





Functional properties of *Lactobacillus* sp. strains for health improvement

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University of Tartu

Bio-Competence Centre of Healthy Dairy Products LLC

Plan of lecture

1. Health indices
2. Age groups : Adults vs. Elderly
3. Human microbial ecology **in health**
4. **Biodiversity of Lactobacillus sp.**
5. Role of Lactobacillus sp. for health biomarkers

6. **Selection** of probiotic strains for functional properties
7. **Three steps** approvement of a LAB strain functionality
8. How to perform food intervention clinical trials
9. A path to Nutritional Health Claim approval

Health **WHO:** A complete physical, mental and social well-being: quality of life

Biomarkers of health

EFSA Journal 2011;9(12):2474

- **Personal questioning**
  - Well being-quality of life
  - Specific complaints
- **Objective clinical indices**
  - BMI
  - Fat storage
  - Mucosal membranes state
  - EKG , MRI

- **BLOOD analyses**

Infammatory indices → **Infection**
us- CRP
WBC, formula
Chronic inflammation

Insulin resistance +BP +BMI

Blood sugar → **MetSyndr**

Chol, LDl, HDL, Triglycerides →

ox-LDL, isoprostanes **CVD, ox-S**

URINE , Faeces

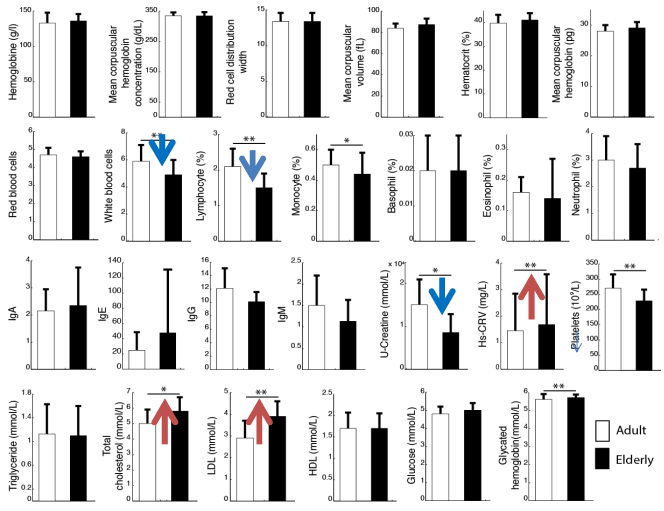
SHIFTS TO INCREASE THE RISK OF

Does aging change these indices?

15-Sep-15 Valencia Beneficial ...

Differences in 42 blood biomarkers between adults n=16 and elderly n=33

Le Roy et al. .accepted Oncotarget



Elderly: down WBC, Lymphocytes, U-creatinine
up hs-CRP, cholesterol, LDL

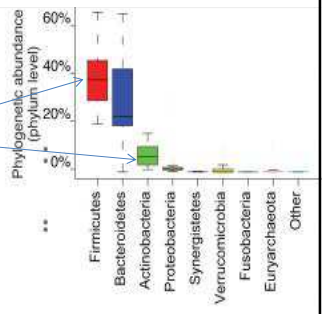
Human microbial ecology of GI tract in health

Vrieze et al. Phylogenetic tree: diversity and abundance by 16S RNA sequencing. Diabetologia.2010 ,53(4): 606–613

Gut microbiome
Superorganism 10% +90%
Viruses, eucarya, bacteria, archaea

MICROBIOTA
Indigenous
- **Beneficial bacteria**
- Opportunistic pathogens
- Transitory microbes

Lactobacillus
Bifidobacteria



In healthy subjects the Homeostasis between the host tissues and its indigeneous microbiota is granted by *conserved proportions between different bacterial phylotypes* .

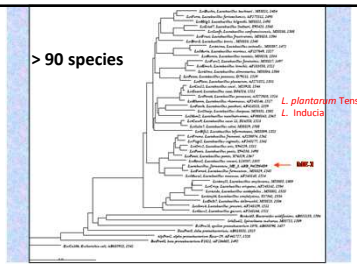
The metabolic adaptation to disturbances is gained by series of interconnected metabolic processes and molecular mechanisms. ˇvan Ommen et al., Genes Nutrition 2014., 9:243

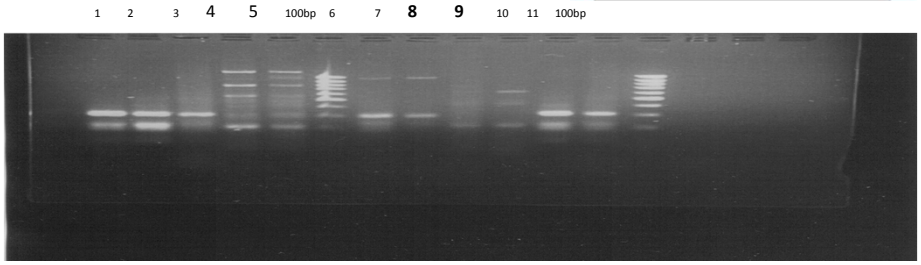
In different diseases, the shifts in abundance of the predominant gut microbiota are accompanied with changes of host health biomarkers Arumugam et al., 2011

LACTOBACILLUS sp. genotypes:

16s RNA gene sequencing,
phylogenetic tree and
species specific PCR products

Genome sequencing, data NCBI genomes

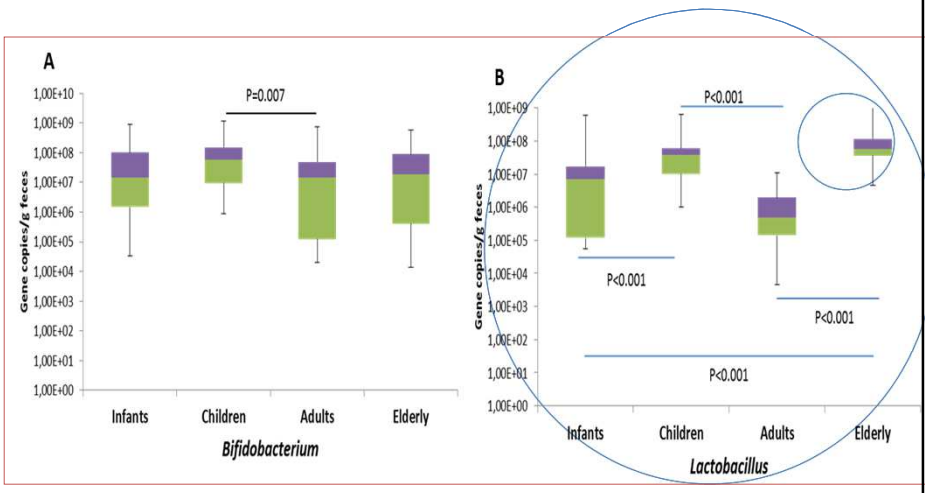




- ITS-PCR products of amplified 16S-23S rRNA gene spacer regions from Lactobacilli using primers16S-1500F and 23S-32R (DNA Technology AS).

1. ***L.casei* 8700:2**; 2. ***L.paracasei* ssp *paracasei*** *DSM20020;* 3. *L.paracasei* ssp *paracasei DSM 5622;*
4. ***L.plantarum* 299V**; 5. *L. plantarum* ATCC14917; 6. ***L.acidophilus* La5**; 7. *L.acidophilus* ATCC4356;
8. ***L.fermentum* ME-3;** 9. *L.fermentum* ATCC14931; 10. ***L.rhamnosus* GG**; 11. *L.rhamnosus* ATCC14931
100 bp DNA ladder

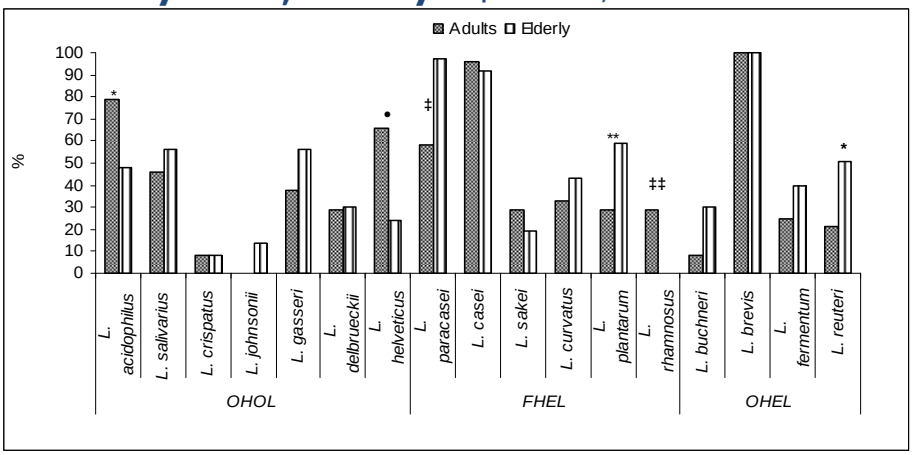
QUANTITATIVE CHANGES OF *LACTOBACILLUS* AND *BIFIDOBACTERIUM* SP. IN HUMAN INTESTINAL TRACT OF DIFFERENT AGE GROUPS (Real time-PCR)



Štšepetova et al. **LAB specific RT-PCR detection of gene copies of *Bifidobacterium* and *Lactobacillus* sp.** ENGHIR Proceedings, 2014

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Lactobacillus sp. in healthy adults vs. elderly 2005, 2007 y Stsepetova et al., Brit.J. Nutr..2011

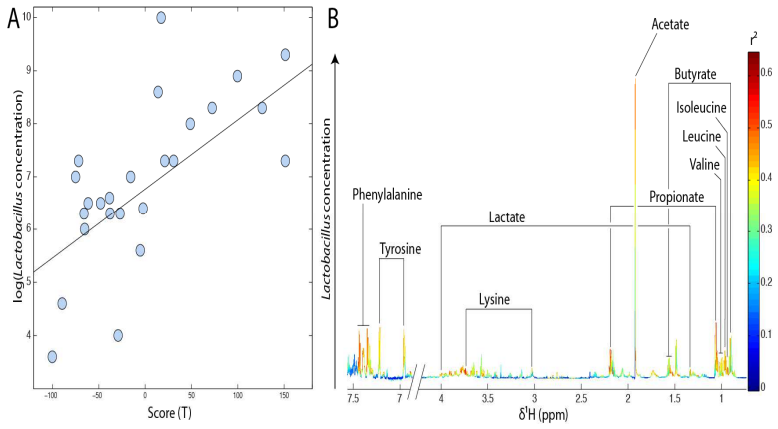


* p=0.031; • p=0.001; ‡ p=0.001; ** p=0.035; ‡‡ p=0.007

Each individual was colonized with **4 to 12 different species**
Adults, were often colonized with ***L. acidophilus*** and ***L. helveticus***.
in Elderly *L. paracasei, L. plantarum* and ***L. reuteri*** prevailed.

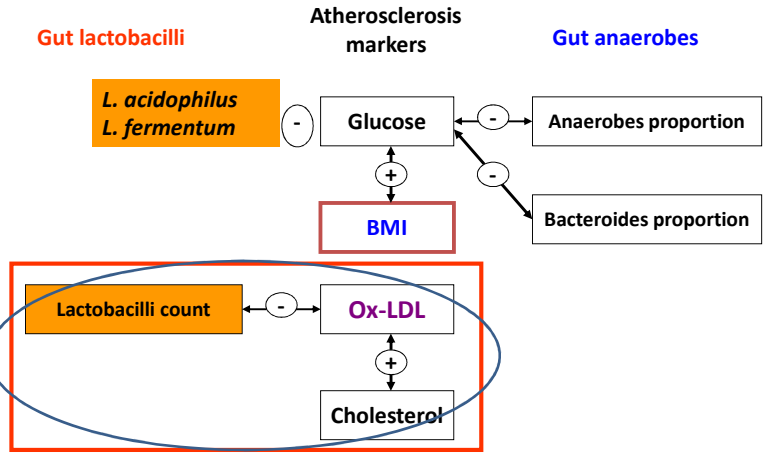
O-PLS scores projection to high total count of *Lactobacillus sp.* is associated with higher faecal SCFAs, lactic acid and amino acid levels.*^{1}H-NMR spectra of faecal waters using the log of total Lactobacillus counts as a response predictor*

Le Roy, et al. Accepted Oncotarget



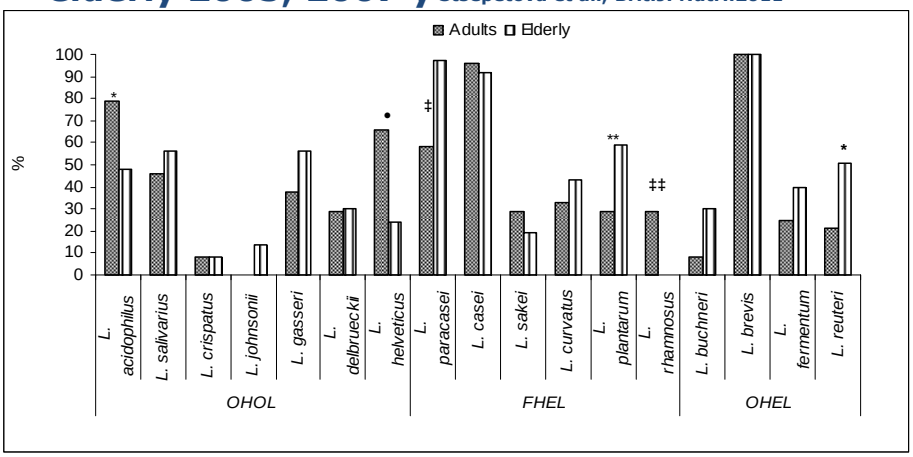
Multiple regression analysis

IMB of elderly and health



Mikelsaar, M.; Stsepetova, J.; Hütt, P.; Kolk, H.; Sepp, E.; Lõivukene, K.; Zilmer, K.; Zilmer, M. (2010). Intestinal Lactobacillus sp. is associated with some cellular and metabolic characteristics of blood in elderly people . Anaerobe, 16(3), 240 - 246.

Lactobacillus sp. in healthy adults vs. elderly 2005, 2007 y Stsepetova et al., Brit.J. Nutr..2011

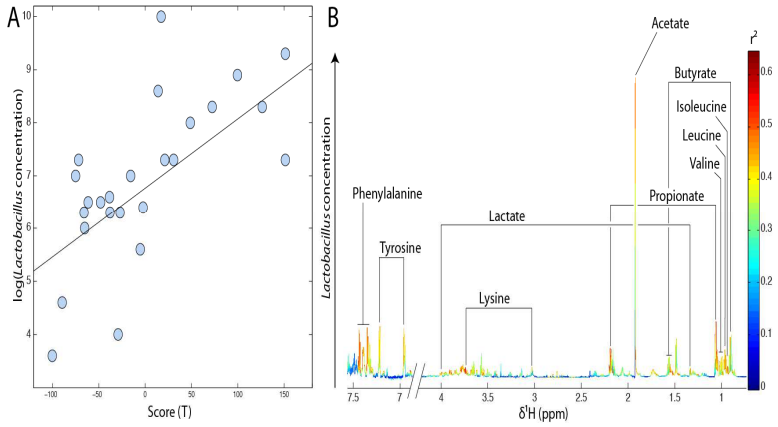


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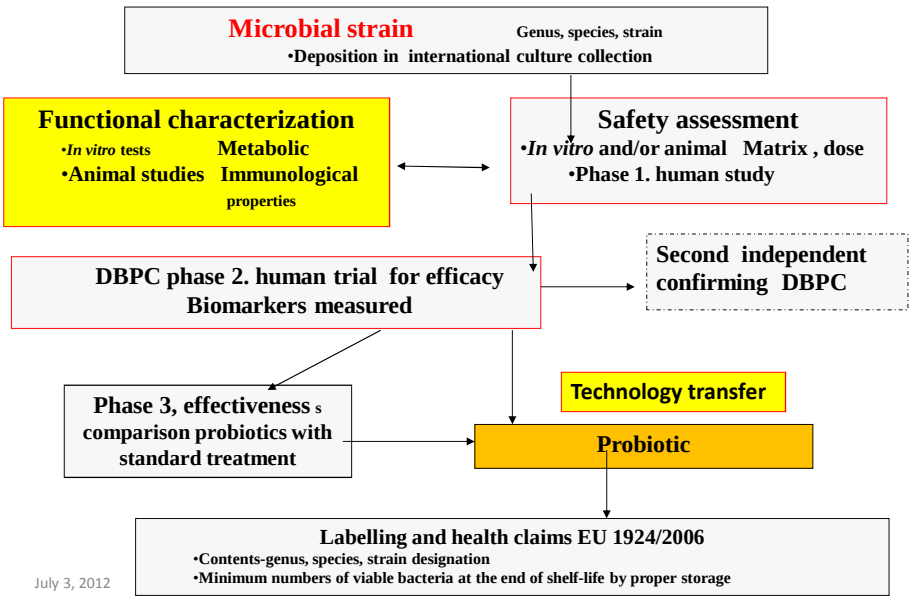
Selection of probiotic strains

- Origin, Identification, Typing
- Colonizing ability: Survival in matrix/host
- Safety: AB sensitivity profile
- **FUNCTIONAL PROPERTIES: special for host?**

Regulations for application of PROBIOTIC BACTERIA in human food

Food and Agriculture Organisation of the United Nations (FAO) and WHO, May 2002

ILSI RECOMMENDATIONS 2008



July 3, 2012

THUS: Beneficial influence of *Bifidobacterium* and *Lactobacillus* sp. to human health

Functional properties:

- SCFA
- polyamines
- NO-, CO2
- biocides
- antioxidative capacity:

Mn- SOD, GSHPX, GSSGRX, scavenger of ROS

Anti-inflammatory cytokines

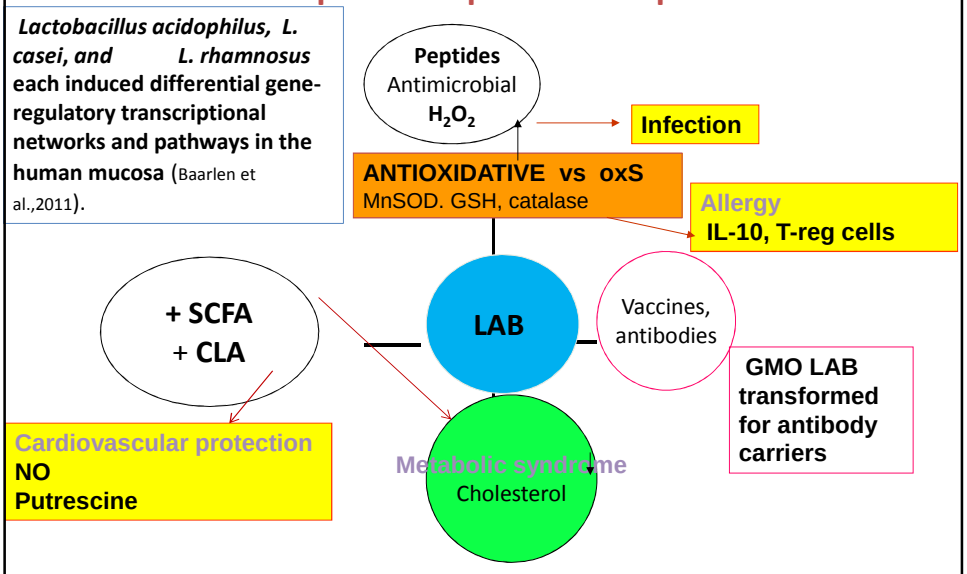
Health effects of probiotics:

- Prevention and reduction of diarrhea
- Lactose intolerance
- Antimutagenic and anticarcinogenic properties
- Host metabolism support
- Increasing mucosal immunity
- Contra oxidative stress
- Reducing serum cholesterol

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LAB bioactive compounds – potential impact for health

Lactobacillus acidophilus, L. casei, and L. rhamnosus **each induced differential gene-regulatory transcriptional networks and pathways in the human mucosa** (Baarlen et al.,2011).

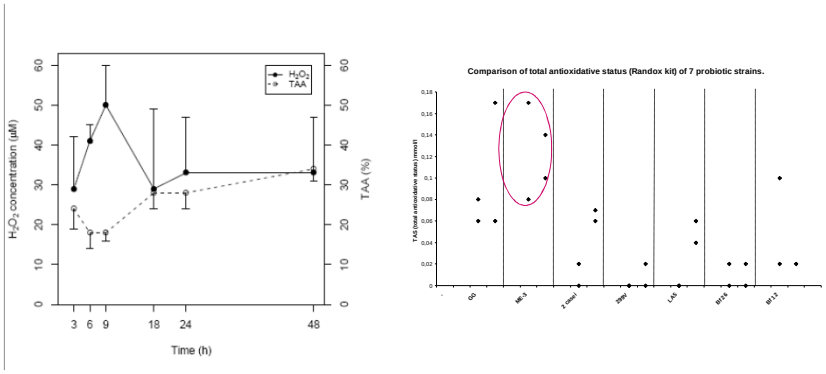


Annuk et al., 2003; Kullisaar et al., 2003; *Kõll et al. 2010 Songisepp et al., 2012; Hütt et al. 2014; Mikelsaar&Zilmer, 2009; Mikelsaar et al., 2013*

15-Sep-15

I. In vitro detection of oxidative/antioxidative properties of *Lactobacillus* sp. strains

Jones RM[1], Mercante JW, Neish AS. Curr Med Chem. 2012;19(10):1519-29



* Kullisaar et al. Two antioxidative lactobacilli strains as promising probiotics. Int. J. Food Microbiol., 2002, 72, 215-24.

*Hütt et al., Antagonistic acitivity of probiotic lactobacilli and bifidobacteria against entero- and uropathogens. JAMA, 2006, 100, 1324-1332

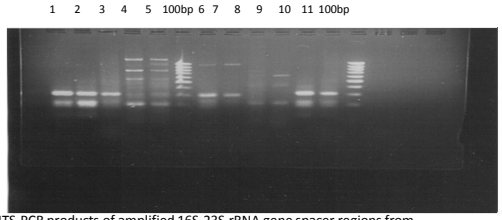
* Kullisaar et al., Complete glutathione system in probiotic *L. fermentum* ME-3. Applied Biochemistry and Microbiology, 2010, 46, 481-486.

15-Sep-15

Valencia Beneficial ...

Lactobacilli EU 5th PF

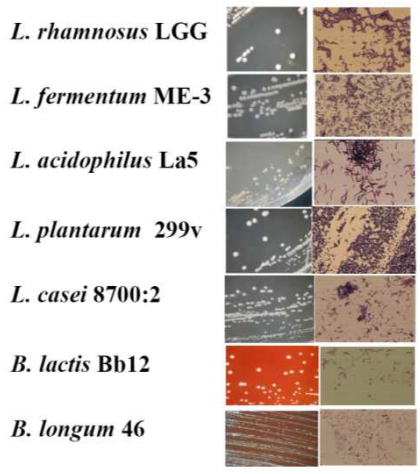
Hütt et al. J.Appl. Microbiol.2006,100: 1324-1332



ITS-PCR products of amplified 16S-23S rRNA gene spacer regions from Lactobacilli using primers16S-1500F and 23S-32R (DNA Technology AS).

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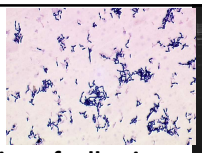
100 bp DNA ladder

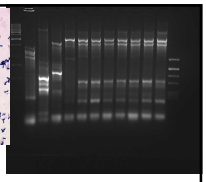


May 16,2012

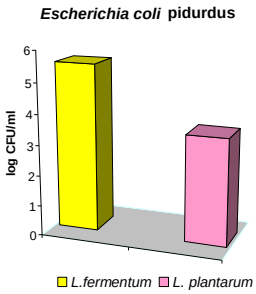
Valencia

Lactobacillus fermentum ME-3
(DSM 14241)





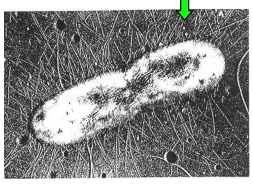
1. Antagonism for pathogen



2. Blocking of adhesines

L. fermentum ME-3: Gal, GalNAc

Escherichia coli ↑ P-pili



3. Natural resistance to

TMP+SMX, metronidazole,
Fluoro-quinolones, cefoxitin

4. Antioxidative defence to mucosa

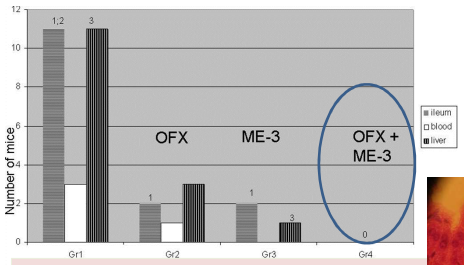
increase of TAA & TAS, capture of radicals

↓ **ox-LDL**, **GSSG/GSH ratio**
isoprostanes

Mikelsaar et al., 2002; Annuk et al., 2001, Kullisaar et al. 2002, Songisepp et al. 2005

Infections: Experimental pathology *with L. fermentum* ME-3

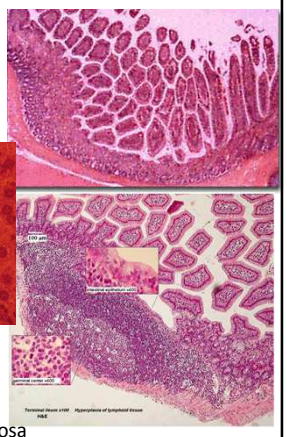
Truusalu et al. MEHD,2004; Truusalu APMIS 2008; Truusalu BMC Microbiology,2010

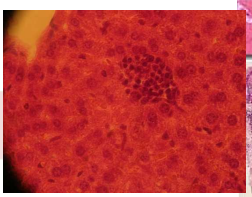


Absence of live *Salmonella* Typhimurium in blood and organs by ME-3 + ofloxacin

1. Immune stimulation:

A.





C

B.

A. Normal mucosa
B. In mice ***L. fermentum*** **ME-3** promotes PP hyperplasia and germ centre lymph follicles

2. Improved mucosal antioxidativity - no granulomas (C)

↑ Total antioxidative activity, TAA

↓ Lipid peroxidation, glutathione red-ox ratio

How to perform clinical intervention trials with functional food ?

Clinical food trials in Tartu

Regulations for application of probiotic bacteria . ***FAO and WHO, May 2002, ILSI RECOMMENDATIONS 2008***

1. **SELECTION OF PROBIOTIC CANDIDATES**

   KÕLL et al. 2009,. Tartu HUMB.www

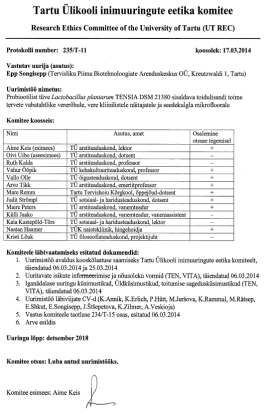
2. **Selection of human target group and biomarkers** *EFSA Journal 2011, 9*

4.

Reduction of the risk of atherosclerosis by

* antioxidative activity
* reduction of LDL-cholesterol
* post-prandial triglycerides levels

3.Registration and ETHICS



Tartu Ülikooli inimuuringute eetika komitee

Research Ethics Committee of the University of Tartu (UT REC)

Protokolli number: 235/T-11 **koosolek: 17.03.2014**

Vastutav uurija (asutus):
Epp Songisepp (Tervisliku Piima Biotehnoloogiate Arenduskeskus OÜ, Kreutzwaldi 1, Tartu)

Uuringuteema nimetus:
Probiootilist tüve *Lactobacillus plantarum* TENSIA DSM 21380 sisaldava toidulisandi toime tervete vabatahtlike veresüsile, vere kliinilisele näitajatele ja seedekulgla mikrofloorale

Komitee koosseis:

Nimi	Asutus, amet	Osalemine otsuse tegemisel
Aime Kels (esimees)	TÜ arstiteaduskond, lektor	+
Orvi Uibo (sekretimees)	TÜ arstiteaduskond, dotsent	–
Ruth Kalda	TÜ arstiteaduskond, professor	–
Vahur Õunpõk	TÜ kehakultuuriteaduskond, professor	+
Valle Ilja	TÜ ägustiteaduskond, dotsent	+
Arvo Tiik	TÜ arstiteaduskond, emeeritprofessor	+
Mare Rezen	Tartu Tervishoiu Kõrgkool, õppejõud-dotsent	+
Judit Nõmpf	TÜ arstiteaduskond, dotsent	+
Maire Peters	TÜ arstiteaduskond, vanemteadur	+
Källi Jaako	TÜ arstiteaduskond, vanemteadur, vanemassistent	–
Kata Kostopõld-Tõru	TÜ arstiteaduskond, lektor	–
Neeme Hinrmar	TÜK, statistikiinik, lektor	+
Kristi Lõuk	TÜ filosoofiateaduskond, projektijuht	–

Komiteele läbivaatamiseks esitatud dokumendid:
1. Uurimistöö avalduse koosküsimuse saamiseks Tartu Ülikooli inimuuringute eetika komiteelt, tähtaeg 06.03.2014 ja 25.03.2014
2. Uuritavate infoleht ja nõusolekuvormid (TEN, VITA), tähtaeg 06.03.2014
3. Igaühelõise uuringu kõsimustikud, Üldkõsimustikud, tõttumise sagedusküsimustikud (TEN, VITA), tähtaeg 06.03.2014
4. Uurimistöö läbivijate CV-d (K.Annik, K.Erlich, P.Hitt, M.Jørlova, K.Rammul, M.Rätsep, E.Sõkut, E.Songisepp, J.Štšepetova, K.Zilmer, A.Veskioja)
5. Vastus komitee taotlusе 234/T-15 osas, esitatud 06.03.2014
6. Arve mõlds

Uuringu lõpp: detsember 2018

Komitee otsus: Luba antud uurimistööks.

Komitee esimees: Aime Kels

ISCRNT43435738 and ISCRNT53154826 **registered trials**

r

PRINCIPLES OF EU nutrition health claims regulation 2006

BENEFICIAL BACTERIA = Probiotics (functional food/food supplements) can be applied in frame of dietary interventions for

1) **maintenance or optimization of host physiological health markers by recruiting healthy volunteers with borderline biomarkers**

2) **reducing the risk for diseases like atherosclerosis, acute infectious diarrhea, antibiotic associated drr, Irritable bowel syndrome, IBS, IBD, colorectal cancer, metabolic syndrome Met-S and CVD by inducing intestinal microbiota changes in association with biomarkers (Sanders et al. 2013)**

Bernhardt et al., 2015 Annales of Nutrition& Metabolism

TARTU Probiotics

Origin of Lactobacillus fermentum ME-3

- 1995 from the GI tract of Estonian 1-year healthy child (Sepp *et al.*, Acta Pediatr.1997, Mikelsaar et al. 2001)
- Inventors: Mikelsaar, Zilmer, Kullisaar, Songisepp, Annuk, 2001
- Deposited in April 2001 in Germany as DSM 14 241, Acronym ME-3
- Patent applications of Univ. of Tartu:
  - Priority application in Estonia 29.06.2001
  - International application PCT/EE02/00006 28.06.2002; EPO examination report 2003
  - Probiotik. RU 22 84354 27.09.2006
  - US patent, US 7,244,424 B2 Jul.17, 2007

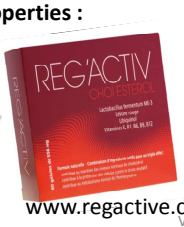


EPO patent 2011

FUNCTIONAL PROPERTIES

Antimicrobial activity ,
Antioxidative properties :





L f ME-3
Vitamins
L-cysteine
Ubiquinol,
Monacolin

www.regactive.com/en/

Origin: ***Lactobacillus plantarum*** **TENSIA ™** INDUCIA DSM 13279 and DSM 13280

Bio-Competence Center of Healthy Dairy Products LLC

Inventors: Songisepp, Mikelsaar, Rätsep, Zilmer M. Hütt, Zilmer K., Üksti, Kõljalg

Priority 2008; Russian patent 2013; EPO patent 2013

FUNCTIONAL PROPERTIES

Antimicrobial activity e.g. listeria, salmonella, shigella **plantaricin**, **H_20**

- **NO**
- **CLA**
- **peptides = ACE-inhibitory properties,**
- **metabolism of putrescine-spermidine**

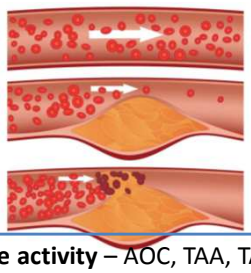
Sharafedtinov et al. BMC Nutr J. 2013, 12:138.
Mikelsaar, M.; Zilmer, M. (2009). Microbial Ecology in Health and Disease, 21(1), 1 - 27.





Kefir trial *ISRCTN 49744186* *with* ***L. fermentum*** **ME-3** contra **ATHEROGENESIS and CVD by lipid metabolism regulation**

atheromes

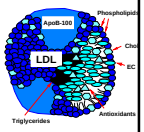


Inclusion criteria: desire to participate, appropriate age (35-65 years),
elevated blood lipids >5.2 mmol/l for the total cholesterol, >3.4 mmol/l for LDL-C,
>3.0 mmol/l for the LDL-C/HDL-C ratio and
>1.7 mmol/l for the level of triglycerides,

Antioxidative activity – AOC, TAA, TAS
Captures free radicals OH* O*, H_2O_2
Reduces glutathione GSSG to GSH and back
Alfa- and beta-galactosidases
Bile salt hydrolysis activity
TDCA discs, precipitation zones 1.5 mm

Outcome measures

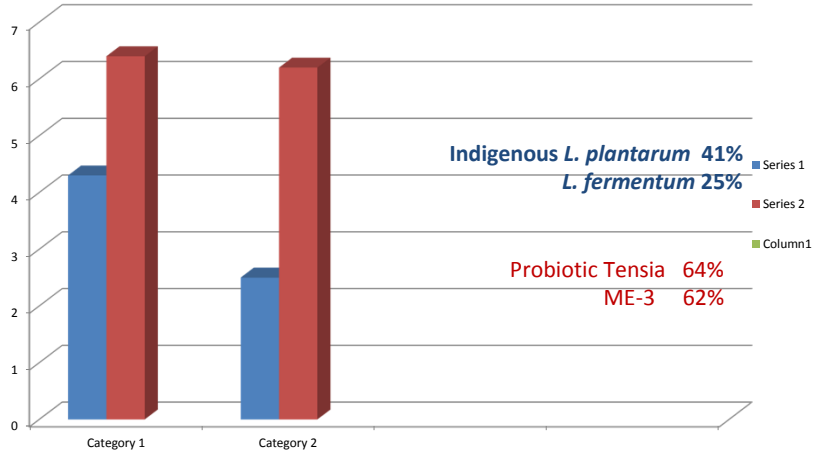
* Primary markers:
LDL -cholesterol
Triglycerides



* Secondary markers:
LDL/ HDL-cholesterol
Ox-LDL

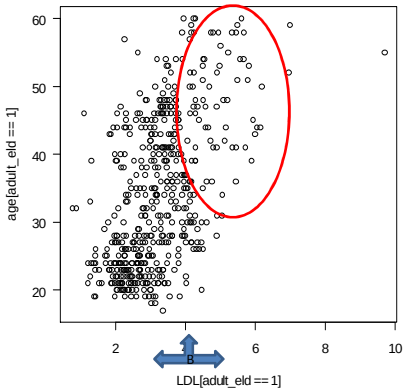
15-Sep-15 Valencia Beneficial ...

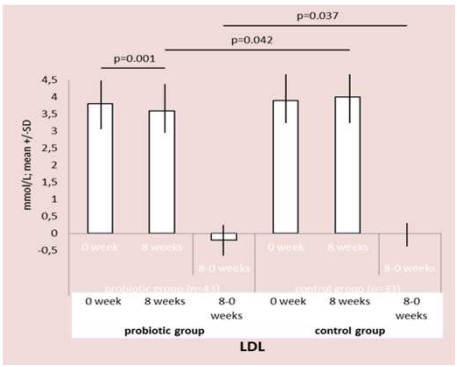
Temporal colonization % by probiotic *L. fermentum* ME-3 and *L. plantarum* Tensia in trials did not depend on the proportion of LAB species in total intestinal microbiota of general population



Tensia **ME-3** ***PCR-specific primers***

5 % Reduction of borderline high > 4 mmol/l content of blood LDL-chol after 8 weeks consumption of kephir with *L. fermentum* ME-3.





Blood **LDL**
18-60y adults n=495

N=66 N= 43

Conclusion

- **Specific species and STRAINS of *Lactobacillus* are bound to several health biomarkers:**
  Chol, LDL, ox-LDL, BMI, BP.
- **Probiotic products can**
  ***shift the biomarkers ON BORDELINE**
  - **OF NORMAL**

*** reduce the risk** of some diseases as proved in clinical trials

Lactobacillus fermentum* ME-3 → **Atherosclerosis:** oxLDL, LDL-C

x

Thank you very much!

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