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Probiotic Bacteria and Enteric Infections

Cytoprotection by Probiotic Bacteria

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Part I
Introduction and History of Probiotics

Chapter 1
Probiotics: From the Ancient Wisdom to the Actual Therapeutical and Nutraceutical Perspective

Giuseppe Caramia and Stefania Silvi

1.1 Probiotics

1.1.1 The Beginnings of Probiotics: The Fermented Milk

The recent history of probiotics began in the early 1900s. Thanks to Metchnikoff (1845–1916) (Fig. 1.1), professor of biology at the University of Odessa, who moved from Ukraine, his homeland, to Messina (Italy) for political reasons after the assassination of Czar Alexander II. In 1882 he discovered the mechanism of phagocytosis and cell-mediated immunity, for which he received the Nobel Prize in 1908, and in 1888, moved to Paris to work at the institute directed by Pasteur, pushed his research on the conditions and the organic alterations that promote aging. At Pasteur's death in 1895, he became the Director of the famous Pasteur Institute and continued his studies in various fields of knowledge and philosophy becoming famous among the general public for his books (*The Nature of Man*, 1904; *The Prolongation of Life*, 1906, etc.).

Starting from the studies of Pasteur on seething microorganisms, and of other researchers on the intestinal bacterial flora (Carre 1887; Tissier 1906), considering that the Caucasian shepherds had a longer average life than the inhabitants of Paris and, according to reports at the time, than the Americans (87 years against 48), he suggested that the shepherds' longevity depended on fermented milk, which they largely consumed, since it was a source of "good" and "anti-putrefactive" microorganisms. It was indeed known that the food wastes ferment in the colon due to some intestinal microorganisms and he was convinced that the putrefactive flora produces toxins, lethal in the long time.

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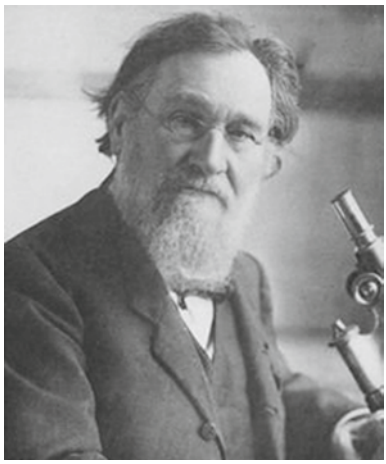

Fig. 1.1 Elias Metchnikoff (1845–1916)

Really, the history of fermented milk and yogurt, with their excellent nutritional properties, was born together with man, in the earliest times of antiquity, most probably 500,000 years ago, when our ancient progenitors learned to light the fire defending themselves from the cold, keeping out animals, lighting the caverns, cooking the game and therefore many millennia before the beginning of the pasture and livestock. The use of fire, fermented milk and yogurt are thus part of human history and their role has been with humanity, to date, between legends and historical data (Flandrin and Montanari 1977; Perles 1977).

The need to keep such a precious food must have been felt since the beginning, and an ancient legend tells of a merchant who, having to cross the desert, brought some foods with him, including milk placed in a bag made with the dried stomach of a sheep. The enzymes remained on the wall of the sheep's stomach used as container, acidified milk and clotted its proteins in small lumps, giving rise to the curd and discovering cheese. The same phenomenon happened to the primordial yogurt derived from the acid fermentation of milk sugars. Thanks to the contamination with special milk enzymes, and a kind of liquid yogurt, used for many millennia by nomadic shepherds and people from the East. Certainly, it was used by the Indians and Sumerians in the fourth century BC, at the beginning of the Egyptian Civilization in the IV–III millennium BC, by the Phoenicians in the III–II millennium BC. The Bible, dated to the thirteenth century BC, reports that "Abraham offered to God, showed in an oakwood, fermented milk" (Genesis 18, 1–8) and Isaiah (VIII BC, 7:15) also says that "you will eat curdled milk and honey."

The Greek historian Herodotus (484–425 BC), Xenophon (430–355 BC), and Aristotle (384–322 BC) have spoken on the use of the yogurt (Bresciani 1977). At the time of the ancient Greeks and Romans, the consumption of fermented milk was recommended as a tonic, especially for children and convalescents, and the Greek physician Galen (129–216 AD), lived in the Imperial Rome, extensively spoke about the yogurt in one of his works, giving to it certain beneficial effects for both the liver and the stomach.

In the Middle Ages, fermented milk and cheese was mainly produced at the abbeys and convents, and they appear in the Crusaders' chronicles; later, we can find them in very distant populations such as Bulgarian shepherds, the Hindus, the Calmucchi, in France, at the court of Francis I (1494–1547), the Zulu, the Russians and other peoples of the Ottoman Empire that used yogurt, a term that derives from the Turkish *yogur* (kneading or mixing with a tool), as a panacea to purify the blood, to prevent tuberculosis, to solve some intestinal disorders and even to help sleeping.

It was known that fermentation is a very important aspect in the formation of yogurt, but the origin of such fermentation was still unclear.

The presence of invisible microorganisms (or micro-Dei), which can creep into our bodies causing diseases, is already present in trace in some Chinese legends and in ancient Egyptian medical texts. Afterwards, Marco Terenzio Varrone (116 BC–27 AD) before and Girolamo Fracastoro (1478–1553) later, talk about it openly. The existence of small organisms, called "animalculi", in the genesis of the diseases and of many other unclear phenomena, was firstly postulated by Lazzaro Spallanzani (1729–1799), who in 1780 coined and introduced into the medical literature the term "germ", so he is considered the founder of the experimental microbiology.

This was opposed to the "spontaneous generation" theory, for which the life is born in a "spontaneous" way from inert or inanimate matter by the effect of some "vital flows", a theory supported until then by the Aristotelian school disciples, by the Epicurean School, by famous philosophers of the Renaissance and in the eighteenth century by Georges-Louis Leclerc, Count of Buffon (1707–1788), and by John Turberville Needham (1713–1781). This dispute continued for many years and was finally permanently settled by Louis Pasteur (1822–1895) in 1864 which made light of that argument confirming the Spallanzani's thesis and thus winning the prize of the Science Academy of Paris for having clearly demonstrated the germs source. Pasteur arrived at such result, thanks to his studies on the fermentation of beer (1854), wine and vinegar (1861–1862) and on the deterioration of the wine by fungi or bacteria (1863–1864); findings confirmed in the following years by studies on silkworm disease (1865–1870), chickens cholera (1880), anthrax in bovines, sheep, horses (1881). In this route it was crucial, of course, the availability of the microscope, "small glasses to see minimal things nearly" that "multiplies things perhaps fifty thousand times" as his discoverer Galileo Galilei wrote (1564–1642) (Saggiatore: 1623), which significantly evolved over the past two centuries mainly thanks to Anton van Leeuwenhoek (1632–1723) and of his successors, thus triumphantly entering in the scientific research field (Caramia 2000).

1.1.2 From the Intuition to the Yogurt

Using bacterial strains selected from the milk of Caucasian and Bulgarian shepherds, through fermentation and acid coagulation of milk by the two microorganisms, *Streptococcus thermophilus* and *Lactobacillus delbruekii* subsp. *bulgaricus* (Fig. 1.2), is obtained a fermented milk, the "Lactobacilline", that in 1906 the

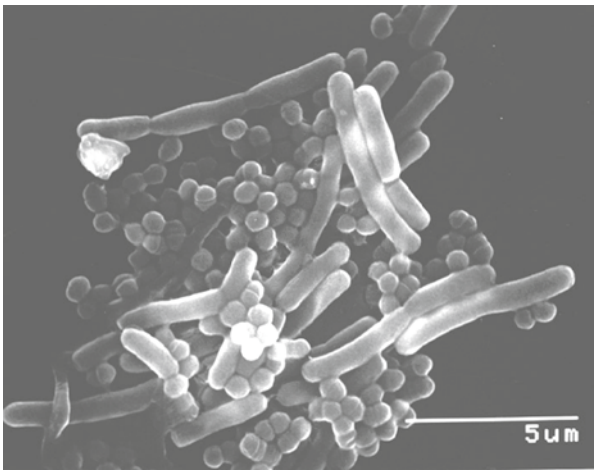

Fig. 1.2 *Streptococcus thermophilus* and *Lactobacillus bulgaricus* from yogurt matrix at scanning electron microscope. (By M. Benevelli—Dept. "Scienze degli alimenti", Bologna University, Italy)

French Society "Le Fermente" began to market and sold in pharmacies, according to the Metchnikoff's idea of helping children suffering from diarrhoea. The product obtained great success among the consumers: today French are the biggest consumers of yogurt compared with other European partners (including Italy), thanks also to the Greek entrepreneurs of Jewish origin, Isac and Daniel Carasso, who was born in Thessaloniki (in Spanish called Mr. Danone).

In 1907/1908 Metchnikoff in his book "The prolongation of life. Optimistic studies" confirms that not all microorganisms are harmful to human health and suggests that "The dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes" (Metchnikoff 1907; Caramia 2008).

Some years later after his death, in 1925 it was sold a product called "yogurt" that rapidly spread in Europe and North America. However, there were also harsh critics since these microorganisms were not found in the faeces of "yogurt" consumers, than someone excluded any beneficial effect of the two seething bacteria. Metchnikoff's intuition, based on empiricism, scientific observations and ingenious intuition, was then mocked by the scientific community, but the beneficial properties of yogurt remained in the collective imaginary, so its use was increasingly widespread.

Always in the 20's, Minoru Shirota, a Japanese microbiologist at the University of Kyoto (Fig. 1.3), discovered that some bacteria of the intestinal flora contribute to bacterial pathogens defence. The following studies led to isolate and cultivate *Lactobacillus casei* (Lc) (Fig. 1.4), afterwards called Lc Shirota, and in 1935 in Japan began the production of a beverage containing this microorganism, called Yakult®, that over the years was spread throughout the world.

An important contribution to the Metchnikoff's theory came in 1936 from two veterinarians, Zobell and Andersen, who suggested the existence in the large intestine of a "microbial film" made by the population of intestinal microorganisms adhering to the intestinal mucosa, which represents a "complex ecosystem with intensive metabolic activities".

Fig. 1.3 Minoru Shirota (1899–1982)

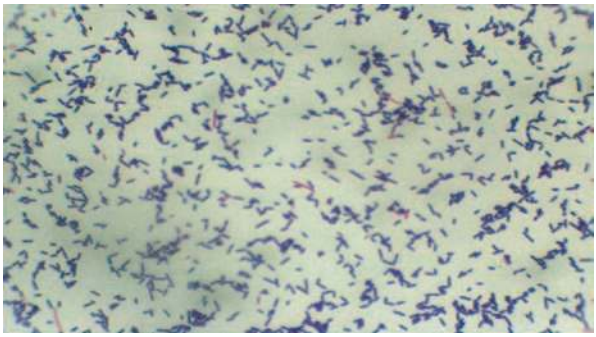

Fig. 1.4 Gram staining of *Lactobacillus casei Shirota*

1.1.3 The term "Probiotic" and its Technical-Scientific Evolution

Metchnikoff has the worth of having introduced the concept of probiotic microorganisms, from the Greek "pro-bios", for life, even if the origin of the term "probiotic" (to be distinguished from lactic ferments that are bacteria of not human origin and producing lactic acid) should be attributed for some to Kollath (1953) and for others to the German researcher Ferdinand Vergin, who in 1954 proposed to use the term "Probiotika" for the "active substances that are essential for a healthy development of life" (Vergin 1954).

In an article published in *Science* in 1962 two veterinarians, Lilly and Stillwell, very likely not knowing the Vergin's proposal, called "probiotics" the so-called "lactic ferments," that is "anaerobic bacteria able to produce lactic acid, starting from different dietary substrates, and to stimulate the growth of other microorganisms" (Lilly and Stillwell 1965).

The last term, also used in contrast to the antibiotic one (against life), which in 1960 was at its peak, thanks to the discovery and development of some important

new drugs with antibacterial action that changed the history of the anti-infective therapy, comes in the current use, not only in medicine. With the advance of knowledge on the physiological and therapeutic role of probiotics, the probiotic definitions became increasingly elaborate and exhaustive. So Parker in 1974 was the first man to use that term to identify the microorganisms- based supplements used for zootechnical feeding, defining them as: "organisms and substances which contribute to intestinal microbial balance" (Parker 1974). This new concept has been successful, especially through the work of a British microbiologist, Roy Fuller, specialized in the study of lactic acid bacteria, who in 1989 deleted from the definition the "substances" giving probiotic capabilities to microorganisms only: "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" (Fuller 1989).

Few years later, two Dutch researchers, Haven and Huis in't Veld, extended the definition including in the beneficial action of the probiotic microorganisms the microflora of both the uro-genital and the upper respiratory system. The probiotics become then: "mono-or mixed cultures of live microorganisms which when applied to animal or man, beneficially affects the host by improving the properties of the indigenous microflora" (Huis in't Veld et al. 1994).

It is currently accepted the probiotic definition formulated in 2001 by FAO/WHO "Live microorganisms which when administered in adequate amount confer a health benefit to the host" (FAO/WHO 2001). Respecting the "Guidelines on probiotics and prebiotics" their characteristics can be summarized as follows:

- Must not lose its properties during storage;
- Must be normally present in the human intestine;
- Must be able to survive, to overcome the gastric barrier, resisting to the action of digestive gastric juice, intestinal enzymes and bile salts and colonize the intestine: for this reason, the minimum effective dose, which is very indicative because it depends on the strain and preparation used, is 10^7 CFU/day;
- Must be able to adhere to and to colonize the intestinal cells: the bacterial membrane structure is involved in the mechanism of adhesion and direct switch with the mucosa, the surface proteins and possibly also the secreted ones. In this respect should be reported the possible apoptotic induction on neoplastic cell lines, recently highlighted, which opens possible therapeutic implications;
- Must exert metabolic functions at the enteric level, with beneficial effects for human health, and antagonism against pathogenic microorganisms by producing antimicrobial substances;
- Should not cause immune or otherwise harmful reactions and then be considered as safe (GRAS status: generally recognized as safe);
- Resistance to antibiotics must be intrinsic or due to genetic mutations, whereas if it is caused by a horizontal gene transfer (i.e. transposons, genomic DNA segments that breaks off to join another, conjugative plasmids carrying genes for resistance, virulent or temperate phages) his choice becomes more problematic;
- Must also be administered in adequate doses and have a favourable cost-efficacy ratio.

1.2 Prebiotics

Prebiotics are predominantly dietary fibers, particularly soluble, also called "colonic food", consisting of specific carbohydrates. Increasingly used by the food industry (beverages, sweets) since 1980 for modifying viscosity, emulsification capacity, gel formation, freezing point and colour of foods, prebiotics have been widely studied since the early 90's, while the spread of the probiotics use, to provide the optimal nutrients to encourage growth of beneficial intestinal microflora (symbionts).

In 1995 Gibson and Roberfroid defined prebiotics as "non-digestible substances that when consumed provide a beneficial physiological effect on the host by selectively stimulating the favourable growth or activity of a limited number of indigenous bacteria in the colon, and thus improves host health" (Gibson and Robertfroid 1995).

As beneficial effect of health by "selective stimulation of the growth" and "activity of a limited number of colonic bacteria" are difficult to verify, in recent years the authors revisited their concept and defined prebiotics as: "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health" (Gibson et al. 2004; Roberfroid 2007; Kelly 2008).

Based on the last definition, prebiotics may have the following characteristics (Gibson and Robertfroid 1995; Gibson et al. 2004; Roberfroid 2007; Kelly 2008; de Vrese and Schrezenmeir 2008):

- must pass, almost undamaged and in adequate amount, the digestive processes occurring in the first section of the digestive tract (mouth, stomach and small intestine);
- must be a nutritional fermentable substrate for intestinal microflora, in order to selectively stimulate the growth and/or metabolism of one or a few bacterial species;
- should positively change the bacterial flora in favour of the acidophile protective one (bifidobacteria, lactobacilli); and finally they should induce systemic or luminal effects that are positive for the human health.

Prebiotics are present in many edible plants such as chicory, artichoke, onions, leeks, garlic, asparagus, wheat, bananas, oats, soybeans and other legumes. Many commercial prebiotics are obtained from vegetable raw materials, while others are produced by enzymatic way through the hydrolysis of complex polysaccharides or the trans-glycosylation of mono- or disaccharides, a beneficial system for mass production starting from simple sugars (sucrose and lactose). Anyway, the addition of prebiotics in foods must comply with the ESPGHAN (European Society for Paediatric Gatroenterology Hepatology and Nutrition) recommendations (Aggett et al. 2003; Roberfroid 2007) including:

- standard methods for the analysis of carbohydrates content in food;
- right labels with the indication of quality and quantity carbohydrates content;
- international databases;
- knowledge of the origin, specific effects and indications for the use of prebiotics.

The natural and commercial prebiotics consisting of oligo- and polysaccharides that are not, or only to a small extent, hydrolized by the digestive enzymes of the human upper intestinal tract and reach intact the colon where they are selectively fermented, particularly from indigenous and exogenous bifidobacteria and lactic acid bacteria, act as a fermentable carbon sources for the colonic microflora.

The most popular, most widely commercially available and the most researched prebiotic compounds are oligosaccharides oligofructose, fructooligosaccharides (FOS), metabolized by the β-fructofuranosidase (β-Fru) enzyme, the polysaccharide inulin, and partly the *trans*-galacto-oligo-saccharides (TOS) metabolized by the β-galactosidase (β-Gal) enzyme (Gibson and Robertfroid 1995; Bouhnik et al. 2006; Kolida and Gibson 2007; Roberfroid 2007; de Vrese and Schrezenmeir 2008; Kelly 2008). Oligofructose, fructooligosaccharides (FOS) (a mixture of oligosaccharides consisting of 3–10 carbohydrate monomers) and inulin (a mixture of fructooligo- and polysaccharides), are bifidogenic, but there is a great deal of intra-individual variability in bifidogenic and anaerobe responses to those inulin-type prebiotics (some experts consider oligofructose, FOS and inulin as synonymous terms for "inulin-type probiotics", oligo- or polysaccharide chains comprised primarily of linked fructose molecules, and inulin HP for the long-chain, high-molecular weight mixes of inulin-type fructans with a degree of polymerization (DP) > 10) (Roberfroid 2007; Kelly 2008, 2009). The effects on other gut microorganisms, as well as pathogenic organisms, are inconsistent but oligofructose and FOS show nutrition and health relevant properties like a low cariogenicity, a low calorimetric value and glycemic index, and a moderate sweetness (30–60% of the sucrose value = 1–2 kcal/g) (Kelly 2008). For this reason they are used as sweeteners in syrup, tablets or powder. Other candidates as prebiotics, for which there are already promising data, but for someone not yet sufficient, are the gluco-oligo-saccharides (GOS) which are oligo or polysaccharide chains comprised primarily of linked galactose units and which stimulate the growth of bifidobacteria and lactobacilli species, the soy-oligo-saccharides (SOS) raffinose and stachiose, metabolized by the α-galactosidase (α-Gal) enzyme, the iso-malt-oligo-saccharides and more (Roberfroid 2007; Kelly 2009; Bruzzese et al. 2009).

1.3 Synbiotics

An alternative chance to modulate or balance the intestinal microflora is the use of pro-and pre-biotic together making synbiotic compounds, that are alimentary or pharmaceutical preparations that containing either one or more probiotic strains and prebiotic ingredients, exploit the synergy between the microorganisms activity and their support for the benefit of the intestinal microflora and, consequently, of the whole body.

In 1995 Gibson and Roberfroid defined synbiotic as "a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract, by selectively

stimulating the growth and/or activating the metabolism of one or a limited number of health-promoting bacteria, and thus improving host welfare".

The simultaneous administration of both probiotics and a substrate that they can metabolize gives to the administered strains greater opportunities for the colonization and survival of probiotic organisms in the colon of the host by increasing or prolonging their beneficial effects: this is really the best strategy for their integration, because it improves the survival (increasing the product shelf life) and on the other hand it provides a specific substrate for the resident bacterial flora.

Theoretically, the synbiotics have better beneficial effect on intestinal flora than pro- and prebiotics by lowering the pH, promoting growth of potentially protective bifidobacteria and inhibiting of potentially pathogenic microorganisms, stabilizing the intestinal environment and releasing short-chain organic acids.

Inulin-type probiotics, FOS or GOS, as well as their synbiotic combination with probiotic bacteria, *L. plantarum, L. paracasei* or *B. bifidum* strains, increased bifidobacteria and lactobacilli and inhibited various human- and animal pathogenic bacterial strains (*Clostridium sp., E. coli, Campylobacter jejuni, Enterobacterium sp., Salmonella enteritidis* or *S. typhimurium*) (Kanamori et al. 2004).

The most used and already marketed synbiotics regard mixtures of oligofructose, FOS, GOS, with probiotic bacterial strains of *L. plantarum*, *L. paracasei*, *L. rhamnosus*, *B. bifidum* or *B. lactis*.

1.4 Various Genera of Probiotics

The majority of probiotic microorganisms belong to the genera *Lactobacillus* (Figs. 1.5 and 1.6) and *Bifidobacterium* (Fig. 1.7). There are also other genera of bacteria and some yeasts widely used and reported in Table 1.1 (Baffoni and Biavati 2008). Lactobacilli and bifidobacteria are Gram-positive lactic acid-producing bac-

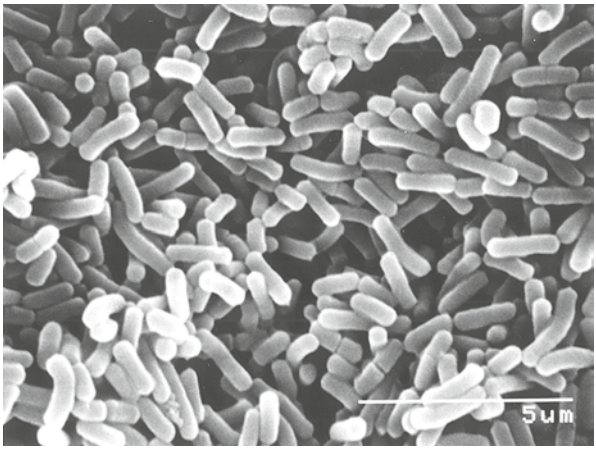

Fig. 1.5 Morphology of *Lactobacillus rhamnosus* at scanning electron microscope. (By M. Benevelli—Dept. "Scienze degli alimenti", Bologna University, Italy)

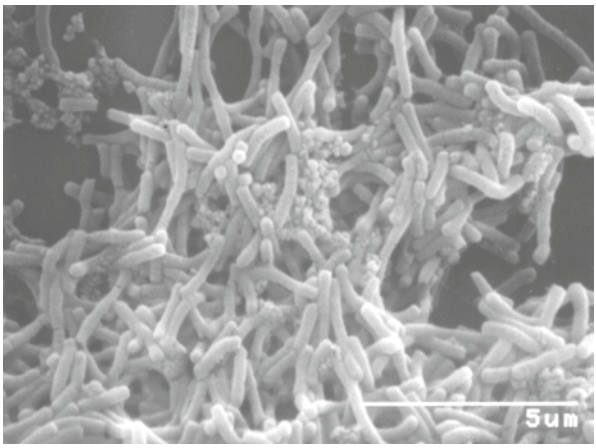

Fig. 1.6 Morphology of *Lactobacillus rhamnosus* from yogurt matrix at scanning electron microscope. (By M. Benevelli—Dept. "Scienze degli alimenti", Bologna University, Italy)

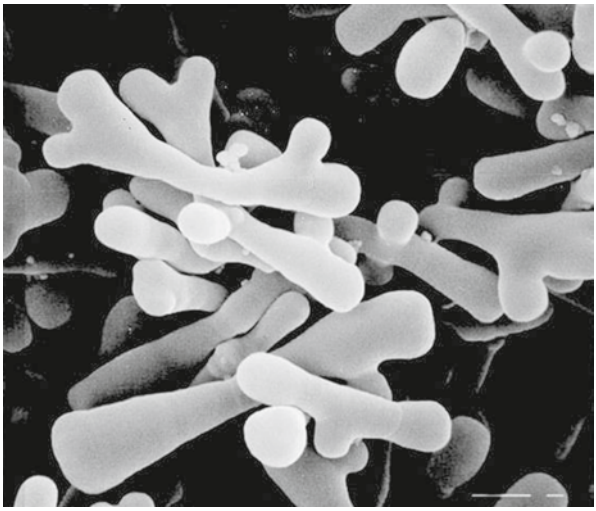

Fig. 1.7 Morphology of *Bifidobacterium* spp. at scanning electron microscope

teria that constitute a major part of the normal intestinal microflora in animals and humans. Lactobacilli are Gram-positive, non-spore forming rods or coccobacilli. They have complex nutritional requirements and are strictly fermentative, aerotolerant or anaerobic, aciduric or acidophilic. Lactobacilli are isolated from a variety of habitats where rich, carbohydrate-containing substrates are available, such as human and animal mucosal membranes, on plants or material of plant origin, sewage and fermented milk products, fermenting or spoiling food. Bifidobacteria constitute a major part of the normal intestinal microflora in humans throughout life. They appear in the faeces a few days after birth and increase in number thereafter. The number of Bifidobacteria in the colon of adults is 10^{10}–10^{11} CFU/g, but this number decreases with age. Bifidobacteria are non-motile, non-spore forming, Gram-positive rods with varying cell morphology. Most strains are strictly anaerobic.

Table 1.1 Microorganisms considered as probiotics. (By Baffoni and Biavati 2008, modified)

Lactobacillus	Bifidobacterium	Enterococcus	Streptococcus	Lactococcus
L. acidophilus	*B. adolescentis*	*E. faecalis*	*S. thermophilus*	*L. lactis subsp. cremoris*
L. brevis	*B. animalis*	*E. faecium*		*L. lactis subsp. lactis*
L. casei	*B. bifidum*			
L. curvatus	*B. breve*			
L. fermentum	*B. infantis*			
L. gasseri	*B. longum*			
L. johnsonii	*B. thermophilum*			
L. reuteri				
L. rhamnosus				
L. salivarius				
Propionibacterium		Yeast		Others
P. freudenreichii		*Kluyveromyces lactis*		*Leuconostoc mesenteroides*
P. freudenreichii subsp. shermanii		*Saccharomyces boulardii*		*Pediococcus acidilactici*
P. jensenii		*Saccharomyces cerevisiae*		

1.5 Probiotics as Therapy

The primordial milk enzymes at the beginning of last century, selected from the milk of the Caucasian and Bulgarian shepherds, have been sold according to the ideas of Metchnikoff and Tissier “to help children suffering from diarrhoea” and sold in pharmacies to bring “good and anti-putrefactive micro-organisms” because “not all microorganisms are harmful to human health”.

In this light over the next few decades lactic acid bacteria with special features, now considered probiotics, kept the primary indication: the preventive-therapeutic use, particularly for some gastroenterological diseases, to try to restore and/or rebalance the functionality of microbiota, the intestinal mucosa and the immunological aspects, keeping in mind the indications listed in the guidelines about the evidence based medicine on the levels of scientific evidence and the strength of clinical recommendations.

1.5.1 Acute Infectious Diarrhoea

In most industrialized countries, acute infectious diarrhoea (AID) is now a minor disease because fatal cases are very exceptional. It is determined in about 70% by viral agents, such as rotavirus, which are responsible for 30–45% of all viral diarrhoea, calicivirus, including norwalk virus, enteric serotypes adenovirus 40 and 41, and Astrovirus; while among bacteria we should mention *Campylobacter jejuni* (main cause of diarrhoeal disease in adults in the US), *Salmonella, Shigella*, enteropathogens *Escherichia coli, and Yersinia enterocolitis*.

Firstly, it should be noted that not all probiotics are the same, because not all of them determine the same therapeutic effects, but, based on the levels of scientific evidence and the strength of clinical recommendations, it is believed appropriate to share in principle what was recently proposed by the ESPGHAN and by the European Society for Paediatric Infectious Disease (ESPID) and by many other scientists: "Probiotics may be an effective adjunct to the management of diarrhoea. However, because there is no evidence of efficacy for many preparations, we suggest the use of probiotic strains with proven efficacy and in appropriate doses for the management of children with acute gastroenteritis as an adjunct to rehydration therapy (levels of scientific evidence II and strength of clinical recommendations B). The following probiotics showed benefit in meta-analyses of RCTs: *Lactobacillus* GG (I, A), *L. reuteri* (I, A) and *Saccharomyces boulardii* (II, B)" (Floch et al. 2008; Guarino et al. 2008; Kligler and Cohrssen 2008).

In particular, *L. reuteri* has shown to shorten significantly the clinical course of rotavirus-induced gastroenteritis, as well as reducing incidence of acute diarrhoea (Figs. 1.8 and 1.9) (Shornikova et al. 1997a, b).

As for prevention of infectious diarrhoea, mostly of viral origin, which can be contracted at nursery schools, kindergartens or during hospitalization for other pathologies, it is not yet clear which probiotic or association of probiotics is more effective. Besides, the dose administered which must be equal to or greater than 5–10 billion CFU/day and the early initiation of therapy are important, so that the probiotic, with appropriate doses and immediately administered, may contrast the action of the pathogen (Floch et al. 2008; Guarino et al. 2008, 2009). More recently,

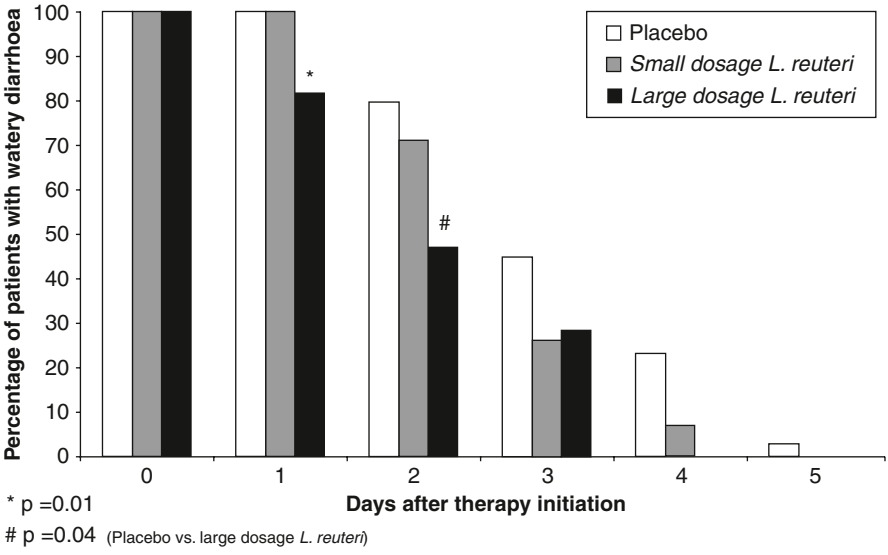

Fig. 1.8 Percentage of patients with persisting watery diarrhoea in the groups receiving placebo ($n = 25$) and small ($n = 20$) and large ($n = 21$) dosage of *L. reuteri* (Shornikova et al. 1997a)

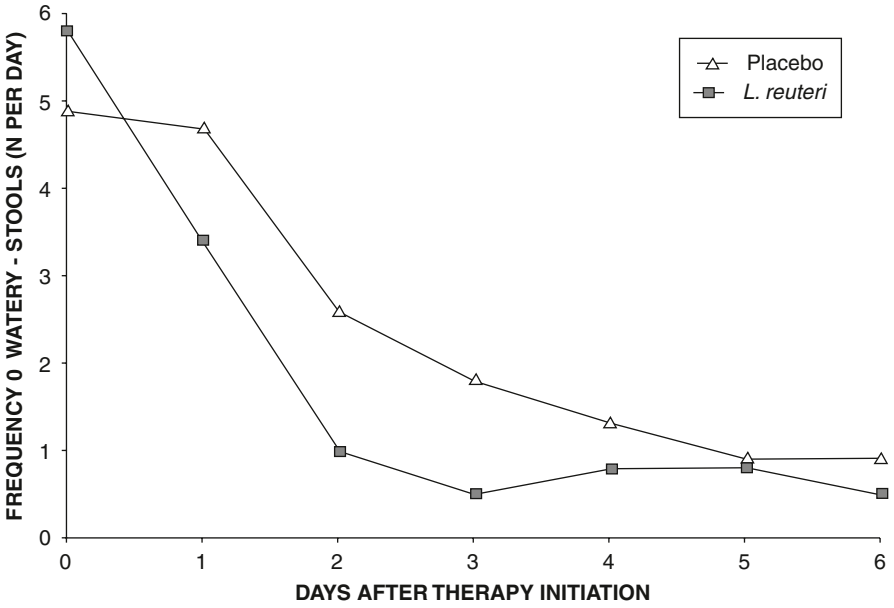

Fig. 1.9 Frequency of watery stools per 24-h period in patients receiving *L. reuteri* and placebo (Shornikova et al. 1997b)

Eom and colleagues showed the therapeutic effect of *L. reuteri*, administered at a dose of 2×10^8 CFU/die only, to significantly reduce acute diarrhoea in children (Eom et al. 2005).

1.5.2 Antibiotic Associated Diarrhoea

Antibiotics, aminopenicillins, cephalosporin, clindamycin etc., are much prescribed in all industrialized countries with several side effects especially in children: among which the most frequent is antibiotic associated diarrhoea (AAD). The resulting alteration of intestinal microflora reduces the development of anaerobic microflora, which leads to a reduced metabolism of carbohydrates and therefore to osmotic diarrhoea, favours the development of pathogens such as *Clostridium difficile, Salmonella, C. perfringens type A, Staphylococcus aureus* and *Candida albicans.* According to the recent studies, even in adults, there is level I of scientific evidence in favour of the use of probiotics in the treatment of AAD (Doron et al. 2008; Floch et al. 2008; Pham et al. 2008; Surawicz 2008). Therefore, there are grounds to recommend their use especially in risky cases, as in subjects where there is repeated use of antibiotics or in subjects with diarrhoeal episodes occurring after the administration of antibiotics. This in an attempt to prevent inflammatory processes of the intestinal mucosa in children that can often lead to chronic inflammatory disease

of the large intestine (Crohn's disease, ulcerative colitis, pouchitis) in subsequent years (Caramia 2008; Floch et al. 2008; Guandalini 2008). In a randomized, double-blind, placebo-controlled pilot study, recently presented at the Clinical Nutrition Week 2009, patients receiving antibiotics were given *L. reuteri* (10^8 CFU b. i. d.) or an identical placebo for 4 weeks. Patients treated with *L. reuteri* had a significantly lower incidence of diarrhoea (only 7.7%) compared to patients receiving placebo (50%) (Cimperman et al. 2009).

1.5.3 Clostridium difficile Associated Diarrhoea

The *Clostridium difficile* is the main cause of diarrhoea caused by antibiotics (CDAD) and of nosocomial colitis. It has been indicated as responsible for between 10% and 20% of all cases of diarrhoea caused by antibiotics, 60% of antibiotic-associated colitis and nearly all cases of pseudo membrane colitis. The diarrhoeal disease caused by *C. difficile* is determined only by the *C. difficile* strains producing the toxin A, who plays a mild cytotoxic activity and causes damage to the mucous, inflammation and intestinal secretion, and by toxin B, one of the most powerful cytotoxin, which determines loss of intracellular potassium, inhibition of protein synthesis and nucleic acids.

Unfortunately, the diversity of probiotics, their doses and the heterogeneity of studies make it difficult to recommend a definitive therapy, and also to indicate which probiotics to use as an antibiotic treatment and for prevention of *C. difficile* associated diarrhoea and/or colitis. For this reason, despite there are many promising data, the level of scientific evidence in favour of the use of probiotics or a combination of antibiotic and probiotic in the treatment of CDAD is currently of type II only (Doron et al. 2008; Floch et al. 2008; Guandalini 2008; Hookman and Barkin 2009; Yangco et al. 2009).

1.5.4 Infection Caused by Helicobacter pylori

Helicobacter pylori (HP) infection affects over 50% of the world's population and covers 80% of the population in the developing countries. HP infection is the main cause of peptic ulcer disease (70–90% of cases), lymphoma and in 1% of infected persons, leads to the development of gastric cancer with remarkable increase in mortality (Kelly and LaMont 2008; Jarosz et al. 2009). In developed countries, the infection starts in childhood, where it seems to have an incidence of 10–15%, then rapidly increasing during evolution (Sabbi et al. 2008). The transmission is oro-faecal as the seed is located in the gingival bags and at the root of the tongue.

Several studies showed that patients treated with probiotics associated with the standard antibiotic therapy had higher rate of eradication with a minor number of side effects.

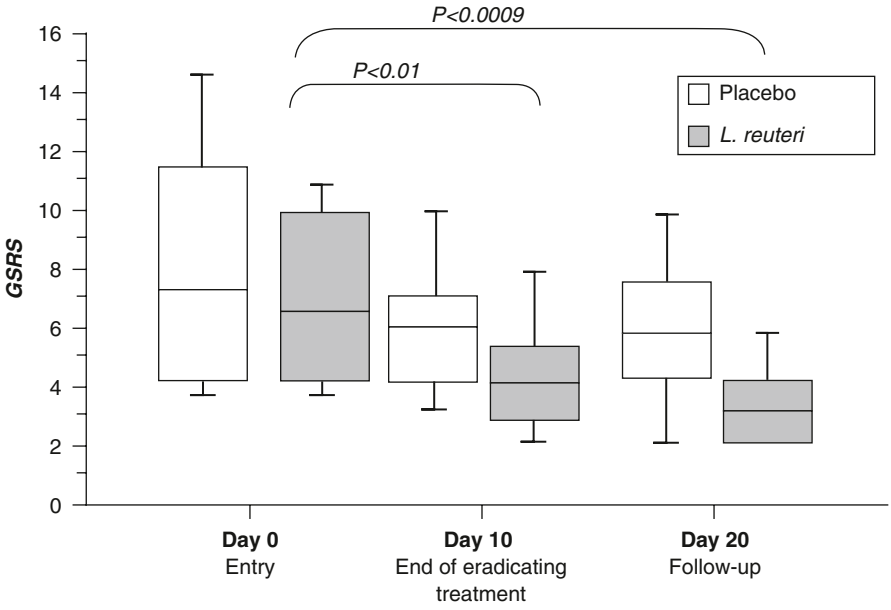

Fig. 1.10 Gastrointestinal symptom rating scale (GSRS) in children receiving *L. reuteri* or placebo. Comparison of continuous variables performed using Mann-Whitney. (Adapted from Lionetti et al. 2006)

A pilot study performed on 30 Hp positive adults treated with omeprazole + placebo or omeprazole + *L. reuteri* for 30 days, showed that 60% of patients in the *L. reuteri* group was eradicated, while none in the placebo group (Fig. 1.10) (Saggioro et al. 2005). Lionetti and colleagues in 2006 showed a reduction in gastro- intestinal symptoms by *L. reuteri* supplementation during and after the eradication therapy in a group of Hp infected children (Lionetti et al. 2006). Finally, Francavilla and colleagues in a recent pilot study conducted on 40 Hp positive adults, undergoing the standard eradication therapy, showed that the pre-administration of *L. reuteri* in the 4 weeks before treatment significantly reduces the bacterial load, and decreases the GI associated symptoms (Francavilla et al. 2008).

Excellent results are also reported by the administration of fermented milk enriched with probiotics (Tong et al. 2007; Sachdeva and Nagpal 2009). The HP infection in adults are related to the type and virulence of HP strain, the production of toxins A and B, the level of infection, the extent of inflammation, and the density of HP colonization. Therefore, there is a considerable interest in developing therapy to prevent HP infection. Probiotics intake, with suppression of HP, may have a favourable effect on HP infection and in decreasing the risk to develop related diseases; however, further, large, long-term and placebo-controlled studies are needed to confirm all those effects (Kelly and LaMont 2008; Selgrad and Malfertheiner 2008; McFarland 2009).

1.5.5 Traveller's Diarrhoea

The traveller's diarrhoea (TD) is the most common pathologic condition among travellers, and can occur in approximately 20–50% of the subjects during or immediately after a trip to a country with hot and humid weather conditions and inadequate sanitary conditions. More than one third of patients with TD had pathogen-negative illness but in the case of food contaminated with pathogens, the most frequent are enterotoxigenic *Escherichia coli, Campylobacter jejuni, Shighella, Salmonella*, sometimes virus, such as *rotavirus, calicivirus, enterovirus*, or parassites such as *Giardia lambdia, Entamoeba histolytica, Cryptosporidium parvum,* and *Cyclospora cayetanensis* are less common causes of TD (McFarland 2007; Johnson et al. 2009).

Sometimes serious but of transitory nature, it is characterized by non formed or liquid evacuations, often accompanied by nausea, vomiting, abdominal cramps, tenesmus, in 10% of cases stool blood, occasionally fever. The evolution is favourable in 1–5 days, but sometimes it needs up to 10 days. It has also been reported that 2–10% of travellers with TD develop persistent diarrhoea and that about 10% of them suffer from post infectious irritable bowel syndrome (Caramia 2008).

In a recent meta-analysis of probiotics for the prevention of TD on randomized, controlled, blinded efficacy trials in humans diarrhoea, several probiotics, *S. boulardii* and a combination of *L. acidophilus* and *B. bifidum*, had significant efficacy (Takahashi et al. 2007; Guslandi 2008).

There are well founded reasons to believe that probiotics may be a safe and effective strategy to prevent TD, to which one can associate racecadotril an inhibitor of intestinal encefalinasis and therefore antisecretive and antidiarrhoeic action, but continued research are needed (Tormo et al. 2008; Vandenplas et al. 2009).

1.5.6 Necrotizing Enterocolitis

The necrotizing enterocolitis (NEC), inflammatory intestine disease with an incidence of 1 to 3/1,000 live births, is relatively common in preterm in the first six weeks of life, and leads to death in 15–30% of those with lower weight to 1,500 g and the 20–40% of cases requiring surgery.

The causes of the disease appear to be multifactorial and are represented by prematurity, hypoxia-ischemia of the intestine, extensive use of antibiotics, reduced exposure to maternal microflora and excessive exposure to the typical sections of neonatal intensive care (NICU) (Staphylococci, Enterobacteriaceae, enterococci, *Candida* spp.), or to the use of sterile food as an alternative to mother's milk.

Based on several multicentre, randomized, double blind investigations there are reasonable grounds to believe that despite the differences between tested probiotics, the beginning of administration, dose and duration of the treatment and groups of participants, the enteral supplementation of some probiotics may reduce the risk

of severe NEC and mortality in premature newborn with weight >1,000 g. There is therefore a level I of evidence, even if a deeper understanding of NEC pathogenesis and the mechanisms by which probiotics prevent this disease is needed (Dani et al. 2002; Bin-Nun et al. 2005; Deshpande et al. 2007; Alfaleh and Bassler 2008; Lin et al. 2008; Caplan 2009).

1.5.7 Bacterial Vaginosis

Bacterial vaginosis (BV) is the most prevalent vaginal infection worldwide and is characterized by depletion of the indigenous lactobacilli. The vaginal microflora is composed by several species of lactobacilli (*L. acidophilus, L. brevis, L. casei* spp. *paracasei, L. rhamnosus*) named "Doderlein" microflora. They create on the mucosa a bacterial biofilm able to inhibit the growth and the adhesion of pathogenic bacteria. This microflora presents variations related to the life style (diet, stress, sexual habits, etc.) which may cause quali-quantitative modifications of the normal environment and the introduction of several kinds of pathogens such as *Gardnerella vaginalis* and *Candida albicans*.

Antimicrobial therapy is often ineffective while the probiotic approach, either topic or combined with the oral administration, gave interesting results. This has been confirmed by *L. rhamnosus* strain (Lcr35) showing the ability to adhere to cervical and vaginal cells and to affect the viability of two main vaginosis-associated pathogens, *Prevotella bivia, Gardnerella vaginalis*, as well as *C. albicans* (Coudeyras et al. 2008).

In a trial eighty-four patients with bacterial vaginosis were randomized to receive either oral metronidazole 500 mg twice a day for seven days, or one vaginal tablet containing freeze-dried *L. rhamnosus* once a week at bedtime for two months starting one week after the last antibiotic administration. Chi-squared analysis showed a significant difference between the two treatment groups at day 90 ($p = 0.05$). Safe and effective long-term vaginal administration of *L. rhamnosus* appears to be a useful complementary approach in the management of bacterial vaginosis (Marcone et al. 2008).

Recently sixty-four women diagnosed with BV were randomly assigned to receive a single dose of tinidazole (2 g) supplemented with either 2 placebo capsules or 2 capsules containing *L. rhamnosus* GR-1 and *L. reuteri* RC-14 every morning for the following 4 weeks. After a treatment of 28 days, the probiotic group had a significantly higher cure rate of BV (87.5%) than the placebo group (50.0%) ($p = 0.001$). In addition, according to the Gram-stain Nugent score, more women were assessed with "normal" vaginal microbiota in the probiotic group (75.0% versus 34.4% in the placebo group; $p = 0.011$) (Martinez et al. 2009a). This study shows that probiotic lactobacilli can provide benefits to women with BV and that probiotic capsules containing *L. rhamnosus* GR-1 and *L. reuteri* RC-14 can increase the effectiveness of an antifungal pharmaceutical agent (fluconazole) (Martinez et al. 2009b).

1.5.8 Irritable Bowel Syndrome

The irritable bowel syndrome (IBS), a disorder of the function of the intestine, affects 3–25% of the general population and is characterized by pain at the abdomen, constipation alternating with periods of diarrhoea, presence of air and a sense of abdominal bloating. This is due to psychological factors, including sexual abuse, because the central nervous system interacts with intestinal neurotransmitters and hormones, to dietary factors resulting in alteration of intestinal motility, and abnormal dismicrobism, colic fermentation by bacteria and sometimes a secondary gastrointestinal infection (e.g. *Campylobacter* or *Salmonella*) (Belaise et al. 2002; Mättö et al. 2005).

Recent reviews of the literature provide interesting data also on gut motility and pain perception, however the results, sometimes contradictory, are not yet very clear and the effectiveness of probiotics in IBS show a level of scientific evidence of type I (Kunze et al. 2009). Very likely the strength of the recommendations of type B is due to the limited number of patients treated with different probiotics (Jiménez 2009). It is therefore considered useful to continue with more appropriate and thorough studies on those probiotics that have shown a more promising result.

1.5.9 Crohn's Disease

Crohn's disease, a gastrointestinal disorder characterized by chronic inflammation or ulceration involving all layers of the intestinal wall, is one of a group of diseases called inflammatory bowel disease (IBD) such as Crohn's disease (CD), ulcerative colitis (UC) and pouchitis. IBD are most common in the developed countries of Europe, the US, and Scandinavia, and less in Southern and Eastern Europe, in Asia or Africa, and the diseases are relatively uncommon in Cuba and Central and South America (Loftus 2004). This has given rise to a number of theories regarding IBD etiology and the significance of diet high in refined sugar, meat, milk, eggs and low in fiber, fruit, and vegetables.

CD can affect any area from the mouth to the anus but often affects the lower part of the small intestine, the ileum, while the colon is the second most common site of involvement. Prevalence of Crohn's disease is approx 0.18% and is more frequent in young adults between 15 and 30 years of age (Wallace 2009).

The intestinal microbiota play an important role in the pathogenesis and maintenance of disease. Although marked alterations occur in faecal and mucosal bacterial communities in CD and others IBD, it is unclear whether they are responsible for causing disease, or are due to changes in the gut environment that result from inflammatory reactions and extensive tissue destruction that later concur to maintain the pathological condition.

A study has been recently conducted on a limited number of patients treated for 13.0 ± 4.5 months with a mixture of probiotics composed of *B. breve* 30×10^9 CFU/

day plus *L. casei* 30×10^9 CFU/day plus *B. longum*, 15×10^9 CFU/day; 3.3 g of psyllium (*Plantago ovata*) were added to probiotics as prebiotics, diluted in 100 mL of water immediately before being given three times a day. The treatment led to satisfactory results with reduction of the CD activity index (CDAI), which takes into account eight parameters (the number of liquid discharges, abdominal pain, general condition, presence of complications of abdominal masses, low hematocrit, loss weight, treatment with opioids or similar), and of the International Organization for the Study of Inflammatory Bowel Disease (IOIBD) score. In particular there was a reduction in diarrhoea and abdominal pain in eight patients, complete remission in six cases and partial in one case, the suspension of cortico-therapy in two cases and dose reduction in four (Fujimori et al. 2007).

A Cochrane survey in the period 1966–2007 on several meta-analysis has found only one reliable study conducted on eleven patients for which it was not possible to draw any conclusion and a subsequent meta has found only two studies in which therapy with probiotics had positive results (Butterworth et al. 2008; Heilpern and Szilagyi 2008; Macfarlane et al. 2009b).

Other evidences suggest that the administration of select prebiotics, probiotics and synbiotics may improve the clinical outcome of patients with IBD. This suggests that there is potential for controlling these diseases through manipulation of the composition of the gut microbiota, and direct interactions with the gut immune system (Kanauchi et al. 2009; Macfarlane et al. 2009a). In addition, IBD patients are well known to carry a higher risk of developing colorectal cancer due to chronic inflammation (Kanauchi et al. 2009).

Therefore, probiotics and/or prebiotics may be appropriate treatments for prophylactic use due to their physiologic characteristics and lack of obvious toxicity.

1.5.10 Ulcerative Colitis

Ulcerative Colitis (UC) is a chronic intestinal inflammatory disease that affects the final part of the colon (sigma and rectum), but can be more extensive and involve the left side of the colon or the whole colon. Severe UC, a serious, potentially life threatening condition and hospitalization, should be considered in all patients who have more than 6–10 bloody stools per day, associated with fever, dehydration, tachycardia malaise and/or increased C-reactive protein.

The main therapeutic remedies are represented by optimal doses of oral 5-aminosalicylic acid (5-ASA), topical therapy with either 5-ASA or steroids, and systemic corticosteroids. Failure to induce remission will occur in only 40% of patients after a first course of oral systemic steroids and only 20% will have no improvement of symptoms whatsoever (Van Assche et al. 2008).

Among the newer agents, a probiotic preparation (VSL#3) may prove helpful in inducing remission also in paediatric patients with ulcerative colitis associated with 5-ASA (Huynh et al. 2009; Miele et al. 2009).

In a recent study three groups of 40 patients were treated with probiotic, prebiotic, or synbiotic therapy. The probiotic group ingested one daily capsule consisting of *B. longum* 2×10^9 CFU and the prebiotic group ingested daily 8.0-g doses of psyllium. The synbiotic group underwent both treatments. All patients completed Inflammatory Bowel Disease Questionnaires (IBDQs) at the onset of the trial, at the 2-week midpoint, and at the 4-week end of the trial.

Total IBDQ scores improved within groups by the end of the trial (probiotics 162–169, NS; prebiotics 174–182, NS; synbiotics 168–176, $p = 0.03$). C-reactive protein decreased significantly only with synbiotic therapy (from 0.59 to 0.14 mg/dL, $p = 0.04$). There were no adverse events and patients with UC on synbiotic therapy experienced greater quality of life changes than patients on probiotic or prebiotic treatment. These data suggest that synbiotic therapy may have a synergistic effect in the treatment of UC (Fujimori et al. 2009).

Probiotics, prebiotics, and synbiotics may improve the clinical outcome of patients with UC and may be appropriate treatments for prophylactic use due to their physiologic characteristics and lack of obvious toxicity (Kanauchi et al. 2009).

In patients with severe ulcerative colitis, the restorative proctocolectomy is often performed, it consists in removing the entire colon and rectum, with severe anatomo-pathological ulcerative lesions, and in making an ileal "reservoir or pouch", using the last ileal handle, which is anastomosed to the anus: it is thus created a pocket (pouch in Anglo-Saxon terminology) like a rectal ampoule, which acts as a reservoir for faecal content.

Pouchitis is the major long-term complication after ileal-pouch anal anastomosis for ulcerative colitis, and broad-spectrum antibiotics are the mainstay of treatment. Recently, it has been shown the efficacy of a highly concentrated probiotic preparation (VSL#3, 900 billions/sachet lyophilized viable bacteria containing four strains of *Lactobacillus*, three strains of *Bifidobacterium* and one strain of *Streptococcus thermophilus*) in preventing pouchitis onset, in preventing relapses of chronic pouchitis and in the treatment of mild pouchitis (Gionchetti et al. 2003, 2007). Patients receiving probiotics have shown a significant improvement in their quality of life and no any side effects or alteration of the laboratory parameters, however further controlled studies are warranted.

1.5.11 Atopic Dermatitis

The prevalence of atopic dermatitis (AD) has risen over the past decades, especially in western societies. This increase, according to the revised hygiene hypothesis, is caused by a changed intestinal colonization pattern during infancy, which has an impact on the immune system, so healthy subjects have an intact functional allergen-specific regulatory T-cell response, while allergic subjects have an impaired response. Manipulating the intestinal microflora with pro-, pre- or synbiotics is an innovative way to prevent or treat AD (von Hertzen et al. 2009).

In 2007 Abrahmsson and colleagues performed a double-blind, randomized, placebo-controlled trial, which comprised 232 families with allergic disease, of

whom 188 completed the study. The mothers received *L. reuteri* (10^8 CFU/die) from the 36th gestational week until delivery (Abrahamsson et al. 2007). Their babies then continued with the same product from birth until 12 months of age and were followed up for another year. Primary outcome was allergic disease, with or without positive skin prick test or circulating IgE to food allergens. The cumulative incidence of eczema was similar in the two groups, however, the *L. reuteri* group had less IgE-associated eczema during the second year (8% versus 20%; $p = 0.02$). The skin prick test reactivity was also less common in the treated than in the placebo group, significantly so for infants with mothers with allergies, 14% versus 31% ($p = 0.02$). A recent prospective, double-blind, randomized, placebo-controlled clinical trial performed on 50 subjects aged 3–47 months with moderate/severe AD showed that 12 months of *L. reuteri* supplementation may be beneficial in the long-term control of eczema (Gromert and Axelsson 2009). Based on a recent review at this moment there is not enough evidence to support the use of pro-, pre- or synbiotics for the prevention or treatment of AD in children in clinical practice (van der Aa et al. 2009). On the contrary, some other published results suggest that the administration of selected strains of probiotics during the perinatal period may be helpful in the prevention of AD; however probiotic strain or the composition of different probiotics and/or prebiotics, the dose and the timing of supplementation still need to be determined (Ji 2009; Kopp and Salfeld 2009; von Hertzen et al. 2009). Results of these trials are still conflicting and more randomized controlled trials specifically designed for children with food allergy, with different probiotic strains, are necessary to draw definitive conclusions (van der Aa et al. 2009).

1.5.12 Dental Caries

Dental caries is a multifactorial disease that includes the participation of cariogenic and non-cariogenic bacteria, salivary components (proteins, enzymes, calcium, phosphate, fluoride) and dietary sources of fermentable carbohydrates (sucrose, glucose). Dental caries is one of the most prevalent diseases in humans, is second only to the common cold, affects 90% of late adolescents and young adults and more than 95% of adults experiencing caries on enamel and root surfaces (Islam et al. 2007; García-Godoy and Hicks 2008). Caries, gingivitis and periodontal disease occur because of the accumulation of pathogenic dental plaque. Prevalence and severity have increased during the past decade in young children, particularly in the primary dentition (García-Godoy and Hicks 2008). The changes in the homeostasis of the oral cavity with an overgrowth of *Streptococcus mutans* is recognized as the primary cause of the disease but cariogenic bacteria include *S. sobrinus, Lactobacillus* species and *Actinomyces* species, as well as to a lesser extent *S. mitis, S. oralis, S. gordonii* and *S. anginosus*. They strongly adhere and releases acids by the fermentation of carbohydrates, leading to the demineralization of the tooth. This attachment is mediated mostly by the interaction of surface proteins and bacterial polysaccharides. Early acquisition of *S. mutans* is a major risk factor for early childhood caries and predicts future caries experience.

Ambiguities in the basic treatment of dental caries, such as the use of fluoride and antibiotics, and increasing problems of resistance to synthetic antimicrobials, vitalize the deployment of probiotic therapies for elimination of the bacterium or suppression of its virulence (Kuramitsu et al. 2007; García-Godoy and Hicks 2008).

The probiotic approach to change the oral environment toward health by the deliberate implantation of specific oral streptococci that naturally lack the ability to produce lactic acid to encourage the shift from a pathogenic to a nonpathogenic plaque or biofilm, is one interesting strategy (Hillman et al. 2009; Zahradnik et al. 2009).

Daily applications of *S. rattus* strain JH145, a naturally occurring LDH-deficient variant of *S. rattus*, can compete with *S. mutans* for its habitat on the tooth surface and has been used to affect the numbers of an implanted *S. mutans* strain in a rat model. It has been then demonstrated that *S. rattus* JH145 competes with *S. mutans* for its habitat on the tooth surface and as a probiotic has a prevention role in dental caries (Hillman et al. 2009).

A pilot clinical trial has been performed in humans to assess the safety and to test the ability of a probiotic "mouthwash" ProBiora containing three specific strains of naturally occurring oral bacteria *S. oralis* strain KJ3sm, *S. uberis* strain KJ2sm, and the spontaneous lactic acid-deficient variant of *S. rattus*, strain JH145, to affect the levels of *S. mutans* and certain known periodontal pathogens in the mouth when administered twice daily over a period of 4 weeks. The results of this pilot human study suggest that the probiotic mouthwash product may be safe for daily use as an aid in maintaining both dental and periodontal health.

Two interesting studies have been also performed on the short-term effect of chewing gums containing the probiotic *L. reuteri* on both the levels of salivary mutans streptococci in young adults (Table 1.2) (Caglar et al. 2006) and more recently

Table 1.2 Distribution of salivary mutans streptococci counts before and after 3 weeks of daily ingestion of a probiotic strain (*Lactobacillus reuteri*) with straw and tablets. The values denote the number of subjects. (Caglar et al. 2006)

Administration/time	n	Mutans streptococci score (cfu)				
		0 No growth	1 $\leq 10^4$	2 10^5	3 $\geq 10^6$	p
Group A *Probiotic straw*						
Baseline	30	6	4	5	15	
End	30	9	6	14	1	<0.05
Group B *Placebo straw*						
Baseline	30	6	9	9	6	
End	30	8	7	8	7	NS
Group C *Probiotic tablet*						
Baseline	30	9	5	5	11	
End	30	17	2	8	3	<0.01
Group D *Placebo tablet*						
Baseline	30	13	5	5	7	
End	30	12	6	6	6	NS

Cfu colony forming units, *NS* not significant

Table 1.3 Concentration of inflammatory mediators (pg/ml) (SD) in gingival crevicular fluid at baseline and at designated time intervals during and after use of chewing gums containing *Lactobacillus reuteri*. (Twetman et al. 2009)

Variable/time	A/P (active + placebo) (n = 13, 26 sites)	A/A (active + active) (n = 13, 26 sites)	P/P (placebo + placebo) (n = 13, 24 sites)
IL-1β			
Baseline	10.5 (10.5)	9.0 (7.6)	10.4 (12.3)
1 week	10.1 (9.8)	4.7 (6.9)	13.8 (14.0)
2 week	9.1 (8.9)	4.4 (4.7)	9.3 (10.5)
4 week	10.2 (11.6)	9.8 (11.1)	10.9 (13.3)
TNF-α			
Baseline	0.23 (0.24)	0.41 (0.29)	0.45 (0.52)
1 week	0.32 (0.31)	0.14 (0.15[a])	0.43 (0.49)
2 week	0.41 (0.34)	0.24 (0.26)	0.39 (0.36)
4 week	0.22 (0.19)	0.33 (0.36)	0.40 (0.38)
IL-6			
Baseline	0.92 (1.67)	1.90 (3.28)	1.53 (2.48)
1 week	0.67 (0.68)	0.94 (1.70)	1.60 (2.49)
2 week	0.76 (1.23)	0.95 (1.69)	1.91 (2.50)
4 week	0.99 (1.20)	0.74 (1.56[a])	1.03 (1.87)
IL-8			
Baseline	116.2 (51.8)	103.3 (36.8)	107.3 (69.9)
1 week	102.2 (43.7)	86.2 (54.4)	82.9 (32.9)
2 week	95.2 (60.2)	77.4 (31.8[a])	85.8 (42.0)
4 week	97.7 (69.0)	105.8 (63.8)	106.7 (60.6)
IL-10			
Baseline	0.28 (0.27)	0.24 (0.38)	0.32 (0.44)
1 week	0.32 (0.38)	0.19 (0.24)	0.28 (0.31)
2 week	0.32 (0.38)	0.22 (0.37)	0.24 (0.26)
4 week	0.20 (0.23)	0.19 (0.20)	0.20 (0.20)

[a] Statistically significant difference compared to baseline ($p < 0.05$, Wilcoxon signed rank test)

on the levels of inflammatory mediators in gingival crevicular fluid (Table 1.3) (Twetman et al. 2009).

Further research is required involving double-blind randomized controlled trials in order to bring further benefits of more effective caries control (Zahradnik et al. 2009).

1.6 Advances Made from the Traditional Use to the Modern Application in Medical Field

In the recent years, probiotics, together with prebiotics, met a growing scientific interest in the context of the so-called functional foods and/or nutriceuticals, attractive and imaging names that reveal their important implications for the human health.

The main function of probiotics, evoked by Metnikoff, has been to reduce the putrefactive intestinal flora responsible of, according to the popular theory of the end of nineteenth century, the auto-intoxication and the occurrence of disease due to the release of substances harmful for the body (LaMont 2000). Today, following the recent scientific knowledge, probiotics are considered as useful to promote a mutual beneficial balance between man and his intestinal bacterial microflora, selectively stimulating the growth and/or the metabolic activity of microbial groups, which are important for digestive processes, intestinal wall permeability, resistance to pathogenic invasion, lipid metabolism, intestinal and general mucosal immunity, etc. The improvement of clinical and biochemical intestinal function was also obtained in some pathologic conditions such as cystic fibrosis patients (Infante et al. 2008).

Prebiotics have received a great scientific interest as well as selectively stimulate the growth and/or metabolism of the microbial groups relevant to the host health (bifidobacteria, lactobacilli) and inhibit the growth and activity of pathogenic microorganisms of the proteolytic putrefactive flora, and lead to an increase in survival and colonization of probiotic microorganisms, administered as commercial products. These effects are important because they help to enhance the beneficial action by preventing the mucosal inflammation and the permeability alterations. It should be noted, however, that by their fermentation probiotics prevent both the formation of amines, which are harmful because alter blood circulation and muscle activity and are mutagenic and carcinogenic, especially in cases where there is a liver failure which compromises the detoxification; and the production of phenols, mainly scatole and indole highly carcinogenic; and the secondary bile acids that may promote colon cancer (Fotiadis et al. 2008).

Even the short-chain fatty acids (SCFA) that are formed (acetic, propionic and butyric acid) play an important role because they are excellent nutrients for a better trophism and function of colonic mucosal cells, they play a protective action against inflammatory bowel diseases and a preventive action on the development of colon cancer as well as reducing the proliferation of pathogens and having antiputrefactive properties. All results in a better absorption of nutrient substances versus the toxic ones playing a preventive role with obvious implications for the whole body health (Parracho et al. 2007).

To confirm these data also in preterm and term infants, a formula was supplemented with a prebiotic mixtures of galactose-based prebiotics, long-chain (GOS) and high-molecular weight mixture of inulin-type fructans containing 0.4–1 g/100 ml in a 9:1 ratio, and it was administered for 3–12 weeks, leading to a significant increase in bifidobacteria amount in the infant faecal flora from 20% to approx 60% (breast-fed babies ~80%), and giving some metabolic and clinical changes similar to those caused by breast milk (Moro et al. 2002; Knol et al. 2005; Boehm and Moro 2008; Kelly 2008).

These and many other metabolic and clinical effects on the immune system, on inflammatory processes, on lipid or mineral metabolism, etc., have induced many researchers to administer in various clinical conditions the associations of probiotics and prebiotics, the so-called synbiotics.

Several studies of the recent literature reveals evidence that support the positive impact of synbiotics on intestinal microflora of preterm and term infants and in adult, on immunonutritional parameters and on prevention of eczema, especially the atopic eczema (Rastall and Maitin 2002; Bartosch et al. 2005; Casiraghi et al. 2007; Kukkonen et al. 2007; Uchida et al. 2007; Panigrahi et al. 2008; Underwood et al. 2009). It has been also reported that synbiotics administered to newborn infants seem to increase resistance to respiratory infections during the first 2 years of life, reduce the incidence and severity of respiratory diseases during the cold season, decrease the incidence of septic complications in patients with severe systemic inflammatory response syndrome and are safe (Kukkonen et al. 2008; Pregliasco et al. 2008; Shimizu et al. 2009). Leyer and colleagues reported that also daily dietary probiotic supplementation for 6 months was a safe effective way to reduce fever, rhinorrea, and cough incidence and duration and antibiotic prescription incidence, as well as the number of missed school days attributable to illness, for children 3–5 years of age (Leyer et al. 2009). An interesting reported activity, that must however be confirmed, is the CD remission (Fujimori et al. 2007). Moreover some studies suggest that synbiotic therapy could prove more effective in the treatment of UC than therapies limited to probiotics or prebiotics, C-reactive protein decreased significantly only with synbiotic therapy and patients with UC on synbiotic therapy experienced greater quality-of life-changes than patients on probiotic or prebiotic treatment suggesting that synbiotic therapy may have a synergistic effect in the treatment of UC (Fujimori et al. 2007; Kanauchi et al. 2009; Macfarlane et al. 2009a).

Recent experimental and clinical studies support the fact that, in critically ill patients, early enteral nutrition enriched with synbiotics should restore the balance of microbial communities in a beneficial way with positive effects on intestinal permeability and bacterial translocation and may reduce systemic inflammation, improve the immunological status of the intestinal mucosa and help prevent infections (Manzanares and Hardy 2008). A positive effect of synbiotics has been noted also in multiple trauma patients and in patients with high-risk operations (Rayes et al. 2009).

So it seems clear that while probiotics have to compete with already established bacterial communities, prebiotics have the advantage that they target bacteria already present in the bowel. This makes prebiotics a potentially more efficient and practical way of manipulating the gut microbiota. However, if for any reason the target bacteria are absent from the gut, either due to disease, aging, or antibiotic therapy, then prebiotics alone are not likely to be effective, at least in the short term.

For this reason synbiotics, a combinations of prebiotic and probiotic, may be useful in several conditions and have also a synergistic role. The rationale for their use is that the prebiotic enhances growth of the probiotic component in the gut, giving it a competitive advantage while also stimulating the growth and metabolism of autochthonous microorganisms.

There are reasonable grounds for believing that, besides seeking new and more effective probiotics that it is hoped will be available in the near future but not so easy to obtain, we must not neglect the development of optimal synbiotics that may prove useful in various clinical-pathological conditions.

1.7 Advances Made from the Traditional Use to the Modern Application in Food Industry

In the industrial field a number of food bio-products have been employed or are in progress of being developed to enhance their usage as delivery vehicles of probiotic cell fed humans. Most of these products are of dairy origin and include fresh milk, fermented milk, powdered milk and cheese. Since few years, the food companies involved in the probiotic field following the new demands of consumers promote the researches on novel probiotic foods such as beverages, cookies, ice-cream, dairy dessert, sausages and others.

The possibility of influencing the composition of intestinal microflora by consuming probiotic bacteria partially depends on the dose level. It is generally recognized that 10^8–10^9 bacteria are necessary at the time of consumption. Therefore the probiotic culture must remain viable in the food carrier up to consumption. Shelf-life of a product is defined as the time that the product can be stored, during which the defined quality of a specified proportion of the goods (in case of probiotic, viability of culture) remains acceptable under expected or specified conditions of distribution, storage and display. In some cases, modification of traditional processing protocol is necessary to enhance the viability of probiotic bacteria. In addition to maintaining the viability of probiotic bacteria in the cultured products through the time of the consumption, it is imperative that the incorporation of the probiotic bacteria does not adversely affect the flavour, the texture and other quality attributes of food product.

In recent years some yogurt have been reformulated to include live strains of *L. acidophilus* and species of *Bifidobacterium* (known as AB-culture) in addition to conventional yogurt microorganism, *S. thermophilus* and *L. bulgaricus*. For the production of AB-yogurt, similar processing procedures to traditional yogurt are applied with the exception of incorporation of live probiotic starter cultures. *L. acidophilus* and *B. bifidum* have to retain viability and activity in the food carrier to meet the "therapeutic minimum" at the time of the consumption. Yogurt acidity, strains selection, co-culture and species interaction, inoculation practice, dissolved oxygen and storage conditions are all factors affecting the viability of probiotic species in yogurt (Lourens and Viljoen 2001).

A study from Estonia (Songisepp et al. 2004) tried to develop an original probiotic cheese based on Estonian open-texture, smear-ripened, semisoft cheese "Pikantne". Cheese was produced by two methods using cheese starter cultures in combination with 0.04% of probiotic *L. fermentum* strain ME-3 with high antimicrobial activity and antioxidative properties. The probiotic *Lactobacillus* was added into milk simultaneously with start cultures (cheese A) and into drained curd (cheese B). Cheese A, B, and the control, the original cheese "Pikantne" where no probiotics were added, were described to be of commercial grade with respect to sensory criteria after one month of ripening. Both cheese variants with probiotic additive were found to have flavour and texture comparable to the control cheese. The probiotic strain was found to withstand the technological processing of cheese, surviving

and sustaining moderate antimicrobial and high antioxidative activity throughout ripening and storage. The ripened cheese contained approximately 5×10^7 CFU/g viable ME-3 cells but the viability of ME-3 strain incorporated into cheese showed a slight decrease between day 24 and 54 after preparation. Hence semisoft cheese "Pikantne" serves as suitable carrier of *L. fermentum* ME-3.

New researches from Brazil (Aragon-Alegro et al. 2007) suggest that, among chilled foods, chocolate mousse may be a suitable vehicle for probiotics, despite the delicate processes involved in the manufacture. For this study, three mousses have been prepared; one containing no live cultures or prebiotic fibres (control mousse), one containing *L. paracasei*, and the third containing *L. paracasei* plus inulin, a prebiotic fiber.

The product has been monitored for 28 days to assess the population of the probiotic *L. paracasei* as well as contaminants, during storage at +5°C. Data reported that probiotic was still viable after 28 days, maintaining population level about seven log CFU per gram. Moreover in this study detrimental effects, due to probiotic organism presence, were not observed on flavour of final product. Forty-two consumers were recruited to taste and evaluate the three chocolate mousses and no significant differences were reported although the probiotic mousse was considered the most preferable. For this reason the authors concluded that chocolate mousse could be an excellent vehicle for the incorporation of *L. paracasei* strain tested and the prebiotic ingredient inulin not interfere in this viability.

To use as probiotic vehicles, great potential has the ice-cream, it is a relatively innovative matrix for the application of probiotics, with the added advantage of being appreciated by people belonging to all age groups and social levels. However, the development of ice-creams containing probiotic bacteria requires the overcoming of certain technological intrinsic requirements related to their processing stages. Also, it is important to confirm if, after long storage periods, the probiotic cultures are still able to confer the same health benefits already observed in other foods with shorter shelf-lives and higher storage temperatures, such as yogurts and fermented milks (Başyiğit et al. 2006; Cruz et al. 2009).

The commercial application of probiotic microorganisms in fermented sausages is not common yet. There are both advantages and disadvantages connected to fermented meat matrices. They are adequate for the carriage of probiotic bacteria since they are usually not or only mildly heated and may promote the survival of probiotic bacteria in the gastrointestinal tract. In contrast, bacterial viability may be reduced due to the high content in curing salt and the low water activity and pH. Therefore, results are expected to be strain-dependent. Up till now, several approaches have been followed but most results are too preliminary to be able to evaluate the effect of probiotic fermented meats on human health. De Vuyst and colleagues obtained through screening for technological requirements among bacteria that are naturally present in the meat or that originate from meat starter cultures, possible probiotic candidates (De Vuyst et al. 2008). Alternatively, existing probiotic bacteria have been applied in meat products. The probiotic strains have been used not only as starters for fermented meat preparation but also for preservation of salami (Pidcock et al. 2002) from the contamination of *Listeria monocytogenes* and the enterohem-

orrhagic *E. coli* (EHEC). As one possible mode of action for probiotics is the production of antimicrobial compounds, lactic acid bacteria may act as both probiotic and bioprotective culture as well as fermenting agent in meat product, such as dry sausage (Työppönen et al. 2003). Also the vegetable foodstuff have been used as carriers for probiotic such as artichokes (Valerio et al. 2006) used in an artichoke human feeding study involving four volunteers, and giving good recovering from stool. Even fibers were used as carriers for *L. rhamnosus* during freeze-drying and storage in apple juice and chocolate-coated breakfast cereals (Saarela et al. 2006) giving good opportunity to solve the problems, often present, of preservation of probiotic into particular kinds of foods and during special producing processes. At last there are authors such as Verdenelli et al. (2009), who use a series of probiotic enriched-foods, from typical kinds of Italian cheese to classical dark chocolate bar, as probiotic intake in a volunteers study on colonisation properties of *L. rhamnosus* IMC 501 and *L. paracasei* IMC 502.

However, considering whatever food as carrier for probiotic it is important to guarantee the viability of the probiotic strains during the whole shelf-life of the product, the maintenance of the beneficial effects and, the preservation of nutritional and quality food characteristics. Moreover in order to respect the new dispositions of the European Community, whatever the health claims made directly or indirectly, companies will need to do the human studies to verify them.

The use of probiotics is also widely diffused in feeds for animals, replacing antibiotic treatments for long time widely used, with the aim of both increasing animal development and improving its health status. While numerous studies were conducted on a large variety of animals (Strompfová et al. 2004; Carnevali et al. 2006; Silvi et al. 2008; Taheri 2009), the major consumption happen on chickens, pigs and ruminants. Different compositions of the probiotic feed may be directed to different kinds of animals and the supplied probiotic may be related to the age of farming animal (Holzapfel and Naughton 2005). In the "animal production", the use of safe and high quality fodders is a concept characterized by close links with the "protection of human life and health." These fodders are used to promote the nutritional efficiency of the food principles of portion, to regulate the processes of digestion and assimilation and to stimulate and increase specific functional and/or productive performance of the animals. These auxinic substances with production purposes, also known as "growth or performance promoters", have been represented for many years by antibiotics used at low dosages, from minerals and other substances whose residues or metabolites, however, could find themselves in foodstuffs or in the manure and then into the environment, representing a risk for humans and encouraging the antibiotic resistance phenomenon in pathogens.

For this reason, since the 1st of January 2006 was banned its use throughout the European Community and has gone to an increasing use of "additives without residues", probiotics and associations between probiotics and prebiotics with the known benefits on the limitation and elimination of pathogens (decreased incidence of diarrhoea and other illnesses and decreased pathogen contamination of livestock products such as meat, eggs, and milk), on the improvement of the intestinal and general health status, on the stimulation of the animal growth and finally on the

decreased of the environmental impact of livestock farming improving security since “auxinics without residues”. A further aspect particularly interesting and in harmony with very recent researches on microbiology applied in space field, is the use of probiotic bacteria as dietary supplement during space missions, with the aim to improve the astronaut health and to protect them against gastro-intestinal disorders (Canganella 2008).

1.8 Final Considerations

During the last decades, the role of the intestinal microflora in health and disease has become increasingly recognized. Much interest exists in modulating the composition of the gut towards a potentially more beneficial community as probiotics and prebiotics that share a unique role in human nutrition, largely centering on manipulation of populations or activities of the bacteria that colonize our body. In fact, our digestive system is a microbiological-chemical and immunological laboratory in a continuous activity from the earliest periods of fetal life. During the physiological birth, the intestinal tract of foetus, sterile until then, undergoes a protective contamination, initially dominated by facultative anaerobic strains such as *E. coli*. Thereafter, differences exist in the species composition that develops, which is largely governed by the type of diet. In a very short time, however, more than 400–500 bacterial species proliferate and, after the first-second childhood, the microbial complex consists of 1–2 kg of regulatory microorganisms in a state of vital balance for the health. In this balance take part chemical substances such as the digestive secretions, which in adults amount to about 10 l per day and are formed from saliva (about 0.5 l), gastric juices (2.5 l), bile (0.5 l), pancreatic juices (1.5 l), small intestinal/colonic juices (1–5 l). Such secretions, together with the microbiota, obviously influenced by all assumed substances, food or not, are essential for the food digestion and both for the immune system activity, located for about 70% in the gastro-intestinal tract, and for stimulating the immune response, represented by immunoglobulins, transferrin, lysozyme, fibronectin, etc.

The better outcome may be achieved by using targeted dietary supplementation with functional foods, dietary ingredients that have a cellular or physiological effect above basic nutritional value. Recognition of the health-promoting properties of specific commensal microorganisms has encouraged modulation of the human intestinal microflora towards a more beneficial composition and metabolism, by using probiotics, prebiotics and synbiotics.

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