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Assessing and Maintaining Probiotics in Food

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Outline

- Trends in the market for probiotic foods
- Maintaining probiotics in food
  - Production & formulation
  - Food processing
  - Storage in food matrix
- Assessing probiotics in food
  - Plate counting methods
  - Flow cytometry
  - Survival through GI-tract

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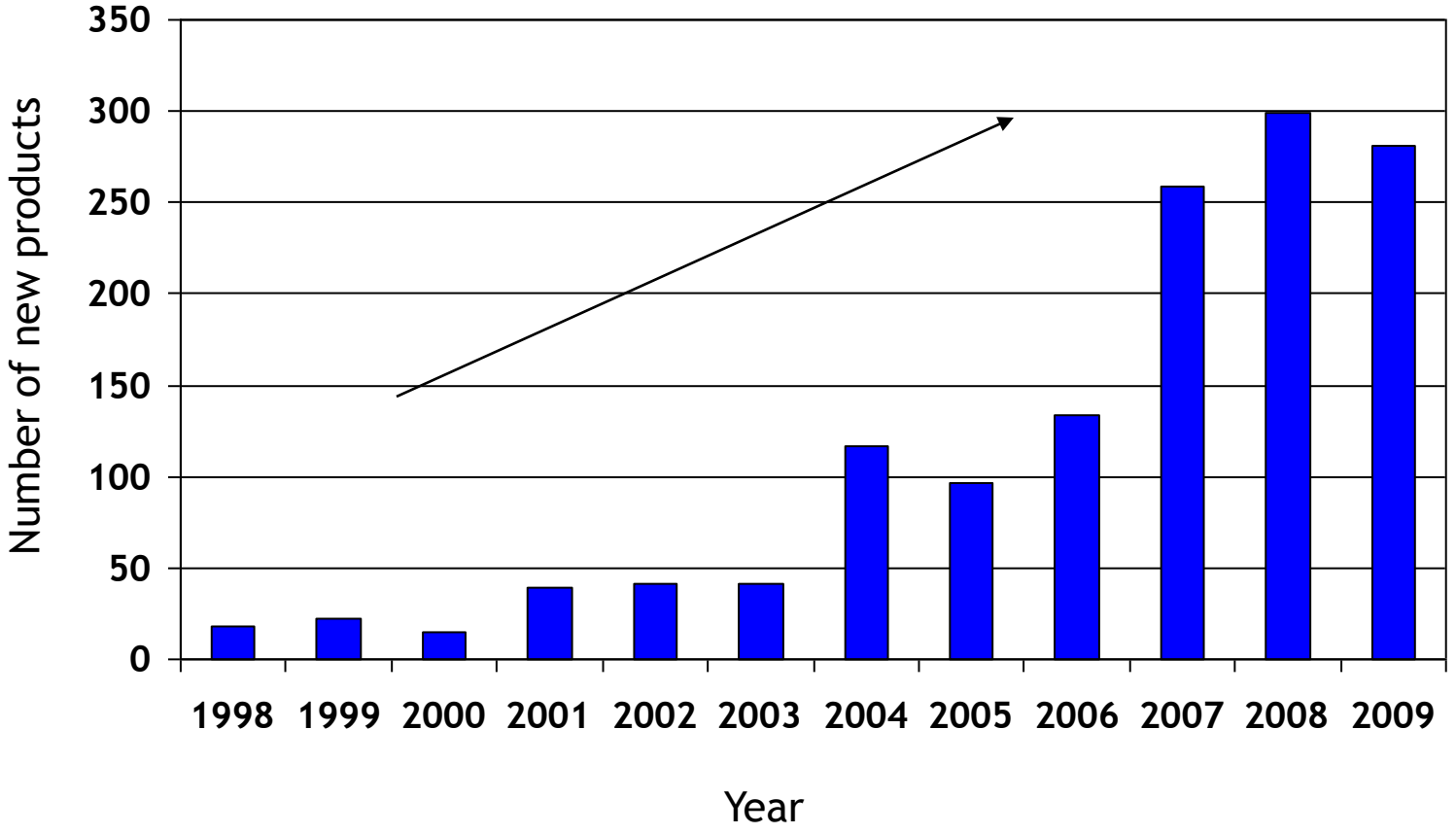
Trends in the market for probiotic foods

Trends within probiotics in food

- Continued growth in most markets
- 95% of current market within fermented milk products
- Probiotics moving into other types of fresh foods
  - Cheese, ice cream, drinking milk, soy based products, fruit juices
- Increased attention from regulatory side
- Increased focus on credibility
  - Clinical documentation, safety, cell count

Continued interest in probiotics

– Number of new probiotic food products launched is growing



Source: Mintel, GNPD 2010
Includes all new products launched worldwide,
excludes extensions and repackaged products

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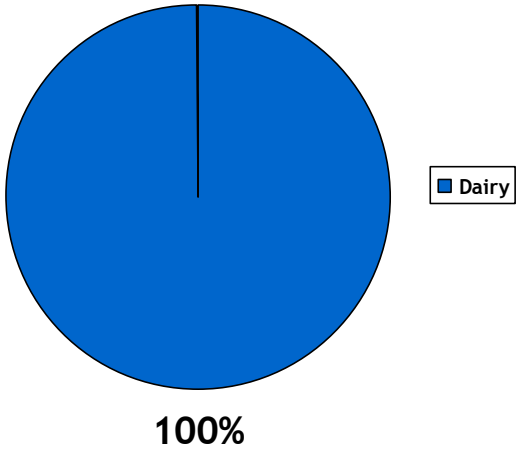
Probiotic food segments

Dairy products, and specifically yoghurt-like products, form the largest segment by far in the market for probiotic foods, probably due to the consumer perception of dairy products:

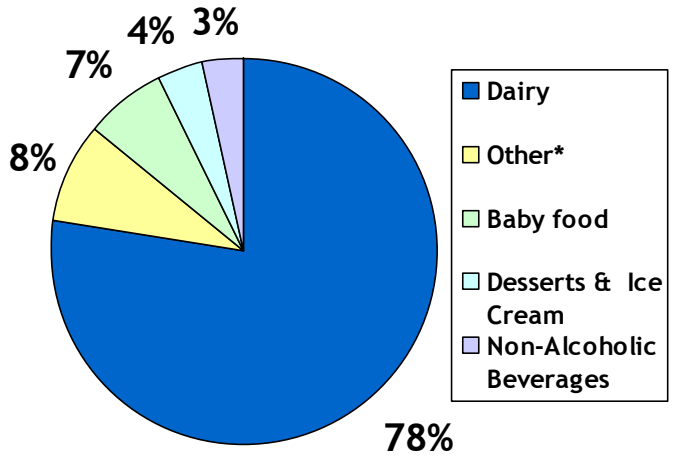
- Generally considered healthy
- Bacteria is a known phenomenon in e.g. yoghurts
- Consumers seem to prefer when functional ingredients are placed in a natural context

1998



18 new launches all in dairy

2009



281 new launches
moving into new categories

Source: Mintel, GNPD 2010
Includes all new products launched worldwide, excludes extensions and repackaged products
*other includes: Bakery, sauces and seasonings, chocolate, spreads and confectionary.

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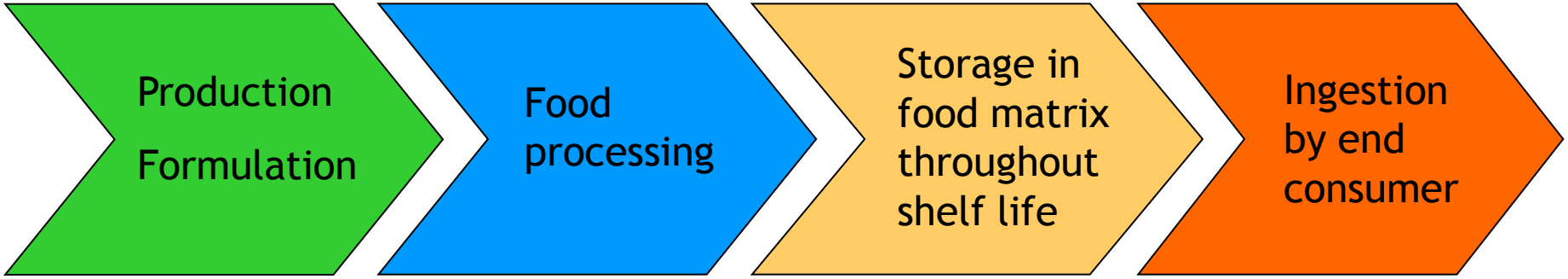
Maintaining probiotics in food

"Live micro organisms which when administered in adequate amounts confer a health benefit on the host"

FAO / WHO 2002

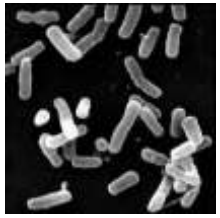
The life journey of a probiotic microorganism



Maintaining viability of probiotics until they reach their final destination in the human gut is key to a successful delivery in foods!

First of all, you need to choose the right strain…

- Chr. Hansen supplies probiotic strains with substantial clinical documentation to the food industry
- More than 450 research papers supporting Chr. Hansen probiotic strains
- More than 80 human clinical studies documenting strain efficacy



Bifidobacterium animalis ssp. *lactis*, BB-12®
Clinical documentation on gastro-intestinal benefits

Lactobacillus acidophilus, LA-5®
Clinical documentation on gastro-intestinal benefits

Lactobacillus paracasei ssp. *paracasei*, CRL-431™
Clinical documentation on immune support benefits

Strain production and formulation

DVS® (Direct Vat Set) concept invented by Chr. Hansen offers our customers consistency, control and convenience in production process

Products available in various forms:

Frozen

Freeze-dried granules

Freeze-dried powder

- ✓ Fermentable in an industrial scale and easy to concentrate
- ✓ Ability to survive freezing and drying
- ✓ Stability in frozen and/or freeze-dried form

Strain production and formulation

A possible way to affect subsequent survival

- Production
  - Adaptation strategies
  - Physiological state/growth phase
- Down-stream
  - Encapsulation
  - Entrapment

Food processing

It is importance to choose the right strain and starter culture

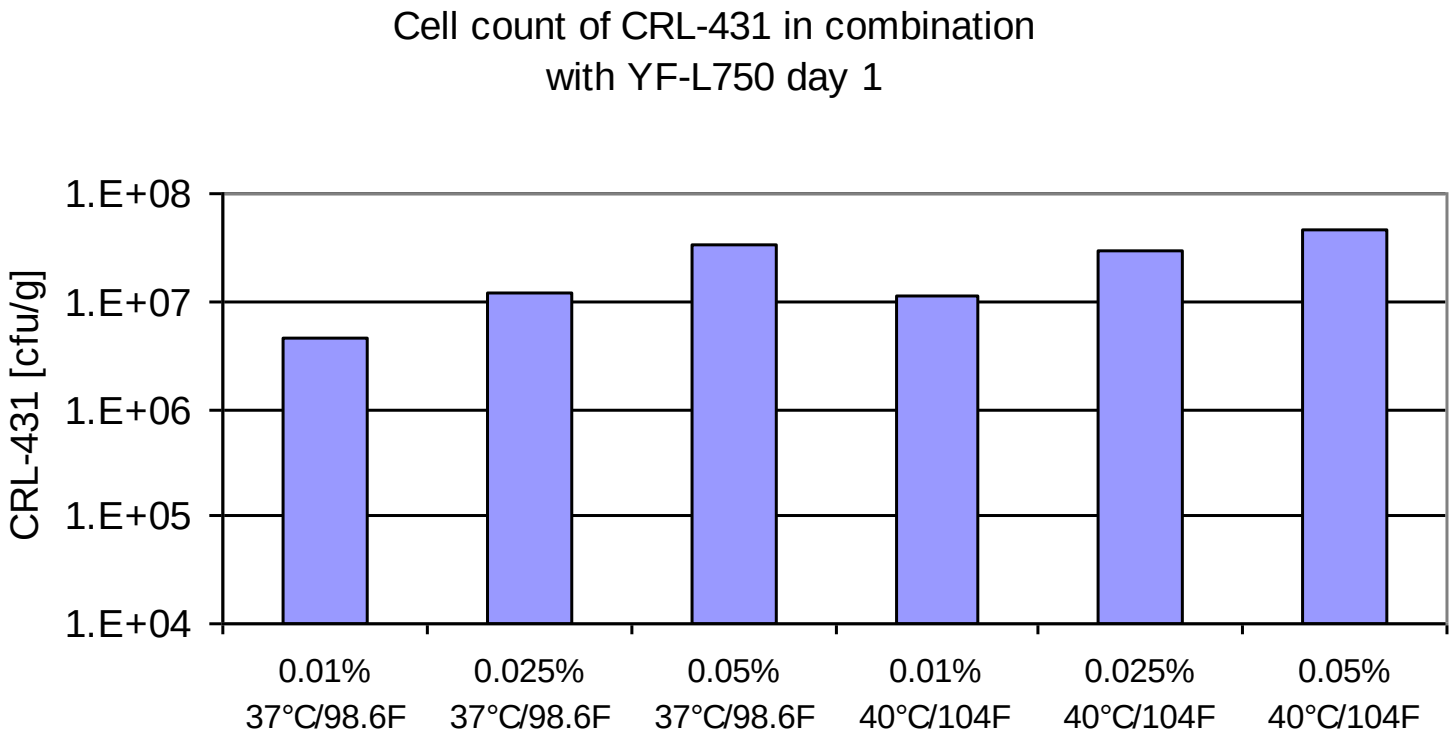
- A strain of *Streptococcus thermophilus* has the unique characteristic that it improves growth of Bifidobacteria
  - (WO2010/023290, Folkenberg, D.M. and Seimandi, C.)

- Specific *Lactococcus lactis* strains improve the growth of Bifidobacteria during fermentation of the milk → 10^8 CFU/g - while other strains negatively influence growth
  - (WO2010/023290, Folkenberg, D.M. and Seimandi, C.)

Yoghurt cultures having proteolytic or oxygen-scavenging properties have shown to be beneficial to Bifidobacteria

Food processing

Inoculation level and fermentation temperature influence cell count



However, dosage response is not always linear!

- Inhibitory food matrix
- Antagonistic activity, e.g. competitive exclusion by starter culture

Storage in food matrix throughout shelf life

Daily serving size and dosages are important

How to ensure correct dosage of probiotic until end of shelf life?

E.g. Bifidobacterium BB-12®: A billion a day keeps the doctor away...'

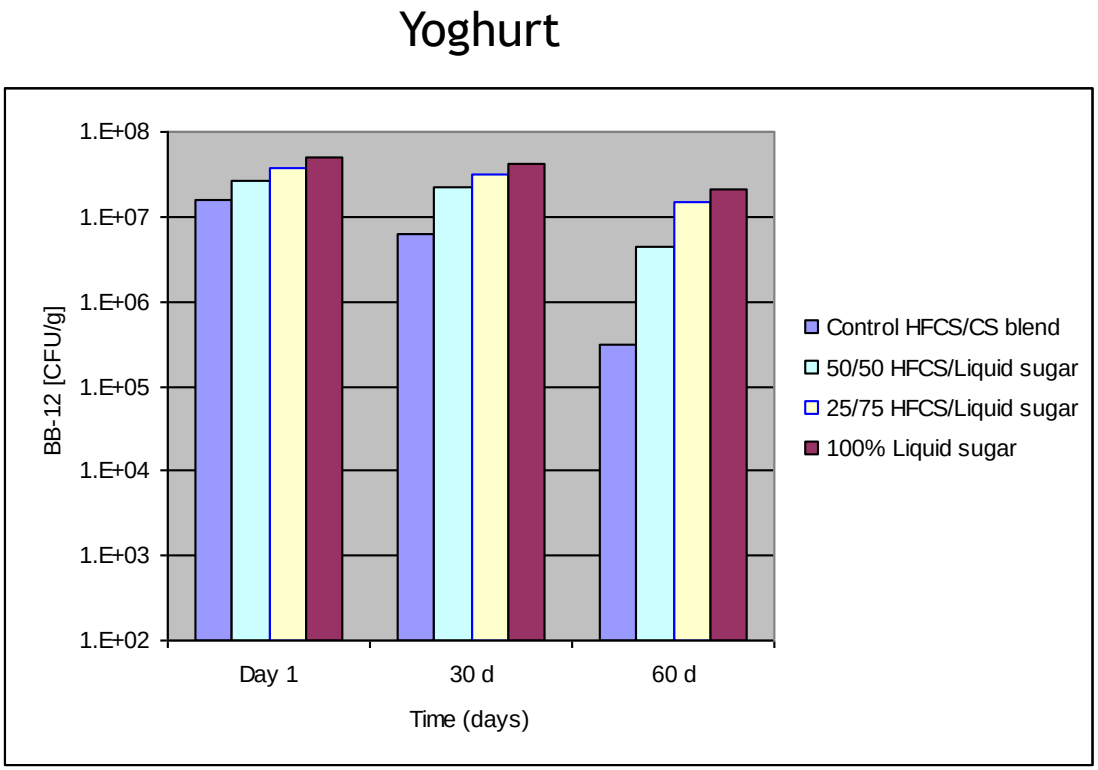
Example of serving size/daily dosage:

Yoghurt serving size	100 g
BB-12 cells required per serving*	10^9 cells
BB-12 cells required per gram* of yoghurt	10^7 cells

* at the end of shelf life.

Food matrix

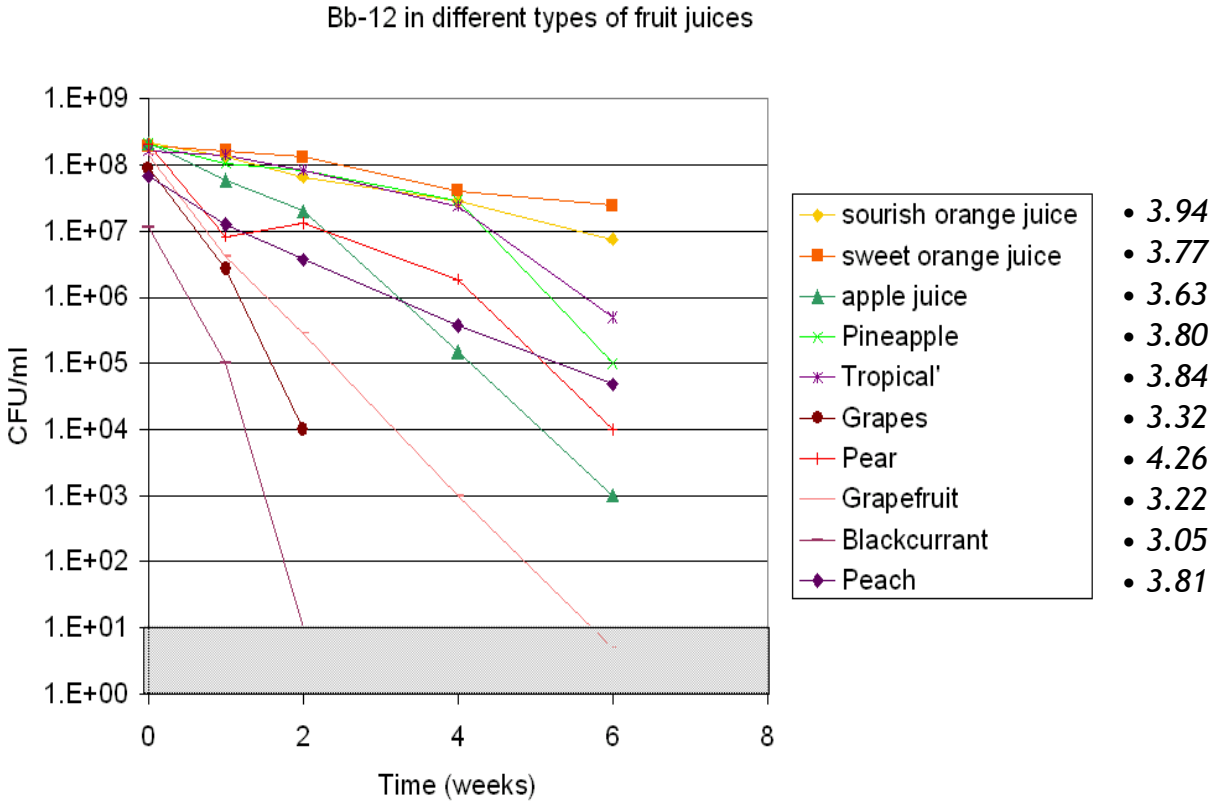
Sugar and protein types and concentrations influence survival



A combination of the direct effect of these ingredients on the probiotics and the indirect effect on the starter culture

Food matrix

Survival is negatively influenced by low pH



Survival seems to depend on pH

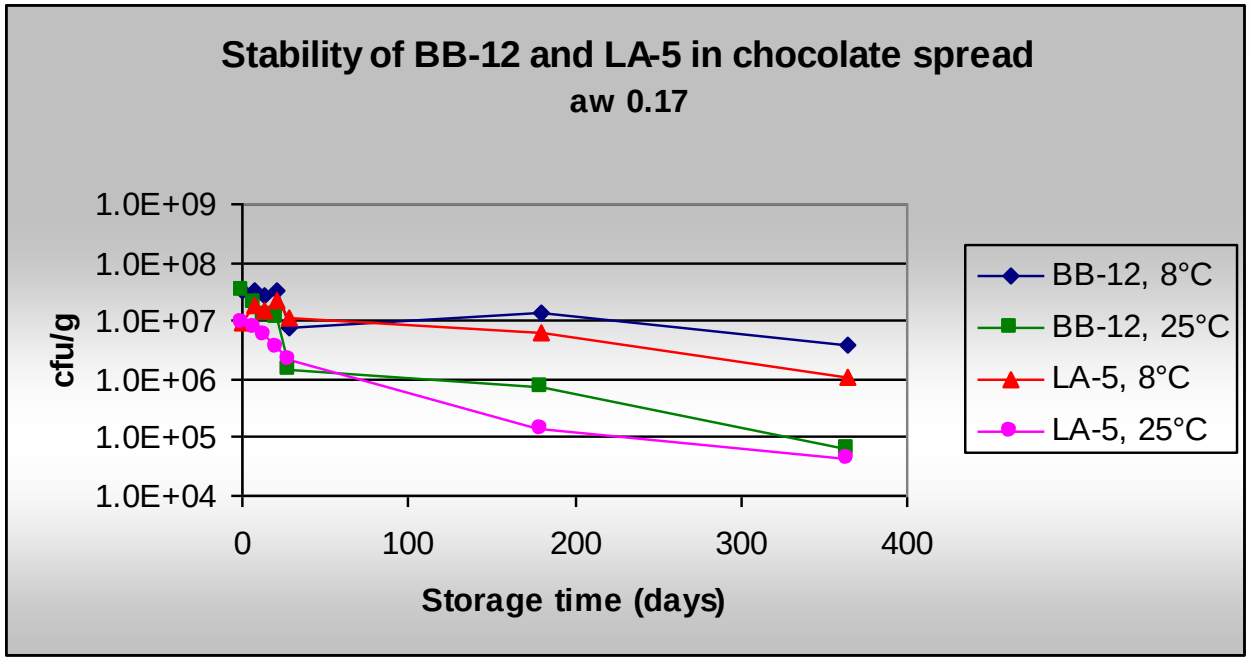
…. but also on the type of acids present, presence of antimicrobial substances and others!

Indication of correlation between degree of postacidification and survival of Bifidobacteria during cold-storage of yoghurt

Food matrix

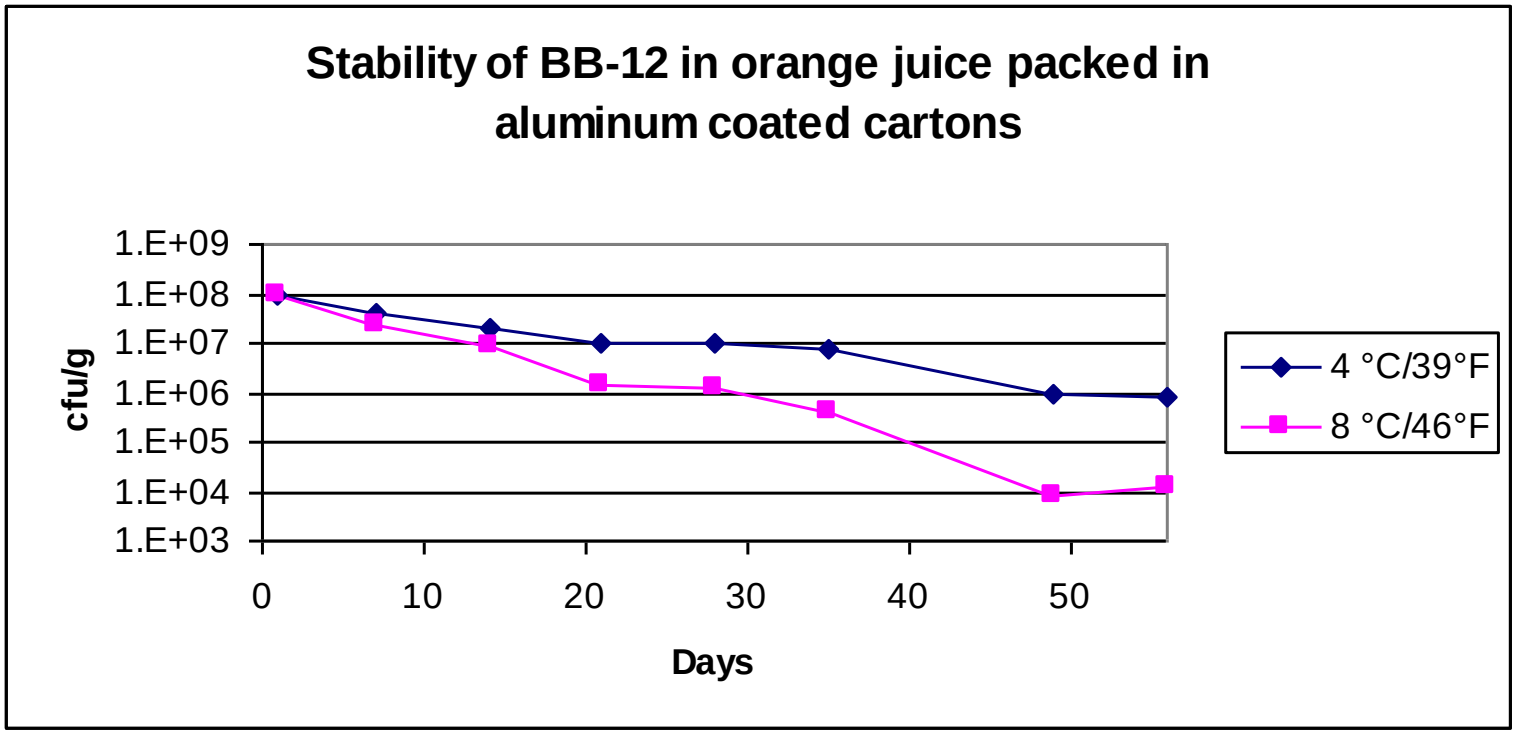
Low storage temperatures positively affect survival

8 C/46 F vs. 25 /77 F



Food matrix

Even slightly lower storage temperatures positively affect survival



Food matrix

Dissolved oxygen should preferably be kept at a minimum

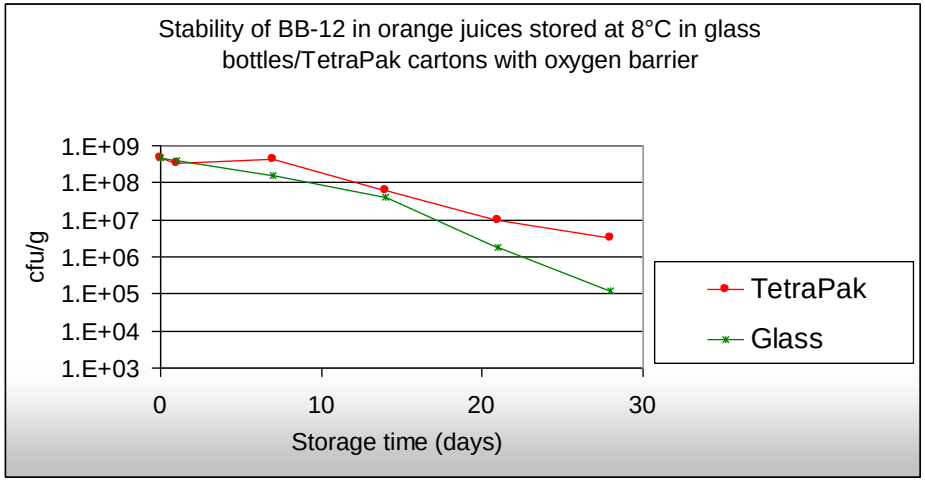
In general Lactobacilli are more oxygen tolerant than Bifidobacteria

O_2

↓ Heat treatment and de-aeration of the milk prior to fermentation

↑ Pumping and filling after fermentation

↑ Permeation through containers during **storage**



Food matrix

Other factors influence probiotic end cell counts

↑ Presence of cereals and cereal-components such as oat and wheat bran

↓ Preservatives, e.g. the use of K-sorbate in US

↓ Water activity (e.g. in dry products)

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Assessing probiotics in food

… and an example of how to evaluate the subsequent survival in GI-tract

Knowing that we have the right strain/(-s) in the right concentrations

Species identification (16S or 23S rRNA, DNA fingerprinting)

Genomics, the ultimate in strain characterization

Enables:

- Comparison of genomes of related strains and variants
- Prediction of full metabolic potential of strain
- Prove absence of undesirable genes
- Analysis of gene expression under various conditions
- Analysis of interactions between strains and compounds in the environment
- Analysis of interactions with host
- Design of primers for specific detection in complex mixtures

Garrigues et al. (2005). Australian J. Dairy Tech. 60: 84-92

Enumeration of probiotics by traditional plate counting

The most commonly used method for food products

Type of enumeration – different methods

- As single strains (optimal method)
- In mixed cultures (selective method)
- In low quality products (contaminated) (selective method)

IDF standard – IDF192

Enumeration of presumptive *Lb. acidophilus*

INTERNATIONAL STANDARD

ISO 20128

IDF 192

First edition 2006-05-15

Milk products — Enumeration of presumptive *Lactobacillus acidophilus* on a selective medium — Colony-count technique at 37 °C

Produits laitiers — Dénombrement de Lactobacillus acidophilus présomptifs sur un milieu sélectif — Technique de comptage des colonies à 37 °C

At Chr. Hansen we follow the IDF standard, slightly modified/adapted to our cultures

Use of antibiotics for selective count, depends on culture composition

- If product contains *L.casei* or *L.rhamnosus* use of both clindamycin and ciprofloxacin is necessary
- When it is known that fermented milk does not contain *L.casei* and *L.rhamnosus* only clindamycin is used

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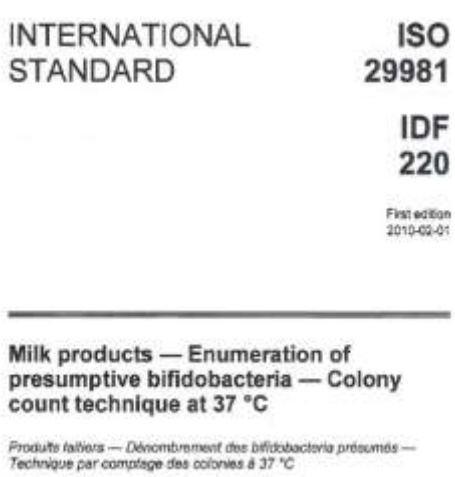
IDF standard – IDF220

Enumeration of Bifidobacteria



INTERNATIONAL STANDARD

ISO 29981

IDF 220

First edition
2010-02-01

Milk products — Enumeration of presumptive bifidobacteria — Colony count technique at 37 °C

Produits laitiers — Dénombrement des bifidobactéria présumés — Technique par comptage des colonies à 37 °C

At Chr. Hansen we follow the guidelines of the IDF standard, modified/adapted to our cultures

- Use of MRS with cystein hydrochloride instead of TOS – we have found that both media support growth of BB-12® equally well

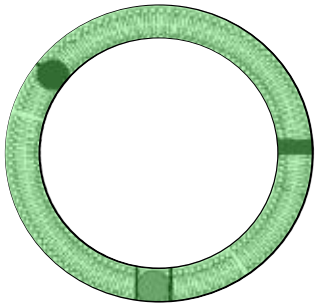
- Reduction in the amount of mupirocin used for inhibition of background culture – we have found it to be sufficient and it significantly reduces cost of analysis

Flow cytometry as an alternative cell count method

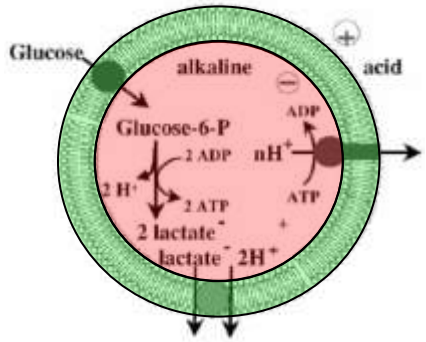
Allows detection of active cells having a membrane potential

DiOC2(3)
- staining and detection of cellular membrane potentials by flow cytometry

Passive (lipophilic) staining by $DiOC_2(3)$



Cellular membrane potential with red-shifted fluorescence



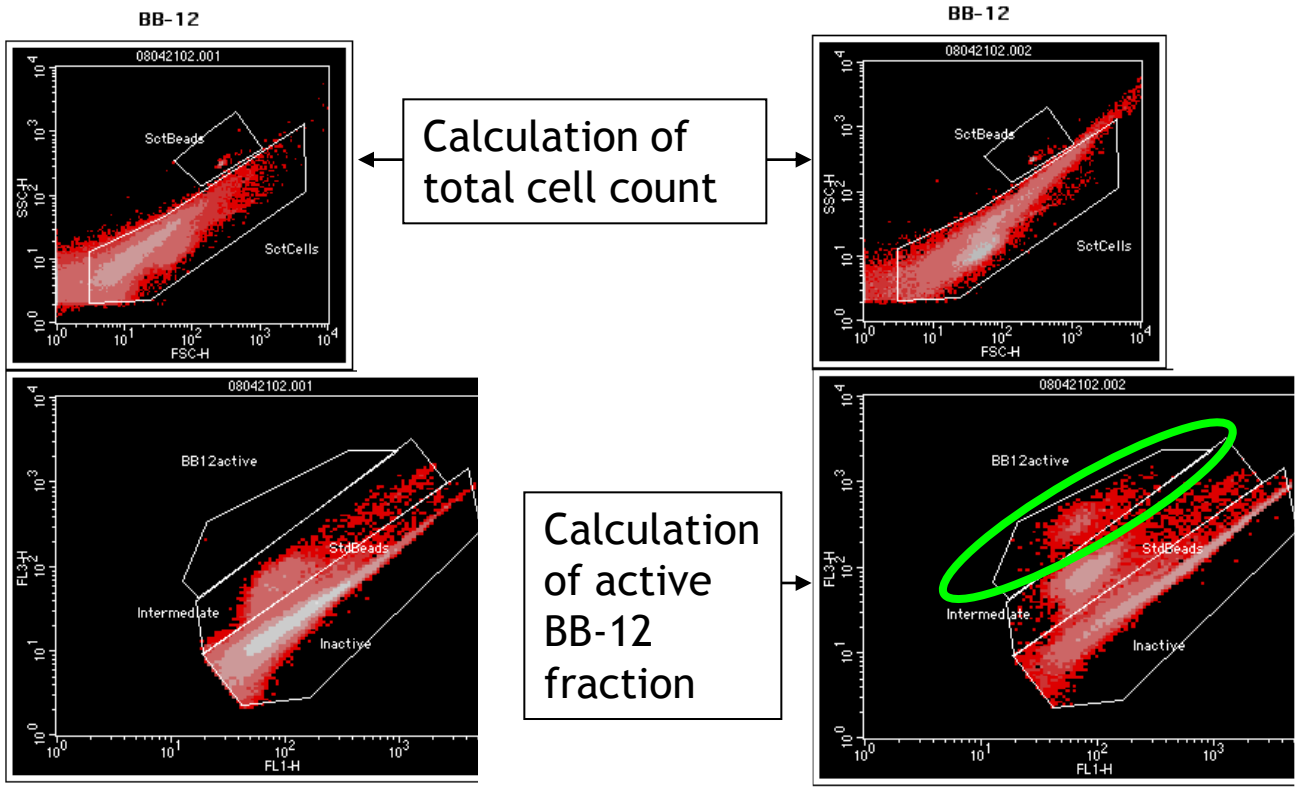
Active cells with membrane potential:
red-shifted fluorescence
(Shapiro 1990, ASM news 56:584)
(Novo et al. 1999, Cytometry 35:55)

Modified from:
Konings 2002, Antonie van Leeuwenhoek 82:3

BB-12® cell counts in yoghurt by flow cytometry

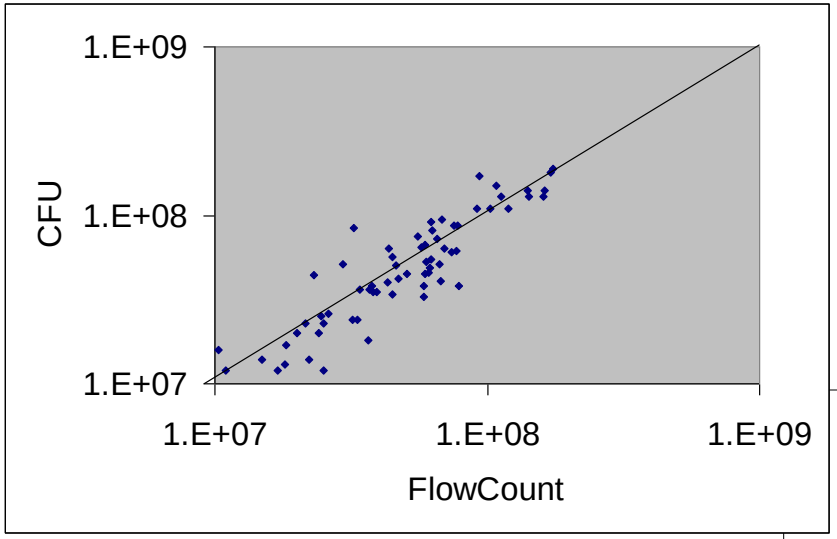
Yoghurt without BB-12

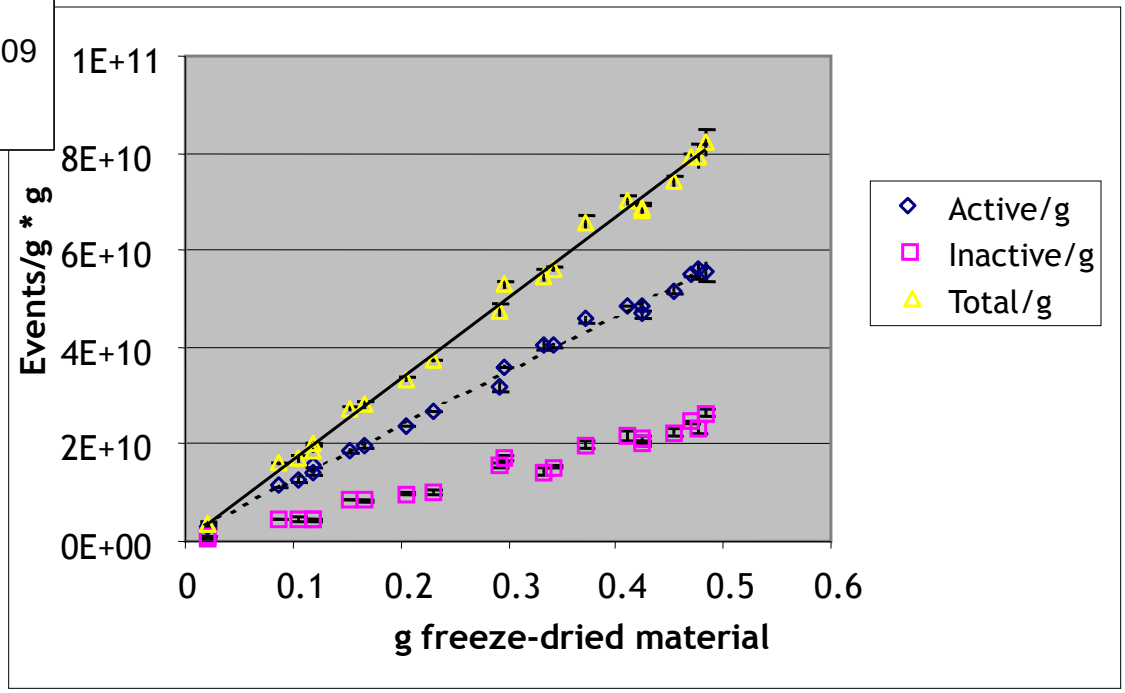
Same yoghurt spiked with 10^8 CFU/g BB-12



Validation of cell count analysis by flow cytometry

Good correlation to CFU measurements and linear dose-response





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Flow cytometry assay for high-through-put analyses

Enables analysis of 96 samples in less than 3½ hours

Step 1:
Rehydration of cells (10 – 30 min; 20 C)

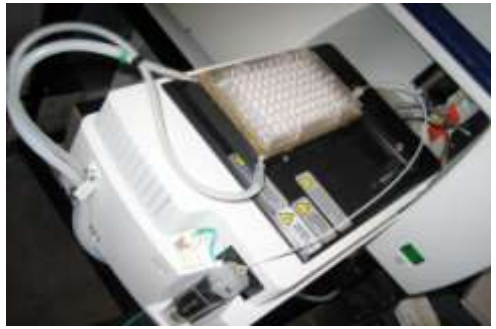
Setup for 96-well microplates
(eg. 48 tubes; assay duplication)

Step 2:
Activation in medium (30 min; 37-40 C)

Automatic assaying by robot (1 h including 30 min incubation)

Step 3:
Cell staining prior to flow cytometry
(30 – 60 min; 20 C)

FCM with autosampler
(96 analyses/25 min)



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An example of how to evaluate subsequent survival potential in GI-tract

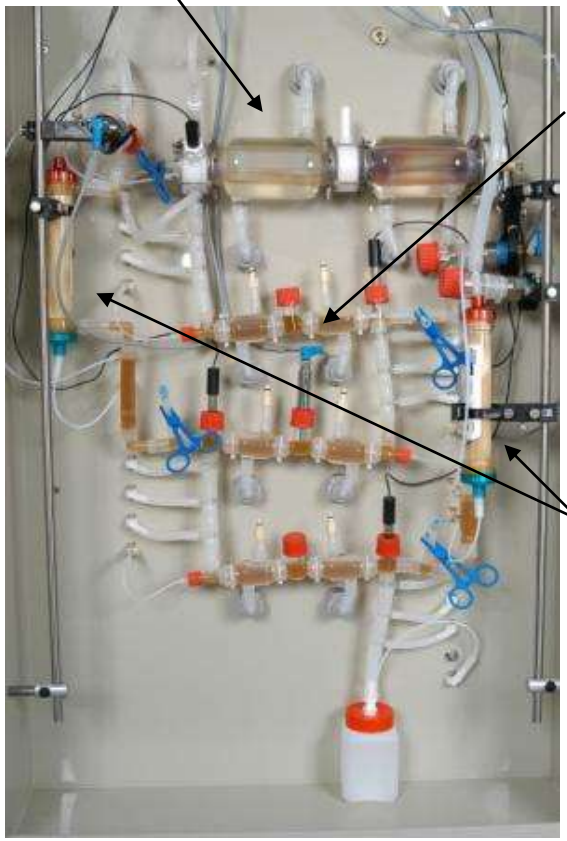
Computer controlled dynamic gastrointestinal model

TIM-1 from TNO

- Simulates the conditions of the human stomach and the small intestine
- Appropriate pH in different GI-compartments
- Sequential input of enzymes, co-factors and bile salts in physiological amounts
- Appropriate mixing at each stage of digestion
- Physiological transit times for each step of digestion
- Removal of the products of digestion

Gastric secretion

1M HCl
Pepsin
Lipase



Jejunum/ileum secretion

Electrolytes
1 M $NaHCO_3$
Pancreatic juice
Bile

Hollow fiber membranes

Cutoff = 5800 Da

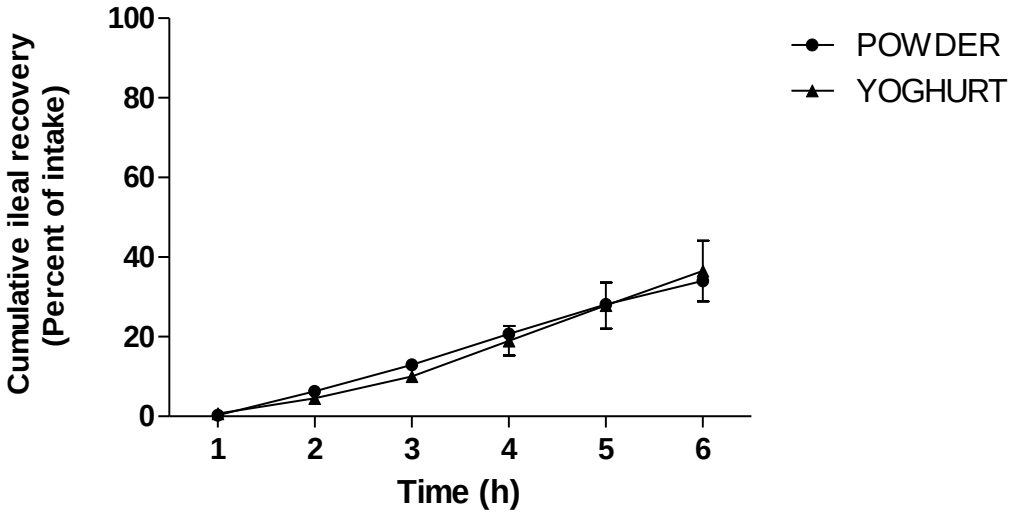
Stability of probiotics in the upper GI tract

TIM enables examination of survival potential for probiotics in humans

TIM-1 is used for product development and R&D to study:

- Resistance to gastric transit
  - Matrix
  - Fermentation and formulation
  - Down-stream processing
  - Multi-strain products
- Characterization of strains
- Probiotics activity in the GI-tract

Yoghurt vs. powder



Take home messages

- The market for probiotics is dominated by fermented milk products but expanding to new non-dairy applications and there is increased attention from regulatory side.

- Maintaining probiotics in food depends on many different conditions during culture production and formulation, food processing and food storage – right strategies must be choosen!

- Assessment of probiotics in food can be based on selective or non-selective traditional plate count methods or high through-put methods such as flow cytometry.

Acknowledgement

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Thank you for your attention!