Carrying out Microbiological Analyses of the Fish, Seafood Products and Fish Habitat

1. The classification and relevant properties of microorganisms

Microbiology is a science that studies life forms that are invisible for unaided eye, that means organisms having dimensions ranging from few µm to few tens of µm. They can be further divided to prokaryotes or bacteria, and protists or eukaryotic microorganisms. Prokaryotes do not have an actual nucleus, but the DNA[1] is free in the cytoplasm as a structure called nucleoid, while protists have a similar cell structure to that of animals and plants. Their DNA is surrounded by a nuclear membrane, and they have a number of specialized structures or organelles in their cytoplasm to carry out some special tasks. Typical prokaryotic and eukaryotic cells are presented in Figure 1.

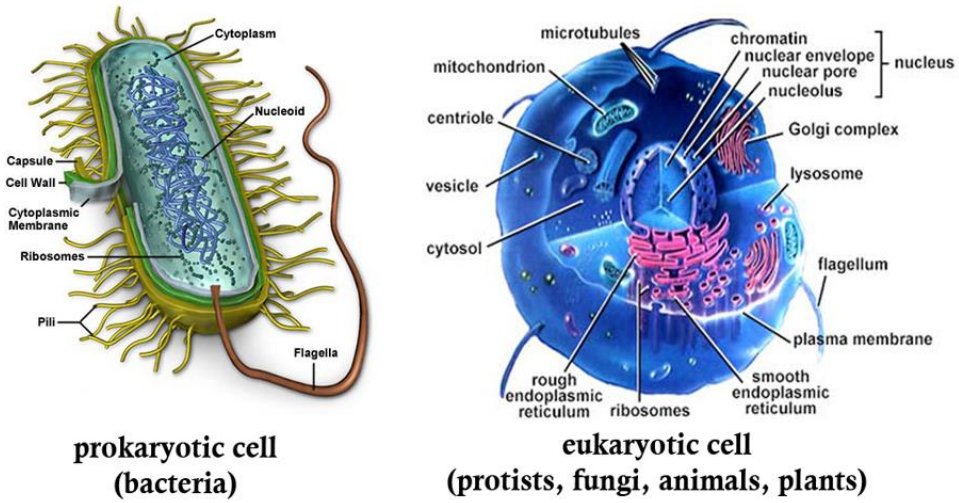


Figure 1. A typical bacterial cell (on the left) and an eukaryotic cell (right). Notice the characteristic nuclear region of the eukaryotic cell, and the lack of it in the bacterial cell. Characteristic bacterial structures include "hairs" (pili) protruding from the cell wall, and the presence (in many species) of one or several flagella, by which the bacteria swim around.

Viruses from the third group of microorganisms in addition to bacteria and protists. They only consist of a nucleocapsid that contains the genome of the virus (See Figure 2). Depending of the virus, either DNA or RNA can form the genome and carry the genetic information. Viruses do not have any metabolism of their own but they have to infect the host cell, take over its metabolism

[1] For the structure and functions of DNA and RNA, see the chapter on Immunological and molecular biological methods

and redirect it to synthetize new viral particles. Plants, animals and also bacteria have their specific viruses. Due to their life cycle, viruses are associated with various human, animal and plant diseases. The study of viruses generally requires specific research facilities and cannot be done in a standard microbiological laboratory, with the exception of bacterial viruses or bacteriophages. he life cycle of a bacteriophage is shown in Figure 2.

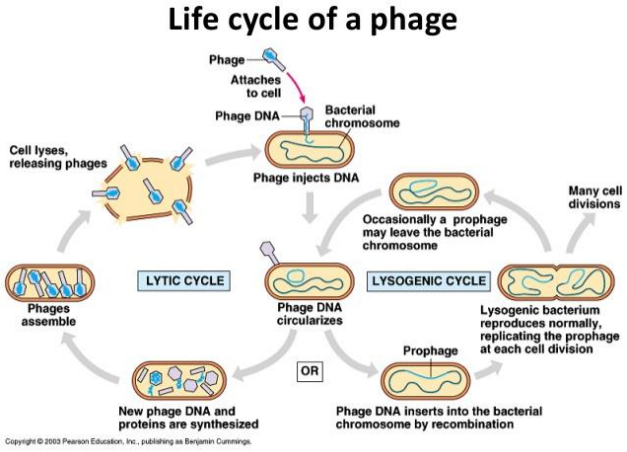


Figure 2. An example of the life cycle of a bacterial virus (Phage). The nucleocapsid of the phage attaches to the surface of the bacterium, the nucleic acid of the phage is injected into the cell, and takes over the cell metabolism. In the lytic cycle the cell is programmed to program to produce new phages, which are released resulting in the disintegration of the cell (lytic cycle). Sometimes the phage DNA may become integrated into the bacterial DNA (lysogenic cycle) and in this case the phage remains "silent" and does not cause the death of the cell, until some events activates the phage again. These silent prophages are present in many bacteria.

1.1. Bacteria

Typical bacteria have sizes of 1 – 5 µm, and the cell shapes can be either round (coccoid bacteria) or rod-like. There is a wide variety of special bacterial groups, but the most common ones that are relevant to food microbiology are Proteobacteria, Firmicutes and Actinobacteria. These groups are metabolically very diverse, but Proteobacteria can be distinguished from Firmicutes and Actinobacteria on the basis of the cell wall structure causing them to react differently in the so called Gram-staining. Proteobacteria are Gram- negative, while both Firmicutes and Actinobacteria are Gram-positive.

The Gram-negative bacteria have a multilayered cell wall consisting of an outer membrane, a murein layer in the so called periplasmic space and the cell membrane. Murein (or peptidoglycan) is a polymer resembling chemically chitin, and is typical to bacterial cell walls. In Gram-positive

bacteria the murein layer is much thicker and situated immediately on the top of the cell membrane, and there is no outer membrane. The structures of Gram-positive and Gram-negative cell walls are shown in Figure 3.

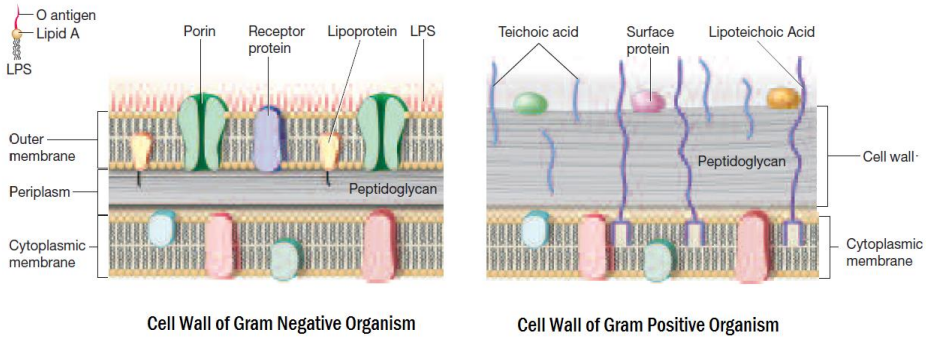


Figure 3. The cell wall structures of Gram negative (left) and Gram positive (right) bacterial cell wall. Notice the thick peptidoglycan (murein) layer characteristic to Gram positive microorganisms.

Firmicutes and Actinobacteria differ from each other by the composition of their DNA. The DNA bases adenine, thymine, guanosine and thymine are the essential building blocks of DNA (see Chapter "Conducting tests on fish and seafood products by means of enzyme-immunoassay and molecular-biological methods"). The guanosine and cytocine contents of the DNA in Actinobacteria are higher than in Firmicutes.

Some bacteria, such as members of *Bacillus* and *Clostridium* genera, can form endospores or very heat resistant survival forms (see Section 2.4.2.)

1.1.1. The bacterial growth conditions

The external factors that limit bacterial growth include temperature, pH and water activity (a_w). Most bacteria are mesophilic, meaning that they prefer temperatures between 25 – 40 °C. Some species tolerate temperatures of 50° C or more and are called thermophiles. Psychrophilic bacteria favor low temperatures (2 – 20°C). Many bacteria associated with water environment and fish are psychrophilic.

The optimal pH for growth for most bacteria is in the neutral range (6–7), but some bacteria grow at pH values 4 -5 or even lower. Likewise, there are species that can grow at pH > 8.

1.1.2. The bacterial metabolism

Bacteria utilize sugars, proteins and lipids as energy sources. This can be done by the respiratory process requiring oxygen, in which case we speak of aerobic bacteria. Many bacteria are able to utilize the energy sources anaerobically in a process called fermentation. When the end products of the respiratory metabolism are water and carbon dioxide, the fermentation end products are various organic acids and alcohols. If a bacterium is able to perform aerobic metabolism in the presence of oxygen and fermentation in anaerobic conditions, it is called a facultative anaerobe. If the organism is capable only for aerobic metabolism, it is called an obligate aerobe, while obligate anaerobes are only capable of fermentation and do not tolerate oxygen. Microaerophilic bacteria are fermentative organisms, which, however, tolerate and even require some oxygen. Generally, respiration is energetically a much more favorable metabolic pathway than fermentation, which means that bacteria relying mainly on fermentation live in nutritionally rich environments (foods, decaying plant and animal material, etc).

Bacteria also synthetize many different compounds, besides their own structural components. They produce pigments, volatile compounds, toxins, antibiotics and various enzymes. Therefore, they are much used as production organisms in biotechnology.

Some bacterial groups/species that are important in food microbiology are listed in Table 1

Table 1) The general characteristics of some important bacteria that may cause problems in food.

Bacterial group	Species	Gram-reaction	Endo-spores	Cell shape	Energy metabolism		Metabolic type
					Respiration	Fermentation	
Proteobacteria	*Escherichia coli*	-	-	rod	+	+	facultative anaerobe
	Salmonella enterica	-	-	rod	+	+	Facultative anaerobe
	Pseudomonas aeruginosa	-	-	rod	+	-	Obligate aerobe
	Campylobacter jejuni	-	-	rod	-	+	Microaerophilic[1]
Firmicutes	*Bacillus cereus*	+	+	rod	+	+	Facultative anaerobe

	Clostridium botulinum	+	+	rod	-	+	Obligate anaerobe[2]
	Enterococcus faecalis	+	-	coccus	-	+	Microaerophilic
	Listeria monocytogenes	+	-	rod	+	+	Facultative anaerobe
Actinobacteria	*Staphylococcus aureus*	+	-	coccus	+	+	Facultative anaerobe
	*Propionibacterium freudenreichii**	+	-	rod	±[3]	+	Facultative anaerobe

[1] Microaerophilics have fermentative metabolism but tolerate oxygen; [2]Obligate anaerobes do not tolerate oxygen; [3]± = Growth mainly fermentative although there is capability of respiratory metabolism

**Lactobacillus plantarum* and *Propionibacterium freudenreichii* are examples of bacteria that are not harmful but are actually useful in certain food processes.

1.1.3. Bacterial genetics

As in other organisms (with the exception of certain viruses) DNA is the carrier of genetic information from one generation to another. Bacteria do not have sexual reproduction, but the DNA simply replicates in a semiconservative fashion at every cell division providing each daughter cell with an identical copy. Despite of this, the bacteria can exchange genetic information. Certain viruses that attack bacteria can convey DNA from an infected bacterium to another in a process called transduction. Some bacteria are also capable of taking in their cells foreign DNA directly from their environment in a process called transformation. Maybe the most important gene exchange process, however, is conjugation, in which a recipient bacterium receives DNA from the donor bacterium by a direct cell to cell contact.

Plasmids, or small independently replicating DNA molecules outside the actual bacterial nucleoid, often are involved in conjugation (Figure 4). They often carry genes for properties like antibiotic resistance and are responsible for the rapid spread of the resistances in bacterial populations, if antibiotics are used indiscriminately. In some bacterial species plasmids also carry toxin genes and virulence factors that are essential for the bacterial pathogenicity.

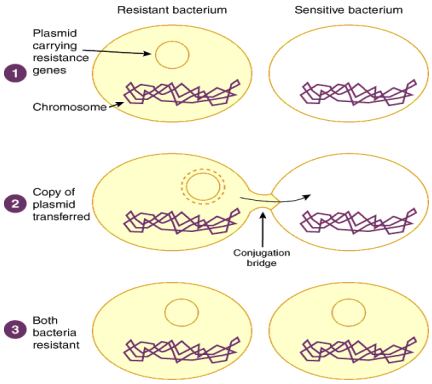


Figure 4. The process of bacterial conjugation. The donor bacterium (on the left) transfers its DNA to the recipient (on the right). The practical significance of conjugation to food and clinical microbiology is associated with the very rapid spread of antibiotic resistances in microbial populations, when resistant donor bacteria spread their resistance genes among the recipients.

1.1.4. Bacterial pathogenicity and food poisonings

By far the vast majority of bacterial species are harmless and have a fundamental role in the normal ecosystems of soil and waters. However, some bacteria cause diseases and infections in humans or animals. The bacterial pathogenicity or ability to cause a disease depends of many virulence factors, such as an ability to colonize the host, to secrete toxins, or to destroy tissues by proteolytic enzymes. Often the genes coding for these properties are grouped together as so called pathogenicity islands in the bacterial genome. The bacterial pathogenicity is often aggravated by antibiotic resistance, which can make the treatment of bacterial diseases difficult, and which is becoming more and more prevalent.

1.1.4.1. Food poisoning bacteria

Certain bacteria can cause food poisonings by contaminating food or water. They usually act either by producing toxic proteins (enterotoxins) causing diarrhea and nausea or other types of toxins, or by producing local or systemic infections.

Some important bacterial pathogens associated with food include:

- *Salmonella enterica* and its different serotypes causing different types of gastroenteritis and sometimes systemic disease (typhus)
- Enteropathogenic *Escherichia coli*, which can produce dysentery-type diseases
- *Campylobacter jejuni* causing gastroenteritis
- *Staphylococcus aureus* that produces enterotoxins causing diarrhea
- *Clostridium perfringens* causing diarrhea
- *Clostridiun botulinum* produces extremely potent neurotoxins causing death by paralysis of the respiratory system.
- *Bacillus cereus* producing diarrhea-associated enterotoxins and the emetic toxin causing vomiting

- *Listeria monocytogenes* causing gastroenteritis, but also meningitis, hepatitis, and damaging the unborn in pregnant women are infected.

Salmonella enterica and *Escherichia coli* belong both to the Enterobacteriacea family and have their habitat in the gastrointestinal tract of warm-blooded animals (it should be noted that the majority of *Escherichia coli* are harmless, natural inhabitants of the gut, and only the enteropathogenic bacteria cause problems). *Campylobacter jejuni* is an intestinal microorganism typical for poultry, but can contaminate water and seafood. *Staphylococcus aureus* is frequently found on skin and mucous membranes, approximately one third of people being symptomless carriers. Bacteria of genus *Clostridium* can be found in anaerobic environments like bottom sediments and intestinal tract. *Bacillus* and *Listeria* bacteria are typical soil organisms. As endospore producing bacteria both *Clostridium* and *Bacillus* species can survive the typical processing temperatures of foodstuffs.

1.1.4.2. Fish pathogens

Certain bacteria cause fish diseases. Common bacterial genera associated with fish diseases include *Pseudomonas, Aeromonas, Yersinia* and *Flavobacterium.* Their characteristics are listed in Table 2.

Table 2. Some important fish pathogens

Bacterial group	Species	Gram-reaction	Shape	Oxygen requirements	Growth temperature	Type of disease
Proteobacteria	*Pseudomonas anguilliseptica* and other psychrophilic *Pseudomonas species*	-	rod	Obligate aerobes	4 – 30 °C	General infections, fin rot
	Aeromonas hydrophila	-	rod	Facultative anaerobe	4 – 30 °C	Ulcers, septicemia, fin rot
	Yersinia ruckeri	-	rod	Facultatively anaerobe	20 – 30 °C	Red mouth disease
Bacteroidetes[a]	*Flavobacterium psychrophilum*	-	rod	Obligately aerobic	< 16 °C	Cold water disease of salmonids

	Falvobacterium columnare	-	rod	Obligately aerobe	20 – 25 °C	Cotton wool disease of salmonids

[a] Bacteroidetes is a bacterial group outside the common Proteobacteria, Firmicutes and Actinobacteria

1.2. Eukaryotic microorganisms or protists

Eukaryotic microorganisms include protozoa or unicellular non-photosynthetic organisms, unicellular photosynthetizing organism or microalgae, filamentous fungi and yeasts. Protozoa and algae have important ecological functions and form the planktonic life forms in aquatic ecosystems. However, filamentous fungi and yeasts are the most important protists in food microbiology.

1.2.1. Filamentous fungi

Filamentous fungi grow as long filaments called hyphae. As prokaryotes they can have different mating types and have a sexual life cycle or produce asexual spores or conidia. The life cycle of a typical filamentous fungus, *Neurospora crassa* is shown in Figure 5. The sexual life cycle involves fusion of two mating types (+) and (-), which leads to formation of eight spores in a structure called ascus. When the spores germinate, they can either start another sexual life cycle or start producing conidia that are located in structures called conidiophores.

The metabolism of filamentous fungi is mainly respiratory, which means that they typically grow on the surfaces. Thus, moldy bread, jam juice and fruit are typical examples of food spoilage caused by filamentous fungi. In addition to affecting the outlook, taste and odor of food, they can actually harm the health of consumer by producing toxic compounds, mycotoxins. Some of them are potent liver toxins and carcinogens (for example aflatoxins) while the others can cause kidney injuries, neurological damage or have hormonal effects. Typical food contaminants and mycotoxin producers include fungal genera *Aspergillus*, *Penicillium* and *Fusarium*. The typical growth of filamentous fungi on solid medium is shown in Figure 6.

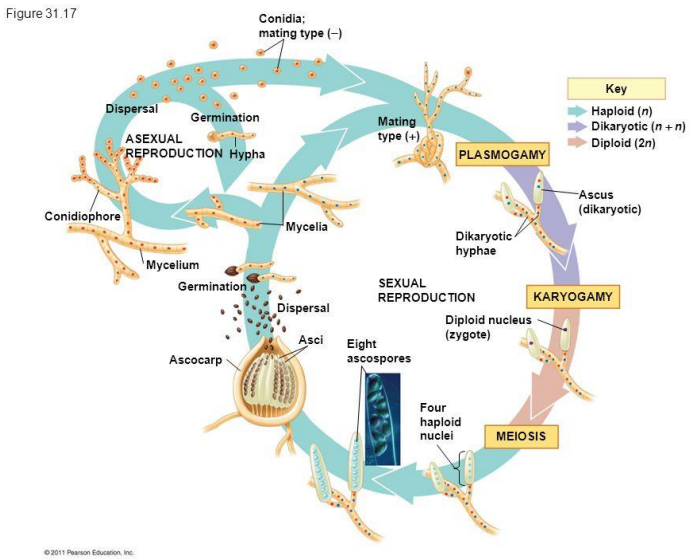


Figure 5. The life cycle of a filamentous fungus. Two fungal filaments, representing different mating types, can join together and start the sexual life cycle, including the fusion of nuclei (karyogamy) and subsequent formation of sexual spores (in this case called ascospores). Asexual spores (conidia) are formed in special structures called conidiophores.

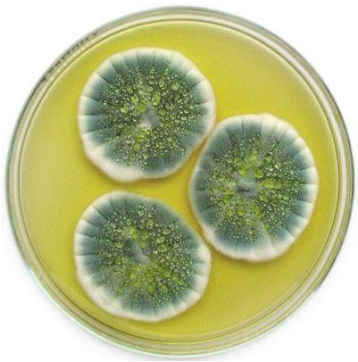


Figure 6. The typical growth of filamentous fungi on a solid medium.

1.2.2. Yeasts

Yeasts are fungi that typically do not produce mycelium but grow as single cells. A typical example is the common baker's yeast, *Saccharomyces cerevisiae* (Figure 7). Baker's yeast has a sexual life cycles (although certain other yeasts do not) producing sexual spores. The cell division occurs by budding, daughter cells appearing as small "buds" protruding from the parental cells.

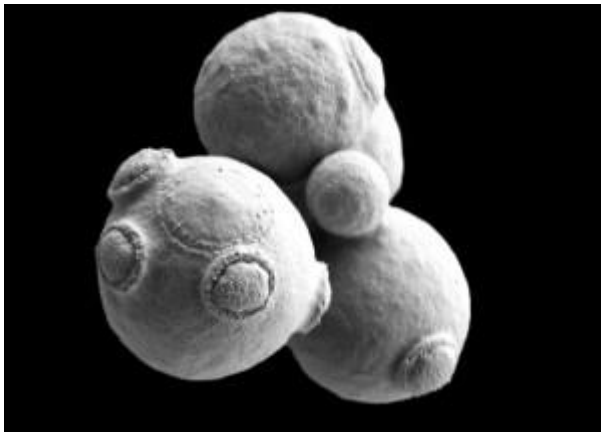


Figure 7. Baker's yeast (Saccharomyces cerevisiae). Notice the lack of filamentous growth and the emergens of "buds" (new yeast cells).

Yeasts can often grow both aerobically or anaerobically producing ethanol and carbon dioxide as fermentation end products. While yeasts are utilized in baking, wine making and beer brewing they can also occur as food contaminants causing spoilage by developing off-flavors and odors.

In contrast to filamentous fungi the yeast cultures cannot be distinguished from bacterial cultures by naked eye but a microscopic examination is needed.

2. Working with microorganisms

Cultivation of microorganisms is the fundamental aspect of microbiology. Particularly important is to prevent the contamination of the samples by microorganisms that are universally around. Therefore, the principles of aseptic techniques are fundamental for all microbiological work. In addition, one has to know the growth requirements of the microorganisms to be cultured, such as the oxygen requirements, optimal temperatures, suitable growth media etc. The basic principles of microbiological methods are outlined in the following sections

2.1. Microbiological laboratory

A microbiological laboratory should be easy to clean with suitable surfaces (ceramics, stainless steel). Separate storage rooms for chemicals and media, refrigerators and freezers belong to the basic infrastructure. Biological safety cabinets (laminar flow hoods) to ensure the aseptic working are required, as well as facilities to autoclave and sterilize media and equipment. Incubators with adjustable temperatures are also essential.

2.2. Aseptic working

The solutions, media, plastics and glassware that are used for microbiological work should be sterile. The most common method for sterilization is to apply either dry heat (suitable for glassware and metal instruments) or moist heat (suitable for liquids). Dry heat sterilization takes place in an oven at temperatures of either 160 °C (for 1.5 – 2 h) or 190 °C (for 12 minutes). Moist heat sterilization is usually done in an autoclave (pressure cooker) with pressurized steam at temperatures of 120 °C for 15 – 20 minutes.

Heat sterilization is not suitable for many biological materials (such as protein solutions) and thy can be sterilized by filtering through 0.2 µ filters that eliminate most microorganisms except viruses.

Laboratory plastics (Petri dishes, single use loops etc) are usually sterilized by γ-irradiation or by treatment by ethylene oxide gas, and are nowadays commercially available in sterile packages.

Laboratory surfaces can be sterilized using disinfectants like 70% alcohol or by UVC radiation (wavelength 290 – 100 nm). It should be noted that many plastics are unstable under UV light and direct exposure to UV is an occupational damage affecting the eyes.

The actual work should be done quickly, avoiding unnecessary opening of the bottles or petri dishes, but also carefully without introducing any contaminants during the procedures.

2.3. Microbial cultures and culture media

When microorganism are grown in laboratory we talk about microbial cultures. A culture is called pure, if it consists of only one species of microorganism. When several different species grow in the same culture, it is called mixed.

Microorganisms can be grown on different types of media. Media can be liquid or solid, and they may be chemically complex containing ingredients like yeast extract, beef extract, tomato juice etc, or they can be chemically defined containing only known ingredients, like some sugar together with necessary salts and trace elements.

Liquid cultures are used in order to cultivate bacteria in large quantities or when the aim is to isolate or enrich certain types of bacteria from a sample.

In liquid cultures the typical growth behavior of microorganisms can be easily observed. After a liquid culture has been inoculated, there typically are no cell divisions during the first few hours. This lag phase is followed by a period of very rapid growth, the so called exponential or

logarithmic phase. When the nutrients of the culture start to be exhausted, the cell divisions cease and the culture enters the stationary phase. Finally, the cells start to die. The typical bacterial growth curve is shown in Figure 8.

Typically, the physiology of the cultured microorganisms differs greatly according to the growth phase. This is an important factor, when microorganisms are used for biotechnological purposes. From the hygienic point of view, it is crucial to note the rapid, exponential growth of bacteria in suitable conditions. The numbers of pathogens in the food can multiply by many orders of magnitude within a few hours, if the food is kept in inappropriate conditions.

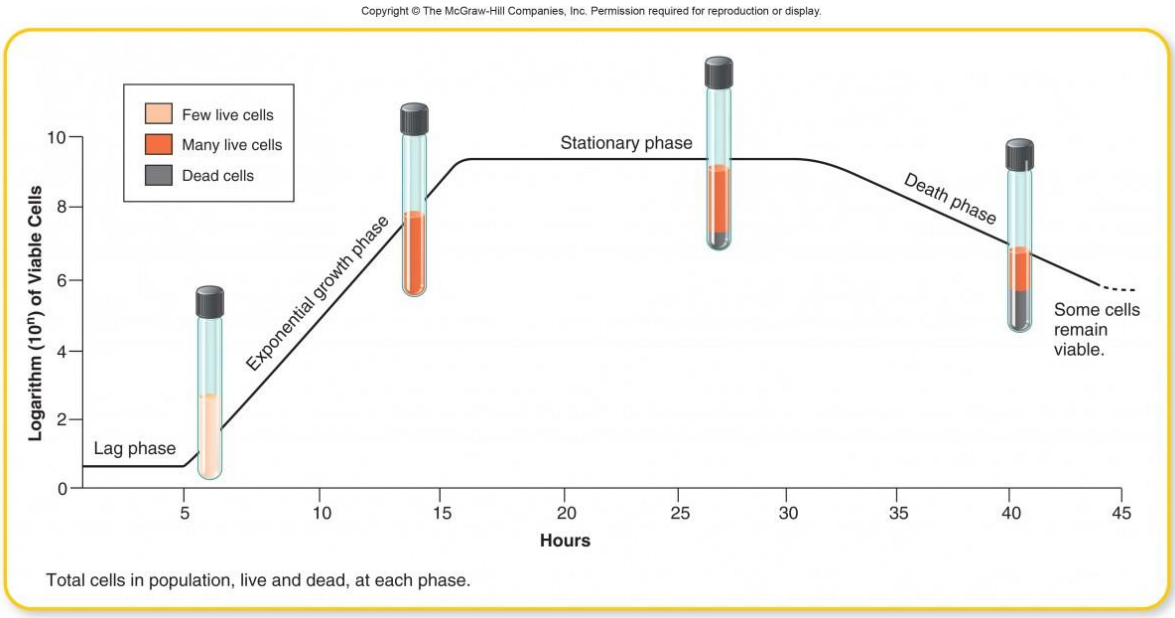


Figure 8. The growth phases of a typical bacterium in a broth culture. Notice the phase of rapid growth (the logarithmic or exponential growth)

Solid media usually contain up to 1.2 % agar (or agar agar) a polysaccharide that gives the medium the solid consistency. Molten medium is typically poured in Petri dishes and allowed to solidify before the inoculation of bacteria. For a typical plate culture a small amount of bacterial biomass or few µl of liquid culture is streaked or criss-crossed across surface of the agar resulting in single colonies after the bacterial growth (Figure 9). A colony is the macroscopic unit of bacterial growth, each colony containing appr. 10^6 bacterial cells, all descendants of a single bacterium that landed on the surface of the agar.

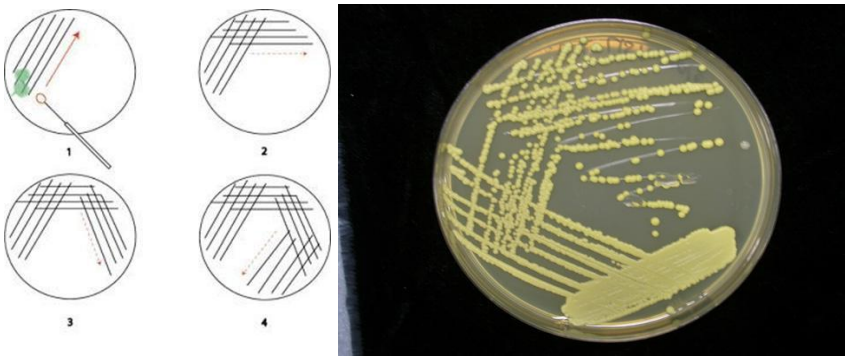


Figure 9. The streak plate of bacteria. The bacteria are streaked on the agar plate (left)in such a way that the bacterial densities decrease every time, when the direction of the streak is changed, until finally single colonies are achieved (photograph on the right)

2.3.1. Special types of cultures and culture media

In food and clinical microbiology it is often important to check, if the sample contain some type of a pathogen or not. The problem is that usually the amples contain a large number of bacteria, yeats and molds, most of them completely innocuous. Therefore different enrichment cultures are often applied. They contain components that are toxic or inhibitory for most microorganisms but tolerated by the bacterium or yeast/mold of interest. Thus mixing the food sample with this type of enrichment medium gives the pathogen or spoilage organism a chance to grow while the other microorganisms die. Some enrichment media for typical food pathogens are listed in Table 3, and practical examples of the microbial enrichment procedures are given in Section 4.1.

Table 3.) Some selection and entrichment media generally used in food microbiology

Medium	Selective agent(s)
Salmonella enrichment broths	Malachite Green
Listeria enrichment broths	Nalidixic acid, Cycloheximide, Acriflavine
Staphylococcus enrichment broths	Lithium chloride, Potassium tellurite
Several broths for *Escherichia coli* and related (coliform) bacteria	Bile salts, Novobiocin, Brilliant Green etc.
Campylobacter selective media	Cefoperazone, Vancomycin, Trimethoprim, Cycloheximide

Selective media for yeasts and filamentous fungi	Agars containing antibiotics like Chloramphenicol and Tetracycline

Enrichment media are different types of broths, while diagnostic media are usually solid. The bacterial colonies that grow on them have a characteristic appearance due to its physiology (utilization of certain carbohydrates, production of enzymes degrading lipids or proteins, tolerance to certain inhibitory agents, production of acid, etc), and this appearance can be used as a diagnostic criterion for the tentative identification of the bacterium.

A typical diagnostic medium is Baird Parker agar on which the lithium chloride resistant *Staphylococcus aureus* cells grow as colonies that are shiny black (due to the reduction of sodium tellurite present in the medium) and surrounded by a precipitation caused by the lecithinase enzyme and a halo due to the lipase produced by the bacterium (Figure 10).

Figure 10. The growth of the food poisoning bacterium Staphylococcus aureus on Baird Parker selective medium. The colonies are shiny black due the tellurite reduction and surrounded by a precipitation and a clear halo, the former caused by the lecithinase and the latter by lipase enzymes

Several other types of diagnostic media have been designed for the demonstration of the different species of Enterobacteriacea (the microbial family covering the coliform bacteria), pathogenic Bacilli etc. For example the Violet Red-Bile-Glucose (VRBG) agar is generally used for the detection of coliforms and related species, and the Mossel agar (containing the antibiotic polymyxin) is specific for the food poisoning organism *Bacillus cereus.*

2.4. Microscopy and stainings

Colonies are the macroscopic observable units in microbiology. In order to study the actual microorganisms themselves, one has to use microscope. The principle of a typical light microscope in shown in Figure 11.

In the standard light microscope the light is directed through the condenser lens to the specimen studied. From there the light passes to the objective lense reaching the projector lense, and finally the eye.

The final magnification depends of the type of objective used. Most microscopes are equipped with a revolving set of objectives (for example, 10 X, 40 X and 100 X), and one can choose the

required magnification according to the estimated size of the specimen. For most microorganisms the 100 X objective is required.

By certain modification of the basic illumination systen, the so called phase contrast microscopy, bacteria and other microorganisms can be directly observed. However, they normally are so transparent that different stainings should be used. The most common stainings in bacteriology are the Gram-staining and spore staining.

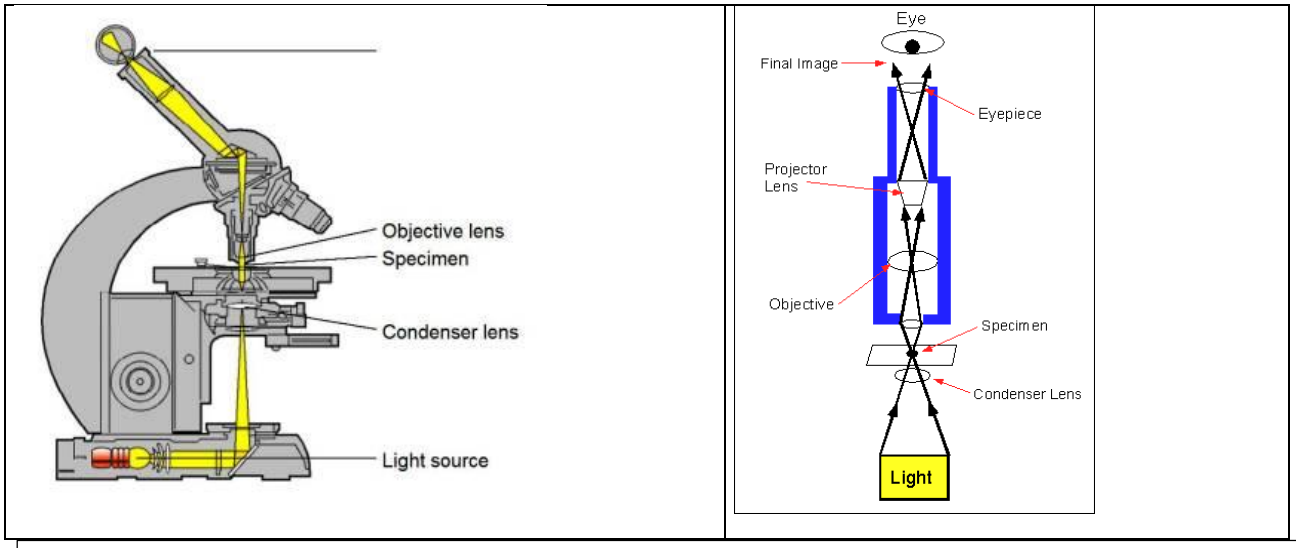


Figure 11. A typical light microscope and its optical system (Koehler illumination)

2.4.1. Gram staining

The division of the bacteria to Gram-positives and Gram-negatives has already been mentioned in Section 1.1 while ediscussing the structure of bacterial cell wall. The Gram-staining is thus an important taxonomic criterion. the outline of the staining procedure is given below (Figure 12):

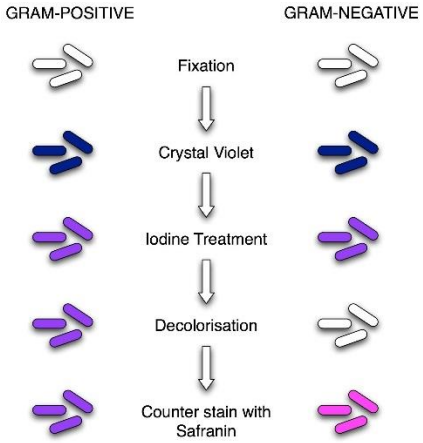


Figure 12. The Gram-staining procedure

For the staining some bacterial mass from a colony is spread thinly on an objective glass slide, the sample is fixed by heating the slide in a flame for a few seconds, and the slide is then stained in a crystal violet solution. The stained slides are treated with iodine and washed with alcohol. At this stage the Gram-negative bacteria loose their deep purple colour. Finally, the slide is counterstained with a red colour, safranin. In the microscopic examination typical gram-positives are dark blue while Gram-negatives are red (Figure 13)

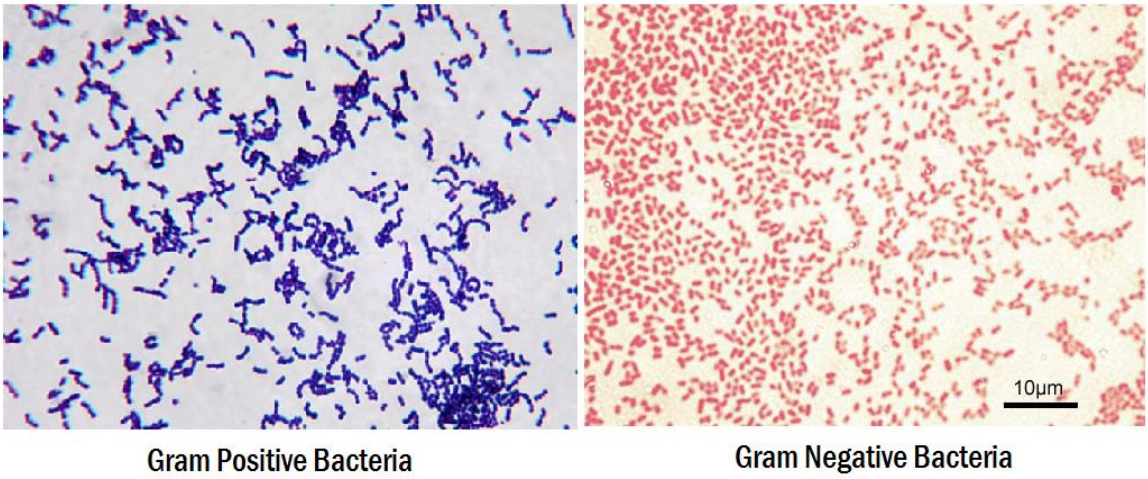


Figure 13. Typical Gram positive (left) and Gram negative (right) bacteria

2.4.2. Spore staining

The intracellular, heat resistant endospores of certain bacteria genera (*Bacillus, Clostridium*) can be stained with malachite green. For the staining the air dried objective slide with bacteria is flooded with malachite green solution, and gently heeated on the flame for up to five minutes (not allowing the malachite green to dry or to boil). The malachite green is washed away with water, and a counterstain is done with safranin. The spores can be seen as green against the red background of the cells (Figure 14)

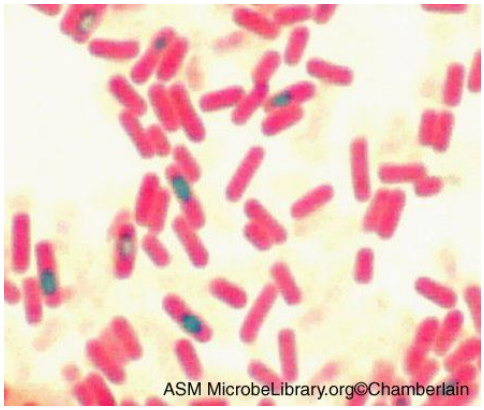


Figure 14. The outcome of a spore staining. The endospores have been stained by malachite green and can be seen inside the bacteria.

2.4.3. Other types of staining

For some special purposes fluorescent dyes are used to stain bacteria (and other microorganisms). These techniques require a special microscope providing fluorescence illumination that triggers the fluorescent signal from the stained cells. The special stains used can indicate, for example, whether the cells are live or dead (viability staining). When antibodies[2] generated towards a specicific bacterium are labelled with a fluorescent dye, and these fluorescent antibodies are used to stain the microbial sample containing multiple microbial species, the specific bacterium can be recognized by the fluorescent signal.

2.5. Estimating the mirobial counts in the sample, the serial dilution method and the most probable number method

Obtaining an estimate of the microbial numbers in the sample is one of the fundamental questions in microbiology, especially in clinical and food microbiology. If a phase contrast microscope is available, this can be done by direct micrsocopy using special counting chambers. In these chambers the microorganisms within a certain space, the volume of which is known, are counted, and the number in the original sample can then be calculated. One cannot, however, apply this methodology to solid samples, and defining the microbial species in the sample is difficult or impossible.

If fluorescent dyes are available they can also be utilized in the direct microscopy. There are also so called cell sorters or instruments that automatically calculate the numbers of fluorescent stained cells. They are, however, not suitable for routine estimation of microbial numbers.

The standard methodology, on which most microbiological guidelines related to food safety are based, is the serial dilution of the sample and the subsequent cultivation.

2.5.1. The serial dilution method

In the serial dilution method, 1 g of the sample (food, soil, water, animal feed etc) is mixed with 9 ml of suitable sterile diluent (0.9 % saline, peptone water etc). This is the 1/10 or 10^{-1} or simply -1 dilution. From this dilution I ml is transferred to another tube containing 9 ml of diluent, and the tube is mixed thoroughly. This is dilution 1/100, or 10^{-2}, or -2. This procedure is repeated untill suitable dilutions are obtained (for example, -3 and -4).

[2] Antibodies are proteins produced by the immune system after an exposure to, for example, an microorganism. These antibodies then specifically recognize and bind to the cells of that microbial species. See also Chapter XX (Enzyme linked immunoassays and molecular biological methods)

An aliquote of 0.1 ml from the tube having the dilution of -3 is then pipetted on a petri dish containing solid medium and spread evenly over the plate using the sterile levelling drag (L-rod). The plate is marked to contain the plate dilution of – 4. The same procedure is done with the tube containing the -4 dilution, and in this case the plate dilution is -5. The procedure is illustrated in Figure 15.

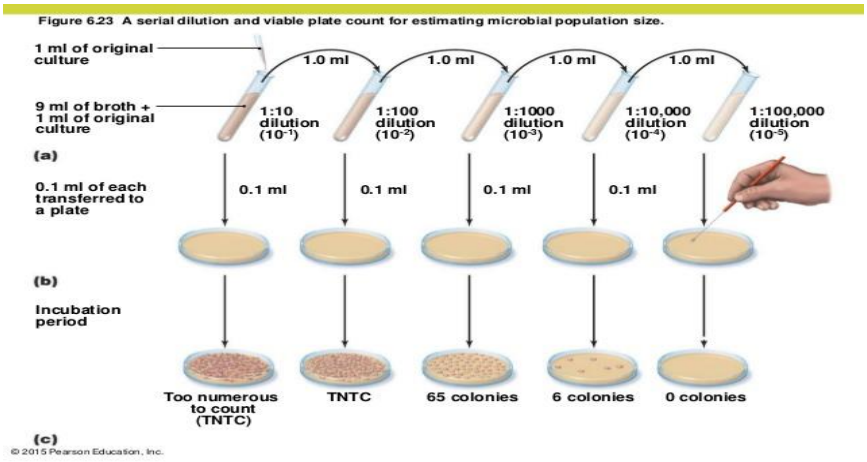


Figure 15. The serail dilution method for the evaluation of the number of bacteria in the sample.

The plates are then incubated and the colonies counted. The number of colony forming units (CFUs)[3] in the original sample can be calculated using the following formula:

$$\text{Number of micro-organisms} = \frac{\Sigma c}{(n_1 + 0.1 n_2)\, d}$$

Σc	=	The sum of colonies counted
n_1	=	The number of dishes retained in the 1st dilution
n_2	=	The number of dishes retained in the 2nd dilution
d	=	The dilution factor corresponding to the first dilution

Round the result calculated to two significant figures. Report the result as the total aerobic count g^{-1} test material.

By using the colony counts indcated in Figure , the number of micrrorganisms in the original sample (assuming that each microorganism gave raise to a colony) is thus :

(65 + 6)/ [(1 + 0.1) x 10^{-4}] or (71/1.1) x 10^4 = 64.5 x 10^4 or 6.5 x 10^5 CFU/ml. The dilution factor in this case was 10^{-4}. In practice, given the inaccuracies of the method, at least duplicate plates are made for each dilution and also used in the final calculations.

Sometimes the method outlined is modified by mixing one ml of sample with 9 ml of molten agar (kept at + 45 °C) and pouring this mixture to a Petri dish. After the agar has solidified the dish can be incubated and colonies within the agar counted. In this case the plate dilution is the same as the

[3] a CFU is often considered to be a single bacterium or spore, but in practice w edo not know, whether an aggregate of cells or a single cell has given raise to colony. Therefore, we speak of CFUs rather than of cells.

tube dilution. This modification may be useful, if the number of CFUs in the sample is expected to be low.

2.5.2. The most probable number (MPN) method

When the number of microorganisms in a sample is expected to be very low, the most probable number (MPN) method can be used. It is the method commonly applied to water quality analyses.

Also the MPN method is based on serial dilution. In a typical procedure a three step dilution series is made, and from each dilution three subcultures are made by transferring 1 ml to 9 ml of a growth medium. After an incubation, the number of tubes in which the bacterial growth has occurred (and which have turned turbid) are counted. If all tubes from all the dilutions are turbid, then the result is marked as 333, if only one replicate in each dilution is turbid, then this is marked 111, if the numbers of turbid tubes are 3, 2 and 1, then the result is 321 (Figure 16) etc. The most probable numbers associated with these different values are tabulated. For example, the value 321 would indicate that the most probable bacterial number in the original sample was 150/ml. MPN tables are included in the official hygienic standards, for which this method is recommended.

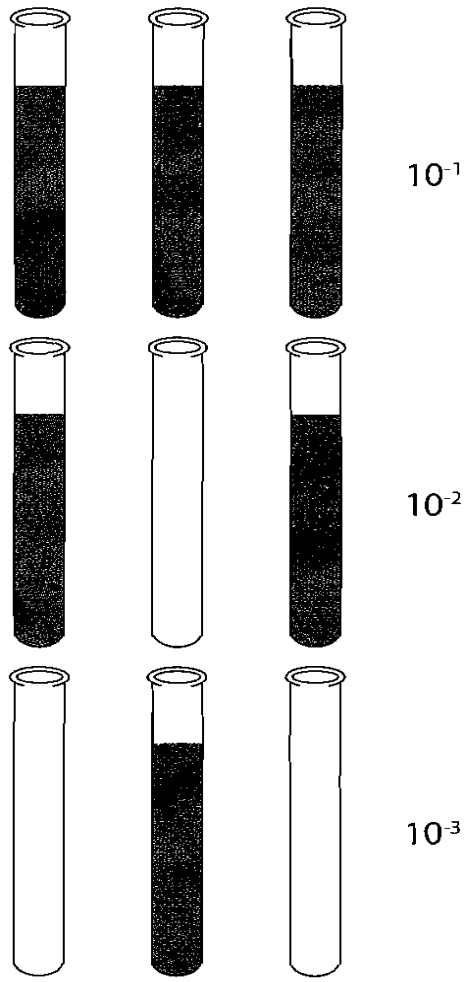


Figure 16. The illustration of the 321 outcome in the MPN determination. The dark tubes are the ones showing microbial growth. 10^{-1}, 10^{-2} and 10^{-3} refer to the dilution of the original sample.

3. Microbial identification

When a microorganism has been isolated and grown as a pure culture in the laboratorium, it should often be identified. Fungal and bacterial colonies are easy to distinguish from each other. From a bacterial isolate an experienced microbiologist can already give rather accurate indications of the identity by its growth conditions (aerobe/anaerobe, optimal growth temperature etc) the appearance of the colony and of the individual cells (rods or cocci, Gram-positive or -negative, spore former or not?). Particularly, if selective or indicator media have been used in the isolation, the identitiy of the bacterium can be concluded with a reasonable, although not absolute, certainty

For filamentous fungi, the exact identification is more complicated beyond the genus level, and an expert is often needed to do the species level identification.

Traditionally, in the final bacterial identification, several biochemical tests, based on the ability of the bacterium to utilize different sugars, have been used. These once laborious tests are now easy to perform routinely because of the specific kits that are commercially available. The API system provided by bio Mérieux, France, is a typical example. On the basis of the range of fermentable sugars and a few physiological tests a bacterium can be tentatively identified, although closely related species may still be difficult to differentiate. Similar systems are also available for yeasts.

Molecular biology and PCR techniques (see the Chapter "Conducting tests on fish and seafood products by means of enzyme-immunoassay and molecular-biological methods") are increasingly used to unequivocally identify microorganisms, mainly bacteria but also yeasts and fungi. For bacteria, the standard procedure is the PCR-multiplication of the variable and species specific region of the so called 16 S- rDNA, the sequence of which provides a species-specific fingerprint of the isolated bacterium.

4. Food hygiene and regulatory microbiology

As outlined in Section 1.1.4. several bacteria can cause food poisonings. Besides these consumer safety problems, the excessive growth of even innocent microorganisms can affect the quality of food by producing off-flavors and bad taste. For these reasons, there are several national and international regulations and guidelines to ensure the microbiological quality and safety of foods.

Although these regulations may vary to a certain degree within different regions there are certain common principles. Usually there is a zero tolerance for serious food pathogens like *Salmonella*.. For

other types of microorganisms, certain limits or breakpoints (defined as certain CFU limits/g) are defined.

An example of an official microbiological guidance is the following excerpt (Table 4) of the Regulation 2073/2005 of the European Union (EU) on the microbiological criteria for foodstuffs. The particular table relates to *Listeria monocytogenes* in different ready-to-eat foods (including fish and seafood). There is a maximum limit of 100 CFU/g. The number of samples that should be analyzed (n) is 5. The analyses should be carried out using a specific microbiological standard (ENISO 111290-2)[4].

Table 4) An example of the microbiological standards of the European Union applied to foods that support the growth of *Listeria monocytogenes* (other than those intended for infants and for special medical purposes)

Food category	Microorganisms/their toxins/metabolites	Sampling plan		Limits		Analytical reference method	Stage, when the criterion applies
		n	c	Vm	M		
Ready-to-eat food able to support the growth of *L. monocytogenes*, other than those intended for infants and for specific medical purposes	*Listeria monocytogenes*	5	0	100 cfu/g		ENISO 11290 -2	Products placed on the market during their shelf life

In the case of less serious pathogens the rules allow a bit more flexibility (Table 5). For example, for cheeses made of milk or whey that has undergone a heat treatment, the rules for *Escherichia coli* are the following:

Table 5) The microbiological criteria for certain cheeses in the European Union

		Sampling plan	Limits	Analytical reference method	Stage, when the criterion applies	Action in case of

[4] Standards are generally available but have a certain price and their reproduction is illegal

Food category	Microorganisms/their toxins/metabolites	n	c	m	M			unsatisfactory results
Cheese made from milk or whay that has undergone heat treatment	*E. coli*	5	2	100 cfu/g	1000 cfu/g	ISO 16649 -1 or 2	At the time of the manufacturing process when the *E. coli* count is expected to be highest	Improvement of production hygiene and selection of raw materials

In this case two limits are applied, 100 cfu/g and 1000 cfu/g. Five samples should still be analyzed, but now two of them (indicated in the "c" column) can exceed the lower breakpoint (m, 100 cfu/ml), but none the maximum limit (M) of 1000 cfu/g.

The reason for applying two limits for certain pathogens/spoilage organisms that are less dangerous than the most serious ones is the inherent inaccuracy of the methodology especially at the lower cfu values and the difficulty to completely exclude these relatively common microorganisms.

In addition to the official guidelines, many enterprises have their internal quality control criteria, which can be even more stringent than those required by the authorities.

Nowadays many food enterprises implement Hazard Analysis Critical Control Point (HACCP) system as a part of their quality control. In this system, which is compulsory in the EU, the microbiologically most vulnerable stages ("Critical Control Points") of the process are identified and specific control measures defined for them. The microbiological or other safety criteria that are then controlled may depend on the process, quality of raw materials etc., and likewise the critical breakpoints applied.

4.1. Practical examples of microbiological analysis of food

The most common microbiological analyses performed on food samples include the so called total aerobic plate count based on a serial dilution method and giving the total number of CFUs /gram food sample, when grown at 30 °C in aerobic conditions. This is often accompanied by the analysis of some specific bacterial group, such as Enterobacteriacea and coliforms plated on VRBG agar. However, in many cases one has to ensure the absence of a food pathogen by performing an enrichment, and the following two examples represent these types of studies illustrating the general principles

4.1.1. Enrichment of *Salmonella* of various foods of animal origin

Salmonella enterica is a serious food pathogen that can cause severe illness in addition to typical gastroenteritis. Therefore, there is a zero tolerance for its occurrence in the food. There are several protocols and standards to demonstrate the presence or absence of *Salmonella*, but generally the first step is a non-selectivce enrichment, meaning that the few *Salmonella* cells that form the hygienic risk have a chance to grow. Usually the sample (25 g) is mixed with 225 ml of Buffered Peptone Water (BPW, a medium allowing the growth of a wide range of bacteria) and then incubated at 37 °C overnight.

Of this non-selective pre-enrichment culture one ml is transferred to 10 ml of a selective medium (for example Sodium Tetrathionate broth), or alternatively, 0.1 ml to 10 ml of another selective broth, the Rappoport-Vassiliadis Soy-Peptone broth (RVS-broth). The former medium contains tetrathionate and the latter malachite green as the special selective agent. The incubation is continued either at 37 °C (tetrathionate broth) or at 41 °C (RVS-broth) overnight.

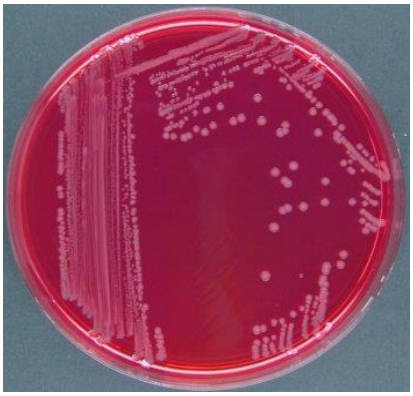
From these selective enrichment cultures loopfuls (10 µl) are spread on selective agars , either on Xylose Lysine Desoxycholate (XLD) agar or Brilliant Green (BGA) agar. The plates are incubated at 37 °C overnight.

The typical *Salmonella* colonies on these two agars are shown in Figure 17

A.

Figure 17: Colonies of *Salmonella* on A) XLD agar (Black colonies) and on B) BGA plates (Colorless colonies).

B



Although the isolation procedure described above give a reasonable certainty whether or not the sample contains *Salmonella*, a serological confirmation and further typing using antisera produced against the different variants of *Salmonella* is usually performed. Often also Salmonella-specific PCR is applied after the enrichment step instead or in addition to palting on agar.

4.1.2. Enrichment of *Listeria monocytogenes*

Listeria monocytogenes is a serious food pathogen that can cause food poisonings, systemic infections, meningitis and damage an unborn child. Therefore it is monitored in several food stuffs and in most cases (though not in all, see Table a zero tolerance (like in the case of *Salmonella*) is required. There are several enrichment methods, and the following description is an overview of the ISO 11290 method.

With *Listeria monocytogenes* there usually is no non-selective pre-enrichment step, but a 25 g sample is directly mixed with a half-strength selection medium (Fraser medium) containing acriflavine and nalidixic acid as selective agent and incubated for 24 hours at 30 °C. Subsequently 1 ml of this broth is transferred to full strength Fraser broth. In addition to the selective agents, the Fraser broth contains esculine which is hydrolyzed by *Listeria monocytogenes*, the reaction giving the broth a dark colour. After a further 24 hour incubation the enrichment broth is plated on a selective Oxford and PALCAM agars, both containing acriflavine and a combination of other selective agents favoring *Listeria monocytogenes.*

Typical *Listeria monocytogenes* colonies on PALCAM agar are shown in Figure 18

Figure 18. Listeria monocytogenes colonies on PALCAM agar.

5. Concluding remarks

Microbiological examination of foods, fish and seafood included, is of fundamental importance in securing safe and nutritious food. The methods reviewed in this Chapter give an overview of the current practices in food microbiology laboratories. Increasingly, these basic techniques are complemented with molecular biological methods, for example, using PCR for the identification purposes, or instead of plate cultivation after an enrichment step.

For many microbiological procedures there are specific official standards with detailed instructions how to perform the analysis. Both the technical and regulatory aspects of food microbiology evolve rapidly, and in the future one can expect a lot more instrumentation and automatization in the control laboratories. However, the fundamental aspects of food microbiology: microbial enumeration, their identification and the detection of pathogens and spoilage organisms will still form the basis of everyday work.

Further reading

Adams, M., Moss, MO., McClure, P. Food Microbiology, Royal Society of Chemistry, 2015, 562 pp

Jay, JM., Loessner, MJ., Golden, DA. Modern Food Microbiology. Springer US, 2000, 790 pp (available also as an e-Book)