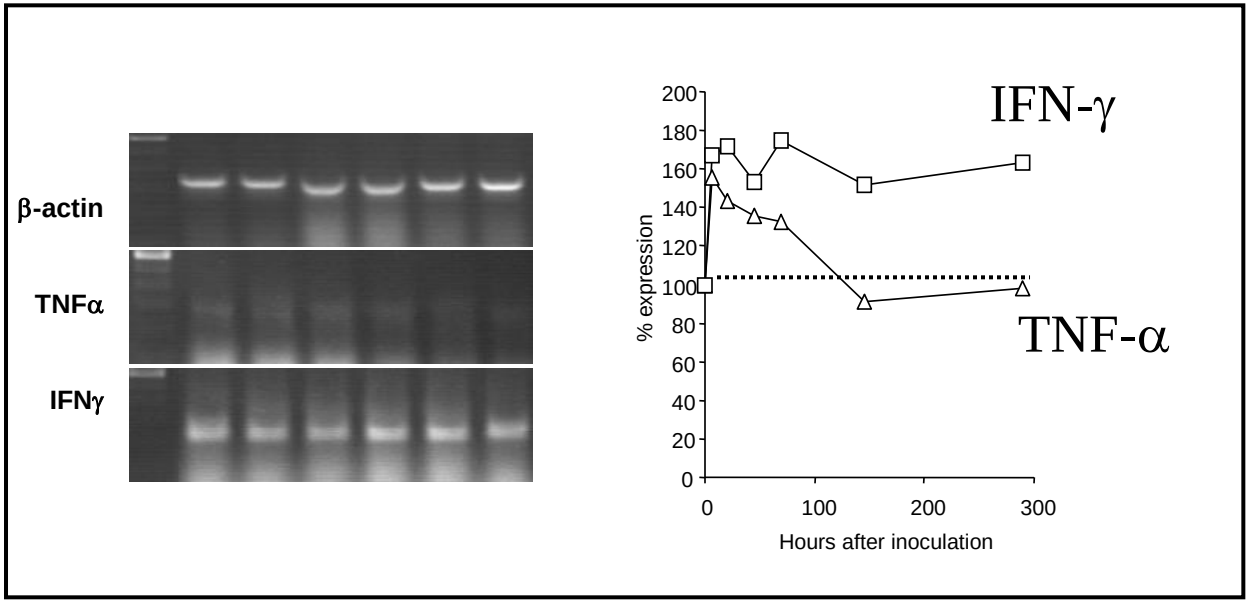
3) Adsorption of toxins in the GI-tract

4) Rebalancing the gut microflora

Innate Immunity

1) Immune Stimulation

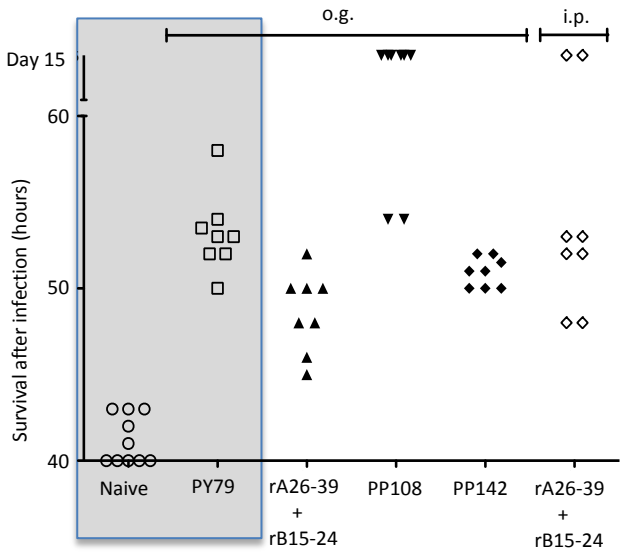
Cytokine mRNA *in vivo*



IFN-γ → Th1 response → Cellular immunity & IgG2a

Evidence for Innate Immunity and Induction by Non-Recombinant Spores carrying no antigen

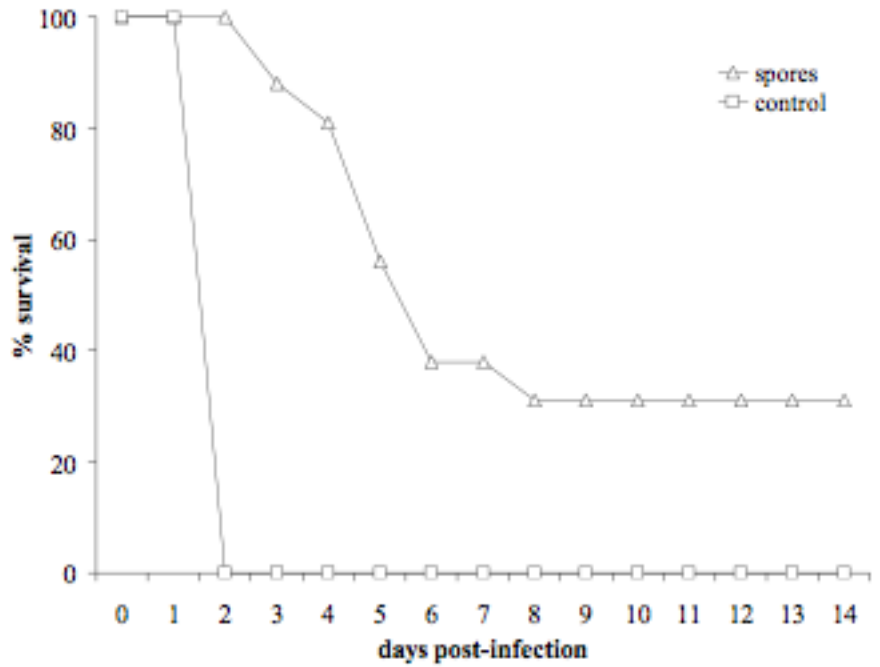
1. *C. difficile* infection



Vaccination experiment

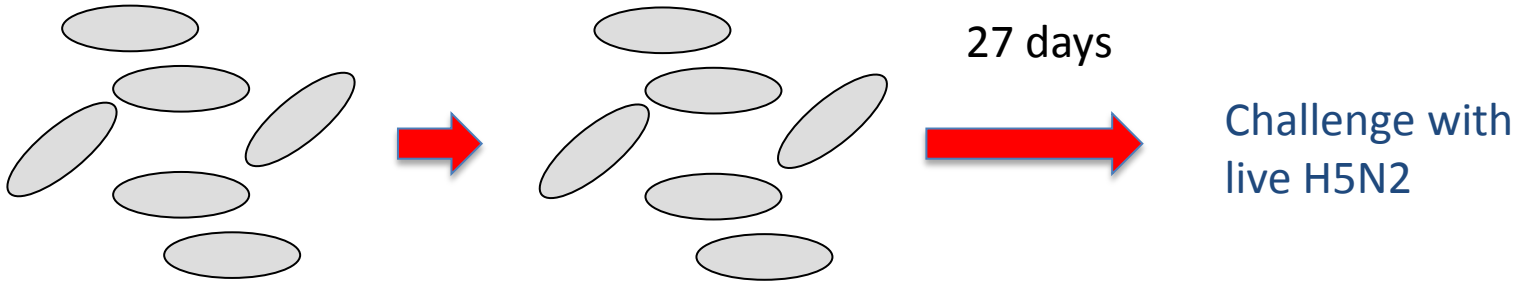
Shaded box shows increased survival of hamsters pre-dosed with *B. subtilis* PY79 spores and then challenged with *C. difficile*

2. Anthrax



In this study the spores were delivered via the intra-peritoneal (i.p.) route to provide protection against an i.p. challenge with *B. anthracis* STI spores. The spores were administered to mice 48h and 24h prior to challenge with *B. anthracis* (approx 100 MLD) and protection afforded against anthrax was monitored. Results showed that pre-exposure administration of the *B. subtilis* spores was able to provide partial protection against anthrax infection in mice (expt done at Porton Down).

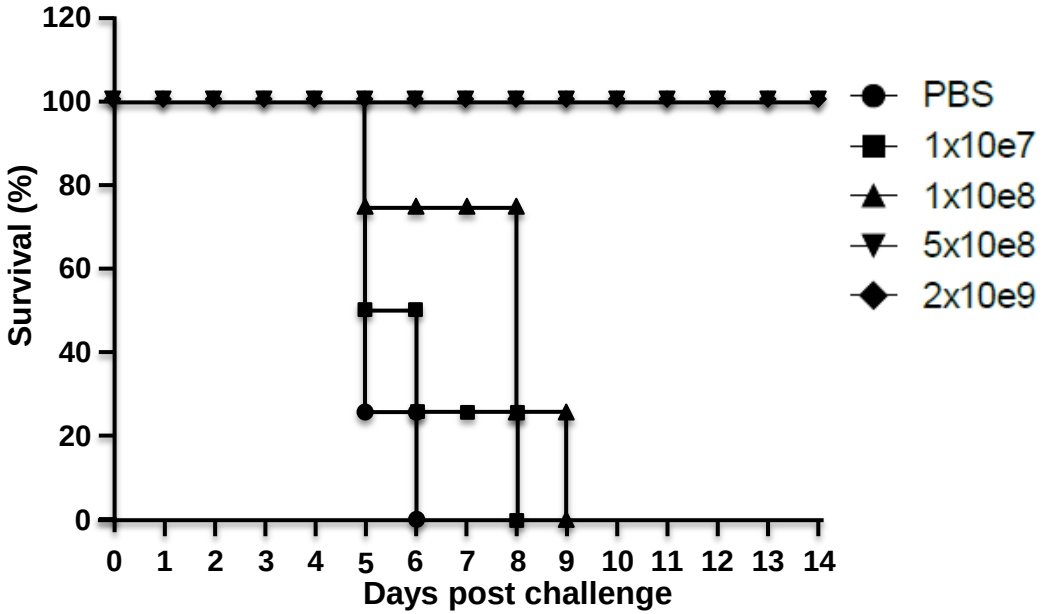
3. Influenza



Day 1/i.n.

Day 14/i.n.

Survival rates



No antigen-immunotherapy

Shinya et al. Virology Journal 2011, 8:97
http://www.virologyj.com/content/8/1/97

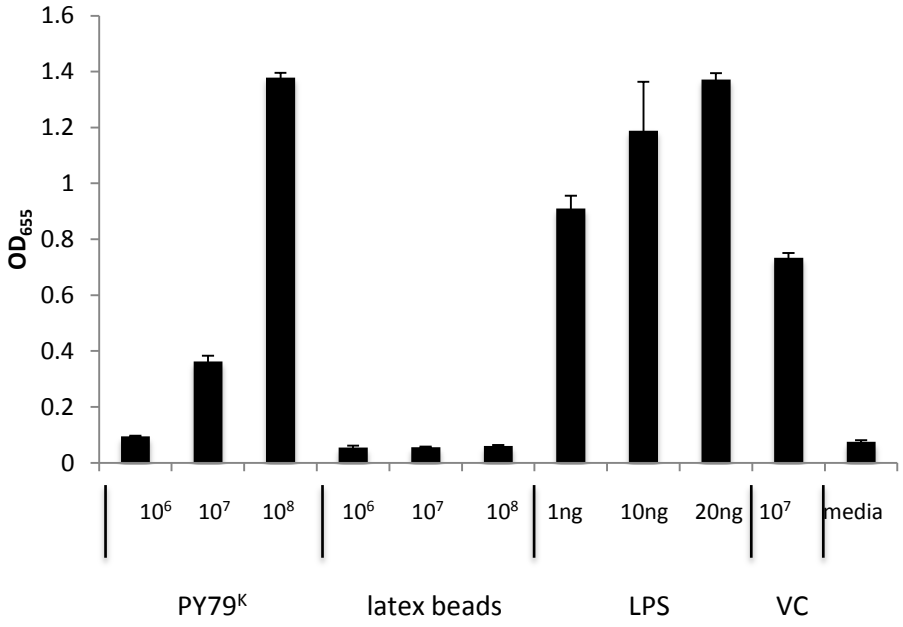
VIROLOGY JOURNAL

RESEARCH Open Access

Toll-like receptor pre-stimulation protects mice against lethal infection with highly pathogenic influenza viruses

Kyoko Shinya[1,2*], Tadashi Okamura[2], Setsuko Sueta[2], Noriyuki Kasai[2], Motoko Tanaka[1], Teridah E Ginting[1], Akiko Makino[1], Amie J Eisfeld[3], Yoshihiro Kawaoka[1,3,4*]

Induction of innate immunity. Spores stimulate NF-Kβ pathway via interaction with Toll-like receptors



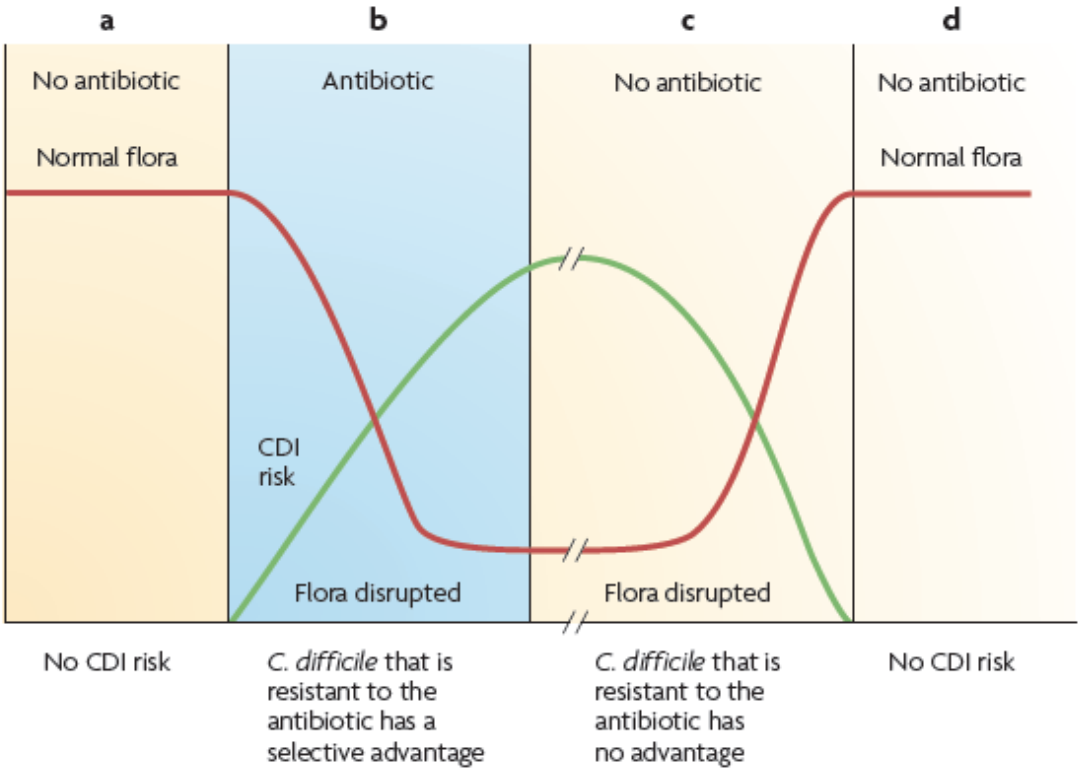
Clostridium difficile Infection (CDI)

Major nosocomial pathogen
~4,000 deaths per annum in the UK
No vaccine

Protective antigen thought to be toxin A and toxin B

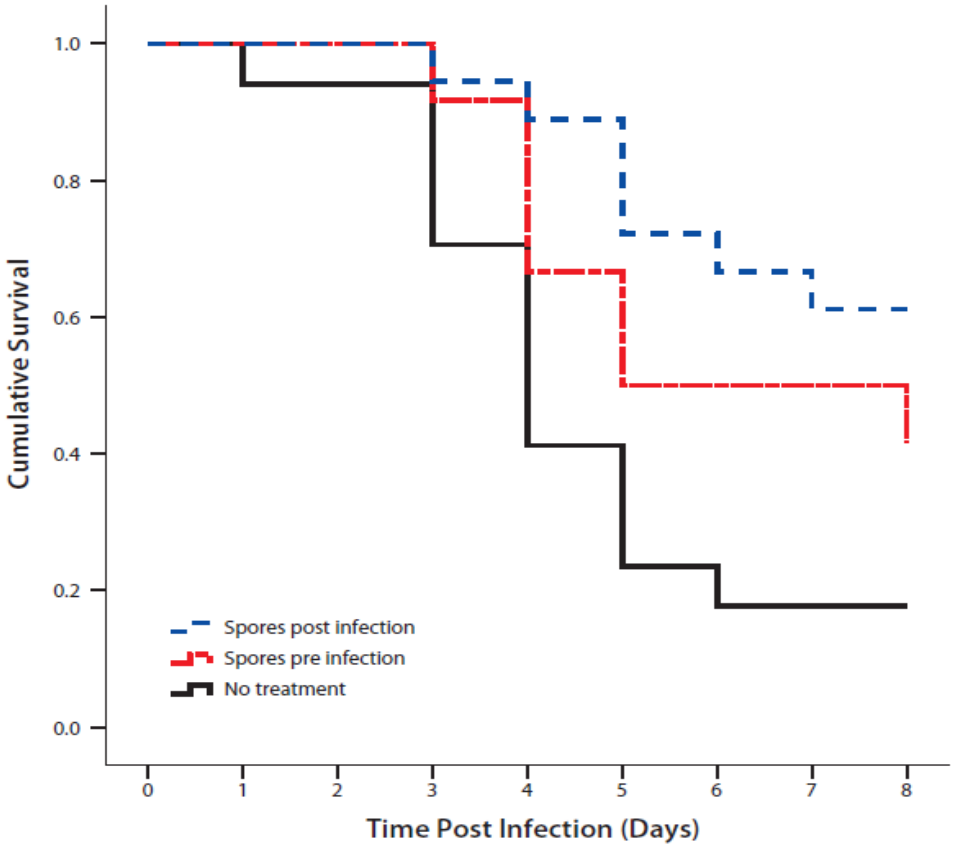
All vaccines under development are parenteral

Emergence of the 078 ribotype from pigs to humans



In ~30% of patients, CDI can return in 2-3 weeks (relapse or recurrence)

Effect on *C. difficile* infection, pre and post dosing

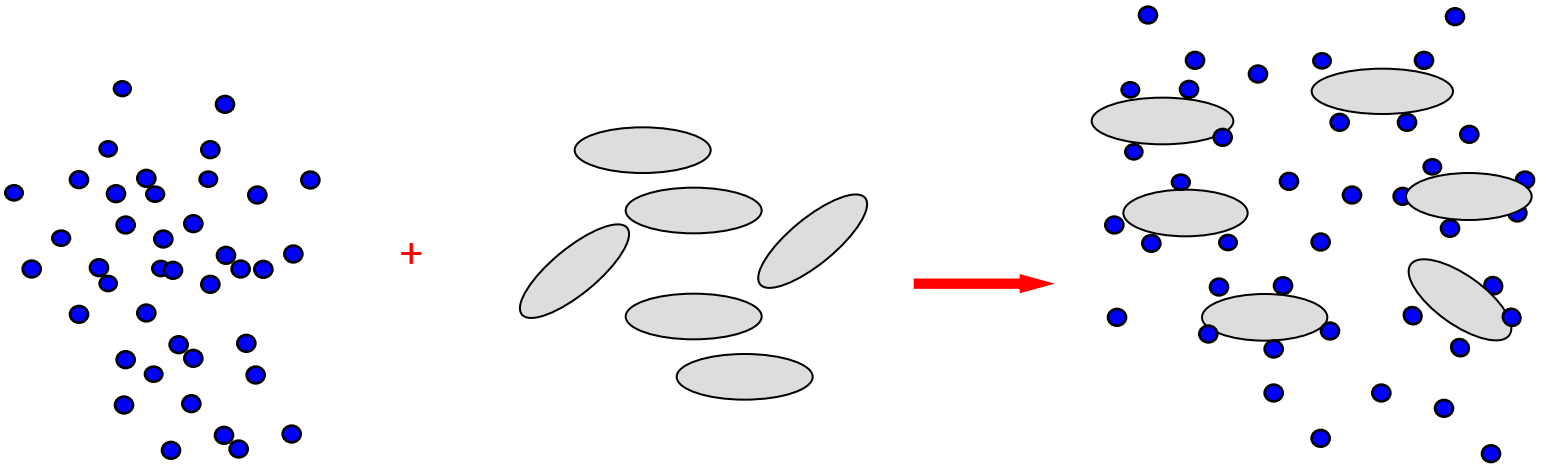


Mice dosed every day for 7 days pre infection or every day post infection Kaplan-Meier survival plot for mice infected with *C. difficile* VPI 10463. Groups received oral doses of live *B. subtilis* PXN21 spores as treatment either pre or post infection. *Post infection slightly better*

Pre-dosing effect is likely to be due to innate immunity

Post-dosing effects could be due to subtraction of toxin the GI-tract?

Adsorption to Spores



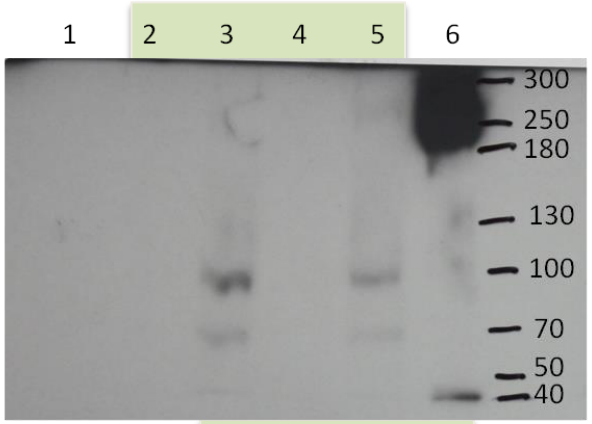
toxin

B. subtilis spores
-Net negative charge
-hydrophobic

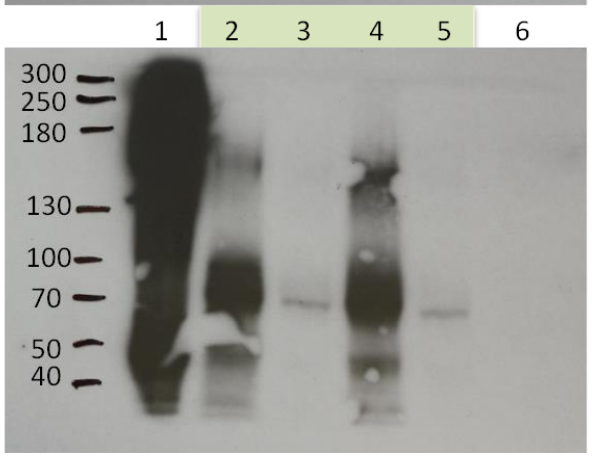
Ionic and hydrophobic interactions

In vitro binding of toxin to spores

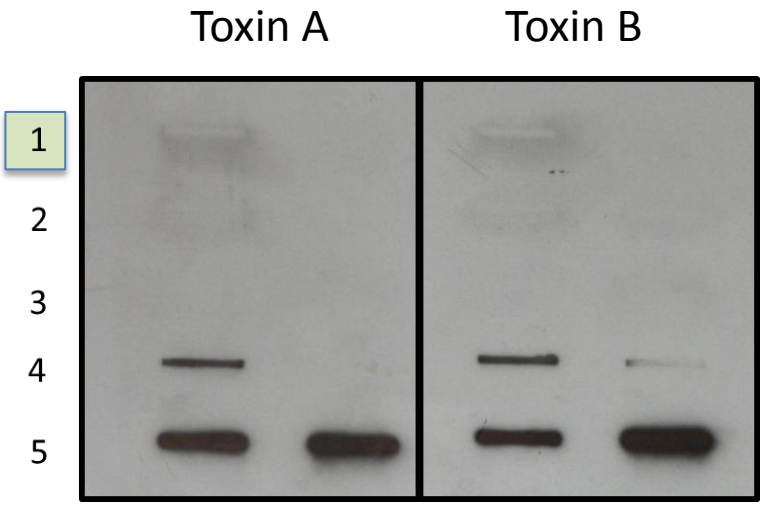
Toxin A



Toxin B



Caecal extracts from post-dosed mice



1 caecum extracts post-dosing
2 caecum extracts no spores
3 caecum extracts no C. difficile
4 purified toxins
5 = toxin detected in supernatants of caecal extracts

Secretion of beneficial molecules in the GI-tract

Most *Bacilli* produce Nattokinase, a protease that reduces blood clotting

Antimicrobials. Depends on species and strain but, Plipastatin, Bacilysin, Subtilosin, Fengycin, Iturins, etc

Many strains can be administered together with antibiotics and this allows temporary restoration of the gut microflora during prophylaxis

Spores, and Specifically Sporulation are Important for Development of the GALT

The Journal of Immunology

Role of Commensal Bacteria in Development of Gut-Associated Lymphoid Tissues and Preimmune Antibody Repertoire[1]

Ki-Jong Rhee, Periannan Sethupathi, Adam Driks, Dennis K. Lanning, and Katherine L. Knight[2]

Intestinal bacteria are required for development of gut-associated lymphoid tissues (GALT), which mediate a variety of host immune functions, such as mucosal immunity and oral tolerance. In rabbits, the intestinal microflora are also required for developing the preimmune Ab repertoire by promoting somatic diversification of Ig genes in B cells that have migrated to GALT. We studied the mechanism of bacteria-induced GALT development. Bacteria were introduced into rabbits in which the appendix had been rendered germfree by microsurgery (we refer to these rabbits as germfree-appendix rabbits). We then identified specific members of the intestinal flora that promote GALT development. The combination of *Bacteroides fragilis* and *Bacillus subtilis* consistently promoted GALT development and led to development of the preimmune Ab repertoire, as shown by an increase in somatic diversification of VDJ-Cμ genes in appendix B cells. Neither species alone consistently induced GALT development, nor did *Clostridium subterminale*, *Escherichia coli*, or *Staphylococcus epidermidis*. *B. fragilis*, which by itself is immunogenic, did not promote GALT development; hence, GALT development in rabbits does not appear to be the result of an Ag-specific immune response. To identify bacterial pathways required for GALT development, we introduced *B. fragilis* along with stress-response mutants of *B. subtilis* into germfree-appendix rabbits. We identified two Spo0A-controlled stress responses, sporulation and secretion of the protein YqxM, which are required for GALT development. We conclude that specific members of the commensal, intestinal flora drive GALT development through a specific subset of stress responses. *The Journal of Immunology*, 2004, 172: 1118–1124.

Summary

Gut commensals
Spore probiotics are ‘live’ entities that germinate in the GI-tract, proliferate and then re-sporulate
They promote a healthy GALT

Stability
They have unique resistance properties enabling them to be stored at room temperature indefinitely

Efficacy
Proven by worldwide use
Emerging clinical trial data

Mechanism
Stimulate innate immunity
Somehow promote GALT development
Adsorption of toxins
Secretion of antimicrobials

Dr. Hong Huynh
Dr. Irene Bianconi
Dr. Lluis Sempere
Dr. Stephanie Willing
Dr. Anil Chandrashekran
Ms. Krisztina Hitri
Ms. Nicola Byrne

Siamed Hasseni
Saba Anwar
William Ferreari
Celia Rodriqgues
Emma Popescu
Jacob Goonesana
Alex Curilovs

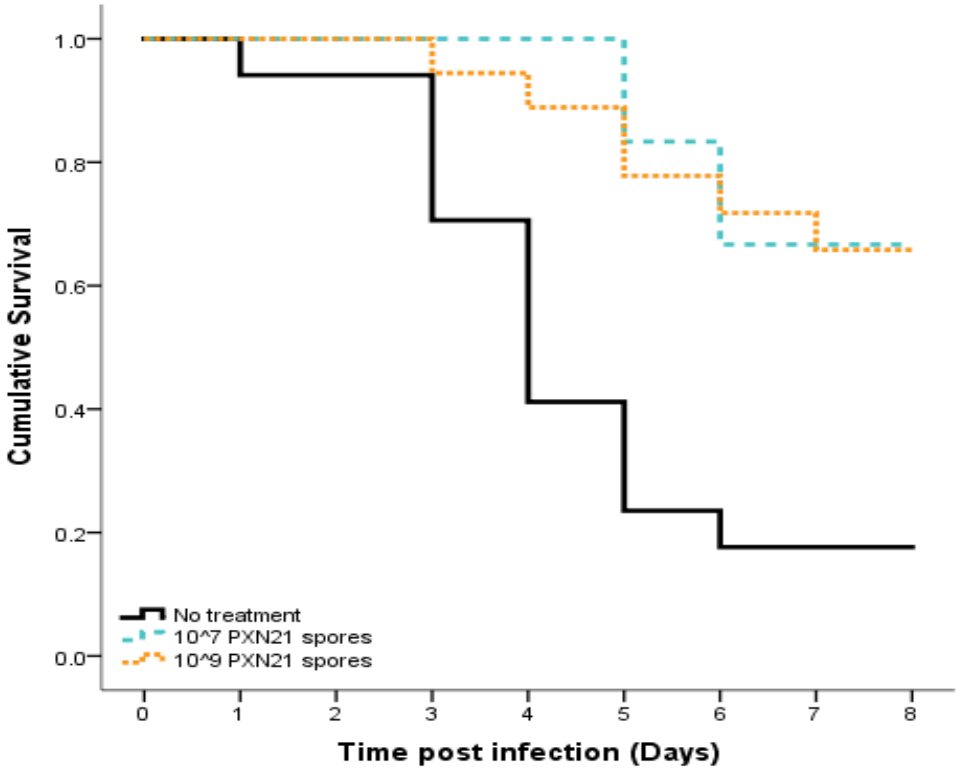
MRC Medical Research Council

European Commission

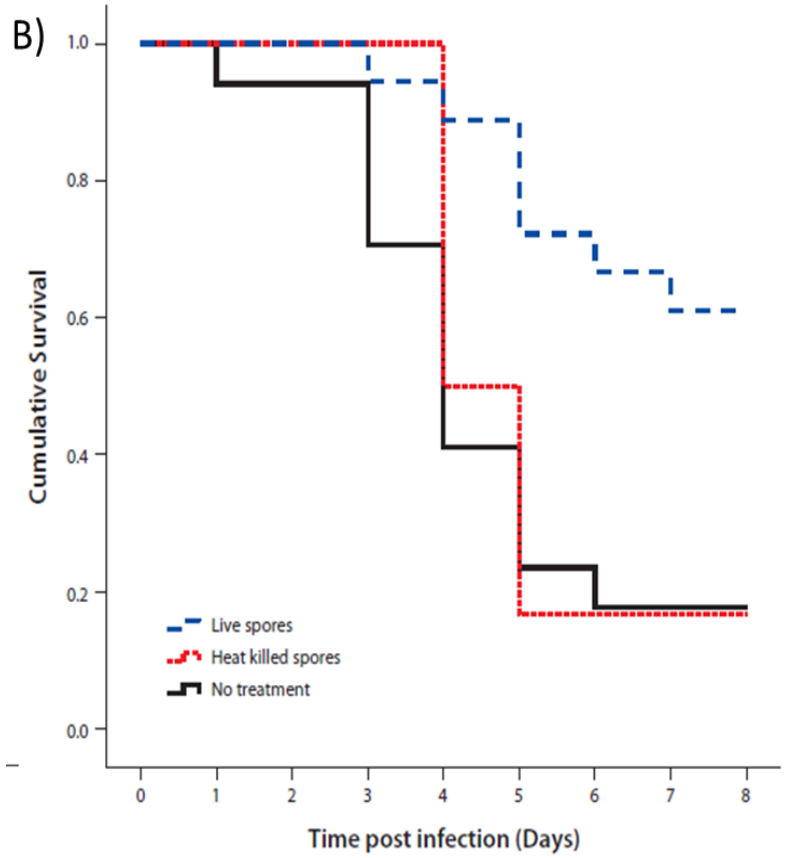
Phase 1/2a clinical trial 2013

www.cdvax.org

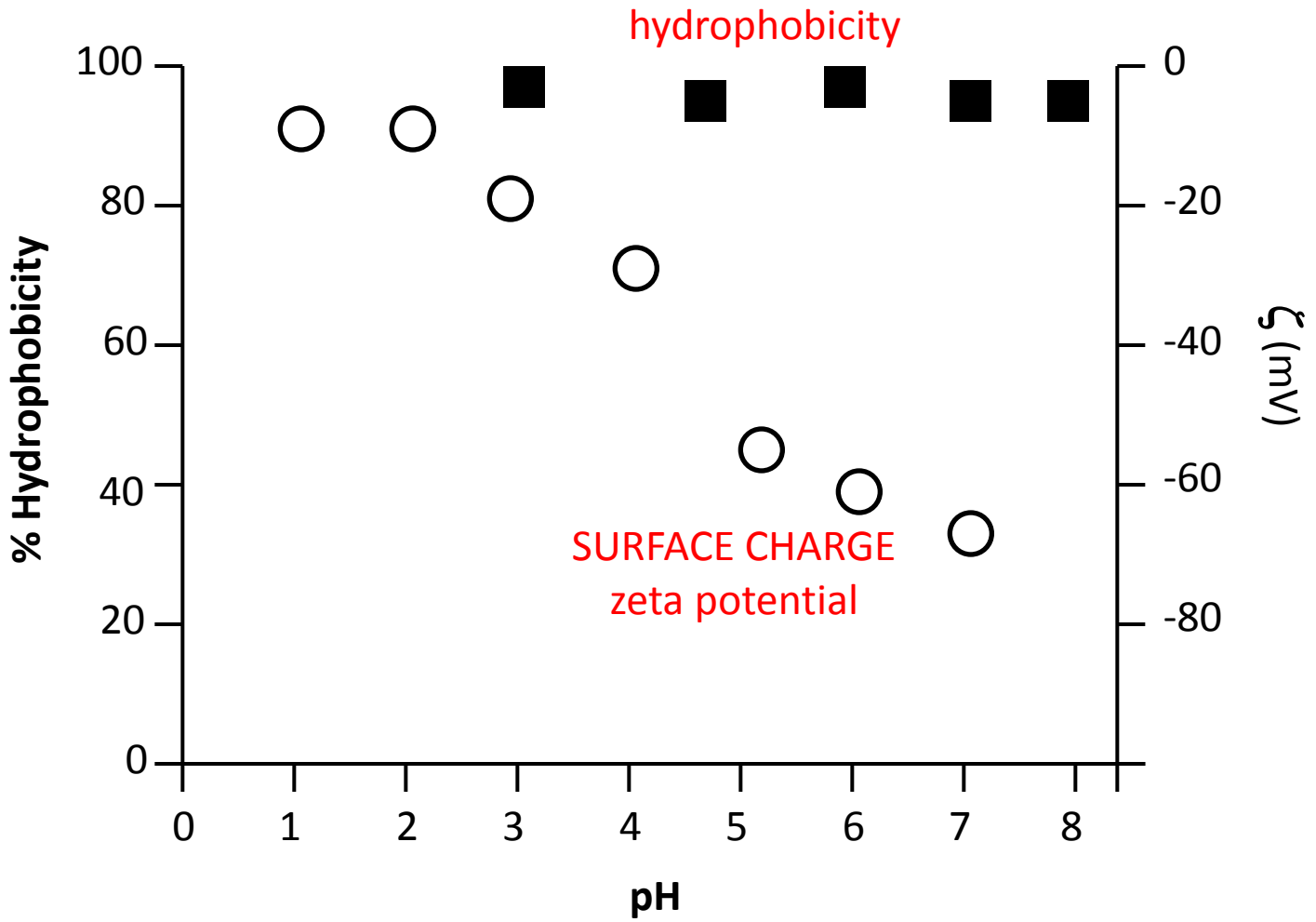
Suppression of *C. difficile* infection is not dose dependent



Kaplan-Meier survival plot for mice infected with *C. difficile* VPI 10463. Groups received oral doses of live *B. subtilis* PXN21 spores post infection at a concentration of either 10^9 or 10^7 spores per dose.



Live spores rather killed spores are needed for suppression of *C. difficile* infection



At all pH values spore is negative charged, but as pH drops below pI protein becomes + charged

At all pH values, spore is hydrophobic and thus able to interact with hydrophobic molecules such as LPS, virions, etc