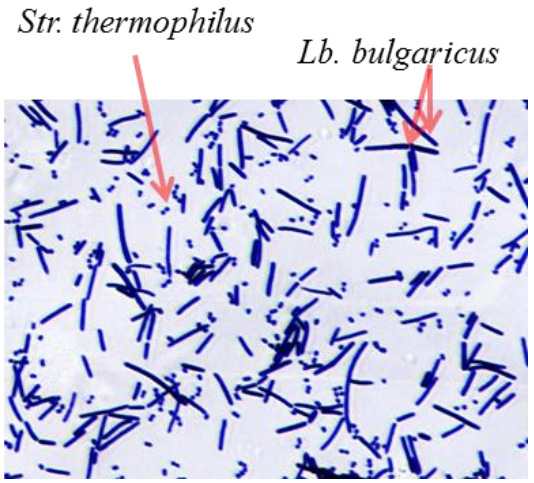
Polysaccharide of lactic acid bacteria as yogurt starter

Junko Nishimura[1] and Seiya Makino[2]

1 Hachinohe Institute of Technology, Japan
2 Meiji Co. Ltd., Japan

Codex Alimentarius Commission



Definition of the yogurt

Fermented milk using both *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*

Fermented by *Streptococcus thermophilus* and every *Lactobacillus* genus

Ministerial Ordinance on Milk and Milk products Concerning Compositional Standards, etc.

Yogurt is a kind of fermented milk

Definition of fermented milk

The products is fermented by lactic acid bacteria or yeast in milk or milk beverage containing solid not fat materials (including past, liquid, and frozen)

There is no the description about bacteria to use fermentation.

Lactococcus lactis subsp. *cremoris*, *Lactobacillus helveticus*, *Lactobacillus rhamnosus*, *Bifidobacterium*, *Lactobacillus gasseri* etc.

Typical lactic acid bacteria producing EPS

Streptococcus genus

Str. thermophilus, Str. phocae

Lactobacillus genus

Lb. delbrueckii subsp. *bulgaricus*, *Lb. plantarum, Lb. helveticus, Lb. rhamnosus, Lb. crispatus, Lb. johnsonii, Lb. fermentum, Lb. confusus, Lb. paracasei, Lb. curvatus, Lb. hilgardii, Lb. fermentum, Lb. reuteri, Lb. kefiranofaciens, Lb. casei, Lb. suebicus*

Lactococcus genus

Lc. lactis subsp. *lactis*, *Lc. lactis* subsp. *cremoris*

Leuconostoc genus

Leuc. cremoris, Leuc. mesenteroides, Leuc. pseudomesenteroides, Leuc. kimchii

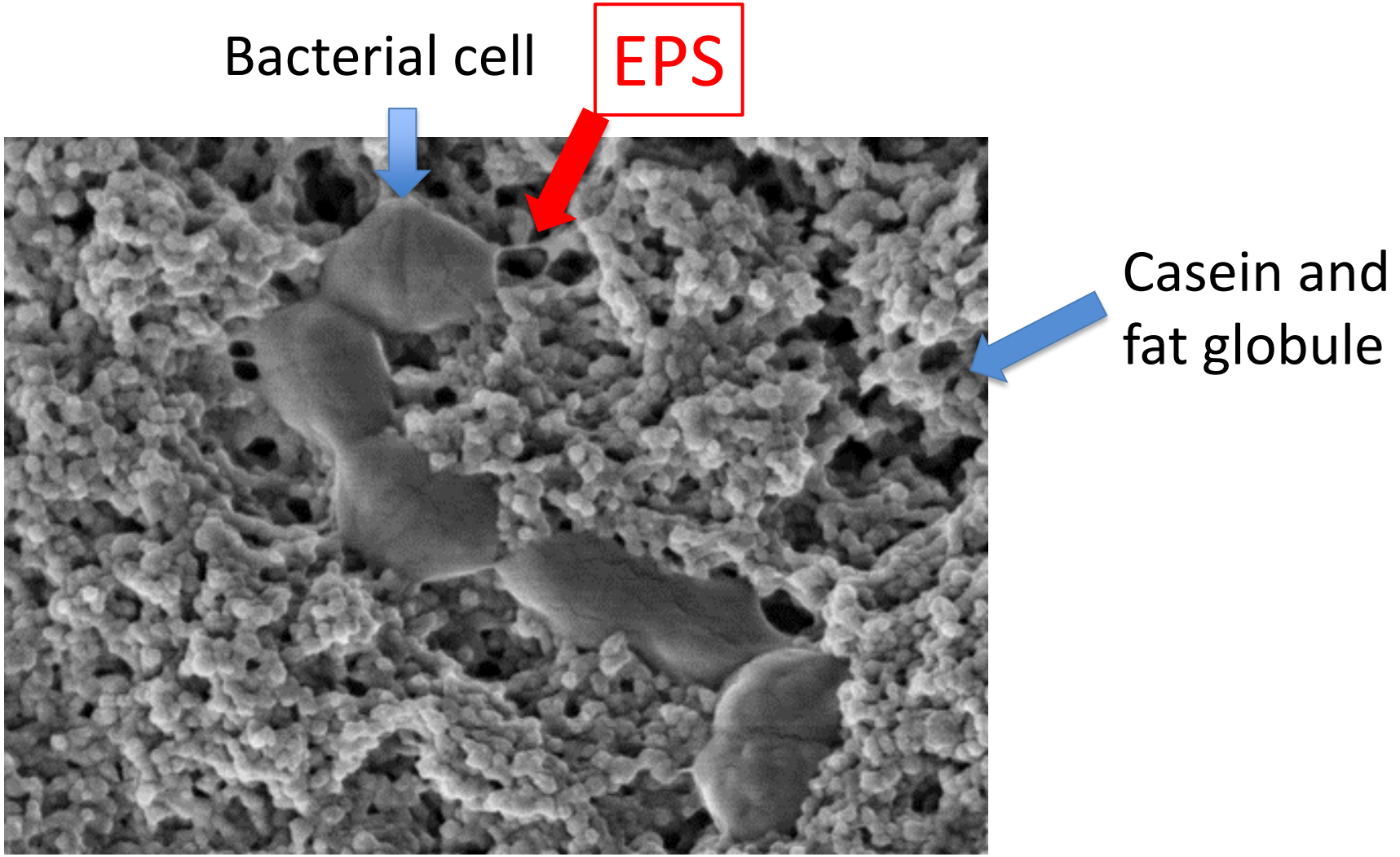
Others

Weissella strains (*W. confusa, W. cibaria*), *Propionibacterium freudenreichii, Pediococcus acidilactici, Enterococcus faecium*

Bifidobacterium

B. animalis

Images of network strand structure in commercial ropy yogurt in Japan



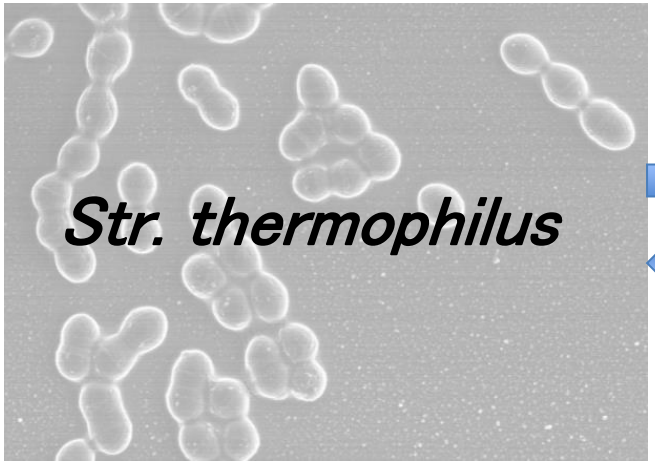
Physiological functions of fermented milk by lactic acid bacteria producing EPS

Lactic Acid Bacteria	Activity
★ *Lactobacillus delbrueckii* subsp. *bulgaricus*	Antitumor activity, Immunostimulatory Anti-influenza virus activity, Enhancement of NK cell activity, Reduction of atopic dermatitis
Streptococcus thermophilus	Prevention of chronic gastritis
★ *Lactococcus lactis* subsp. *cremoris*	Antitumor activity, Immunostimulatory Hypocholesterolemic activity
Lactobacillus helveticus	Antitumor activity, Antiviral activity
Lactobacillus casei	Anti-inflammatory action
Lactobacillus kefiranofaciens	Antitumor activity, Immunostimulatory

Comparing with EPSs of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*

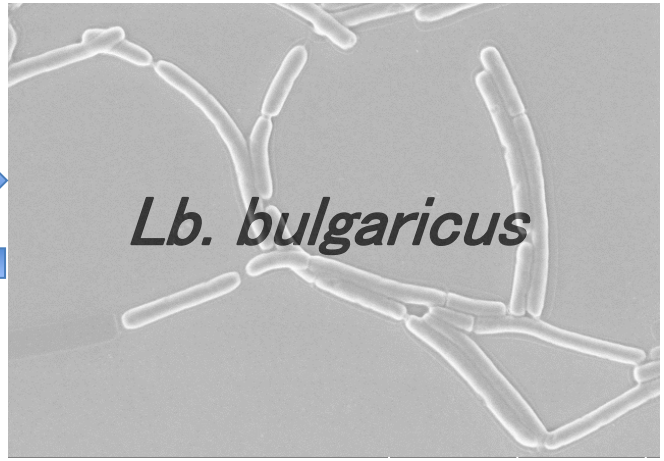
Relation with *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* in yogurt

Symbiosis



Formic acid, Folic acid,
Pyruvic acid, CO_2

Peptide, Amino acids



While these species have been used as yogurt starter…

Str. thermophilus

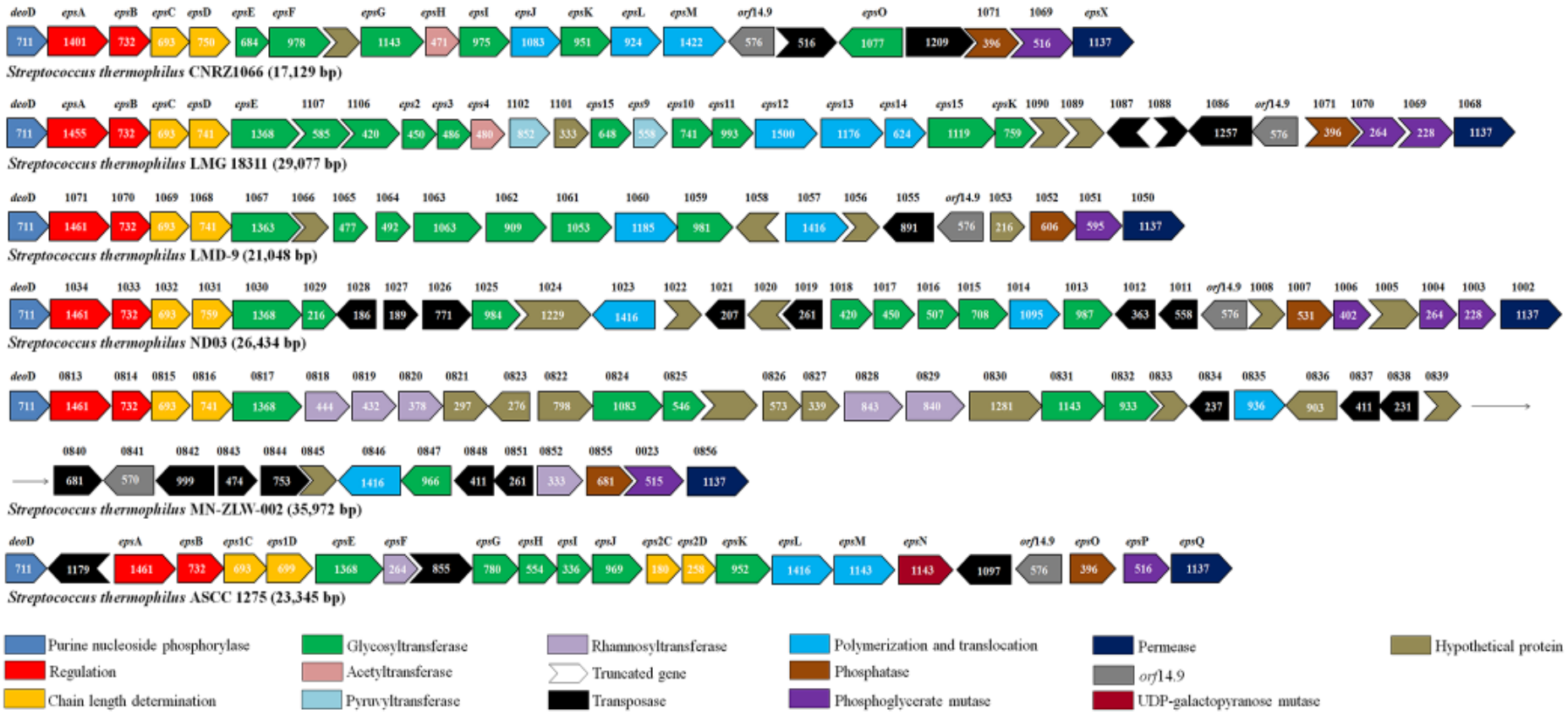
Pseudogenes (approximately 10%)
→Most are carbohydrate-associated genes

Adaptation to milk

Horizontal gene transfer

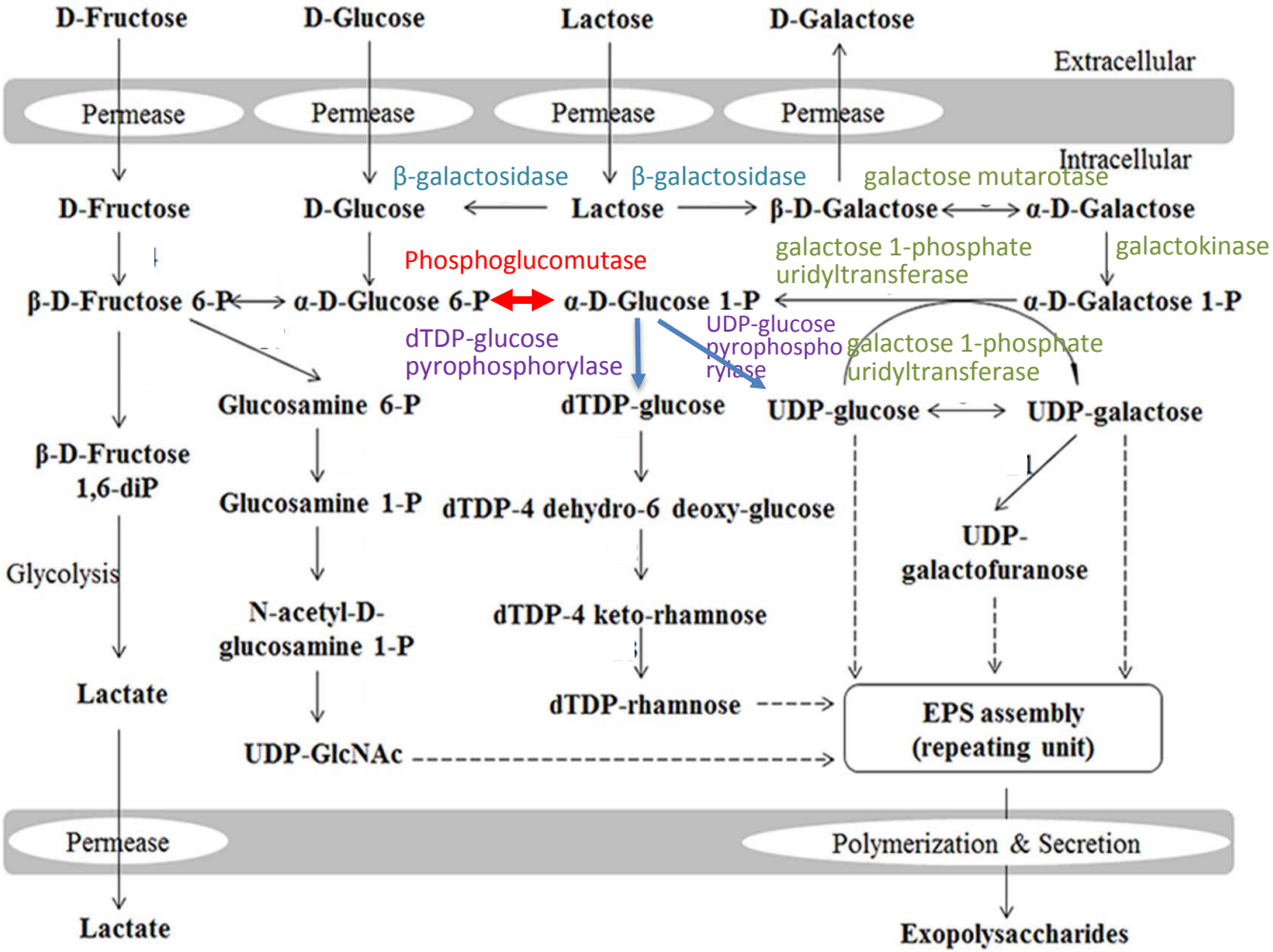
EPS synthesis-associated genes : from *St. thermophilus to Lb. bulgaricus*

Genes of EPS produced from *Streptococcus thermophilus*



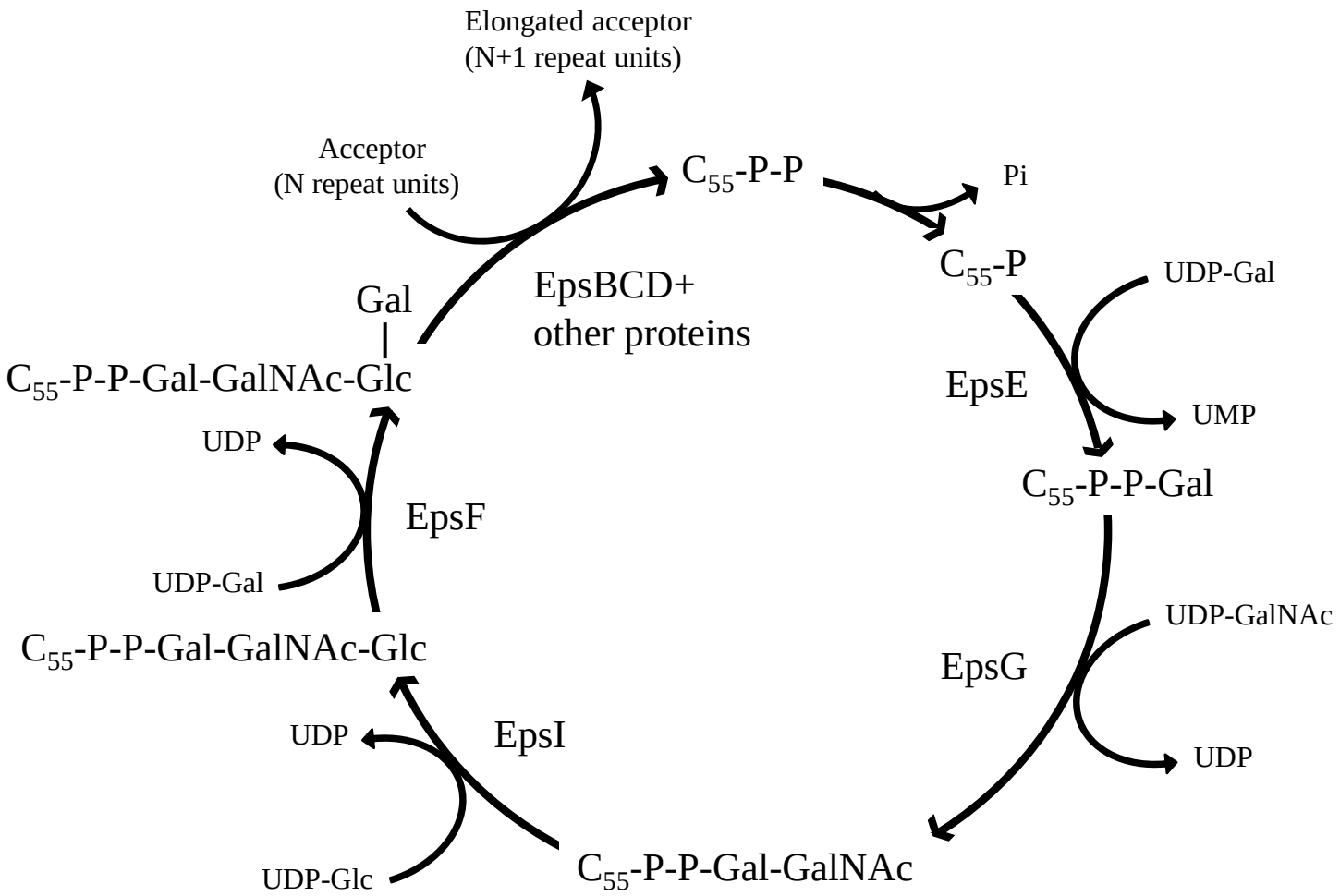
Qinglong Wu, *et al.*, Scientific Reports, 2014, 4, 4974. doi: 10.1038/srep04974

Biosynthesis of EPS produced from *Streptococcus thermophilus*



Qinglong Wu, *et al*., Scientific Reports, 2014, 4, 4974. doi: 10.1038/srep04974

EPS biosynthesis of EPS in *Streptococcus thermophilus*

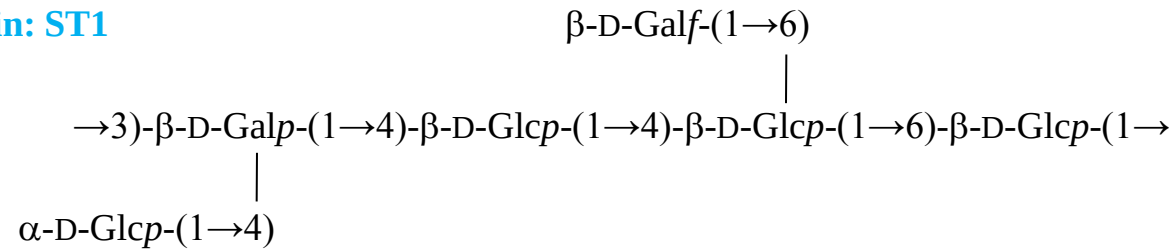


Nishimura J, *et al*., Advances in Microbiology, 2012, 2(3), 208-215.

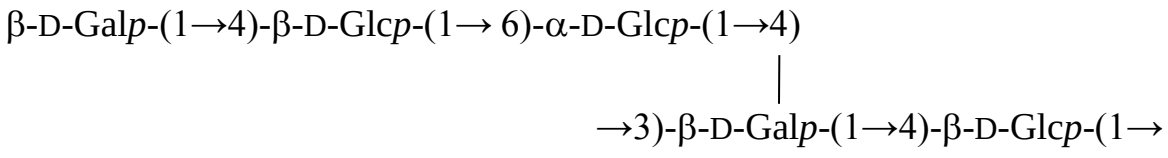
Welman AD, *et al*., TRENDS in Biotechnology, 2003, 21(6), 269-274.

Chemical structure of EPS produced from *Streptococcus thermophilus* strains (1)

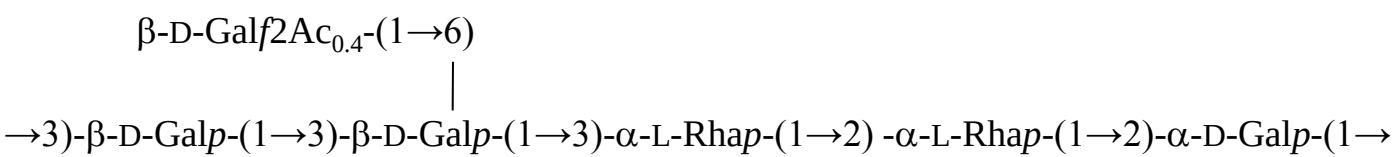
Strain: ST1



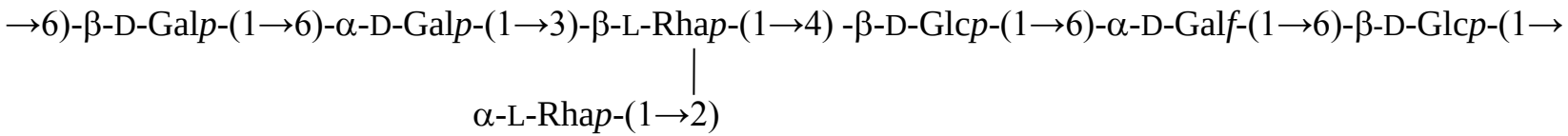
Strain: THS



Strain: S3



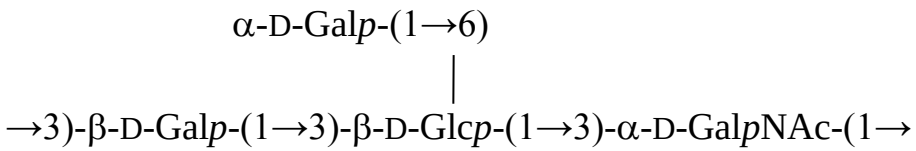
Strain: EU20



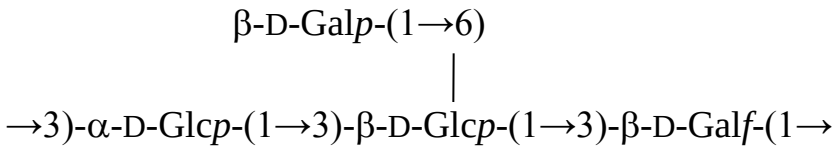
Nishimura J, *et al*., Advances in Microbiology, 2012, 2(3), 208-215.

Chemical structure of EPS produced from *Streptococcus thermophilus* strains (2)

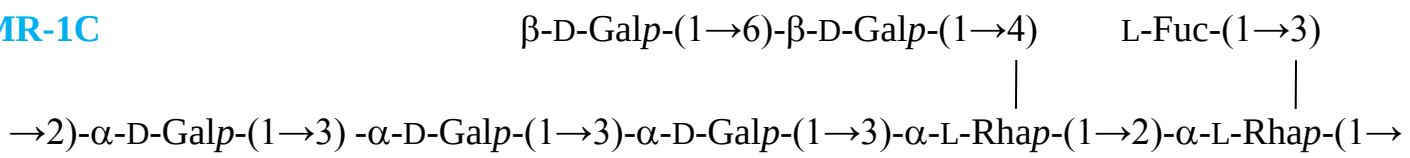
Strain:Sfi6, Sfi20, IMDO1,2,3, NCFB859, 21



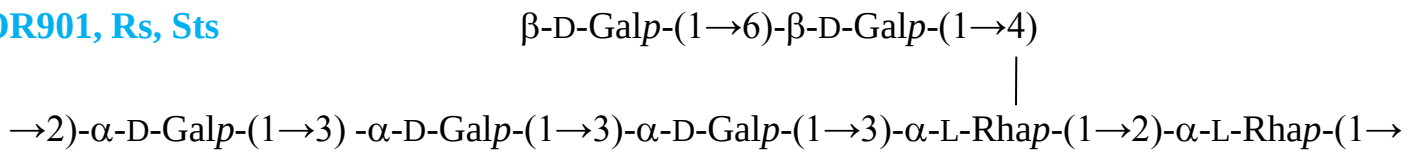
Strain: Sfi39, SY89, SY102



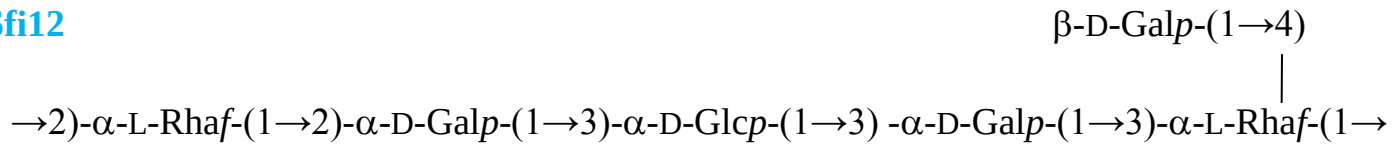
Strain: MR-1C



Strain: OR901, Rs, Sts



Strain: Sfi12



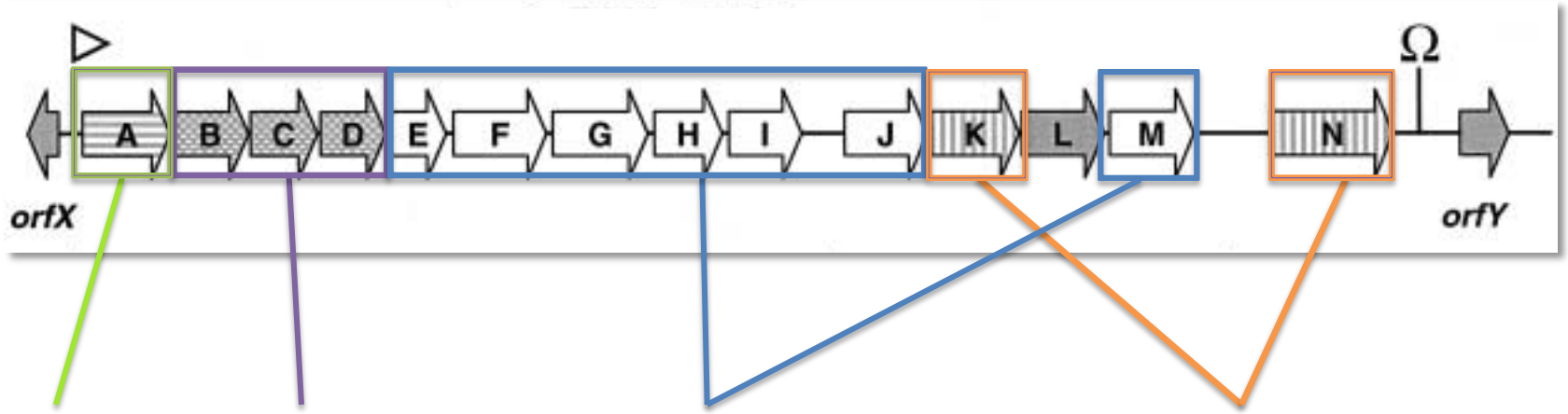
Nishimura J, *et al*., Advances in Microbiology, 2012, 2(3), 208-215.

Genes of EPS produced from *Lactobacillus delbrueckii* subsp. *bulgaricus*

Lb. bulgaricus Lfi5

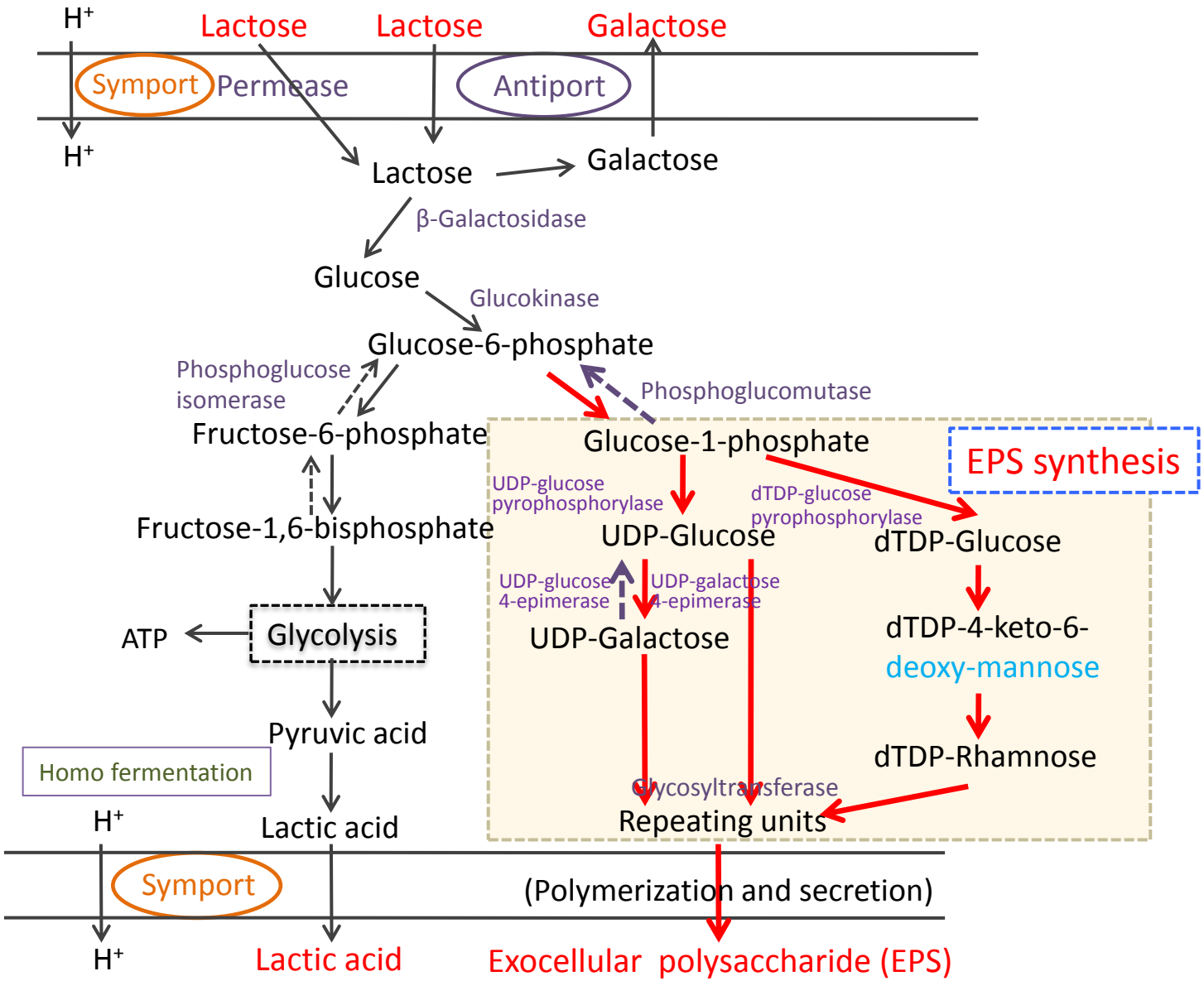
EPS associated genes exists on genomic DNA

The cluster is consisted of 14 genes (*epsA* to *epsN*) and transferred as one mRNA



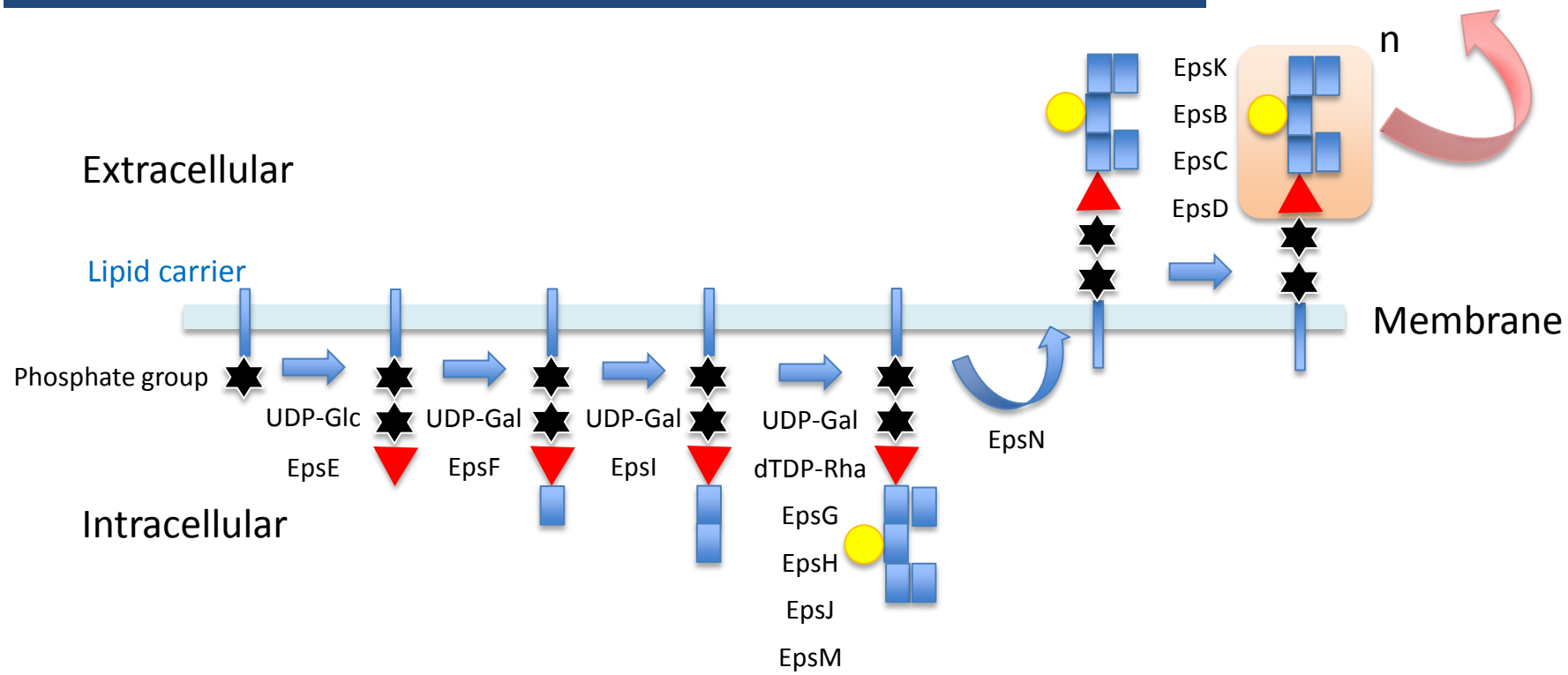
Lamothe GT, *et al*., Archives of Microbiology, 2002, 178(3), 218-228.

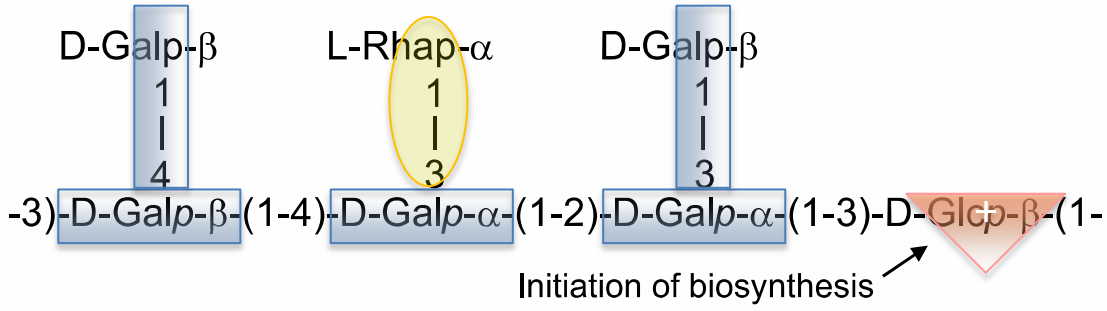
Biosynthesis of EPS produced from *Lactobacillus delbrueckii* subsp. *bulgaricus*



Welman AD, *et al*., TRENDS in Biotechnology, 2003, 21(6), 269-274.

The model of EPS synthesis of *Lactobacillus delbrueckii* subsp. *bulgaricus*

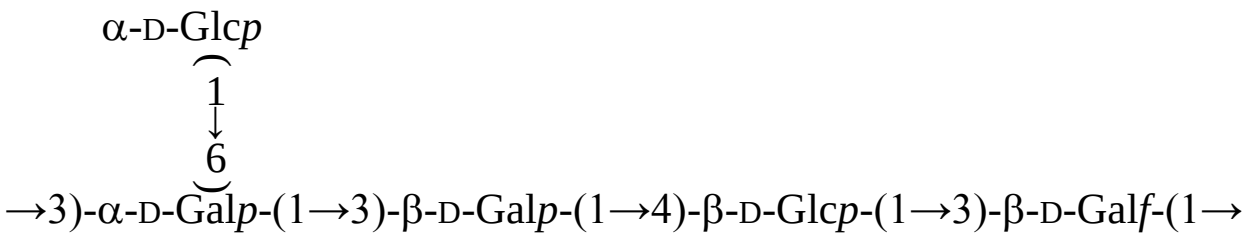




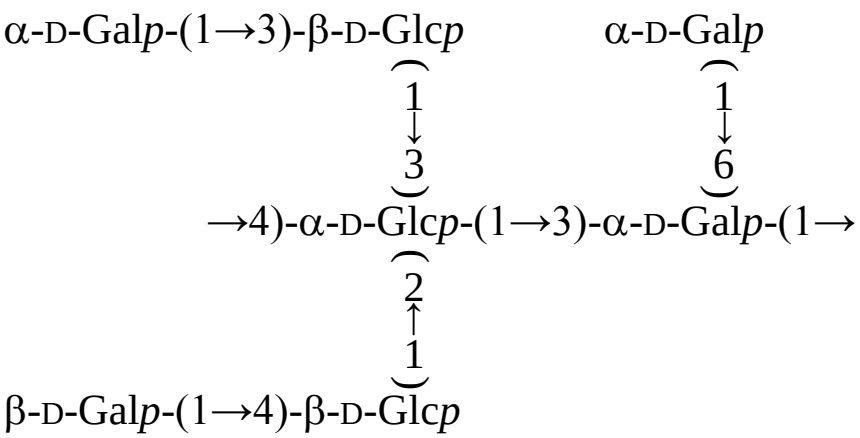
Lamothe GT, *et al.*, Archives of Microbiology, 2002, 178(3), 218-228.

Chemical structure of EPS produced from *Lactobacillus delbrueckii* subsp. *bulgaricus* strains (1)

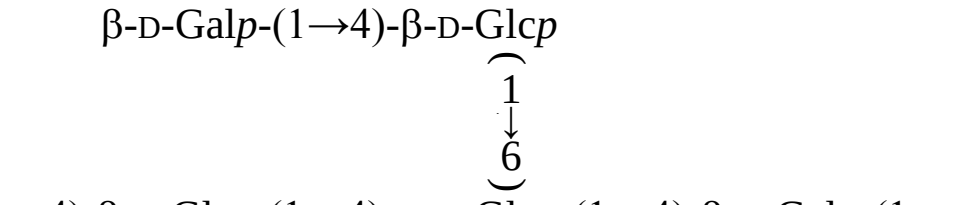
Strain: LBB. B26



Strain: NCFB2074



Strain: 291



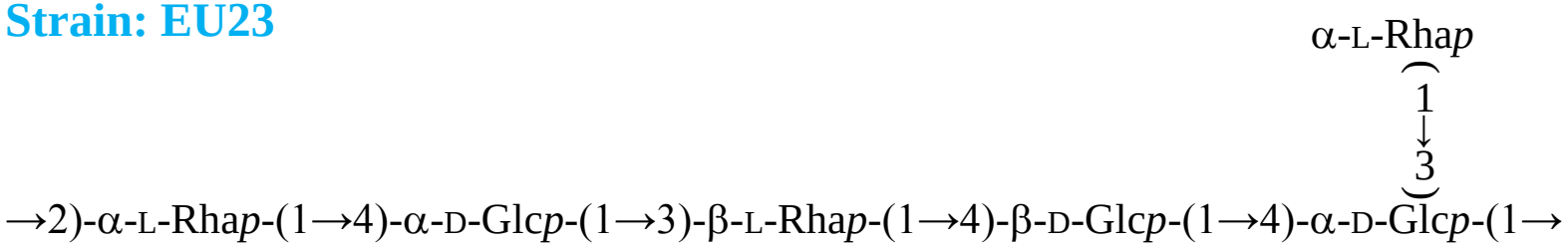
Nishimura J, Advances in Microbiology, 2014, 4(14), 1017-1023.

Chemical structure of EPS produced from *Lactobacillus delbrueckii* subsp. *bulgaricus* strains (2)

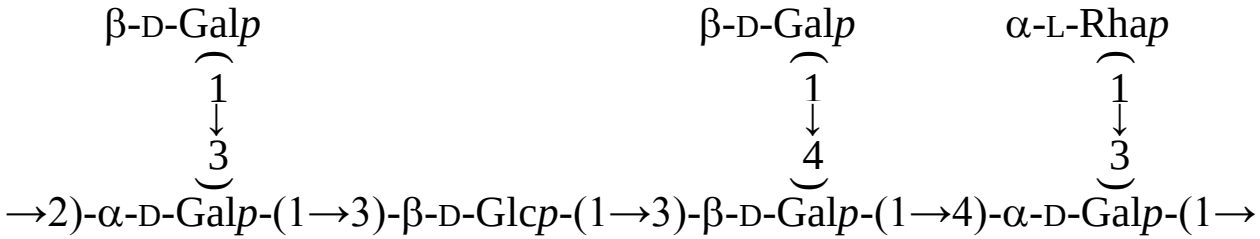
Strain: LBB. B332

→3)-α-D-Glc*p*-(1→3)-α-D-Gal*p*-(1→3)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→2)-α-D-Gal*p*-(1→

Strain: EU23



Strain: rr, Lfi5

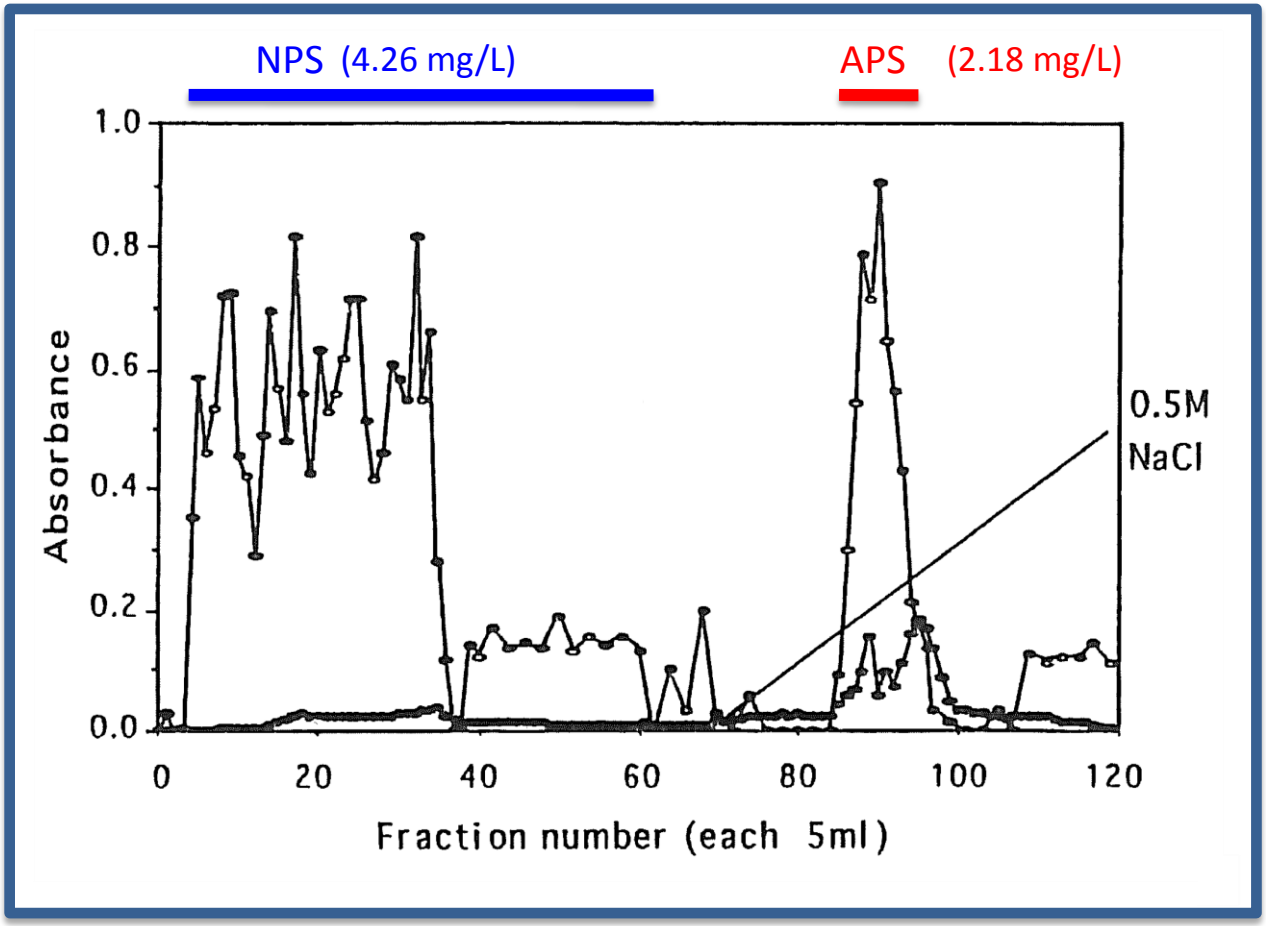


Nishimura J, Advances in Microbiology, 2014, 4(14), 1017-1023.

Chemical structure and physiological function of EPS produced from *Lactobacillus delbrueckii* subsp. *bulgaricus* OLL 1073R-1

Chemical structure of EPS produced from *Lactobacillus delbrueckii* subsp. *bulgaricus* OLL 1073R-1

Anion-exchange chromatogram of EPS from *Lb. bulgaricus* OLL 1073R-1



＜Elution conditions＞
Column: DEAE-TOYOPEARL 650 M (2.6 x 20 cm)
Eluate: 50 mM Tris-HCl buffer (pH8.6)
Gradient solution: 0-0.5M NaCl (−)
Flow rate: 1.0 ml/min
Detections: Neutral saccharides 490 nm (○)
Protein 280 nm (●)

Production of NPS and APS

(Uemura (Nishimura) J, *et al*., *Milchwissenschaft*, 1998)

Molecular weights, chemical compositions, and molar ratio of monosaccharide in NPS and APS



＜Conditions＞

Column : Asahipak GS-710 (7.6 mm x 500 mm)

Mobile phase : 5mM Triethyl acetic acid buffer (pH5.0)

Temperature : Room temp.

Flow rate : 1.0 ml/min

Detections : RI, UV(220nm)

NPS

1.2×10^6 Da

APS

1.1×10^6 Da



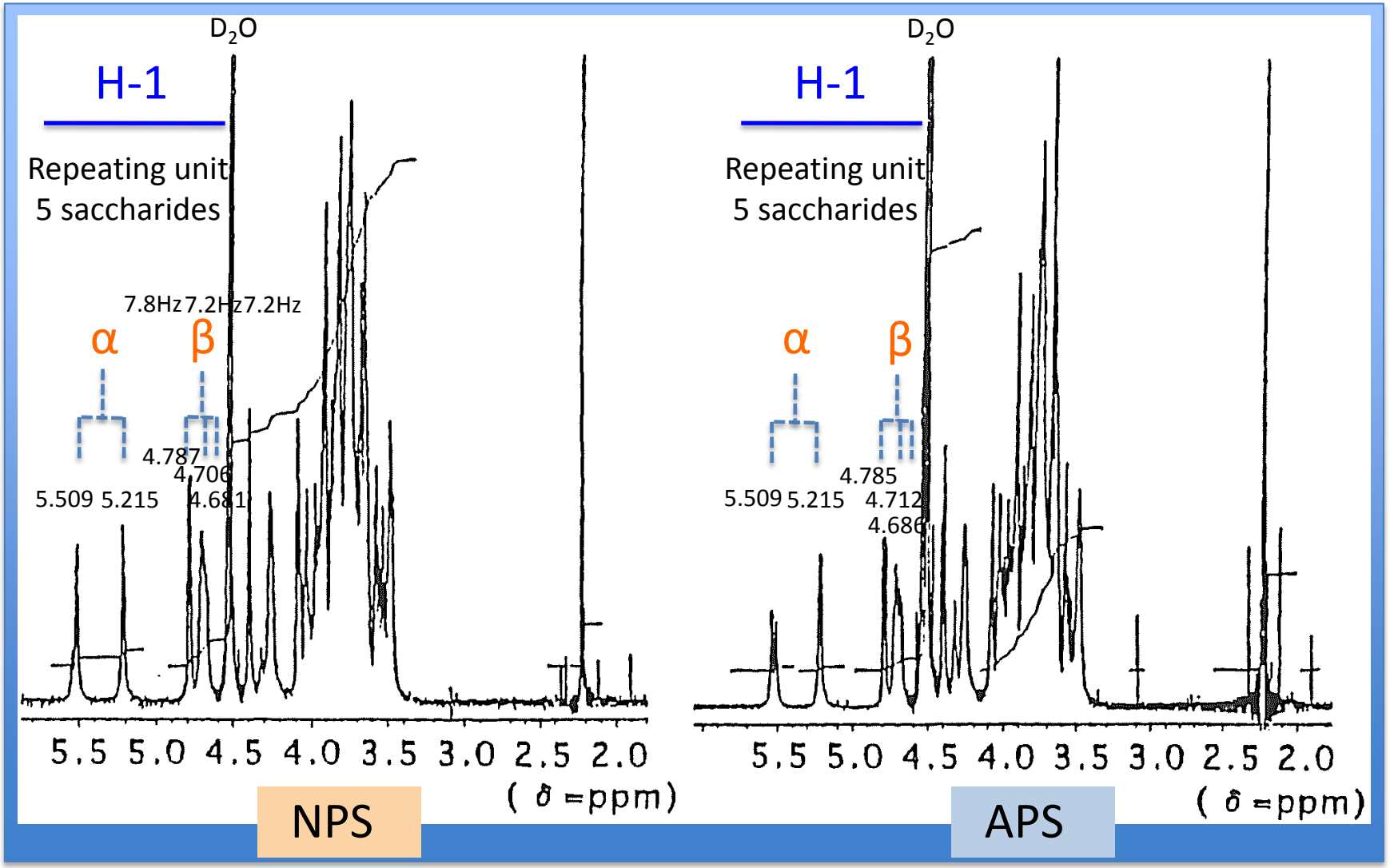
HPLC chromatograms of NPS and APS

	Molar ratio	
	NPS	APS
Glucose	1.0	1.0
Galactose	1.3	1.3
	Contents （%）	
Phosphorus	0.0	0.1

Only phosphorus contents are different between NPS and APS

(Uemura (Nishimura) J, *et al*., *Milchwissenschaft*, 1998)

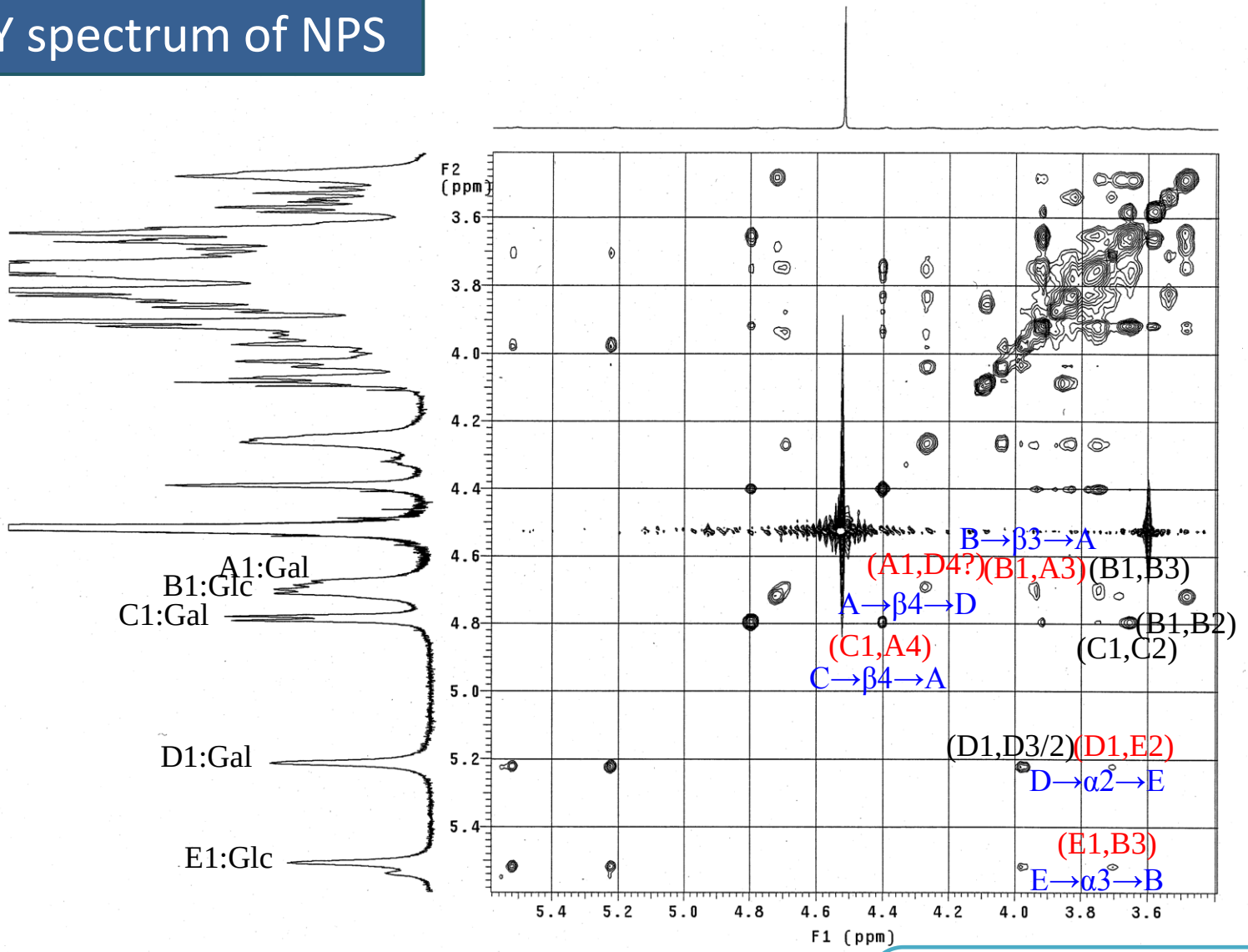
^{1}H-NMR spectra of NPS and APS



(Uemura (Nishimura) J, *et al*., *Milchwissenschaft*, 1998)

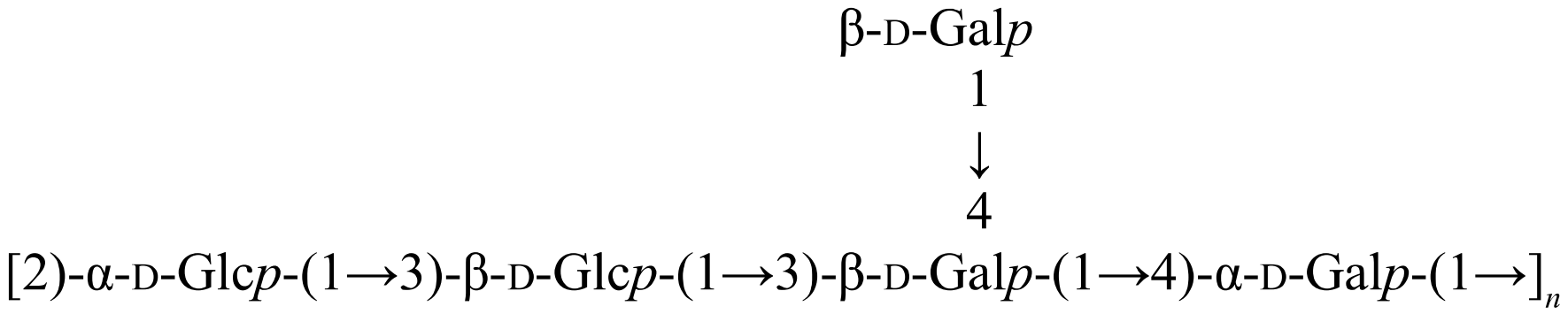
Same carbohydrate structure

NOESY spectrum of NPS



C
↓
β4
↓
B→β3→A→β4→D→α2→E→α3→

Chemical structure of NPS produced from *Lactobacillus delbrueckii* subsp. *bulgaricus* OLL1073R-1



Van Calsteren MR, *et al*., Carbohydrate Research, 2015, 413, 115-122.
doi: 10.1016/j.carres.2015.05.015

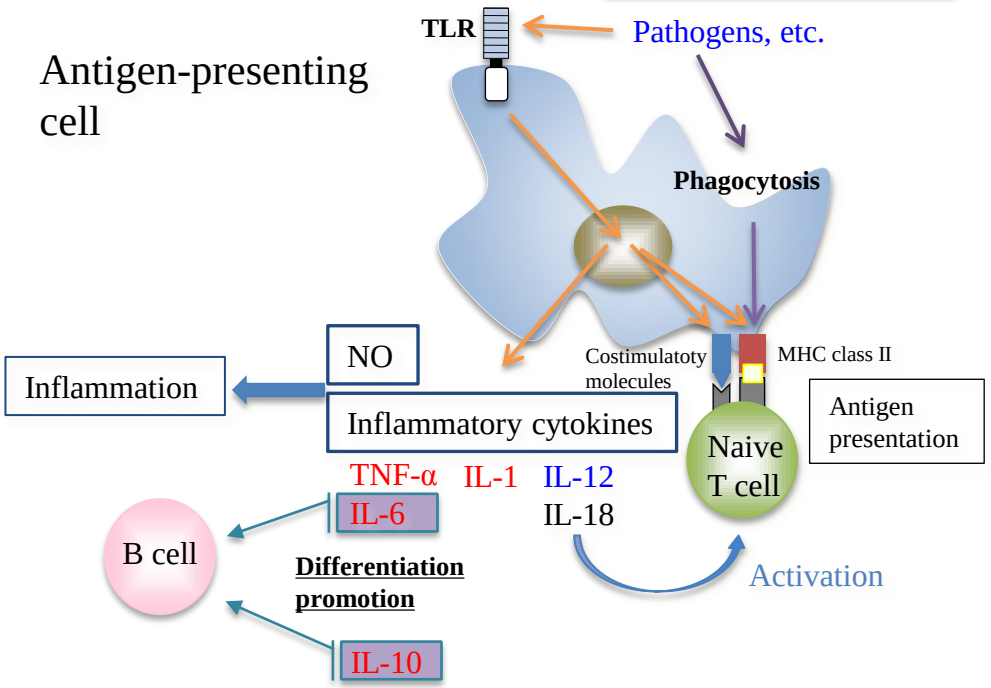
Conclusion

1. *Lb. bulgaricus* OLL 1073R-1 strain produced NPS and APS which phosphorus content was different. The common repeating unit was decided.

β-D-Gal*p*
1
↓
4
[2)-α-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→3)-β-D-Gal*p*-(1→4)-α-D-Gal*p*-(1→]$_n$

2. The physiological functions were different between NPS and APS. → Phosphorus

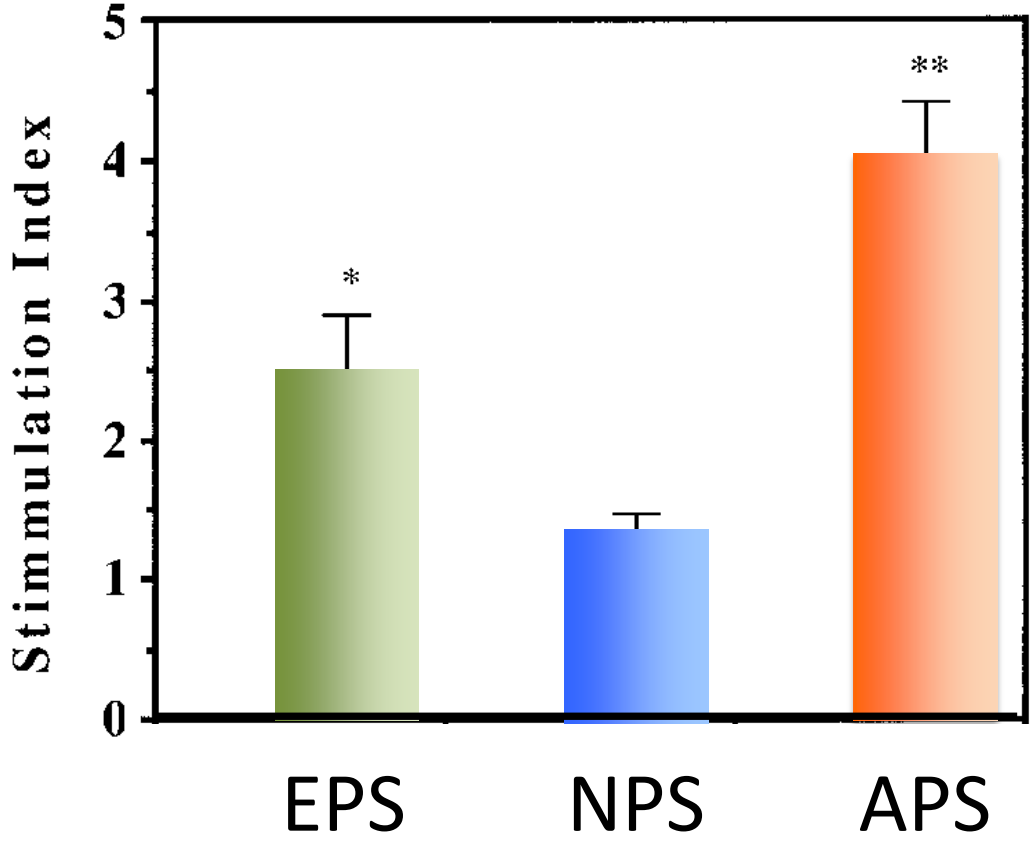
	NPS	APS
Mitogenic activity (Spleen: B cell)	-	+
Morphological change(Mϕ)	+	+
NO production(Mϕ)	-	-
Anti-influenza virus effects(mice)	-	+




Thank you for your attention.

Physiological function of EPS produced from *Lactobacillus delbrueckii* subsp. *bulgaricus* OLL 1073R-1

Mitogenic activity of polysaccharide preparations from *Lb. bulgaricus* to spleen cells



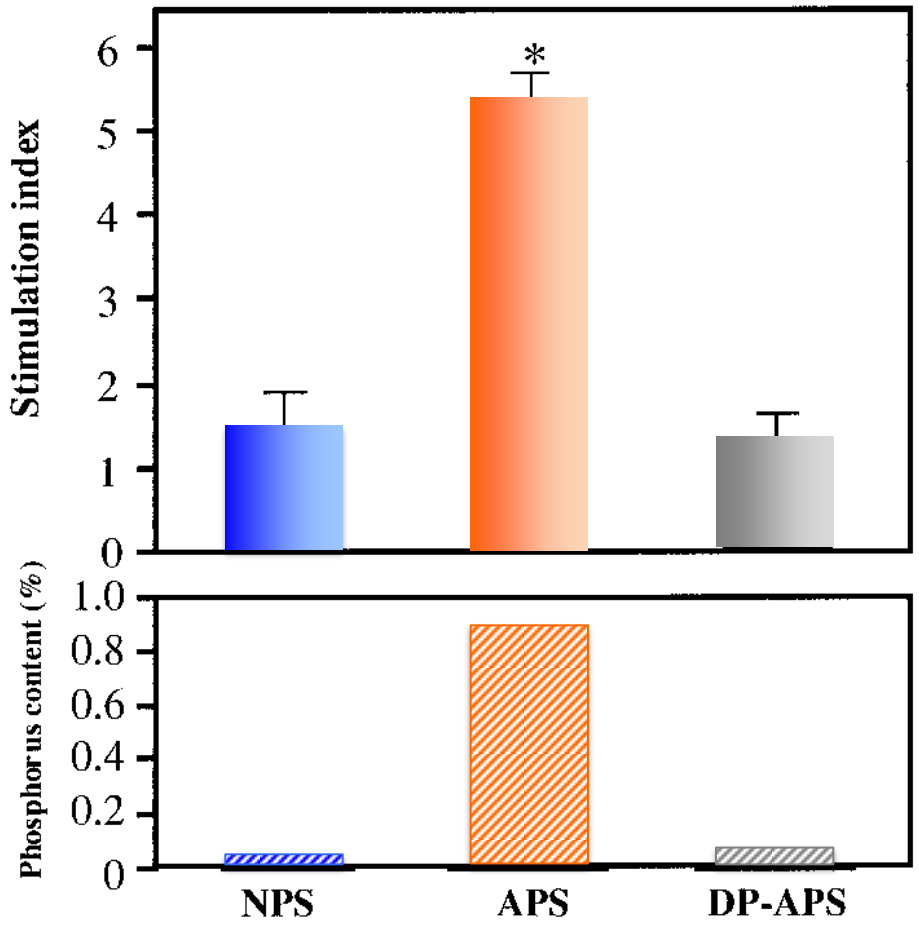
(concentration of 200 µg/ ml)

Kitazawa H, *et al.*, International Journal of Food Microbiology 40 (1998) 169 –175.

Effect of dephosphorylation of APS on the mitogenic response

A.

B.



Kitazawa H, *et al.*, International Journal of Food Microbiology 40 (1998) 169 –175.

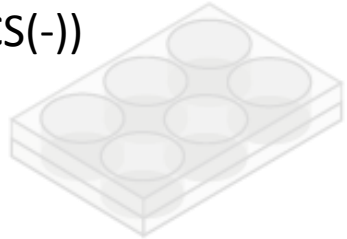
Physiological function of *Lactobacillus delbrueckii* subsp. *bulgaricus* OLL1073R-1

Materials and Methods

J774.1 cells
(Macrophage-like cell line)
(RPMI-1640 medium : FCS(-))

→ Adhesion and wash





← NPS, APS
(100～200 μg/ml)
or LPS (*E.coli* O111:B4)
(20 μg/ml)

Cultivation with CO_2 incubator
(37℃, 5% CO_2)

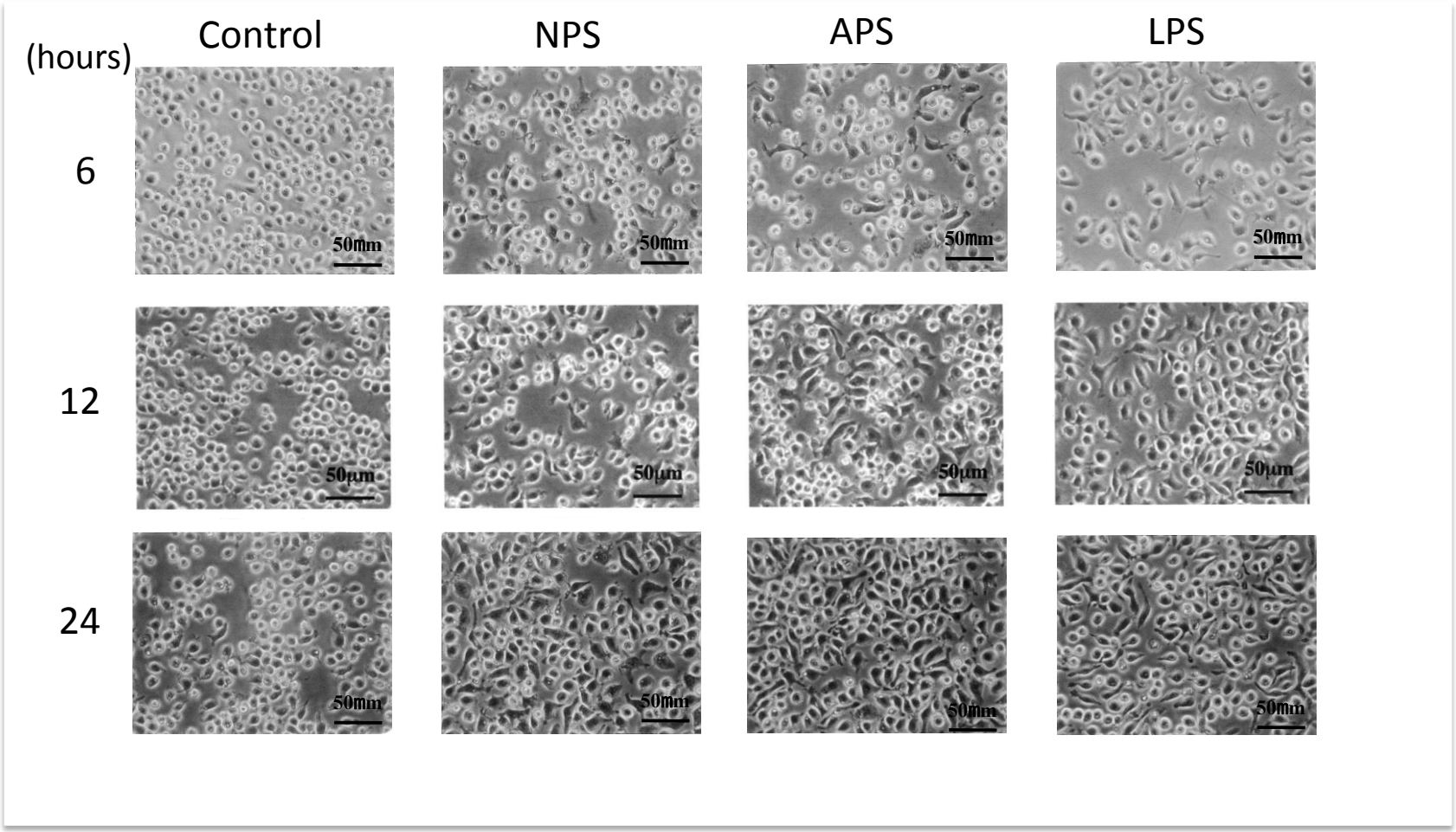
6, 12, 24 hrs

Culture supernatants : Determination of NO
(Azo-dye method)

Cells : Alternation of cellular morphology (x 200)

Uemura (Nishimura) J, *et al*., Food Microbiology, 2003,20(3), 267-273.

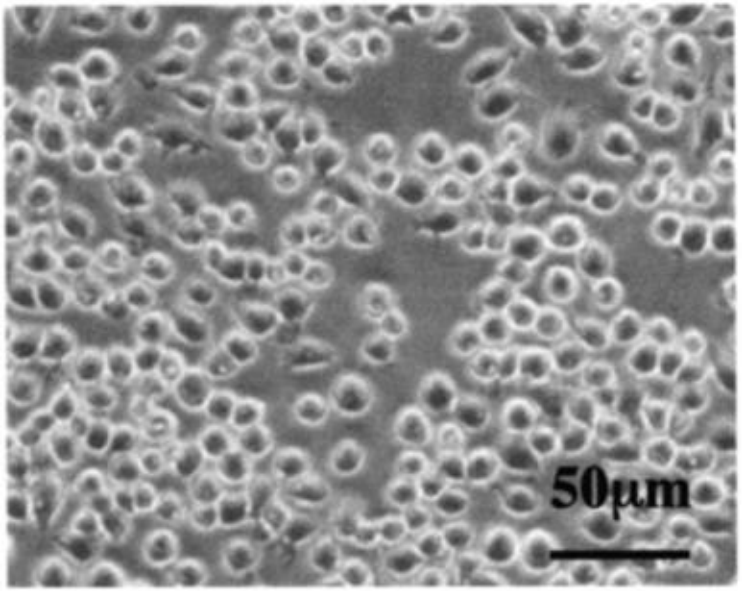
Morphological change by the stimulation of NPS, APS and LPS on J774.1 cells



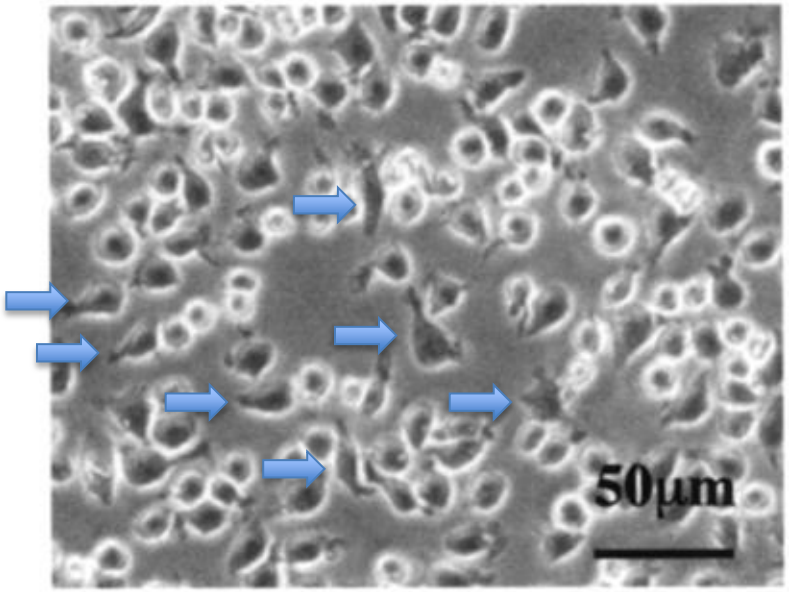
Uemura (Nishimura) J, *et al*., Food Microbiology, 2003,20(3), 267-273.

Expansion of the stmulation for 12 hrs

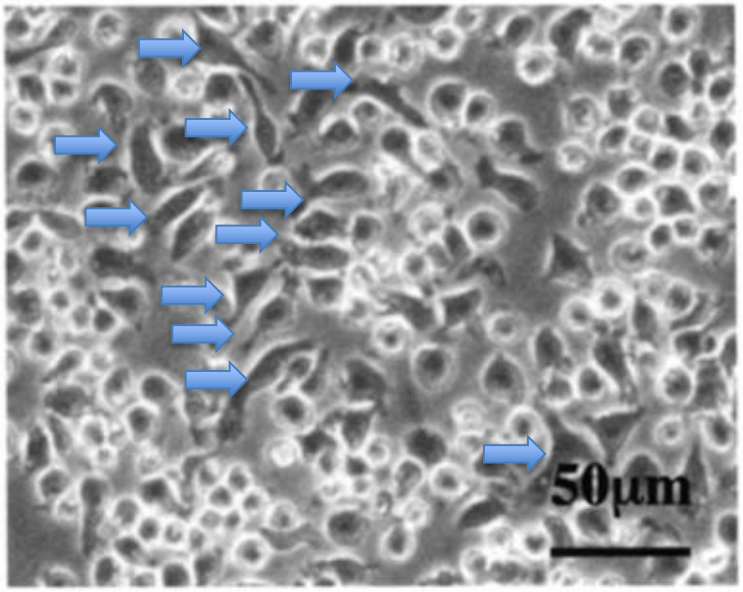
Control



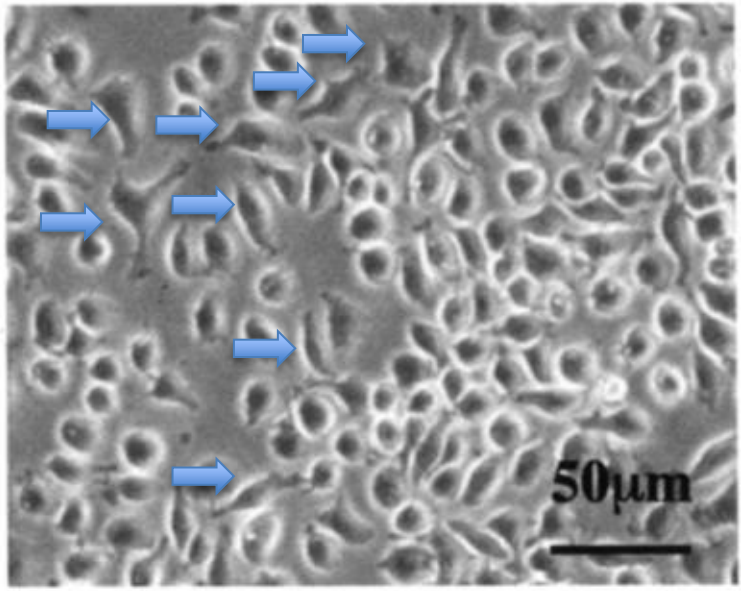
NPS



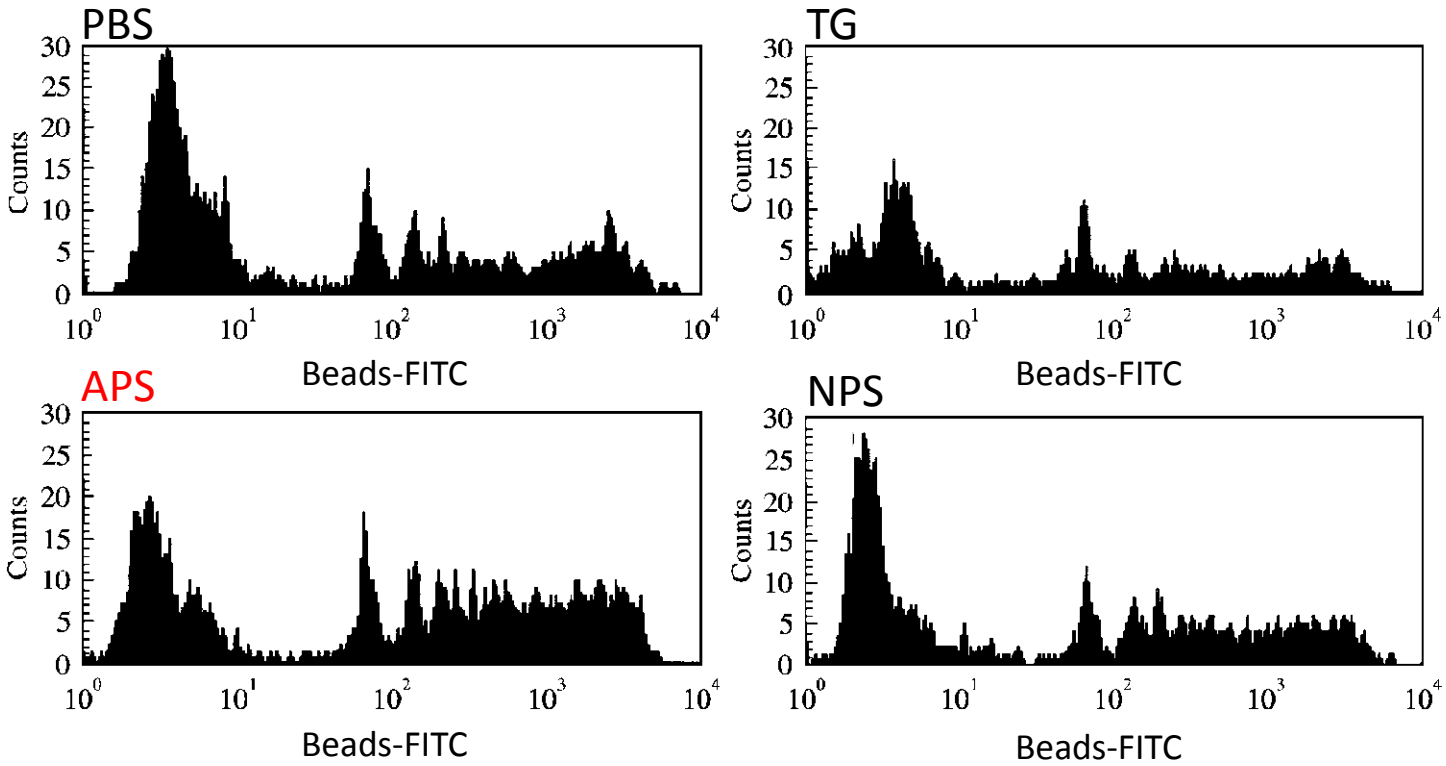
APS



LPS



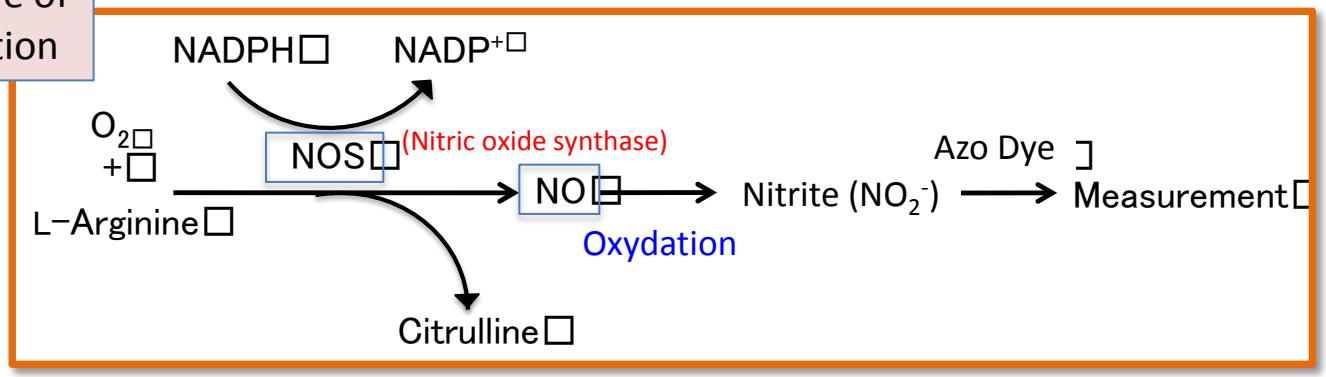
Analysis of macrophage phagocytosis augmented with NPS and APS



H. Kitazawa et al., Food Microbiology, 17, 109-118, 2000.

Generation of nitric oxide by the stimulation of NPS, APS, and LPS on J774.1 cells

The principle of determination



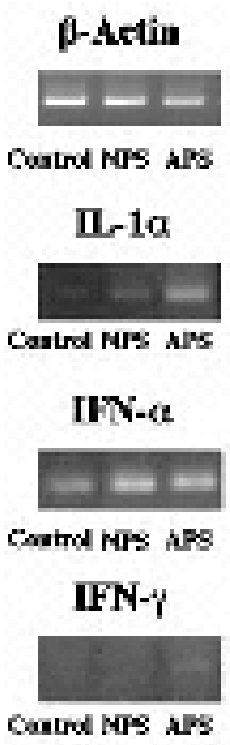
Concentration of NO_2-N (ng/ml)			
	Incubation time (hours)		
	6	12	24
Control	14.1±0.8	16.1±2.1	32.3±3.4
NPS	14.1±2.0	12.4±0.9	23.3±2.3
APS	14.9±1.1	13.4±0.8	22.0±1.4
LPS	33.5±5.4*	42.1±2.3*	67.9±3.6**

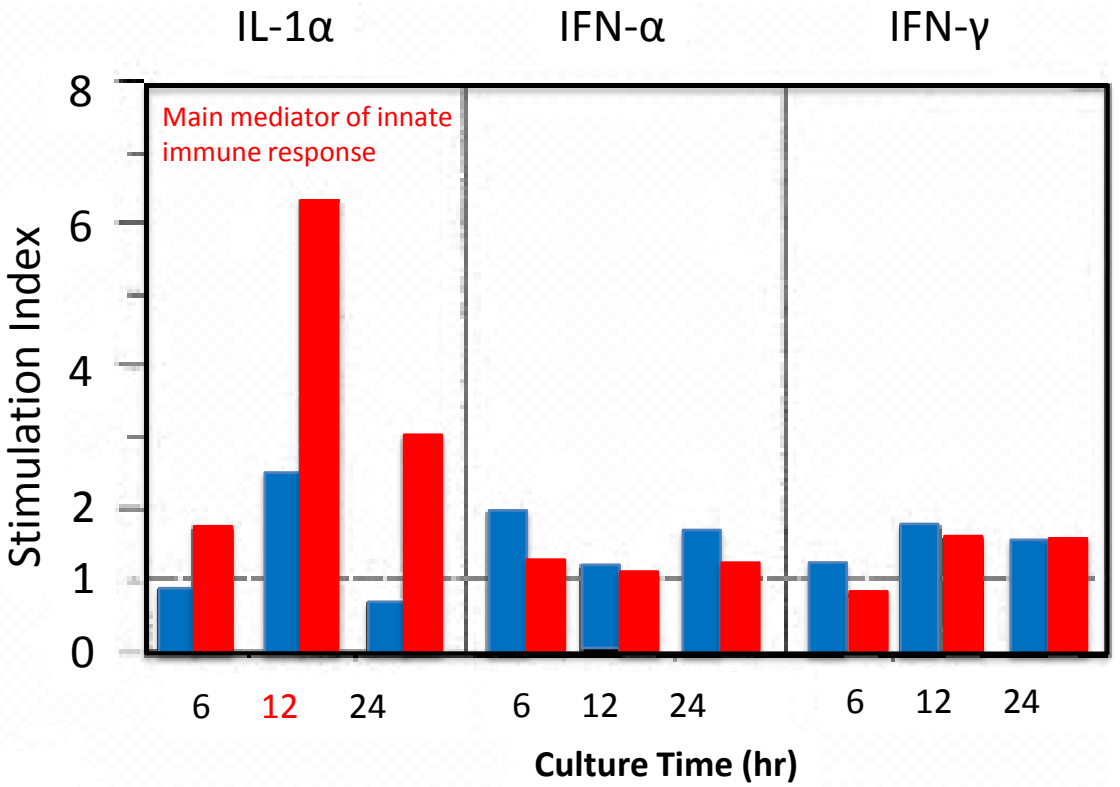
(n=3, means±s.d., *p<0.01, **p<0.001)

NPS and APS does not induce NO generation (Unlike LPS)

Uemura (Nishimura) J, *et al.*, Food Microbiology, 2003,20(3), 267-273.

Gene expression of cytokines by the stimulation with NPS and APS (1)

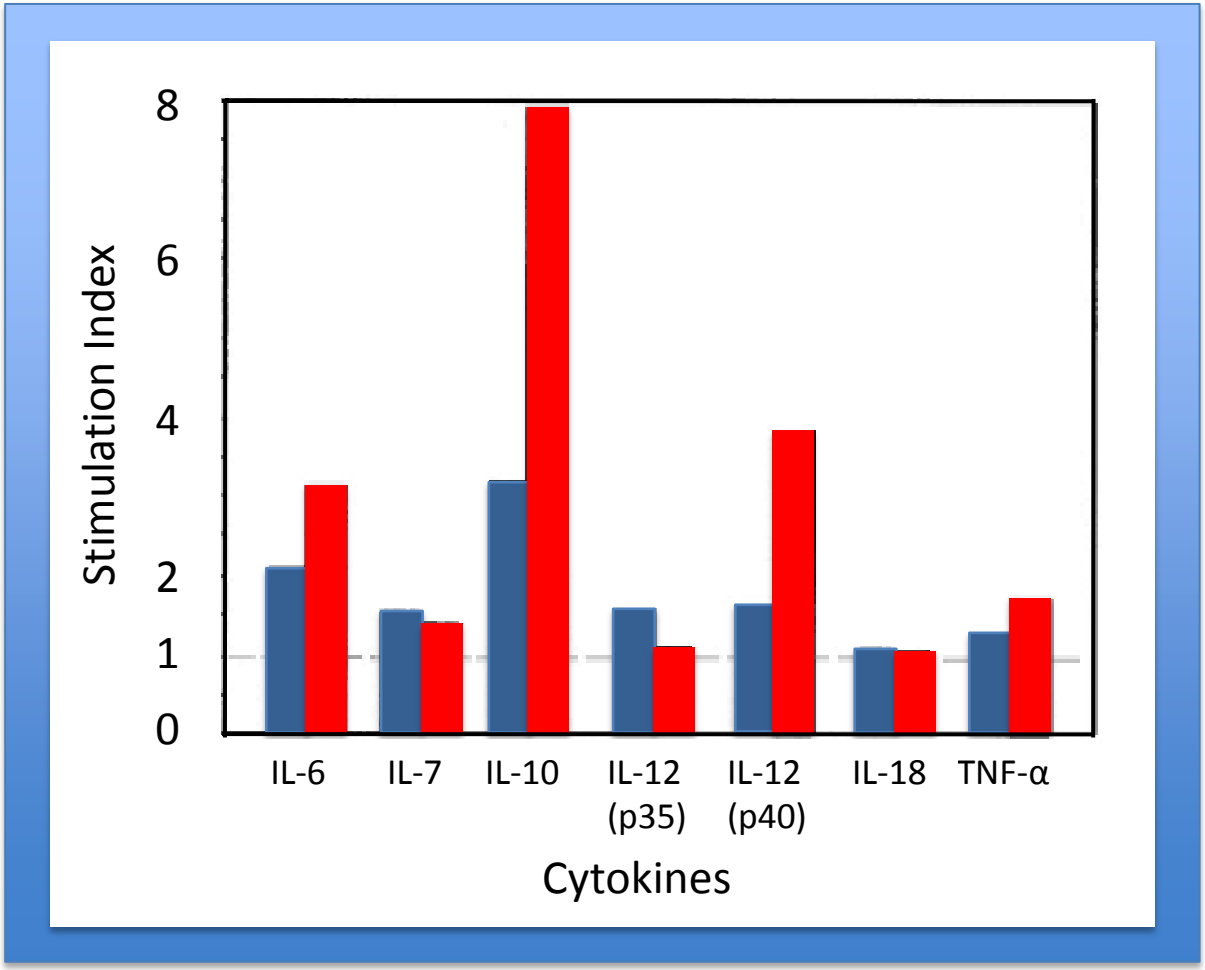




■ : NPS (100 μg/ml) ■ : APS (100 μg/ml) (Uemura (Nishimura) J, *et al.*, *Food Microbiology*, 2003)

Gene expression of IL-1α was augmented by the stimulation with APS (maximum at 12 hr).

Gene expression of cytokines by the stimulation with NPS and APS (2)

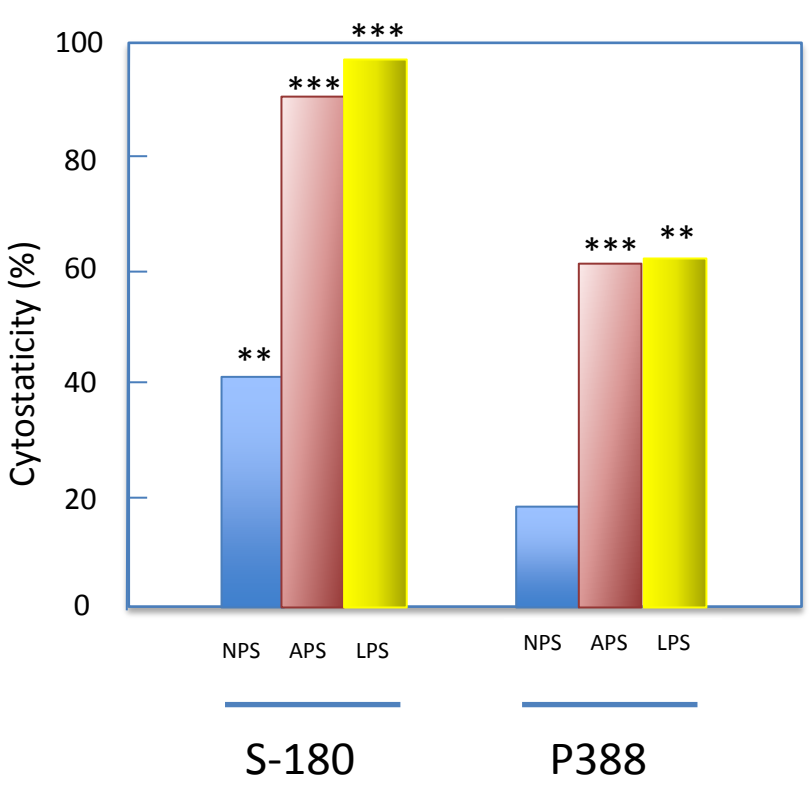


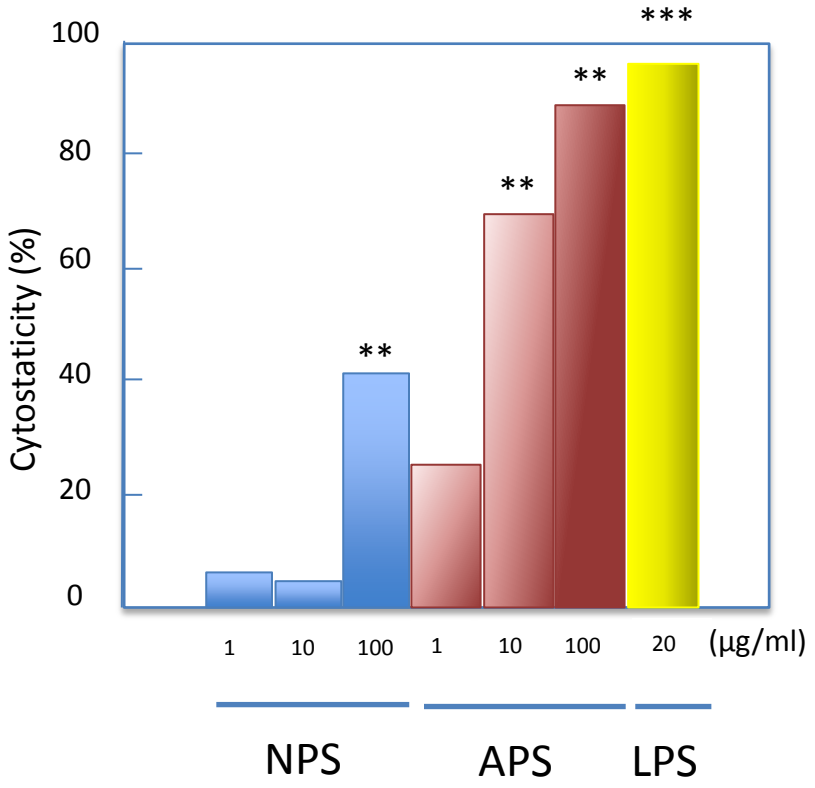
Stimulation: 200 μg/ml (for 12hr)

(Uemura (Nishimura) J, *et al.*, *Food Microbiology*, 2003)

Gene expressions of IL-6, 10, 12p40, and TNF-α were also enhanced by APS.

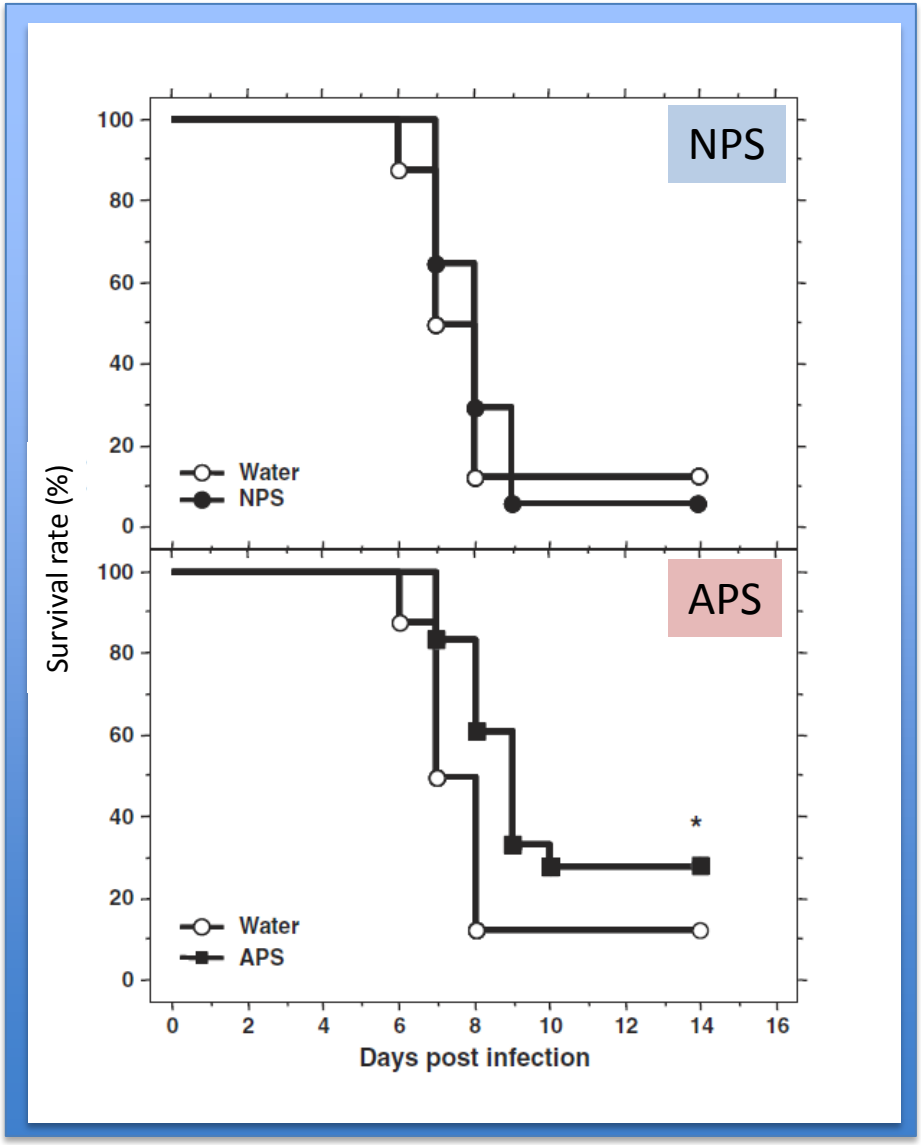
Cytostatic activity of macrophages stimulated with NPS and APS to tumor cells





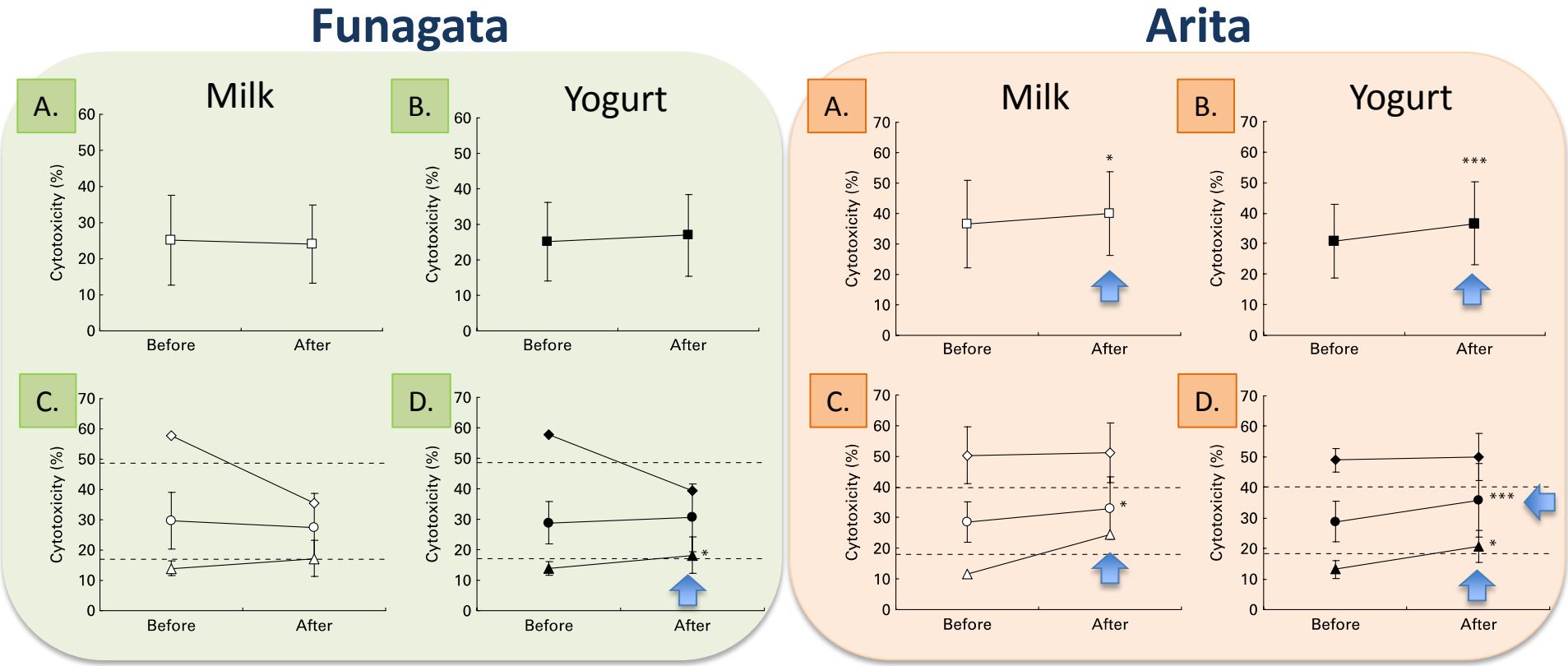
H. Kitazawa et al., Food Microbiology, 17, 109-118, 2000.

Effect of NPS and APS on the survival rate of influenza virus-infected mice



Nagai T, *et al*., International Immunopharmacology, 2011,11, 2246-2250.

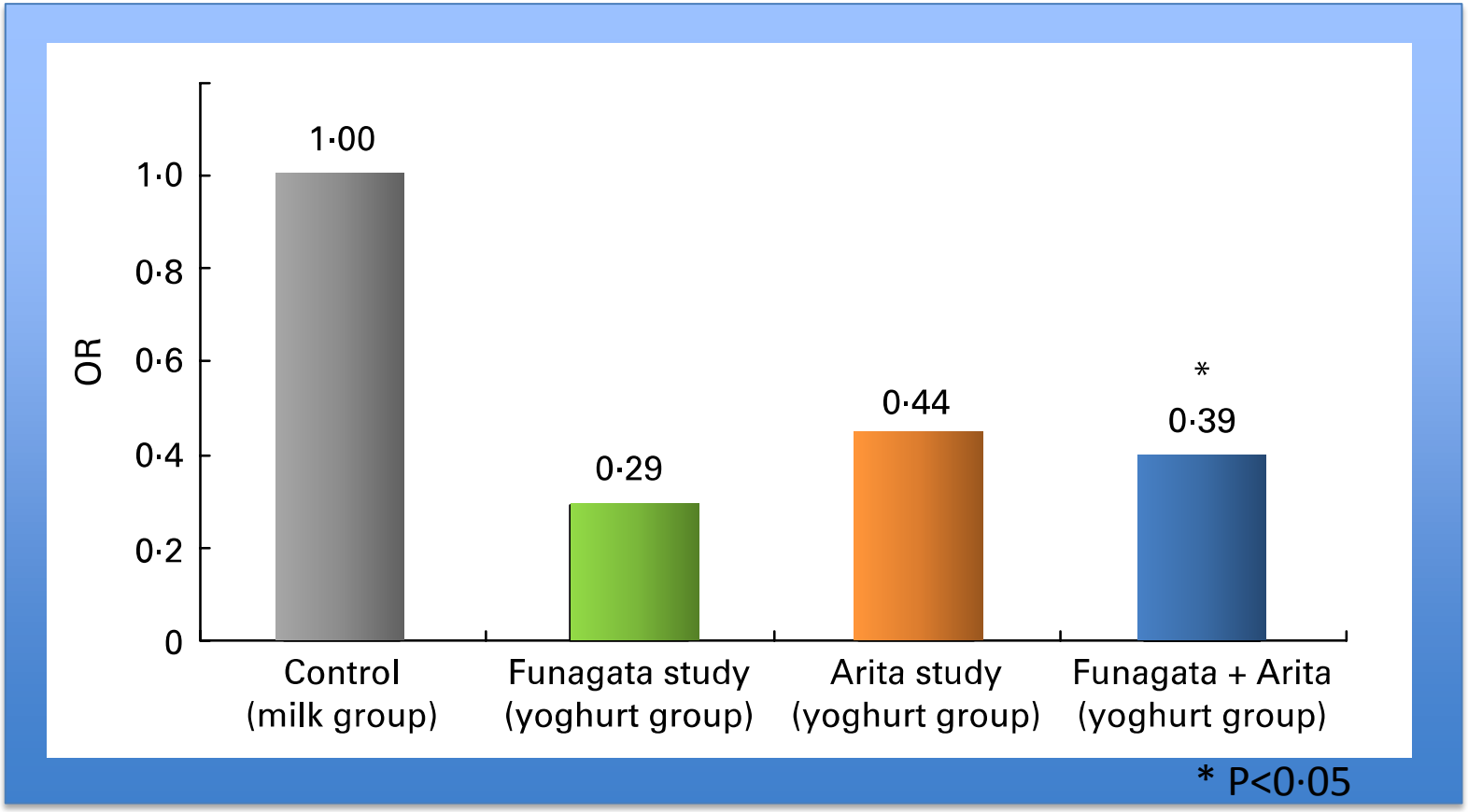
Natural killer cell activity of in the milk and yogurt fermented with *Lb. bulgaricus* OLL1073R-1



Makino S, *et al.*, British Journal of Nutrition, 2010, 104, 998-1006.

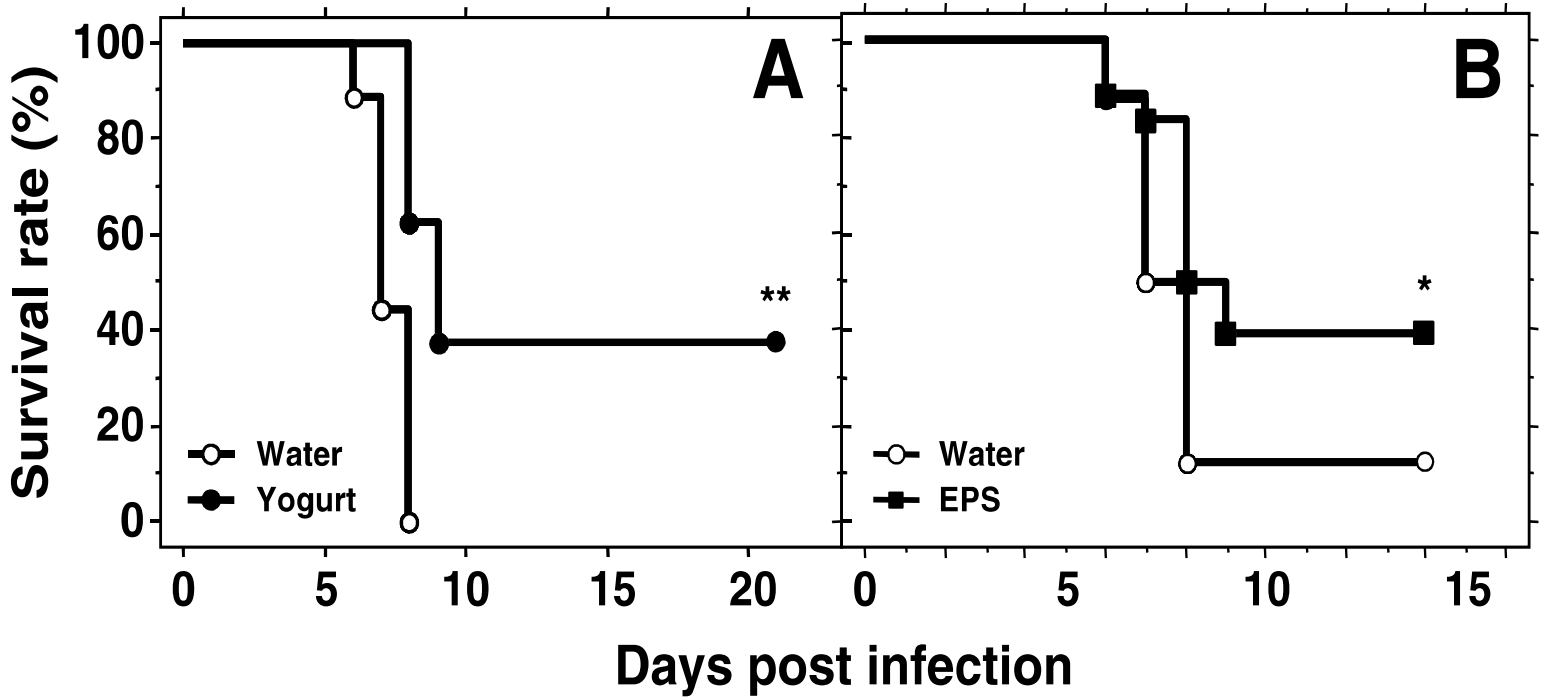
Development as functional yogurt fermented by *Lactobacillus delbrueckii* subsp. *bulgaricus* OLL 1073R-1

Physiological function of *Lactobacillus delbrueckii* subsp. *bulgaricus* OLL1073R-1



Makino S, *et al*., British Journal of Nutrition, 2010, 104, 998-1006.

Effect of yogurt and EPS on the survival rate of influenza virus-infected mice



(*, p<0.05; **, p<0.01, Kaplan–Meier method logrank test)

Group A:
Water, and yogurt fermented with *Lb. bulgaricus* OLL1073R-1 and *Str. thermophilus* OLS3059

Group B:
Water, and EPS prepared from the culture supernatant of *Lb. bulgaricus* OLL1073R-1

Nagai T, *et al*., International Immunopharmacology, 2011,11, 2246-2250.