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
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

<table>
<thead>
<tr>
<th>Year</th>
<th>Number of new products</th>
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<tr>
<td>1998</td>
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<td>2009</td>
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Source: Mintel, GNPD 2010
Includes all new products launched worldwide, excludes extensions and repackaged products
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

18 new launches all in dairy

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.
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

Production  
Formulation  
Food processing  
Storage in food matrix throughout shelf life  
Ingestion by end consumer

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
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:

<p>| | |</p>
<table>
<thead>
<tr>
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<tbody>
<tr>
<td><strong>Yoghurt serving size</strong></td>
<td>100 g</td>
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<tr>
<td><strong>BB-12 cells required per serving</strong></td>
<td>$10^9$ cells</td>
</tr>
<tr>
<td><strong>BB-12 cells required per gram of yoghurt</strong></td>
<td>$10^7$ cells</td>
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* 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°C/77°F

Stability of BB-12 and LA-5 in chocolate spread
aw 0.17

cfu/g

Storage time (days)
Food matrix
Even slightly lower storage temperatures positively affect survival

Stability of BB-12 in orange juice packed in aluminum coated cartons

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Food matrix
Dissolved oxygen should preferably be kept at a minimum

In general Lactobacilli are more oxygen tolerant than Bifidobacteria

\[ \text{O}_2 \]

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

↑ Pumping and filling after fermentation

↑ Permeation through containers during storage

Stability of BB-12 in orange juices stored at 8°C in glass bottles/TetraPak cartons with oxygen barrier
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)
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

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)
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.
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 DiOC2(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

Calculation of total cell count

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

Calculation of active BB-12 fraction
Validation of cell count analysis by flow cytometry
Good correlation to CFU measurements and linear dose-response
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)
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
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 chosen!

- Assessment of probiotics in food can be based on selective or non-selective traditional plate count methods or high throughput methods such as flow cytometry.
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Thank you for your attention!