

J.J. Malago
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R. Marinsek-Logar
Editors

Probiotic Bacteria and Enteric Infections

Cytoprotection by Probiotic Bacteria



Springer

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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*

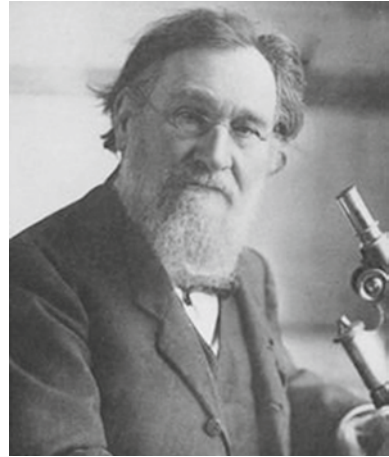
The recent history of probiotics began in the early 1900s. Thanks to Metchnikoff (1845–1916) (Fig. 1.1), professor of biology at the University of Odessa, who moved from Ukraine, his homeland, to Messina (Italy) for political reasons after the assassination of Czar Alexander II. In 1882 he discovered 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 Cal-mucchi, 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.

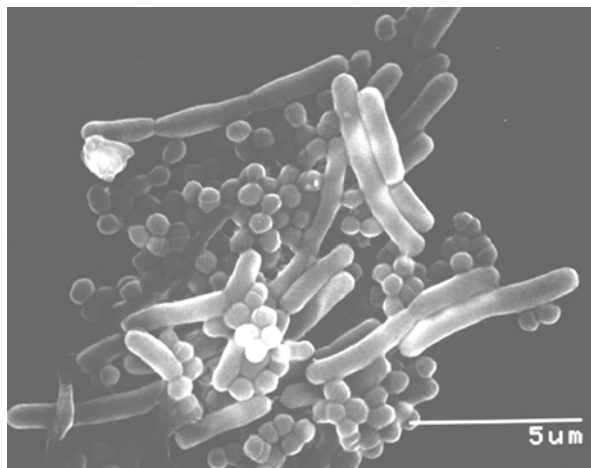
The presence of invisible microorganisms (or micro-Dei), which can creep into our bodies causing diseases, is already present in trace in some Chinese legends and in ancient Egyptian medical texts. Afterwards, Marco Terenzio Varrone (116 BC–27 AD) before and Girolamo Fracastoro (1478–1553) later, talk 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.

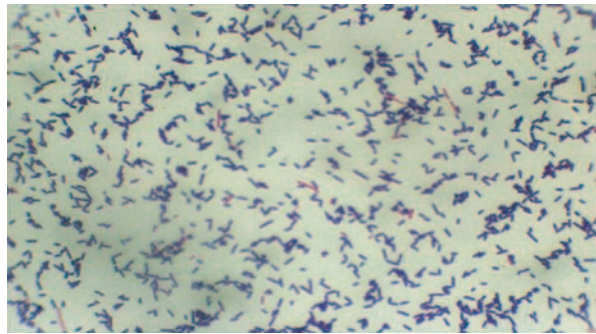
Always in the 20’s, Minoru Shirota, a Japanese microbiologist at the University of Kyoto (Fig. 1.3), discovered that some bacteria of the intestinal flora contribute to bacterial pathogens defence. The following studies led to isolate and cultivate *Lactobacillus casei* (Lc) (Fig. 1.4), afterwards called 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 Gastroenterology 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, hydrolyzed 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 Roberfroid 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 prebiotics”, 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-

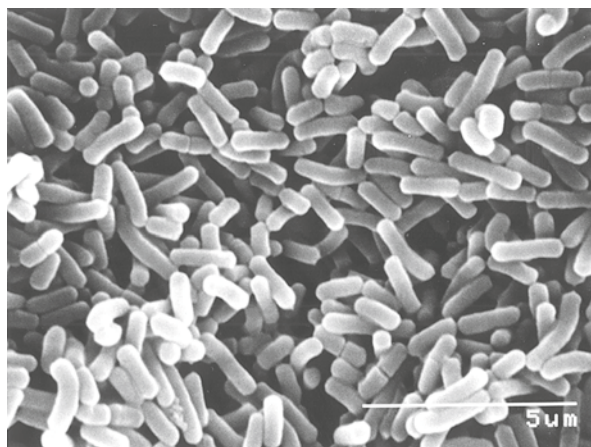


Fig. 1.5 Morphology of *Lactobacillus rhamnosus* at scanning electron microscope. (By M. Benevelli—Dept. “Scienze degli alimenti”, Bologna University, Italy)

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

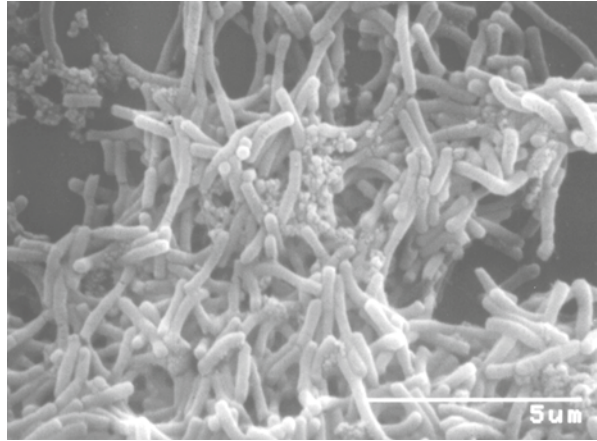
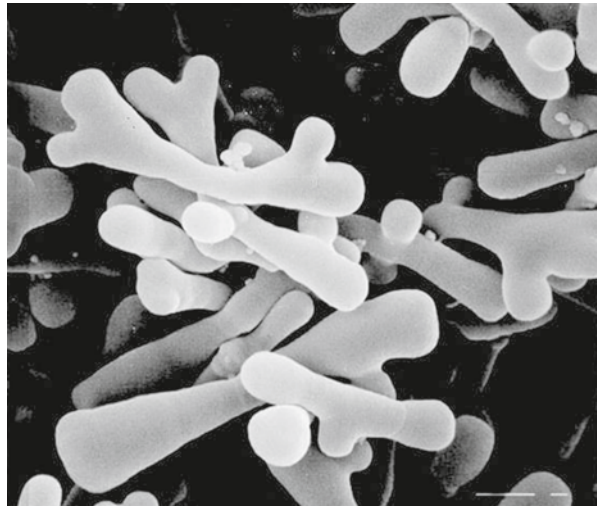


Fig. 1.7 Morphology of *Bifidobacterium* spp. at scanning electron microscope



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.