Synbiotic 2000 - preclinical documentation

Selection procedures.

355 *Lactobacillus* strains were isolated from the colonic mucosa of healthy individuals and

Another 180 from ecologically cultured rye. They were all screened for

- binding of porcine
- mucin,
- expression of cell surface hydrophobicity
- binding of collagen,
- fibronectin and
- other extracellular matrix proteins.

The ability to survive in an environment similar to that of upper GI tract - e.g. the time required for transport through the gastrointestinal (GI) tract to the colon was also studied:

- Survival when exposed to a pH of 2.5 for 2 hrs.
- Survival when exposed to 20% bile for 1 hr

Seven strains met the criteria and were selected for further studies: *Pediococcus pentosaceus* 16:1, *Leuconostoc mesenteroides* 77:1, *Lactobacillus paracasei* F19, *Lactobacillus paracasei* 50:1, *Lactobacillus paracasei* 50:1, *Lactobacillus plantarum* F5, *Lactobacillus plantarum* F26

Fig 1. shows the data for each of the seven strains.

Properties of selected LAB strains

Strain designation	pH tolerance	Bacteriocin	Hydrophobicity SAT	Mucin binding	β-galactosidase
P. pentosaceus 16:1	2.5	1:8	<0.1	1.05	+
Leu.mesenteroides 77:1	2.5	1:4	<0.1	1.27	+
L. paracasei F19	2.5	1:32	0.1	0.52	-
L. plantarum 2592	2.0 + pepsin	1:16	1	0.71	-
L. paracasei 50:1	2.5	1:16	2	0.91	-
L. plantarum F5	2.0 + pepsin	1:32	1	0.56	+
L. plantarum F26	2.0 + pepsin	1:16	1	0.58	-

Fig 1.

Characteristics of the chosen seven strains.

All seven strains chosen demonstrated in vitro ability

- to utilize inulin or amylopectin as the only carbon source
- produce β-galactosidase, - important to alleviate symptoms of lactose intolerance.
- produce antimicrobial substances with activity against gram-positive, some strains also against gram-negative microorganisms.
- activity against *Candida* strains and the gastric pathogen *Helicobacter pylori.*

Four of the studied seven strains were, based on information from these studies selected for a planned synbiotic composition aimed to consist of lactic acid bacteria and four bioactive fibres- the composition was given the name of Synbiotic 2000,

Ability to control inflammation

The ability to activate pro- and anti-inflammatory was separately studied – the effects are summarized in fig 2.

Cytokine induction by the different strains of lactic acid bacteria with or without heat treatment.

Strains/treatment	IL-1β	IL-8	IL-10
2592 untreated	31.0	32.8	30.7
heated	26.8	31.9	28.1
16:1 untreated	28.8	27.3	28.8
heated	27.5	27.9	27.3
F19 untreated	28.2	27.6	28.5
heated	28.0	28.1	27.6
77:1 untreated	26.9	31.9	27.5
heated	28.9	29.2	28.5

Strains were cultured on MRS agar under aerobic (2592 *L. plantarum*; 16:1 *P.pentosaceus*) or in anaerobic (F19 *L. paracaser*, 77:1 *L. mesenteroides*) conditions at 35°C for 20 hours.
U937 monocytic leukemia cells (10^6) were incubated with either bacterial suspension (10^7 cfu) or PBS (control) in a total volume of 500 ml at 37°C for 2 hours, and 50 ml of the supernatant was dot blotted.
Reaction against anti-interleukin immunoglobulins on dot blots was scored by densitometry.

Fig 2.

NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) is a protein complex that controls transcription of DNA, cytokine production and cell survival. NF-κB, found in almost all animal cell types, is involved in cellular responses to challanges such as severe infections, trauma, mental and physical

stress, heavy metals, ultraviolet irradiation, oxidized LDL, various pro-inflammatory cytokines, free radicals and, most important bacterial or viral antigens. NF-κB is known to play a key role in regulating the immune response to insults like those mentioned above. Impaired regulation of NF-κB has been linked to cancer, inflammatory and autoimmune diseases, septic shock, viral infection, and impaired immune functions.

Ability to transcribe NF-κB indicates ability to counteract inflammation and associated diseases. The effects of the various strains to transcribe NF-κB were studied on the nucleus of macrophage U 937. The outcome of these studies are summarized to fig 3.

Expression of nuclear NF-kB in U937 monocytic cells by different strains of lactic acid bacteria.

Strains	Control	*L. plantarum* 2592	*P. pentosaceus* 16:1	*L. paracasei* F19	*L. mesenteroides* 77:1	2592 [a]	16:1 [a]	F19 [a]	77:1 [a]
NF-kB p65[b]	2	4	3	4	3	3	2	2	2
NF-kB p50[b]	1	2	2	2	2	2	2	2	1
IkBa[c]	1	2	1	0	1	1	1	1	1

Strains were cultured on MRS agar under aerobic (2592 *L. plantarum*; 16:1 *P.pentosaceus*) or in anaerobic (F19 *L. paracasei*; 77:1 *L. mesenteroides*) conditions at 35°C for 20 hours. The U937 cells from monocytic leukemia cell line (10^5 cells) were incubated with either bacterial suspension (10^7 cfu) or PBS (control) in a total volume of 500 ml at 37°C for 2 hours. Reaction against anti-human NF-kB subunit and IkBa immunoglobulins on dot blots were scored according to the density.

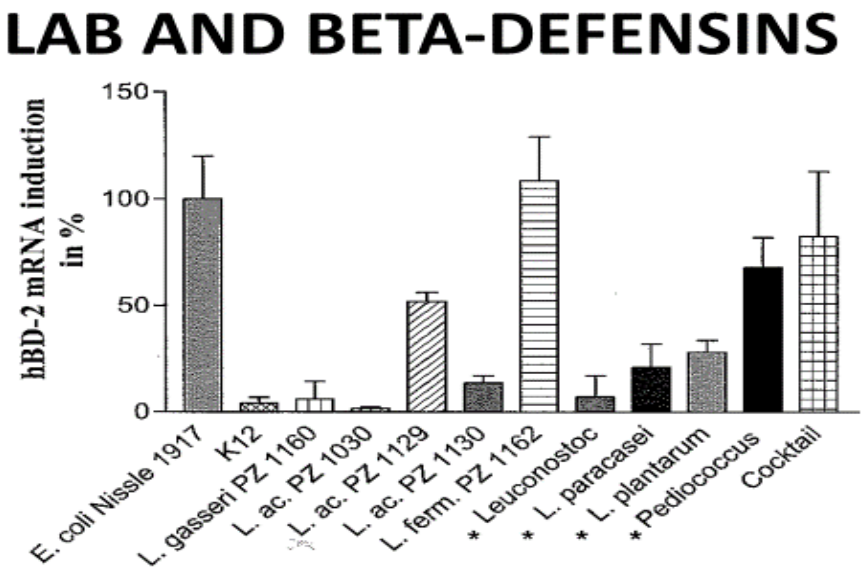
[a] Bacterial strains were heat-treated at 100°C for 30 minutes.

[b] Nucleous extract.

[c] Cytosole extract.

Fig 3.

Beta defensins are a family of peptides with impact the function of the innate immune system and involved in the creation of resistance of epithelial and other surfaces to microbial colonization – avtive against many Gram-negative and Gram-positive bacteria, fungi, and enveloped viruses, β-defensins found in white blood cells such as macrophages, granulocytes and NK-cells, but also i epithelial cells. The effects of the four chosen lactic acid bacteria were studied *in vitro* and and accumulative effects observed when combining the four lactic acid bacteria – see fig 4.



.Fig 4.

Association to antibiotics.

It is important for optimal function of probiotics that the supplemented lactic acid bacteria are not totally sensitive to antibiotics but complement each other. Of that reason extensive studies were undertaken to study the abilities of each of the strain to resist elimination by various antibiotics – see fig 5.

Table 2. Antibiograms of LAB

Antimicrobial agent**	*L.plantarum* 2592	*L.paracasei* supsc *paracasei* F:19	*P.pentosaceus* 16:1	*Leu.mesenteroides* 77:1
PeO	4	0.50	4	0.5
PeV	6	0.75	6	0.75
AMP	0.38	0.75	0.19	0.19
AMC	0.50	0.75	0.19	0.38
CRO	48	64	0.25	8
CXM	4	4	1	6
IMP	1	2	0.023	3
GEN	6	6	3	0.25
ERY	0.25	0.125	0.25	0.125
CLI	3	0.19	3	0.032
TEL	48	2	12	3
LZD	2	1	2	1.50
CIP	>64	1.5	>64	>64

- ***L plantarum*** **and** ***L paracasei*** **are relatively resistant.**
  Pediococcus **is the most sensitive, except against quinolon.**
  All LAB are relatively resistant to erythromycin, linezolid and amikacin
  e.g. the antibiotics often used against gram-positives.

Fig 5.

As summarized both *L plantarum* och *L paracasei* are relative to antibiotics whereas *Pediococcus* is relative sensitive. A condition for subsequent clinical studies was that the studies could be undertaken in parallel to often extensive antibiotic treatment in severe sick intensive care patients. The clinical resistance

of the chosen lactic acid bacteria is supported by the fact that excellent clinical results were obtained by the symbiotic composition.

Experimental studies in animals.

Peritonitis induced by a technique called ceacal ligation (Ceacum is separated from the colon and punctured, which leads to leakage of content into the open peritoneum) was used in order to study the effects on induced sepsis. Two separate types of experiments were used. Fifty rats were used to study the effects of oral pre-treatment with the with the composition Synbiotic 2000 and eighty rats to study the effects of live subcutaneous injection of the four lactic acid bacteria used in the composition. The results were almost identical – the results reported below are from the study using oral supply, which is the method intended for use in clinical patients (Fig 6).

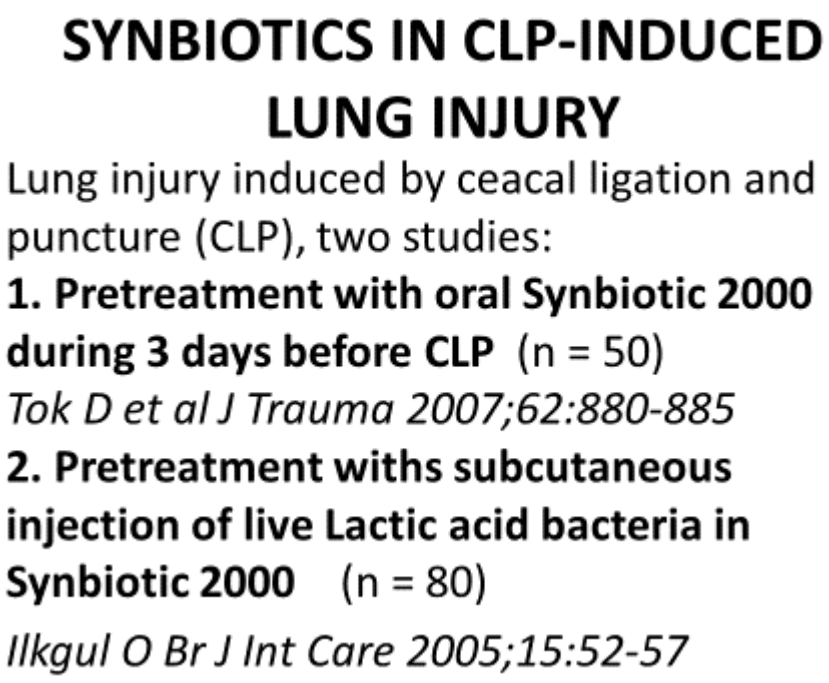


Fig 6.

The treatments with either the full Synbiotic-composition or only the lactic acid bacteria in the composition inhibited totally the reaction to sepsis as demonstrated in no or low increases in neutrophils in tissues such as lung tissues – known to be severely affected in sepsis – a common condition cared in Intensive Care Units (Fig 7).

NEUTROPHILS IN LUNG TISSUE

Tok D et al J Trauma 2007;62:880-885

- **Synbiotic 2000 9.00±0.44**
- Only LAB 8.40±0.42
- Only the fibres 31.20±0.98
- Placebo 51.10±0.70
- $p < 0.05$

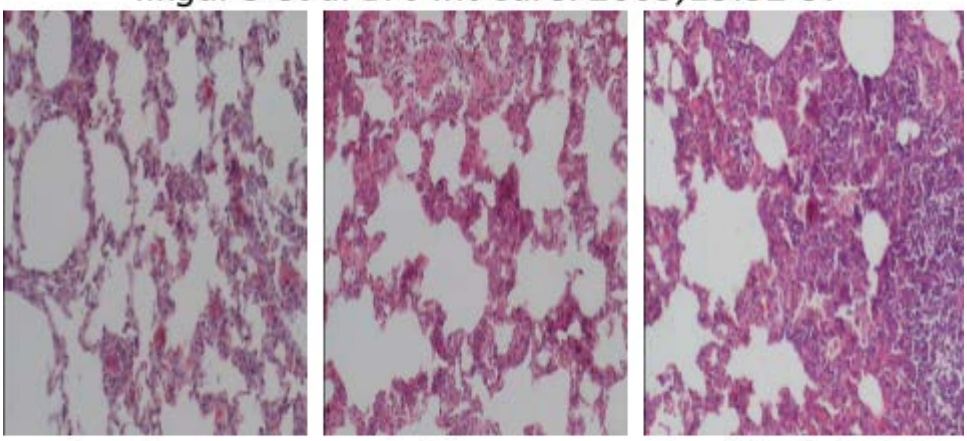
Fig 7.

An important observation is that treatment with Synbiotic 2000 did effectively prevent the tissue destruction regularly observed in the lungs and and most often creating need for ventilator treatment, common in the

Intensive Care Units (Fig 8).

SYNBIOTIC 2000 IN LUNG INJURY

Ilkgul O et al Br J Int Care. 2005;15:52-57



- **Placebo** **Only fibres** **Synbiotic 2000**

Fig. 8.

Myeloperoxidase (Fig 9), Malonaldehyde (Fig 10) and Nitric Oxide (Fig 11) are standard measuments of general inflammation in the body – as demonstrated below – all these were statistically significantly reduced – all signs of the efficacy of Synbiotic 2000 to inhibit the inflammatory reactions to sepsis.

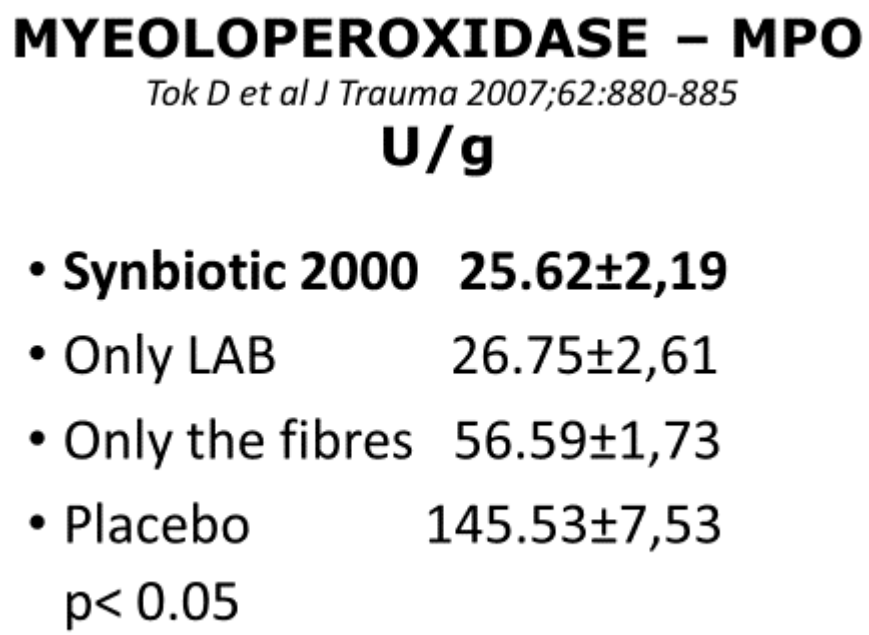


Fig 9.

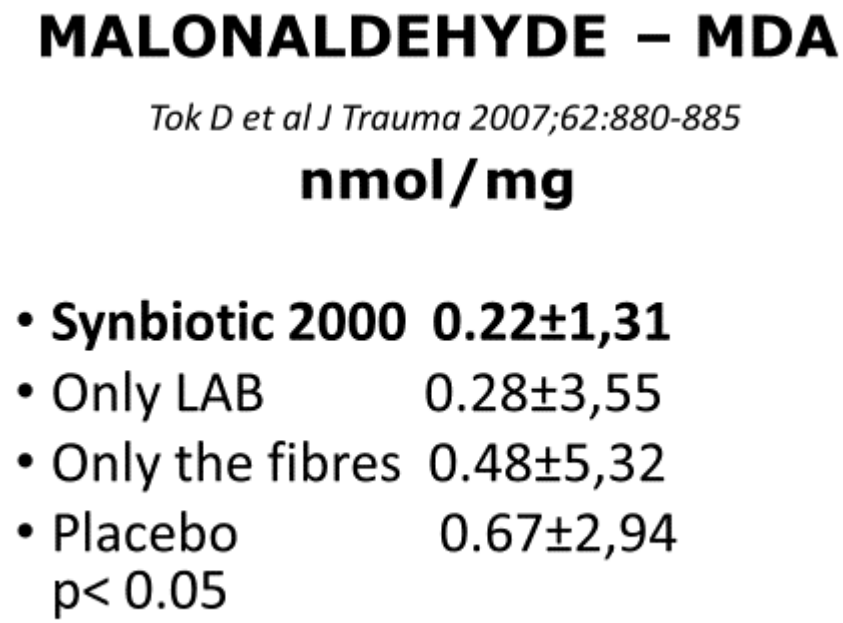


Fig 10.

NITRIC OXIDE
micromol/g

• **Synbiotic 2000**	**17.16±2,03**
• Only LAB	8.91±2,24
• Only the fibres	47.71±3,20
• Placebo	66.22±5,92

$p < 0.05$

Fig 11.

Many incidental observations have been made during the subsequent clinical studies such as ability to inhibit Helicobacter pylori, Candida and viral infections and also multi-resistant pathogens (Fig 12, fig 13).

Multi-resistant *Acinetobacter baumanii* inhibited by two of the Synbiotic-2000 strains Professor *Val Edwards-Jones, Manchester, UK*

Fig 12.

Inhibition and Synergy of the four Synbiotic 2000 strains in multi-resistant *Klebsiella* *Professor Val Edwards-Jones, Manchester, UK*

Fig 13.

Safety aspects.

Governmental in various countries, Food and Drug Administration – FDA – in the US have declared Probiotics as Generally Regarded As Safe – GRAS. We decided, however, to study the eventual side-effects in the consecutive phases. Immuno-compromized mice received during 4 weeks daily supply of Synbiotics 2000 – no negative effects were observed. This was follow by daily supplementing 50 healthy individuals during three months with Synbiotic 2000 – again no side effects were reported (Fig 14.) After that – several hundred severely sick patients have been treated, often in postoperative and Intensive Care Units. No side effects have during these more than10 years of treatment been reported.

SAFETY ASPECTS

A. LAB in Synbiotic 2000 fed during 4 weeks to immuno-compromized mice - no negative effects observed.

B. Synbiotic 2000 fed to 50 healthy volunteers in 3 months – no negative effects observed.

C. Synbiotic 2000 given to:

- 340+250 critically ill and with 38 with HIV

- About 100 liver liver transplant patients,

- About 100 with liver cirrhosis,

- About 100 patients undergoing extensive abdominal operations,

- patients with ulcerative colitis,

app 15 severe trauma patients

No negative effects observed

Fi14.

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- Tillfälliga tekniska problem.