بسمه تعالی

معيارهاي ارزيابي ايمني سويه هاي پروبيوتيك

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Definition of "Probiotics"

- The World Health Organization (WHO) and the Food and Agriculture Association (FAO) define probiotics as:

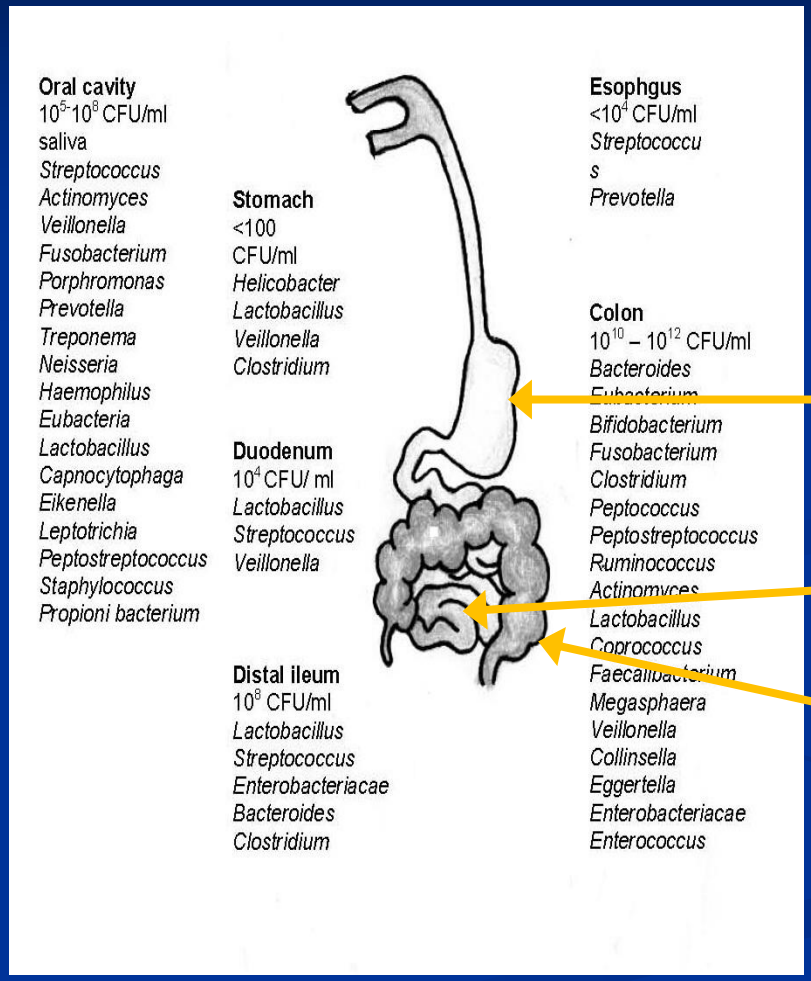
"Live microorganisms that when consumed in adequate amounts confer a health benefit on the host."

Human Microbiome Project

- The **Human Microbiome Project (HMP)** aims to characterize the microbial communities found at several different sites on the human body, including nasal passages, oral cavities, skin, gastrointestinal tract, and urogenital tract, and to analyze the role of these microbes in human health and disease.

http://commonfund.nih.gov/hmp/

Numbers and species of the most common bacteria in the various parts of the alimentary tract



Microbiota 10^{14} bacteria , more than 500 different species

$>10^{3}$ CFU/g

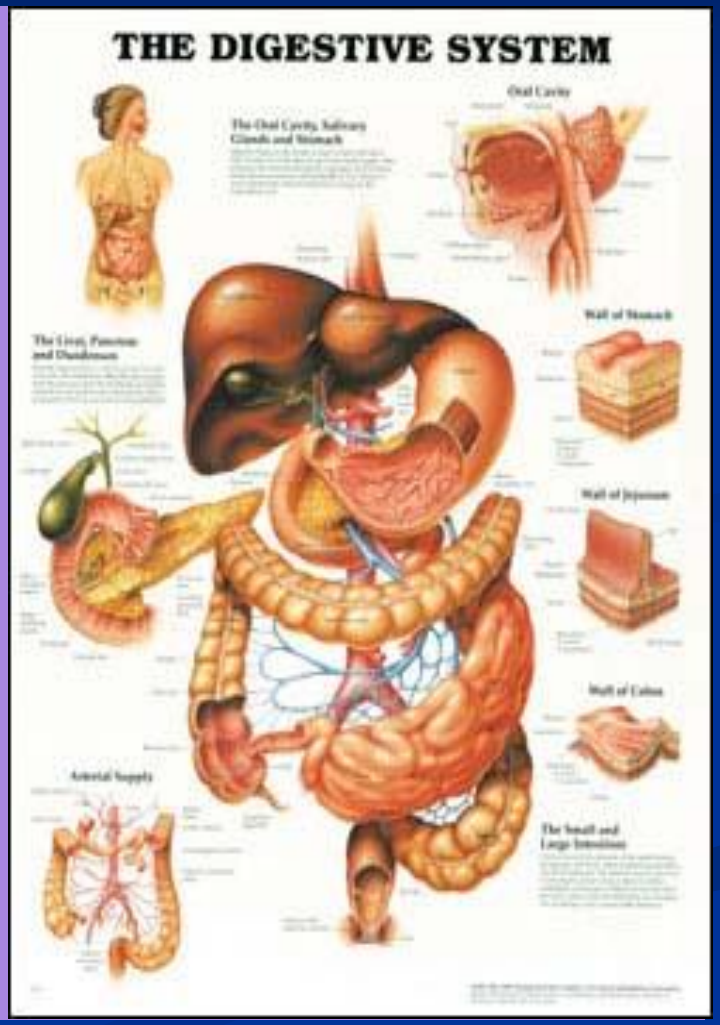
$>10^{3-5}$ CFU/g

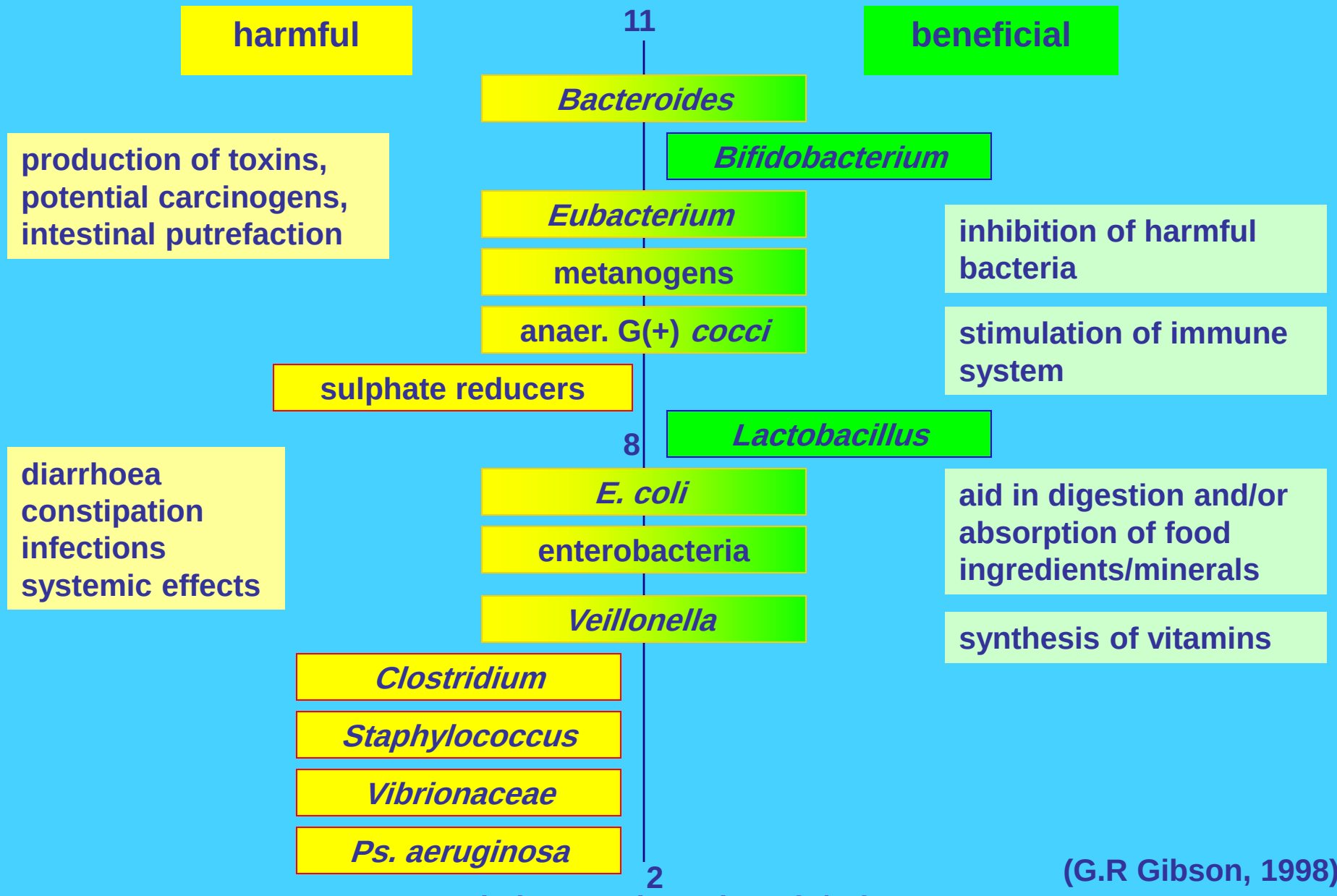
$>10^{12}$ CFU/g

The number of hosted microbes is considered to be 10 times more than the total number of human cells

The Digestive System

Normal intestinal flora contains100 trillion organisms from 400 different species




harmful
beneficial
11
Bacteroides
Bifidobacterium
production of toxins, potential carcinogens, intestinal putrefaction
Eubacterium
metanogens
anaer. G(+) cocci
inhibition of harmful bacteria
stimulation of immune system
sulphate reducers
Lactobacillus
8
diarrhoea constipation infections systemic effects
E. coli
enterobacteria
Veillonella
aid in digestion and/or absorption of food ingredients/minerals
synthesis of vitamins
Clostridium
Staphylococcus
Vibrionaceae
Ps. aeruginosa
2
Population numbers, log cfu/g faeces
(G.R Gibson, 1998)

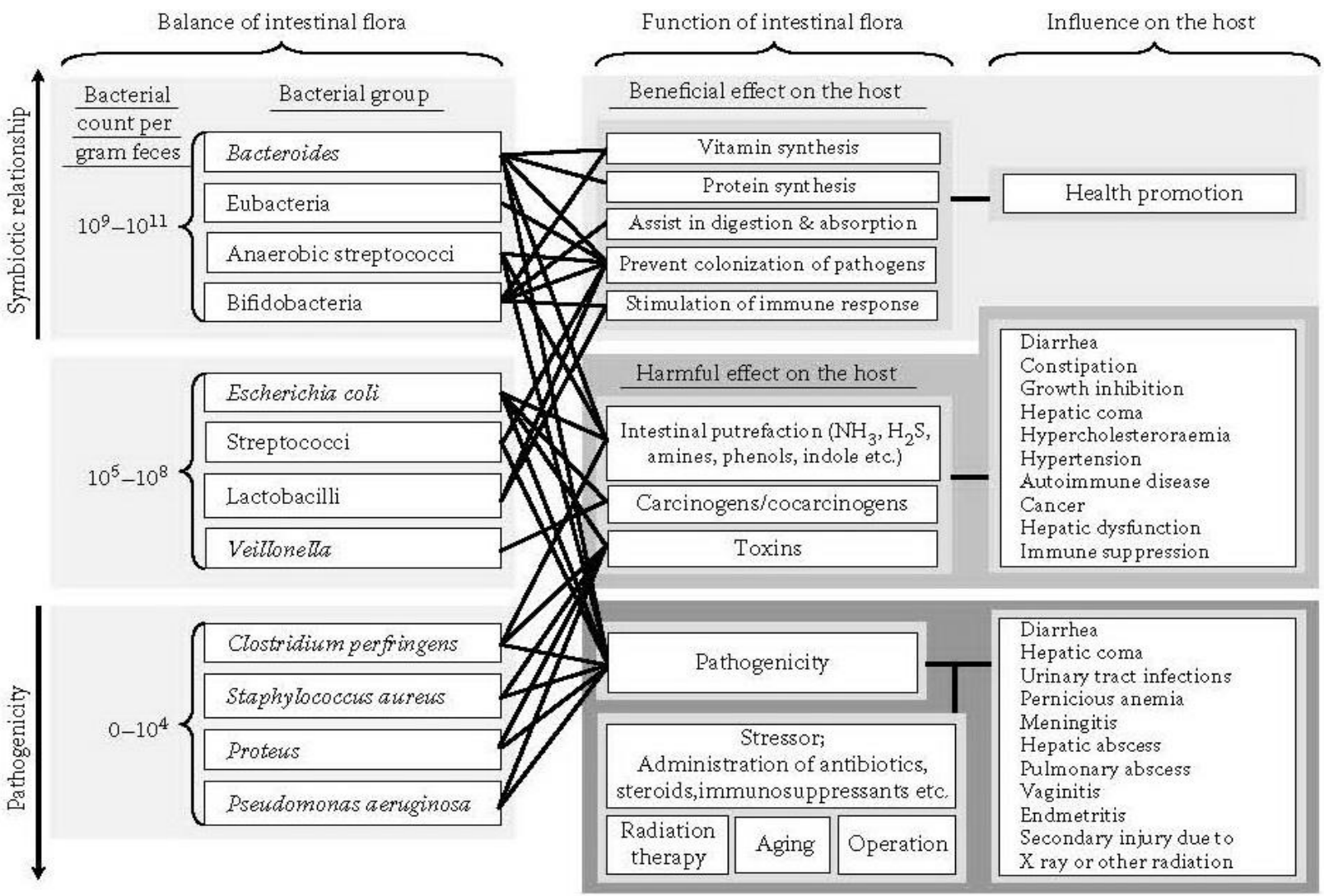


FIGURE 19.3
Interrelationship between intestinal flora and the human body (Reference 13).

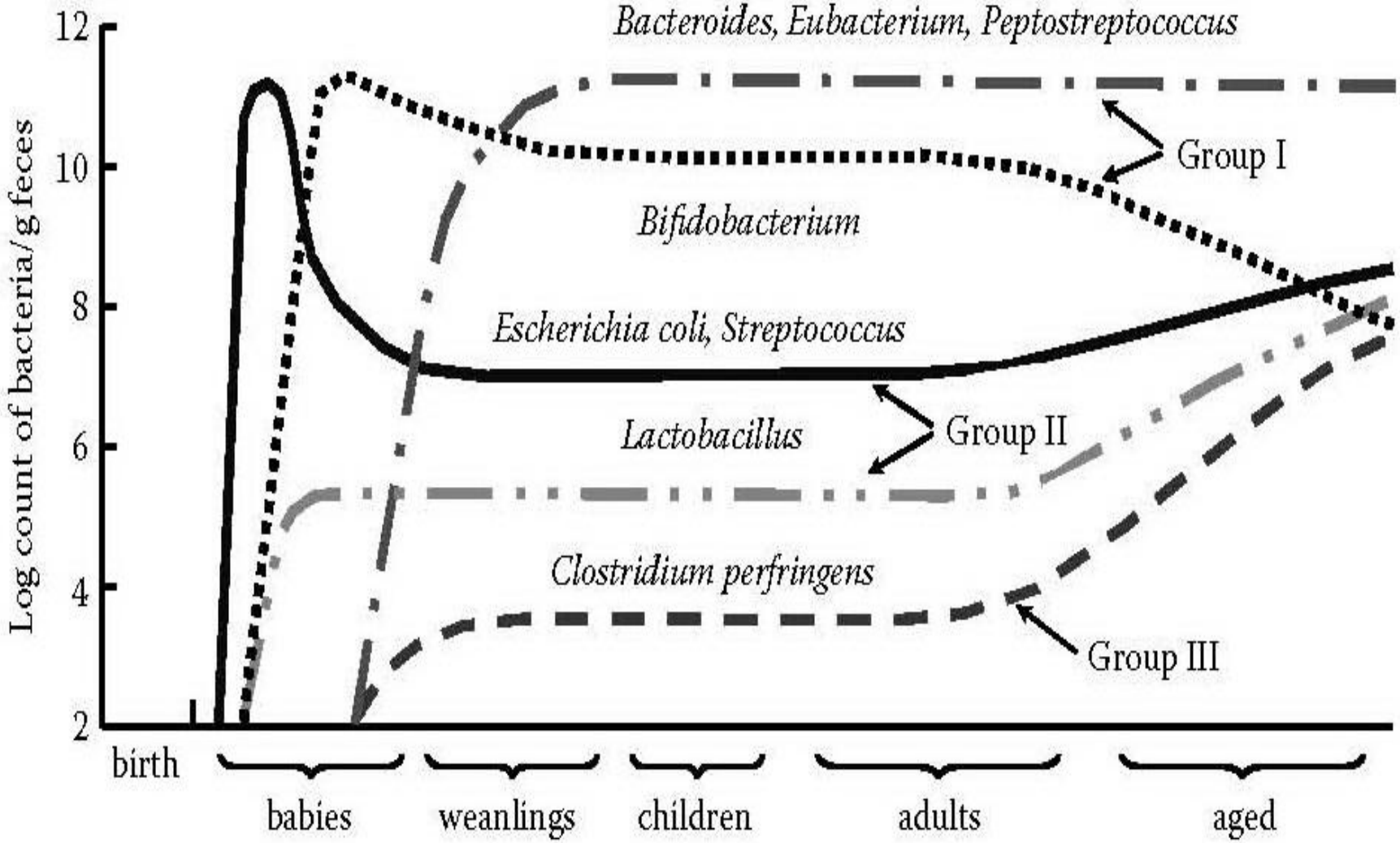
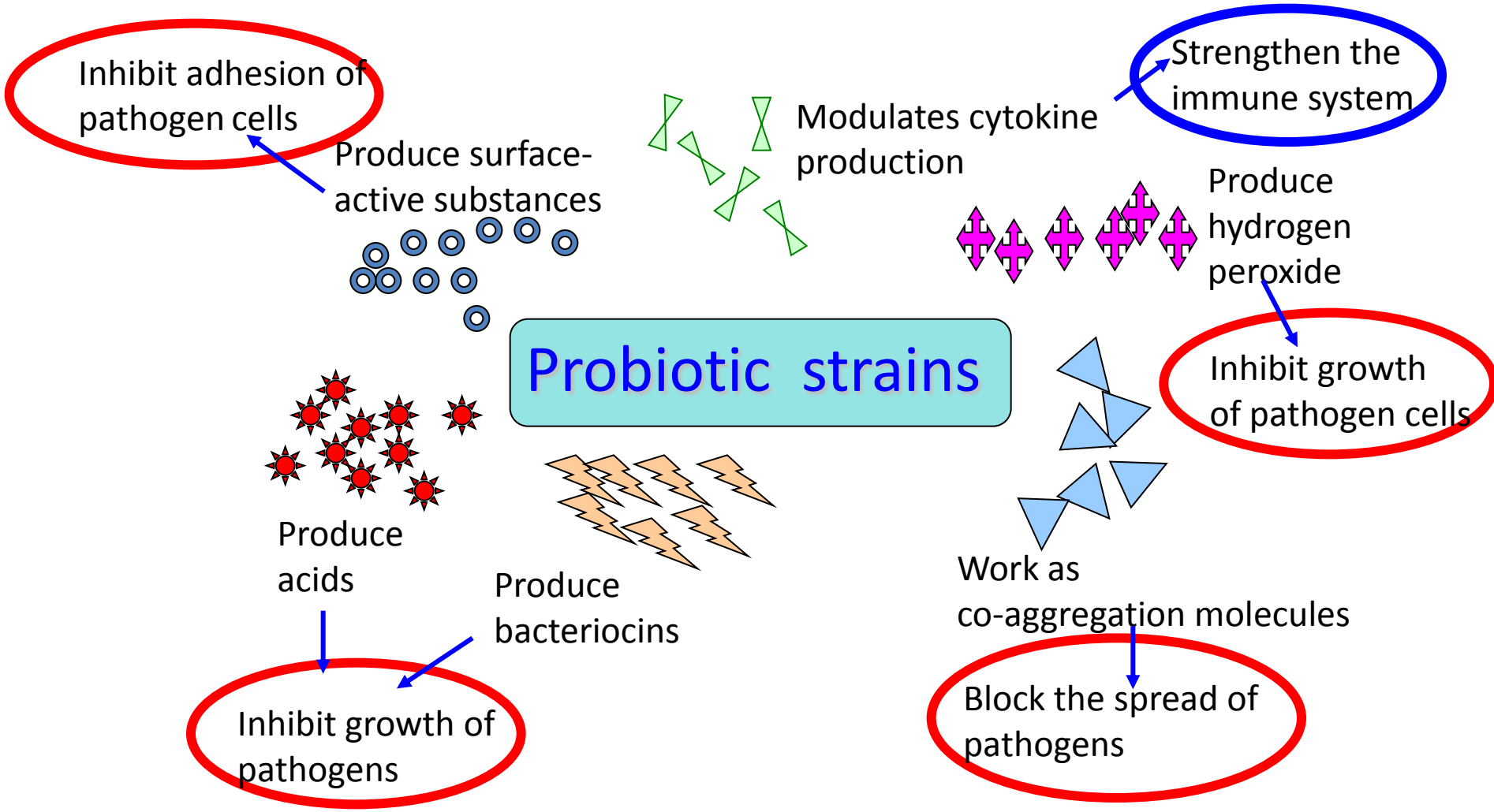


FIGURE 19.4
Changes in the fecal flora with increased age (Schematic model from Reference 14.)

Probiotics – Mechanisms of Action



Some probiotic strains with scientific documentation on beneficial effects

- lactobacillus johnSonii LA1
- lactobacillus rhamnosus GG
- lactobacillus acidophilus NCFB 1748
- lactobacillus acidophilus 74-2
- Lactobacillus gasseri ADH
- Lactobacillus reuteri ATCC 55370
- lactobacillus paracasei subs. paracasei Shirota
- L. paracasei suhsp. subs. paracasei Actimel
- lactobacillus plantarum DSM 9843
- Streptococcus thermophilus
- L. delbl: subsp. bulgaricus
- Bifidobacterium lactis BB-12
- Bifiaidobacterium longum BB- 536

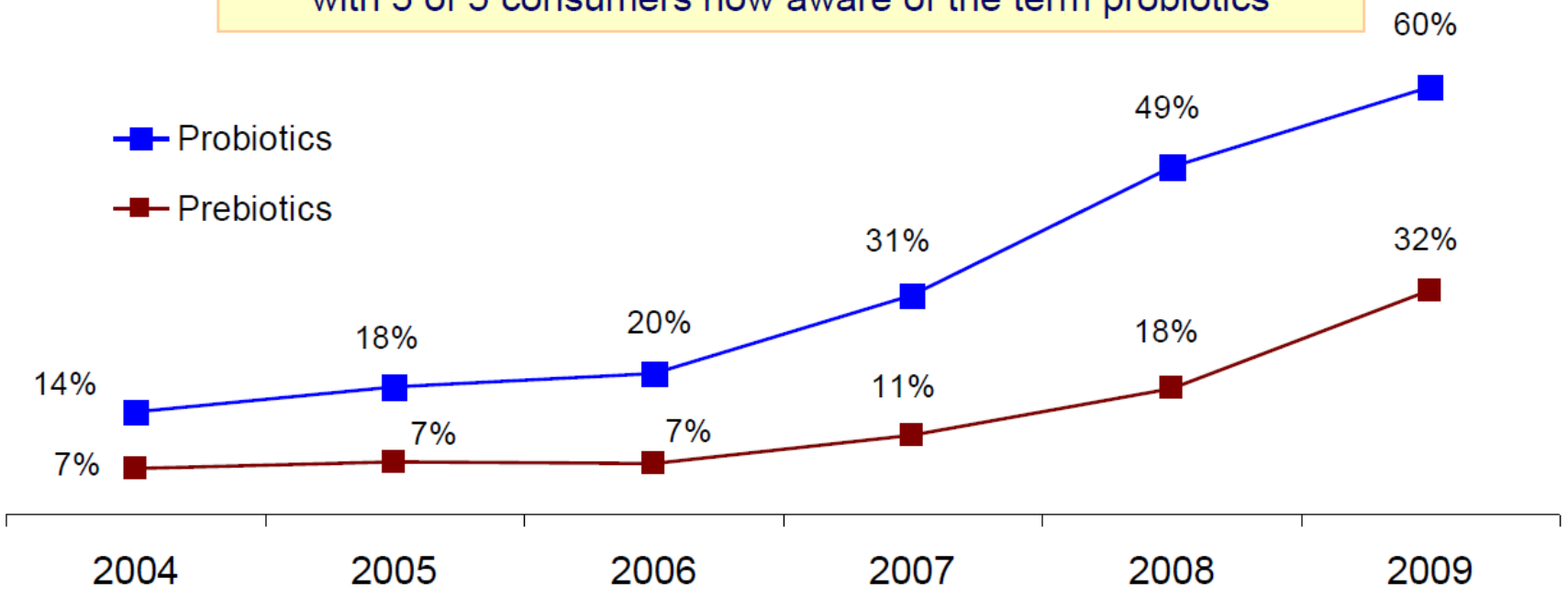
Probiotics in the Current Marketplace

- Growing public and scientific interest in probiotics
- Global probiotic market estimated at billions of dollars per year
- Hundreds of probiotics available as food, dietary supplements, skin and pet products

Awareness of Probiotics in the Current Marketplace

Awareness of the terms are moving into "mainstream"…

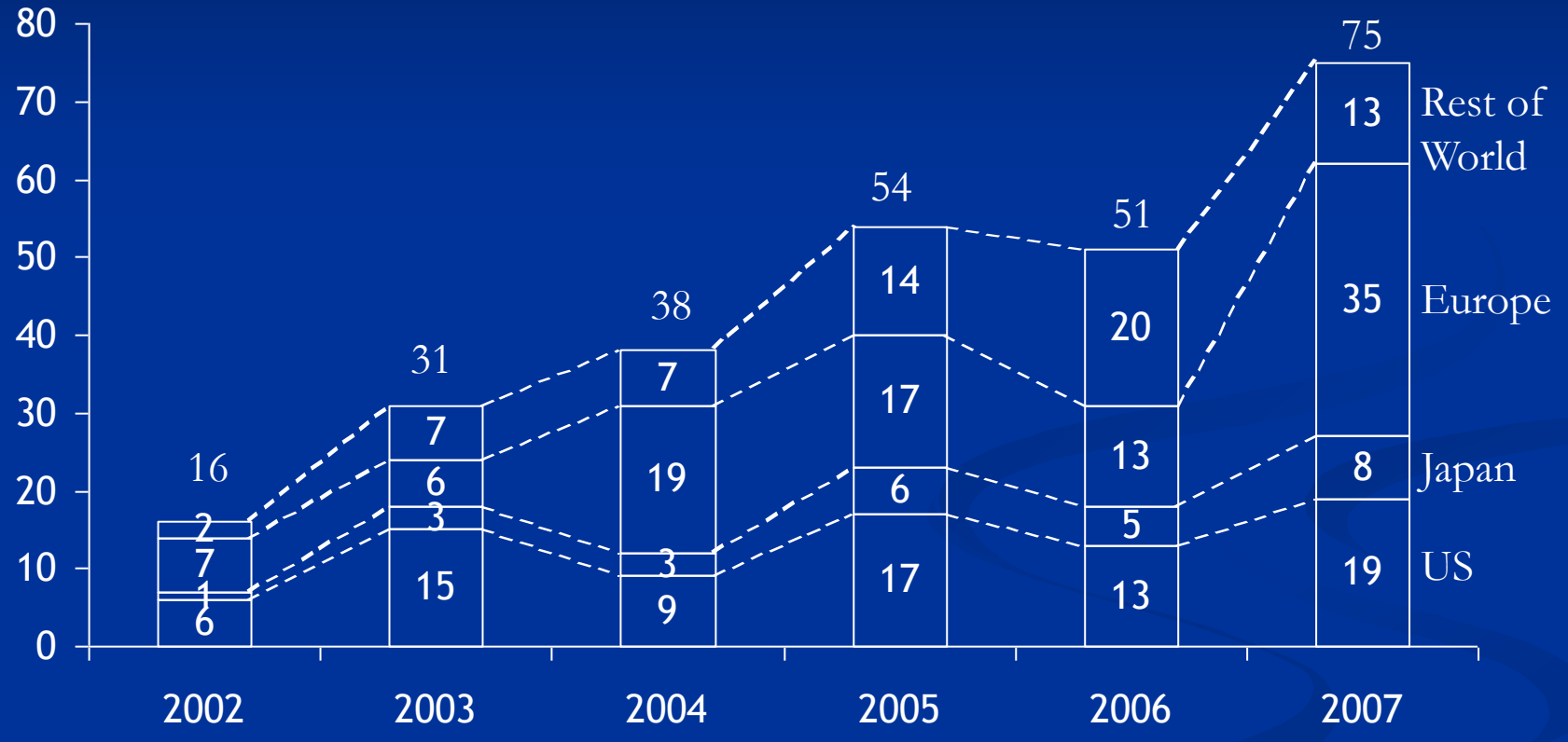
with 3 of 5 consumers now aware of the term probiotics



Natural Marketing Institute (NMI) 2009 Supplement/OTC/Rx Database™

Increasing number of dietary supplement probiotic product launches in 2007 (driven by Europe)

Probiotic product launches globally; dietary supplements (#)



Source: Mintel database; Global launches

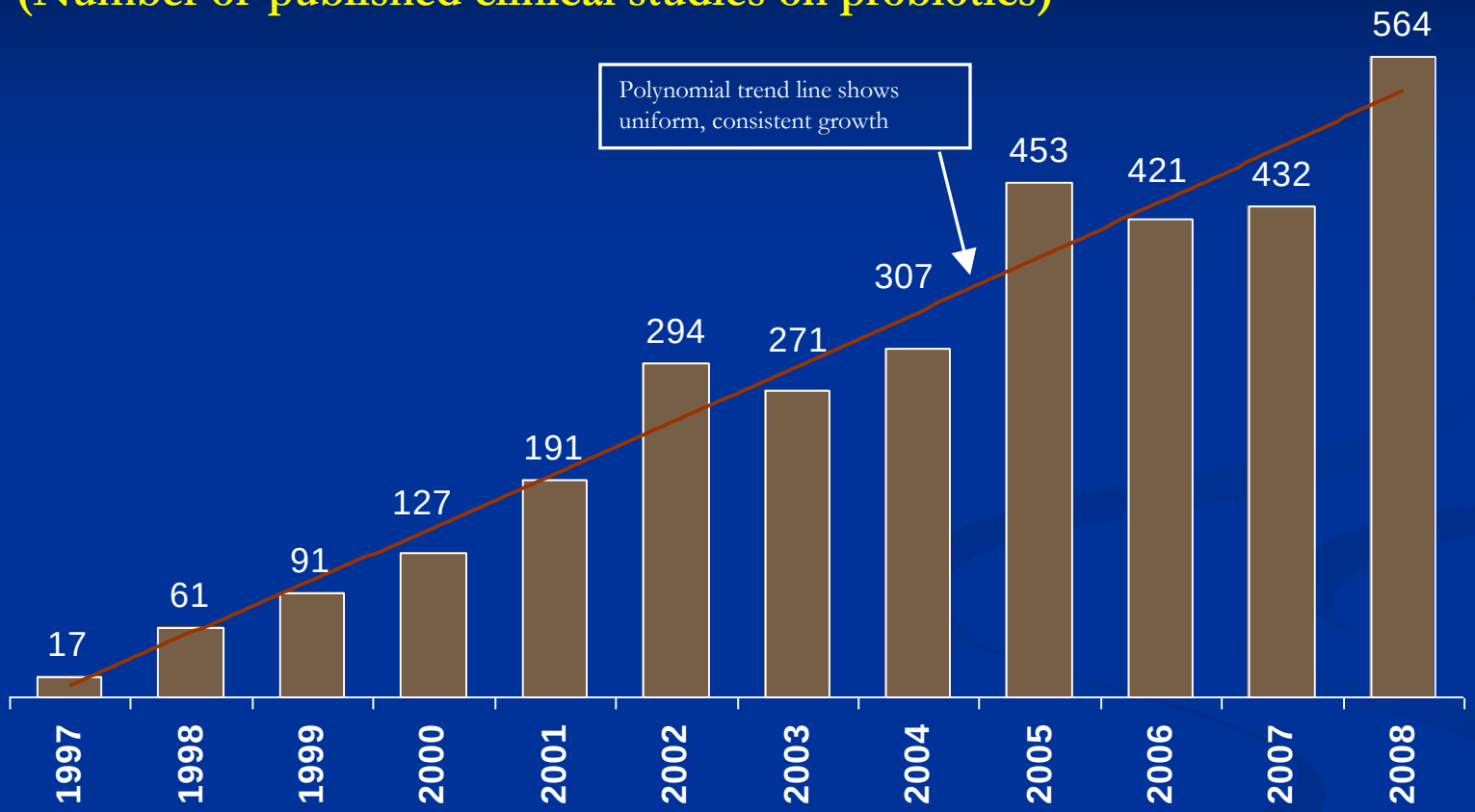
~35% average annual growth last 5 years

Scientific interest in probiotics has grown significantly since 2000…

PubMed Articles on Probiotics (#)

Clinical Research Activities Trended

(Number of published clinical studies on probiotics)



Source: www.ncbi.nlm.nih.gov/pubmed

Not Allowable Claims

The FDA does not allow any statements on a **food or supplement** that would communicate benefits on:

- ✓Reducing the risk of acute diseases (colds, flu, GI infections)
- ✓Managing symptoms in people who are not healthy (in-patients)
- ✓Improving therapeutic efficacy of a drug
- ✓Managing side effects of a drug (e.g., antibiotics)
  (Even if such use is recognized as safe in the target populations)

- Probiotics in the US are food or dietary supplements and therefore regulated by the Dietary Supplements Health and Education Act (DSHEA)

- Claims are required by FDA to be “truthful and not misleading” and supported by “competent and reliable scientific evidence”

DSHEA: Probiotic Claims

Supports a healthy immune system*
Helps keep your microflora in balance*
Helps build and maintain a healthy digestive system*

Disclaimer:

*This statement has not been evaluated by the Food and Drug Administration.

This product is not intended to diagnose, treat, cure, or prevent any disease.

DSHEA: Dietary Supplements Health and Education Act

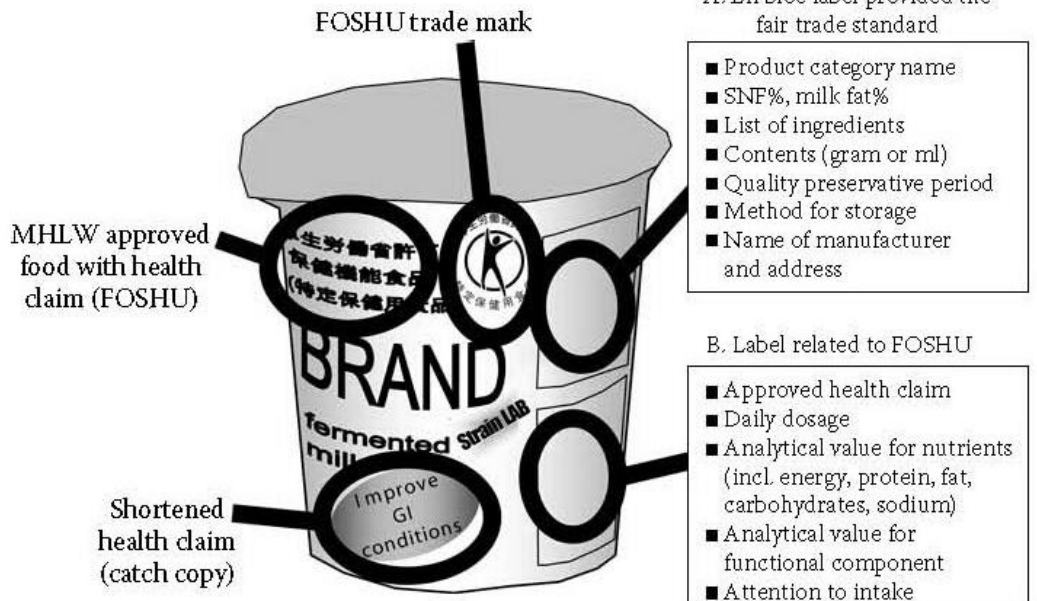


FIGURE 19.2
Package example for FOSHU product.

Probiotics: Quality Control

- Source (animal vs human; normal vs diseased)
- Formulation (vehicle)
- Safety (in at risk populations)
- Characterization (strain purity)
- Viability & Activity (Cfu delivered)
- Dose (Dose-response studies)
- Combinations/cocktails (Different effects of different bacterial strains)

Safety of Probiotics

- The safety of probiotics is strain-specific.

- The genus and species of the microbe being used should be assessed with respect to:
  - **Genetic stability,**
  - **Metabolic activities,**
  - **Potential for pathogenicity or toxicogenicity**
  - **Method of administration**
  - **Level of exposure,**
  - **Health status of the users**
  - **Physiologic functions**

- Although probiotics marketed as foods and dietary supplements should be safe for the generally healthy population, their safety has not been asserted on individuals with underlying health conditions.

safety assessments

There have been some reports that have identified LAB associated with clinical pathological conditions such as bacteraemia (Bayer *et al. 1978) and occasionally endocarditis and abscess* (Aguirre and Collins 1993), it is unlikely that LAB were the causative agents of these cases (Gasser 1994).

- Safety concerns have been raised, however, because probiotic LAB have been isolated from patients with
- endocarditis (Presterl et al., 2001; Wallet et al., 2002; Zé-Zé et al., 2004),
- sepsis (Salminen et al., 2004; Ha et al., 1999; Aguirre and Collins, 1993),
- liver abscesses (Rautio et al., 1999; Cukovic-Cavka et al., 2006)
- urinary tract infections (Aznar et al., 2004).
- Recently, probiotics were associated with an increased risk of mortality in patients with severe acute pancreatitis (Besselink et al., 2008).

Side Effects of Probiotics

- Rare cases cause bloating, diarrhea, abdominal pain.
- People having on underlying disease or compromised immune system cause potential health problems like skin rash, fever, bloody stools etc.
- Sometimes interact with immuno-supressive drugs leading to life threat conditions. So people taking such drugs should avoid it.
- Although there have been some reports that have identified LAB associated with :
- bacteraemia (Bayer *et al. 1978)*
- *endocarditis and abscess* (Aguirre and Collins 1993),

Thus,

safety assessments

are recommended for

probiotic strains

that are aimed to be incorporated into

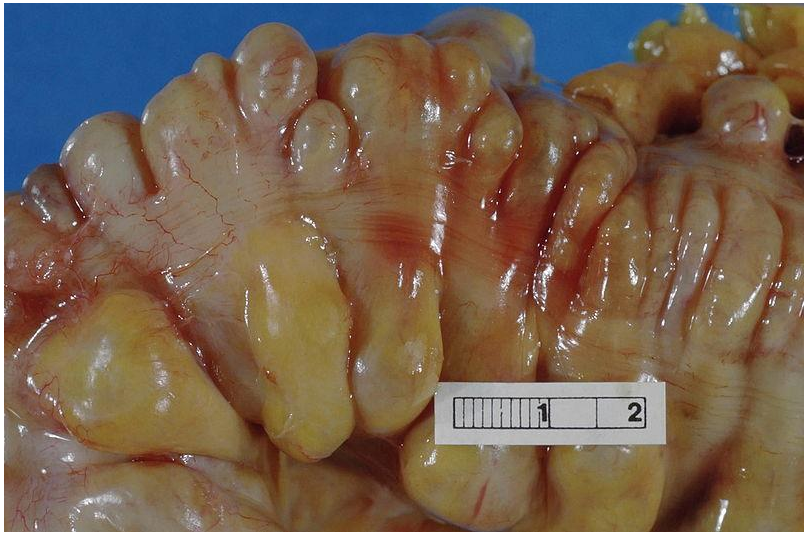
food products .

Translocation by intestinal bacteria

- **Translocation by intestinal bacteria is facilitated by numerous factors including :**
- intestinal mucosal injury,
- immunodeficiency,
- gut prematurity
- and abnormal bacterial flora (e.g. overgrowth)
- Risk factors for translocation have been studied in the mouse model. Zhou et al. evaluated whether 3 probiotics (*Lactobacillus rhamnosus HN001* (DR20(TM), *Lactobacillus acidophilus HN017 and Bifidobacterium lactis HN019 (DR10)) could translocate and cause invasive infection* in an oral mouse model.

Safety of probiotics

- adhesion of *Lactobacillus spp, to* human intestinal mucosa of patients with diverticulitis, rectal carcinoma, and irritable bowel disease (IBD) (Ouwehand et al.)
- All strains were more adherent to mucus than whole tissue.



However, it is not clear whether the in-vitro finding of greater adherence to mucus predicts ability of the bacteria to translocate.

- In February 2008, Besselink et al. reported the results of a randomized clinical trial in which a 16 percent mortality rate was observed in patients with predicted acute pancreatitis who were treated with a multispecies probiotic preparation consisting of 4 *Lactobacillus species and 2 Bifidobacteria* species, vs. 6 percent mortality in the placebo treated group .
- There were 9 cases of bowel ischemia in the probiotic treated patients, 8 of whom died, vs. no cases in the placebo group.
- The reason for the excess deaths is still unanswered

TABLE 1
Cases of bacterial sepsis temporally related to probiotic use in humans[1]

Study	Age	Risk factors	Probiotic	Method of identification[2]	Form of sepsis
Rautio et al (24)	74 y	Diabetes mellitus	LGG	API 50 CH, PFGE of DNA restriction fragments	Liver abscess
Mackay et al (25)	67 y	Mitral regurgitation, dental extraction	*Lactobacillus rhamnosus,* 3×10^9 CFU/d	API 50 CH, pyrolysis mass spectrometry	Endocarditis
Kunz et al (26)	3 mo	Prematurity, short-gut syndrome	LGG	No confirmatory typing	Bacteremia
	10 wk	Prematurity, inflamed intestine, short-gut syndrome	LGG	PFGE of DNA restriction fragments	Bacteremia
De Groote et al (27)	11 mo	Prematurity, gastrostomy, short-gut syndrome, CVC, parenteral nutrition, rotavirus diarrhea	LGG, 1/4 capsule/d	rRNA sequencing	Bacteremia
Land et al (28)	4 mo	Cardiac surgery, antibiotic diarrhea	LGG, 10^{10} CFU/d	Repetitive element sequence-based PCR DNA fingerprinting	Endocarditis
	6 y	Cerebral palsy, jejunostomy feeding, CVC, antibiotic-associated diarrhea	LGG, 10^{10} CFU/d	Repetitive element sequence-based PCR DNA fingerprinting	Bacteremia
Richard et al (29)	47 y	Not stated	*Bacillus subtilis,* 8×10^9 spores/d	Antibiotic susceptibility	Bacteremia
	25 y	Not stated	*B. subtilis,* 8×10^9 spores/d	Antibiotic susceptibility	Bacteremia
	63 y	Neoplastic disease	*B. subtilis,* 8×10^9 spores/d	Antibiotic susceptibility	Bacteremia
	79 y	Not stated	*B. subtilis,* 8×10^9 spores/d	Antibiotic susceptibility	Bacteremia
Oggioni et al (30, 31)[3]	73 y	Chronic lymphocytic leukemia	*B. subtilis,* 10^9 spores/d	16S rRNA sequencing	Bacteremia

[1] Where no dose is given, there was no precise dose described in the original publication. CVC, central venous catheter; rRNA, ribosomal RNA; PFGE, pulsed-field gel electrophoresis; PCR, polymerase chain reaction; LGG, *Lactobacillus rhamnosus* GG; CFU, colony forming units.

[2] API 50 CH; BioMerieux, Hazelwood, MI.

[3] Fatal outcome not clearly related to probiotic sepsis.

TABLE 2
Cases of fungal sepsis temporally related to probiotic use in humans[1]

Study	Age	Risk factors	Probiotic[2]	Method of identification[3]	Form of sepsis
Hennequin et al (32)	30 mo	Cystic fibrosis, CVC, poor nutritional state, intestinal surgery	*Saccharomyses boulardii*, 750 mg/d	PFGE of mitochondrial DNA restriction fragments	Fungemia
	36 y	HIV infection, CVC, diarrhea	*S. boulardii*, 1.5 g/d	PFGE of mitochondrial DNA restriction fragments	Fungemia
	47 y	Antibiotic-associated diarrhea, upper GI surgery for malignancy	*S. boulardii*, 2 g/d	PFGE of mitochondrial DNA restriction fragments	Septic shock
	78 y	Peptic ulcer, chronic renal failure, pneumonia, COPD	*S. boulardii*, 1.5 g/d	PFGE of mitochondrial DNA restriction fragments	Fungemia
Cassone et al (33)[4]	34 y	CVC, intensive care unit	No direct treatment	PFGE of undigested chromosomal DNA	Fungemia
	48 y	CVC, intensive care unit	No direct treatment	PFGE of undigested chromosomal DNA	Fungemia
	75 y	CVC, intensive care unit	No direct treatment	PFGE of undigested chromosomal DNA	CVC colonization
	35 y	Intensive care unit	Unclear	PFGE of undigested chromosomal DNA	Fungemia
Perapoch et al (34)	3 mo	CVC, diarrhea, parenteral nutrition	*S. boulardii*, 100 mg/d	PFGE of mitochondrial DNA restriction fragments	Fungemia
	Infant	Short-bowel syndrome, CVC, parenteral nutrition	Not received directly (no direct treatment)	PFGE of mitochondrial DNA restriction fragments PFGE of undigested chromosomal DNA	Fungemia
Lherm et al (35)[5]	50–82 y	Acutely unwell on intensive care unit with respiratory failure, CVC	*S. boulardii*, 1.5–3.0 g/d	PFGE of nuclear and mitochondrial DNA restriction fragments	Fungemia
Bassetti et al (36)	51 y	Immunosuppression, *Clostridium difficile*–associated diarrhea, CVC	*S. boulardii*, 1 g/d	PFGE of DNA restriction fragments	Fungemia
Riquelme et al (37)	42 y	Kidney and pancreas transplant, immunosuppression, *C. difficile*–associated diarrhea	*S. boulardii*, 1 g/d	PFGE of DNA restriction fragments	Fungemia
	41 y	HIV, diarrhea	*S. boulardii*, 750 mg/d	PFGE of DNA restriction fragments	Fungemia
Fredenucci et al (38)	49 y	Antibiotic-associated diarrhea, immunosuppression	*S. boulardii*, 200 mg/d	PFGE of undigested chromosomal DNA API 32C	Fungemia
Cesaro et al (39)	8 mo	Acute myeloid leukemia, CVC, neutropenia	*S. boulardii*	API 32C	Fungemia
Cherifi et al (40)	89 y	*C. difficile*–associated, colitis, gastrostomy	*S. boulardii*, 300 mg/d	No formal identification described	Fungemia
Henry et al (41)	65 y	Malignancy, immunecompromise, mucositis, diarrhea, parenteral nutrition	*S. boulardii*	No formal identification described	Fungemia
Niault et al (42)	78 y	Antibiotic-associated diarrhea, intensive care unit, intragastric feeding	*S. boulardii*, 1.5 g/d	No formal identification described	Fungemia
Viggiano et al (43)	14 mo	Burns, diarrhea, gastrostomy	*S. boulardii*, 200 mg/d	No formal identification described	Fungemic shock
Zunic et al (44)	33 y	Inflammatory bowel disease, intensive care unit, parenteral nutrition	*S. boulardii*, 1.5 g/d	No formal identification described	Fungemia
Pletincx et al (45)	1 y	Parenteral nutrition, antibiotic-associated diarrhea, CVC	*S. boulardii*, 600 mg/d	No formal identification described	Septicemia
Rijnders et al (46)[5]	74 y	Colitis, nasogastric feeding	*S. boulardii*, 600 mg/d	No formal identification described	Fungemia
Lestin et al (47)[6]	48 y	Diabetes, *C. difficile*–associated diarrhea	*S. boulardii*, 150 mg/d	API 32C	Fatal fungemia

[1] CVC, central venous catheter; COPD, chronic obstructive pulmonary disease; PFGE, pulsed-field gel electrophoresis; GI, gastrointestinal.
[2] 250 mg *S. boulardii* = 5.425×10^{13} live cells.
[3] API 32C; BioMerieux, Hazelwood, MI.
[4] Cases thought to be related to *S. boulardii* treatment of neighboring intensive-care-unit patients.
[5] Fatal outcome ($n = 3$) not clearly related to probiotic sepsis.
[6] Fatal fungemia in association with toxic megacolon; death thought to be related to probiotic sepsis.

The Safety of Probiotics in Foods in Europe and Its Legislation

- in Europe the annual consumption of pure LAB biomass, from fermented dairy products alone is estimated to be 3400 t

Safety Assessment of Probiotics

- 1) Acute Toxicity
- 2) Endocarditis
- 3) Effect on the Immune System
- 4) Metabolism
- 5) Hemolysis
- 6) Resistance to Human Serum-Mediated Killing
- 7) Induction of Respiratory Burst
- 8) Platelet Aggregation
- 9) Adhesion to Extracellular Matrix Proteins and Intestinal Mucus
- 10) Adhesion to Tissue Culture Cells
- 11) Phosphatidyl-inositol-Specific Phospholipase C (PI-PLC) Activity
- 12) Mucinolytic Activity
- 13) Antibiotic Resistance
- 14) Taxonomy

1) Acute Toxicity

- The toxicity of LAB strains to the GI tract mucosa is considered an important aspect of safety, as most probiotics are intended for oral consumption.
- Balb-c, Swiss mice or Lewis rats are commonly used to assess acute toxicity.
- Bacteria (10^7–10^{10} CFU/ml) can be added to the drinking water for 8 to 10 d, 50 µl of bacterial suspension can be orally fed for 28 d, or 1 ml bacterial suspension can be given by gavages for 7 d.

Acute Toxicity

- 1) Changes in activity, behavior, and general health are observed daily.
- 2) Water intake, feed intake, and body weight are measured weekly.
- 3) pathological changes: The animals are euthanized after the experiments, and the organs are examined for gross pathological changes .
- 4) mucin degradation: The contents of the distal part of the ileum, cecum, and colon can be dissolved and homogenized, after which mucin degradation can be determined colorimetrically .
- 5) Bacterial translocation can be measured by plating blood samples taken by cardiac puncture and by plating homogenized tissue samples of the mesenteric lymph nodes, spleen, and liver.

  A : Microbiological :The plates are incubated at 37˚C for 72 h.

  B: The organisms are identified by randomly amplified polymorphic DNA (RAPD) polymerase chain reaction (PCR) .
- 6) Histological examination: part of the stomach, distal part of the ileum, cecum, and colon are removed, fixed for histological examination

Acute Toxicity of Probiotic Lactic Acid Bacteria in Mice

Species	Strain	LD_{50} (g/kg body weight)
Bifidobacterium lactis	HN019	>50
Bifidobacterium longum	[a]	25
Bifidobacterium longum	BB-536	>50 (0.52 via intraperitoneal route)
Enterococcus faecium	AD1050 [b]	>6.60
Lactobacillus acidophilus	HN017	>50
Lactobacillus casei	Shirota	>2.00
Lactobacillus delbrueckii subsp. bulgaricus	[a]	>6.00
Lactobacillus fermentum	AD0002[b]	>6.62
Lactobacillus helveticus	[a]	>6.00
Lactobacillus rhamnosus	GG (ATCC 53103)	>6.00
Lactobacillus rhamnosus	HN001	>50
Lactobacillus salivarius	AD0001 [b]	>6.47
Streptococcus equinus	[a,b]	>6.39

[a] Strain not known.

[b] Nonviable, heat-inactivated, preparations.

Source: Modified after Reference 104.

2) Endocarditis

- *L. casei and L. rhamnosus are the organisms most commonly associated with* infective endocarditis (IE), although the incidence of *Lactobacillus endocarditis* is very low .
- Nevertheless, a European Union-sponsored workshop organized by the Lactic Acid Bacteria Industrial Platform proposed that the safety of each strain should be assessed .
- Several models of IE have been developed, such as a rabbit model using specific-pathogen-free male Japanese white rabbits.

Endocarditis

- In this model, a polyethylene catheter is passed down the artery and positioned in the left ventricle.
- During 7 to 10 d, small vegetations of platelets and fibrin can form on the tricuspid valve or the endocardium at points of contact with the catheter.
- After this period, 1 ml of bacterial suspension, (10^9 CFU/ml), is injected into the marginal ear vein.
- Blood samples are taken at intervals from the opposite marginal ear vein.
- The animals are killed, and small pieces of liver, spleen, and vegetations at the heart valve are removed, weighed, and homogenized in 1 ml of saline.
- The blood samples and organ samples at 0.1 ml are plated, and colony counts are performed.

3) Effect on the Immune System

cytokine profiles

Several models have been developed to study the effect of LAB on the immune system.

Table 14. Summary of ELISA results

	TNFα	TGFβ	IFNγ	IL-4	IL-2	IL-5	IL-6	IL-8	IL-10	IL-12
433118	+	+/-	+/-	+	+/-	-	-	-	+/-	+/-
42319	+	+/-	+	+/-	+/-	+	+/-	-	+/-	+
35624	+	+/-	+	+	+	-	+/-	-	+/-	-
4331	-	+/-	+/-	+	+	+/-	-	+/-	+/-	-
43338	+/-	+/-	+/-	+/-	+/-	-	-	-	+/-	-
42361	+	+/-	+/-	+	+/-	-	-	+/-	+/-	+/-
LJ1	+/-	-	+/-	+/-	+/-	-	-	-	+/-	+/-
BB12	+	+/-	+/-	+	+/-	-	+/-	+	+/-	+/-
Danon	+	-	+	+/-	+/-	+	+/-	-	+/-	+
299V	-	-	+/-	+/-	+/-	-	-	-	+/-	+/-
Shirota	+	+/-	+	+/-	+/-	+	+/-	+/-	+/-	+/-
GG	-	-	+/-	+/-	+/-	-	-	-	+/-	-

+ = Greater than control value.
- = Less control value.
+/- = Similar concentration to control.

Local cytokine profiles either T helper cell type 1 (Th1) immunogenic responses, which may be the predominant response in autoimmune diseases, or T helper cell type 2 (Th2) tolerogenic responses, which may contribute to diseases such as atopic allergy .

The pro-inflammatory cytokines secreted by the epithelium, such as TNF-α, IL-1, IL-6, IL-8, and IL-12, are the hallmarks of the inflammatory responses of the intestine .

TEST: ELISA &

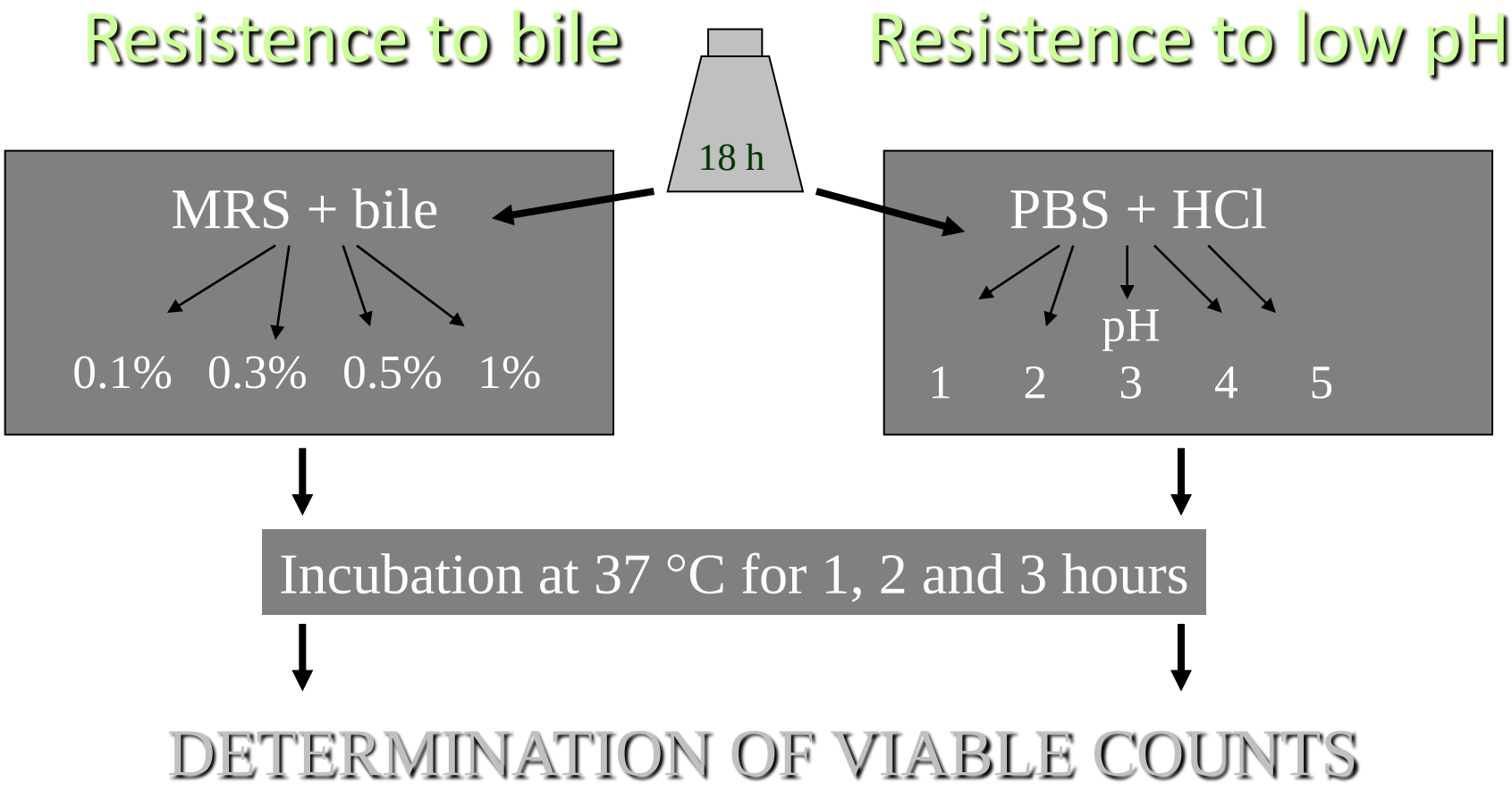
Experimental Autoimmune Encephalomyelitis

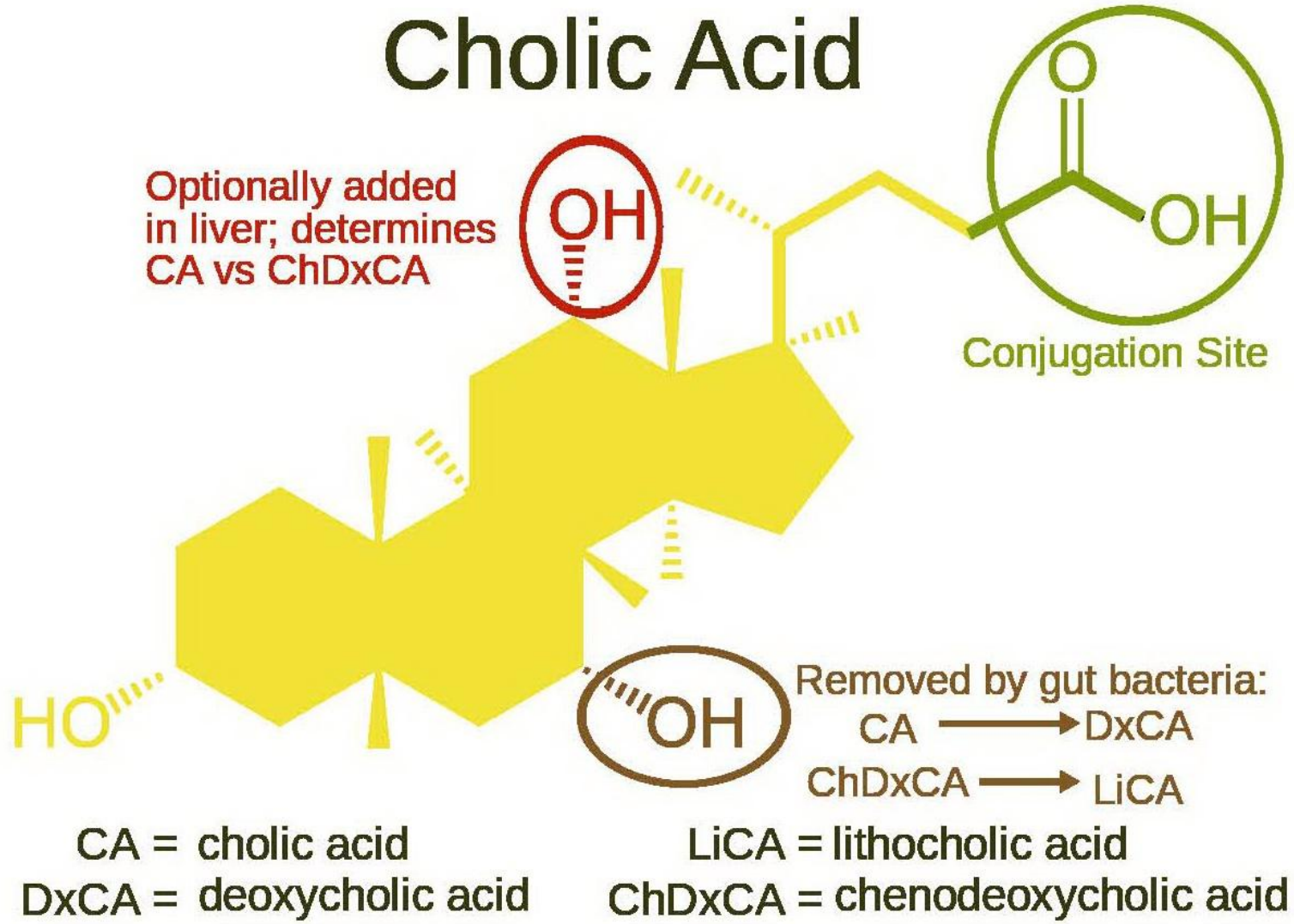
- In mice, strain-dependent cytokine profiles are induced after oral administration of *Lactobacillus* .
- In SJL/J mice, lactobacilli are able to enhance or inhibit the development of disease after induction of experimental autoimmune encephalomyelitis (EAE) .
- Female SJL/J mice 6 to 12 weeks old, which have a low microbiological burden and low IgE levels, or BALB/c mice, which are Th2 biased, are used.
- The mice receive a 10^{10} CFU/ml bacterial suspension intra gastrically on 5 alternating days to create a continuous high level of lactobacilli.
- On day 0, the mice are subcutaneously immunized with 50 to 300 µg proteolipid protein (PLP 139-151) in Freund's complete adjuvant to induce acute EAE.

- On days 0 to 1 and days 2 to 3 the mice are intravenously injected with 10^{10} CFU/ml of *Bordetella pertussis to affect the integrity of the blood-brain barrier.*
- *The severity of the* EAE can be scored using the disability scale:
- 0 = no clinical signs,
- 1 = tail weakness,
- 2 = mild paralysis and ataxia of the hind legs,
- 3 = severe paralysis or ataxia of the hind legs,
- 4 = moribund
- 5 = death due to EAE.
- EAE develops at days 12 to 16 after immunization with a highest score of 3.

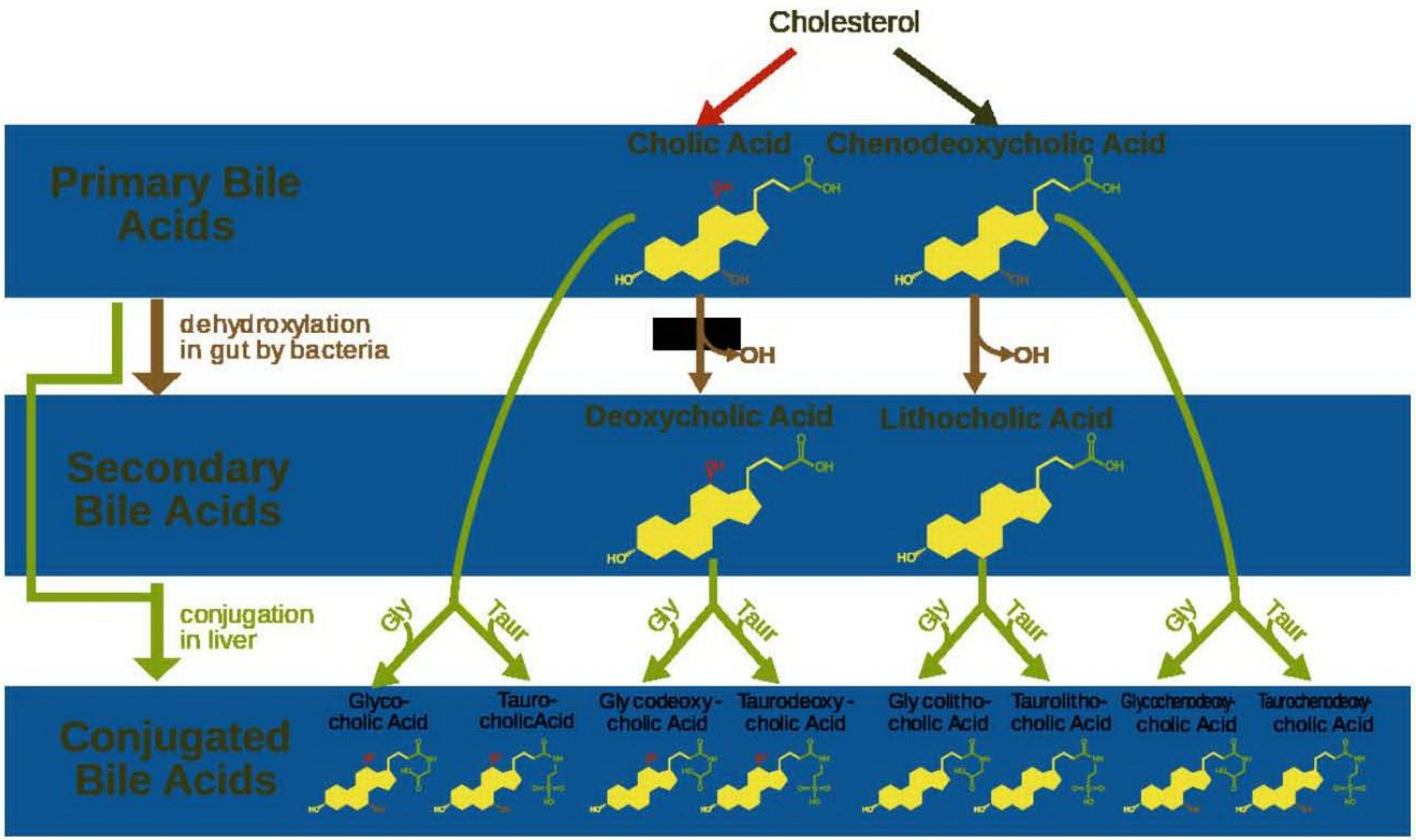
- Blood samples are taken by tail vein puncture on different days.
- ELISA is used to detect anti-PLP (Proteolipid Protein) antibodies.
- Immunohistochemical analysis is performed on cryosections (8 µm) from snap frozen material.
- For the detection of *cytokine* profiles(IL-1α, IL-1β, IL-2, IL-4, IL-10, IFN-γ, and TNF-α), monoclonal antibodies with an anti-mouse/rat Ig-biotin (Dako) and an HRP-labeled avidin-biotin complex (Dako) are used .

4) Metabolism





Structure of cholic acid showing relationship to other bile acids



Synthesis of the remaining 10 bile acids (2 secondary, 8 conjugated) from the two primary bile acids.

Metabolism

- Lactobacilli are capable of transforming conjugated primary bile salts into free deconjugated and secondary dehydroxylated bile salts in the small bowel .
- The free bile salts may induce diarrhea and intestinal lesions , whereas secondary bile salts may exhibit carcinogenicity .
- Excessive deconjugation or dehydroxylation of bile salts by probiotics, through production of the enzyme bile salt hydrolase, could therefore be a potential risk .

- Amounts of bile salts deconjugated can be determined using high-performance liquid chromatography (HPLC).
- Samples can be taken before introduction of bile salt into the bacterial suspension, then every 60 min for 6 h and after 24 h.
- For quantification of conjugated bile salts, 200 µl of sample is mixed with 50 µl of internal standard (dexamethasone); 20 µl of the mixture is then passed through a 0.45-µm nylon filter and injected into the HPLC.
- For quantification of free bile salts, 200 µl of cholic acid is filtered through a 0.45-µm polysulfone filter and mixed with 50 µl internal standard (testosterone). Of this mixture, 20 µl is then injected into the HPLC.
- For quantification of deconjugated bile salts, 2 ml of sample is resuspended in 8 ml 0.9% NaCl in 0.1 *M NaOH and 6 ml* of mobile phase. The bile salts are recovered by passage through a Sep-Pac cartridge. Dexamethasone is added to the mobile phase as an internal standard.

- LAB can produce two stereo-isomers of lactic acid, D and L lactic acid. Since some LAB also have racemases, some strains will produce D/L lactate.
- L-lactate is readily metabolised whereas the D-isomer is not.
- There have been concerns about infants, in particular, ingesting high levels of D-lactic acid and there is a maximum recommended intake level for D-lactate.
- Because Lb. acidophilus produces D-lactic acid there is some interest in using other probiotic lactobacilli or bifidobacteria in products for babies and young children.
- Several cases of D-lactic acid acidosis have been described in patients that have had intestinal bypass surgery.
- This condition is associated with transient neurological symptoms including headaches, weakness and visual disturbances
- . The D-lactic acid acidosis has been shown to be due to the overgrowth of Lb. acidophilus in the small intestine, generally due to its selection by antibiotic therapy.

Iranian Biomedical Journal 6 (2 & 3): 69-75 (April & July 2002)

Comparison of Lactic Acid Isomers Produced by Lactic Acid Bacteria

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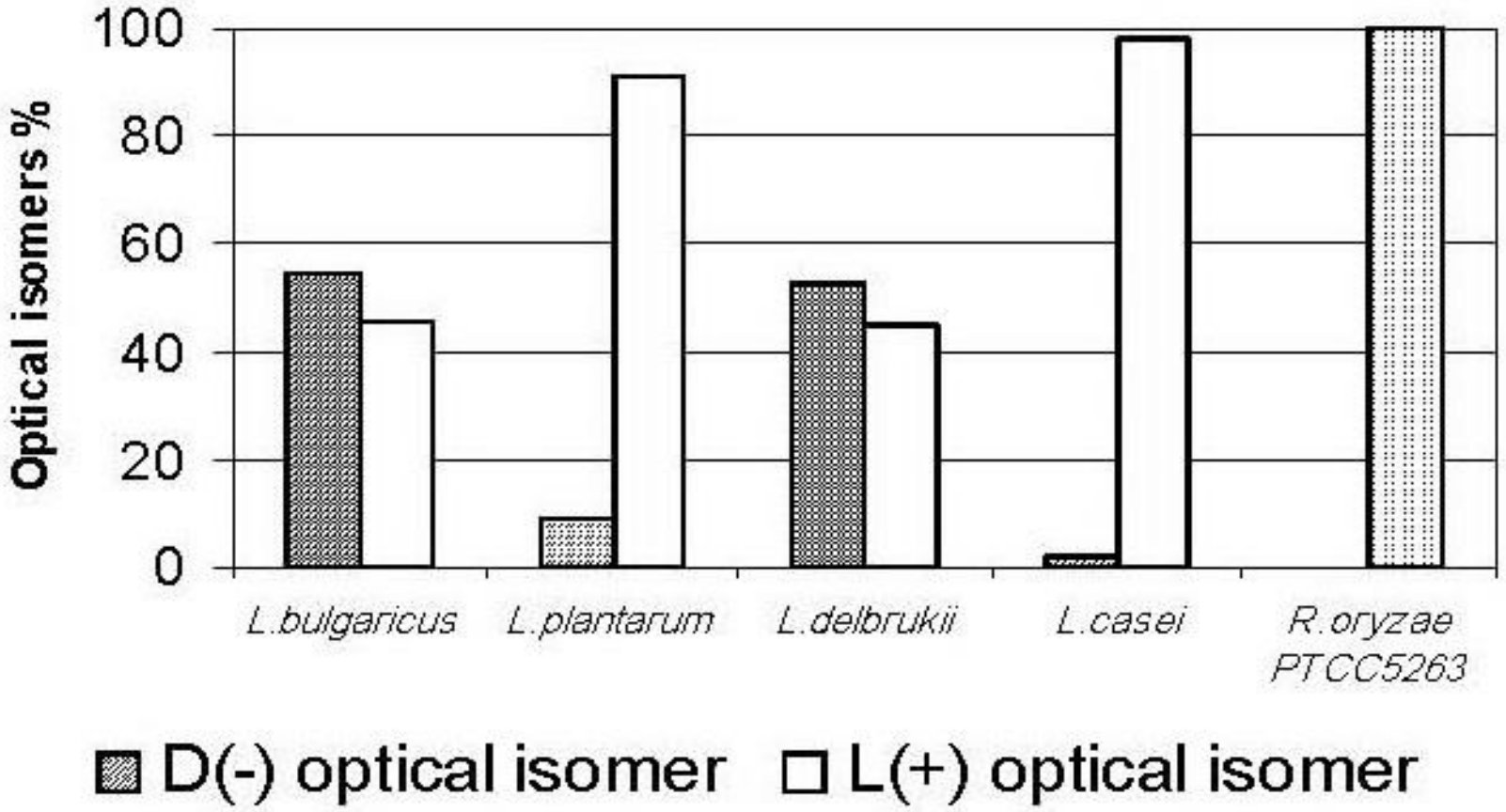
ABSTRACT

Many organisms produce lactic acid by fermentation, but most industrially important strains are from the genus *Lactobacillus* and *Rhizopus oryzae*. L(+)-Lactic acid is the only optical isomer for use in pharmaceutical and food industries because human body is only adapted to assimilate this form. In this research, six strains of *Lactobacillus* and four strains of *R. oryzae* (known as high producer) were examined for optical isomers of lactic acid. The production of lactic acid was improved and lactic acid produced in submerged media on rotary shaker incubator. The optical isomers of lactic acid were examined by L(+) and D(-) lactate dehydrogenase kit. All the *R. oryzae* strains tested produced only L(+) isomer of lactic acid. The highest fungal and bacterial producer strains were *R. oryzae* PTCC 5263, *Lactobacillus plantarum* PTCC 1058, *L. Bulgaricus* PTCC 1332 *and L. delbruekii subsp delbruekii* PTCC 1333. *Lactobacilli* strains produced combination of both optical isomers of lactic acid. Among them, *L. casei subsp. Casei* produced the low amount of D(-)-lactic acid (2%). The optimum concentration of glucose for lactic acid production by *R. oryzae* and *Lactobacillus* strains were 180 g/l and 80–120 g/l, respectively. *Iran. Biomed. J. 6 (2 & 3): 69-75, 2002*

Keywords: Lactic acid, Production, Optical isomers


Optical isomers %
100
80
60
40
20
0
L.bulgaricus
L.plantarum
L.delbrukii
L.casei
R.oryzae PTCC5263
D(-) optical isomer
L(+) optical isomer

($Y_{x/s}$ · g biomass produced/g substrate consumed , $Y_{P/s}$ · g product formed/g substrate consumed , productivity · g product formed/ h fermentation time · l volume of fermentation medium)

Strain	Dry Mass g/l	Production g/l	$Y_{x/s}$ g/g	Productivity g/lh	$Y_{p/s}$ g/g
L. bulgaricus PTCC 1332	9.00	24.00	0.15	0.33	0.40
L.plantarum PTCC 1058	9.12	29.00	0.15	0.40	0.83
L.delbruekii subsp delbruekii PTCC 1333	8.76	15.00	0.14	0.21	0.25
L.lichmannii PTCC 1057	7.86	1.68	0.13	0.02	0.02
L. casei PTCC 1055	8.22	6.18	0.13	0.08	0.10
L.casei subsp casei PTCC 1608	8.40	19.00	0.14	0.26	0.31
L.lactis subsp lactis PTCC 1403	7.80	2.91	0.13	0.04	0.04
R. oryzae PTCC 5263	22.28	55.00	0.18	0.92	0.46
R.oryzae PTCC 5174	19.25	41.00	0.16	0.59	0.34
R.oryzae PTCC 5175	21.22	51.00	0.17	0.85	0.45
R.oryzae PTCC 5176	20.35	31.00	0.17	0.45	0.26

Table 2: Effects of glucose concentration on lactic acid production in optimized medium.

Glucose g/l	*R. oryzae* PTCC 5263			*L. bulgaricuse* PTCC 1332			*L.plantarum* PTCC 1058		
	Production g/l	Productivity g/lh	Yield %	Production g/l	Productivity g/lh	Yield %	Production g/l	Productivity g/lh	Yield %
40	33	1.37	83.3	35.8	1.50	89.55	38.10	1.60	95.67
60	49	2.00	81.0	51.8	2.20	86.34	55.90	2.33	93.24
80	64	2.60	80.0	76.6	2.10	95.83	77.80	2.40	97.31
100	80	3.30	80.0	89.6	2.00	89.69	89.10	2.80	89.19
120	106	3.40	85.0	76.6	1.60	63.39	100.00	2.10	83.33
140	116	3.80	82.0	63.6	1.33	45.46	66.60	1.40	47.57
160	133	4.00	83.0	50.0	1.00	31.25	42.00	0.87	26.25
180	160	4.10	84.0	30.0	0.62	16.67	27.00	0.56	15.00
200	85	3.50	42.0	17.0	0.35	11.76	15.00	0.31	7.5.00

- Some LAB can produce
- biogenic amines such as putrescine, cadaverine, histamine, tyramine and 2-phenylethylamine.
- Some of these can cause unpleasant reactions (nausea, headaches, and respiratory disorders)
- including dangerously high blood pressure particularly in individuals with reduced monoamine oxidase (MAO) activity or those taking MAO inhibitors, an older class of antidepressant medication. Providing strains are screened properly biogenic amine formation should not be a problem

5) Hemolysis

- Hemolysis is a common virulence factor among pathogens that serves mainly to make iron available to the microbe and causes anemia and edema in the host.
- Strains to be tested are grown on suitable solid media containing 5% human blood and incubated under appropriate conditions. *Staphylococcus aureus and Bacillus cereus should be included as positive controls for α- and* β-hemolysis, respectively.

6) Resistance to Human Serum-Mediated Killing

- The complement system in blood can opsonize bacteria and facilitate their phagocytosis by leukocytes.
- The activated complement system can also form a complex that kills bacteria .
- Evasion of this will therefore enhance the survival of the bacteria after translocation.

- Blood is collected from at least 10 healthy adult donors and allowed to clot, and the serum is pooled in equal amounts.
- Part of the serum has to be heated to 56˚C for 20 min to inactivate the complement system.
- The bacteria (10^7 to 10^8 CFU/ml) were mixed with serum, heat-inactivated serum or PBS, to a final serum concentration of 80% and incubated aerobically 90 min at 37˚C.
- The reaction is stopped by incubating the sample 10 min on ice and making serial dilutions in PBS.
- Viablity test:

  A: Dilutions are plated on appropriate media and incubated.

  B: Alternatively, viability can be assessed using flow cytometry

7) Induction of Respiratory Burst

- Upon phagocytosis, peripheral blood mono-nucleocytes (PMNs) produce a burst of reactive oxygen species; this will kill and digest the phagocytosed particles.
- The ability to avoid the induction of such a respiratory burst may enhance the survival of a translocated microbe and hence the risk for infection.

- Bacteria are grown under appropriate conditions, washed twice with HEPES-buffered Hank's balanced salt solution (HH; 10 m*M HEPES; pH 7.4),* and the absorbance (600 nm) is adjusted to 0.5 ± 0.01.
- PMNs were collected by lysing erythrocytes in freshly collected human blood with 0.8% NH4Cl. PMNs are washed and resuspended in Hank's balanced salt solution.
- The concentration of PMNs is determined by flowcytometry
- The measurement of the respiratory burst is then performed as described by Lilius and Marnila :

Lilius and Marnila method

- To gelatin-coated microtiter plate wells (Cliniplate; Labsystems, Helsinki, Finland), 25 ml Hank's balanced salt solution containing 0.1% gelatin, 20 ml 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol; 10 m*M), 40 ml bacterial suspension were added and incubated* 30 min at 37˚C.
- Subsequently, 40 ml PMN suspension is added. For background measurements, PMNs are incubated without bacteria. The plates are incubated at 37˚C, and luminescence is measured for at least 2 h with 3-min intervals, e.g., with a Victor2 multilable reader (PerkinElmer, Turku,Finland).
- Results are presented as the maximum signal (mV/100,000 PMNs) after subtraction of the background and as the average of at least three independent observations with PMNs from different donors.

8) Platelet Aggregation

- The spontaneous aggregation of platelets leads to the formation of thrombi and edema and has been implicated in infective endocarditis .
- Platelet-rich plasma (PRP) is obtained by centrifuging fresh citrated blood 10 min at 100 . *g.*
- *Platelet-poor plasma (PPP) is obtained by further centrifuging* 10 min at 2350 . *g.*
- *The PRP is adjusted with PPP to an absorbance* (660 nm) of 0.5 ± 0.01 to give a platelet concentration of approximately 2.27 , 10^8/ml.
- The ability to induce platelet aggregation by the test strains was determined by adding 25 µl bacteria (2 . 10^9 CFU/µl) to 250 µ PRP or PPP, pre-incubated at 37◦C for 5 min.
- A lag phase of more than 25 min can be assumed to be a negative aggregation .
- Platelet aggregation is carried out in an aggrego-meter,

9) Adhesion to Extracellular Matrix Proteins and Intestinal Mucus

- Adhesion to mucus and human intestinal epithelium has been proposed as one of the main criteria for selecting potentially promising probiotics for use in humans .
- It is, however, also the first step in pathogenesis and deserves close attention.
- Collagens are the major proteins of the extracellular matrix and may be exposed in injured tissue. Pathogens often have affinity for these proteins as this affinity will give them access to host tissues .
- Fibrinogen is present in high concentrations in plasma and forms the structure of the blood clot.
- It also coats the outer surface of implanted biomaterials.
- Its presence in wounds and on foreign bodies makes fibrinogen an important substratum for microbial adhesion.

- Human collagen type IV and fibrinogen can be purchased from, e.g., Sigma ; intestinal mucus can be isolated from feces of healthy adult volunteers by aqueous extraction and dual ethanol precipitation .
- The substrata are passively immobilized (0.5 mg/ml) on microtiter plate wells by overnight incubation at 4˚°C.
- Bacteria are grown under appropriate conditions, to the culture media 10 µl/ml of tritiated thymidine (methyl-1,2-[3H]thymidine, 120 Ci/mmol) is added to metabolically radiolabel the bacteria. After growth, the bacteria are washed twice with PBS, and the absorbance (600 nm) is adjusted to 0.25 ±0.01.
- The bacteria are added to the wells and incubated for 1 h at 37꞉C.
- Nonbound bacteria are removed by washing twice with HH.
- Bound bacteria are released and lysed with 1% sodium dodecyl sulfate (SDS) in 0.1 *M NaOH* for 1 h at 60꞉C.
- Radioactivity is determined by liquid scintillation and the adhesion expressed as the percentage of radioactivity recovered after adhesion,relative to the radioactivity in the bacterial suspensions added to the immobilized collagen, fibrinogen, or mucus .

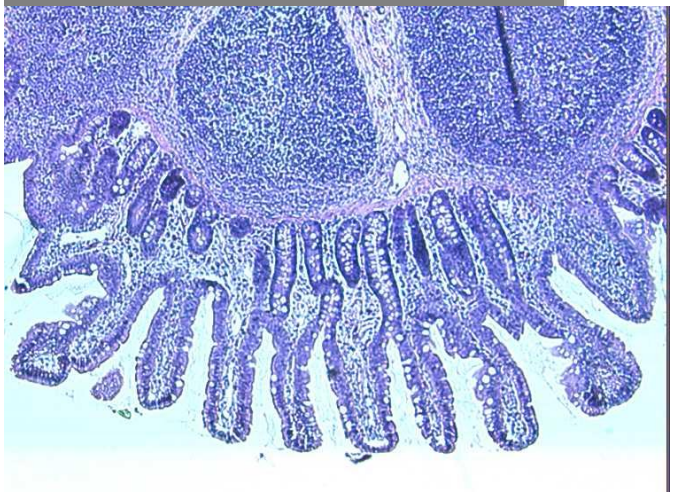
10) Adhesion to Tissue Culture Cells

Adhesion ability →

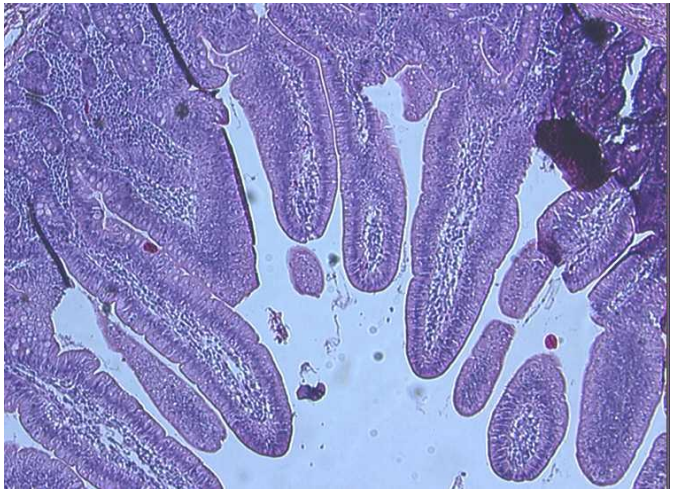
In vitro
Caco-2 cells

↓

Ex vivo



Ileum



Deodenum

Adhesion to Tissue Culture Cells

in vitro models

- ***Culturing of Intestinal Cells***
- *in vitro models* involving human intestinal epithelial cell lines (derived from colonic adenocarcinomas) have been used extensively to assess the adhesive properties of probiotics: HT-29 (ATCC 38-HTB) and Caco-2 (ATCC 2102-CRL) cells .
- The cells are cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10 to 20% heat-inactivated (56°C for 30 min) fetal calf or bovine serum (Life Technologies GIBCO, BRL, Rockville, MD) in 10% CO2/90% O2 air at 37°C . The use of 100 µg/ml streptomycin and 100 U/ml penicillin is recommended.
- The medium can also be supplemented with 1% nonessential amino acids. For the HT-29MTX cell line, DMEM with glutamax is recommended. For maintenance purposes, the cells are passaged weekly using trypsin/EDTA and seeded in 6-well plates.
- The medium is changed every 2 to 3 d and replaced by DMEM without any supplements at least 1 h prior to use for adhesion experiments.
- The cells are used at 2 weeks post confluence for the adhesion assays to allow full morphological and functional differentiation .

Adhesion to Intestinal Cells

- Bacteria are grown under appropriate conditions. Prior to the adhesion assay all bacterial strains are washed twice with PBS and resuspended in DMEM to a final concentration of 10^8 CFU/ml.
- Before the adhesion experiment the cells are washed three times with PBS.
- Different methods can be used to test the adhesion of bacteria to intestinal cells.
- **For light microscopy**, two-chamber slides or 6-well tissue culture plates containing glass coverslips are used on which the cells are seeded. Bacterial suspensions of 0.5 to 3 ml are added to each of the wells and incubated for 1 to 3 h at 37°C in 10% CO2/90% air.
- The cells are then washed three to five times with PBS to remove unbound bacteria, fixed with acetone, and stained with Giemsa or fixed with methanol and stained with Gram.
- The number of adherent bacteria are counted in 20 random microscopic areas using oil immersion.
- The assay is performed in duplicate or triplicate over several successive passages. Adhesion can be expressed as the number of bacteria adhering to 100 cells.

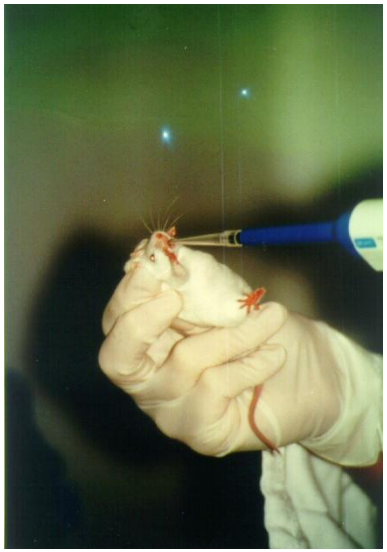
- **For scanning electron microscopy**, the adhesion experiment is performed as for light microscopy, but the cells are fixed with 2.5% glutaraldehyde in 0.1 *M phosphate buffer for 1 h at room temperature.*
- *After two washes with* phosphate buffer, cells are postfixed for 30 min at room temperature with 2% osmium tetraoxide in phosphate buffer and washed three times with the same buffer.
- The cells are dehydrated in graded series of ethanol and amyl acetate. Finally, the cells are dried in a critical point dryer and coated with .

- **For radiolabeling,** the bacteria can be labeled by addition of 14C-uracil and 14C-adenine or 14C-acetic acid or methyl-1,2-[3H] thymidine (Amersham International, UK). To remove excess radioactivity, the bacteria are washed with PBS + 0.1% sodium azide. The cells are prepared on glass coverslips in

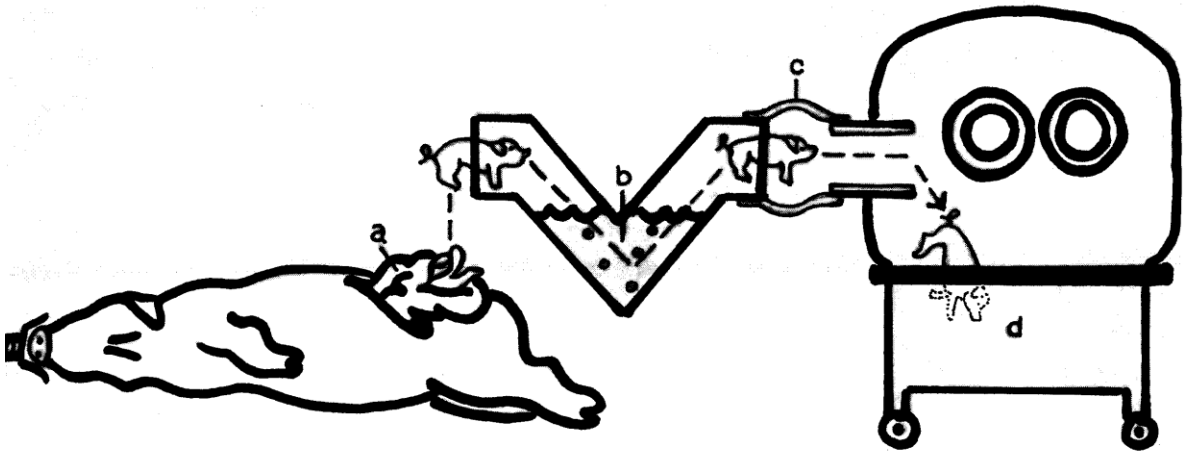
- 6-well tissue culture plates. To each well, 50 µl to 2 ml of radiolabeled bacteria are added and incubated for 1.5 h at 37°C in 10% CO2/90% air. The monolayers are washed three times with PBS to remove unattached bacteria. The cell-associated bacteria and intestinal cells are dissolved in 0.1% SDS/0.1 to 0.9 *M NaOH.*
- Radioactivity is determined by liquid scintillation and the adhesion expressed as the percentage of radioactivity recovered after adhesion, relative to the radioactivity in the bacterial suspensions added to the cells.
- The number of attached bacteria can also be enumerated by plate counting.
- After 1-h incubation, the monolayers are washed three times with PBS to remove nonadherent bacteria, and the monolayers are lysed with sterilized distilled water. Serial dilutions are then plated to count the number of viable bacteria.
- The adhesion ratio can be calculated by dividing the number of adherent bacteria by the number of added bacteria). For the interpretation of the results, the adhesion index is strongly dependent on the *in vitro conditions used. The percentage of adhesion does not* constitute an absolute value and has to be seen in relation to the nonadhesive control strain.

In vivo TRIALS

- Survival in the GIT
- Adhesion
- Competitive exclusion
- Immune stimulation



Open hysterotomy



a. Neonate is removed
b. Passed through a germicidal trap
c. Transfer sleeve
d. Sterile rearing isolator

11) Phosphatidy-linositol-Specific Phospholipase C (PI-PLC) Activity

- PI-PLC activity will damage the host cell membrane and is thought to be important for translocation by some microbes .
- Strains to be tested are spot inoculated on appropriate solid media and incubated until colonies are visible.
- Thereafter, PI-PLC activity can be determined by applying an overlay with 20 mg/l L-a-phosphatidyl-inositol in 1.4% (w/v) agarose.
- PI-PLC activity is visible as a turbid halo of insoluble diacylglycerol around the colony.
- *B. cereus is included as a positive control.*

12) Mucinolytic Activity

- An ability to degrade mucus or its glyco-proteins is considered one of the valuable indicators of potential pathogenicity and local toxicity of lumen bacteria .
- Both *in vitro and in vivo assays can be used to evaluate toxicity.*
- Bacteria are grown under appropriate conditions.

- **For the *in vitro assay,***
- mucin degradation can be evaluated in liquid medium or in a petri dish.
- For the first method, 200 µl of bacterial suspension (10^7 CFU/ml) is incubated at 37°C for 2 to 5 d with 10 to 25 ml of PBS, basal medium with or without 1 to 3% glucose, basal medium containing 0.3% hog gastric mucin (HGM) with or without 1 to 3% glucose, or basal medium containing 0.3% human intestinal glycoproteins (HIG).
- After incubation the supernatants are collected and heat treated to inactivate mucinolytic enzymes.
- The mucin pellets are precipitated by the addition of chilled ethanol.
- The pellets are washed and resuspended in 0.5 ml 10 m*M Tris-HCl buffer.*
- *The decrease of total carbohydrates and* protein content in the mucin residues is measured by SDS-PAGE .
- the degradation ratio can be calculated as :
- [concentration in test samples/concentration in control sample] . 100%

- **For the second method,**
- 10 µl of bacterial suspension is inoculated onto 0.5% HGM(hog gastric mucin) and 1.5% agarose incorporated into basal medium.
- The plates are incubated at 37°C for 72 h and subsequently stained with 0.1% Amido Black in 3.5 *M acetic acid for 30 min.*
- *They are then washed with 1.2 M acetic* acid until a discolored halo around the colony appears.
- The mucin degradation activity can be defined as the size of the mucin lysis zone.

13) Antibiotic Resistance

- Resistance to antibiotics can be intrinsic or acquired via specific genetic elements.
- Antibiotic resistance can be assessed using standard disk diffusion tests on appropriate solid media or antibiotic dilution in broth culture.
- Intrinsic resistance is usually species dependent and will be present in the majority of strains from a given species.
- Acquired resistance, on the other hand, will usually be strain dependent. Because transferable antibiotic resistance is a serious safety concern, it needs to be distinguished from intrinsic resistance, as outlined by the Scientific Commission on Animal Nutrition ,

Suggested Experiments to Distinguish between Intrinsic and Acquired Antibiotic Resistance, As Suggested by the Scientific Commission on Animal Nutrition

Test	Intrinsic	Acquired
Resistance present in most strains of a given species	+	-
In vitro transfer of resistance	-	+
Known resistance genes	-	-/+
Isolation and sequencing of the resistance gene	+	+
Localization of chromosome	-/+	-/+
House keeping genes flanking the resistance gene	+	-/+
Insertion sequences flanking the resistance gene	-	+

RISK OF TRANSFER OF ANTIMICROBIAL RESISTANCE

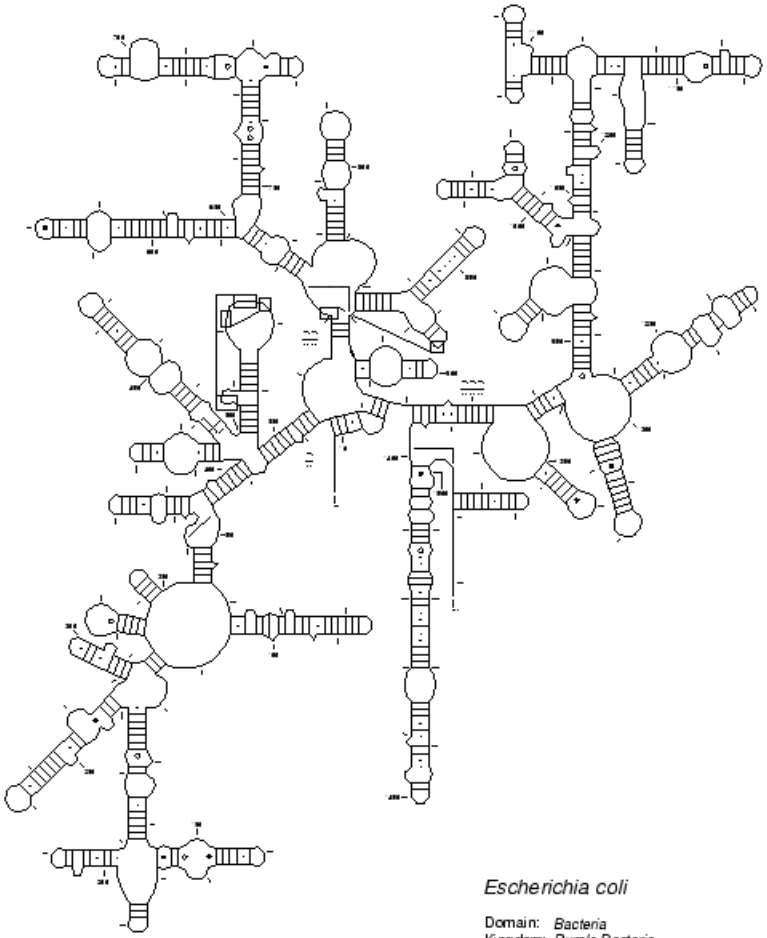
- Ideally, probiotic strains would not harbour antimicrobial resistance genes on transmissible elements that are capable of transfer to pathogenic or opportunistic pathogenic bacteria , but many existing probiotic strains already do.
- Antibiotic resistance can be located on mobile genetic elements such as plasmids or transposons, or on the bacterial chromosome (where transfer, is difficult, at least for lactobacilli .
- Plasmids are common in most of the probiotic bacteria, but not all antimicrobial resistance is harboured on plasmids.
- Ammor et al. recently found resistance genes in several lactic acid bacteria and Bifidobacterium - specifically resistance to tetracycline [tet(M), tet(W), tet(O) and tet(O/W)], erythromycin and clindamycin [erm(B)] and streptomycin [aph(E) and sat(3)].
- Most of the resistance determinants were located on the bacterial chromosome, except for tet(M), which was identified on plasmids in *Lactococcus lactis.*

- *Given the increasing clinical importance of invasive infections* with vancomycin resistant enterococci and threat of emergence of vancomycin resistant *Staphylococcus aureus, attention has been* focused on the potential for transfer of vancomycin resistance to and from probiotic bacteria.
- Many strains of lactobacilli are naturally resistant to vancomycin.
- In the *Lactobacillus strains studied to date,* the vancomycin resistance genes appear to be chromosomally located and are not easily transferable to other genera .
- Mater et al. demonstrated transfer of vancomycin resistance (VanA cluster) from *Enterococcus to a commercial strain of Lactobacillus* acidophilus, both in vitro and in the gut of mice .

14) Taxonomy

- The accuracy and reliability of the species identification of probiotic strains and the label correctness of the probiotic products have long been ignored as true safety aspects.
- Triggered by the exponential growth of the functional food market in general, it is only since the mid-1990s that commercial probiotic strains have been included in taxonomic studies to obtain an identification up to the species level. Regardless of the country in which studies

Secondary Structure: small subunit ribosomal RNA

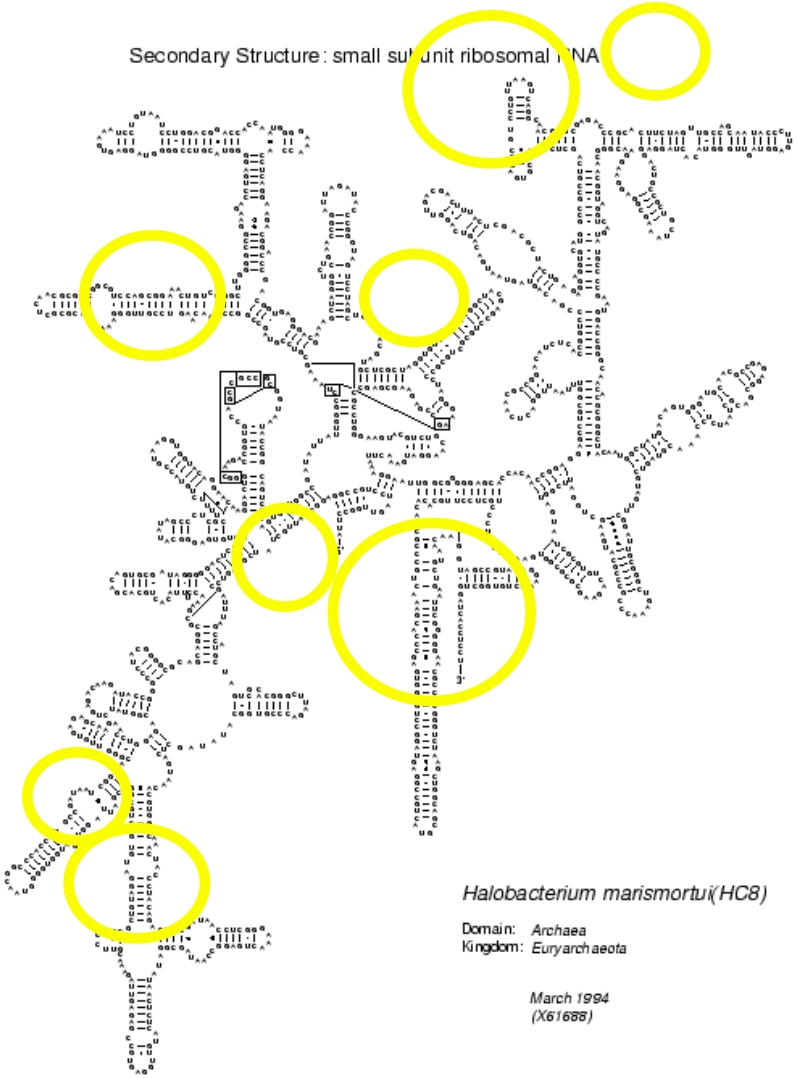


Escherichia coli

Domain: *Bacteria*
Kingdom: *Purple Bacteria*
Order: *gamma*

August 5, 1994 v3.0
(J01695)

Secondary Structure: small subunit ribosomal RNA



Halobacterium marismortui(HC8)

Domain: *Archaea*
Kingdom: *Euryarchaeota*

March 1994
(X61688)

Score = 2800 bits (1516), Expect = 0.0
Identities = 1519/1520 (99%), Gaps = 1/1520 (0%)
Strand=Plus/Plus

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Query  793    GAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGAGT  852
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Query  1093   GTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTATCA  1152
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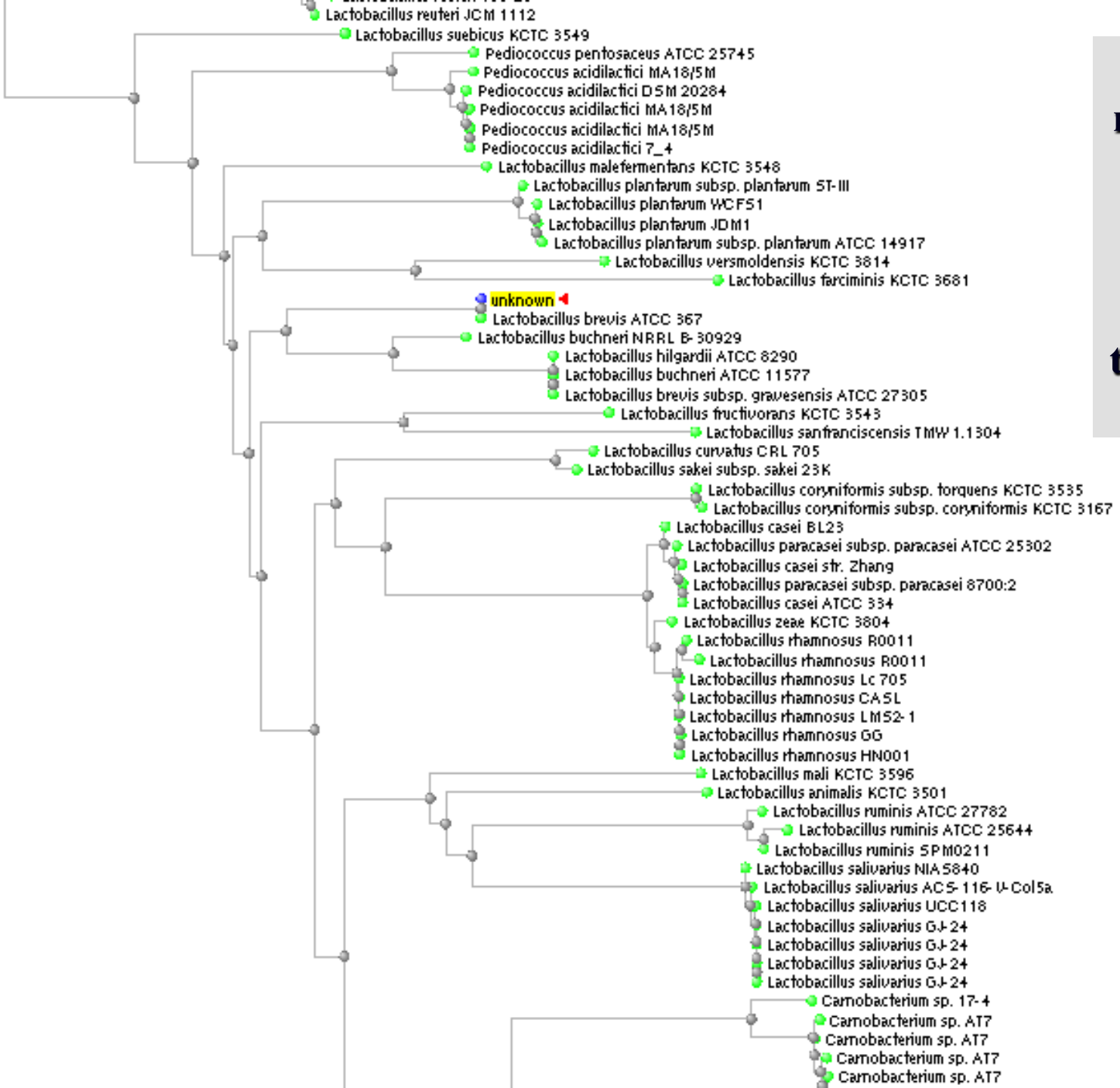
Legend for links to other resources: UniGene GEO Gene Structure Map Viewer PubChem BioAssay

Sequences producing significant alignments:

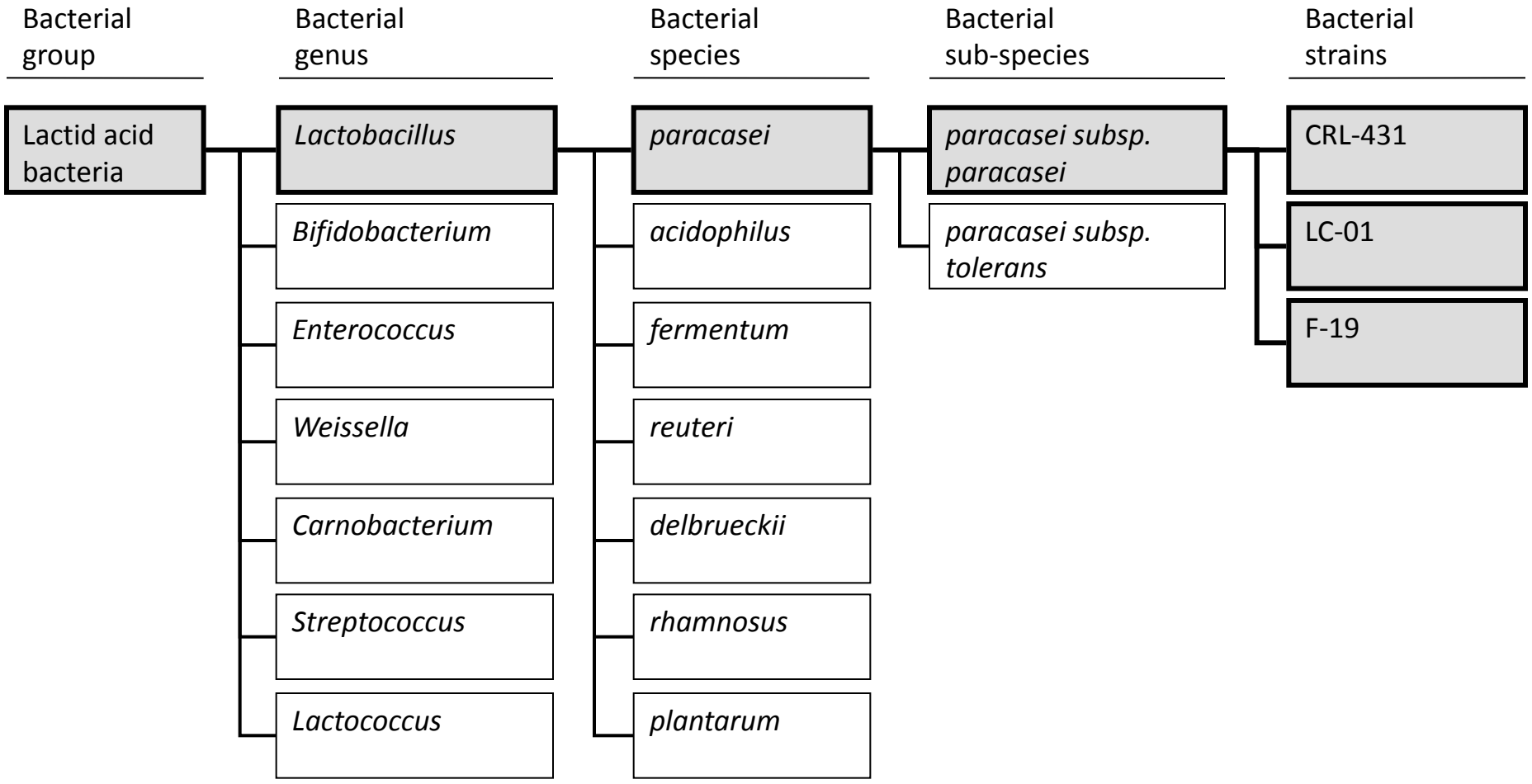
Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
NC_008497.1	Lactobacillus brevis ATCC 367, complete genome	2800	1.396e+04	99%	0.0	99%	
NC_015428.1	Lactobacillus buchneri NRRL B-30929 chromosome, complete genome	2338	1.164e+04	99%	0.0	94%	
ACGP01000200.1	Lactobacillus hilgardii ATCC 8290 contig00272, whole genome shotgun sequence	2337	2337	99%	0.0	94%	
ACGH01000101.1	Lactobacillus buchneri ATCC 11577 contig00136, whole genome shotgun sequence	2337	2337	99%	0.0	94%	
ACGG01000095.1	Lactobacillus brevis subsp. gravesensis ATCC 27305 contig00186, whole genome shotgun sequence	2337	2337	99%	0.0	94%	
NC_014554.1	Lactobacillus plantarum subsp. plantarum ST-III chromosome, complete genome	2311	1.152e+04	99%	0.0	94%	
NC_012984.1	Lactobacillus plantarum JDM1, complete genome	2305	1.153e+04	99%	0.0	94%	
NC_004567.1	Lactobacillus plantarum WCFS1, complete genome	2305	1.151e+04	99%	0.0	94%	
ACGZ02000033.1	Lactobacillus plantarum subsp. plantarum ATCC 14917 Contig84, whole genome shotgun sequence	2300	2300	99%	0.0	94%	
BACN01000105.1	Lactobacillus malefermentans KCTC 3548 DNA, contig: contig00105, whole genome shotgun sequence	2290	2290	99%	0.0	94%	
AEEG01000012.1	Pediococcus acidilactici DSM 20284 contig00019, whole genome shotgun sequence	2268	2268	99%	0.0	94%	
AGKB01000005.1	Pediococcus acidilactici MA18/5M contig5, whole genome shotgun sequence	2263	2263	99%	0.0	94%	
AGKB01000010.1	Pediococcus acidilactici MA18/5M contig10, whole genome shotgun sequence	2263	4520	99%	0.0	94%	
ACXB01000026.1	Pediococcus acidilactici 7_4 cont1.26, whole genome shotgun sequence	2263	2263	99%	0.0	94%	
AGKB01000006.1	Pediococcus acidilactici MA18/5M contig6, whole	2257	2257	99%	0.0	93%	



Evolutionary relationships of representative bacteria based on the sequences of their 16S rRNA genes

Strain Taxonomy

Note: Overview not exhaustive



Overview of Studies Reporting on Species Identification of Probiotic Strains or Label Correctness of Probiotic Products

Probiotic Strains or Products Tested	Identification Technique(S) Used	Reported Discrepancies with Original Strain or Product Label Information[a]	Ref.
Commercial probiotic strains	16S rDNA sequencing, fatty acid analysis, carbohydrate fermentation	14/29 strains displayed nonmatching species designations	76
Probiotic (fermented milk) products	16S rDNA sequencing, fatty acid analysis, carbohydrate fermentation	6 products contained other species than those stated	76
Food supplements and fermented products	API	13/29 food supplements lacked one or more stated species; 2/5 fermented products contained one additional species	93
Dried *Bacillus* spore probiotics	16S rDNA sequencing, API 50 CH	4/5 products were mislabeled	81
Mild and probiotic yogurts	DNA-DNA hybridization, carbohydrate fermentation	9/15 yogurts contained other species than those stated	77
Bifidobacterium dairy products	DNA-DNA hybridization, carbohydrate fermentation	3/6 products contained other species than those stated	78
Novel-type yogurts	DNA-DNA hybridization	4/16 yogurts contained other species than those stated	79
Dairy products and food supplements	Whole-cell protein profiling	14/30 food supplements were mislabeled; 10/25 dairy products were mislabeled	71
Dairy products and food supplements	16S rDNA-DGGE	2/4 dairy products were mislabeled; 3/5 food supplements were mislabeled	84
Yogurts and lyophilized products	16S rDNA-DGGE, species-specific PCR	1/5 yogurts contained other species than those stated; 6/7 lyophilized products lacked one or more species stated	85

[a] Discrepancies were only mentioned for those products that contained sufficient information on their bacterial species composition.

Postmarketing Surveillance

1) The manufacturer of a probiotic product has the final responsibility for its safety.
2) Once a probiotic product has been introduced into the market, surveillance for any cases of disease related to the probiotics is important to be able to continue to ascertain the safety of the product.
3) Any *Lactobacillus, Bifidobacterium or other LAB isolate associated with disease should be* reported, stored, and eventually compared to probiotic strains of the same species .
4) This also illustrates that accurate taxonomic identification of both probiotic and clinical isolates is essential;
5) unfortunately, this is not always the case. Genotyping should reveal clonal relatedness of the clinical isolate and probiotic of LAB.
6) For future reference and research, it is important that the clinical LAB isolates are minimally subcultured to avoid adaptation to laboratory culture conditions and are stored properly.
7) This makes it possible to investigate the characteristics of these isolates and obtain information on potential risk Factors .

Next-Generation Probiotics

New probiotic microorganisms and new applications are likely to be introduced into the market.

Although genetically modified organisms (GMOs) are not particularly appreciated by the European consumer, this may be different in the case of clear medical applications.

GMO probiotics expressing antigens from pathogens would be safe vaccines and would be preferable over attenuated pathogens .

Legislation for the assessment of the safety of GMOs is very strict and falls outside the scope of this review.

-Most probiotics currently on the market belong to the genera *Lactobacillus* and *Bifidobacterium, although species from other genera are also used*

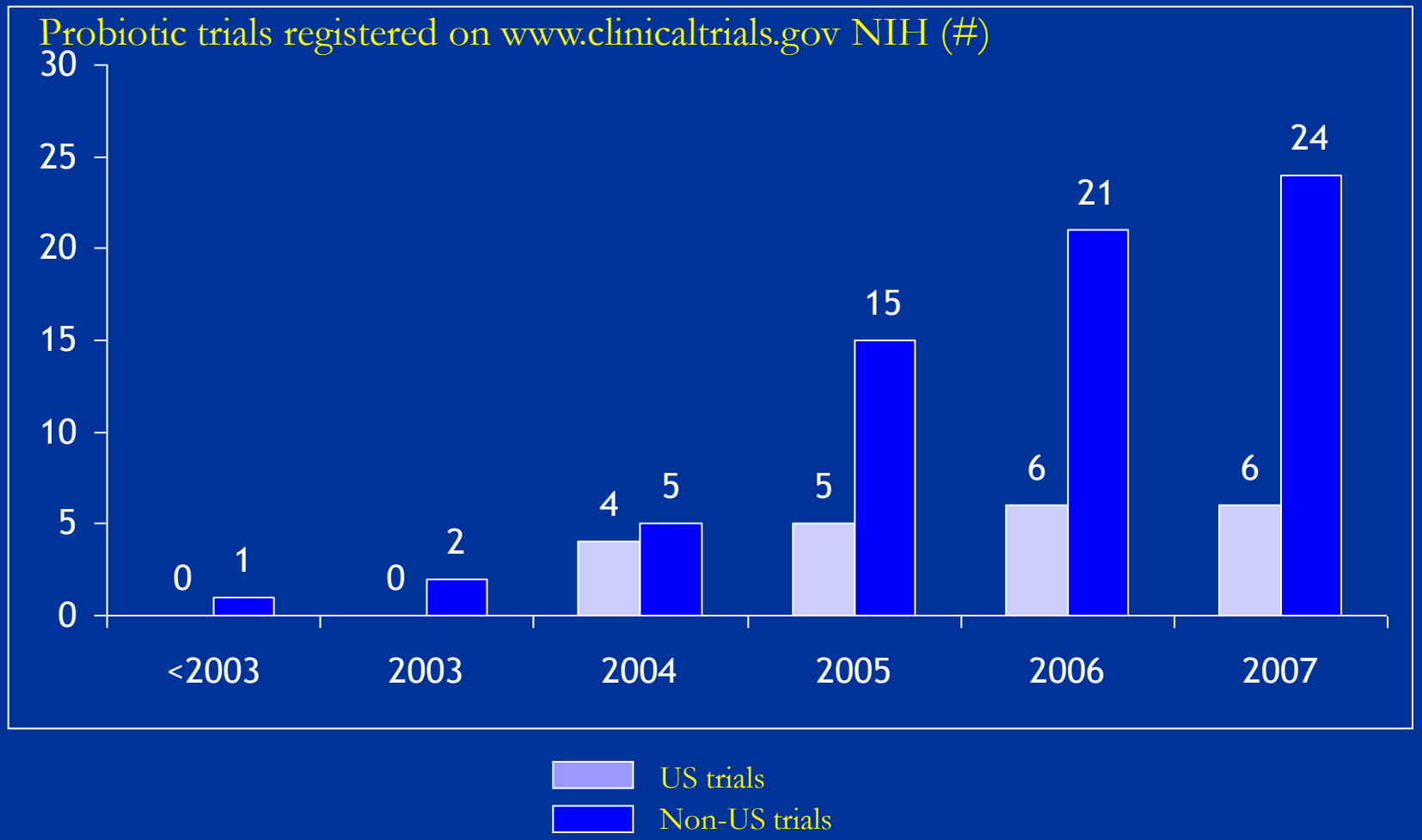
-*Oxalobacter formigenes to reduce the risk for kidney stones*

-Probiotics producing anti-inflammatory cytokines (interleukin-10) *in situ in the intestine may provide novel strategies for the treatment of i*nflammatory bowel disease*,*

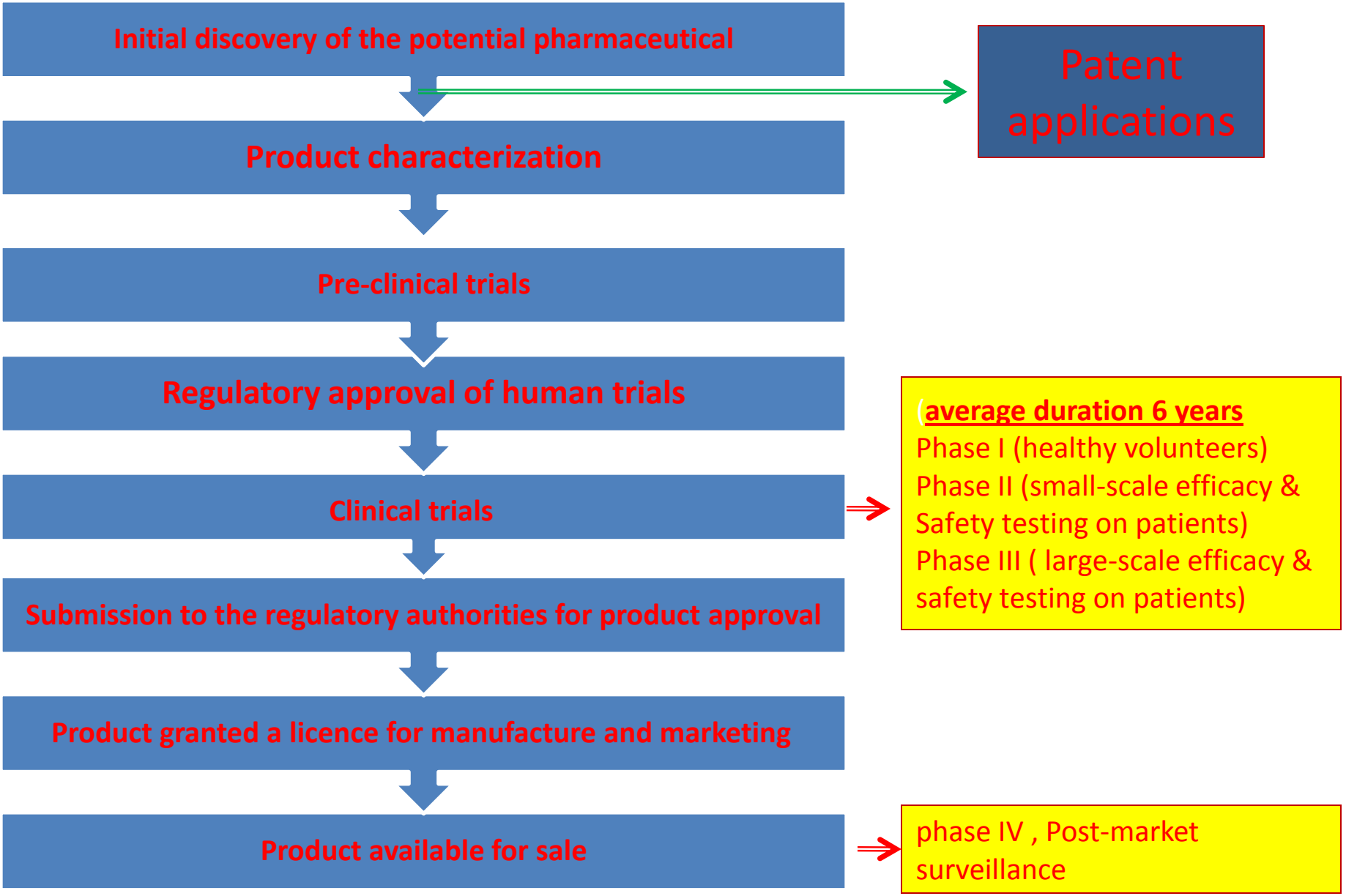
-Recent publications indicate that intranasal application of probiotics (α-hemolytic streptococci) may be a feasible option to prevent recurrent otitis media.

It is obvious that such applications, which are very different from traditional probiotic use, require different standard and safety assessment procedures.

…and so has the number of probiotic trials



Development of a pharmaceutical products



Suggested Steps for the Assessment of the Safety of Probiotics and Starter Cultures

- **1. Strains that are not properly taxonomically described (DNA-DNA hybridization, rRNA sequence determination) should not be marketed.**
- **2.Strains for which the origin is not known should not be marketed.**
- **3. Strains carrying transferable antibiotic resistance genes should not be marketed.**
- **4. Strains with a long history of safe use by humans can be used safely as probiotics.**
- **5. Strains that belong to species for which no pathogenic strains are known but which do not have a history of safe use are likely to be safe as probiotics but are nevertheless novel foods.**
- **6 Strains that belong to species for which pathogenic strains are known should be treated as novel foods.**
- **7. Strains that are genetically modified are novel foods and should be treated as such.**
- **8. Epidemiology and post marketing surveillance should be carried out.**

Microbial Genera Commonly Used as Probiotics

Genus (species)	Remark
Bifidobacterium	*B. dentium* has been found to be associated with dental caries.
Bacillus	Some strains with a history of safe use are known.
Carnobacterium	Some strains are pathogenic for fish.
Enterococcus	Some strains with a history of safe use are known.
Escherichia coli	Some strains with a history of safe use are known.
Lactobacillus	
Lactococcus	Some strains are pathogenic for fish.
Propionibacterium	Only species of the classical or dairy propionibacteria, cutaneous propionibacteria have been associated with disease.
Saccharomyces cerevisiae	*S. boulardii* is likely to be a variant of *S. cerevisiae.*
Streptococcus	Only *S. thermophilus* has a history of safe use.

Legislation Evaluation of Lactic Acid Bacteria

- LAB are difficult to classify and regulate. Food supplements containing LAB
- could be classified as foods or food additives. They could also be classified
- as drugs, particularly if one makes health claims or medicinal claims concerning
- their use.
- Regular foods or food supplements containing LAB could fall under European
- novel food regulation, in case the bacterium in question has not been
- used before in the food in question. Novel foods by legal definition are foods
- and food ingredients that have not been used for human consumption to a
- significant degree within the Community before 15 May 1997. Regulation
- EC 258/97 (102) of 27 January 1997 of the European Parliament and the

- Council lays out detailed rules for the authorization of novel foods and novel
- food ingredients.
- Companies that want to place a novel food on the EU market need to
- submit their application in accordance with Commission Recommendation
- 97/618/EC (103) that concerns the scientific information and the safety
- assessment report required. Novel foods or novel food ingredients may
- follow a simplified procedure, only requiring notifications from the company,
- when they are considered by a national food assessment body as
- “substantially equivalent” to existing foods or food ingredients.

Japan

- The concept of “Functional Foods” started in Japan where the world’s first health-claim approval system, Foods for Specific Health Uses (FOSHU), and the market for functional foods were developed in the last decade.
- Japanese traditionally consume fermented foods with viable microorganisms such as “natto” (steamed soybean fermented with bacilli), “miso” (soy paste fermented with yeast and fungi), and sour vegetables fermented by lactobacilli.
- The total production of fermented milks and LAB drinks in Japan reached 916,000 and 550,000 KL per family in 2003, respectively. A typical Japanese family spends approximately $100 per year on average for both product categories.

History of Fermented Milk in Japan

- Fermented milk has been consumed by humans for at least 4000 years and throughout civilizations such as the Egyptians, Mesopotamians, Aryans in central Asia, Greeks, and Romans. In Japan, the culture of dairy product intake started about 1350 years ago.
- Medical literature from the year 984 indicates that milk products were used to cure total debilitation and constipation and improve skin. Long before Metchnikoff discovered in 1902 that putrefaction in the intestines causes early aging and death in humans and that this could be prevented by consumption of sour milk .
- In the medical field, tablet and powdered forms of viable LAB were also introduced in 1971 as a drug for curing intestinal illnesses.

Product name	Manufacturer	Volume	LAB	Additives or enrichment *	FOSHU health claim**
Fermented Milks					
Bulgaria Yogurt LB81	Meiji Milk	120 g, 500 g	*L. delbrueckii* ssp. *bulgaricus, S. salivarius* ssp. *thermophilus*		1
Bifidus Yogurt	Morinaga Milk	300 g, 500 g	*B. longum, L. bulgaricus, S. thermophilus*		1
Nature Pro GB	Nippon Milk Community	200, 500 g	*L. gasseri, B. longum, L. bulgaricus*		1
Probio Yogurt LG21	Meiji Milk	120 g, 100 ml	*L. gasseri, S. thermophilus*		-
LC1 Yogurt	Nestlé Snow	120 g, 90 g	*L. johnsonii, S. thermophilus*		1
Lactoferrin Yogurt	Morinaga Milk	120 g	*L. bulgaricus, S. thermophilus*	a	-
Dannon BIO	Calpis Ajinomoto Danone	120 g	*B. animalis, L. bulgaricus, S. thermophilus*		-
Aloe Yogurt	Morinaga Milk	80 g, 130 g	*L. bulgaricus, S. thermophilus*		-
Sofuhl Plain	Yakult	100 g	*L. casei*		1
Yogurt Onaka-e-GG!	Takanashi Milk	100 g	*L. rhamnosus*		1
Fermented Milks (drink type)					
Yogurt Onaka-e-GG!	Takanashi Milk	100 ml	*L. rhamnosus*		1
Bulgaria Nomu Yogurt LB81	Meiji Milk	100 ml, 240 ml, 500 ml, 1000 ml	*L. bulgaricus, S. thermophilus*		1
Milmil	Yakult	100 ml	*B. breve, B. longum, L. acidophilus*	b	1
Joie	Yakult	125 ml	*L. casei*		1
Interbalance L-92	Calpis Ajinomoto Danone	150 ml	*L. acidophius*		-
Milk Products LAB Drinks					
Yakult	Yakult	65 ml	*L. casei*		1
Yakult 400	Yakult	80 ml	*L. casei*	c	1
LC1 Yogurt Drink	Nestlé Snow	120 ml	*L. johnsonii, S. thermophilus*		-
Milk Products LAB Drinks (sterile)					
Calpis	Calpis	500 ml ***	*L. helveticus, S. cerevisiae*		-
Sour Milk Ameal S	Calpis	160 ml	*L. helveticus*	d	2
Pretio	Yakult	100 ml	*L. casei, L. lactis*	e	2
LAB Drinks					
Rolly Ace	Kagome Rabio	65 ml	*L. casei*		-
Calpis Kids	Calpis	100 ml	*L. helveticus, L. acidophilus*		1

* Health benefit through additives : a) lactoferrin, b) lactoferrin, DHA, vitamin D, c) galactooligosaccharides, calcium, and vitamins.
Health benefit through fermented products : d) lact-tripeptides, e) γ-amino butyric acid.

** Health claim : 1) Improve gastrointestinal conditions; 2) For people with mild hypertension.

*** need to dilute before drinking

- Japanese pioneers developed unique LAB drinks, one of which is a concentrated
- type of sterilized fermented milk drink called “CALPIS,” that was
- introduced in 1919. Another is the LAB drink or “YAKULT” which was
- introduced in 1935. After a sweetened fermented milk hardened by agar and
- named yogurt was industrialized in 1950, a “yogurt” product corresponding
- to the European definition was at last introduced in 1971 to Japanese consumers.

Codex and Japanese Standards of Lactic Acid Bacteria and Ingredients for Fermented Milks

Description	Japanese regulations				Previous Codex Standard (1975)		Conditions		Current Codex Standard for Fermented Milks (2003) *2						
	Fermented Milks	Milk Products LAB Drinks	LAB Drinks	Milk Products LAB Drinks (sterile)	Yogurt	Flavored Yogurt			Yogurt	Alternate Culture Yogurt *3	Acidophilus Milk	Kefir	Kumys	Fermented Milk	Flavored Fermented Milks *4
Incorporation *1	A	A	A	A	E	E	*Streptococcus. thermophilus*		E	E	A	A	A	A	A
							L.. delbrueckii subsp. *bulgaricus*			A			E		
					A	A	Other *Lactobacillus*		A	E	A	A	A		
								L. acidophilus			E				
							L. kefiri, Leuconostoc, Lactococcus, Acetobacter			A	A	E			
							Yeast						E		
	A	A	A	A	E	E	Pasteurized milk products	Milk and/or products obtained from milk, Water	A	A	A	A	A	A	A
					A	A	Dehydrated milk products								
					N	A	Non-dairy ingredients *5 and Flavors		A	A	A	A	A	A	max 50%
					N	A	Food additives *6		N	N	N	N	N	N	A
Composition	min 10^7 LAB or Yeast (cfu/ml)		min 10^6 LAB or Yeast (cfu/ml)	no bacteria	viable and abundant		LAB viable count *7	Total count (cfu/g)	min 10^7			min 10^7	min 10^7	min 10^7	min 10^7
								Labeled microorganisms (cfu/g)	min 10^6			-	-	min 10^6	min 10^6
								Yeasts (cfu/g)	-			min 10^4	min 10^4	-	-
	min 8%	min 3%	less than 3%	min 3%	min 8.2%		Solid not-fat (SNF)	Milk protein (%w/w)	min 2.7%			min 2.7%	-	min 2.7%	min 2.7%
	-	-	-	-	Yogurt : min 3% Partially skimmed yogurt : 0.5 - 3% Skimmed yogurt : max 0.5%		Milk fat (MF)		less than 15%			less than 10%	less than 10%	less than 10%	less than 10%
	-	-	-	-	-	-	Acidity (% w/w lactic acid)		min 0.6%			min 0.6%	min 0.7%	min 0.3%	min 0.3%
	-	-	-	-	-	-	Ethanol (% vol/w)		-			-	min 0.5%	-	-

Codex and Japanese Standards of Lactic Acid Bacteria and Ingredients for Fermented Milks (continued)

*1 E = essential, A = allowed, N = not allowed.

*2 Sweeteners can be added onto all the fermented milk categories and the label may be described as "sweetened" + designation of Fermented Milks (e.g. sweetened yogurt).

Concentrated Fermented Milk is described as a Fermented Milk the protein of which has been increased prior to or after fermentation to min 5.6%.

Whey removal after fermentation is not permitted in the manufacture of fermented milks, except for Concentrated Fermented milk.

*3 Alternate culture yogurt shall be named through the use of an appropriate qualifier in conjunction with the work "yogurt". (e.g. "mild" and "tangy") and the term "alternate culture yogurt" shall not apply as a designation.

*4 "Flavored Fermented Milks" are composite milk products defined at CODEX STAN 206-1999.

*5 Codex defines non-dairy ingredients as nutritive and non nutritive carbohydrates, fruits and vegetables as well as juices, purees, pulps, preparations and preserves derived therefrom, cereals, honey, chocolate, nuts, coffee, spices and other harmless natu

*6 Codex defines food additives as colors, sweeteners, emulsifiers, flavor enhancers, acids, acidity regulators, stabilizers, thickeners, preservatives, and packaging gases. Stabilizers and thickeners can be used in Fermented Milks (Plain) when national leg

*7 "Fermented Milks Heat Treated After Fermentation" described at Codex is not applied to the requirement for viable microorganisms count.

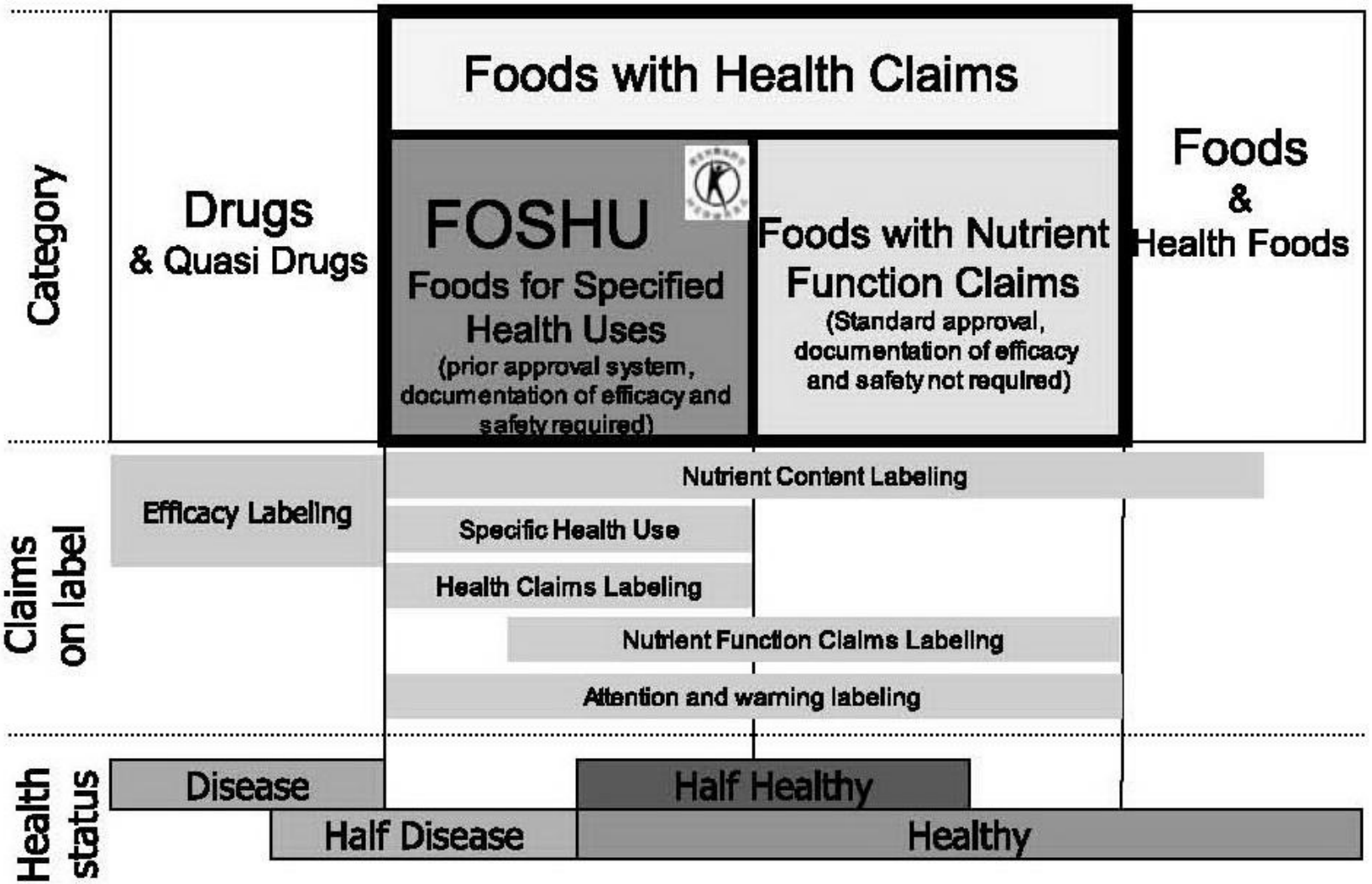


FIGURE 19.1
Food with health claims including FOSHU as of 2004.

The Labeling Standards for Foods with Health Claims

1. In agreement with the nutritional targets and health policy of the nation.
2. Expressed to supply nutritional components or to contribute to specific health use (including being helpful to promote or maintain health by influencing the structure or function of the body).
3. Scientific evidence is adequate and factually described.
4. Clearly expressed using understandable and correct sentences or terms to convey information to consumers.
5. Obliged to indicate attention, including appropriate intake manner for prevention of health risk from excess intake or contraindication.
6. Comply withapplicable Laws including the Food Sanitary Law, the Nutrition Improvement Law, the former Health Promotion Law, and the Pharmaceutical Affairs Law.
7. Clearly indicateFoods with Health Function (FOSHU or Food with Nutrient Function Claims) as to distinguish products from drugs, and from indicating on the label any referenceto diagnosis, cure, or prevention of diseases.

List of FOSHU Products

Health Claim and Functional Component	Number of Product	Product Form
Improve Gastrointestinal Conditions		
Lactic acid bacteria	58	Fermented milk, lactic acid bacterium drink
Lactosucrose	25	Soft drink, jelly, cookie, table sugar
Fructo-oligosaccharides	11	Table sugar, soft drink, candy, pudding
Galacto-oligosaccharides (GOS)	8	Table sugar, vinegar
GOS + polydextrose	1	Soft drink
Soybean oligosaccharides	7	Soft drink, table sugar
Xylo-oligosaccharides	5	Soft drink, vinegar, chocolate, candy
Isomalt-oligosaccharides	3	Table sugar
Raffinose	1	Powdered soup
Lactulose	1	Soft drink
Indigestible dextrin	28	Soft drink, dessert, fish meat paste,
Psyllium husks	20	Noodle, soft drink, cereal, soft drink
Na-Arg + corn fiber	1	Soup
Hydrolyzed guar gum	4	Porridge
Wheat bran	4	Cereal food
Agar	3	Jelly
Beer yeast	1	Fermented milk
Polydextrose	2	Soft drink
Whey fermented products	1	Tablet
For those with high blood pressure		
Sardine peptides	11	Soft drink
Dried bonito oligopeptides	6	Soup, tea, tablet
Lactotripeptides	3	Lactic acid bacteria drink, tablet
Casein dodecapeptides	3	Soft drink
Eucommia leaf glycoside	2	Soft drink
For those with high serum cholesterol (CHO)		
Soy protein	18	Soft drink, hamburger, sausage, soya milk, dessert, soup
Low molecular weight sodium alginate*	9	Soft drink, soup
Chitosan	4	Biscuit
Plant phytosterol	3	Margarine, cooking oil
Soy peptide	2	Soft drink
Psyllium husks*	2	Noodle, soft drink
EPA, DHA	1	Soft drink
Difficult to cling body fat/For those conscious of body fat /For those high serum tryacylglycerol (TG)		
Diacylglycerol (DG)	5	Cooking oil
DG + phytosterol **	4	Cooking oil
Tea cathekine	2	Tea drink
Globin protein digest	3	Soft drink
MCT	1	Cooking oil

List of FOSHU Products (continued)

Health Claim and Functional Component	Number of Product	Product Form
For those concerned about blood sugar level		
Indigestible dextrin	30	Soft drink, tofu, soup, steamed rice,
Wheat albumin	3	Soup
Guava polyphenols	1	Soft drink
Touchi extract	1	Soft drink
L-alabinose	1	Table sugar
For those concerned about bone health / Promote mineral absorption		
Fractooligosaccharides	5	Soft drink, table sugar
Isoflavone	4	Tea drink
Vitamin K2	3	Natto (fermented soybean)
Milk basic protein	1	Soft drink
Casein phosophopeptide	3	Soft drink, chewing gum, tofu
Calcium citrate malate	2	Soft drink
Heme iron	3	Soft drink, jelly
Non-cariogenic / reinforce demineralization		
CPP-ACP	7	Chewing gum, tablet
Xylitol, Calcium phosphate, fnoran	6	Chewing gum, candy
Sugar alcohols + tea polyphenols etc	3	Chocolate, chewing gum
Maltitol	2	Candy
POs-Ca	1	Chewing gum
Total	339	

As of March 31st, 2003.

* Some products with the health claim "for those who high in blood cholesterol" are also approved the health claim "improvement of GI conditions" in the same product.

** The approved health claim is "for those high in TG and CHO, and mild obesity."

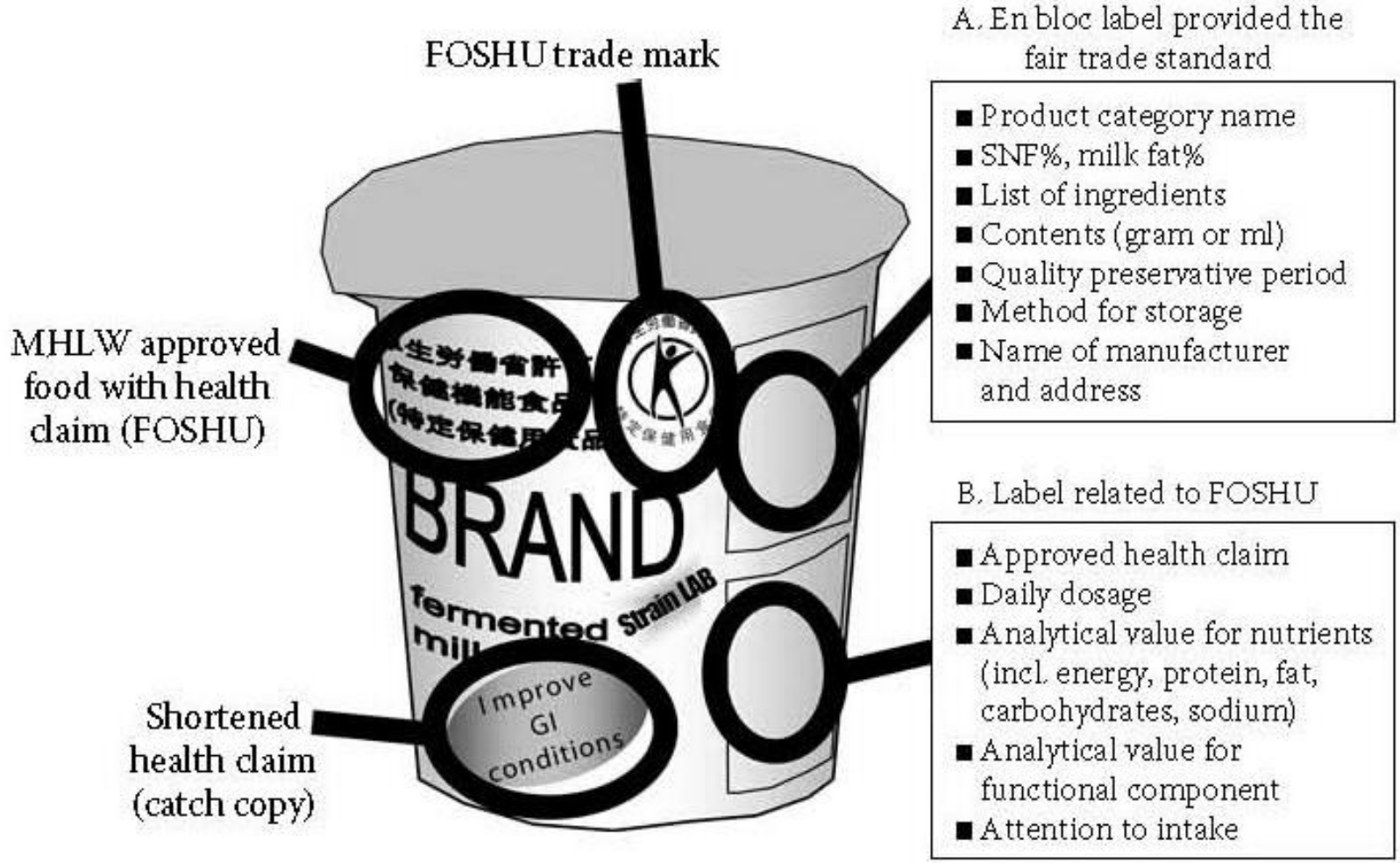


FIGURE 19.2
Package example for FOSHU product.

TABLE 19.3

Guideline for Evaluation of FOSHU Application for Products with Oligosaccharides (OS)

Test Items		Requirements
Efficacy Confirmation		
In Vitro *and Animal Study on the OS*		
1)	*In vitro* digestive study	Indigestiblity in the intestinal conditions with digestive juice
2)	*In vitro* utilization test by intestinal bacteria	Selective utilization by bifidobacteria or LAB
3)	Animal study	Capability to reach into the colon without digestion
Human Study Using Final Product		
1)	Intestinal flora analysis	Improvement of intestinal flora to show with increase in beneficial bacterial (%) like bifidobacteria and decrease in harmful bacteria (%) like *Clostridium perfringens*
2)	Defecation frequency	Increase in subject with mild constipation
3)	Fecal conditions or intestinal environment	Improvement of the quantity, hardness, color or shape of fecal sample, or decrease in pH or putrefactive compounds like ammonia
Safety Confirmation		
1)	Acute, sub-acute, sub-chronic toxicity tests*	No side effect in rodent
2)	*In vitro* mutagenesis test*	Negative
3)	Maximum ineffective dose test in human	No temporary diarrhea
4)	Excess dose study on final product in human	No side effect on general health status

* Toxicity test can be excluded when the related functional component has adequate dietary history in humans.

Items for FOSHU Application Documents

1. Name of the applicant (representative) and the address
2. Name and address of head office and factory
3. Product name
4. Shelf life
5. Content amount
6. Reason for seeking approval and how the intake contributes to the improvement of one's diet and the maintenance/enhancement of health of the entire population
7. Health claims labeling seeking approval
8. List of ingredients and composition
9. Manufacturing process
10. Profile for nutrients and energy
11. Daily amount of intake
12. Considerations and precautions at intake
13. Instructions for preparation, storage, or intake of the product
14. Others
15. Attachments
    - a The articles on an association with corporate seal
    - b Package sample
    - c Explanation how the product how the intake contributes to the improvement of one's diet and the maintenance/enhancement of health of the entire population, daily amount of intake, and considerations and precautions at intake
    - d Summary of scientific literatures filed for each item including Specific Health Uses
    - e List of scientific literatures
    - f Certificate of nutrient analysis and energy
    - g Record of qualification and quantification for the related functional component in the food, and the method for the measurement
    - h Documents for quality control
    - i Reason why some documents are not attached (if necessary)
    - j Scientific literatures for each item including specific health use and safety

TABLE 19.7

Documented Efficacies in Healthy Humans on Probiotic Strains in Food Sold in Japan

Probiotic Strains	Product Name in Japan	Improve GI conditions Microflora and Defecation	Immune Reinforcement	Regulate *Helicobacter pylori* and Gastrititis	Diarrhea Prevention	Improve / Prevent Atopic Dermatitis	Ref.
Lactobacillus delbrueckii ssp. bulgaricus 2038 (LB81 strain)	Meiji Blugaria Yogurt Lb81 *	➳					15
Bifidobacterium longum BB536	Bifidus Plain Yogurt *	➳					16
Lactobacillus gasseri OLL2716 (LG21 LAB)	Meiji Probio Yogurt Lg21			➳			33
Lactobacillus johnsonii La1 (LC1 LAB)	Nestlé LC1 Yogurt	➳†	➳†	➳†			17,34-37
Bifidobacterium animalis DN-173 010	Danoe Bio	➳‡					18
Lactobacillus gasseri SBT2055, Bifidobacterium longum SBT2928	Nature Pro GB *	➳					19
Lactobacillus rhamnosus	Yogurt E-GG! *	➳			➳†	➳†	20,38-40
Lactobacillus casei Shirota YIT9029	Yakult *	➳	➳				21,41
Bifidobacterium breve YIT4006 (?)	Milmil *	➳					22
Bifidobacterium lactis Bb12	Nan F **	➳	➳		➳†	➳†	23,40,42,43

The results in healthy human subjects have been published in scientific journal(s).

† Efficacy confirmation study was performed with a randomized double blind placebo controlled design.

‡ A double blind study was conducted on colonic transient time, which is not corresponded to the Japanese standard to evaluate GI conditions.

* FOSHU approved with health claim of improvement of gastrointestinal conditions.

** The same type of follow-up formula are currently sold outside Japan.

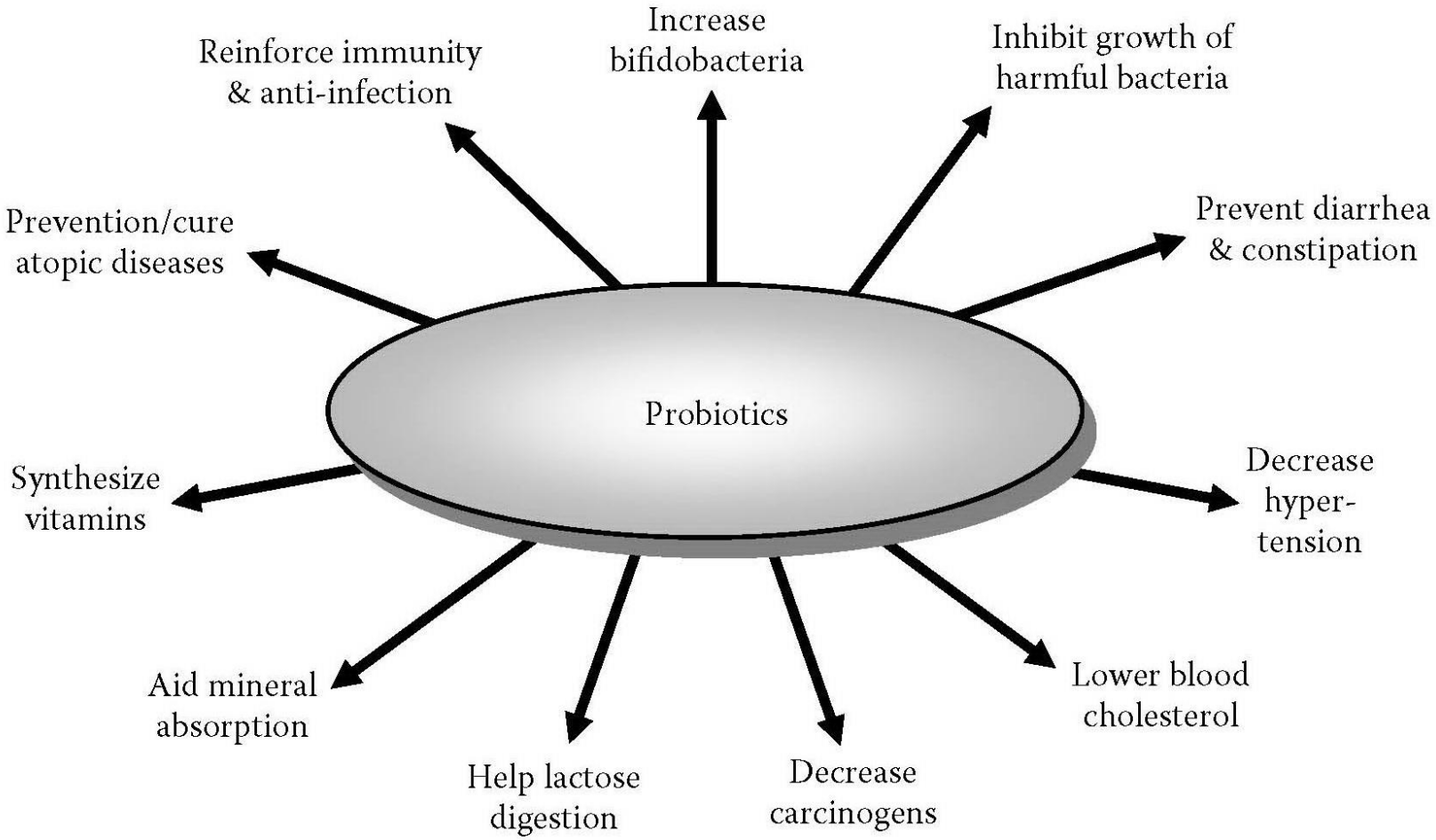


FIGURE 19.5
Beneficial effect of probiotics.

TABLE 19.8

Possible Expression on FOSHU Product

	<Possible Expression>	<Impossible Expression>
A. Biomarkers on body conditions, which are easily measurable, to be maintained or improved. (Biomarkers measurable by self-check or annual medical examination)	This product contains component (or as main component), thereby	This product improves high blood pressure (hypertension).
	This product helps to maintain blood pressure (or blood sugar level, neutral fat, cholesterol) normal.	The marker should not be directly related to improvement of symptoms or diseases.
B. Physiological functions and organic functions of the body to be satisfactory maintained or improved.	This product satisfactorily maintains (or helps to improve) defecation.	This product is effective on detoxication or promotion of fat metabolism.
	This product heightens (or promotes) absorption (or deposit) of calcium	Apparently related to improvement of diseases.
C. Changes in physical conditions whose body status is subjective and momentary but not continues nor chronic be improved.	This product is suitable (helpful) for people those who feel physical fatigue.	This product is helpful for prevention of aging.
		Scientific evidence is inarticulate.

* MHLW notice in 2001.