Probiotic microorganisms in the microbiota of traditional fermented foods

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Institute of Technology of Agricultural Products


FOOD QUALITY & SAFETY


FOOD PROCESSING AND FOOD PRESERVATION TECHNOLOGIES


FOOD MOLECULAR MICROBIOLOGY


GENOMICS


WINES & SPIRITS


PRESERVATION TECHNOLOGIES OF FRESH PRODUCTS


FOOD BIOTECHNOLOGY & RECYCLING OF AGRICULTURAL BY-PRODUCTS


EDIBLE FUNGI


DAIRY PRODUCTS

Sector

FOOD PROCESSING AND PRESERVATION

Research activities

- Application of novel technologies for ensuring food safety and quality
- Natural antimicrobials/ preservatives (essential oils, hydrosols), biopreservation
- Mild treatments/ non-thermal technologies such as High Hydrostatic Pressure (with a laboratory scale equipment installed in ITAP)
- New packaging systems (e.g. active packaging, edible films)
- Fermentation and production of high added value food products/ functional foods (e.g. probiotic/starter cultures, prebiotics, bioactive compounds)
- Exploitation of Greek/ traditional foods as a source of microorganisms with probiotic/ technological/ protective properties and application in laboratory, pilot and industrial scale
- Application of modern and rapid techniques for food analysis (e.g. GC-MS, FTIR, HPLC, PFGE, PCR, RT-PCR, DGGE) combined with data analytics

Structure of presentation

- Probiotic microorganisms Definition, benefits, mode of action
- Probiotic microorganisms in microbiota of fermented foods
- Applications in Foods
- **Legal framework**

Beginning of story

“All diseases start from the stomach”

“Let food be your medicine and medicine be your food”

Hippocrates (460-377 B.C.)

The evolution of the story

460-377 B.C.

Hippocrates

Elie Metchnikoff

Russian Nobel laureate, Pasteur Inst., 1st introduced the relation of m/o yogurt with health

1907

1930

Minoru Shirota

He made Yakult, with *Lactobacillus casei* Shirota.

1953

Werner Kollath

Probiotic (pro and Bios*)*
"promotes life"

1965

Lilly and Stillwell

"substances secreted by one organism which stimulate the growth of another»

1992

Fuller

"a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance."

2002

FAO/WHO

"live microorganisms which when administered in adequate amounts confer a health benefit on the host"

2013

Hill et al.*

Expert consensus document on the scope and appropriate use of the term probiotic according to FAO.
"functional foods"

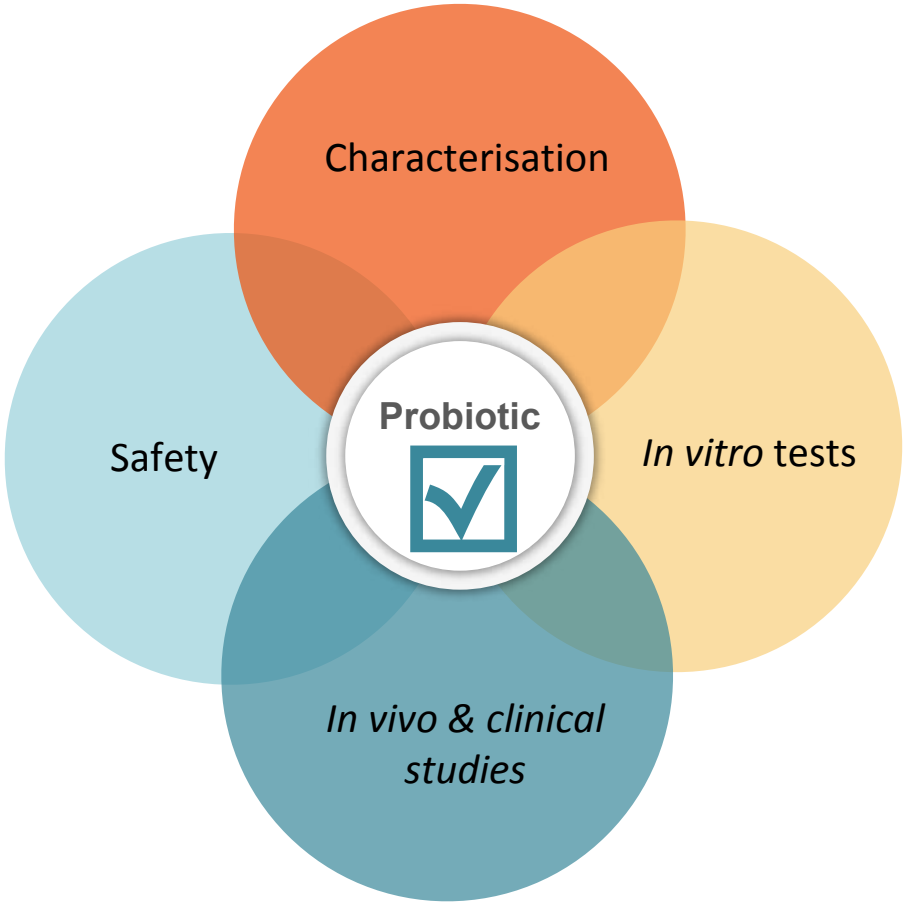
2021

ISAPP
Marco et al.

Consensus statement on fermented foods

*Hill C, Guarner F, Reid G, et al. Nat Rev Gastroenterol Hepat. 2014;11:506–514.
Marco et al. *Nature Reviews Gastroenterology & Hepatology* **2021; 18, 196–208

Minimum requirements for the definition of probiotics



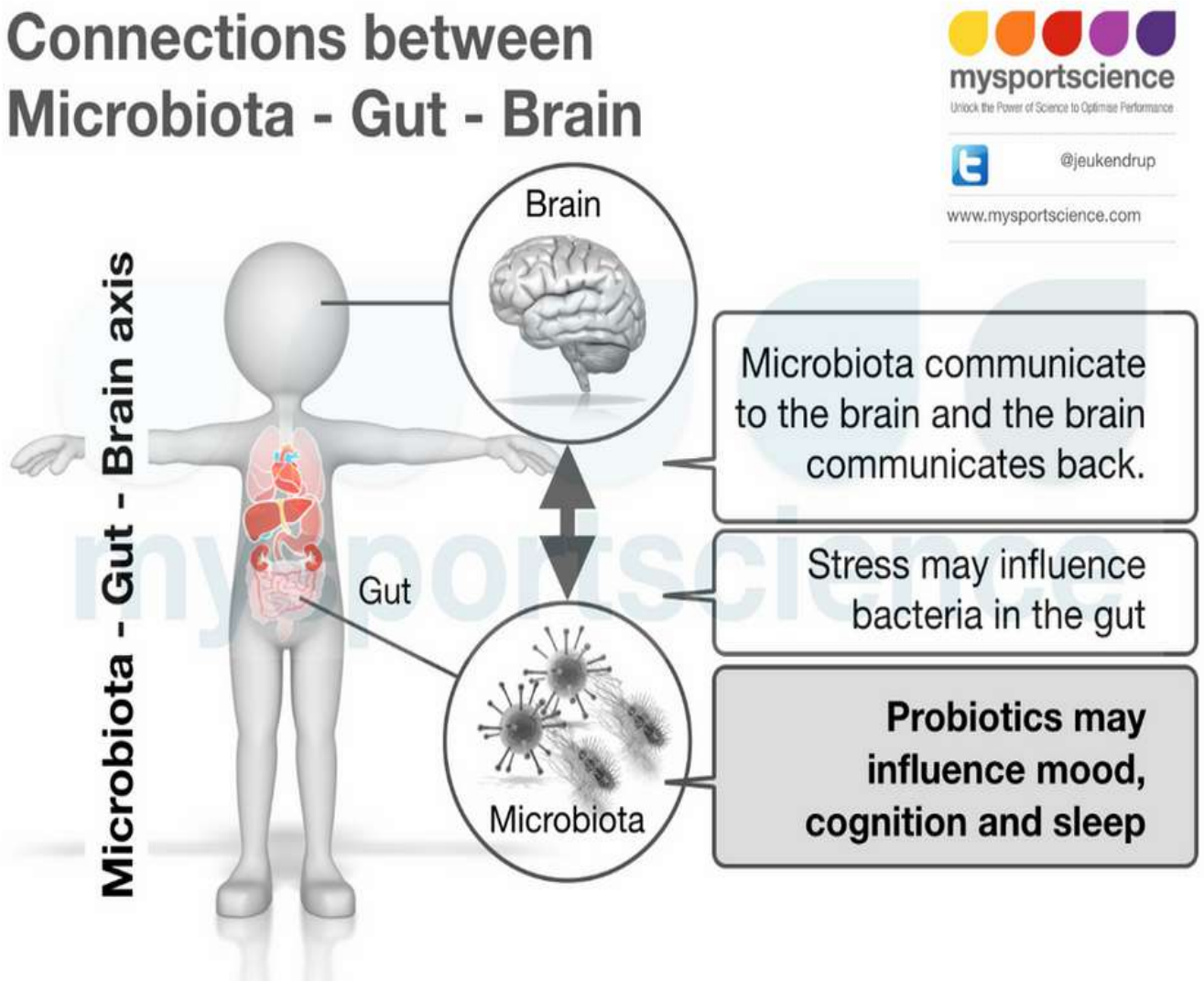
Characterisation/identification (genus, species, strain)

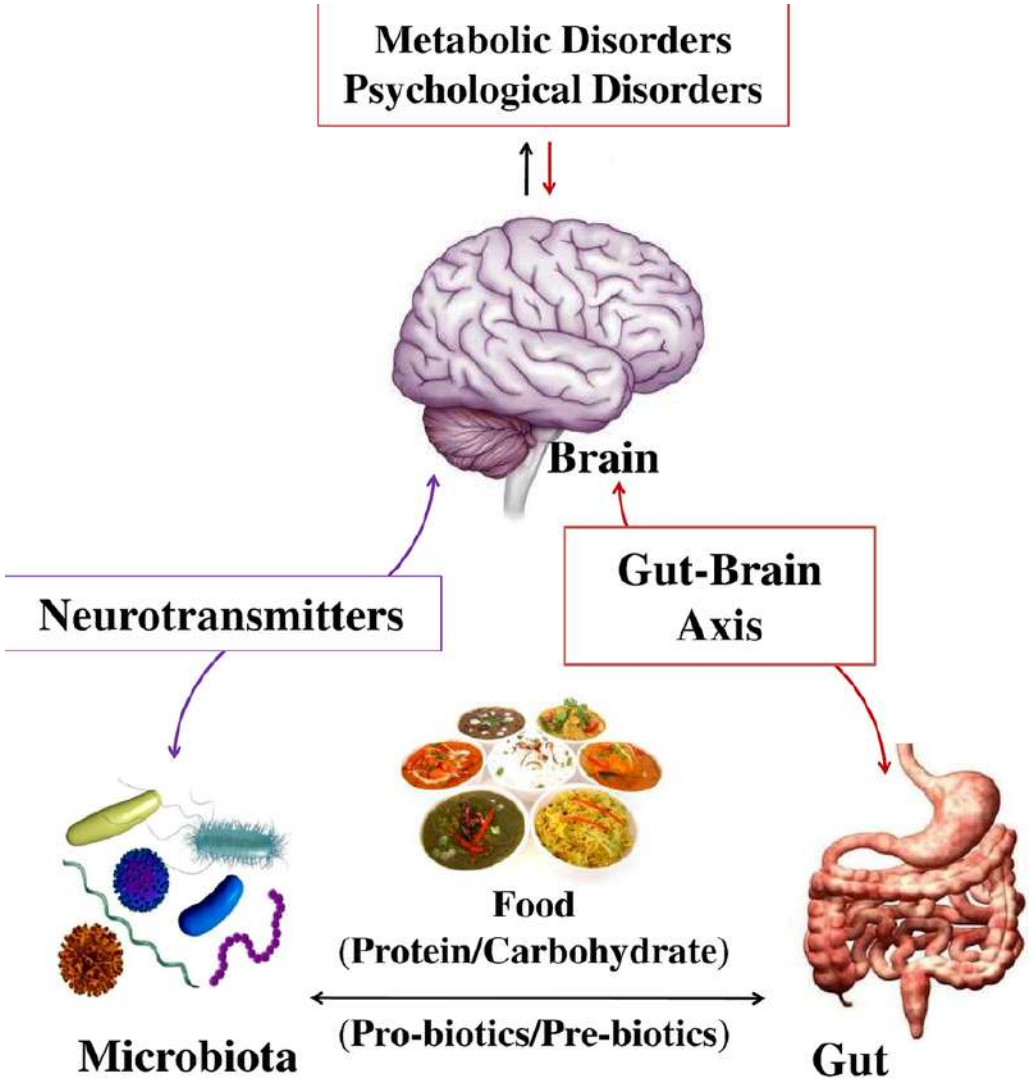
In vitro tests : eg. resistance to low pH, digestive enzymes, bile salts, antimicrobial activity on pathogens

In vivo and clinical studies for the documentation of the beneficial activity on health

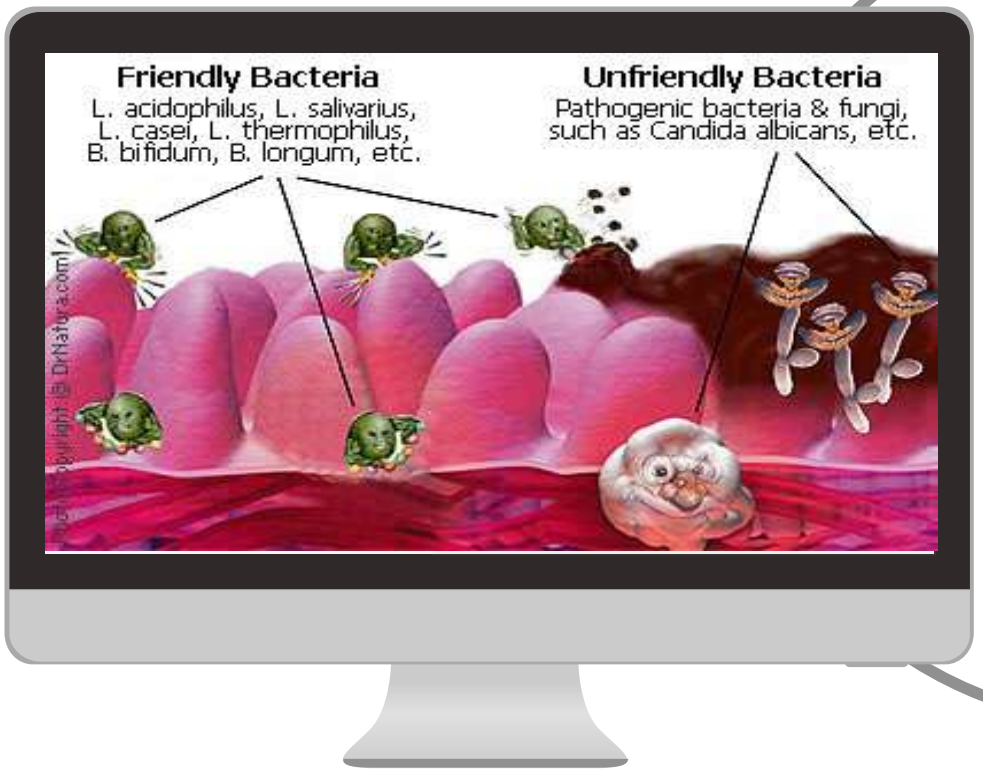
Safety Evaluation of the probiotic strain without contaminants in the administered form

Probiotics and Gut-Brain Axis





Beneficial activity of probiotics



Intestinal microflora differentiation,
Antibiotic-related diarrhea control,
Reduced chance of bowel cancer

Lactose intolerance relief,
Cholesterol lowering,
Antihypertensive effect

Immunoregulation-stimulation of the immune system
Increased resistance to infections, treatment of allergies, etc.

Beneficial activity– mode of action

Antimicrobial activity:

Production of lactic acid and antimicrobial substances such as bacteriocins.

Reduction of intestinal pH and inhibition of pathogens eg. *Clostridium, Salmonella, Shigella, E. coli*, etc.

Maintaining gut barrier function - Prevents the attachment of pathogens to the intestinal epithelium.

Biochemical activity:

Increased intestinal acidity helps absorb trace elements, especially calcium.

Production of the enzyme *β*-D-galactosidase for the breakdown of lactose.

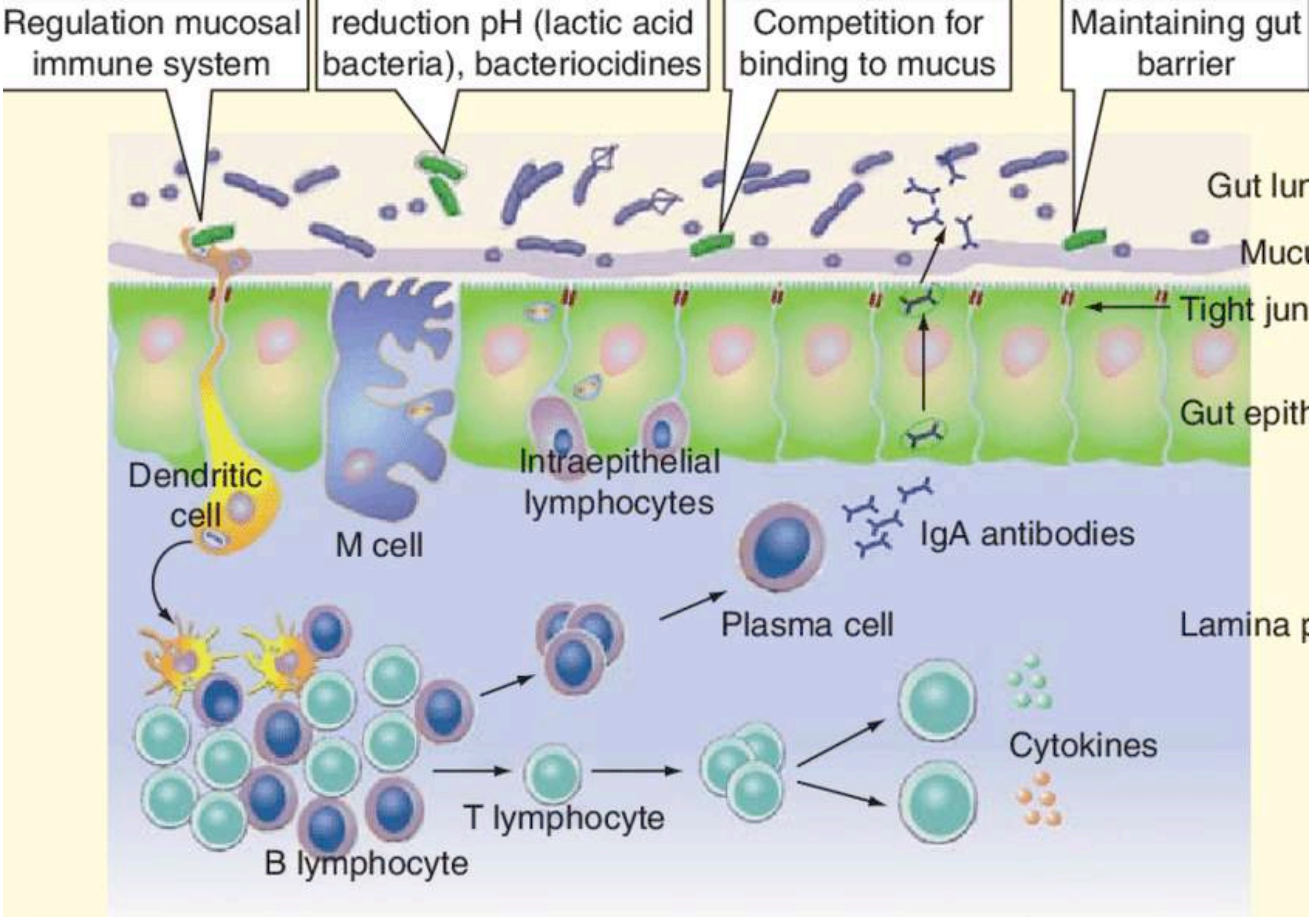
Production of vitamins (B & K vitamins)

Reduction of the production of various toxic and carcinogenic metabolites.

Immunological / anti-inflammatory action:

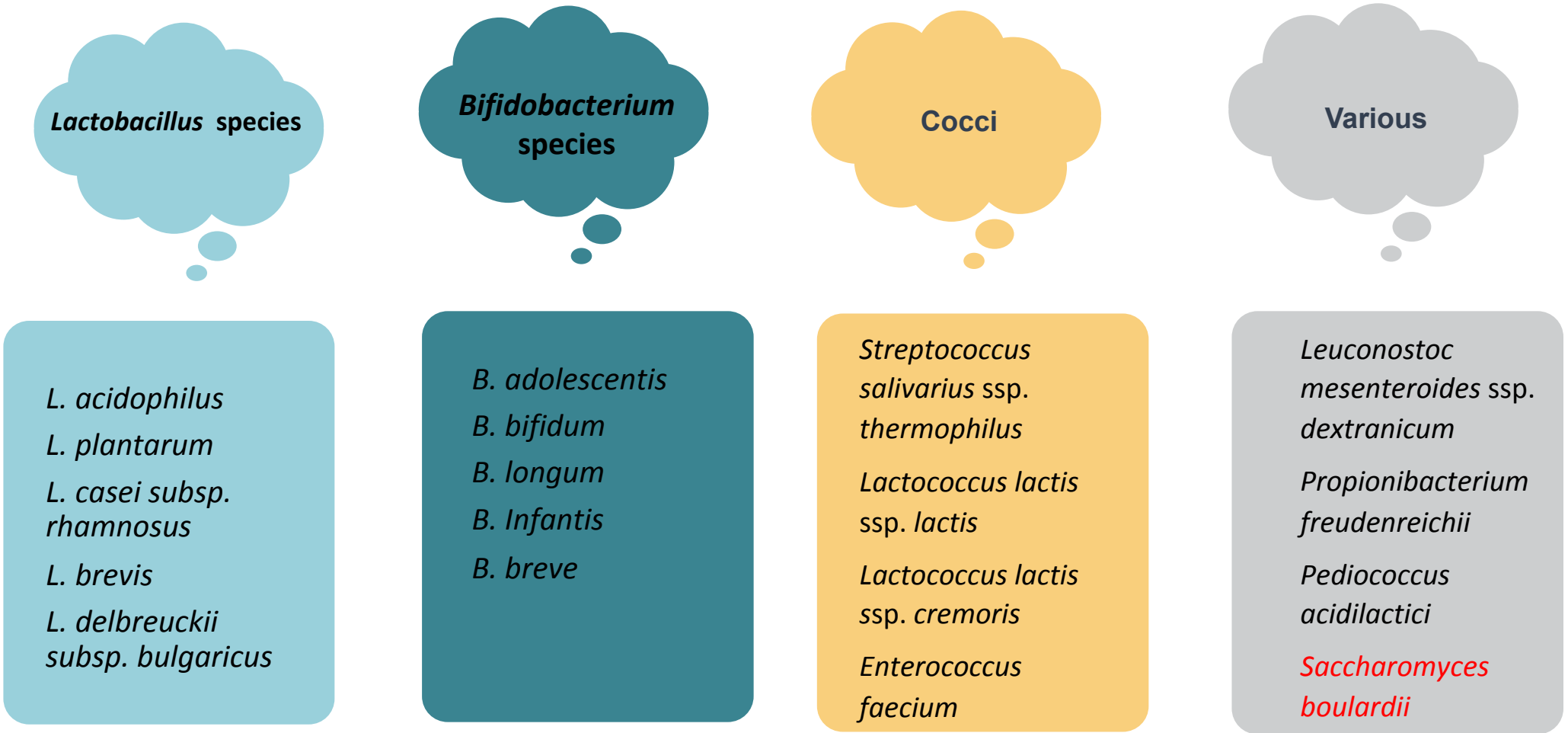
Increased immunoglobulin A (IgA) secretion

Modes of action



Probiotic microorganisms are:

- Mainly lactic acid bacteria but also yeasts .
- They are used in fermented foods, mainly dairy, but also as food additives.



Probiotics as medicines

Poly-probiotics as medicines

Lactobacillus gasseri PA16/8,
Bifidobacterium bifidum MF20/5,
Bifidobacterium longum SP07/3

Lactobacillus acidophilus,
Bifidobacterium longum

Lactobacillus acidophilus, Bifidobacterium bifidum,
Bifidobacterium longum

Lactobacillus plantarum, Lactobacillus acidophilus,
Lactobacillus casei, var. rhamnosus, Enterococcus faecium

- Minimum consumption (indicative):

  100g of probiotic food with **10^7 cfu/g.**

- Most probiotics do not adhere permanently to the epithelium, but exert their action by metabolizing and grow through their passage in the intestine.

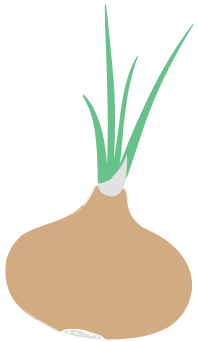
- Thus, daily consumption of these bacteria is probably the best way to maintain their action and effectiveness.

Food is the best source of probiotics

There is synergistic action among food constituents (**prebiotics**) and the probiotic cultures

The natural balance of the acidic environment in the stomach from food also increases the stability of probiotics

Prebiotics



01 Non-digestible ingredients beneficial to humans

02 They selectively stimulate the growth and / or action of intestinal bacteria

03 They improve the survival of probiotics in the digestive system and they become more effective

04 Examples of foods with prebiotics: garlic, onion, asparagus, wheat, barley, rye

- To date, some dairy products are commonly referred to as probiotic foods
- However, most fermented foods of plant and animal origin contain probiotic microorganisms
- Such foods are the traditional Greek fermented products e.g. olives, pickles, sausages, cheeses

Probiotic microorganisms in the microbiota of traditional fermented foods

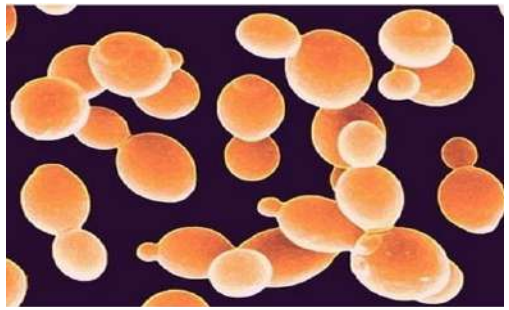
Fermentation

- It is a complex process of reactions resulting from the presence of microorganisms and is a method of biopreservation
- These microorganisms are either already present in the raw materials used for fermentation, or are added by humans (eg. yeast)

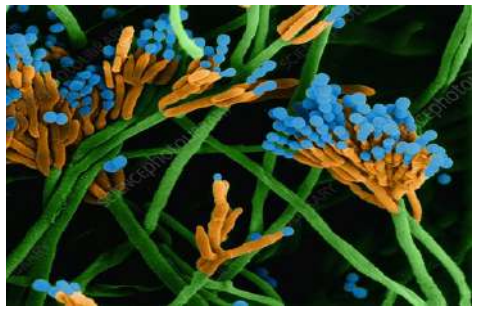
- It was applied empirically from ancient times, but the involvement of microorganisms in the process became known by Pasteur
- The microorganisms involved in fermentation can be:

Bacteria

Yeasts



Fungi



Fermented foods already offer:

- traditional method of preservation preferred by consumers

- valuable elements for health eg. fruits and vegetables with high levels of antioxidants, vitamins, dietary fiber, trace elements

- enhancing them by adding probiotic lactic acid bacteria can give them additional health-promoting properties.

Probiotic microorganisms in the microbiota of traditional fermented foods

Usefulness of fermentation microorganisms

Starter cultures

- ✓ Drive the fermentation
- ✓ Ensure the control and prediction of fermentation
- ✓ Improve the digestibility and nutritional value of foods
- ✓ Improve sensory characteristics and “identity” to the product

Bioprotective cultures

- ✓ May contribute in fermentation
- ✓ Increase the shelf life and safety (compete to spoilage and pathogens)
- ✓ Decrease product recalls
- ✓ Waste decrease
- ✓ Natural products– consumer acceptance

Probiotic cultures

- ✓ May contribute in fermentation
- ✓ Survive during gut passage
- ✓ May colonise the gut epithelium
- ✓ Use prebiotics and nutrients of the daily diet
- ✓ Exert beneficial action when consumed regularly

Table 1 | **Distinctions between probiotics, fermented foods and probiotic fermented foods**

Probiotic substance	Definition	Format	Evidence for health benefit	Claim that is consistent with category[a]	Microbial composition		
					Alive and present in levels demonstrated to provide benefit	Taxonomically defined to strain level	Genome sequence available
Probiotic	Live microorganisms that, when administered in adequate amounts, confer a health benefit on the host	No specific format required	Required	"Probiotic" can be used on the label along with a health benefit claim, such as "helps to reinforce the body's natural defences", if the claim is supported by evidence	Required	Required	Required
Fermented food	Foods made through desired microbial growth and enzymatic conversions of food components	Food	Not required	If live microorganisms are not present: "Foods made by fermentation"; if live microorganisms are present: "Contains live and active cultures"	Not required	Not required	Not required
Probiotic fermented food	Food fermented by or containing probiotic(s) with strain-specific evidence	Food	Required	Same as for probiotic	Required for probiotic but not for fermentation microorganisms	Required for probiotic but not for fermentation microorganisms	Required for probiotic but not for fermentation microorganisms
	Food fermented by or containing probiotic(s) without strain-specific evidence	Food	Required	"Contains probiotics"	Required for probiotic but not for fermentation microorganisms	Required for probiotic but not for fermentation microorganisms	Required for probiotic but not for fermentation microorganisms

[a]As allowed by local or regional regulations.

Research Targets ...

01 Isolation of probiotic bacteria from Greek traditional fermented foods

02 Use of selected probiotic bacteria as starter cultures in fermentation

03 Survival of probiotics at the end of fermentation and during shelf life

04 Safety assessment of final products

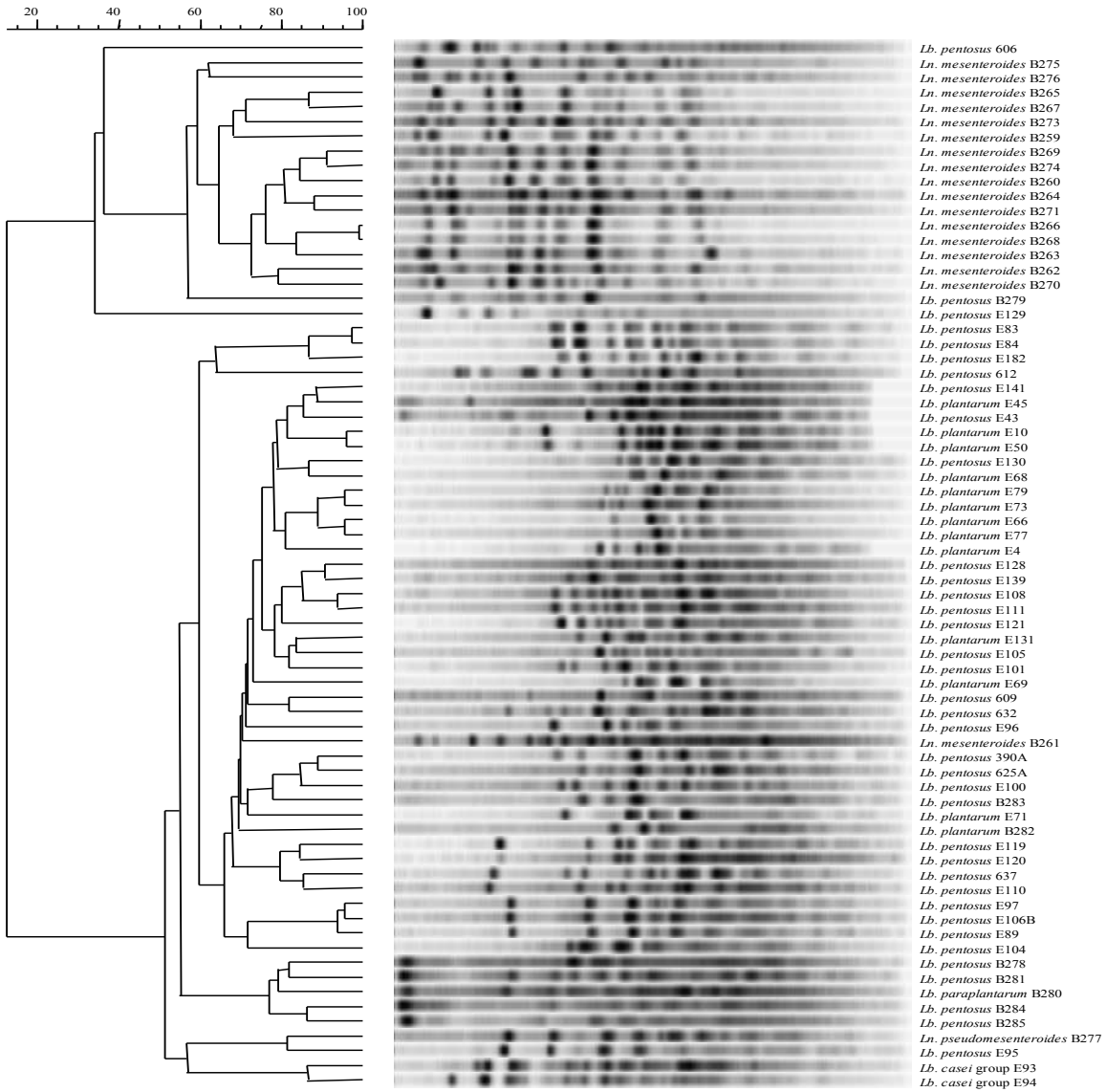
05 Encapsulation of probiotics and use in foods

Research Targets

01

Isolation of probiotic bacteria from Greek traditional fermented foods

Isolation of probiotic lactic acid bacteria from olive microbiota



Doulgeraki, A.I., Pramateftaki, P., Argyri, A.A., Nychas, G-J.E., Tassou, C.C. and Panagou, E.Z. (2013). Molecular characterization of lactic acid bacteria isolated from industrially fermented Greek table olives. LWT- Food Research and Technology, 50: 353-356.

71 different strains of lactic acid bacteria have been isolated from Greek fermented olives

13 *Lactobacillus plantarum*
37 *Lb. pentosus*
1 *Lb. paraplantarum*
2 *Lb. casei* group (*Lb. casei*, *Lb. paracasei*)
17 *Leuconostoc mesenteroides*
1 *Ln. pseudomesenteroides*

9 out of 71 showed PROBIOTIC PROPERTIES *IN VITRO*

Argyri A., Zoumpopoulou G., Karatzas K.A., Tsakalidou E., Nychas G.J., Panagou E. & Tassou C. (2013) *Food Microbiology* 33 (2), pp. 282-291, doi:10.1016/j.fm.2012.10.005

In vitro tests compared to *Lb. casei* Shirota & *Lb. rhamnosus GG*

Strains	Test					
	Low pH (SR%)[a]	Bile salts (SR%)[b]	Bile salts hydrolysis	Haemolytic activity[d]	Antibiotic resistance [e]	Caco-2 (Adherence%)
Lb. pentosus B281	95.64	94.78	0 [c]	α	K, C, S	37.21
Lb. pentosus E97	89.69	96.79	0	γ	K, C, S	39.76
Lb. pentosus E104	92.52	97.64	0	γ	K, G	33.72
Lb. pentosus E108	91.08	100.59	0	γ	K, A	60.78
Lb. plantarum B282	87.79	100.09	1	γ	K, G, E	68.94
Lb. plantarum E10	89.95	98.67	1	γ	K, G	44.75
Lb. plantarum E69	98.36	100.02	0	γ	K, G	30.51
Lb. paracasei subs. *paracasei* E93	89.41	96.55	0	γ	K, G, S	41.92
Lb. paracasei subs. *paracasei* E94	82.75	88.80	0	γ	K, G, S	74.02
Lb. casei Shirota	82.83	100.20	0	γ	S, E, P, T, C	31.41
Lb. rhamnosus GG	64.02	100.61	0	γ	K, A, P	34.00

Isolation of probiotic bacteria from Greek traditional fermented dairy and meat products

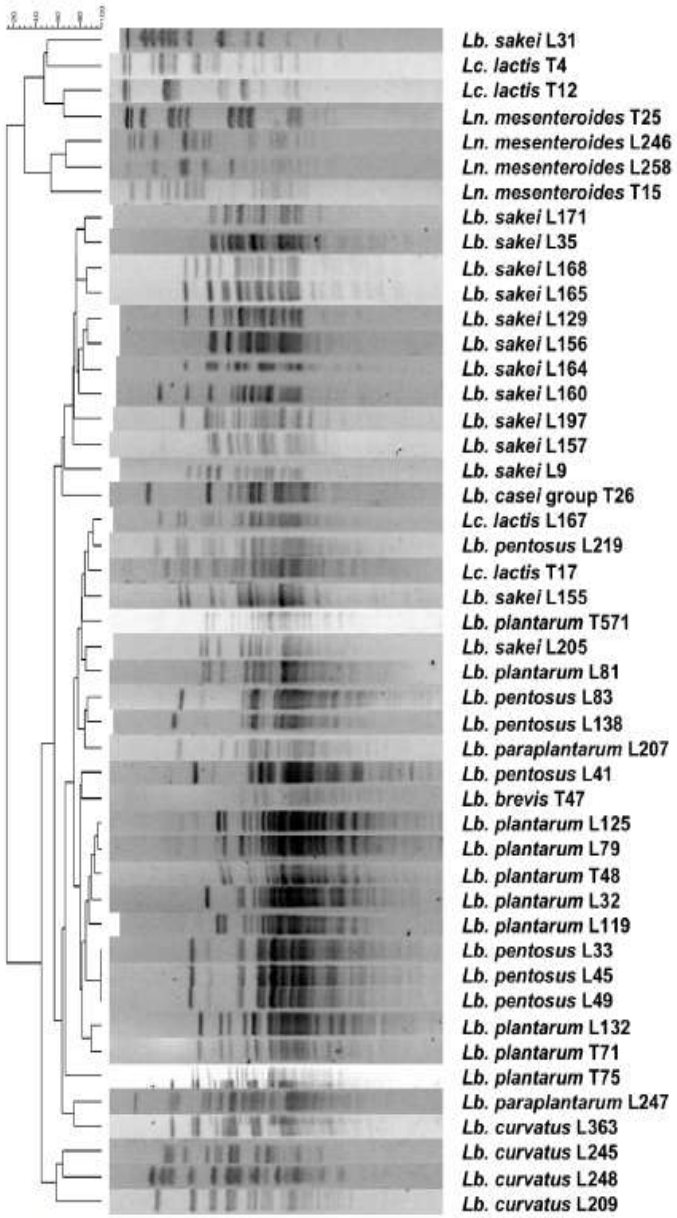


Figure 1: Cluster analysis of PFGE results. Smal digestion fragments of the lactic acid bacteria recovered from different dairy and meat samples calculated by the unweighted average pair grouping method. The distance between the pattern of each strain is indicated by the mean correlation coefficient (r%).

Traditional Greek dairy products such as feta cheese, manouri cheese and xerotyri cheese, and traditional meat products such as sausages, fermented sausages from Lefkada region, cured beefs and soutzouki (a dry spicy product) were obtained from local markets in Greece.

In conclusion, certain strains were found to possess desirable probiotic properties *in vitro*. In more detail, 19 strains (*Lb. brevis* T47, *Lc. lactis* T4, *Lb. sakei* L35 and L165, *Lb. paraplantarum* L207, *Lb. plantarum* T73, T71, T48, T571, L119, L32, L79, L125 and L132 and *Lb. pentosus* L45, L41, L49, L33 and L83) were found to have desirable probiotic properties alike or superior of the 2 reference probiotic strains examined, too. The selected strains are good candidates for further investigation with *in vivo* and *in situ* studies, to elucidate their potential health benefits and their performance as novel probiotic starters and adjunct starters in food fermentation processing.

Pavli F.G., Argyri A.A., Papadopoulou O.S., Nychas G-J.E., Chorianopoulos N.G. & Tassou C.C. (2016) *Journal of Probiotics & Health* 4:3 DOI: 10.4172/2329-8901.1000157

ProbioDairyMeat

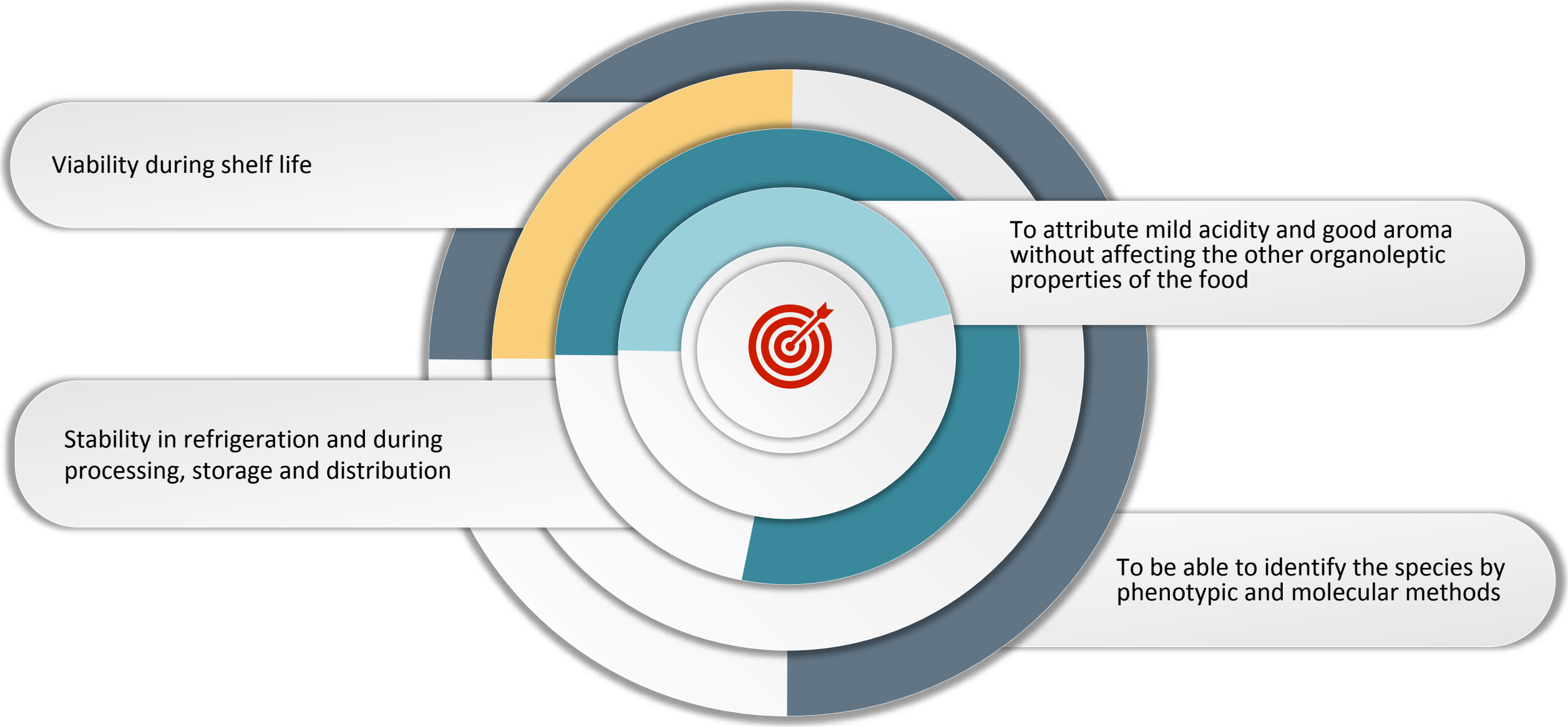
Research targets

02 Use of selected probiotic bacteria as starter cultures in fermentation

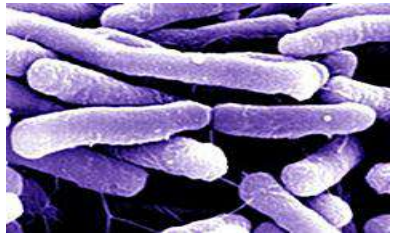
03 Survival of probiotics at the end of fermentation and during shelf life

04 Safety assessment of final products

Assessment of technological properties of probiotics



Probiotic bacteria in table olive fermentation



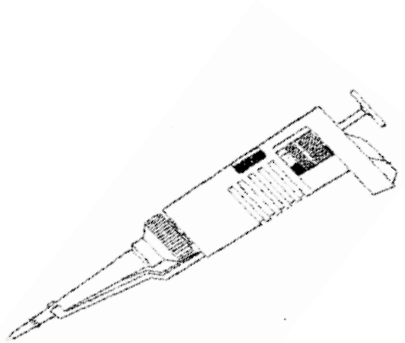
Probiotic bacteria from olive microbiota

Safety assessment of final product

Olives fermented with *Lb. pentosus* B281

Brine NaCl 6% (w/v)

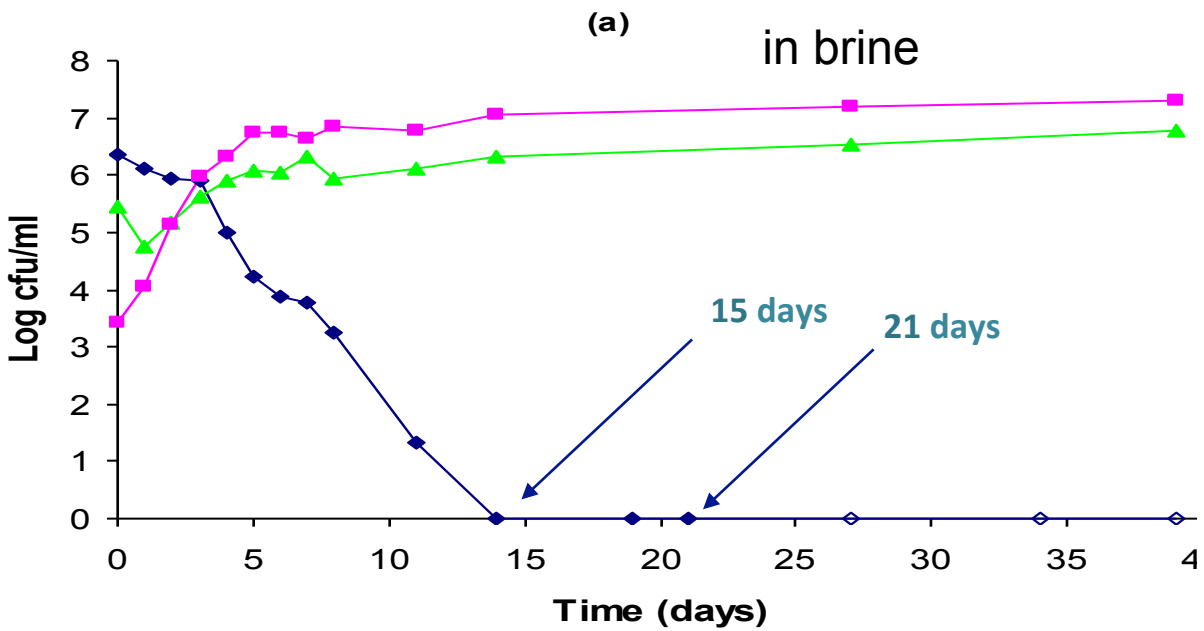
Storage at 20°C

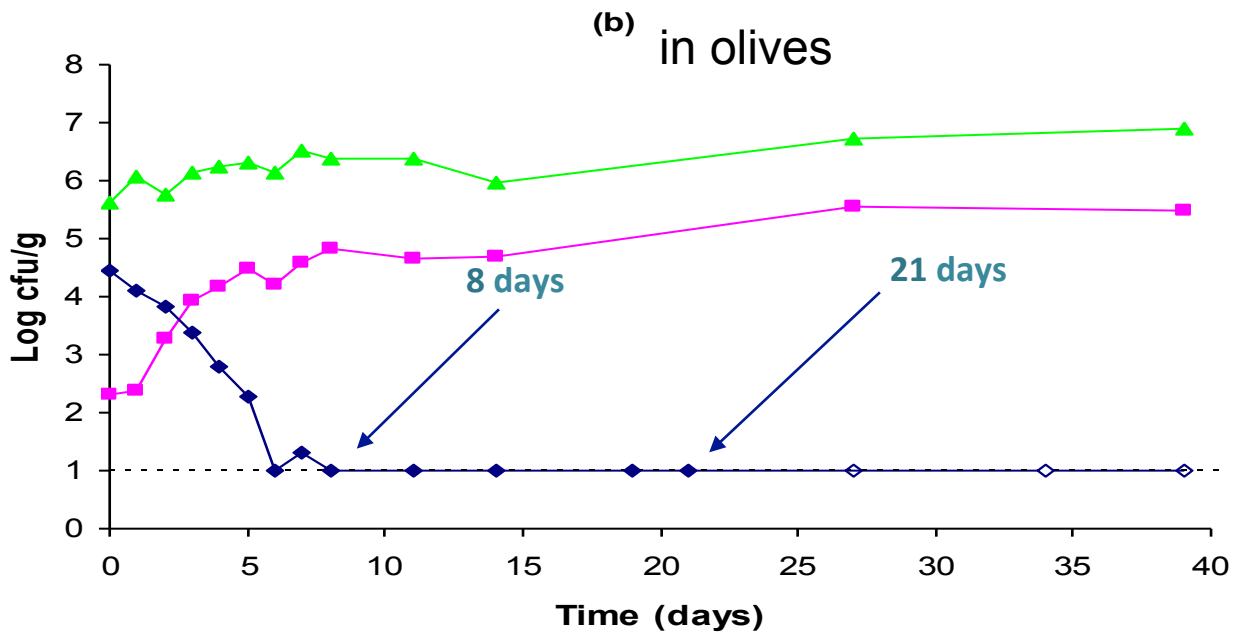


Addition of the pathogenic bacteria
(Cocktail of 5 strains of each bacterium)

- *E. coli* O157:H7
- *Salmonella* Enteritidis
- *Listeria monocytogenes*

Survival of *Salmonella* Enteritidis





Changes in the population of LAB (▲), yeasts (■) and *Salmonella* Enteritidis (◆) in brine (a) and olives fruits (b), during storage of green table olives previously fermented with *Lb. pentosus* B281. (⋯): detection limit, (◊): pathogen not detected after enrichment

Argyri A., Lyra E., Panagou E. & **Tassou C**. (2013) Fate of *Escherichia coli* O157:H7, *Salmonella* Enteritidis and *Listeria monocytogenes* during storage of fermented green table olives in brine. *Food Microbiology* 36(1):1-6 DOI: 10.1016/j.fm.2013.04.001

PROBIOLIVES

Probiotics as adjunct cultures in yogurt at 4°C

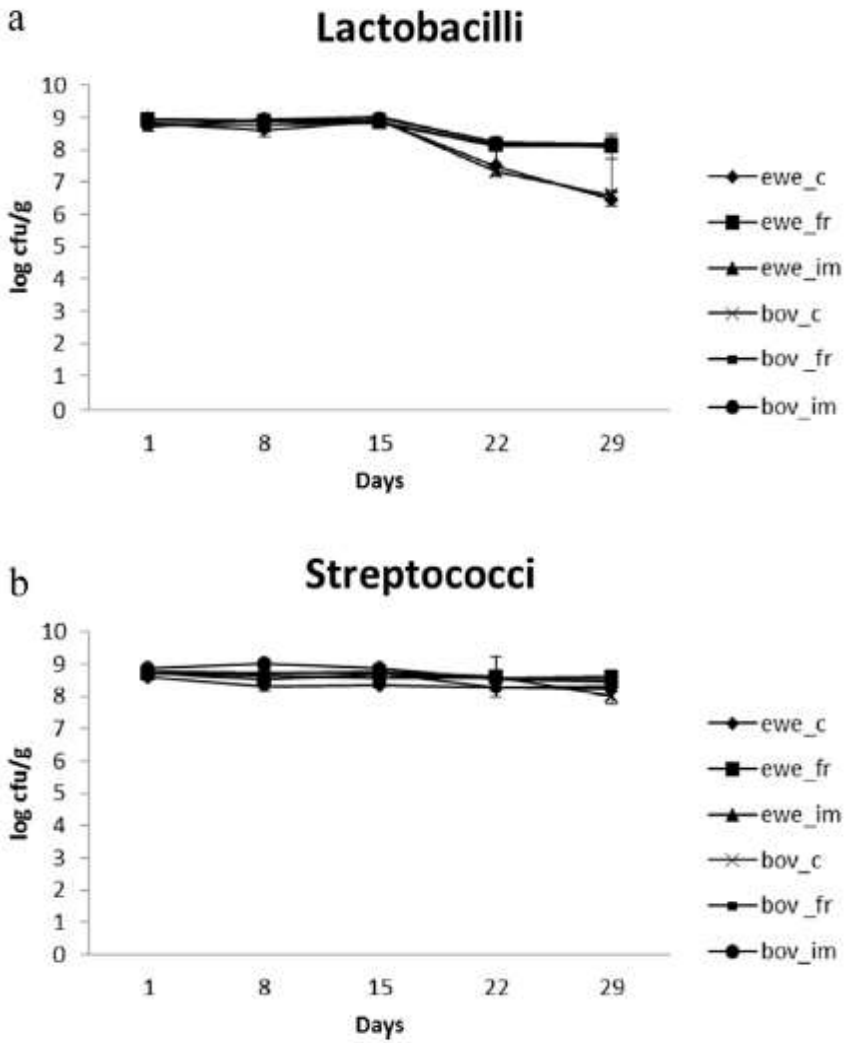
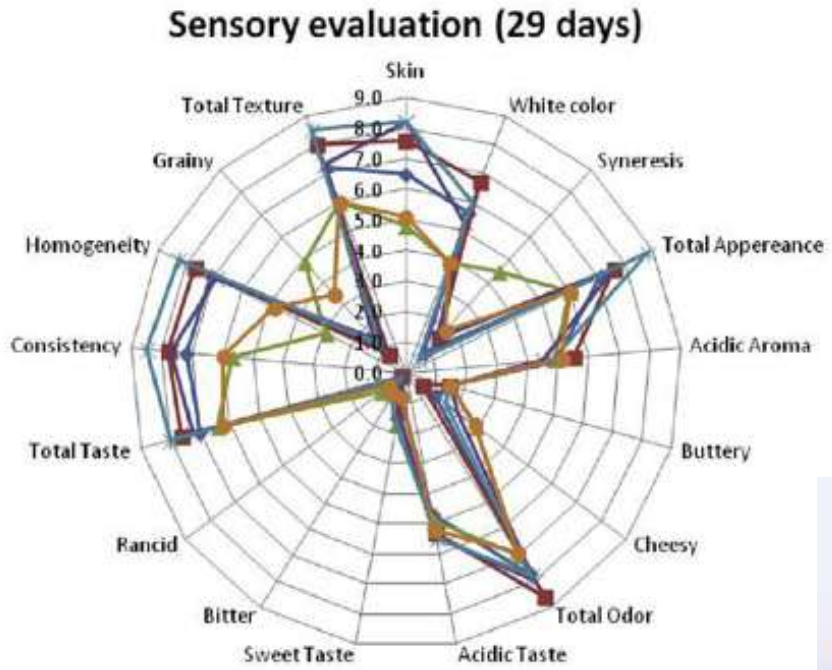
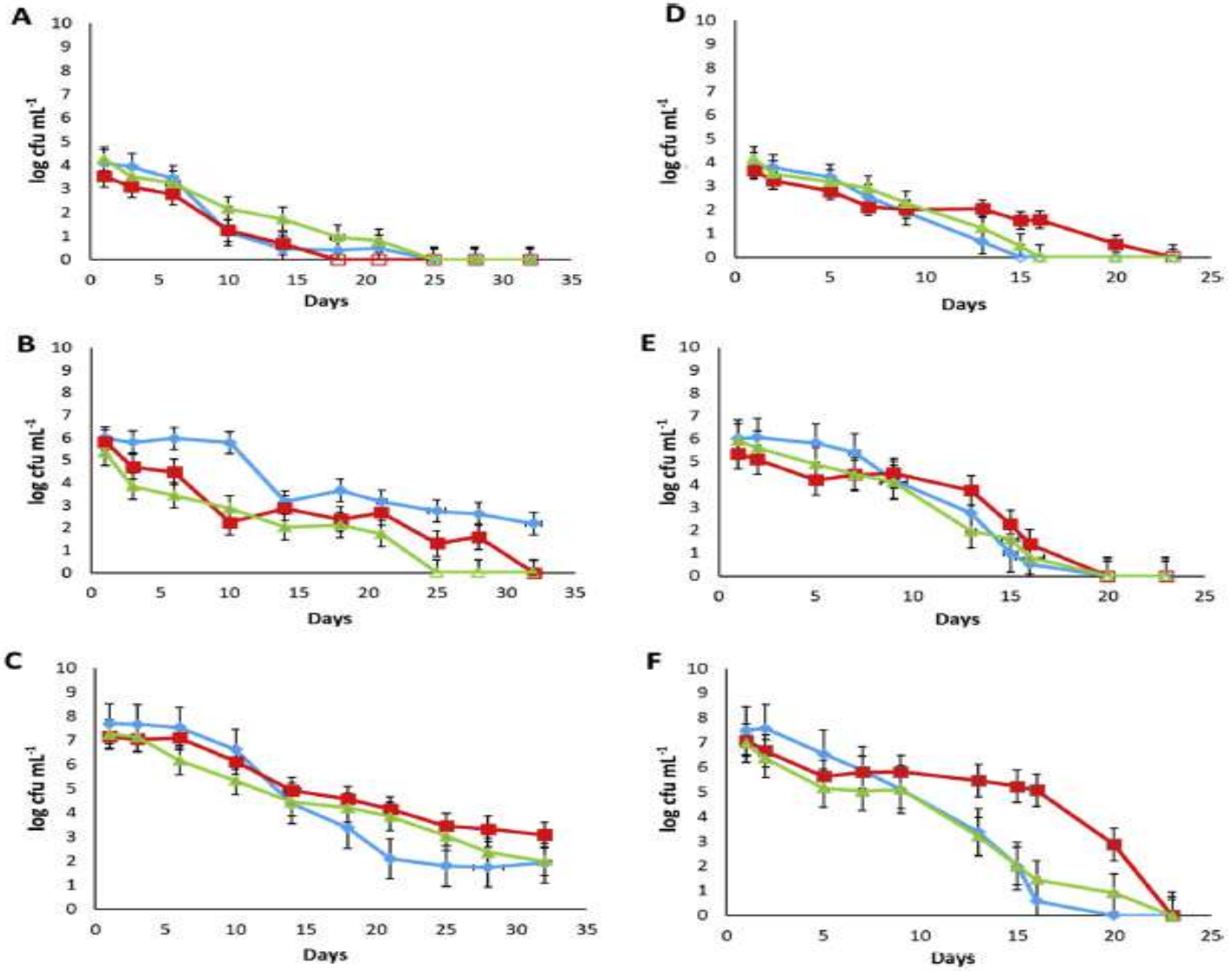


Fig. 2. (a) Lactobacilli and (b) streptococci counts in probiotic yoghurts during refrigerated storage. Ewe: ewe's yoghurt, bov: bovine yoghurt, c: yoghurt with no probiotic culture (control), fr: yoghurt containing free *L. plantarum* 2035, im: yoghurt containing immobilized *L. plantarum* 2035 on whey protein.



Sidira, M., Santarmaki V., (….), **Tassou C.,** et al. (2017) *LWT - Food Science and Technology*, 75: 137-146. DOI: 10.1016/j.lwt.2016.08.026

Probiotics in sour milk – survival of *L.monocytogenes*, low, medium, high inoculum at 4ºC (ABC) and 12ºC (DEF)



. Growth curves of *Listeria monocytogenes* during storage of fermented milk at A, B and C, low, medium and high inoculum, 4 °C, respectively and D, E, and F, low, medium and inoculum, 12 °C, respectively: ◆, control sample; ■, *Lb. plantarum* 2035 (probiotic case A); ▲, *Lb. plantarum* T571 (probiotic case B). Empty markers indicate absence of *nocytogenes* after enrichment. Filled markers indicate presence of *L. monocytogenes* after enrichment. The bars represent the mean values ± standard deviations (two biological les, each sample analysed three times).

Papadopoulou O., Argyri A., Varzakis E., Sidira M., Kourkoutas Y., Galanis A., **Tassou C**., Chorianopoulos N., (2019) *Int. Dairy Journal*, https://doi.org/10.1016/j.idairyj.2019.06.006

Probiotics as adjunct cultures in feta cheese (maturation –survival) at 4º (B) & 12ºC (D)

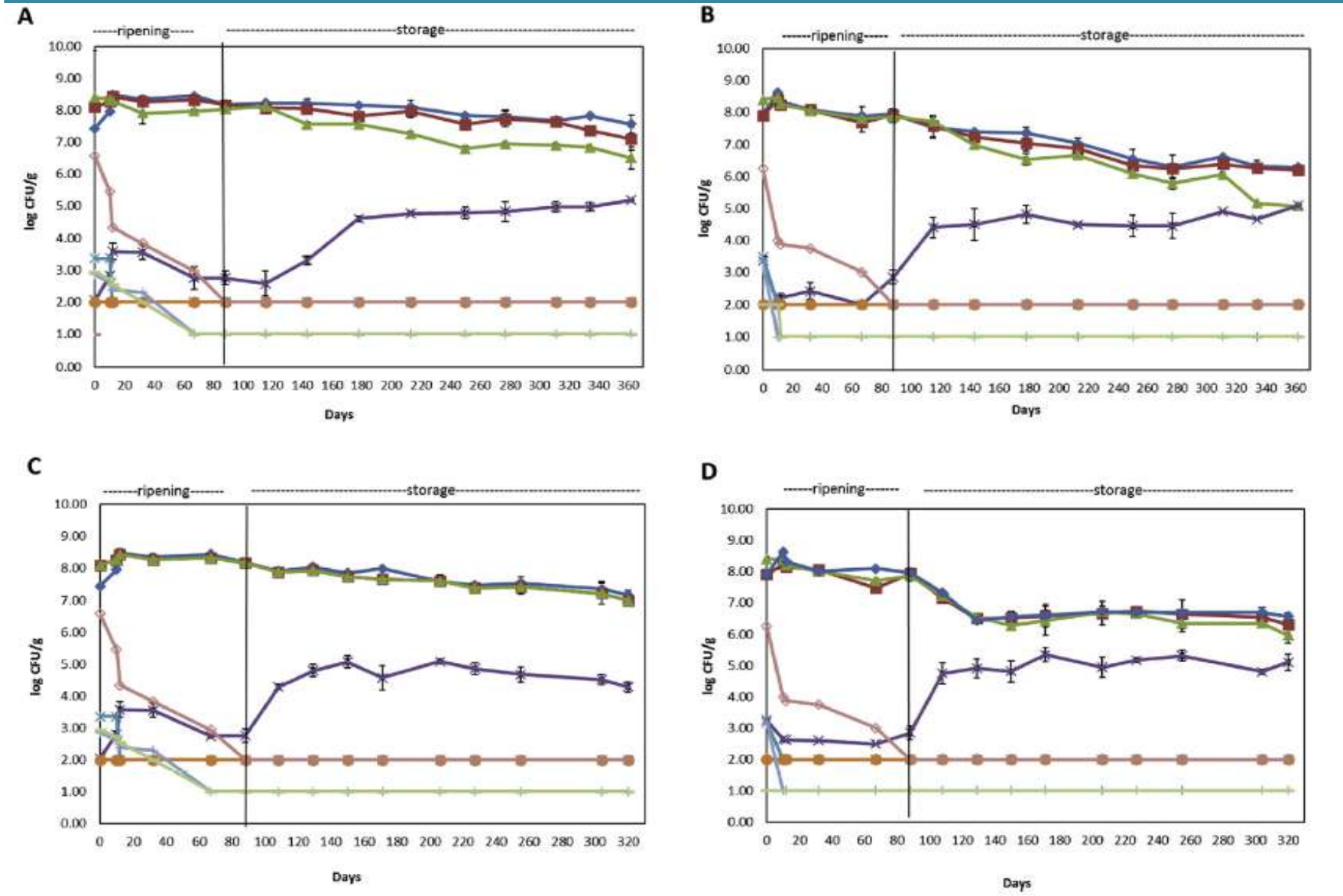


Fig. 1. Growth curves of the examined microflora of cheese samples during ripening and storage of Feta cheese. A: control samples stored at 4 °C, B: probiotic samples stored at 4 °C, C: control samples stored at 12 °C and D: probiotic samples stored at 12 °C. (◆): Total viable counts, (■): Mesophilic lactic acid bacteria, (▲): Lactic cocci/streptococci, (x): Yeasts, (•): *Pseudomonas* spp., (●): *Brochothrix thermosphacta*, (+): *Enterobacteriaceae*, (◇): *Staphylococcus* spp., and (–): coliforms. The bars represent the mean values ± standard deviations (two biological samples, each sample analyzed three times).

Papadopoulou O., Argyri A., Varzakis E., **Tassou C**., Chorianopoulos N. (2018) *Food Microbiol*. 74, 21-33, https://doi.org/10.1016/j.fm.2018.02.005

Probiotics as adjunct cultures in feta cheese – survival of *Listeria monocytogenes* at 4 (A) and 12°C (B)

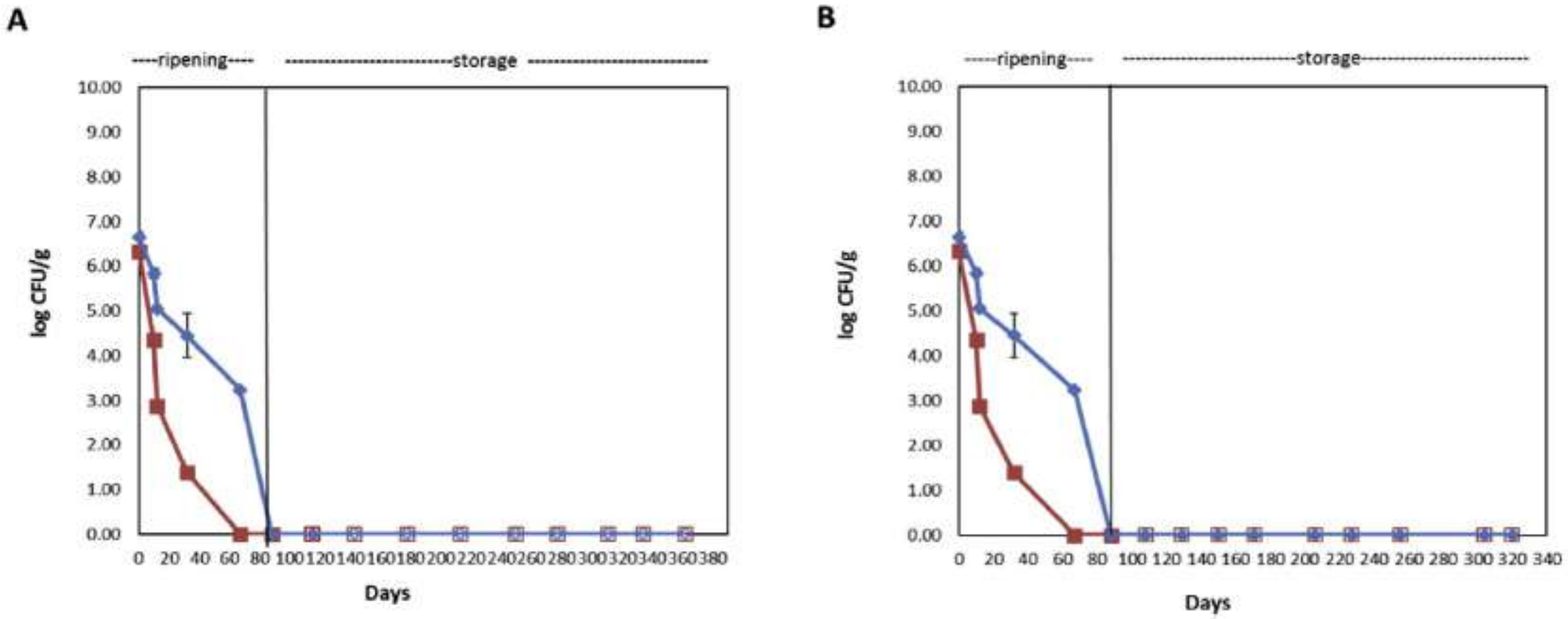


Fig. 2. Growth curves of *Listeria monocytogenes* cocktail strains during ripening and storage of Feta cheese without (♦) or with (■) the addition of *Lb. plantarum* T571 at 4 (A) and 12 °C (B). Open symbols (◊), (□) indicate absence of *L. monocytogenes* after applying enrichment method. The bars represent the mean values ± standard deviations (two biological samples, each sample analyzed three times).

Papadopoulou O., Argyri A., Varzakis E., **Tassou C**., Chorianopoulos N. (2018) *Food Microbiol*. 74, 21-33, https://doi.org/10.1016/j.fm.2018.02.005

Probiotic cultures in fermented sausages

Probiotic culture

- Increase of safety and shelf life
- Production of functional foods with health benefits

Research targets

05

Micro-encapsulation of probiotics for use in foods

Encapsulation system WPI/GA for improvement of survival

Probiotic strain

↓

Encapsulation solution

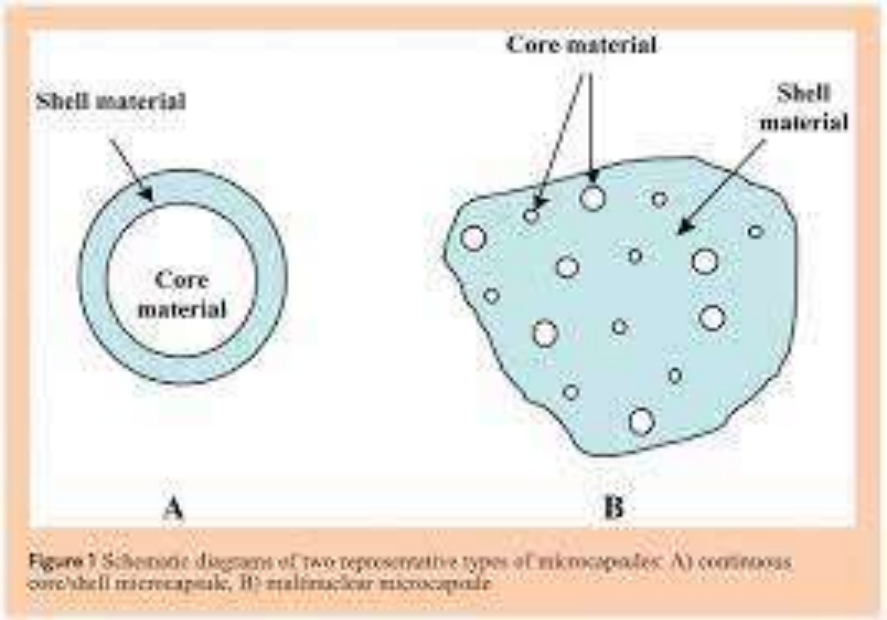
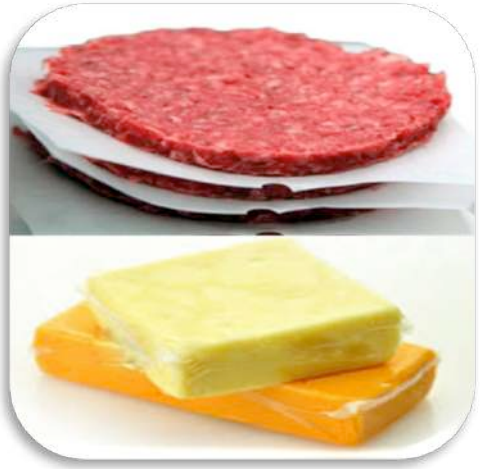


Figure 1 Schematic diagrams of two representative types of microcapsules: A) continuous core/shell microcapsule, B) multinuclear microcapsule

Encapsulation of probiotic cultures in edible membranes

Probiotics



- Increase of safety and shelf life
- Production of functional foods with health benefits
- The material can either be consumed or discarded - biodegradable

In vivo tests

Curr Microbiol
DOI 10.1007/s00284-017-1285-z

Survival, Intestinal Mucosa Adhesion, and Immunomodulatory Potential of *Lactobacillus plantarum* Strains

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In conclusion, oral administration of either *L. plantarum* 2035 or *L. plantarum* ACA-DC 2640 in a rat model resulted to (a) survival of the probiotic bacteria during transit through the GIT, (b) transient distinct adhesion to the intestinal mucosa at a dosing scheme- and time-dependent manner and (c) modulation of the systemic immune response. Daily administration proved to be a prerequisite for maintaining ample levels of the bacteria in the intestinal tissue. These findings add novel information on the characteristics of these probiotic bacterial strains further clarifying their properties and promoting their potential role in delivering health benefits.

EU legislation and health claims for probiotic foods

EC regulation No 1924/2006: of the European Council and the council of 20 December 2006, regarding the nutrition and health claims for foods
http://ec.europa.eu/food/food/labellingnutrition/claims/index_en.htm

EC Guidance on the Regulation N° 1924/2006 on nutrition and health claims made on foods

A claim is a **nutrition claim** if in the naming of the substance or category of substances, there is only factual information (Nutrition claims examples: Contains lycopene; Contains lutein)

A claim is a **health claim** if in the naming of the substance, or category of substances, there is a description or indication of a functionality or an implied effect on health.

EU legislation and health claims for probiotic foods

EC regulation No. 432/2012: committee of 16 May 2012, establishing a list of permitted health claims made on foodstuffs

After the **14th of December 2012** (entry into force of Reg. 432/2012) the use of the term "probiotic" is prohibited because it is a health claim (http://ec.europa.eu/nuhclaims)

Probiotic Food in the EU: the regulatory framework

Almost **400 health claim applications with probiotics** have been submitted for authorization. **Only one was authorized** – an article 13.1 claim on live yoghurt and improved lactose digestion for the microorganisms L. delbrueckii subsp. bulgaricus and Streptococcus thermophilus (minimum 10^8 CFU/g).

Scientific Opinion on the substantiation of health claims related to live yoghurt cultures and improved lactose digestion (ID 1143, 2976) pursuant to Article 13(1) of Regulation (EC) No 1924/2006 (Link).

All other probiotic health claims applications have been either rejected or withdrawn due to the uncertainty of the assessment of EFSA. The most common reason for rejection was the insufficient characterization, but in some cases, application on well-characterized microorganisms were also rejected. As there is no health claim authorized for individual strains (excep. the yoghurt microorganisms), probiotic is considered by the European Commission as a health claim.

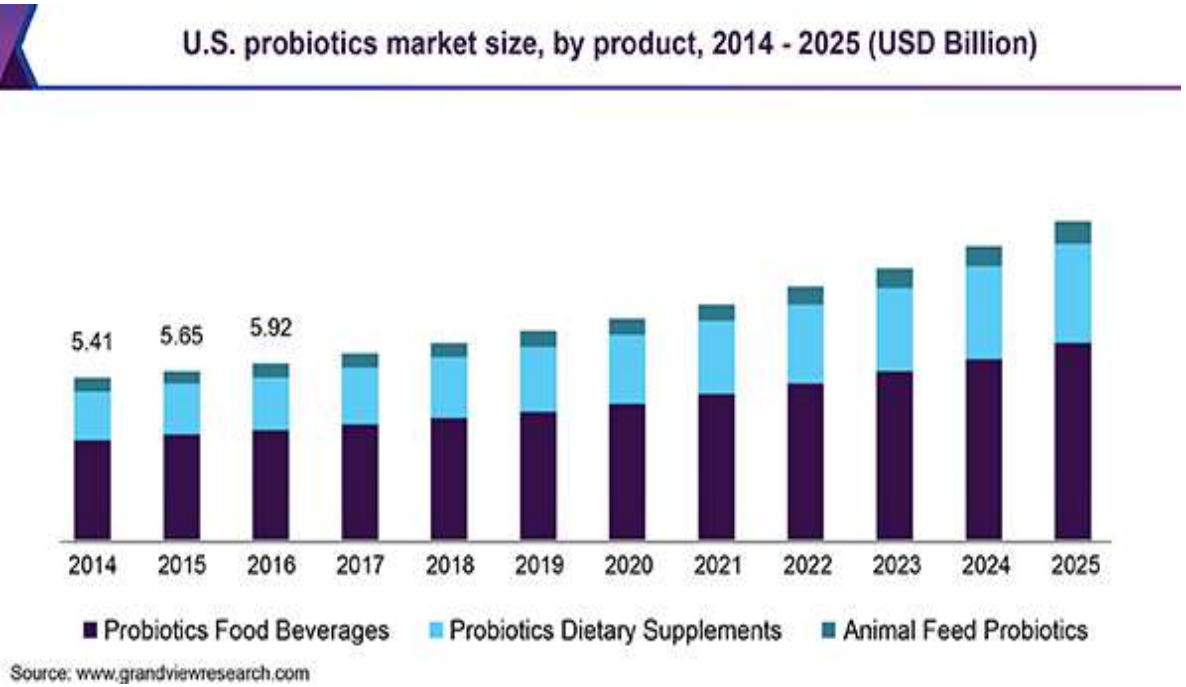
However, in recent years different approaches have been developed by EU Member States.

Rejected applications (http://ec.europa.eu/nuhclaims) because ...

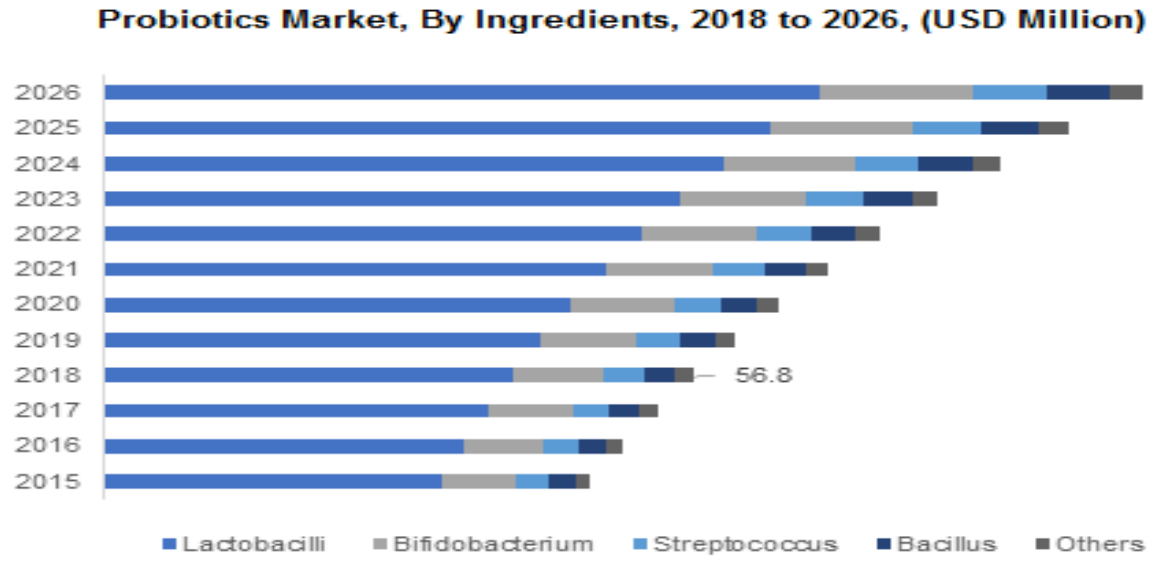
.... there are no reliable clinical trials to substantiate the cause-effect relationship and therefore the health claim

Art.14(1)(a)	ACTIMEL® *Lactobacillus casei* DN-114 001 plus yoghurt symbiosis	Fermented milk containing the probiotic Lactobacillus casei DN-114 001 and yoghurt symbiosis decreases presence of Clostridium difficile toxins in the gut (of susceptible ageing people). Presence of Clostridium difficile toxins is associated with the incidence of acute diarrhoea	Non-compliance with the Regulation because on the basis of the scientific evidence assessed, the evidence provided is insufficient to substantiate this claimed effect for this food		Q-2009-00776	Commission Regulation (EU) 1160/2011 of 14/11/2011	Non-authorised
Art.13(1)	Sacharomyces cerevisiae var boulardii	- probiotic -helps maintain intestinal flora/comfort.	Non-compliance with the Regulation because on the basis of the scientific evidence assessed, this claimed effect for this food has not been substantiated.	Saccharomyces cerevisiae var. boulardii CNCM I-1079 and defence against pathogenic gastro-intestinal microorganisms	2009;7(9):1247, 2012;10(6):2717		Non-authorised (expiry of transitional period 02/01/2014) [...]
Art.13(1)	Symbiotic: Probiotics (*Lactobacillus helveticus* CNCM I-1722, Bifidobacterium bifidum CNCM I-3426, Bifidobacterium infantis CNCM I-3424) and fructoligosaccharides from sucrose	Participates in healthy microflora balance essential for body's natural defences Stimulates the specific and non specific immune system Reinforces the barrier effect against pathogens	Non-compliance with the Regulation because on the basis of the scientific evidence assessed, this claimed effect for this food has not been substantiated.	Contribution to immune defence against pathogens	2010;8(2):1470, 2012;10(8):2852		Non-authorised (expiry of transitional period 02/01/2014) [...]
			Conditions of use of				

Nevertheless, the market for probiotics continues to grow.....



Source: www.grandviewresearch.com



Probiotics in the EU: Specific approaches in EU Member States

Italy: The Italian Guidelines on Probiotics and Prebiotics are a comprehensive guideline for probiotics in food, allowing the use of the term “probiotic” if specific conditions are fulfilled eg. indication for use in food and food supplements of probiotic microorganisms traditionally used for intestinal microflora balance.

Czech Republic: The Czech Republic has issued national guidelines allowing the use of the term “contains probiotics” as a nutrition claim, subject to the fulfillment of the conditions of use for nutrition claims defined in the NHCR.

France, Portugal and Belgium: The use of the term “probiotic” as a non-specific health claim, when accompanied by a specific health claim, is accepted.

Spain: The Spanish Agency for Food Safety and Nutrition (AESAN) will accept the use of the term “probiotic” on the labels of food and food supplements produced and commercialized in the country.

Netherlands: in March 2021 a Nutrition and claims handbook released informing the EU that the term probiotic will be used in the country until the EU makes a statement on this.

Thank you for your attention!