Probiotics
Myth or Reality?

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Athens - Greece
1. Introduction

2. The Probiotics in Athens

*In vitro* and *in vivo* inhibition of *Helicobacter pylori* by *Lactobacillus casei* strain Shirota

*Lactobacillus fermentum* ACA-DC 179 displays probiotic potential *in vitro* and protects against TNBS induced colitis and *Salmonella* infection in murine models

Feed supplementation of *Lactobacillus plantarum* PCA 236 modulates gut microbiota and milk fatty acid composition in dairy goats

Lactic acid bacteria efficiently protect human and animal intestinal epithelial and immune cells from enteric virus infection

3. Conclusions
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3. Conclusions
Health is the Greatest Wealth

➢ In Europe,
  patterns of life and thus the concepts of food and nutrition
  have changed significantly the past 55-60 years,
  because of economic, social and technological changes

➢ From a former emphasis on
  - survival
  - food safety

➢ Food sciences now aim at
  - developing foods to promote well-being and health
  - reducing the risk of major chronic & degenerative diseases
    (e.g. cancer, cardiovascular diseases, obesity, GI tract disorders)
Health is the Greatest Wealth

 ➢ Scientific evidence supports the hypothesis that,
   by modulating specific target functions in the body,
   diet can have beneficial physiological and psychological effects
   that go beyond basic nutritional effects

 ➢ “Let your food be your medicine and your medicine your food”
   Hippokrates of Kos (ca. 460 - 370 BC)
Functional Foods

- Development and growth
- Regulation of energy balance and body weight
- Physical performance and fitness
- Mental performance
- Cardiovascular function
- Intestinal function and the gut microbiota
Human gut flora

> $10^{14}$ cells and up to 1000 different species

- Pseudomonas aeruginosa
- Proteus
- Staphylococci
- Clostridia

**Beneficial**

- Enterobacteriaceae
- Escherichia coli

- Lactobacilli
- Eubacteria
- Bifidobacteria

**Pathogens**

- Bacteriodes

$10^2$

$10^{12}$
The Probiotics

“Bifidobacteria for a balanced and healthy intestinal flora”

Henry Tissier, 20th Century

“Consumption of lactobacilli for longevity without ageing”

Elie Metchnikoff, 20th Century

“Live microorganisms that, when consumed in an adequate amount as part of a food, confer a health benefit on the host”

FAO / WHO Experts, 2001
The Modes of Action

- **Inhibition of adherence**
  - Pathologic
  - Non-pathologic

- **Antibacterial substances**

- **Enhanced sIgA**
  - Without Probiotics
  - With Probiotics

IgA-producing Paneth cells Non-pathogenic flora IgA
The Benefits

✔ Alleviation or prevention of diverse GI tract disorders
✔ Management of food allergies
✔ Cholesterol lowering effect
✔ Anti-tumor activity

In vitro – Ex vivo
Animal trials
Clinical trials
The Rules

FAO / WHO Expert Group, Argentina 2001

- Strain identification

  - Functional characterization
    - (in vitro & in vivo)

  - Safety assessment
    - (in vitro & in vivo)

- Clinical trials
  - Phases I, II, III, IV

- Probiotic food / supplement

- Labeling of probiotic food / supplement
Outline

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   by *Lactobacillus casei* strain Shirota

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   Feed supplementation of *Lactobacillus plantarum* PCA 236 modulates gut microbiota and milk fatty acid composition in dairy goats

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Agricultural University of Athens
(est. 1920)

Crop Sciences
Animal Sciences
Agricultural Biotechnology
Food Science and Technology
Natural Resources Management & Agricultural Engineering
Rural Economics & Development
General Sciences

Laboratory of Dairy Research
The Projects

**Greek General Secretariat for Research and Technology**

*EPET II (1999-2001)*

Development of a probiotic type of yogurt

**European Commission**

*PROPATH – FP5-01179 (2002-2005)*

Molecular Analysis and Mechanistic Elucidation of the Functionality of Probiotics and Prebiotics in the Inhibition of Pathogenic Microorganisms to Combat Gastrointestinal Disorders and to Improve Human Health

**European Commission**

*PATHOGENCOMBAT - FP6-007081 (2005-2010)*

Development of biotechnological tools for risk assessment at intra- and inter-cellular level throughout the food chain

**Greek Ministry of Health**

*MH-43 (2009-2911)*

Bacteriocins active towards microorganisms involved in periodontitis. Laboratory and clinical study
PhD Theses

Petros Maragkoudakis (2004)
Probiotic and technological potential of lactic acid bacteria.
In vitro and in vivo impact on Helicobacter pylori

Scientific Project Officer
European Commission
Joint Research Centre (JRC)
Institute for Health and Consumer Protection (IHCP)
Systems Toxicology Unit
Ispra, Italy

Georgia Zoumpopoulou (2008)
Probiotic properties of lactic acid bacteria.
In vitro and in vivo study of their antimicrobial and immunoregulatory activity

Post-doc
Laboratory of Dairy Research
Agricultural University of Athens
Athens, Greece
In vitro and in vivo inhibition of Helicobacter pylori

by Lactobacillus casei strain Shirota
**In vivo H. pylori inhibition – An animal trial**

*H. pylori* SS1 infection established

*L. casei* Shirota daily supplemented at 10^8 CFU/ml in animals’ drinking water

5 animals per time point, per group

Animals sacrificed after 1, 2, 3, 6 & 9 months

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<table>
<thead>
<tr>
<th><strong>H. pylori SS1</strong></th>
<th><strong>L. casei Shirota</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>In the stomach</td>
<td>In the intestine and faeces</td>
</tr>
<tr>
<td>Urease test</td>
<td>API</td>
</tr>
<tr>
<td>Species-specific PCR</td>
<td>Species-specific PCR</td>
</tr>
<tr>
<td>Histopathology</td>
<td>RAPD-PCR</td>
</tr>
</tbody>
</table>
Decrease of *H. pylori* colonization in gastric mucosa

Control group
*H. pylori* SS1

Study group
*H. pylori* - *L. casei* Shirota
Histopathologic evaluation of antral gastric samples

H. pylori SS1 group
Giemsa stain

H. pylori SS1 group
H & E stain

Arrows:
H. pylori in the gastric pits

Normal mucosa

L. casei Shirota group
H & E stain

L. casei Shirota - H. pylori SS1 group
H & E stain

Arrows:
- Inflammation
- Intraluminal abscesses

Arrows:
Absence of gastritis
Decrease of chronic gastritis in the antrum

(a) control group

H. pylori SS1

(b) study group

H. pylori - L. casei Shirota
Decrease of active gastritis in the antrum

(a) control group
H. pylori SS1

(b) study group
H. pylori - L. casei Shirota
Anti-IgG *H. pylori* antibodies in mice

**Graphical Representation:**

- **H. pylori SS1 group** (●; control group)
- **H. pylori - L. casei Shirota group** (close triangles; study group)
- **L. casei Shirota group** (open triangles; control group)

**Legend:**

- O.D. @ 492 nm
- Months post-infection

**Key Points:**

- The graph shows a comparison of antibody levels over time in different groups.
- The *H. pylori SS1* group (●) is shown in the control group.
- The **H. pylori - L. casei Shirota** group (close triangles) is the study group.
- The **L. casei Shirota** group (open triangles) is the control group.

**Statistical Notations:**

- NS: Not Significant
- p=0.037
Conclusions

- Significant reduction in the levels of *H. pylori* colonization in the *L. casei* Shirota treated study group

- Significant decline in the associated chronic and active gastric mucosal inflammation

- A trend towards a decrease in the anti-*H. pylori* IgG response
Lactobacillus fermentum ACA-DC 179 displays probiotic potential in vitro and protects against TNBS induced colitis and Salmonella infection in murine models.
**L. fermentum ACA-DC 179** and **S. macedonicus ACA-DC 198** inhibit *in vitro* pathogenic streptococci

<table>
<thead>
<tr>
<th>Target strain</th>
<th>Inhibition (diameter, mm±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>L. fermentum ACA-DC 179</td>
<td></td>
</tr>
</tbody>
</table>
| S. agalactiae LMG 14694T | - | 7.7±0.58  
| S. anginosus LMG 14502T | - | 8.7±1.53  
| S. bovis LMG 8518T | - | 7.7±0.58  
| S. equinus LMG 14897T | - | 10.0±1.00  
| S. gordonii LMG 14518T | - | 10.0±0.00  
| S. mutans LMG 14558T | - |  
| S. oralis LMG 14532T | 7.0±0.00 | 9.3±1.15  
| S. pneumoniae LMG 14545T | 7.0±0.00 | 12.7±0.58  
| S. pyogenes LMG 21599T | - | 13.0±1.00  
| S. salivarius LMG 11489T | - | 5.7±1.15  
| S. sobrinus LMG 14641T | - |  
| S. thermophilus LMG 6896T | - | 14.7±1.53  
| S. thermophilus ACA-DC 27 | 14.0±0.58 | 16.0±1.15  
| S. thermophilus ACA-DC 40 | 11.0±1.00 | 16.0±1.00  
| S. thermophilus ACA-DC 1502a | 12.0±1.00 | 16.0±0.58  | 25  

L. fermentum ACA-DC 179 and S. macedonicus ACA-DC 198 inhibit *in vitro* pathogenic streptococci.
L. fermentum ACA-DC 179 inhibits in vitro Salmonella typhimurium SL 1344
**L. fermentum ACA-DC 179 induces the secretion of anti-inflammatory cytokines in human PBMCs**

<table>
<thead>
<tr>
<th>Strain</th>
<th>pg mL⁻¹± SEM</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-10</td>
<td>IL-12</td>
<td>TNF-α</td>
<td>IFN-γ</td>
<td>IL-10/IL-12</td>
</tr>
<tr>
<td>Unstimulated control</td>
<td>13±1</td>
<td>9±2</td>
<td>1±0</td>
<td>3±2</td>
<td>1±0</td>
</tr>
<tr>
<td><em>L. fermentum</em> ACA-DC 179</td>
<td>2815±1018</td>
<td>10±3</td>
<td>3579±1467</td>
<td>115±75</td>
<td>141±51</td>
</tr>
<tr>
<td><em>L. plantarum</em> ACA-DC 287</td>
<td>1498±430</td>
<td>153±51</td>
<td>19307±7466</td>
<td>28694±18439</td>
<td>25±10</td>
</tr>
<tr>
<td><em>S. macedonicus</em> ACA-DC 198</td>
<td>777±153</td>
<td>337±67</td>
<td>36721±11680</td>
<td>37684±13569</td>
<td>4±1</td>
</tr>
</tbody>
</table>
Lactobacillus fermentum ACA-DC 179 protects against TNBS induced colitis in murine model

Day 1-5 Intragastric administration of
- L. fermentum ACA-DC 179
- S. macedonicus ACA-DC 198
once daily (10⁸ cfu/animal)

Day 5 Intrarectal administration
of TNBS = Acute colitis

Day 7 Mice sacrificed
Wallace inflammation scores
Lactobacillus fermentum ACA-DC 179 protects against Salmonella infection in murine model

Day 1-5  Intragastric administration of  
L. fermentum ACA-DC 179 (■)  
L. plantarum ACA-DC 287 (◆)  
once daily (10^8 cfu/animal)

Day 5  Intragastric gavage of  
S. typhimurium C5  
(once, 5×10^4 cfu per mouse)

Week 3  Mortality recorded

Animals not treated with lactobacilli (▲)
Conclusions

*Lactobacillus fermentum* ACA-DC 179

- Active against *S. oralis & S. pneumoniae*, *Salmonella*
- Induces elevated levels of the anti-inflammatory IL-10 in PBMCs
- Reduces significantly TNBS-induced colitis in mouse model
- Successful in an experimental *Salmonella*-infection mouse model
Feed supplementation of *Lactobacillus plantarum* PCA 236 modulates gut microbiota and milk fatty acid composition in dairy goats.
Animal feeding trial

- Dairy goat farm - Nicosia, Cyprus, May to June 2008
- 24 female goats - Damascus breed
- Animals within their lactation period
- Control (n=12) & Probiotic (n=12) groups housed and milked separately
- Standard nutritional scheme during the morning and evening milking
- *L. plantarum* PCA 236 (isolated from Kasseri cheese) as lyophilised powder
- 4 g daily (12 log CFU/goat) during the morning and evening milking time

Analysis on Day 0, 7, 14, 21 and 35

- Microbiological analysis of faeces
- Follow up of *L. plantarum* PCA 236 in faeces
- Milk composition analysis
- Milk fatty acid content
- Milk antioxidant capacity
- Blood antioxidant capacity
- Immunoglobulins IgA, IgM and IgG in goat plasma
**Animal feeding trial**

- No adverse effects observed in the animals receiving *L. plantarum* PCA 236

- *L. plantarum* PCA 236 recovered in faeces of all animals in the study group

- Better feed digestion in the study group

- No differences in
  - milk antioxidant capacity
  - blood antioxidant capacity and
  - immunoglobulins IgA, IgM and IgG in goat plasma
**Lactobacillus plantarum PCA 236**
modulates gut microbiota in dairy goats

- **Total aerobic flora**
- **Clostridia**
- **Streptococci**
- **LAB and L. plantarum PCA236 (●)**

(□) Control treatment
(●) Feed supplementation with *L. plantarum* PCA236
**Lactobacillus plantarum PCA 236**
modulates milk fatty acid composition in dairy goats

White bars: Control treatment
Grey bars: Feed supplementation with *L. plantarum* PCA236

Polyunsaturated

Vaccenic acid (C18:1 t11)

Rumenic acid (C18:2 c9-t11)

Linolenic (C18:3 n3)

Linoleic acid (C18:2 n6)
Conclusions

Feed supplementation with *Lactobacillus plantarum* PCA 236

- modulates gut microbiota
- modulates milk fatty acid composition

in dairy goats
Lactic acid bacteria efficiently protect human and animal intestinal epithelial and immune cells from enteric virus infection
### Lactic Acid Bacteria

Lactic acid bacteria strains used in this study.

<table>
<thead>
<tr>
<th>Designation</th>
<th>Species</th>
<th>Origin</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>ACA-DC 146</td>
<td><em>L. plantarum</em></td>
<td>Feta cheese</td>
<td>Maragkoudakis et al. (2006)</td>
</tr>
<tr>
<td>ACA-DC 179</td>
<td><em>L. fermentum</em></td>
<td>Kasseri cheese</td>
<td>Zoumpopoulou et al. (2008)</td>
</tr>
<tr>
<td>ACA-DC 4037</td>
<td><em>L. paracasei</em></td>
<td>Kasseri cheese</td>
<td>Maragkoudakis et al. (2006)</td>
</tr>
<tr>
<td></td>
<td>tolerans</td>
<td></td>
<td>Franz et al. (1999)</td>
</tr>
<tr>
<td>BFE 900</td>
<td><em>E. faecium</em></td>
<td>Bean sprouts</td>
<td>Yousif et al. (2005)</td>
</tr>
<tr>
<td>BFE 2207</td>
<td><em>E. faecium</em></td>
<td>Fermented food (Sudan)</td>
<td>Patrignani et al. (2006)</td>
</tr>
<tr>
<td>BFE 5092</td>
<td><em>L. plantarum</em></td>
<td>Fermented milk (Kenya)</td>
<td>Nissen et al. (2009)</td>
</tr>
<tr>
<td>PCA 142</td>
<td><em>L. fermentum</em></td>
<td>Kasseri cheese</td>
<td>Nissen et al. (2009)</td>
</tr>
<tr>
<td>PCA 185</td>
<td><em>L. gasseri</em></td>
<td>Feta cheese</td>
<td>Nissen et al. (2009)</td>
</tr>
<tr>
<td>PCA 227</td>
<td><em>L. pentosus</em></td>
<td>Unknown</td>
<td>Unpublished</td>
</tr>
<tr>
<td>PCA 236</td>
<td><em>L. plantarum</em></td>
<td>Kasseri cheese</td>
<td>PathogenCombat data</td>
</tr>
<tr>
<td>PCD 71</td>
<td><em>Ent. faecium</em></td>
<td>Sausage</td>
<td>PathogenCombat data</td>
</tr>
<tr>
<td>PCS 20</td>
<td><em>L. plantarum</em></td>
<td>Slovenian cheese</td>
<td>Nissen et al. (2009)</td>
</tr>
<tr>
<td>PCS 22</td>
<td><em>L. plantarum</em></td>
<td>Slovenian cheese</td>
<td>Schillinger and Villarreal (2010)</td>
</tr>
<tr>
<td>PCS 25</td>
<td><em>L. plantarum</em></td>
<td>Slovenian cheese</td>
<td>Nissen et al. (2009)</td>
</tr>
<tr>
<td>PCS 26</td>
<td><em>L. plantarum</em></td>
<td>Slovenian cheese</td>
<td>Nissen et al. (2009)</td>
</tr>
<tr>
<td>LcS</td>
<td><em>Lb. casei</em> Shirota</td>
<td>Human</td>
<td>Known probiotic</td>
</tr>
<tr>
<td>LGG</td>
<td><em>L. rhamnosus</em> GG</td>
<td>Human</td>
<td>Known probiotic</td>
</tr>
</tbody>
</table>

### Cell Lines

Intestinal and monocyte/macrophage derived cell lines used in this study.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Type</th>
<th>Organism</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>H4</td>
<td>Epithelial</td>
<td>Human</td>
<td>University of Maribor</td>
</tr>
<tr>
<td>TLT</td>
<td>Monocyte/macrophage</td>
<td>Human</td>
<td>University of Maribor</td>
</tr>
<tr>
<td>PoM2</td>
<td>Monocyte/macrophage</td>
<td>Pig</td>
<td>University of Maribor</td>
</tr>
<tr>
<td>CLAB</td>
<td>Enterocyte like</td>
<td>Pig</td>
<td>University of Maribor</td>
</tr>
<tr>
<td>PSI</td>
<td>Epithelial</td>
<td>Pig</td>
<td>University of Maribor</td>
</tr>
<tr>
<td>GIE</td>
<td>Epithelial</td>
<td>Goat</td>
<td>University of Maribor</td>
</tr>
</tbody>
</table>

### Viruses

**RV Rotavirus**

**TGEV Transmissible Gastroenteritis Virus**
Protective effect of LAB on intestinal CLAB porcine cell line challenged with rotavirus (RV)

Results presented as % of monolayer integrity compared to a healthy growing cell line

- □ Monolayer challenged with RV
- □ Monolayer challenged only with LAB strains
- □ Monolayer pre-incubated with selected LAB strains and then challenged with RV
Protective effect of LAB on intestinal and macrophage cell lines challenged with RV and TGEV

Results presented as % of monolayer integrity compared to a healthy growing cell line

**GIE**: epithelial goat; **H4**: epithelial human; **PSI**: epithelial pig; **TLT**: macrophage human

![Graph](image)
Conclusions

- Strains with moderate to complete monolayer protection against viral RV or TGEV disruption

- Possible mechanisms:
  - ability of the LAB strains to adhere to the cell line monolayer?
  - ability of the LAB strains to induce the release of NO & H$_2$O$_2$ (ROS)?

- Good indications on the nature of the antiviral effect were evident only for
  - *L. casei* Shirota
    against TGEV
    on the human macrophage cell line TLT
  - *L. plantarum* PCA 236
    against both RV and TGEV
    on the goat epithelial cell line GIE
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3. Conclusions
The Conclusions
What is a nutrient? Defining the Food – Drug Continuum
Rowe, 1999

2000 BC
Here, eat this root

850 AD
That root is heathen; here, say this prayer

2000 AD
That antibiotic doesn’t work anymore; here, eat this root

1000 AD
That prayer is superstition; here, drink this potion

Atermon Cochlea
(Archimedes Screw)

1940 AD
That potion is snake oil; here, shallow this pill

1985 AD
That pill is ineffective; here, take this antibiotic

2000 AD
That antibiotic doesn’t work anymore; here, eat this root
The Conclusions

Industrialized World

In the past
An aging population accepted
- getting older
- passing the baton to the next generation

Today
The aging generation (baby boomers)
- “forever-young”
- less willing to give in to the aging process

Global Food Security Crisis
(WHO, 2009)
- 3.5 million people die annually from malnutrition
- 21 countries with high levels of acute and chronic undernutrition
- Poor people spend over half their disposable income on food, depleting resources for health
The Collaborations

**Dionyssis Sgouras & Andreas Mentis**  
Laboratory of Medical Microbiology  
Hellenic Pasteur Institute, Athens, Greece

**Katerina Petraki**  
Laboratory of Pathology  
Hippocration Hospital, Athens, Greece

**Corinne Grangette & Bruno Pot**  
Laboratory of LAB and Mucosal Immunity  
Institute Pasteur of Lille, Lille, France

**Kostas Mountzouris**  
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Agricultural University of Athens, Athens, Greece

**Andreas Hadjipetrou & Giorgos Theofanous**  
Pittas Dairy Industries Ltd, Nicosia, Cyprus

**Avrelija Cencic**  
Faculty of Agriculture & Medical Faculty  
University of Maribor, Maribor, Slovenia
Thank you for your attention!

Ευχαριστώ!