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«Οι σύγχρονες τεχνικές βιο-ανάλυσης στην υγεία, τη γεωργία, το περιβάλλον και τη διατροφή»

APPLIED FOOD MICROBIOLOGY

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CHAPTER 1. MICROBIAL CONTAMINATION OF RAW MATERIALS

1.1. SOURCES OF CONTAMINATION

Raw materials used in the food industry may be contaminated via soil, water, plants, equipment and utensils, humans, animals and air. In the upper layer (+30cm²) of fertile soil, 10^6-10^7 bacteria are present. They mineralise organic material, which makes it possible for plants to absorb it. Water is also microbially contaminated. Potable water contains 10^2 bacteria/ml; waste water on the other hand contains 10^8 bacteria/ml.

Plants themselves are microbially contaminated and this is determined by the soil they are cultivated in and the water they come in contact with. By treatment of the soil with liquid manure, plants may be contaminated with faecal micro-organisms, including pathogens.

Insufficiently cleaned and disinfected equipment and utensils are culture mediums for microorganisms. In addition to this they are important sources of cross-contamination. The qualitative and quantitative aspect of those sources of contamination is determined by the type of food and use of those equipment and utensils.

Human beings may be a source of contamination because of lack of personal hygiene. Via skin, hairs and respiratory tract of people employed in the food industry, numerous micro-organisms find their way into food products.

Animals are a source of contamination via intestines, skin, feathers, hooves and droppings, which are extremely microbially contaminated.

Finally, the microbial contamination of air will also influence the microbial quality of food.

1.2. CONTAMINATION OF THE DIFFERENT TYPES OF FOOD

1.2.1. MEAT

1.2.1.1. Fresh meat

A. ***Quantitative contamination***

a. **Slaughter**

As a rule, carcass meat is sterile immediately after slaughter. If the animal has suffered from "stress" before slaughter, the meat may be contaminated. The number of bacteria may then amount to 10^3/g, depending on the type of animal. In

exceptional cases, bacteria may end up in the mussels by way of the intestines, and cause "bone taint" (discolouration of the meat near the bone). The meat may also be contaminated via the blood stream, if contaminated knives were used during slaughter. If GMP is applied during slaughter, there is 1 bacterium/10-100 g of meat present.

b. **Dressing**

During dressing a contamination takes place via skin or hide, knives, hands, clothing, water and equipment. If GMP is applied, the total number of bacteria and the total number of Enterobacteriaceae is as follows:

- beef:	total count	:	10^3-10^5/cm^2
	Enterobacteriaceae	:	10-10^2/cm^2
- mutton:	total count	:	10^3-10^6/cm^2
	Enterobacteriaceae	:	10^3/cm^2
- pork:	total count	:	10^3-10^6/cm^2

the total number of ***Enterobacteriaceae*** is high because the skin is not removed.

c. Chilling

Quick chilling at low temperatures with high air speeds will reduce the total viable count (TVC) on the surface of the carcass. Under less rigorous conditions, the TVC rises, but the number of mesophiles will increase faster than the number of psychrophiles, and since pathogens have a mesophilic character, the risk of food poisoning will increase. GMP for chilling means that fast chilling to below 3°C is required.

d. Cutting

Cutting and boning enlarges the relative surface and this causes an increase in the TVC/g, depending on the initial contamination of the carcass, the conditions of hygiene and the temperature/time in which those actions occur. Good results are achieved at 10°C and if efficiently cleaned and disinfected equipment is used.

B. ***Qualitative contamination***

a. Saprophytes

Gram-negative rods: ***Acinetobacter-Moraxella, Aeromonas, Alcaligenes, Flavobacterium, Pseudomonas, Enterobacteriaceae;***

Gram-positive rods: ***Corynebacterium,*** **lactic acid bacteria,** ***Microbacterium thermosphactum, Bacillus, Brochothrix;***

Gram-positive cocci: ***micrococci, staphylococci,*** **faecal** ***streptococci;***

Yeasts and moulds

b. Pathogens

Salmonella, Escherichia coli, Campylobacter jejuni-coli, Yersinia enterocolitica, Listeria monocytogenes, Staphylococcus aureus, Clostridium perfringens, Clostridium botulinum.

Remark: organ meat has a higher degree of contamination than meat, because organs have a physiological cleaning function (filter for micro-organisms) and because the pH of organ meat is higher than the pH of meat.

1.2.1.2. Meat products

In meat products two groups must be distinguished prepared meats and meat preparations. Prepared meat is cured meat whose anatomic structure is preserved and consequently, it has a low degree of contamination. A meat preparation on the other hand is cured meat whose anatomic structure is altered, in other words, comminuted meat.

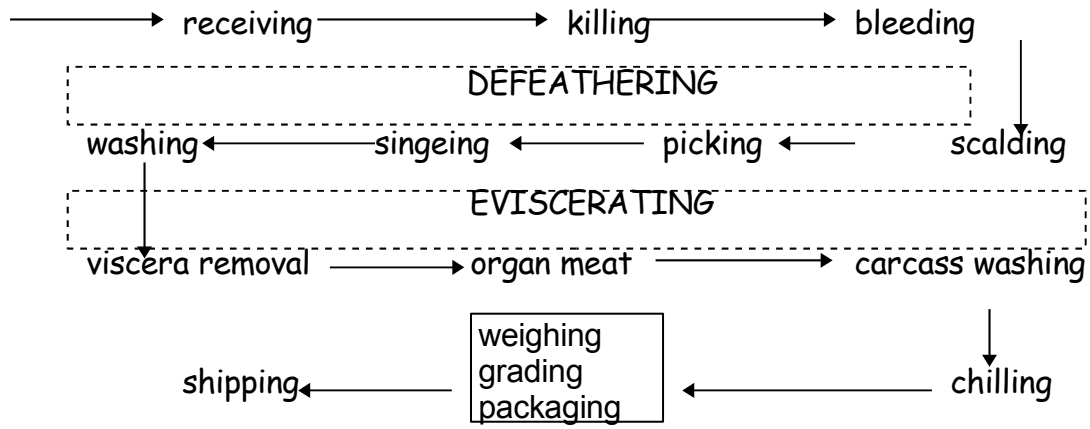
The TVC of contaminated meat is high for the following reasons:

- the meat usually originates from various carcass parts that have been treated more frequently, and that are therefore more microbially contaminated;
- by comminuting the meat, its relative surface has increased and thus also the number of bacteria/g;
- because of contaminated equipment;
- sometimes a highly contaminated part may be processed;
- because of the high microbial charge of some ingredients (herbs, spices, milk products);
- the casing (for sausages).

The types of micro-organisms that are found in meat products depend on the type (= technology). These aspects will be discussed in this course later on.

1.2.2. POULTRY

The killing process of poultry is as follows:



A. ***Quantitative contamination***

a. Arrival

At the arrival of poultry, in the slaughterhouse cages, feathers and legs are highly contaminated with: ***Acinetobacter-Moraxella, Pseudomonas, Corynebacterium, Micrococcus, Staphylococcus, Flavobacterium*, and yeasts**. Very specific is the faecal contamination that occurs due to the contraction of the cloaca as a result of the electrical shock. During this operation, an amount of faeces is excreted, and it ends up on the carcass.

b. Scalding

Scalding is an immersion of poultry in water at 50°C during a few minutes. This is necessary for the removal of feathers. The water in the scald tanks contains 50.000 bacteria/ml. The total count in the scald tank rises during the slaughter process, and reaches a constant value later on. Because of this heat treatment, the number of psychrotrophic bacteria on the carcass drops.

3. Defeathering

Defeathering causes a rise in the total count. Especially in the follicles there is a build-up of micro-organisms. Nowadays, defeathering is done by machines (rubber fingers). By this, the number of mesophiles increases from 10^4 tot $10^5/cm^2$. The number of psychrophiles remains the same. This stage in the slaughter process is also critical for the cross-contamination with *Salmonella*.

d. Evisceration

If this stage in the slaughter process occurs efficiently, it cannot influence the microbial contamination quantitatively. Qualitatively however, a contamination with ***Enterobacteriaceae*** may occur. Moreover, there is a possibility of cross-contamination with various micro-organisms via humans and equipment.

e. Washing

Washing of carcasses usually happens by means of spraying with water, which may contain 40-60 ppm chlorine to disinfect it. Water contaminated with ***Pseudomonas*** may be harmful for the microbial quality of poultry. Spray washing with water removes 50 to 90 % of the bacteria mechanically.

f. Cooling

Cooling of poultry carcasses may occur in different ways:

static tank chilling (1/3 ice, 1/3 water, 1/3 carcasses). Duration: 4 to 24 hrs. This causes a rise in the number of psychrophiles, but there is cross-contamination with ***Salmonella***;

-continuous immersion chilling: in pre-chilled water, with or against the direction, during less than 1 hr. at 4°C. The total count remains the same, but there is cross-contamination with ***Salmonella***;

-spray chilling with pre-chilled water: causes a mechanical removal of the bacteria. This method however is not fit for commercial use.

-air chilling. The advantage of this method is that the skin is slightly dehydrated. The total count remains the same;

-carbon dioxide cooling: the total count remains the same or drops slightly, but this is an expensive method.

g. Weighing and packaging

The increase in the total count is determined by GMP. An important problem is the cross-contamination with ***Salmonella***.

B. ***Qualitative contamination***

At the end of the slaughter process, the microbial contamination of poultry is qualitative and quantitative as follows:

-the meat is sterile;

-on the skin the total count is 10^4-10^8/cm², and it consists of the following types : ***Pseudomonas***, ***Acinetobacter-Moraxella***, ***corynebacteria***, ***micrococci*** and ***Enterobacteriaceae*** (10^2-10^3/cm²); and for the pathogens: ***Salmonella***, ***Campylobacter***, ***Yersinia enterocolitica***, ***Listeria monocytogenes***, ***Staphylococcus aureus***, ***Clostridium perfringens*** and ***Escherichia coli***.

Remark

Edible viscera (hearts, livers and gizzards) contain larger numbers of bacteria than the carcasses and moreover, there is a greater possibility of contamination with ***Salmonella*** and ***Listeria monocytogenes***.

The total count of industrial poultry is also higher than that of carcasses. Cross-contamination with ***Salmonella*** and ***Listeria monocytogenes*** occurs frequently.

Necks and backs are deboned mechanically. The resulting paste has a large number of bacteria (10^5-10^6/g), of which 10^2-10^3 ***Enterobacteriaceae***. The following pathogens are also found: ***Staphylococcus aureus, Clostridium perfringens, enterococci, Salmonella, Yersinia enterocolitica, Campylobacter*** and ***Listeria monocytogenes.***

1.2.3. FISH, SHELLFISH AND MOLLUSCS

A. ***Quantitative contamination***

a. Freshly captured fish

The meat of freshly caught fish is sterile. Skin, viscera and gills are contaminated and the degree of contamination depends on their environment (table 1).

TABLE 1. Quantitative contamination of fish.

Environment	skin	gills	viscera
pure cold water	10^2/g	10^3/g	10^3/g
polluted tropical and subtropical water	10^7/g	10^9/g	10^9/g

The additional contamination occurring on board and on land is determined by the GMP. Contamination mainly takes place via hold, tools, ice and human beings. The treatments on board, during which contamination can appear are: eviscerating, rinsing (with seawater) and storage in ice. Contamination on land can occur as result of the following operations: unloading, sorting, filleting, gutting, portioning, packing and transporting.

b. Shellfish

Shellfish originating from cold water contains 10^5 bacteria/g and Shellfish originating from warm water contains 10^5 to 10^6 bacteria/g.

c. Molluscs

In molluscs such as oysters and mussels there are 10^4 to 10^6 bacteria/g present.

B. ***Qualitative contamination***

a. Fresh fish

Fresh fish originating from cold water is mainly contaminated with psychrophilic Gram-negative bacteria such as: ***Pseudomonas***, ***Shewanella***, ***Acinetobacter-Moraxella***, ***Flavobacterium*** and ***Alcaligenes***. Fish originating from warm water on the

other hand, is contaminated with Gram-negative bacteria such as: ***Corynebacterium***, ***Bacillus***, ***Micrococcus***. When stored in ice, over 90 % ***Pseudomonas*** spp. and ***Shewanella*** spp. are present.

Fresh fish, caught in polluted areas, or fish that was not in hygiene treated on board or on land, may be contaminated with pathogens: ***Salmonella***, enterococci, ***Staphylococcus aureus***, ***Clostridium botulinum type E***. In living fish, 2 pathogens may survive, namely ***Clostridium botulinum type E*** and ***Vibrio parahaemolyticus*** (in warm water).

b. Shellfish

Shellfish that is cultivated or captured in cold water is contaminated with ***Shewanella***, ***Pseudomonas***, ***Acinetobacter-Moraxella*** and ***Flavobacterium*** (Gram-negative psychrotrophes). Shellfish originating from warm water is contaminated with Gram-positive corynebacteria and micrococci. The pathogens that may occur in shellfish are the same ones that are found in fish.

c. Molluscs

In molluscs, little or no Gram-positive bacteria occur. The following Gram-negative bacteria appear: ***Vibrio***, ***Pseudomonas***, ***Acinetobacter-Moraxella***, ***Flavobacterium*** and ***Cytophaga***.

Molluscs sometimes are cultivated in estuaries, the water of which is highly contaminated with enteropathogens originating from human and animal faeces. As a result the molluscs will also be contaminated with: ***Salmonella***, ***Shigella***, ***Vibrio cholerae***, ***Escherichia coli***, ***Clostridium botulinum***, ***Clostridium perfringens***, ***Staphylococcus aureus*** and some viruses. Special attention should be paid to contamination of warm water molluscs with ***Vibrio parahaemolyticus***.

1.2.4. MILK

Fresh raw milk may be contaminated via the udder, the exterior surfaces of the animal, equipment and other sources. The contamination strongly differs, both quantitatively as qualitatively.

a. Udder

Milk rests that remain in the teat after milking may cause development of streptococci and especially micrococci in the teat. Via the teat they enter in the udder, and consequently a contamination of fresh raw milk with 10^2-10^3 bacteria/ml may be considered as normal. In cases of mastitis the milk may contain 10^6 bacteria/ml. The largest numbers are found in the early stage of the disease. If the animal suffers from chronic mastitis, fluctuations occur. Other diseases that occur by deceased dairy cows may also shed specific micro-organisms in the milk.

The microflora in the milk of healthy dairy cows is composed as follows:

-small numbers of mastitis streptococci (***Streptococcus agalactiae***, ***Streptococcus disgalactiae***, ***Streptococcus uberis***);

-large numbers of micrococci: ***Micrococcus spp.*** (10^5/ml), mainly due to use of unclean milking machines, and ***Staphylococcus spp.***

b. The exterior surfaces of the animal

Materials such as soil, feed residues, bedding and manure stick on the animal. This causes contamination of the udder, and consequently, of the milk, with ***Bacillus*** (soil), clostridia (silage fed) and Enterobacteriaceae (mainly coliforms coming from bedding and manure). The contribution of these sources of contamination is 10^2 to 10^3/ml, depending on the method of cleaning and disinfecting the udder, prior to milking.

c. Equipment

Milking machines, cooling tanks and tank trucks, used to transport milk, may be contaminated due to insufficient cleaning and disinfection. The following types find via this source their way into raw milk : streptococci from the lactis-group (***S. lactis***, ***S. cremoris***, ***S. lactis*** var. diacetylactis), coliforms and Gram-negative psychrophiles (***Pseudomonas***, ***Alcaligenes***, ***Flavobacterium***, ***Chromobacterium***). More resistant and slower growing micrococci, enterococci and lactobacilli occur in milk stone (10^5/ml milk).

d. Other sources

During hand milking, a contamination through air with micrococci and spores of ***Bacillus*** and ***Clostridium*** takes place. These types survive heat processes and may cause problems for pasteurisation of milk.

Milk handling personnel may contaminate the milk via skin, nose and mouth, with micrococci, staphylococci, and, because of lack of personal hygiene, with ***Salmonella*** and other bacteria that cause gastro-intestinal infections.

1.2.5 VEGETABLES

Vegetables are mainly contaminated via the soil in which they are cultivated and via water, air, insects and other vermin. The degree of contamination depends of course on the structure of the plant. Humans may raise the degree of contamination during harvest. Harvesting often injures the produce, with as results: (a) nutrients stimulating microbial growth are released and (b) an invasion of micro-organisms in the interior tissues takes place. During subsequent processing, in other words, washing of the vegetables, the total count will drop, but cutting may cause a new increase in the total count. Use of insecticides during cultivation of vegetables may decrease the total count.

Unlike meat, fish and poultry, the internal tissue of fresh and intact vegetables is contaminated with Gram-negative rods.

A. ***Quantitative contamination***

Vegetables are mainly contaminated with large numbers of bacteria (Table 2). Via soil and air, relatively large numbers of fungal-spores may occur (<10^3-10^5 spores/g).

TABLE 2. Quantitative bacterial contamination of some important vegetables.

Vegetable	Degree of contamination
carrots	10^5/g
beets	10^6/g
cabbage	10^3/g-10^6/g
beans	10^3/g-10^5/g
spinach	10^6/g-10^7/g
peas	10^5/g-10^7/g
snap beans	10^5/g-10^6/g
potatoes	10^5/g-10^7/g

B. ***Qualitative contamination***

a. Saprophytes

-Bacteria: ***Pseudomonas*** (typical bacteria that are present in air, water and soil), coliforms, corynebacteria, lactic acid bacteria, micrococci, spore-forming bacteria.

-Moulds: ***Aerobasidium***, ***Fusarium*** and ***Alternaria***.

b. Pathogens

These micro-organisms end up on the vegetables due to contact with human or animal faeces (e.g. liquid manure). The following types may thus be found on vegetables: ***Salmonella***, ***Shigella***, ***Vibrio cholerae***, ***Listeria monocytogenes***, ***Bacillus cereus***, hepatitis virus and amoebas.

Pseudomonas aeruginosa and ***Klebsiella***, both responsible for hospital infections may also be found in vegetables.

1.2.6. FRUITS

The sources of contamination of fruits are similar to those of vegetables. The largest contamination occurs during transport and ripening process. Injury due to picking can cause an invasion of micro-organisms in the slightly contaminated flesh. One of the most important sources of contamination is the water that is used for washing, cooling and conveying the fruit. Such water is often recycled and a build up of a number of bacteria may occur. Water will not only contaminate fruit surfaces, but may also bring micro-organisms into the interior of the fruit, where they are

protected from subsequent treatments.

A. ***Quantitative contamination***

There are few bacteria in and on fruit. Moulds are the most important micro-organisms in fruit (10^3-10^5/g).

B. ***Qualitative contamination***

a. Saprophytes

As stated previously, the primary spoilage organisms are moulds. The following types are important and play a role in fruit spoilage: ***Penicillium***, ***Alternaria***, ***Rhizopus***, ***Fusarium***, ***Mucor***, ***Phoma***, ***Botyris***.

b. Pathogens

Especially the pathogenic mould ***Penicillium expansum***, that grows on apples and produces the toxic patuline, is important. Other mycotoxin-producing moulds may also be found on fruit.

1.2.7. NUTS

Nuts have a natural protection against microbial contamination. The mesocarp covering the shell (e.g. coconuts and walnuts), a rigid shell (e.g. walnut), or a seamless shell (e.g. hazelnut) offer a very good protection.

1.2.7.1. Groundnuts

A frequently cultivated groundnut is the peanut. The saprophytic moulds ***Aspergillus***, ***Penicillium*** and ***Fusarium***, and also the aflatoxin producing ***Aspergillus flavus*** are typical contaminations.

1.2.7.2. Treenuts

Intact nuts on the tree are sterile. Damage to the shell due to mishandling, or to birds or insects, will predispose the treenuts to contamination. Nuts shaken from the tree, fall onto the soil, where they may be more or less contaminated, depending on soil moisture levels and length of time on the ground. In addition to this, faeces of domestic animals lying on the soil, may cause faecal contamination of the nuts.

a. Saprophytes

-Bacteria: ***Pseudomonas***, ***Acinetobacter-Moraxella***, ***Xanthomonas***, ***Enterobacteriaceae***, ***Clostridium***, ***Bacillus***, ***Corynebacterium***, ***Micrococcus***;

-Moulds: ***Penicillium***, ***Aspergillus***, ***Trichotecium***.

b. Pathogens

A frequently occurring pathogen is ***Salmonella***. Mycotoxin- producing moulds may

contaminate the nuts both on the tree as during harvest and storage. Important is here ***Aspergillus flavus***.

1.2.8. CEREALS

Cereals are mainly contaminated in the field via dust, water, insects, fertiliser, droppings of animals and diseased plants. Moreover, this contamination can rise as a result of microbial growth, depending on the moisture level and the ambient temperature. The result of all this, is that the grains are already contaminated prior to harvest.

A. ***Quantitative contamination***

The microbial contamination consists mainly of ***actinomyces*** (>10^6/g). Psychrotrophic bacteria (10^4-10^5/g) and aerobic spore-formers (10-10^5/g) are of course also present.

After harvest the grains are dried, either by air or mechanically. In both cases the TVC drops. Still, the best result is achieved if dried mechanically, since the air may also be a source of contamination.

During transport and storage, the TVC may increase as a result of contamination via dust. If the grains are dried during storage, the total count may decrease.

B. ***Qualitative contamination***

a. Bacteria

The following bacteria are found on grains of various cereals: ***Pseudomonas***, ***Micrococcus***, ***Bacillus***, ***Lactobacillus***, ***Enterobacter***, ***Clostridium*** and mainly actinomyces. However only a small number of faecal bacteria are found because there is only little contact with the soil.

b. Moulds

A number of types of moulds are typical contaminators of cereals, e.g. ***Alternaria***, ***Fusarium***, ***Rhizopus***, ***Penicillium***, ***Aspergillus***.

c. Yeasts

A lot of types of yeasts are present in cereals, but they are not important because of the low water activity (low moisture level) of the grains.

On cereals, little or no pathogenic micro-organisms occur, except for ***Aspergillus flavus***, which occurs after injuries due to larvae.

1.2.9. EGGS

The interior of freshly laid eggs is sterile, though bacteria can enter via the oviduct. Such entry is common with ducks.

The principal sources of contamination of the egg shell are intestines, nest box (faeces), dust, soil, feedstuffs, transport and human beings.

A. ***Quantitative contamination***

The egg shell contains 10^2-10^7 bacteria, depending on the effect of the previously discussed possible sources of contamination.

B. ***Qualitative contamination***

The most important group of micro-organisms on eggs are bacteria.

a) Gram-positive cocci: ***micrococci***, ***staphylococci***

b) Gram-negative rods: ***Arthrobacter***, ***Bacillus***

c) Gram-negative rods: ***Pseudomonas***, ***Acinetobacter-Moraxella***, ***Alcaligenes***, ***Escherichia***, ***Enterobacter***, ***Aeromonas***

CHAPTER 2. MICROBIAL GROWTH IN FOOD

2.1. INTRINSIC FACTORS

2.2.1. ACIDITY (pH)

Micro-organisms are able to grow in an environment with a specific pH. Their growth is characterised by a minimal, an optimal and a maximal pH-value. Those values are different for bacteria, yeasts and moulds. (table 3).

TABLE 3. Microbial growth as a function of pH.

micro-organisms	pH-value (min.)	pH-value (opt.)	pH-value (max.)
Bacteria	4.4	7.0	9.8
Yeasts	1.5	4.0-6.0	9.0
Moulds	1.5	7.0	11.0

However, there are exceptions. Acidophillic bacteria, such as lactic acid bacteria (min. pH = 3.3 and max. pH = 7.2) and acetic acid bacteria (min. pH = 2.8 and max. pH = 4.3) are often found in acid food. In addition to this, there are also basophilic bacteria, which grow at low pH-values, such as ***Vibrio parahaemolyticus*** (min. pH = 4.8 and max. pH = 11.0) and ***Enterococcus*** (min. pH = 4.8 and max. pH = 10.6).

The optimal pH for growth is not always the optimal pH for other cell activities, such as toxin production. Another concept is **"biological pH"**. This is the pH found in food as a result of fermentation (see further). Table 4 shows a summary of the most important types of food and their pH-values.

TABLE 4. pH-values of some food products.

food	pH-value
Meat*	5.6-6.2
Fish*	5.2-6.8
Shellfish*	6.8-7.0
Poultry*	5.9-6.3 (turkey) 6.2-6.4 (chicken)
Eggs	6.8 (yolk)-9.3 (white)
Milk	6.3-6.5
Cheese	4.9-7.4
Vegetables	4.2 (tomato)- 6.5 (beans)
Fruit	1.8 (lemon)-6.7 (melon)
Cereal products	5.3-8.0

* The pH of this group of food products depends on the "***rigor mortis***" period.

During this period, the glycogen, that is present in the muscle, is converted into lactic acid. This process is called glycolysis. The more glycogen the muscle contains at the moment of death, the more lactic acid can be build up, and the lower the pH will be. This can be achieved by slaughtering or killing animals, which have done little or no work and did not suffer from stress prior to their death. The conversion of glycogen into lactic acid takes place in meat, shellfish and poultry. In molluscs on the other hand, a weak lactate dehydrogenasis may be seen in the muscle, and this causes the conversion of lactic acid into pyruvic acid and succinic acid (Krebs cycle).

2.1.2 WATER ACTIVITY (a_w)

The water activity is a measure for the amount of water micro-organisms dispose of. This is expressed by the following formula:

$$a_w = P/P_0$$

P= water vapour pressure of the solution, P_0 = water vapour pressure of pure water

The growth of the various groups of micro-organisms is limited because of min. a_w-values (table 5).

TABLE 5. Min. $a_w$values for microbial growth.

Microorganisms	Min. a_w values
Bacteria Gram -	0.95
Gram +	0.91
Yeasts	0.88
Moulds	0.80

Exceptions are: halophilic bacteria (min. a_w = 0.75), xerophilic moulds (min. a_w = 0.60) and osmophilic yeasts (min. a_w = 0.60).

The a_w-value of raw unprocessed food is higher than 0.98, except for cereals and nuts, whose a_w is situated between 0.60 and 0.70, depending on the degree of dehydration.

2.1.3 REDOX POTENTIAL (Eh)

Microbial growth in food depends on the way the growth medium will act as electron donor or acceptor. The reduced and oxidised substances play of course an important role. Their ratio is expressed in mV. The more oxidised substances, the higher the Eh; the more reduced substances, the lower the Eh.

Micro-organisms that grow at high Eh are called aerobes and those that grow at low Eh-values are anaerobes. Micro-organisms that grow both at high and low Eh are facultative anaerobes. Micro-organisms growing at low Eh-values, such as lactic acid bacteria, are micro-aerophilic.

Fresh plant and animal tissues are characterised by a low internal Eh and a high Eh on the surface. Diffusion of oxygen causes a rise in Eh. The Eh in the interior of fresh meat amounts to -200 mV. Cominuted meat on the other hand has an Eh of +200 mV due to the impact of and/or the increased contact with air as a result of grinding. The Eh of fresh meat "***pre-rigor***" is + 250mV; this value decreases "***post-rigor***" to -130 mV and further to -205 mV because of microbial growth.

2.1.4. THE COMPOSITION OF THE FOOD PRODUCT

In addition to water, micro-organisms need an energy source, a source of nitrogen, minerals and vitamins in order to grow. The type and the availability of those nutrients will thus determine the microbial growth.

a) Energy source

The most obvious sources of energy are the carbohydrates (mono-, di-, tri-, and polysaccharides), organic acids, amino acids and alcohols. Amino acids, di-, tri-, and polypeptides (proteins) can also be used as energy sources. Some micro-organisms are even able to use lipids as energy source.

b) Nitrogen source

Micro-organisms need an N-source for their synthesis. The following food substances can be applied for this: amino acids, peptides, proteins, nucleotides, urea and ammonia.

c) Minerals

Minerals are indispensable for various cell functions. Minerals occur as salts. The qualitative and quantitative composition depends on the type of food.

d) Vitamins

Certain micro-organisms are unable to produce certain indispensable vitamins (auxotrophic). This means that their growth depends on the presence of one or several vitamins in the food. For example it is known that Gram-positive bacteria need more vitamin B than Gram-negative bacteria.

2.1.5. PRESENCE OF NATURAL ANTIMICROBIAL SUBSTANCES

Some food products contain by nature antimicrobial substances. For instance, fresh milk contains lactenin and an anticoliform factor. Cranberries contain benzoic acid. Spices contain some essential oils with antimicrobial effect (eugenol in clove). Cinnamon contains cinnamon aldehyde.

Another important natural antimicrobial substance is lysozyme in protein. The effect of lysozyme and of other natural antimicrobial systems such as lactoperoxydase and the glucose oxidase system will be discussed in detail later on.

2.1.6. BIOLOGICAL STRUCTURES

Certain food structures offer protection against microbial invasion, e.g. the skin of fish, and carcasses, the testa of seeds, the peel of fruit and vegetables, and the shell of eggs and nuts.

2.2. EXTRINSIC FACTORS

Extrinsic or external factors are the properties of the environment in which the food product is stored, i.e. temperature, relative humidity and the presence of some gases.

2.2.1. TEMPERATURE

Microbial growth is possible between temperatures from - 18 °C to 70 °C. Micro-organisms can be divided into three groups, depending on the temperatures at which they are able to multiply (table 6).

TABLE 6. Micro-organisms and temperature ranges of growth.

Group	T° Min.	T° Opt.	T° Max.
Psychrophiles	-18	10	20
Mesophiles	5	30-37	50
Thermophiles	37	55	70

Micro-organisms that are able to grow at low temperatures but that do not necessarily need those low temperatures for their growth are called psychrotrophes e.g. some mesophiles.

2.2.2 RELATIVE HUMIDITY

There is a relation between the a_w of a food product and the R.H. of the environment in which it is stored. This connection is expressed as follows:

$$a_w \times 100 = \% \text{ R.H.}$$

If this condition is fulfilled, there will be a balance between the food product and its environment, in other words, the food product will neither take up liquid nor dehydrate.

2.2.3. PRESENCE OF GASES

The replacement of air by one or more gases influences microbial growth. This will be discussed in detail later on.

2.3. IMPLICIT FACTORS

Implicit factors are the results of mutual interactions in mixed microbial populations. As a result of the microbial ecology in foods, competition, growth stimulation and growth associations may occur.

2.3.1. COMPETITION

a) Nutrient depletion

Micro-organisms of high metabolic activity consume certain nutrients very quickly. This leads either to inhibition or to stimulation of other micro-organisms. Examples are the depletion in O_2 due to aerobe micro-organisms, and the accumulation of CO_2 resulting in an accelerated growth of anaerobes and facultative anaerobes and an inhibition of aerobes. (e.g. ***Staphylococcus aureus*** in meat).

b) Change of intrinsic factors

A change of specific internal factors may stimulate or inhibit microbial growth. Addition of 3.5 % NaCl causes a decrease in a_w and as a result, the saprophytes are inhibited whilst the growth rate of ***Staphylococcus aureus*** increases.

c) Production of antimicrobial substances

Lactic acid bacteria and acetic acid bacteria produce H^+ ions and organic acids. Because of this, the pH increases and some micro-organisms are inhibited. Moreover, some organic acids have an antimicrobial activity.

-CO_2 depletes O_2 and lowers the pH through formation of carbonic acid in the liquid phase of the food, resulting in an inhibiting effect.

-Peroxides, formed from lipids and carbohydrates, by some bacteria, they show an antimicrobial activity.

-Some micro-organisms are capable of producing antibiotics and/or bacteriocins in the food product itself ; this causes an inhibition of growth of other micro-organisms (e.g. nisin produced by ***Streptococcus lactis*** which is active against Gram-positive bacteria).

2.3.2. GROWTH STIMULATION

a) Metabolites

Yeasts produce high amounts of vitamin B and this stimulates the growth of lactic acid bacteria. Another example is the hydrolysis of starch by moulds, providing mono- and disaccharides. This causes an accelerated growth of yeast.

b) Changes in pH

Lactic acid fermentation lowers the pH of a food product. This causes surface growth of moulds, which will raise the pH again. Subsequently, this stimulates the growth of spoilage organisms.

c) Change in Eh or a_w

Examples are:

Eh; the growth of ***Clostridium perfringens*** in meat lowers the Eh hence a stimulation of the growth of ***Clostridium botulinum***.

a_w; due to their lipolytic activity, xerophilic moulds can liberate H_2O, causing a rise in a_w. This results in growth of less xerotolerant bacteria.

d) Hydrolysis

Hydrolysis of polymers in plant and animal tissues facilitates the invasion of tissues by spoilage organisms.

e) Inhibitors

Microbially formed inhibitors can be broken down again by other micro-organisms, and as a result they lose their inhibitory or lethal effect. For instance, H_2O_2 that is broken down by catalase-positive bacteria ($2\ H_2O_2$ catalase $2\ H_2O + O_2$).

f) Symbiosis

There are symbiotic associations between two species of bacteria where growth is not possible unless both species are present. This is the case with yoghurt: ***Lactobacillus bulgaricus*** in yoghurt produces valine, histidine and glycine, which are indispensable for the growth of ***Streptococci thermophilus***. The latter produces formate which is an important growth factor for ***Lactobacillus bulgaricus***.

2.3.3. SUCCESSIONS - ASSOCIATIONS

In meat, poultry and fish, the initial flora consists of ***Pseudomonas*** and ***Acinetobacter-Moraxella***. During spoilage, ***Pseudomonas*** will dominate as a result of mutual interactions. Numerous of other successions and associations may occur in food. This is determined by internal and external flora and by the ecology of the food product.

CHAPTER 3. MICROBIAL ASPECTS OF PRESERVATION

3.1. REDUCED pH

3.3.1. MECHANISM

The pH of a food product can be lowered by adding strong acids. As a result, the pH rises and consequently the pH of the cytoplasm drops. Addition of weak lipophylic acids causes leakage across the cell membrane. This way H^+ ions can penetrate the cell easily, and because of this the pH of the cytoplasm decreases. Moreover, those weak acids can diffuse under undissociated form (acid) in the negatively charged cell. Subsequently, those molecules are dissociated and the anions then react with essential cell substances, with inhibition as a result. Some acid sensitive ions such as SO_3^- and NO_2^- show an inhibitory effect at reduced pH.

3.1.2. EFFECT ON SPOILAGE MICRO-ORGANISMS

In general inorganic acids show a lower inhibitory effect than organic acids. The inhibitory effect depends on type of acid and concentration.

a) Non-spore-forming bacteria

Gram-negative bacteria such as ***Pseudomonas*** and ***Acinetobacter-Moraxella*** are inhibited at pH < 5.3.

Gram-positive bacteria on the other hand are only inhibited at pH <4.

b) Spore-forming bacteria

Reduced pH causes an inhibition of germination and development. Most spores are acid sensitive, except for ***Clostridium pasteurianum*** (butyric acid producer), that germinates at pH > 3.5 and results in growth and spoilage.

c) Yeasts and moulds

Yeasts and moulds are acid tolerant and grow at pH < 4.0 (table 3). In acid foods, yeasts will outgrow the lactic acid bacteria, depending on the type of acid. The growth ability of yeasts depends on the energy-requiring systems that prevent acidification of the cell interior. For instance, acid that penetrates the ***Saccharomyces baillii***, will be pumped out with an inducible transporting system produced in the cell. Since the pump requires energy in the form of glucose, the organism will only be acid resistant if there is enough glucose present. Yeasts are extremely acid tolerant and they can only grow in aerobic conditions.

d) Pathogens

Gram-negative pathogens such as ***Salmonella spp.*** may be controlled by pH below

4.0., but even lower pH-values or combinations of low pH and other factors, such as low temperature, are required to control coliforms.

Staphylococcus aureus is inhibited by pH < 4.0 whereas spore-forming ***Clostridium botulinum*** and ***Bacillus cereus*** are inhibited respectively at pH < 4.7 and pH < 5.0. However, the min. pH for growth is lower than the min. pH for toxin production. In practice it is advisable to determine the min. pH by experiment for each food product. This value can differ considerably depending on the other internal and external factors.

3.1.3. USE OF pH TO CONTROL MICRO-ORGANISMS IN FOOD

A. ***Organic acids***

Organic acids can be added directly in the form of food acidulants, whether or not combined with chemical preservatives. This can also be done indirectly by fermentation (lactic acid fermentation).

a) Food acidulants

Food acidulants are usually applied in relative large concentrations (> 0.5). The ones that are most frequently used are: acetic acid, citric acid, lactic acid, malic acid, tartaric acid, gluconic acid in the form of glucono-delta-lactone (GDL). This mainly results in a reduced pH, and occasionally an antimicrobial activity of the molecule may be observed.

b) Preservatives

Preservatives "***sensu stricto***" such as benzoic acid, sorbic acid and propionic acid are applied at low levels (+ 0.1 %). The effect is mainly based on the antimicrobial activity of the molecule, which has to be present in undissociated form. Lipophylic undissociated acids dissolve in the cell membrane and as a result the permeability alters. By this the substrate transport and oxidative phosphorylation are uncoupled from the electron transport system. This leads to an increase in pH of the cytoplasm with cell destruction as a result. Some undissociated acids are water-soluble and dissolve in the cytoplasm with again an increase in pH as a result.

c) Types

The most frequently applied types of chemical preservatives and their pK-values and solubility are given in table 7.

TABLE 7. Types of organic acids applied for chemical preservation in foods.

Acid	pK	Water-solubility
Acetic acid	4.75	very soluble
Benzoic acid	4.2	0.3 %

Acid	pK	Water-solubility
Citric acid	3.1	very soluble
Lactic acid	3.7	very soluble
Propionic acid	4.9	very soluble
Sorbic acid	4.8	0.2 %

d) Factors that influence the choice

Spectrum (table 8).

TABLE 8. Spectrum of the chemical preservatives (x).

Preservative	Bacteria	Yeasts	Moulds
Benzoic acid	++	+++	+++
Sorbic acid	+ (xx)	+++	+++
Propionic acid	+	++	++
Parabens	++	+++	+++
Acetic acid	++	+++	++
Lactic acid	++	++	++
Citric acid	++	++	++

(x) + little active ++ medium active +++ highly active
(xx) The weak antibacterial activity of sorbic acid is due to the fact that sorbic acid is inactive towards catalase-negative bacteria such as lactic acid bacteria and clostridia, which frequently appear in foods.

Substrate factors

- Acidity

The antimicrobial activity of chemical preservatives is influenced by the pH. Since the cell has a negative charge, only the undissociated molecules can diffuse in the cell and subsequently dissociate in the cell, and thus have an antimicrobial effect. By means of this formula:

pH = pK + log [salt]/[acid]

it can be determined that with a neutral or slightly acid pH, the degree of dissociation will be high, with a small uptake in the cell as a result. Table 9 shows the amount of undissociated acid at specific pH-values of the main preservatives.

TABLE 9. Percentage of undissociated acid at specific pH-values.

Preservative	pH 3	pH 4	pH 5	pH 6
Acetic acid (pK = 4.75)	98.5	84.5	34.9	5.1

Preservative	pH 3	pH 4	pH 5	pH 6
Benzoic acid (pK = 4.2)	93.5	59.3	12.8	1.44
Citric acid (pK = 3.2)	53.0	18.9	0.41	0.006
Lactic acid (pK = 3.7)	86.6	39.2	6.05	0.64
Propionic acid (pK = 4.9)	98.5	87.6	41.7	6.67
Sorbic acid (pK = 4.8)	97.4	82.0	30.0	4.1

On the basis of table 9 it can be deducted that preservatives have to be applied in foods with a low pH. In foods with a neutral or weak pH, which for sensory reasons cannot be lowered by adding organic acids, esters of para-hydroxybenzoic acid (parabens) can be applied. Those esters only dissociate in alcaline conditions whereas in neutral and slightly acid foods they occur in undissociated form.

There are different esters of para-hydroxy-benzoic acid, that are all inhibitors of moulds, yeasts and bacteria. The antimicrobial activity rises as the chain length of the ester molecule rises, but the solubility decreases. The addition of 0.1 % ethyl para-hydroxybenzoic acid appears to be sufficient to inhibit most of the micro-organisms.

- The distribution coefficient

The influence of the distribution coefficient is especially important in fatty foods. Since microbial spoilage always occurs in the liquid phase, each part of the preservative that ends up in the lipid phase has to be considered as a loss of antimicrobial activity. This means that for these foods we have to opt for preservatives with a low distribution coefficient. Important is that salt, sugar and other water-soluble substances raise the distribution coefficient, because by this the water-solubility of the preservative is reduced. On the other hand, at reduced pH, the distribution coefficient will decrease, since only undissociated acid molecules are soluble in the lipid phase and vice versa.

- Water activity

The addition of substances that reduce the water activity of food products causes an increase in the effect of preservatives.

B. ***Lactic acid fermentation***

Some food products if necessary, submitted to a lactic acid fermentation in order to reduce the pH in a microbial manner. Typical examples are milk (yoghurt), meat (dry sausage) and vegetables (sauerkraut).

a) Yoghurt

Skimmed milk or milk with a low fat level is, after adding possible stabilisers, pasteurised. Subsequently the milk is inoculated with a starter culture consisting of a

mixture of ***Streptococcus thermophilus*** and ***Lactobacillus bulgaricus*** which live in symbiosis (see 2.3.2.f.). These bacteria convert the lactose in the milk into lactic acid, which causes a decrease in pH to 4.5-4.8 which results in coagulation of proteins.

b) Dry sausage

Commercial starter cultures used for manufacturing dry sausages are usually a mixture of two or three micro-organisms, consisting of one or two strains of lactic acid bacteria (***Lactobacillus spp.***, ***Pediococcus spp.***) and one strain of micrococci (***Micrococcus spp.***, ***Staphylococcus spp.***) or a yeast (***Debaryomyces hansenü***). Some types of dry sausage are inoculated at the surface with moulds in order to obtain specific flavour.

- ***Lactic acid bacteria***

Lactobacillus spp.

Most of the lactobacilleae that are applied in commercial starter cultures for meat products are homofermenters; heterofermenters may be present in raw flesh as contaminators. The most frequently used lactobacilleae in starter cultures are ***Lactobacillus plantarum***, ***Lactobacillus sake*** and ***Lactobacillus curvatus***. The most important properties are summed up in Table 10.

Pediococci

Pediococci are all homofermenters. The most frequently used species are: ***Pediococcus pentosaceus***, ***Pediococcus acidilactici***. The specific properties are given in Table 11.

TABLE 10. Important properties of *lactobacilleae* used in starter cultures.

Characteristic	***Lactobacillus plantarum***	***Lactobacillus sake***	***Lactobacillus curvatus***
Form	rods	rods	rods
CO_2 from glucose	-	-	-
Fermentation of			
Glucose	+	+	+
Saccharose	+	+	seldom
Lactose	+	+	seldom
Maltose	+	-	usually
Gluconic acid	+	+	-
Starch	-	-	-
Mannitol	+	-	-

Characteristic	***Lactobacillus plantarum***	***Lactobacillus sake***	***Lactobacillus curvatus***
D-ribose	+	+	+
Lactic acid isomer	DL	DL	DL
Acetoin production	+	usually	seldom
Nitrate reduction(a)	+	-	-
Nitrite reduction(a)	slight or -	slight or -	-
H_2O production (b)	-	usually	usually
Breakdown of arginine	-	usually	usually
Growth 4°C	slight or -	+	+
Growth 10°C	+	+	+
Growth 8 % NaCl	+	+	+
Growth 10 % NaCl	-	slight or -	slight

(a) in media with sugar and high pH, (b) in media without haemine

- ***Micrococci***

Next to lactic acid bacteria, Micrococcaceae are applied in fermentation of dry sausage, because of their catalase- and nitrate-reducing activity. Both the genera ***Staphylococcus*** and ***Micrococcus*** are used. The mutual differences between 2 genera are very small (table 12). The only difference lies in the fact that micrococci are aerobic and ferment glucose oxidatively, whereas Staphylococci are facultative anaerobic and form acid by anaerobic fermentation of glucose. The predominant representatives that are used in starter cultures are: ***Staphylococcus carnosus***, ***Staphylococcus xylosus*** and ***Micrococcus varians***.

TABLE 11. Important properties of *pediococci* used in starter cultures.

Characteristic	***P. pentosaceus***	***P. acidilactici***
Form	cocci	cocci
CO_2 from glucose	-	-
Fermentation of		
Glucose	+	+
Saccharose	+	+
Lactose	+	-
Maltose	+	-
Gluconic acid	-	-
Starch	-	-
Mannitol	-	-
D-Ribose	+	+

Characteristic	***P. pentosaceus***	***P. acidilactici***
Lactic acid isomer	DL	DL
Acetoin production	+	+
Nitrate reduction (a)	-	-
Nitrite reduction (a)	-	-
H_2O production (b)	-	-
Breakdown of arginine	+	+
Growth 4°C	-	-
Growth 10°C	+	-
Growth 8 % NaCl	slight or -	+
Growth 10 % NaCl	-	+

(a) in media with sugar and high pH, (b) in media without heamine

- ***Yeasts***

In certain specific cases, yeasts are applied. In starter cultures ***Debaryomyces hansenii*** is often added because of its high salt tolerance. Because they require oxygen in order to grow, yeasts only develop at the surface of the product.

- ***Moulds***

For the development of characteristic odours and flavours, yeasts can be inoculated on the surface of dry sausage. The strains that are available on the market are ***Penicillium nalgiovense*** and ***Penicillium chrysogenum***.

TABLE 12. Important properties of *Micrococcaceae* used in dry sausage

Characteristic	***S. carnosus***	***S. xylosus***	***M. varians***
Anaerobic growth with glucose	weak to +	weak	weak
Aerobic acid production from			
Glucose	+	+	+
Saccharose	-	+	-
Lactose	usually	+	weak
Mannitol	+	+	-
Maltose	-	+	-
Nitrate reduction	+	+	+
Acetoin production	+	+	-
Gelatine splitting	-	weak	-
Growth at 15°C	weak to +	+	+
Growth at 15 % NaCl	+	+	+

FUNCTIONS

- ***Lactic acid bacteria***

Lactic acid bacteria are mainly applied in fermentation of dry sausage, with different purposes.

Lactic acid production

Lactic acid bacteria in starter cultures have to be homofermenters, in other words, in accordance with the Embden-Meyerhof-Parnas pathway, glucose is converted into pyruvate, which again is converted into lactic acid and NAD by lactic acid hydrogenase and $NADH_2$.

Important is the rate of acid production and the value to which the pH can drop. The rate of acid production of a specific lactic acid bacterium is higher as a) there are more lactic acid bacteria initially present, b) the temperature is higher and c) the lactic acid bacteria can ferment the C-source faster. Monosaccharides are broken down more rapidly, whereas polysaccharides have to be split up in their monomers first, which takes some time.

An extra fermentable C-source has to be added to meat, which mostly contains low levels of carbohydrates due to the "post-mortem" phenomenon, by which glycogen is converted into glucose (glycolysis), and the latter is again broken down into lactic acid.

Lactic acid production is an important process in dry sausage fermentation. It causes a decrease in pH and as a result a) the undesired bacteria are inhibited in their growth; b) the iso-electrical point of the meat proteins is achieved and the proteins thus become less soluble and denaturate (coagulation or gel formation) - this results in the drying out of the sausage (reduced a_w); c) a solid product is obtained and d) nitrite is converted into NO, which contributes to colour formation and -stability.

Other antagonistic effects

Other ways in which lactic acid bacteria have an inhibitory effect on the present bacteria are :

a) lactic acid has in its undissociated form a certain bacteriostatic effect;

b) acetic acid produced in small quantities has also an inhibitory effect;

c) lactic acid bacteria are demanding regarding their nutrients, and because of this a lack of nutrients for other micro-organisms may arise;

d) lactic acid bacteria produce hydrogen peroxide and this causes premature rancidity and colour deviations;

e) some lactic acid bacteria produce bacteriocins.

- ***Micrococci***

Micrococci are applied both for fermentation of dry sausages as for cured meat products because they fulfil of a number of specific functions.

Colour formation

During the production of raw meat products, nitrite and/or nitrate are added to obtain an extended storage life but also for colour formation.

Myoglobin is the most frequently found pigment in the muscles and is mainly responsible for the desired or undesired colour of meat. Myoglobin is a haemprotein that consists of a protein (globin) attached to a haematin nucleus with a central Fe^{2+} atom. The reactions of those haempigments determine the colour of fresh meat and meat products.

The cause of colour formation in meat products is the reaction of nitric oxide with myoglobin which forms nitroso- metamyoglobin, that is further reduced (in the presence of ascorbic acid and/or other reducing compounds) to nitrosomyoglobin. The latter provides a pink colour and protects the Fe^{2+} better from oxidation. Subsequently heat and/or an acid environment are required for the formation of the more stable nitrosohaemochrome.

Hydrogen peroxide produced by lactic acid bacteria has a negative effect on the colour formation. Hydrogen peroxide reacts with nitroso- myoglobin and forms cholemyoglobin, which shows a grey-green colour. This proves also the value of catalase positive bacteria (***micrococci*** and ***lactobacilleae*** are haem-depending).

Nitrate is converted into nitrite by microbial reductases. This reduction occurs during the first 24 to 28 hrs. of fermentation, depending on chosen starter cultures, rate of pH reduction and initial nitrate concentration. Minimum pH for nitrate reduction is 5.2. Nitrate reduction in dry sausage is not optimal at low pH, since microbial reduction is an NADH-depending process. Synthesis of NADH is inhibited at low pH. This results in an insufficient production of nitrate if the pH rapidly decreases to below 5.4. The most favourable pH is 6.4.

Breakdown of nitrite strongly depends on the pH. The lower the pH, the stronger the breakdown. To stimulate the reduction of nitrite to nitric oxide, ascorbic acid is used. In addition to this the present oxygen will partly reconvert the nitric oxide to nitrate. It has to be said however, that if during the preparation of dry sausage, nitrite is added to the cutter, the oxymyoglobin formed due to the impact of air, is very rapidly oxidised to metamyoglobin, and by this a partial oxidation of nitrite to nitrate occurs.

An extremely important reaction is the oxidation of myoglobin to metamyoglobin. During this process Fe^{2+} is oxidised to Fe^{3+}, and the latter causes a brown colour. A low pH reduces the stability of the myoglobin compounds and the autooxidation. In the presence of nitrate and/or nitrite, a number of different processes may occur, and they may lead to the discoloration of raw meat products (figure 1).

Antioxidant

Since micrococci protect the product from harmful influences of O_2, they raise the chemical stability, in other words, through catalase (= antioxidant) they break down the rapidly oxidising peroxides, and this way they slow down the rancidity process. The nitrite, formed out of nitrate by Micrococci also has an inhibitory effect on the breakdown of lipids. This effect is based on complexing prooxidative substances, like iron. The required amount of nitrite is still unknown.

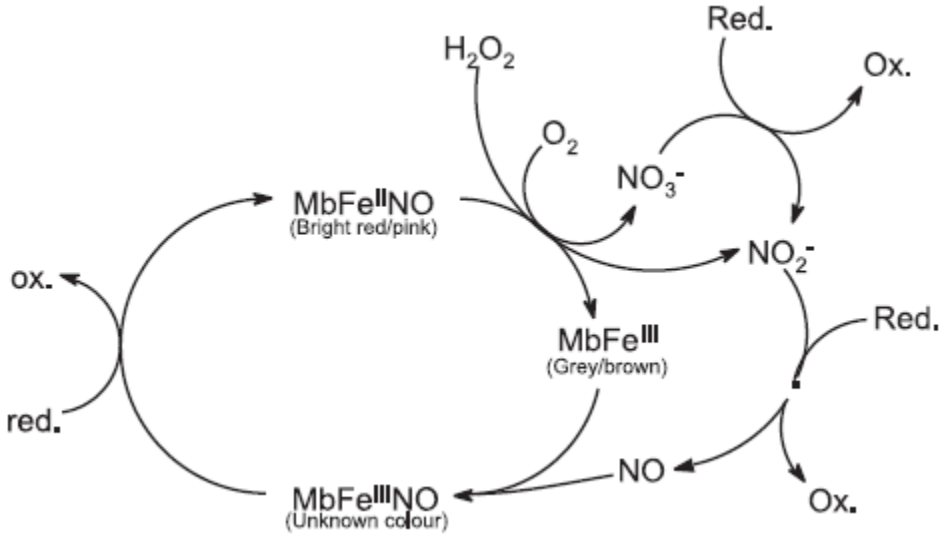


FIGURE 1. Colour cycle of myoglobin derivatives in cured meat during storage. The attractive pink nitrosylmyoglobin ($MbFe^{II}NO$) can be oxidised both by H_2O_2 or by molecular oxygen (autoxidation) to yield grey/brown metmyoglobin ($MbFe^{III}$) and nitrite or nitrate, respectively. In meat added reducing agents the pools of nitrate/nitrite can be reduced back to NO, which binds to metmyoglobin yielding the complex nitrosylmetmyoglobin ($MbFe^{III}NO$). This complex can undergo reduction, thereby returning to $MbFe^{II}NO$. During storage of nitrite-cured meats such discoloration followed by reformation of pink pigment is directly observable.

The formation of flavour

Because of their lipolytic activity, micrococci contribute to the formation of flavour. The nitrite formed by micrococci out of nitrate, reacts with the flesh components (alcohols, aldehydes, inosin and sulphur-containing molecules) and forms specific flavours. In order to obtain sufficient flavour, a quantity of 20 to 40 ppm is

required.

Shelf life

Nitrite is generally known as an inhibitor of numerous micro-organisms. The growth of various food pathogens such as ***Clostridium botulinum***, ***Salmonella spp.***, and ***Staphylococcus aureus*** is inhibited by 80 to 150 ppm nitrite.

Since nitrite inhibits various types of bacteria, there is a possibility that added and/or formed nitrite inhibits the starter cultures, and this results in a less imposed or unsuccessful fermentation.

- ***Yeasts***

By consuming oxygen and breaking down peroxides, ***Debaryomyces*** protects the product from breakdown of fat, and thus improves the quality of the fermented product.

- ***Moulds***

Moulds have an antioxidative effect. By consuming oxygen they provide a reduction of the oxygen pressure against the surface. In addition to this, they break down peroxides. They only grow at the surface and this way they protect the product from the harmful influences of light. This will generally improve colour stability and slow down the rancidity process. Moreover, moulds create a favourable "micro-climate" at the surface, which prevents dehydration of the surface of the sausage or loss of fat during maturing process. Finally, moulds have a lipolytic and proteolytic effect, and they also break down lactic acid. This contributes to the development of a typical flavour.

c) Sauerkraut

To prepare sauerkraut, chopped white cabbage is mixed with 2 to 3 % salt and then it is anaerobicaly fermented at 18.3-21.1 °C. The fermentation process occurs in 3 steps, due to the lactic acid bacteria, belonging to the natural flora of white cabbage.

1st step Leuconostoc mesenteroides breaks down the hexoses in a heterofermentative way to lactic acid (50 %), ethanol, acetic acid, and mannitol. By this the pH drops to 4.4-4.6, and 0.67 % acid in the form of lactic acid is formed.

2nd step Lactobacillus plantarum converts the remaining hexoses via heterofermentation into lactic acid. This causes a decrease in pH to +4.0. This corresponds with a concentration of 1.20 to 1.25 % acid in the form of lactic acid.

3rd step Lactobacillus brevis converts the remaining sugars via heterofermentation into lactic acid, acetic acid, CO_2, ethanol until a pH= 3.6 is

achieved, which corresponds with 1.50 to 4.70 % acid in the form of lactic acid.

This way a microbially stable end product is obtained.

3.2. REDUCED a_w

As stated previously: $a_w = P/P_0$ **and** $a_w \times 100 =$ **% R.H.**

3.2.1. RELATION OF a_w TO % H_2O

The connection between % H_2O and a_w is represented by the water sorption isotherm. For many foods the isotherm obtained by adsorption of water differs from that obtained by desorption (dehydration). This effect is termed hysteresis (figure 2).

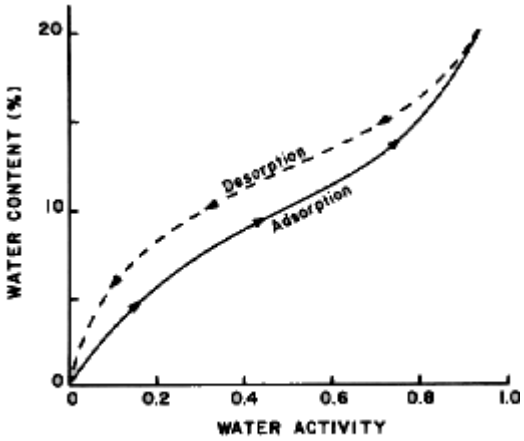


FIGURE 2. Hysteresis-curve

In practice a desorption takes place. In foods showing hysteresis at high a_w-levels, micro-organisms may grow more rapidly in systems adjusted by a desorption process, than in those prepared by an adsorption process at the same a_w.

3.2.2. RELATION OF a_w TO TEMPERATURE

As P is affected by temperature, so is the a_w. The rise of P is small in most foods in function of increasing temperature, unless the solutions are saturated. High temperatures increase the solubility, and hence P increases.

At temperatures below freezing point, P decreases and consequently the a_w drops. Table 13 illustrates this for meat.

TABLE 13. Connection between freezing temperature and a_w in meat.

T° (C)	a_w
-1	0.990
-10	0.907

T° (C)	a_w
-20	0.823
-30	0.746

3.2.3. MECHANISMS OF ACTION

Since micro-organisms are small, do not contain water-impermeable barriers and are in close contact with the environment, they tend to come rapidly into osmotic equilibrium with their surroundings. If, in a particular food, the a_w is low, and the major solutes that are present (NaCl, glucose, sucrose) are not ready to penetrate the cell membrane, the present micro-organisms lose water by osmosis until equilibrium is re-established. According to the amount of water loss, the metabolic activity is reduced or inhibited, and growth ceases. However, all micro-organisms have evolved some capacity to regain the lost water, and thus tolerate some levels of reduction in a_w (osmoregulation). This mechanism is not equally effective with all micro-organisms. That is why it is important to determine the minimum a_w-value for growth for the different strains, species and genera.

The water flow across microbial cell membranes is a passive process and therefore follows rapidly any osmotic gradient. Changes in the cell water content can therefore only occur as a result of changes in osmolarity of the medium or of the cell cytoplasm, or as a result of some physical force such as a decreased pressure in the cell membrane or wall. The basic water regain mechanism is based on the increase of the quantities of cytoplasmic solute so as to raise the internal osmotic pressure to just exceed the external osmolarity, and thus encourage the flow of water back into the cell. These internally accumulated solutes allow continued activity of cytoplasmic enzymes at a_w-values lower than the external solutes commonly presented in foods, generally by interfering with proteins and other cytoplasmic macromolecules.

Examples of such osmoregulatory components are:

- cations (K^+)

- amino and amino acids, amines (glutamic acid and g-aminobutyric acid, glutamine and proline)

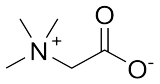
- quaternary amines (glycine, betaine)

- polyols, carbohydrates and derivatives.

This accumulation process is energy-depending, so that a reduced a_w causes an increased maintenance metabolism with a reduced cell yield and a reduced growth speed as a result.

Growth at low a_w-values also leads to changes in membrane lipid composition: the levels of anionic lipids such as phosphatidylglycerol and diphosphatidylglycerol

increase relative to the levels of neutral, or "zwitterionic" lipids such as phosphatidylethanolamine. These characteristic phospholipid changes occur when the osmolarity is raised by charged and also by uncharged solutes, so the phenomenon is not a reaction to specific solute components such as Na^+ or Cl^-, but to the raised osmolarity per se.



3.2.4. EFFECT OF REDUCED WATER ACTIVITY ON MICRO-ORGANISMS

A. ***On specific groups of organisms***

a) Gram-negative bacteria

The min. a_w for growth of proteolytic bacteria amounts to 0.96, whereas the min. a_w for growth of Enterobacteriaceae amounts to 0.93. Exceptions are the halotolerant ***Enterobacter*** (min. a_w = 0.90) and ***Vibrio parahaemolyticus*** (min. a_w = 0.94)

b) Gram-positive non-spore-forming bacteria

As a general rule this group of organisms includes many that are more tolerant than Gram-negative bacteria are to low levels of a_w. The min. a_w for lactic acid bacteria amounts to 0.94 and for micrococci to 0.90 ***Staphylococcus aureus*** however still grows at a_w = 0.83-0.86 with min. a_w for toxin production = 0.93.

c) Gram-positive spore-forming bacteria

The min. a_w for growth of aerobic spore-formers (***Bacillus spp.***) amounts to 0.90-0.91. For anaerobic clostridia, this value is higher. For ***Clostridium botulinum***, min. a_w for growth is 0.94 and for ***Clostridium perfringens*** it is 0.95.

Min. a_w for yeasts and moulds are 0.88 and 0.80 respectively (table 5).

As stated previously, there are exceptions. The halophile ***Halobacterium halobium*** grows at min. a_w = 0.75. The min. a_w for growth of the osmophilic yeast ***Saccharomyces rouxii*** is 0.60. The min. a_w for growth of xerophilic mould ***Xeromyces bisporus*** also amounts to 0.60. It has to be said however, that no mycotoxins are produced at $a_w < 0.83$. Yet in general, min. a_w sometimes depends or does not depend on the type of solutes. Moreover, all the above values were determined under optimal growth conditions (internal and external factors). The knowledge of the min. a_w for microbial growth is important for the understanding of the microbial ecology.

B. ***On resistance of micro-organisms***

If the a_w decreases, the lethal effect of heat decreases. This effect disappears if the pH decreases. The heat resistance of spores is maximal at a_w = 0.2-0.4, but this

is not as pronounced for vegetative cells.

3.2.5. EFFECT OF REDUCED a_w ON SPOILAGE AND ON THE DEVELOPMENT OF PATHOGENS

a) a_w 0.98 (< 3.5 % salt or 26 % sucrose in the liquid phase)

This group of food products includes: meat, fish, poultry, fruit, vegetables, milk, canned fruit, canned vegetables, et al. Under aerobic conditions, spoilage is mainly caused by Gram-negative proteolytic bacteria, which will produce volatile amines, ammonia and esters of organic acids. Under anaerobic conditions lactic acid bacteria predominate, causing souring of foods rich in carbohydrates.

All pathogens are capable of growth at those a_w-values, depending on the surrounding flora which may have an antagonistic effect. Some pathogens develop even without organoleptic changes. Mycotoxin-producing moulds will only develop if bacteria are unable to grow, as it is the case with acid food.

b) a_w = 0.98 - 0.93 (10 % salt or 50 % sucrose in the liquid phase)

This group of food products includes: meat (not dried), cheese, bread, tomato paste et al. Spoilage in this group of food products is caused by Gram-positive lactic acid bacteria, ***bacillaceae*** and ***micrococci***. Sometimes halotolerant ***Enterobacteriaceae*** are able to grow. Moulds can also grow when the bacterial growth has ceased.

At a_w = 0.95 to 0.98, all pathogens are able to grow and at a_w = 0.950 to 0.93 ***Staphylococcus aureus*** and toxin-producing moulds may develop.

c) a_w = 0.93 - 0.85 (17 % salt in the liquid phase or saturated sucrose solution)

This group of food products includes: meat products (dried), condensed and sweetened milk, matured cheese, et al. Spoilage is caused by cocci, yeasts and moulds.

As pathogens only ***Staphylococcus aureus*** and toxin-producing moulds are able to grow.

d) a_w = 0.85 - 0.60

This group of food product includes: "Intermediate Moisture Foods" (I.M.F.), dried fruits, flour, cereals, jam, nuts, et al. Spoilage is caused by halophilic bacteria, xerophilic moulds and osmophilic yeasts.

Toxin-producing moulds are capable of growing, however without toxin production (min. a_w = 0.83).

e) a_w < 0.60

This group of food products includes: confections, chocolate, honey, pasta, cookies,

crispy chips, dried vegetables, milk powder, egg powder, et al. This group of food products is microbially stable. Pathogens can survive and may start to grow again after rehydration.

3.2.6. REDUCED a_w AND CONTROL OF MICRO-ORGANISMS IN FOODS

The a_w of a food product can be lowered by decreasing P. This can be achieved by evaporation of H_2O and/or by adding solutes like salt and sugar.

3.2.6.1. Dehydration

Dehydration occurs in the form of evaporation, the rate of which depends on the increase in temperature. Another drying-technique is lyophilisation or freeze-drying. This process takes place in 2 stages. First the water in the food is frozen, and then the ice is directly converted into vapour (sublimation), leaving a dry product. The survival of micro-organisms is determined by the drying rate: the faster the drying rate, the higher the amount of cells which will be distracted.

3.2.6.2. Addition of solutes

The solutes commonly added to foods to reduce a_w are sugars and salt. Depending on the nature of the food, this may be accompanied by a drying process, which causes a further decrease in a_w. Table 11 shows the link between on the one hand the concentration of NaCl, sucrose and glucose in water and on the other hand the a_w.

Remark : In homogenous foods the a_w may vary during transport and storage, and due to this a gradient arises, depending on the moisture level of the environment. In heterogeneous foods, an equilibrium is achieved during transport and storage. A number of factors play here a role : a) the a_w of the various ingredients and b) the concentration of these ingredients.

3.2.7. INTERACTION OF a_w AND OTHER FACTORS

As stated previously there is a relation between the min. a_w for microbial growth in food products and the other growth conditions, such as pH, t°C, etc. The more any of these remaining conditions are sub-optimal, the higher will be the min. a_w. An example of this interaction is the preservation of meat products (table 15).

TABLE 14. Concentration of NaCl, sucrose and glucose in water and the a_w.

A_w	NaCl (%)	Sucrose (%)	Glucose (%)
0.995	0.88	8.52	4.45
0.990	1.74	15.45	8.90
0.960	6.57	39.66	28.51
0.920	11.90	54.36	32.87
0.880	16.28	62.77	53.05
0.860	18.18	65.63	58.45

A_w	NaCl (%)	Sucrose (%)	Glucose (%)
0.850			61.84
0.840	19.94		
0.820	21.59		
0.800	23.13		
0.753	26.50		

TABLE 15. Relation of a_w, pH and T°C for the preservation of meat products.

Category	pH	a_w	T°
easily perishable	> 5.2	> 0.95	> + 5°C
perishable	5.2-5.0	0.95-0.90	> 10°C
shelf-stable	< 5.2	< 0.95	no chilling
	< 5.0	-	
	-	< 0.90	

Another example is yeast that grows in a liquid medium with a_w = 0.75-0.62. Growth of the same yeast in a solid medium is only possible at high a_w-values.

As stated previously (3.1.3.) the action of preservatives will increase if the a_w drops. If the concentration of preservatives in a food product rises, the min. a_w for growth increases.

3.3. MODIFICATION OF Eh

3.3.1. ADDITION OF ADDITIVES

Specific additives in food can alter the Eh. Nitrite for example, which has an oxidative effect, will raise the Eh, whereas vitamin C and reducing sugars will lower the Eh.

3.2.2. PACKAGING

The choice of packing material also influences the Eh of the food product, depending on the physico-chemical O_2-permeability. Moreover, the O_2-permeability of a packing film influences the microbial interactions resulting in a change of Eh.

3.4. USE OF TEMPERATURE

To preserve food products, in other words to inhibit growth or to kill micro-organisms, the external factor "temperature" can be decreased or increased. Because temperature controls the rate of all physico-chemical reactions, it will also have a profound effect on the biological systems. Temperature influences fundamental properties such as solubility of molecules, viscosity, density, osmotic

properties of cell membranes, surface tension, hydrogen bonds...

In living cells, the effects of temperature are manifested by its influence on such processes as growth rate, metabolic activity, nutritional requirements, chemical composition, substrate uptake rates.

3.4.1. REDUCED TEMPERATURE

3.4.1.1. Chilling

Chilling means a reduction of temperature to -1 - + 7 °C.

A. ***Effect***

a) Growth rate

By chilling, the log-phase is extended and/or the logarithmic growth phase is slowed down. In addition to this, a modification of the microbial ecology may occur. In raw milk kept at 10°C streptococci of the lactic group will grow, whereas at 0°C primarily a psychrophilic flora develops. A commonly held belief is that microbial growth becomes extremely slow at low temperatures. However this does not hold for psychrofilic bacteria e.g. ***Vibrius marinus*** that has a mean generation time of 3-7 hours at 3°C, and ***Bacillus psychrophilus*** that has a generation time of 6.3 hours at -5°C. Besides the specific characteristic of optimum growth at low temperatures, psychrophilic bacteria are unable to grow at moderate temperatures. The optimum growth temperature fluctuates between 4°C and 15°C, whilst the maximum growth temperature fluctuates between 18°C and 20°C and sometimes even lower (13-14°C).

To achieve rapid growth it is essential that substrate uptake is not a limiting process. The ability of microorganism to grow at low temperatures is governed by its capacity to transport solute molecules across the cytoplasmic membrane. Psychrophiles and psychrotrophs differ from mesophiles in being able to transport solutes such as sugars and amino acids into the cell at temperatures < 5°C. Possible explanations for this are :

- permeases in the cytoplasmic membrane of psychrophiles are less sensitive to low inactivation than those in mesophiles ;

- permeases in mesophilic organisms are not unusually cold-sensitive, but owing to changes in the molecular architecture of the lipid bilayer at low temperatures, they are unable to combine with their respective substrates ;

- at low temperatures there is insufficient energy available to support the active transport of solute molecules across the cytoplasmic membrane in mesophiles.

b) On cell membranes

Micro-organisms are able to alter their membrane lipid composition in response to

changes in environmental temperature. Lipids constitute some 40-70 % of the total membrane dry weight and thus qualitative and/or quantitative changes in fatty acid composition in response to shifts in growth temperature are likely to influence profoundly the molecular architecture of the lipid bilayer and its physiological properties.

Within the cell membrane, the phospholipids are organised in the form of a bi-layer:

- the polar head groups are exposed at the intracellular and extracellular surfaces, and are therefore able to interact with the aqueous environment on the inside and outside of the cell.
- the hydrophobic acyl- chains are arranged to the plane of the membrane.

Alterations in membrane lipid composition usually involve changes in the fatty moieties and less commonly the polar head groups. This is because changes in fatty acid composition are much more effective in modifying and maintaining membrane fluidity than changes in head group composition. The physiological consequence of these changes is that optimum membrane fluidity and function are maintained over a range of growth temperatures. This property is termed "homeoviscous" adaptation : membrane fluidity is maintained relatively constant by modulating the viscosity of membrane lipids.

Alteration in membrane lipid composition is a clear and important adaptation in numerous psychrophiles, which enables them to grow at low temperatures. Some psychrophiles however do not have this adaptation, so it may be concluded that it is not an essential requirement for growth at low temperatures.

The "cold shock" is the phenomenon that occurs at a sudden cooling of a bacterium cell. The rapid cooling alters the membrane lipids and hence injures the cytoplasmic membrane. This results in an alteration of permeability which disturbs the uptake of nutrients and the release of metabolites.

Mesophiles are sensitive to cold shock. Gram-negative bacteria are more sensitive than Gram-positive bacteria, except for ***Clostridium perfringens*** and ***Bacillus subtilis***, which are susceptible to cold. ***Staphylococcus aureus*** is very resistant to cold shock. Psychrophilic and psychrotrophic bacteria are less sensitive to cold shock. The susceptibility of a microorganism to "cold shock" depends on the physiological situation. There are 3 categories of susceptibility:

- a steady decline in viability 1) upon chilling stationary phase cells or 2) upon slowly cooling exponential phase cells ;
- "cold shock" on rapidly cooling exponential phase cells, which causes some loss of

cell interior and hence loss of viability ;

- "cold osmotic shock" on (re)suspending exponential phase cells in cold distilled water which causes leaking of essential cell substances.

c) On membrane structure

The lipid bilayer structure is a feature common to all biological membranes. In order to ensure correct membrane function (solute transport, assembly of transport proteins, enzyme activity) it is essential that the membrane lipids are in the liquid-crystalline state. At low temperatures, the membrane lipids are in the gel state, and in this state the lipid bilayer is essentially rigid and the solute transport is severely limited. With increasing temperature, the liquid-crystalline state comes into being, characterised by a loosely packed, fluid and relatively permeable structure, thereby facilitating solute transport.

The temperature at which the transition from the gel to the liquid-crystalline state occurs depends on the nature of the present fatty acids :

- the melting point of saturated straight chain fatty acids decreases with decreasing chain length ;

- a similar effect can be achieved by inserting a double bound into longer chain fatty acids ;

- the introduction of a methyl group at the anteiso position results in fatty acids with lowered melting point, whereas introduction of the methyl group at iso position results in fatty acids with increased melting point.

B. ***Physiological adaptation to low temperature***

a) Morphological changes

Exposure to cold may increase the cell size (***Candida utilis***, ***Escherichia coli***). In addition to this, filaments may be formed (***Escherichia coli***). Mesosome deterioration and double cell wall formation are typical phenomena occurring in ***Bacillus subtilis*** at low temperatures.

b) Differential changes in the activities of enzymes

At low temperatures, a retardation of the activities of enzymes in the bacterium cell may occur, and hence change metabolic processes, which results in the formation of other end products.

Examples are :

- increased pigment production by ***Pseudomonas*** ;
- increased dextran production by ***Leuconostoc*** and pediococci ;

- increased lipase and proteinase production by various micro-organisms : some of these enzymes are thermoresistant and may cause spoilage of enzymes in chilled food products.

Change in lipid composition: if the temperature drops, the quantitative lipid composition remains the same, whereas that of yeasts increases. Qualitatively however, the proportion of unsaturated fatty acids increases both with bacteria and with yeasts. If the proportion of unsaturated fatty acids increases, the temperature at which the membrane lipids freeze, will drop. This is essential for membrane function at low temperatures.

c) Change in macromolecular composition

Psychrophilic ***Pseudomonas spp.*** have 40 % more RNA and 11-14 % more protein in the cells at 0°C than in cultures grown at 14°C, the optimum growth temperature. Similar results have been reported for psychrophilic ***Vibrio spp.*** There was also an increase in cell size and cell volume. Most of the additional RNA synthesised at low temperatures, is ribosomal RNA.

C. ***Effect on spoilage organisms***

At chill temperatures (-1 to +7°C) the growth of mesophilic and thermophilic bacteria is completely inhibited, but psychrophilic and psychrotrophic bacteria may still grow and hence cause spoilage. The lower the temperature however, the longer the generation time and the longer the shelf life.

D. ***Effect on pathogens***

Most pathogens are mesophiles. This means that growth does not occur at temperatures below 7°C. Some pathogens even die. Still some pathogens show a psychrotrophic character e.g. ***Vibrio parahaemolyticus*** (5-8°C), enteropathogenic ***Escherichia coli***, ***Yersinia enterocolitica***, ***Clostridium botulinum*** type E and non-proteolytic ***Clostridium botulinum*** type B and F which grow and produce toxin at 3.5-5°C. The minimum growth temperature for ***Listeria monocytogenes*** is 0°C.

E. ***Effect on indicators of faecal contamination***

Some indicators of faecal contamination such as ***Enterobacteriaceae***, coliforms and faecal streptococci are able to grow at low temperatures. This means that a food product after chilled is considered to be unacceptable storage, in spite of the fact that the initial faecal contamination was acceptable. This way ***Escherichia coli*** and faecal streptococci are able to grow at min. temperatures of 8-10°C, ***Klebsiella***, ***Enterobacter*** and ***Hafnia*** (coliforms) even grow at 0°C.

F. ***Maximum temperatures for the growth of psychrofiles***

The maximum growth temperature is determined by the thermolability of one or more essential chemical or structural components.

- If ***Vibrio psycchroerythrus*** is exposed to 37°C for 2 hours, an extensive lysis of the cellwand can be seen, and the cells become distorted.

- In ***Bacillus insolitus*** filamentous cells without cross-walls are formed, and this implies that cell division is impaired at this low temperature.

- In ***Vibrio marinus*** MP-1 cells, both leakage and lysis occur at temperatures, above the maximum for growth. Once lysis has commenced, it cannot be halted by transferring cells to a lower temperature.

Although loss of intracellular components is considered as the principal cause of cell death of psychrophiles, experiments have shown that this is rather a secondary cause. Several studies demonstrated that enzymes of psychrophiles are abnormally susceptible to temperature as well in vivo as in vitro.

3.4.1.2. Freezing

Freezing means a decrease in temperature to below the freezing point of the food product. The freezing point is different for each food product.

A. ***Effect of freezing and freezing temperatures on micro-organisms***

Firstly, there are a number of lethal effects. The free water in the microbial cell is converted into ice-crystals. This causes a decrease in a_w and an increase in the viscosity of the cytoplasm. In addition to this, the growing ice-crystals will injure the cell. During freezing a loss of cytoplasmic gases (O_2 and CO_2) occurs, and also the pH may be altered. The concentration of cellular electrolytes increases to toxic levels. Essential proteins (enzymes) are denaturated. Finally, there is the effects of cold shock.

Secondly, there are sublethal effects in other words, a reduction in the total number of bacteria. This reduction is not due to death. The micro-organisms are latently present as a result of freeze injury. Cells injured by freezing are still viable in ideal circumstances (after resuscitation). This phenomenon is always a subject of discussion in the microbial analysis of frozen food.

B. ***Factors affecting the antimicrobial effect of freezing***

The following factors are determinant for the antimicrobial effect of freezing :

* type of microorganism and physiological situation ;

- sensitive: vegetative cells of Gram-negative bacteria, yeasts and moulds, depending on the possible presence of protective factors ;

- medium resistant: Gram-positive bacteria, except for ***Clostridium perfringens***

and ***Bacillus subtilis*** ;

- resistant: sporeformers ;

- extremely sensitive: protozoa, cestodes and nematodes ;

** rate of freezing: rapid freezing has better results than slow freezing

*** freezing temperatures: high freezing temperatures are more lethal (more death at -4 to -10°C than at -15 to -30°C) ;

**** shelf-life: during storage there is a slow death of micro-organisms, depending on the temperature ;

***** type of food: sugar, salt, proteins, fats, colloids offer protection, whereas high a_w and low pH stimulate death ;

****** effect of thawing: thawing should happen slowly ;

******* effect of alternately freezing and thawing: from microbial point of view, this has a favourable effect, because death increases, but this method is unfavourable for the quality of the food product.

Remark: Microbial growth is not possible at t° < -18°C. Activities of enzymes however are possible. Therefore a long shelf-life of deep-frozen products preferably takes place at 24°C, since at that temperature both bacterial growth and activities of enzymes are completely inhibited.

C. ***Growth after thawing***

Growth after thawing depends on the type of food and the numbers and types of the surviving cells, since the flora present before freezing has changed during the freezing process.

A minimum growth occurs in the following thawing processes :

- in air : low t° (10°C) and low air velocity (0.25 m/sec.)
- in water : running water (10°C)
- vacuum thawing : at 10°C.

Forcing thawing is very harmful, since this causes spoilage, and a possible growth of any present pathogenic bacteria with possible toxin production. As a result of thawing, protective layers may be more or less damaged, thus making the food more susceptible to microbial attack. Thawing of packaged food in their package causes condensation on the surface of the food product during thawing, which results in accelerated microbial growth.

Generally it can be assumed that there is no difference between the rate of spoilage of a fresh food product and that of a thawed food product by preservation

at low temperature.

3.4.2. INCREASE IN TEMPERATURE

As the temperature increases above that at which growth of micro-organisms ceases, injury and, even death occurs.

A. ***General definitions***

The ***D-value*** is the time (in minutes) required to destroy 90 % of the microbial population at a given temperature.

Thermal death time (TDT). A TDT-curve can be constructed by platting the D-values against exposure temperatures. This curve in fact shows the resistance at several temperatures. The **Z-value** can be derived from this curve.

The **Z-value** is the degrees Celsius required for lowering the TDT by a factor of 10. The D and Z-value are necessary to calculate heat processes.

The ***F_o-value*** is the equivalent time (in minutes) at 121°C of all heat processes to destroy spores or cells.

The ***12D-concept*** is the accepted standard requirement for sterilisation in the preserving industry in other words, if $\log_{10}$ of the total count has decreased by a factor of 12, the product is considered to be sterile.

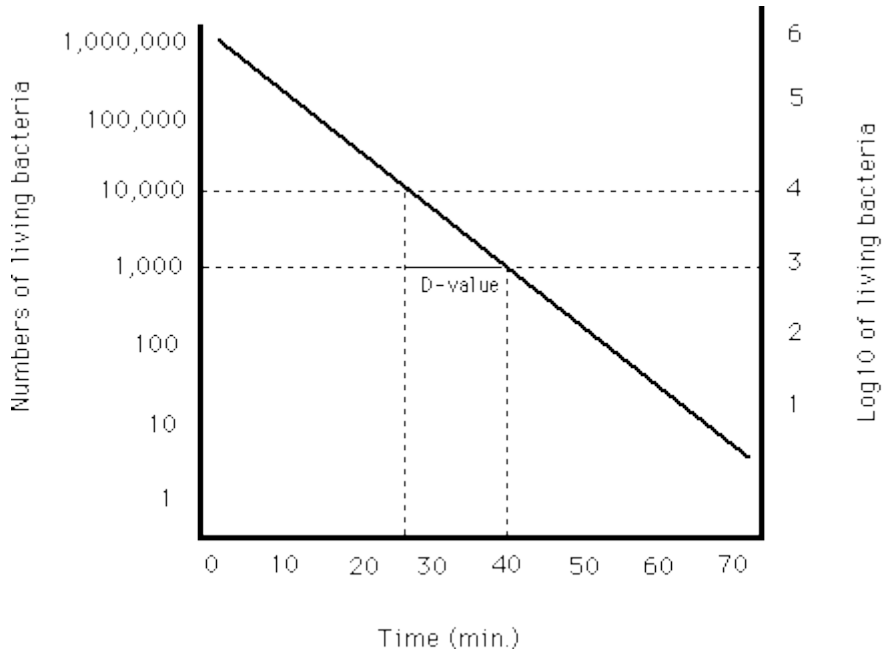


FIGURE 3. D-value

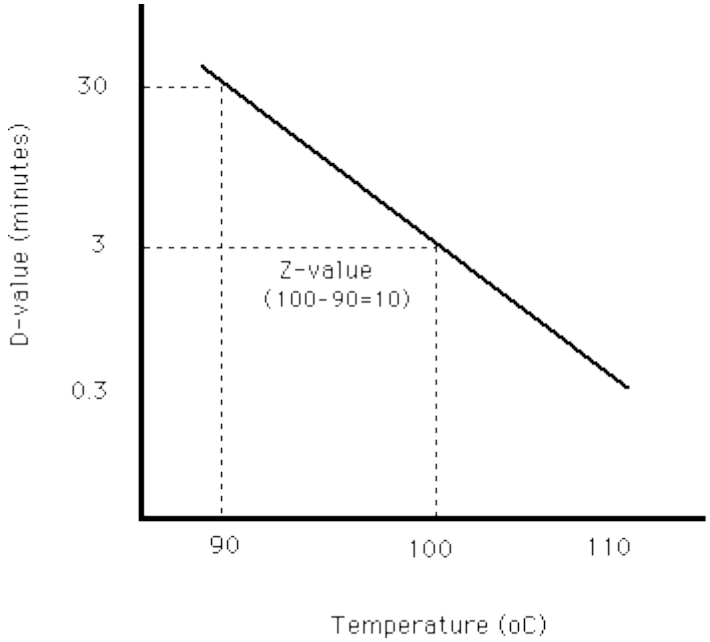


FIGURE 4. TDT-curve

B. ***Factors affecting the heat resistance of micro-organisms***

Factors that can effect heat resistance are of three general types. A first type is the inherent resistance, that is different between species and even between strains within the same species. A second type is the resistance determined by environmental influences active during growth and formation of cells and spores (age of cells, growth temperature, growth medium). A third type of resistance, is the one that depends on the environmental influences acting during the heat process (pH, a_w, type of food, presence of salt and other organic and inorganic substances).

a) Type of microorganism

The majority of vegetative bacteria, yeasts and moulds are killed after a few minutes at 70-80°C and none survive at 100°C. Vegetative cells show a constitutive and an adaptive resistance.

- Constitutive resistance

It is most important in particular to consider changes to DNA, because, although non-DNA damaged cellular components can be replaced by newly synthesised ones, this is only so if DNA remains sufficiently functional to provide the correct genetic information. Consequently repair of DNA or its protection from denaturation, is fundamentally necessary to vegetative cell heat resistance.

There seems to be a correlation between membrane composition and heat tolerance of vegetative cells, and change in membrane composition, by preincubation at increased temperatures, has been shown to modify heat resistance. Cells that contain the highest levels of saturated fatty acids in this membrane are capable to

grow at the highest temperatures and are consequently the most heat resistant. Resistance results from the tertiary structures of proteins that have evolved in the tolerant organisms. Small differences in the primary structure can bring about large differences in the tertiary structure and thus increase the thermal stability of proteins.

- Adaptive resistance

Vegetative cells exposed to a variety of stresses, react homeostaticely by adapting to these stresses in a variety of ways. Therefore practical uses of heat (sterilisation, pasteurisation) involve relatively rapid heat-up rates, so that the opportunity for adaptation does not rise.

Spores are more resistant than vegetative cells. The heat resistance of bacterial endospores has two distinct components. The first of these is an intrinsic property of the strain and is related to the resistance of the corresponding vegetative cell, in other words, spores formed by psychrophilic bacteria are generally much less thermotolerant than those formed by thermophiles. The second component is the additional resistance that results from the particular structure and the composition of the spores. The most of the cytoplasmic content is intrinsic non-heat resistant, but is protected by the compositional and structural factors within the spores, which causes au increase in heat tolerance by 35-45°C.

- Structure

Bacteria endospores are internally compartmentalised in a manner that is unique in prokaryotic cells. The central membrane-bounded cytoplasmic compartment (protoplast or core) is surrounded by a wide cortex, which consists mainly of peptidoglycan. The cortex itself is surrounded by a number of protein-rich coats. This structure is thought to hold the key to spore resistance.

- Composition

Calcium dipicolinate: Spores contain high levels of calcium and dipicolinic acid (2-10 % of dry weight). These components reside predominantly on the central core, probably as insoluble calcium dipicolinate. A direct role of the levels of dipicolinic acid and calcium in heat resistance seems less likely, but a role in the establishment and maintenance of resistance and dormancy is most probable.

Peptidoglycan: Spore cortex peptidoglycan has a similar structure in all spores. The presence of peptidoglycan is linked to the heat resistance of spores. For instance lysozyme rapidly reduces the heat resistance of spores, the coats of which have been made permeable to this enzyme. Some naturally occurring coatless spores or coatless mutants are very lysozyme-sensitive. Mutants with low levels of peptidoglycan have low resistance. Endogenous spore enzymes hydrolyse peptidoglycan during

germination, accompanying the loss of heat resistance.

Basic proteins: Spores contain a number of core-located, acid-soluble, basic proteins. Loss of one of the proteins, results in a loss of heat resistance.

Mineralisation and ion stasis: Besides high levels of calcium^{++}, spores often also contain magnesium^{++} and manganese^{++}, that may also be complexed and precipitated as dipicolinates. They also normally contain high levels of K^+ and Na^+, that are not normally complexed and precipitated by biological molecules. Spores contain high levels of net vegetative charge, and they thus behave like cation-exchange particles releasing Ca^{++} and taking up H^+ if incubated at low pH-values or re-accumulating Ca^{2+} or other cations if incubated with salts of those cations at higher pH-values. The 'H-form' spores made in this way were relatively heat sensitive, and the other forms more resistant, with the Ca^{2+} forms having the greatest resistance. A study in which the spores could be almost completely "stripped" of cations without loss of viability, showed that such spores lost much, but not all, of their additional heat resistance. These data indicate that mineralisation or ion stasis is an important contributing part of the heat resistance.

Water content: There is a correlation between heat resistance and water content of the spore : spores which have the highest heat resistance have the lowest water contents and vice versa. Moreover, if the compartmentalisation within spores is taken into account, an even better correlation is emerging: this is between the ratio of core volume to core plus cortex volume and heat resistance. Spores with a small ratio (and a highly condensed core) have a higher heat resistance than spores with a large ratio, and this implicates that the lower the core water content is, the higher the resistance to heat will be. Further evidence for a low water content in the core of the resting spore, derives from its rapid expansion (1 or 2 minutes) during germination, at a time when it is losing dry matter in the form of Ca-dipicolinate, and breakdown products of basic polypeptides, etc. Furthermore, heat sensitive newly germinated spores can be made heat-resistant again by suspension in solutions of high osmolarity that presumably remove the recently regained water. There are spores of ***Clostridium perfringens*** that are resistant to heat up to 100 °C during 6 hrs. The heat resistance of ***Clostridium botulinum*** is a few minutes at 120 °C.

b) Number of cells

The higher the number of cells, the longer the heating process.

c) Age of cells

The heat resistance is the highest in the early lag phase, the late exponential phase and the early stationary phase. But the heat resistance is low during the logarithmic (exponential) growth phase.

d) Growth temperature

The lower the temperature for growth, the more the microorganism is susceptible to heat. Therefore raw milk is chilled prior to pasteurisation.

e) Composition of the food product

In food products which have a low a_w, the heat resistance of micro-organisms will increase. Fat offers a protection to cells, for example the heat resistance of ***Escherichia coli*** in growth media with different fat levels (table 16).

TABLE 16. Heat resistance of *Escherichia coli* as a function of the fat level.

Growth medium	Thermal death point °C (heating time = 10 min.)
Cream	73
Whole milk	69
Skimmed milk	65
Whey	63
Broth	61

Salts also influence the heat resistance of micro-organisms, depending on the type of salt, the concentration and other factors. Some salts increase the heat resistance because they have an a_w-reducing effect, such as Na and K salts. However some salts lower the heat resistance because they have an a_w-increasing effect, like Ca and Mg salts.

Dissolved carbohydrates lower the a_w and hence the heat resistance increases.

A decrease in pH in the food products is accompanied by a reduction of the heat resistance. Applications of this are semi-acid and acid canned foods.

Proteins and other colloidal substances have a protective effect, and therefore the heat resistance increases.

Specific microbial inhibitors, both the natural ones as the ones added for technological reasons, such as nitrite and some antibiotics, lower the heat resistance.

C. ***Relative heat resistance among various groups of non-spore-forming and spore-forming bacteria, occurring in food products***

The relative heat resistance of a number of non-spore-forming and spore-forming bacteria, frequently occurring in food products is shown in table 17 and table 18.

TABLE 17. Relative heat resistance of non-spore-forming bacteria.

	D-value		
	65 °C	**55°C**	**45°C**
Thermophiles	100	-	-

	D-value		
	65 °C	**55°C**	**45°C**
Mesophiles			
- ***faecal streptococci***	5-30	-	-
- ***Salmonella***	1-30	-	-
- ***Pseudomonas aeruginosa***	-	2	-
- ***Escherichia coli***	-	5	-
Psychrotrophs			
- ***Pseudomonas fluorescens***	-	3-4	-
Psychrophiles			
- ***Vibrio fischeri***	-	-	-
- ***Serratia***	-	-	-

TABLE 18. Relative heat resistance of spore-forming bacteria.

species	D-value	
	120 °C	**100 °C**
Clostridium thermosaccharolyticum	3-4	-
Bacillus stearothermophilus	4-5	3,000
Clostridium sporogenes	0.1-1.5	-
Clostridium botulinum	0.1-0.2	50
Clostridium perfringens	-	0.3-20
Bacillus cereus	-	5

The relative heat resistance of pathogens is low. In food products with a_w > 0.93, they are all killed by heating at 65 °C during 10 minutes. Spore-forming bacteria, however, will survive this heat treatment.

D. ***Mechanisms of heat inactivation and injury***

Although heat has a multiplicity of effects on the cell, four cell compounds for primary sites for lethal heat-induced damage and for non-lethal injury are clearly identifiable: DNA, RNA, ribosomes, cytoplasmic membranes and specific enzymes.

a) DNA

Heat causes single strand breaks in DNA. The effects are much more pronounced during dry heating, because under these conditions, higher temperatures are needed to cause inactivitiaton, than during moist heating. Heat also causes depurination and

depyrimidation of spore DNA. The action of endonucleases after germination on this DNA causes direct damage, followed by enzymatic amplification of the damage that was originally caused. Besides single strand breaks, heat also causes double strand breaks in vegetative cell DNA, either directly or indirectly by accelerating the action of endogenous nucleases so that the damage occurs after heating.

b) RNA and ribosomes

Ribosome destruction results from loss of Mg^{2+} by leakage from the membrane-damaged heated cells. Ribosome degradation and hydrolysis of RNA often accompanies, or follows, mild heating, but is probably not a key lethal event.

c) Cytoplasmic membranes

Heated vegetative cells cause leaks in the cell membrane and lose ions, amino acids and low molecular nucleic acid components. Raising the levels of environmental solutes may reduce the level of injury. Still, it is an important factor for injury and recovery of heated cells (resuscitation).

d) Specific enzymes

Heat inactivates spores by destroying the activity of one of more enzymes on the germination pathway. Since the mechanism of germination has not been elucidated yet, the identity of the heat-sensitive steps is not known.

3.5. USE OF RADIATION

In view of destroying micro-organisms, two categories of radiation can be applied. The first is radiation of wavelengths longer than those of visible light, e.g. IR. light of which the effect is based on a rise in temperature. The second category are radiation of wanelenghts shorter than visible light. By use of those rays, a rise in temperature does not occur in the food. Examples are the lower frequency UV-rays and the higher frequency ionising rays. The last two types are commonly used in food industry, and will be discussed in detail.

3.5.1. UV-rays

UV-rays have an l< 450 nm with l = 260 nm for optimal antimicrobial activity. UV-rays with □< 200 nm do not show an antimicrobial activity and are absorbed by the atmospheric O_2, that is converted into undesired ozone (O_3).

UV-rays from the near UV (l = 360-450 nm) are used to demonstrate fluorescent ***Pseudomonas*** spp. in eggs, based on their ability to excite fluorescence.

The intensity of irradiation is measured by the energy absorbed per unit area and is expressed as ERGS/sec. or mW/cm² (10^7 ERGS/sec. = 1 WATT). A low pressure

mercury lamp gives an intensity of 100 mW/cm^2 at a distance of 1 M. An irradiation-dose of 10^5 mW/sec. does not cause a rise in temperature.

A. ***General properties of UV-rays***

At 260 nm, there is an absorption of UV-rays at the nucleic acid bases

The penetration of UV-rays is small. Therefore they are virtually never used for treatment of food products, but they are used for killing microbes suspended in air or exposed on surfaces.

Micro-organisms are resistant to UV-radiation if they are covered by films of protective substances as in aerosols, or on the surface of wet or greasy foods.

B. ***The effects of UV on micro-organisms***

The antimicrobial effect of UV-radiation is expressed in D-value, in other words, the dose required for 1 decimal reduction. This D-value, however, is hard to determine, because the effect of UV-radiation also depends on the degree of absorption, the nature of the food and the physiological situation in the cell. In table 19, some D-values for the most important groups of micro-organisms in foods are given.

TABLE 19. D-values for the most important groups of micro-organisms.

Group	**D-value (m W sec. x 10^3)**
Gram-negative bacteria	1-5
Gram-positive bacteria	5-20
Yeasts	20
Moulds	200
	1-10

C. ***Use of UV-rays in the food industry***

a. Air

The greatest application of UV-treatment in the food industry is the decontamination of air. This is mainly applied in industrial bakeries. In tanks filled with sugar syrups, condensation in the head space leads to diminished sugar concentration at the surface, which permits superficial growth of moulds. UV-radiation will inhibit this growth of mould efficiently.

b. Liquids

Thin layers of liquid can be disinfected by UV-radiation. The effect depends on the purity and the composition of the liquid layer. This is mainly applied for water treatment. However, this technique is seldom used in food industry.

c. Surfaces

If the surfaces have been cleaned thoroughly beforehand, they can be disinfected by UV-radiation. For example the sterilisation of packages by UV-radiation: untransparent package material has to be treated from the inside, whereas transparent material can be treated from the outside.

d. Solid foods

Sugar contains approximately 10 spores of ***Bacillus stearothermophilus*** per gram. 30 minutes of UV-radiation in layers of 4 mm deep, will destroy the spores.

In chill rooms used for storage of carcasses, uv-radiation may lower surface contamination. However, spoilage of fatty meat may occur as a result of rancidity caused by oxidation of unsaturated fatty acids. This technique is more applicable to mutton- and beef carcasses than to pork carcasses. Another disadvantage is the formation of spots on green vegetables.

D. ***Combination effects and interactions***

In dry atmospheres, UV-resistance of micro-organisms increases. At relative humidity > 60 % the resistance decreases. Micro-organisms exposed to UV-radiation have reduced heat resistance. Oxygen does not influence the sensitivity to UV.

Micro-organisms exposed to sublethal doses have to be subjected to photoreactivation in order to trace them in the laboratory. The recovery is best on poor media and at temperatures subnormal for growth.

3.5.2. Ionising radiation

A. ***Definition***

Ionising radiation is an emanation of sufficiently high photoenergy to displace electrons from target molecules. The electron may be replaced by a positive ion or by another structure, so that a negative ion can be formed. The ability to create positive and negative ions characterises the action of ionising radiation.

B. ***Types***

There are 2 classes of ionising radiation: electromagnetic and particulate radiation.

a) Electromagnetic radiation

Electromagnetic radiation is represented by l-rays and x-rays.

l-rays are produced during the decay of an isotope such as ^{60} Co.

X-rays are produced when fast moving particles such as electrons are used to bombard a suitable target material.

b) Particulate radiation

Particulate ionising radiations include electrons, a-particles (helium nuclei ; $^{4}_{2}He^{2+}$),

protons (hydrogen nuclei; $^{1}_{1}H^{+}$) and neutrons. Electrons, a-particles and protons (all being charged particles) are essentially identical to X- and l-rays. They all cause ionisation by electron ejection and the latter can cause secondary ionisation. However, because of their relatively large mass for a given amount of energy, a-particles and protons have a much lower velocity than X-, l- and γ-rays. Moreover, they are readily stopped by biological material.

Neutrons have no charge and are not repelled by orbital electrons or positively charged nuclei. They cause ionisation by ejecting protons. The kind of ionising radiation which is used to inactivate bacteria commercially include l-rays (usually from a ^{60}Co-source), X-rays and electrons.

C. ***Units of measurement***

The most frequently applied unit is the GRAY = 100 rad. Formerly, Mrad (10^6 rad) and krad(10^3 rad) were applied.

D. ***Properties***

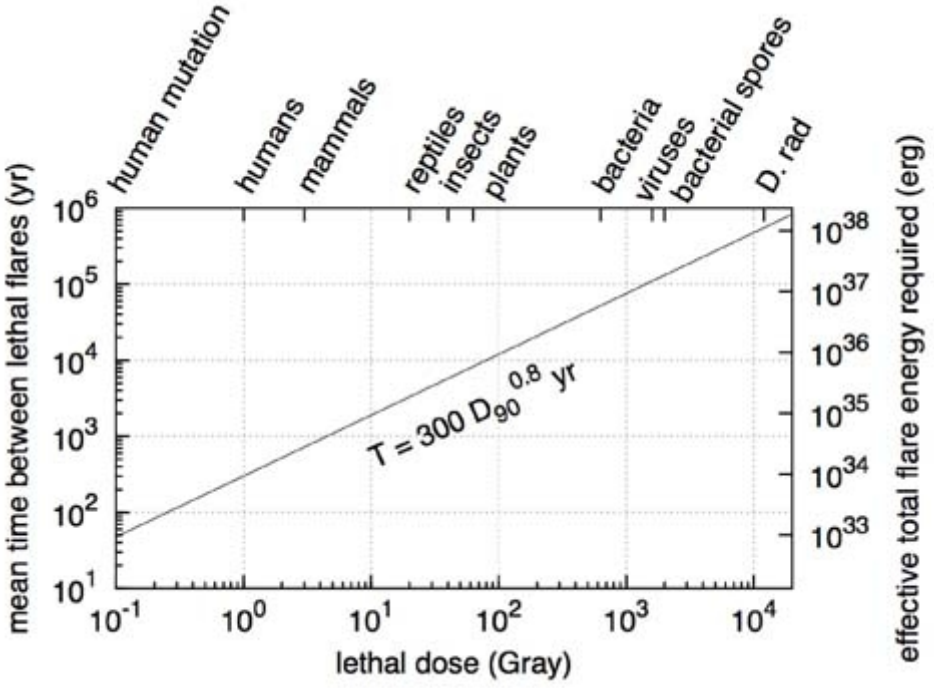
When high energy radiation strikes atoms, it excites them, causing ejection of electrons and the formation of ion pairs, consisting of the negatively charged electrons and the positively charged residues. Ionisation may proceed further as ejected electrons strike other atoms, giving rise to a chain effect.

Water is readily ionised and may be the primary source of ionisation in food. In the presence of oxygen, highly reactive compounds may be produced such as H_3O^+ , H^+, H_2O_2 and OH^-. These substances show an antimicrobial activity. The hydroxyl radical (OH^-) is the major effective species and reacts with organic molecules either by adding to a double bond or by extracting an H-atom from a C-H bond to form H_2O and a C-radical.

In practice, low doses are used, which means that no radioactivity remains in the food. Ionising rays have a great penetrating power. Larger organisms are more easily killed than smaller ones (table 20).

TABLE 20. Approximate lethal radiation doses for various organisms.

Organism	Dose (krad)
Higher animals and man	0.5-1
Insects	1-100
Vegetative bacteria and yeasts	50-1,000
Bacterial spores	1,000-5,000
Viruses	1,000-20,000



E. ***Effect on micro-organisms***

a) Lethal effect

The radiation effects on biological material are ascribed to the sum of two processes: a direct and an indirect process. The direct process includes the chemical reactions occurring in the target molecule, e.g. the ejection of electrons from atoms in the DNA-structure (genome) as a result of the passage of an ionising photon.

The indirect process occurs as a consequence of reactive, diffusible free radicals formed from the radiolysis of water, reacting with the DNA. DNA strand breakage occurs following indirect action on the deoxyribose moiety of the sugar-phosphate backbone of the DNA. About 20 % of the hydroxyl radical reactions with DNA result in the abstraction of an H-atom from any of the five C-atoms of the deoxyribose moiety. The remaining 80 % of the injury is on bases (sensitivity: thymine > cytosine > adenine > guanine). Doses that are lethal to micro-organisms do not inactivate the enzymes and do not alter the proteins.

The maximum sensitivity is achieved when the species is harvested in the exponential phase of growth, resuspended in phosphate buffer, irradiated while bubbling oxygen through the suspension and than plated on a rich medium. For a strict comparison of the resistance between different species, it is important that these parameters are respected. The bacterial species may be listed in the following order of increasing resistance:

Salmonella typhosa, ***Bacteroides vulgatus***, ***Bacteroides ovatus***

Proteus vulgaris, ***Serratia marcescens***, ***Aerobacter aerogenes***, ***Micrococcus pyogenes var. albus***

Escherichia coli, ***Leuconostoc mesenteroides***, ***Leuconostoc dextranicum***, ***Alcaligenes viscous***, ***Micrococcus pyogenes var. aureus***

Klebsiella pneumoniae, ***Corynebacterium acnes***

Diplococcus pneumonia, ***Sarcina lutea***, ***Streptococcus pyogenes***

It is clear that there is some overlap in resistance between the Gram-negative and Gram-positive bacteria, although the most resistant vegetative bacteria such as ***Streptococcus*** and ***Sarcina*** belong to the latter group, whereas the most sensitive such as ***Pseudomonas*** and ***Salmonella*** belong to the former. In general bacterial endospores are more resistant than vegetative bacteria of the same or other species, although considerable differences may also occur between them. Among the clostridia, ***Clostridium botulinum*** type A spores appear to be the most resistant and spores of ***Clostridium perfringens*** are relatively sensitive. Among the aerobic spore-formers ***Bacillus pumilus*** is as resistant as the most resistant clostridia spores, whereas in general ***Bacillus***-spores are more sensitive than those of clostridia. The vegetative cells of ***Deinococcus*** and ***Deinobacter*** species are even more resistant than bacterial spores. In both groups there are exceptions (table 21).

TABLE 21. D-value of irradiation-resistant bacteria.

Species	6D-value
Moraxella	700 krad
Streptococcus faecalis	700 krad
Streptococcus faecium	2,000 krad
Bacterial spores	3,000 krad
Micrococcus radiodurans	> 3,000 krad

Moulds have the same irradiation-resistance as vegetative bacteria.

Yeasts are more resistant than moulds (60 to 1,000 krad). ***Candida krusei*** is extremely irradiation-resistant (6 D = 2,000 krad).

Viruses have a D-value of > 3,000 krad.

If the microbial population of one specific type of microorganism is irradiated, cell death occurs in logarithmic mode.

b) factors affecting the effect of irradiation

- Oxygen: oxygen is a scavenger for electrons; removal of oxygen increases the irradiation-resistance, in other words oxidising reactions are important. Radiation causes formation of radicals in DNA. These radicals react with molecular O_2 to form peroxy radicals. These formations prevent repair and fix damage, there where the radical will combine with a hydrogen atom in the absence of oxygen, and the damage will be repaired.

- Water content: removal of water increases the irradiation-resistance.

- Freezing increase the irradiation-resistance.

- Type of food: there may be local anaerobic conditions in food products, even when oxygen is present externally; this affects irradiation -resistance.

- Irradiation protecting agents: compounds possessing SH-groups are good protecting agents (e.g. cysteine, cysteamine, gluthatione,…). They donate H-atoms to the free radicals in the DNA and they are capable of reacting with hydroxyl- and alkoxyl radicals (RO°). In a similar manner, antioxidiants react with peroxy (ROO°) radicals.

F. ***Use of ionising radiations to control micro-organisms in food***

Food is irradiated with ionising rays to:

a) destroy spoilage organisms; this process is called radurisation,

b) to destroy pathogens; this process is called radicidation,

c) to sterilise the food product; this process is called radappertisation.

Radappertisation is irradiating food products with high doses, e.g. the 12D for ***Clostridium botulinum*** amounts to 4,500 krad. Such high doses have harmful effects on the sensory quality of the treated food product. However, this 12 D dose can be lowered by combining the irradiation with a decrease in pH, addition of common salt and nitrite and use of chemical preservatives.

The common spoilage bacteria (***Pseudomonas***) of proteinaceous foods under cool conditions are destroyed by a dose of 100 krad, and this prolongs the shelf life of the food product. Much the same applies to the moulds (***Mucor***, ***Botrytis***), which commonly spoil dry or acid foods. Pathogens (***Salmonella***, ***Botrytis***), and pathogenic moulds (***Aspergillus flavus***) can be eliminated by doses of 200 to 500 krad. The species with the most resistant spores is ***Clostridium botulinum***, which has consequences for the 12 D concept for sterilisation. The most irradiation-resistant vegetative bacterium is ***Micrococcus radiodurans***, but this species has no significance in foods.

Irradiation in foods is also used against germination, insects and parasites.

3.6. CHEMICAL PRESERVATION

3.6.1. PRESERVATIVES

Chemical preservation is based on the addition of substances with antimicrobial activity. Preservation in the broadest sense of the meaning is based on the use of additives in concentrations above 0.5 %, such as common salt and sugar (see 3.2.4.2.) and acids (see 3.1.3.). Chemical preservation in the narrow sense means addition of substances in concentrations below 0.5 %. The effect of a number of preservatives has already been discussed previously (see 1.3.1.). The legal preservatives are included in a positive list (E-list) in which for each group the permitted types and amounts of preservatives are mentioned. A summary of the legal preservatives and their applications are given in table 22.

TABLE 22. List of preservatives and their applications

Preservative	E-number	Applications
Sorbic acid	E 200	In acid foods with pH < 5.0
Sodium sorbate	E 201	
Potassium sorbate	E 202	
Calcium sorbate	E 203	
Benzoic acid	E 210	
Sodium benzoate	E 211	
Potassium benzoate	E 212	
Calcium benzoate	E 213	
Ethylparabens	E 214	In neutral foods. When the
Sodium methylparabens	E 215	chain length of the ester group
Propylparabens	E 216	increases, the antimicrobial
Sodium propylparabens	E 217	activity rises, but the water-
Methylparabens	E 218	solubility drops.
Sodium methylparabens	E 219	
Sulphur dioxide	E 220	
Sodium sulphite	E 221	In neutral and acid foods.
Sodium bisulphite	E 222	
Sodium metabisulphite	E 223	
Potassium metabisulphite	E 224	
Calcium sulphite	E 226	
Calcium bisulphite	E 227	
Potassium bisulphite	E 228	

Preservative	E-number	Applications
Biphenyl	E 230	
Othophenylphenol	E 231	Anti-mold on citrus fruit.
Sodium ortophenylphenolate	E 232	Residue in fruit and vegetables.
Thiobendazol	E 233	
Nisin	E 234	Cheese, canned fruit.
Natamycine	E 235	Dry sausage, hard cheese.
Formic acid	E 236	In acid foods
Sodium formate	E 237	
Calcium formate	E 238	
Hexamethylene tetramine Acid environment	E 239	In foods with pH > 4.5. Mainly applied to kill bacteria, less to kill moulds and yeasts.
Formaldehyde	E 240	For pipes and equipment.
Potassium nitrite	E 249	Meat products.
Sodium nitrite	E 250	
Sodium nitrate	E 251	
Potassium nitrate	E 252	
Acetic acid	E 260	Acid foods (see 1.3.1.).
Potassium acetate	E 261	
Socium acetate	E 262	
Calcium acetate	E 263	
Lactic acid	E 270	
Propionic acid	E 280	In bread and pastry.
Sodium propionate	E 281	
Calcium propionate	E 282	
Potassium propionate	E 283	

3.6.2. USE OF GASES

3.6.2.1. Gas packaging

A. ***Principle***

The principle of gas packages is based on the replacement of air by a suitable gas mixture, in order to extend shelf life. The most frequently applied gases are CO_2, N_2 and O_2. Each component has a specific function and the composition of the gas mixture depends on the type of food. The choice of gas mixture requires a thorough knowledge of food technology, -microbiology, and -chemistry. If this technique is applied judiciously, it is possible to meet the consumer's demand for more fresh products with a long shelf life. Moreover, this technique can replace the use of classical preservatives.

B. ***Techniques***

Two types of gas preservation technique are applied for food products: controlled atmosphere and modified atmosphere. If controlled atmosphere is applied, the food products (mostly bulk) are stored in a closed space and the concentration of gas initially introduced, is maintained throughout the period of storage. If modified atmosphere is used, the amount and/or composition of gas in the gas-impermeable package is altered. In food industry, three techniques for modified atmosphere are applied: a) vacuum packaging, b) reduction of atmospheric pressure in the package and c) alteration of composition of gaseous atmosphere by injection of a suitable gas mixture into the package.

C. ***Effects***

a) Nitrogen (N_2)

This gas has no antimicrobial effect. Replacing the air inside the package by nitrogen is only used to expel oxygen, and this inhibits the growth of aerobic spoilage organisms.

b) Carbon dioxide (CO_2)

Micro-organisms need CO_2 for their own metabolism. The own CO_2-production usually covers the demand, and therefore micro-organisms seldom have a net need for CO_2. At high concentrations (from 10 %), micro-organisms are inhibited, depending on type of organism, CO_2 concentration, storage temperature, water activity of the food product and growth phase of the micro-organisms at the moment of packing. CO_2 expels the oxygen from the package and this way microbial growth is inhibited. However, this is not the main cause of inhibition, since a replacement of CO_2 by N_2 leads to a smaller inhibition. This leads to the conclusion that other mechanisms play a role. The inhibitory effect of CO_2 is based on several factors, but not all of them have been sufficiently elucidated yet. CO_2 readily dissolves in the liquid phase of the food product and consequently it will lower the acidity by formation of carbonic acid. The water-solubility of CO_2 increases if the temperature drops. The reduction of acidity and the exclusion of O_2 cannot only account for the

inhibitory effect of CO_2. Different hypothesises are given to explain the antimicrobial effect of CO_2. A number of researchers claim that the antimicrobial effect of CO_2 is based on an interaction with the cell membranes. Others claim that some membrane processes are only slightly inhibited, and that the membrane structure is not really perturbated. Many authors defend that CO_2 has a direct influence on of enzymes, via induction or repression of enzyme synthesis. The primary sites at which CO_2 exert its effects are the enzymatic carboxylation and decarboxylation reactions although the effects on enzymes are not limited to carboxylation-decarboxylation reactions. However, at the moment, a clear theory explaining the antimicrobial effect of CO_2 does not exist yet. It is very likely that the inhibition of micro-organisms is a combined action of all the above mentioned mechanisms.

c) Oxygen (O_2)

Oxygen is mainly applied in gas mixtures for colour retention of red muscular tissue. If concentrations above 5 % are used, myoglobin is converted into oxymyoglobin, which gives muscular tissue a bright red colour. In addition to this, irreversible conversion of myoglobin into metamyoglobin is prevented. The use of O_2-concentrations above 50 %, stimulates the fresh odour of packed food. It is also known that high concentrations of O_2 inhibit the growth of specific psychrotrophic spoilage organisms (***Moraxella-Acinetobacter***).

d) Carbon monoxide (*CO*)

The use of 1 to 5 % *CO*, which is not legal yet, inhibits the discoloration of red meat (irreversible production of the brown metamyoglobin), by formation of carboxymethyl-myoglobin, comparable to oxymyoglobing. Moreover, rancidity of fat will be inhibited by reduced concentration of free myoglobin, which is a catalyst for fat oxidation.

D. ***Microbial aspects of modified atmosphere packaging.***

One of the major objectives of modified atmosphere packaging, especially where packaging of food with high a_w-values is concerned (except for fruit and vegetables), is the inhibition of bacterial spoilage. This will extend shelf life. Two aspects are important here: a) the CO_2-level required for optimal inhibition of bacterial growth (spoilers) and b) possible ability of growth of pathogens in the presence of high O_2-concentrations.

a) Inhibition of microbial spoilage

The antimicrobial effect of CO_2 is based on the extension of a) the lag phase and b) the generation time of bacteria. The extension of the lag phase is a very important inhibitory mechanism. The reduced growth rate after the lag phase also

contributes to an extended shelf life. This reduced growth increases if the temperature drops, also due to the fact that solubility of CO_2 increases if the temperature drops. CO_2 mainly inhibits Gram-negative bacteria which are mainly responsible for proteolytic psychrophilic and psychrotrophic spoilage. Gram-positive bacteria on the other hand are less inhibited and lactic acid bacteria appear to be the least sensitive.

b) Risks of growth of pathogens

The risks of growth of pathogens by use of modified atmosphere packaging can mainly be minimalized by respecting the cold chain. A temperature control during storage, distribution and sale is therefore extremely important. At low temperatures in the presence of CO_2, growth of ***Staphylococcus aureus***, ***Salmonella*** and ***Listeria*** is inhibited, but at high temperatures growth may occur. Germination of spores of ***Clostridium botulinum*** is stimulated by a CO_2-pressure below 1 atmosphere, whereas CO_2-pressure above 1 atmosphere inhibits germination of spores and consequently toxin production. In addition to this, vegetative cells are killed. Special attention should be paid to the risks of growth of psychrotrophic and nonproteolytic ***Clostridium botulinum*** type B and especially type E (in fish). These anaerobic bacteria are capable of growth and toxin production at temperatures above 3.3 °C. It has been found that toxin production for spoilage may occur at 100 % CO_2, whereas ***Campylobacter*** does not grow, but still survives. ***Vibrio parahaemolyticus***, however, is sensitive to CO_2.

E. ***Applications in food products***

Modified atmosphere packaging is already applied on a large scale for preservation of meat and meat products, poultry, fish and fish products, cheese, bread and pastry, vegetables and fruit.

3.6.2. Sulphur dioxide (SO_2)

Sulphur dioxide is applied in the form of gas, sulphite, bisulphite and metabisulphite.

A. ***Effect***

The effect depends on the pH (figure 5).

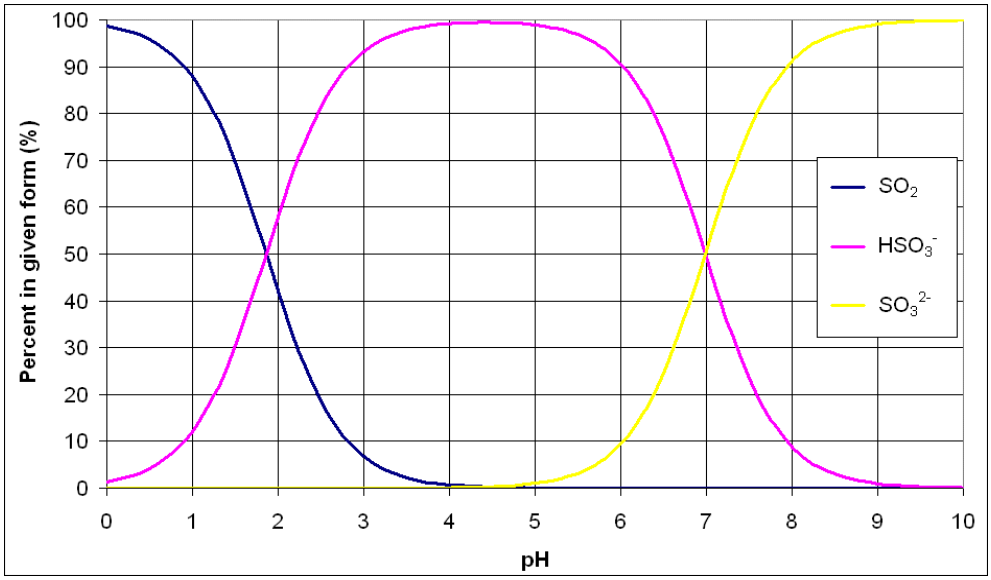


FIGURE 5. Percentage distribution of sulphite, bisulphite and molecular SO_2 as a function of pH in an aqueous solution.

The figure shows that SO_2 is only effective at pH < 4. The effect is based on a reaction of SO_2 with many vital cell components such as SH-groups, enzymes, cofactors, vitamins, nucleic acids and lipids.

B. ***Spectrum***

SO_2 is very active against yeasts and moulds. Bacteria on the other hand are more resistant, but the Gram-negative bacteria are more sensitive than the Gram-positive ones.

C. ***Applications in foods***

In acid foods (fruit, wine, pickles) SO_2 is applied to control the undesirable growth of micro-organisms. In sausage and fresh shrimp, SO_2 will inhibit spoilage of enzymes.

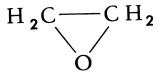
3.6.2.3. Ethylene oxide ($(CH_2)_2O$)

Ethylene oxide is a gas that will penetrate most organic materials (plastics, rubbers, paper, textiles, soil, powder). It is an alternative for sterilisation of heat-, moisture-, and radiation-sensitive objects.

A. ***Effects***

The lethal effect occurs following first-order kinetics, in other words, linear as a function of time. The time required for sterilisation depends on (1) number of cells, (2) concentration of gas, (3) temperature and relative humidity (30 %) and (4) resistance of the material to penetration. The mechanism is based on the reaction of ethylene oxide with bacterial protein at the amino, mercapto, carboxyl

and hydroxyl group:


= $(CH_2CH_2)O$

$R\text{-}SH + (CH_2CH_2)O \rightarrow R\text{-}S\text{-}CH_2\text{-}CH_2\text{-}O\text{-}H$

$R\text{-}NH_2 + (CH_2CH_2)O \rightarrow R\text{-}NH\text{-}CH_2\text{-}CH_2\text{-}O\text{-}H$

$R\text{-}COOH + (CH_2CH_2)O \rightarrow R\text{-}COO\text{-}CH_2\text{-}CH_2\text{-}O\text{-}H$

$R\text{-}OH + (CH_2CH_2)O \rightarrow R\text{-}O\text{-}CH_2\text{-}CH_2\text{-}O\text{-}H$

B. ***Spectrum***

Yeasts and moulds are sensitive. Vegetative bacterial cells are twice as resistant as yeasts and moulds. Bacterial spores are 10 times as resistant as vegetative cells.

C. ***Applications in foods***

Ethylene oxide is widely used to reduce microbial contamination in various dried foods, such as herbs, spices, gums, dried fruit, dried vegetables. Protein-rich foods will not be treated with ethylene oxide because vitamins and essential amino acids would be destroyed. Moreover, toxic residues are formed, and this occurs as follows:

$(CH_2)_2O + H_2O \rightarrow CH_2\ OH\ CH_2\ OH$ (ethyleneglycol)

$(CH_2)_2\ O + H_2O + Cl \rightarrow CH_2\ Cl\ CH_2\ OH$ (ethylenechlorehydrine-chloorethanol)

$+OH^-$

$CH_2\ OH\ CH_2\ OH$ (ethyleneglycol)

3.6.2.4. Ozone (O_3)

A. ***Effect***

Ozone has an effect on dehydrogenases which causes an interference with the cell respiration. Ozone oxidises the SH-groups of essential amino acids and unsaturated fatty acids of the cell wall and the cytoplasmic membrane, which causes leakage. A series of factors affecting the effect of O_3:

- The stage of growth at which ozone is applied: cells in the exponential phase are more resistant than cells in the stationary phase.
- pH: the effect of O_3 increases if pH decreases.
- Temperature: decreasing the t°, increases the effect of O_3.
- Relative humidity: O_3 has the best effect at RH = 60 -80 %
- Organic material: organic matter surrounding cells protects them from the destructive action of ozone.

B. ***Spectrum***

Bacteria are more sensitive to O_3 than yeasts and moulds. Bacterial spores are 10 to 15 times as resistant as the similar vegetative cells. Gram-positive cocci are more sensitive than Gram-negative rods, but the latter are more sensitive than Gram-positive rods.

C. ***Applications in foods***

Ozone is applied in treatment of water, disinfection of air and surfaces.

3.7. NATURAL ANTIMICROBIAL SYSTEMS

3.7.1. LACTOPEROXIDASE SYSTEM (LPS)

The peroxidases are a class of enzymes that do not poses any antimicrobial activity per se, but in combination with appropriate cofactors, mediate the formation of antimicrobial compounds. One example of such an enzyme is lactoperoxidase (LP), the most abundant enzyme in milk. With a halide or the pseudohalide SCN^- and H_2O_2, LP forms the powerful antimicrobial LPS.

3.7.1.1. Components of LPS

Lactoperoxidase (LP). LP is a glycoprotein that constitutes approximately 1 % of the whey proteins. The levels in milk amount to 10-30 mg/ml.

Thiocyanate (SCN^-). SCN^- is present in animal tissues and secretions and is formed from the detoxification reactions between thiosulphates and metabolic products of sulphur amino acids or cyanide or via the diet from foods containing SCN^-.

Hydrogen peroxide (H_2O_2). H_2O_2 is normally not present in foods. H_2O_2 is generated by a) catalase negative organisms (such as lactic acid bacteria) under anaerobic conditions, and b) the action of xanthine oxidase, glucose oxidase, ascorbic acid and sulphydryl oxidase in the presence of oxygen. However, it is immediately neutralised by catalase, peroxidase and superoxide dismutase present in milk.

3.7.1.2. Lactoperoxidase catalysed oxidations

Lactoperoxidase catalyses the oxidation by H_2O_2 of certain halides (Br^+ and I^-) and the pseudohalide SCN^-. The oxidation occurs as follows:

$$H_2O_2 + X^- \rightarrow H_2O + OX^-$$

The mechanism of the lactoperoxidase catalysed oxidation of thiocyanate is as follows:

$$2SCN^- + H_2O_2 + 2H^+ \rightarrow (SCN)_2 + 2H_2O$$

$$(SCN)_2 + H_2O \rightarrow HOSCN + SNC^- + H^+$$

$$HOSCN \rightarrow OSCN^- + H^+$$

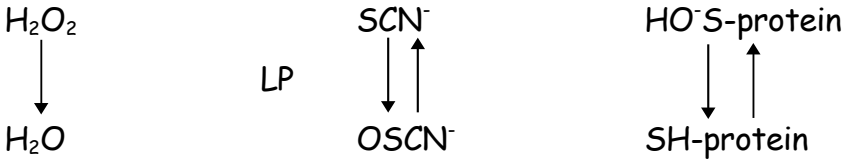
SCN^- may also be oxidised directly to $OSCN^-$:

$$SCN^- + H_2O_2 \rightarrow OSCN^- + H_2O$$

The anions OX^- and SCN^- may oxidise microbial components.

Hypothiocyanate ($OSCN^-$) is the major oxidation product of the lactoperoxidase catalysed oxidation of SCN^-. There is an equilibrium between $OSCN^-$ and HOSCN (pK_a = 5.3). Hypothiocyanate ($OSCN^-$) oxidises free SH-groups of bacterial cells to the corresponding disulphides (S-S), sulphenylthiocyanates (S-SCN) or sulphenic acids (S-OH).

The reaction may be summarised as follows:



$OSCN^-$ oxidises NADH and NADPH to NAD^+ and $NAPH^-$. The functioning of cellular systems is thus perturbed and damage caused to the cytoplasmic membrane, sugar and amino acid transport system or glycolytic enzymes. This may lead to cell death or stasis due to inhibition of vital metabolic functions such as respiration. A threshold concentration of $OSCN^-$ is required before significant inhibition occurs. Once attained, inhibition rises with a small increase in $OSCN^-$. This suggests that a minimal (critical) number of cellular components must be oxidised (10^5 - 10^7) before significant inhibition can occur.

A wide range of bacteria is affected by LPS. Gram-negative bacteria such as coliforms, ***Pseudomonas spp.***, ***Salmonella spp;***, and ***Shigella spp.*** are only temporarily inhibited. This different response is due to a different structure and to the cell wall.

3.7.1.3. Applications

LPS is used for short-term preservation of raw milk. LPS is latent in milk. The normal SCN^--concentrations of 3-5 ppm. are to low for optimal activity and H_2O_2 present in freshly drawn milk rapidly breaks down. This means that the system must be activated by the addition of 10-12 ppm SCN^- and 8-10 ppm H_2O_2. The addition of H_2O_2 may happen directly or indirectly, in other words, enzymatically in situ by glucose oxidase and xanthine oxidase, or chemically from sodium percarbonate. The concentration of H_2O_2 required to activate LPS is far lower than the FAO-approved level of 300-800 ppm. Low concentrations of added SCN^- are not harmful to human beings.

3.7.2. LYSOZYME

Lysozyme is a 1.4 ˜N- acetylmuramidase, cleaving the bond between the C-1 of N-acetylmuramic acid (NAM) and n-acetylglucosamine (NAG). Both sugars are basic units of the bacterial cell wall.

3.7.2.1. Mode of action of lysozyme

A) ***Substrate***

The basic unit of the bacterial cell wall is a disaccharide of two types of modified glucose, a-1-4 glycosidic bond. N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG). Its polymer is called peptidoglycan or murein. It is the major component of the cell wall of Gram-positive bacteria. This explains the specific sensitivity of those bacteria to lysozyme.

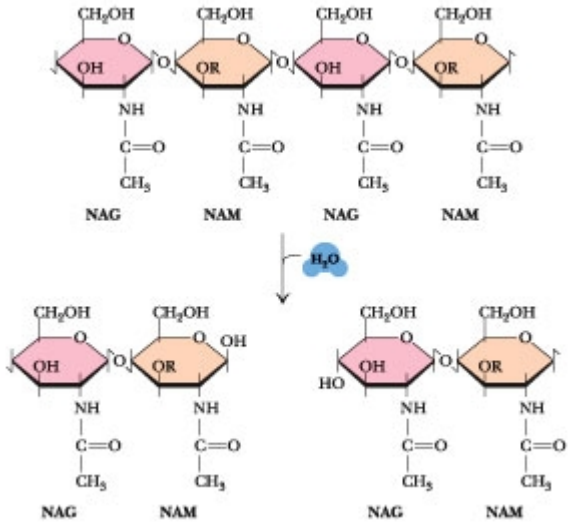


FIGURE 6. Effect of lysozyme on peptidoglycan.

The cell wall of Gram-negative bacteria on the other hand consists at the surface of lipoproteins an lipopolysaccharides, so as to better protect the underlying peptidoglycan (especially Enterobacteriaceae). Moreover, the sensitivity increases if EDTA is added to the growth medium. Chitin* (poly NAG) is also a substrate for lysozyme. Lysozyme cleaves the a-1-4 glycosidic bond between NAM and NAG (figure 6).

3.7.2.2. Factors that influence the effect of lysozyme

A)***pH***

The optimum pH of the lysozyme depends on the origin of the enzyme and on the substrate (table 23).

TABLE 23. pH optimum of lysozyme

Substrate	Fig lysozyme	Papaya lysozyme	Egg white lysozyme

Micrococcus lysodeiticus	4.5-4.7	4.5-4.7	5.0-5.7
chitin	4.1-4.6	4.2-6.4	4.5-6.0

B) ***Ion strength***

The optimal ion strength also depends on the origin of the lysozyme. The lysis of bacteria is inhibited by 5 % NaCl at pH> 7.6 and <5.7. The optimum salt concentration appears to be 0.365 % NaCl. NaCl has a positive effect on the fixation of the enzyme on the substrate. The chlorine ion functions as co-enzyme. The growth phase of the bacteria is also important for the effect of NaCl. The lysis of the cells in the stationary phase is caused by the presence of NaCl. Bacteria in the logarithmic growth phase are better lysed in the presence of NaCl.

C) ***Temperature***

Lysozyme is remarkably heat resistant. In hen's eggs, lysozyme is only partially activated at t° > 80 °c. The effect of heat is a reversible denaturation of the secondary and tertiary structure. The heat stability also depends on the pH of the medium. The heat-sensitivity increases as the medium becomes more alkaline.

D) ***Inhibitors***

The monomer NAG inhibits the action of lysozyme. Next to this, lysozyme can be inhibited by degradation products of lysis, and by histamine and other imidazol compounds. Since each amino acid has a specific role in the protein chain of the lysozyme, a change of these amino acids results in a change of the properties of lysozyme. Acetylation of lysine, nitration of tyrosine and oxidation of tryptophan cause an increase in the activity of lysozyme.

E) ***Applications of lysozyme in foods***

Lysozyme is quite often applied in cheese manufacturing against "blown" defect, caused by ***Clostridium*** spp. Lysozyme has a lytic effect on the vegetative form of ***Clostridium tyrobutyricum*** and on the clostridia in general. Basically, lysozyme can be applied in food products subject to spoilage by Gram-positive bacteria such as heated foods, salted and/or dried foods, fermented foods, etc.

3.7.3. GLUCOSE OXIDASE - GLUCOSE SYSTEM

The enzyme glucose oxidase is a β-D-glucose: oxygen oxido- reductase. It catalyses the oxidation of β-D-Glucose by oxygen with formation of β-D-glucono-d-lactone and H_2O_2. The β-D-glucono-d-lactone hydrolyses spontaneously with formation of gluconic acid.

$$\text{b-D-glucose} + O_2 \rightarrow \text{b-D-glucono-d-lactone} + H_2O_2$$

$$\text{b-D-glucono-d-lactone} + H_2O_2 \rightarrow \text{D-gluconic acid}$$

The reaction proceeds until glucose or the present oxygen have disappeared. If catalase is present, H_2O_2 is converted into H_2O and O_2.

3.7.3.1. Mechanism of action

The mechanism of action of glucose oxidase would be a changing oxidation and reduction of the prosthetic group FAD, as it is the case with other flavone nucleotide containing enzymes. The oxidised form of the enzyme E. FAD acts as a dehydrogenase and frees two hydrogens from the □D-glucose, with formation of the reduced enzyme E. FAD H_2 and β-D-glucono-d-lactone. Subsequently, the d-lacton (nonenzymatic) hydrolyses the D-gluconic acid and the reduced enzyme is again oxidised by molecular oxygen as terminal hydrogen acceptor (figure 7).

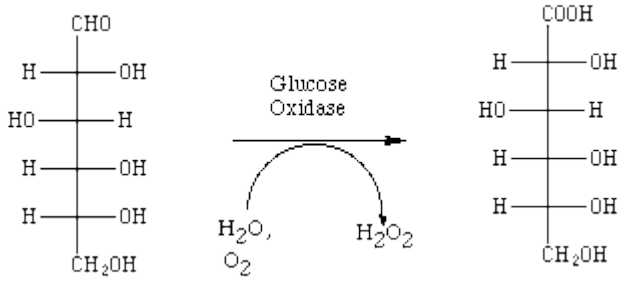


FIGURE 7. Oxidation of β-D-glucose to D-gluconic acid by glucose oxidase.

3.7.3.2. Activity

A) ***Substrate specificity***

Glucose oxidase shows a large specificity for β-D-glucose. The enzyme oxidises nearly 200 times faster the β-D-glucose than the β-anomer. Also the activity of glucose oxidase with β-D-glucose is 100 times bigger than with any other natural monosaccharide. Both the α- αβ-s the anomer of glucose are subject to mutarotation in an aqueous solution, and this results in a mixture with an equilibrium composition of 38% α-anomer and 62% β-anomer. Because of the presence of mutarotase in commercial preparations the equilibrium between α- and β-anomers is always maintained.

Table 24 gives a summary of the substrate specificity of glucose oxidase for different substrates.

TABLE 24. Substrate specificity of glucose oxidase

Substrate	Relative rate
b-D-glucose	100
a-D-glucose	0.64
2-Deoxy-D-glucose	3.30
D-Mannose	0.98
3-Deoxy-D-glucose	1.00

Substrate	Relative rate
D-Galactose	0.50
4-Deoxy-D-glucose	2.00
5-Deoxy-D-glucose	0.05
L-Glucose	0.00
6-Deoxy-D-glucose	10.00

B) ***Effect of pH***

The pH activity curves, both of glucose oxidase from ***Penicillium*** spp. as from ***Aspergillus*** spp. show a large activity between pH of 4.5 and 7.5 with a strong decrease outside this range.

c) ***Effect of temperature and dissolved oxygen***

In general the optimal temperature for the enzymatic activity is determined there, where the activity contribution of a specific temperature is cancelled by an increased destruction rate. In the case of glucose oxidase, change in temperature also means change in oxygen concentration: the solubility of oxygen decreases if the temperature increases. At temperatures above 30 °C, the benefit of a faster reaction rate goes lost because of the small solubility of oxygen. Table 25 proves that the reaction rate remains relatively unaltered between 30 °C and 60°C. Glucose oxidase shows an activity between chilling temperatures and 50 °C.

D) ***Inhibitors and activating substances***

Sodium nitrate and bisulphite show a partial inhibition. Copper ions also have an inhibitory effect. Arabinose can form a compound on the allosteric centre, which prevents binding of glucose. Formaldehyde is a strong inhibitor.

E) ***Stability***

Glucose oxidase is relatively stable in a wide range of pH and temperatures. One of the major stabilisers is the substrate (glucose). Within 10 minutes, 90% of the activity goes lost at pH=8.1 in the absence of glucose, whereas only 20% loss of activity occurs at the same pH within 40 minutes in the presence of glucose.

In practice, thermal stability is a very important property. Glucose oxidase is used amongst others for the removal of oxygen in hot, filled cans. This way, methods to stabilise glucose oxidase during heat treatment by keeping the enzymes dry, were developed. Stabilisation is obtained by preparing the enzyme in a tablet and subsequently encapsulating it in a hot water-insoluble capsule. This way pasteurisation temperatures up to 87 °C can be overcome. During the cooling-off period the enzyme is released, and the removal of oxygen can start.

TABLE 25. Effect of temperature on the activity of glucose oxidase.

Temperature, °C	Relative activity	Q10
0	0.51	1.15
10	0.59	1.30
20	0.77	1.30
30	1.00	1.10
40	1.10	1.10
50	1.05	0.95
60	1.02	0.97

3.7.3.3. Antimicrobial effect

The use of glucose oxidase is important in the glucose oxidase system, because of the production of hydrogen peroxide, which has antimicrobial properties. In order to obtain optimal results, there has to be a correct ratio between the enzyme and the glucose substrate. Excessive glucose oxidase causes rapid exhaustion of the amount of glucose, and in time, there will be no microbial activity left. For maximal activity, a continued production of hydrogen peroxide is required.

The inhibitory effect causes an extended lag phase and a retarded exponential growth.

A) ***Hydrogen peroxide and gluconic acid***

Hydrogen peroxide is a powerful oxidant, which rapidly dissociates in H_2O and O_2:

$$2\ H_2O_2 + O_2 \rightarrow \quad 2\ H_2O + O_2$$

The dissociation is accelerated by the presence of heavy metals, catalase and lactoperoxidase. Changes in factors of environment, such as pH and temperature, also influence the rate of dissociation.

The activity of H_2O_2 doubles for each rise in temperature of 10 °C, but increased temperature also causes accelerated dissociation. The stability of hydrogen peroxide decreases as the pH increases. Dissociation occurs rapidly at pH = 10. The antimicrobial effect of hydrogen peroxide can be attributed to the oxidative properties. The effectiveness varies depending on temperature, pH, H_2O_2 concentration, microbial change and the species present. An important oxidation is the peroxidation of membrane lipids, with formation of a hydroxyl radical (OH°), by the reaction of hydrogen peroxide with a superoxide anion:

$$O_2^- + H_2O_2 \rightarrow \quad OH^- + OH^\circ + O_2$$

Hydrogen peroxide would interfere with the electron transport system or react with cell proteins or break down specific molecular cell structures. Another action would consist of the injury of genetic material of the bacterial cell.

Hydrogen peroxide reacts with glucose, which results in a loss of activity. Hence the importance of the optimal ratio between glucose oxidase and glucose in practice.

Gluconic acid is used in food as complex-former with metal ions and as sweetener in diabetic products. The inhibition of microbial spoilage by gluconic acid is due to the decrease in pH, by which the organoleptic properties of the product are maintained. An example of this is the preservation of fish and shellfish. The preservative effect of sorbate and sorbic acid can be raised via a decrease in pH by gluconic acid, because those organic acids show a better antimicrobial effect at low pH-values.

B) ***Antimicrobial effect of hydrogen peroxide***

Bacterial spores are the most resistant to hydrogen peroxide, followed by Gram-positive bacteria. Gram-negative bacteria, especially coliforms are less resistant. An important factor in the protection against hydrogen peroxide is the presence or absence of catalase. The succeptibility of bacteria to H_2O_2 is in general related to the catalase levels of the bacteria. Bacteria which do not dispose of catalase are the most sensitive to hydrogen peroxide (mostly anaerobes and facultative anaerobes).

Among bacterial spore-formers, ***Clostridium*** spp. are less resistant than ***Bacillus*** spp. Spores of ***Bacillus subtilis*** are extremely resistant. The tolerance of ***Staphylococcus aureus*** to hydrogen peroxide is situated between that of bacterial spore-formers and that of Gram-negative bacteria. This also applies to lactic acid bacteria.

Lactic acid bacteria are catalase-negative. They can only protect themselves form hydrogen peroxide by means of their NADH-peroxidase, which breaks down hydrogen peroxide to 2 water molecules. In fact, it is a defence against self-destruction, because lactic acid bacteria are capable of producing hydrogen peroxide.

C) ***Applications***

Next to the previously mentioned properties of H_2O_2 formed by the glucose oxidase system, this peroxide may also be used as substrate of the LPS (see 3.7.1.).

3.7.4. BACTERIOCINS

Bacteriocins are proteinaceous antimicrobial substances produced by bacteria with an intraspecies antagonistic effect. To consider an inhibitory substance to the group of bacteriocins, a number of criteria has to be fulfilled:

- compared to other species, bacteriocins have a narrow spectrum;
- they need to have an essential, biological part in order to be active;
- they are absorbed by specific cell receptors present on the cell wall and acts as a lethal biosynthesis;

- the genetic information for bacteriocin production and for the immunity to the proper bacteriocin is plasmid encoded.

3.7.4.1. Properties of bacteriocins

A) ***Chemical properties***

Bacteriocins form a heterogeneous group of compounds with a protein structure. Some bacteriocins are pure proteins, whereas others form complicated complexes. These complexes may consist of a protein part and a lipid and/or carbohydrate part.

The nature of the bacteriocin molecule can be determined by means of enzymes such as proteases and lipases. These enzymes act on the molecule by the release of essential components, and hence the bacteriocin is inactivated.

B) ***Physical properties***

There are bacteriocins with low molecular weight (lactacin B) as well as bacteriocins with high molecular weight (helvecitin J).

C) ***Stability***

Bacteriocins of ***Lactobacillus*** spp. may be divided into two groups: heat-labile and heat-stable bacteriocins.

- Heat-labile bacteriocins. These bacteriocins are inactivated at high temperatures. Examples are acidofilucine A, inactivated if heated at 60 °C for 10 minutes and helvecitin J, inactivated if heated at 100 °C for 30 minutes.

- Heat-stable bacteriocins. These bacteriocins are not inactivated by heating at high temperatures. This group includes amongst others lactacin F (no inactivation after 15 min. at 121 °C) and sakacin A (no inactivation after 20 min. at 100 °C).

Both types of bacteriocins are inactivated by proteolytic enzymes. Besides temperature, pH of the environment has also a great effect on the stability of the bacteriocins. Some bacteriocins are stable within a narrow pH-range (e.g. nisin), whereas others remain stable over a wide pH-range (e.g. pediocin AcH).

D) ***Spectrum***

Some lactic acid bacteriocins are only active against closely related species (lacticin B), whereas other bacteriocins have a broad spectrum (nisin, pediocin A).

3.7.4.2. Mechanisms of action of bacteriocins.

Bacteriocins kill sensitive cells in a "single hit" process. This means that one bacteriocin molecule is sufficient to kill a sensitive cell. However, the possibility that one single molecule will kill a cell is very small, since there is a large number of non-lethal adsorption places on the cell wall. The mechanism of action has two phases: the

adsorption phase and the lethal phase.

- Adsorption phase. This phase includes the physical adsorption of the bacteriocin particles to the receptors of the cell wall. Damage to the cells does not occur during this phase.

- Lethal phase. During this phase, the major actions interfere with energy production, synthesis of the macro-molecules, membrane transport and membrane permeability.

3.7.4.3. Applications of bacteriocins

Up to now, only the World Health Organisation (WHO) allows nisin as preservative in food industry. Use of nisin is mainly used for the preparation of cheese spread and other melted cheeses, to inhibit sporulation of ***Clostridium***. Especially butyric acid bacteria (***Clostridium butyricum*** and ***clostridium tyrobutyricum***) are undesired, since they cause the "blown" defect.

Other applications of nisin are:

- in low-acid and semi-acid canned foods to inhibit sporulation of thermophilic spores of ***Bacillus stearothermophilus*** ("flat sour") and of ***Clostridium thermosaccharolyticum***;
- in strong-acid canned foods (pH < 4.5) nisin is used to inhibit sporulation of acid tolerant spores of ***Clostridium*** and ***Bacillus macerans*** ;
- to extend shelf life of foods and alcoholic beverages such as meat, salads, beer and wine.

In beer and wine, nisin could be used to inhibit spoilage caused by lactic acid bacteria. Nisin could also be used in the manufacturing of brandy. The sometimes bad sensory quality of this product is amongst others due to the development of micro-organism that produce buthanol or ethylacetate. Nisin could inhibit growth of these micro-organisms without influencing the yeast fermentation, and this way a higher alcohol level could be obtained.

Shelf life of raw meat preparations such as ham is extended if nitrite is added. The toxicological safety of nitrite becomes more and more doubtful (formation of nitrosamines that are carcinogenic), so that other preservative techniques are searched. A possible alternative for nitrite is nisin. However, experiments have demonstrated that the activity of nisin in meat preparations is far below expectations. This small activity is amongst others explained by adsorption of nisin to meat and fat particles, small solubility, unequal division and possible interference of phospholipids with the mechanisms of action of nisin. Next to this, the presence of nisinase, an enzyme that is produced by some lactic acid bacteria and that is

responsible for inactivation of the polypeptide, may also cause a smaller activity.

The use of bacteriocin-producing lactic acid bacteria as starter cultures for the preparation of fermented food products is going under extended research to industrial applications.

The raw materials used for fermentation of vegetables are by nature strongly contaminated. Inactivation of undesired micro-organisms by heating cannot be applied, either for economical reasons or for quality reasons. Hence, the used starter cultures have to be very competitive in order to dominate in the fermentation culture. It is mainly the natural occurring lactic acid bacteria flora that cause impure starter cultures. Use of bacteriocin-producing strains can thus inhibit the undesired flora, and this way a pure fermentation culture can be obtained.

In meat industry, lactic acid bacteria are mainly applied as starter cultures for preparation of fermented sausages. Lactic acid bacteria contribute amongst others to flavour formation, but also cause decrease in pH, resulting in an extended shelf life. The raw materials that are used, are by nature contaminated with spoilage organisms, among which numerous lactic acid bacteria. Hence, an important function of the used starter cultures is inhibiting these competitive flora.

CHAPTER 4. SPOILAGE OF FOOD PRODUCTS

4.1. MEAT AND MEAT PRODUCTS

4.1.1. FRESH MEAT (CARCASS)

Fresh meat has a high a_w and a pH between 5.5 and 7.0. Moreover, meat is rich in proteins, vitamins and minerals and low in carbohydrates (< 1%). This means that meat is an ideal substrate for microbial growth.

DFD-meat ("dark, firm and dry") has a pH > 6.2, and as a result, bacterial spoilage will occur sooner than in PSE-meat ("pale, soft and exudative"), its pH being < 6.2.

At temperatures < 10 °C and R.H. > 95 %, spoilage of meat is mainly due to growth of psychrotrophic bacteria, especially ***Alteromonas putrefaciens***, ***Acinetobacter***, ***Moraxella***, and ***Alcaligenes***.

At a R.H. <80 %, the surface layers of the carcass lose the greater part of their water. By this the a_w drops and moulds may grow. Spoilage usually begins to be evident when the number of bacteria reaches $10^7/cm^2$. This is accompanied by discoloration, slime formation and typical spoilage odours.

4.1.2. VACUUM PACKED PRIMAL JOINTS

Inside a vacuum package, the residual oxygen is consumed by tissue respiration and is replaced by CO_2. Because of this, inhibition of growth of ***Pseudomonas***, ***Acinetobacter***, and ***Moraxella*** occurs, and hence an extended shelf life is obtained. These aerobic bacteria will consume all of the residual oxygen and subsequently ***Microbacterium thermosphactum*** and ***Enterobacteriaceae*** (facultative anaerobes) will grow. Finally, growth of Gram-positive flora of lactic acid bacteria, the so called "atypical streptobacteria" commences.

In general it is believed that growth of Gram-positive bacteria in vacuum packed meat occurs slower than growth of Gram-negative bacteria in meat packed in air-permeable packs. It can be determined that in meat with pH < 6.0 (presence of glucose) and stored at 0-2 °C the total count may attain 10^7-$10^8/ cm^2$ after about two months, with only a general souring. On further storage the odours become more cheesy, because of the presence of short-chain fatty acids (C_2-C_6) such as acetic acid and butyric acid. In meat with pH > 6.0 (contains less glucose) spoilage is mainly characterised by breakdown of amino acids by ***Pseudomonas***, ***Aeromonas*** and lactic acid bacteria. Especially H_2S (greening), amines and fatty acids are formed. For these reasons, DFD-meat is regarded as unsuitable for vacuum packing.

4.1.3. RETAIL CUTS

Retail cuts are subjected to aerobic spoilage because of the greater surface to volume ratio. These spoilage processes are similar to those of carcass meat. Spoilage of vacuum packed retail cuts resembles that of vacuum packed primal joints (see 4.1.2.).

4.1.4. COMMINUTED MEAT

Comminuted meat is more perishable than intact meat because of the greater availability of meat juice and because surface microbes are distributed throughout the mass during mincing. The total count of comminuted meat is > 10^5/g. If it reaches 10^8/g, spoilage becomes evident.

Sometimes SO_2 is fraudulently added to minced meat, to preserve the red meat colour. Thus SO_2 misleads the consumer as to the freshness of the meat. SO_2 also slows down bacterial spoilage (see 3.6.2.2.).

In aerobically stored meat, there are aerobic conditions at the surface, and hence the colour of freshly minced meat is red (oxymyoglobin). This means that growth of ***Pseudomonas***, ***Acinetobacter*** and ***Moraxella*** will occur, which will increase the pH due to proteolytic activities.

4.1.5. DEEP-FROZEN MEAT

In deep-frozen meat stored at - 18 °C, microbial spoilage is not possible. If the initial count (before freezing) is high, the microbially formed enzymes may be active and cause spoilage. Hence, it is preferable to store the deep-frozen meat at - 24 °C, as at this low temperature enzymatic activities are not possible. Exposure of meat to temperatures in the range -5 °C to -10 °C permits development of moulds (black spots) but there is little off-odour or odd-flavour.

4.1.6. MEAT PRODUCTS

4.1.6.1. Raw cured meat

Typical examples of raw cured meat are ham and bacon. These are products with low a_w, obtained by addition of salt, combined with drying. At those low a_w-values only salt-tolerant micrococci, lactic acid bacteria and moulds can grow. Those micrococci and lactic acid bacteria, however, are little proteolytic and mostly lipolytic, so that the question arises whether they are not more beneficial to flavour than they are spoilage organisms. Spoilage caused by these micro-organisms is accompanied by slime formation and off-odour and off-flavour.

4.1.6.2. Raw meat preparation

A typical example of raw meat preparation is dry sausage. The extension of shelf life is based on a reduced pH by lactic acid fermentation combined with a decrease in a_w by drying. Only moulds are capable to grow on such stable products.

4.1.6.3. Heated meat products

A) ***Pasteurised meat products***

Pasteurised meat products were subject to temperatures below 100 °C. In the first place, this can be done in the final package, as it is the case with sausages and paté. Thermoresistant spores (***Bacillus*** and ***Clostridium***) and some vegetative cells (***lactobacilli*** and ***streptococci***) can survive this heat treatment and thus cause spoilage in this group of meat products. Factors that may play a role are:

(a) pasteurisation temperature: the higher, the less possibility of surviving.

(b) storage temperature: low temperatures will prevent the spores from germination and any surviving vegetative cells (mostly thermophiles) from growing; psychrotrophic lactic acid bacteria and faecal streptococci, however, can develop at chilled temperatures.

(c) a_w-value: the lower, the slower the spoilage.

(d) pH-value: the lower, the slower the spoilage, except for the thermoresistant lactic acid bacteria which sometimes have a psychrotrophic character.

If stored under refrigeration, spoilage will be nonproteolytic but rather be characterised by acidification, combined or not with gas formation from the present (added!) carbohydrates by psychrotrophic lactic acid bacteria (***Leuconostoc***, faecal streptococci) or by ***Microbacterium thermosphactum***.

A second group of pasteurised meat products are those that are manipulated after heating (cooling, portioning, packing) and this causes spoilage. Similar factors as those discussed with meats heated in the final package, play here an important role. In practice, this means that under refrigeration the spoilage process of these heated meat products with low a_w an pH can be compared to spoilage of raw meat. If stored under refrigeration, spoilage can thus be both proteolytic (by gram-negative bacteria from the postprocessing contamination) as an acidificaton with or without gas formation (due to recontamination with lactic acid bacteria). During storage at high temperatures, thermoresistant spores of ***Bacillus spp.*** will germinate and form gas from the present carbohydrates, and clostridia may produce H_2S from proteins and gas from carbohydrates.

B) ***Sterilised meat products***

Sterilised meat products have been subject to temperatures above 100 °C in a hermetically sealed package. Following spoilage phenomena may occur in sterilised meat products:

(a) Spoilage prior to heating: this can be determined microscopically by the presence of a large number of death micro-organisms.

(b) Spoilage occurring between filling and sterilisation: this is usually accompanied by gas formation and as a result the containers will swell.

(c) Spoilage as a result of the survival of heat resistant spores (e.g. ***Clostridium sporogenes***), this indicates under-sterilisation.

(d) Spoilage as a result of leakage: by this the organisms find their way into the product via cooling-water and/or air that may be microbially contaminated.

4.2. POULTRY

4.2.1. FRESH POULTRY (CARCASS)

The intrinsic properties of poultry are as follows: high a_w-value, pH between 5.7 and 6.7, rich in proteins, low carbohydrate level, rich in vitamins and minerals. This leads to the conclusion that microbial spoilage may occur.

At low preservation temperatures, spoilage is typically psychrotrophic, by ***Pseudomonas spp.***, ***Alteromonas putrefaciens*** and ***Acinetobacter-Moraxella***. For example, spoilage of turkey, preserved at 1 °C, will be caused by ***Pseudomonas spp.***. If the total count on the skin attains 10^7-10^8/cm², a typical spoilage odour will develop. With total counts above 10^8/cm², slime formation occurs, which shows a fluorescent green colour under an UV-lamp, due to the production of fluorescent pigments by ***Pseudomonas spp.***. By preservation at 10 °C, the spoilage pattern of turkey completely differs from that at 1 °C, since especially ***Enterobacter liquefaciens*** and atypical lactic acid bacteria will grow.

Meat of poultry is sterile immediately after slaughter. Only after advanced damage to the skin, micro-organisms will migrate through the meat. A distinction should be made between the meat of the legs (pH = 6.4 - 6.7) and that of the breast (pH = 5.7 - 5.9). As to spoilage, ***Acinetobacter*** and ***Alteromonas*** grow better in legs than in breasts, ***Pseudomonas spp.*** grow well in both.

4.2.2. PREPACKED POULTRY

By use of high oxygen-permeable packing film, mainly ***Pseudomonas spp.*** will develop. If an oxygen-impermeable film is applied, growth of ***Alteromonas***, ***Microbacterium thermosphactum*** and atypical lactic acid bacteria occurs. This is due to the fact that there is an accumulation of CO_2, which has an inhibitory effect on the aerobic ***Pseudomonas spp.*** and hence stimulates the growth of facultative anaerobic and micro-aerophilic bacteria. In fully vacuum-packed poultry, mainly ***Enterobacter*** will develop.

4.2.3. DEEP-FROZEN POULTRY

Deep-frozen poultry preserved at - 18 °C is not subject to microbial spoilage. By preservation at - 7 °C only growth of moulds is possible. The following types are qualified: ***Cladosporium herbarum*** (black spots), ***Thamnidium elegans*** and ***Thamnidium chaetocladioides*** (hairy growth) and ***Sporotrichum carnis*** (white spots).

4.2.4. HEATED POULTRY PRODUCTS

The spoilage processes of this group of food products are similar to those of heated meat products (see 4.1.6.3.).

4.3. FISH, SHELLFISH AND MOLLUSCS

The intrinsic properties of fish, shellfish and molluscs are as follows: high a_w, neutral pH, depending on the glycolysis during the "rigor mortis" period, high level of N-substances, rich in vitamins and minerals. The level of carbohydrates in fish and shellfish is low (< 1%) whereas that of molluscs is high (> 3 % glycogen).

The N-substances can be divided into, on the one hand the nonprotein N-fraction (trimethylamine oxide, (TMAO) ; dimethylamine oxide (DMAO) ; urea ; peptides ; amino acids ; nucleic acids) and on the other hand the protein-fraction which is converted into peptides and amino acids by proteolytic activities. This leads to the conclusion that the intrinsic factors of fresh fish are optimal for microbial growth.

4.3.1. SPOILAGE OF FISH (MARINE)

Marine fish contains TMAO as osmoregulator. Fresh water fish does not contain TMAO. Spoilage of fish (bony fish) is for 95 % microbial (the remaining 5 % is autolytic). Microbial spoilage immediately sets in after the "rigor mortis" and occurs mainly in two phases :

- 1st phase : breakdown of TMAO

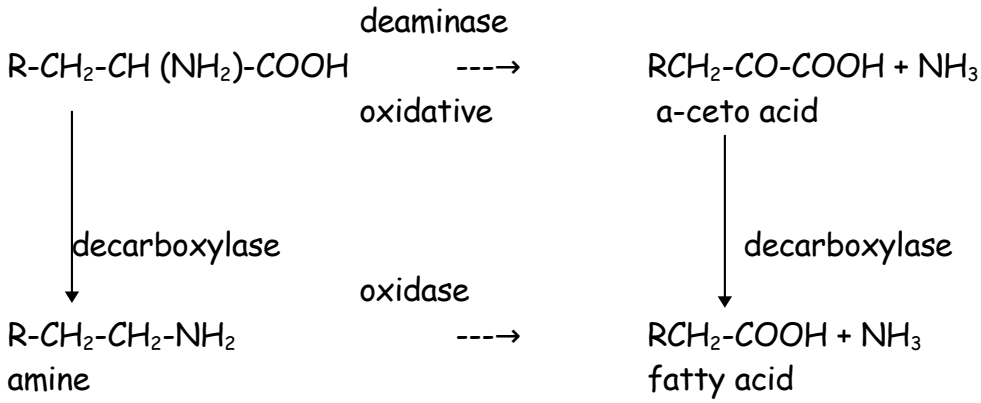
$$\text{TMAO} \xrightarrow[\text{by lack of oxygen (6 days in ice)}]{\text{TMAO-reductase}} \text{TMA}$$

TMAO-reductase is formed by some ***Pseudomonas***, ***Shewanella***, ***Acinetobacter-Moraxella***, Enterobacteriaceae and some Clostridia.

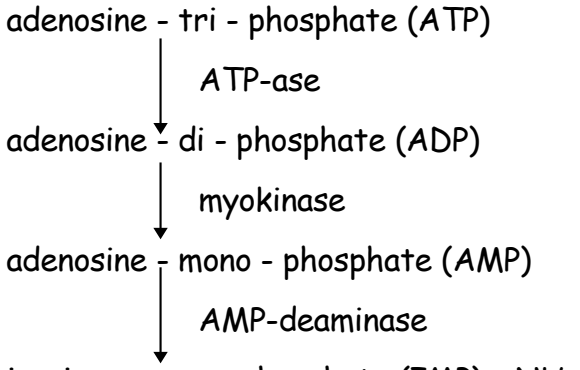
$$CH_3\,CHOH\,COOH + (CH_3)_3\,NO \rightarrow CH_3\,CO\,COOH + (CH_3)_3\,N + H_2O$$
lactic acid TMAO pyruvic acid TMA

$$CH_3\,CO\,COOH + (CH_3)_3\,NO + H_2O \rightarrow CH_3\,COOH + (CH_3)_3\,N + CO_2 + H_2O$$
pyruvic acid TMAO acetic acid TMA

- 2nd phase : breakdown of amino acids



In addition to this, there is the breakdown of adenonine triphosphate (ATP)



Other microbial metabolites may also be formed, e.g. histamine from histidine (scombroid poisoning) by ***Proteus morganii*** at pH = 6 to 4 and t° = 20 to 30°C. This is typical for tuna and mackerel.

Hydrogen sulphide and volatile sulphur compounds are formed from sulphur containing amino acids (cysteïne, cystine, methionine).

Spoilage of elasmobranches (e.g. ray, sharklike) is characterised by an rapid enzymatic formation of NH_3 from urea. In a later stage, spoilage is characterised by TMA production and formation of NH_3 and amines from amino acids by bacteria. The total of TMA, DMA, amines and ammonia is called the total volatile basic nitrogen-fraction (TVB) and is expressed in mg N/100 g fish flesh. For marine fish the proposed levels are as follows : 12-15 mg N/100 g for TMA and 35-40 mg N/100 g for TVB for fish stored in ice.

4.3.2. SPOILAGE OF SHELLFISH

The spoilage pattern of shellfish is similar to that of marine fish, but the spoilage occurs faster because there are more non protein N-substances present, thus accelerating the growth of spoilage organisms.

4.3.3. SPOILAGE OF MOLLUSCS

Molluscs (e.g. oysters, mussels) do not contain TMAO and contain 3 % glycogen. This means amino acids will be broken down to NH_3 and amines, whilst glycogen is converted into acids. This results in a reduced pH. The pH of fresh oysters is 6.2-6.5, and due to spoilage it decreases to 5.8 or below that value.

4.3.4. DEEP-FROZEN FISH

Deep-frozen fish is preserved at -18°C and microbial spoilage does not occur. However, at -7°C growth of moulds may occur.

4.3.5. SEMI-PRESERVES OF FISH

Fish marinades are subjected to spoilage by lactic acid bacteria, yeasts and moulds due to low pH (pH = ± 4.0). Salted and dried seafood, smoked or not, are mainly subjected to spoilage by gram-positive bacteria and moulds, due to reduced a_w-value.

4.4. MILK AND MILK PRODUCTS

4.4.1. RAW MILK

Souring of low milk results from growth of mainly ***Streptococcus lactis*** and also of coliforms, enterococci, lactic acid bacteria and micrococci. Ropy milk is caused by growth (with increased pH) of ***Alcaligenes viscolactis***.

4.4.2. PASTEURISED MILK

Pasteurised milk is subjected to spoilage because of growth of thermoresistant bacteria, such as some micrococci, enterococci, ***Streptococcus thermophilus***, lactic acid bacteria, aerobic spore-formers and anaerobic spore-formers. Those thermoresistant bacteria grow slowly at temperatures < 5°C. In the presence of large numbers however, pasteurised milk has only a shelf-life of 10-14 days at 5°C. Spoilage is only manifested by a acidification and gas formation. Spoilage may also occur due to post-pasteurization contamination with Gram-negative psychrotrophic bacteria. Finally spoilage in pasteurised milk may occur due to thermoresistant spore-formers, such as ***Bacillus cereus***.

Souring of pasteurised milk is caused by enterococci, ***Streptococcus thermophilus***, and lactic acid bacteria, which convert lactose into lactic acid. Micrococci, ***Microbacterium*** and ***Bacillus*** may also form lactic acid if there is no competition with lactic acid bacteria. ***Clostridium*** spp. form butyric acid, H_2 and CO_2 if there are no lactic acid bacteria present.

Gas formation in pasteurised milk is due to coliforms, clostridia and ***Bacillus***, which produce H_2 and CO_2 if there is no competition with lactic acid bacteria. Heterofermentative lactic acid bacteria and yeasts form CO_2.

Proteolysis in pasteurised milk occurs :

1) at low temperatures or

2) by destruction of lactic acid bacteria and other acid producers by heat or

3) by breakdown of acids formed by moulds and yeasts

4) by neutralisation of acids formed by alkaline metabolites formed by other micro-organisms.

There are several forms of proteolysis.

a) Acid proteolysis, caused by bacteria that survive pasteurisation such as micrococci, ***streptococcus faecalis var. liquefaciens***, spores of ***Bacillus*** : this type of proteolysis is characterised by acid production accompanied by proteolysis.

b) Proteolysis with little acid formation or with formation of alkaline substances by micrococci, ***Pseudomonas***, ***Alcaligenes***, ***Proteus***, ***Flavobacterium***, ***Serratia***.

c) Coagulation by enzymes, comparable to the activity of the rennet.

d) Enzymatic proteolysis by intracellular enzymes released after lysis.

e) Residual proteolytic activity by thermoresistant bacterial proteinases, produced during an undesired growth in pasteurised milk prior to pasteurisation. A typical example is ***Pseudomonas fluorescens***.

Another less frequently occurring spoilage phenomenon in pasteurised milk is ropiness. This phenomena may occur at the surface of milk as a result of growth of ***Alcaligenes viscolactis*** (typical ground water bacterium) at 10°C or of ***Micrococcus freudenreichii***. Ropiness in the entire, mass of milk is caused by some coliforms, some lactic acid bacteria, micrococci, streptococci and ***Bacillus***. Coliforms, micrococci and ***Bacillus*** can only cause ropiness if they are not suppressed by lactic acid bacteria.

4.4.3. MILK FAT

Milk fat can be broken down by lipase-producing bacteria, yeasts and moulds. Moreover ***Pseudomonas fragi*** and ***Staphyloccocus aureus*** produce heat-resistant lipases. Alkaline substances may produced in milk fat as a result of non-proteolytic bacterial conversions. Ammonia is also formed from urea, carbonates from citric acid. Alkaline substances are produced by ***Pseudomonas fluorescens*** and ***Alcaligenes viscolactis***.

4.4.4. CONDENSED MILK

Bulk condensed milk (not sweetened) has been subjected to pasteurisation by preheating and condensation. This means that thermoresistant vegetative cells and spores can survive. After preheating and condensation, the condensed milk is canned

and sterilised. If the milk was insufficiently sterilised, early spoilage may occur. This spoilage is manifested in various forms:

- gas formation by clostridia, this however should not be confused with overfilling or hydrogen-swelling ;

- coagulation by ***Bacillus*** spp. ;

- bitter taste due to proteolysis by ***Bacillus*** spp. and sometimes by ***Clostridium*** spp.;

- growth of non-spore-formers, that found their way into the can via leaks and

- cause gas formation coagulation and bitter taste.

Sweetened condensed milk contains 50-60 % sugar, in other words, this product has a low a_w-value. Moreover, the milk is subjected to a preheating process at 71-100°C, followed by a mild condensation process. Subsequently, the milk is canned and sterilised. The logical result of all this is a stable product. Leakage may cause contamination and growth of osmophilic yeasts with gas formation, of ***micrococci*** that make the sweetened condensed milk viscous and of surface growth of moulds.

4.4.5. BUTTER

The cream used to make butter is contaminated with lactic acid bacteria, gas formers and other spoilage organisms. Growth of those bacteria is accompanied by a decrease in pH to values that allow growth of moulds. For the preparation of butter, cream is pasteurised. Butter has a low water content and a high salt content in the liquid phase. This means that growth of bacteria is virtually impossible. Sometimes moulds may develop.

4.4.6. CHEESE

If cheeses are subject to abnormal fermentation, anaerobic spore-formers, yeasts and moulds may grow. Mould growth occurs on cheese surfaces and extend into the interior of the cheese along cracks or fissures. ***Clostridia*** form gas and this may cause internal voids and cracks (***Clostridium tyrobutyricum*** and ***Clostridium butyricum***).

4.4.7. FERMENTED MILK

4.4.7.1. Yoghurt

Yoghurt results from lactic acid fermentation in milk. (lactose is converted into lactic acid). By this a low pH (4.5-4.7) is obtained, allowing (surface) growth of yeasts since yeasts are not inhibited by the lactic acid. On the contrary, yeasts can convert the lactic acid to CO_2 accompanied by an unacceptable yeast and fruit-odour.

4.4.7.2. Buttermilk

Buttermilk is prepared from milk that has been inoculated with a starter culture, consisting of three types of lactic acid bacteria. ***Streptococcus lactis*** and ***Streptococcus cremoris*** are inoculated for lactic acid production while ***Streptococcus lactis diacetylactis*** will be responsible for flavour formation. The function of the first two listed is to produce lactic acid rapidly and that of the latter is to produce flavour compounds. However, these cultures may be contaminated with yeasts and other spoilage organisms, which may cause physical defects in the final product.

4.4.8. ICE-CREAM

As ice-cream is preserved in the deep-freezer, spoilage cannot occur. If the mix is not frozen promptly, spoilage can occur in the mix.

4.5. EGGS

4.5.1. SHELL EGGS

The micro-organisms that are usually present on the shell, can via the pores of the shell and through the membrane, end up in the egg white. Here a number of antimicrobial factors occur that can inhibit the growth of numerous types of micro-organisms (table 27). Yeasts can grow on the egg shell. "Pin spots" are formed in and on the shell. If the relative humidity increases, growth of moulds becomes evident in and on the shell. At a certain moment the mould mycelium reaches the egg white and causes a real mould rot.

4.5.2. LIQUID EGGS (BROKEN EGGS)

Eggs are broken, then homogenised as whole liquid egg or separated into white and yolk for further processing. Salt of sugar may be added and then these products should be pasteurised, chilled, filled into cans or tanks and shipped (chilled of frozen). Shelf-life of liquid egg-products depends on the GMP (breaking, pasteurisation, chilling and packing). Unpasteurised milk products have a shelf-life of 5-7 days at refrigerating temperatures. If GMP is respected during pasteurisation, the refrigerated shelf-life is 20 to 22 days ; but if this is not the case, shelf-life is only 2 to 3 days. The odours of spoilage are much more intense in yolk or whole egg than in egg white.

TABLE 26. Antimicrobial factors in the egg white

Component	Activity
Lysozyme	Lysis of cell walls of Gram-positive bacteria. Flocculation of bacterial cells.

Component	Activity
	Hydrolysis of β-1,4-glycosidic bonds.
Conalbumine	Chelation of iron, copper and zinc especially at high pH.
Riboflavin	Chelation of cations.
Glucose	Repression of respirartory capacity of facultative anaerobes.
pH 9.1-9.6*	Enhances chelating potential of conalbumin.
	Unsuitable pH for many organisms.
Avidin	Binds biotin, making it unavailable to bacteria that require it.
Aproprotein	Combine with riboflavin.
Ovoinhibitor	Inhibits fungal proteases.
Uncharacterized proteins	Combine with vitamine B6.
	Chelate calcium.

* The pH of egg white in newly laid eggs is 7.6-7.8. After a few days at room temperature, during which CO_2 evolves, the pH rises to 9.1-9.6.

TABLE 27. Various types of bacterial rot in eggs

Type of rot	Microorganism
Green	***Pseudomonas fluorescens***
Colourless (fruit odour)	***Pseudomonas***
	Alcaligenes
	Coliforms
Black (H_2S-odour)	***Proteus***
Pink (after green rot)	***Pseudomonas***
red (no odour)	***Serratia***

Salted yolks (> 7.5 % NaCl) are microbially stable except for surface growth of moulds. Dried products are microbially stable because of their low a_w.

4.6. VEGETABLES, FRUITS AND NUTS

4.6.1. VEGETABLES

4.6.1.1. Raw vegetables

Raw vegetables are characterised by high a_w, slightly acid to neutral pH, high Eh, high level of carbohydrate, presence of vitamins and minerals. From this it can be concluded that mainly spoilage by moulds and bacteria will occur. Soft rot is caused by ***Erwinia carotovora*** and ***Pseudomonas fluorescens***. Organisms causing spoilage

other than soft rot are ***Corynebacterium***, ***Xanthomonas*** and ***Pseudomonas***. If moulds develop on vegetables, the tissue becomes moist and weak.

4.6.1.2. Vegetable products

Vegetable juices are sterilised by high temperature during short time (HTST). If the juice is insufficiently sterilised, ***Bacillus coagulans*** can survive and cause spoilage. The typical spoilage phenomenon is termed "flat sour", in other words acid formation without gas formation (the containers do not swell).

Fermented and acidified vegetables can be contaminated and spoiled by lactic acid bacteria, and by yeasts and moulds (at the surface).

4.6.2. FRUITS

4.6.2.1. Raw fruits

Fruits are characterised by high a_w, acid to weak acid pH, high level of carbohydrates, vitamins and minerals. Because of this, the primary spoilage organisms of fruits are fungi, which will cause a breakdown of the structural components.

4.6.2.2. Fruit products

Fruit juices have low pH and reduced a_w because of the added sugars. The primary spoilage organisms are : yeasts and osmophilic yeasts in fruit juices containing a lot of added sugar. Surface growth of moulds may also occur. Some moulds are more heatresistant than yeast and resist pasteurisation temperatures above 80°C. Mainly ***ascospores*** and ***sclerotes*** are heatresistant and may predominate in pasteurised fruit juices. Heterofermentative lactic acid bacteria such as some ***Lactobacillus*** and ***Leuconostoc*** types can grow in fruit juices with pH > 3.5. Finally, ***Gluconobacter*** (***Acetomonas***) can also cause spoilage in fruit juices (souring).

Deep frozen food is microbially stable. After thawing, spoilage by yeasts will occur, as yeasts survive the freezing process and preservation at low temperatures.

Dried fruit is also microbially stable. By rehydration (increase in a_w) xerophilic moulds may start to grow. Growth of ***Xeromyces bisporus*** is already possible from a_w = 0.60.

As it is the case with fermented vegetables, fermented fruits are subject to spoilage by lactic acid bacteria, yeasts and moulds. The olive fermentation is quite similar to that of sauerkraut. Olives contain oleuropein that breaks down into aglycone and elenolic acid ; both inhibitory to lactic acid bacteria. Hence those compounds will influence the fermentation process negatively. So the olives have to

be treated with alkali first in order to extract oleuropein. If this treatment was insufficient, ***Leuconostoc mesenteroides***, responsible for fermentation, will be inhibited, and spoilage will occur as a result of growth of yeasts and coliforms.

In low salt brines of olives, yeasts such as ***Hansenula*** and ***Saccharomyces*** may form gas whereas ***Rhodotorula*** will soften the olives as a result of pectinolytic activity. Lactic acid bacteria can also grow in such brines and cause acid and gas formation.

4.6.3. NUTS

Nuts have a low a_w and are relatively microbially stable. If the a_w increases by rehydration, fungal growth may occur on the nuts.

4.7. SPICES AND HERBS

Spices and herbs are dried and consequently have a low a_w-value. This means that the products are microbially stable. As a result of their origin and of the way of drying they are highly microbially contaminated. After rehydration by addition of moist ingredients, the end products are susceptible to spoilage.

4.8. MAYONNAISE, DRESSINGS AND SALADS

4.8.1. MAYONNAISE

Mayonnaise is an emulsion of oil (80 %), egg yolk (7.5 %) and water (vinegar), to which salt and other flavourings are added. As pH is acid and a_w is low, mayonnaise is microbially stable. If the amount of added salt is to little, moulds (***Geotrichum***) and yeasts (***Pichia bini***) will cause spoilage.

4.8.2. DRESSINGS

Dressings are acid products with pH-values comparable to those of mayonnaise, but the liquid phase is much larger, and this way a higher a_w is obtained. Thus, spoilage may occur due to growth of a) lactic acid bacteria such as ***Lactobacillus fructivorans*** and ***Lactobacillus brevis*** that are heterofermenters and produce gas b) yeasts (***Saccharomyces baii***) and c) numerous mould types.

4.8.3. SALADS

Salads are mixtures of mayonnaise or dressing with various ingredients such as vegetables, meat, poultry, eggs, fish, shellfish and molluscs. Those products are characterised by an acid to semi-acid pH and a relatively high a_w. As a result, spoilage will occur by lactic acid bacteria which cause gas formation and by yeasts and moulds.

4.9. DRINKS

4.9.1. Non-alcoholic drinks

A. ***Natural mineral waters***

The autochtonous microflora of mineral waters is composed of : ***Flavobacterium***, ***Cytophaga***, ***Micrococcus***, ***Nocardia***. These autochtonous bacteria have low nitrogen requirements ; they need only small amounts of organic compounds and are chemo-organotrophic rather than autotrophic.

In general the TVC is small (10-100/ml). These flora are mainly aerobic or psychrotrophic. Shortly after bottling when the open system (the source) is exchanged for a closed system (the bottle), multiplication of the autochtonous bacteria begins. This growth is characterised by an alternating increase and decrease in the number of bacteria. Each new population of cells is often composed of another species, using the death cell of the previous growth as nutrient, i.e. crytic growth. The characteristic critical increase in the bacterial counts, just before bottling, is probably caused by a) the adsorption of organic compounds to the bottle surface, thus increasing their local concentration sufficiently to permit growth, and b) the increase in dissolved oxygen during the filling operation. During the distribution and storage of mineral water in bottles, flavobacteria and related members of the autochtonous microflora grow to counts of up to 10^4-10^5/ml. Such growth is typical of mineral waters bottled in plastic bottles and is believed to be due to the fact that the plastic film the bottles are made of have a certain O_2-permeability.

Mineral water is contaminated with allochthonous bacteria prior to bottling and during bottling. ***Leuconostoc*** en ***Lactobacillus*** will die off soon because of their high nutrient requirements. ***Pseudomonas*** spp. will survive and have the save physiological properties as the autochthonous types.

B. ***Soft drinks***

Soft drinks have a low pH and they furthermore contain CO_2. To some soft drinks relative large amounts of sugar are added, which lowers the a_w. CO_2 inhibits fungi growth. However, spoilage by yeasts (***Torulopsis*** and ***Candida***) is possible.

C. ***Fruit juices***

Spoilage of fruit juices has been discussed in 4.6.2.1.

4.9.2. Alcoholic drinks

Alcoholic drinks have a pH = 4-5 and sometimes they contain CO_2. The usual CO_2 concentration is mainly active against moulds, but less against bacteria and yeasts.

A. ***Beer***

Spoilage processes of beer can be divided into 4 groups :

a) Ropiness caused by ***Acetobacter***, ***Lactobacillus***, ***Pediococcus*** which turn beer sour and make it viscous.

b) Sarcinae-disease caused by diacetyl-producing ***Pediococcus cerevisiae*** that combined with the beer-odour, gives a honey-odour.

c) Souring acidification by ***Acetobacter*** as a result of the oxidative conversion of C_2H_5OH to CH_3COOH.

d) Turbidity as a result of yeast growth (***Saccharomyces***).

B. ***Wine***

Wine is mainly spoiled by yeast and bacteria.

a) Yeasts ***Candida mycoderma*** grows at the wine-surface, which causes the formation of a film.

b) Bacteria Wine can be acidified by ***Acetobacter*** that oxidatively converts C_2H_5OH into CH_3COOH.

A frequently occurring spoilage pattern is spoilage caused by facultative anaerobic or aerobic bacteria which produce volatile acids, make the wine turbid and produce bad odours. This spoilage phenomenon is termed "tourne disease". Lactic acid bacteria can produce acids such as lactic acid and acetic acid, CO_2 and mannitol (from fructose) from the present sugars. They also produce acetamide which gives a characteristic off-odour. Tartaric acid can be broken down to acetic acid and CO_2 by lactic acid bacteria. Breakdown of glycerol leads to formation of acetic acid, lactic acid and propionic acid. Malic acid is broken down to lactic acid and CO_2.

4.10. CEREALS, FLOUR AND BREAD

4.10.1. CEREALS

Cereals have a low a_w-value (10-12 % moisture) and consequently cereals are microbially stable. If the moisture level increases, problems may occur (fungi-growth). Fungi growth depends on the ambient temperature and the relative humidity of the environment.

4.10.2. FLOUR

Flour is a milled cereal product and also microbially stable if the moisture level < 12 %. If the a_w-value increases because of uptake of moisture, fungi growth will occur from 15 % and yeasts and bacteria are able to grow from 17 %.

4.10.3. DOUGHS

Doughs in chill storage owe their stability to the following factors :

1) conditions are anaerobic, thus inhibiting the growth of moulds and other aerobes ;

2) formulations are designed to obtain a low a_w ;

3) refrigeration shows microbial and enzyme activity ;

4) the pH for most products is low and therefore unfavourable for most bacterial growth;

5) the leavening agent produce CO_2.

4.10.4. BREAD

Bread may have a sufficiently high a_w in the core to enable ***Bacillus subtilis*** and ***Bacillus lichinefornus*** to germinate and grow causing ropiness ("ropy bread").

The bread crust on the other hand is rather dry and consequently has a low a_w, which makes microbial growth impossible. If the R.H. of the environment is high, the crust will absorb water, causing a rise in a_w and mould growth.

4.10.6. PASTA

Pastas such as macaroni, spaghetti, noodles, et al. are highly microbially stable because of their high a_w-values.

4.11. SUGAR AND CONFECTIONERIES

This group of food products is characterised by a low a_w-value and thus microbially stable. If the a_w increases because of storage in atmospheres with high R.H., spoilage by osmophilic yeasts (***Torula*** and ***Zychosaccharomyces***) or by some osmophilic ***Bacillus*** and ***Clostridium*** types may occur.

Leuconostoc mesenteroides hydrolyses sucrose and forms dextran, thick and slimy substance that may clog the pipes of the sugar processing industry.

4.12. CANNED FOODS

4.12.1. GROUPS

A. ***Weak acid canned foods (pH > 5.3).*** This group of food products includes canned meat, fish and poultry and some vegetables. In these canned foods, thermoresistant ***Bacillus stearothermophilus*** and H_2S-producing spore-formers may survive, especially by under-sterilisation.

B. ***Semi-acid canned foods (pH = 5.3-4.5).*** This group of food products includes some vegetables, spaghetti, soup and sauces. In these canned foods ***Clostridium thermosaccharolyticum*** and spores of rotting anaerobes may be found.

C. ***Acid canned foods (pH = 4.5-3.7).*** The major product in this group is canned fruit. ***Clostridium pasteurianum***, ***Bacillus coagulans***, lactic acid bacteria, butyric acid-producing anaerobes are still able to grow here.

4.12.2. SPOILAGE PHENOMENA

A. ***Spoilage by thermophilic spore-forming bacteria***

a) "Flat sour" bacteria

"Flat sour" bacteria form acid without gas (swelling of the cans does not occur). This type of spoilage is characteristic for slightly acid canned foods and is caused by ***Bacillus coagulans***, ***Bacillus stearothermophilus*** and ***Bacillus pepo***. These types grow slowly at 25°C but well at 30-55°C.

b) Thermophilic anaerobes that do not form H_2S

This group is mainly responsible for spoilage of weak acid and semi-acid canned foods. The major type is ***Clostridium thermosaccharolyticum*** that produces CO_2 and H_2 (swelling of the cans) and does not grow at 30°C but grows at 37-55°C.

c) Thermophilic anaerobes producing H_2S

Thermophilic anaerobes producing H_2S cause spoilage of weak acid canned foods. This group includes ***Clostridium nigrificans***. The spores of this bacterium, however, are less thermoresistant, which means that their presence in weak acid canned foods always indicates insufficient heating or leakage. Moreover, ***Clostridium nigrificans*** is obligate thermophilic, which means that growth is only possible at temperatures > 37°C.

B. ***Spoilage by mesophilic spore-forming bacteria***

a) Anaerobic spores : ***Clostridium***

Saccharolytic clostridia are capable to deteriorate semi-acid to acid canned foods. Typical types are : ***Clostridium pasteurianum*** and ***Clostridium butyricum***. Proteolytic clostridia mainly grow in weak acid canned foods. Especially ***Clostridium sporogenes***, ***Clostridium putrefaciens*** and ***Clostridium botulinum*** are responsible for this.

b) Aerobic spores : ***Bacillus***

Bacillus subtilis will mainly grow in acid and semi-acid canned foods. The presence

of these anaerobic and aerobic spores indicates underprocessing or leakage.

C. ***Spoilage by non-spore-forming bacteria***

Spoilage by non-spore-forming bacteria (especially lactic acid bacteria) always indicates underprocessing or leakage (several types).

D. ***Spoilage by yeasts and moulds***

If canned foods are spoiled as a result of growth of yeasts and moulds, this indicates leakage or underprocessing.

CHAPTER 5. FOOD POISONING

5.1. FOOD INTOXICATIONS

A bacterial food intoxication refers to food-borne illness caused by the presence of a bacterial toxin formed in the food. The major food intoxications are caused by ***Staphylococcus aureus***, ***Clostridium perfringens***, ***Clostridium botulinum***, ***Bacillus cereus*** and mycotoxin-producing moulds.

5.1.1. *STAPHYLOCOCCUS AUREUS*

A. ***Morphology and growth.***

S. aureus is a nonmotile, non-sporeforming Gram-positive coccus. In liquid culture media or suspended on a slide ***S. aureus*** arranges itself in grapelike clusters, in small groups or in pairs. There are 8 serotypes, depending on the type of enterotoxin that is produced : A,B,C_1,C_2,C_3 and E. Types A and D cause food intoxications to human beings. It is an aerobic or facultative anaerobic bacteria. The optimum T° for growth is 35-37°C with a min. t° 5-6°C and max. t° = 45°C. ***S. aureus*** has a low heat-resistance, and hence it is killed by pasteurisation. Growth occurs in a pH range between 4.5 and 9.3, with optimum pH for growth being 7.0 to 7.5. Min. a_w for growth is 0.86.

B. ***Staphylococcus aureus enterotoxines (SE)***

a) Synthesis of enterotoxin

S. aureus is capable of producing enterotoxins. These toxins are proteinaceous and have a M.W. between 26.000 and 30.000. Furthermore, they have an antigenic structure which means that specific antitoxins may be produced. The enterotoxin production depend on a series of factors given in table 22.

TABLE 28. Factors that influence enterotoxin production

Factor	Optimum	Limits
a_w	0,99	0,93-0,99(x)
pH	6-7	4,5-9,8
t°	40-50	10-46
% NaCl	0,0	0,0-10,0
O_2	aerobic	aerobic-anaerobic
(x) Remark : min. a_w for toxin production is higher than min. a_w for growth of ***S. aureus*** (0.86).		

In food products containing glucose, the production of SE will be inhibited by

growth of lactic acid bacteria, that lower the pH of food products as a result of acid formation (pH < 5.0).

b) Characteristics of the toxin component

The enterotoxins are single polypeptide chains that contain relatively large amounts of lysine, aspartine and glutamic acid and tyrosine. The amino acid composition of SEB and SEC are similar, but then again differ from those of SEA and SED. They all have two residues of half-cystin and one or two tryptophan residues. SEB has 239 amino acids. The two residues of half-cystin are situated close to the centre of the chain, mainly near position 92 and 112. These 2 residues create a loop in the chain because the SH-group of these amino acids is missing (fig.6). This loop is termed "cystine loop".

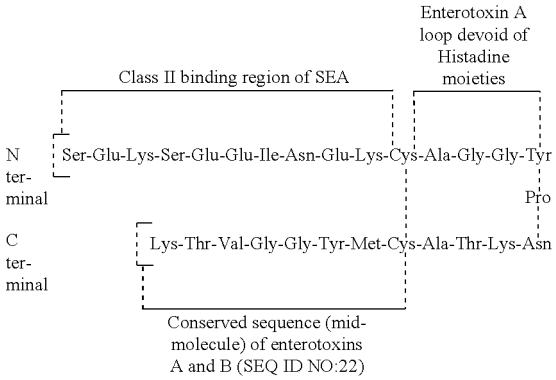


FIGURE 6. The cystine loop

This loop is common to all ***S. aureus*** enterotoxins, although the sequence of amino acids is not the same for the different enterotoxins. Both in the loop of SEA as in the one that of SEB a sequence of 7 amino acids occurs, that is the same for both, namely : -Cys-Met-Tyr-Gly-Val-Thr-Leu-His-.

This segment causes the toxicity of the molecule and the antigenic property is associated with the free terminal nitrogen group. As it is the case for all active proteins, the environment is an essential factor for biological activity of the enterotoxin.

c) Stability of enterotoxin

The ***S. aureus*** enterotoxines are in many respects more stable than other proteins. They are resistant to proteolytic enzymes such as trypsin, chymotrypsin, rennin and pepsin. However, pepsin will inactivate SEB at pH < 2. At higher pH values the effect of pepsin fails. This resistance to proteolytic enzymes explains why enterotoxins are still active after ingestion. The pH of the stomach increases as the amount of ingested food increases and hence the inactivating effect of pepsin disappears. Irradiation doses applied for pasteurisation and sterilisation of food are

not sufficient to inactivate SE. Enterotoxines are remarkably resistant to heat-inactivation. The activity of the SE drops by 50 % when heated during 50 minutes. The higher the concentration of SE, the more heat is required to inactivate the enterotoxin. A major loss in activity of the SE occurs after pasteurisation (700 bar, 15 sec) of milk, skimmed milk and cream . Air drying of milk only slightly influences the toxicity of the present SE. Common boiling process is not sufficient to inactivate the toxin. The time/temperature combination that are applied for treatment of canned food are sufficient to denaturate the amounts of toxin usually present with food intoxication (< 0.5 to 10 mg/100 g).

C. ***Conditions for intoxication***

1. The food has to be contaminated with an enterotoxin producing ***S. aureus*** strain.
2. The food has to be an appropriate substrate for growth and toxin production.
3. The temperature needs to be sufficiently high and the period sufficiently long for toxin production : sufficient amounts of toxin are produced if the total count reaches 10^5-10^9/g food.
4. The enterotoxin has to be absorbed.

D. ***Disease***

a) Symptoms= Symptoms occur after an incubation period of 3 to 5 hrs. The disease is characterised by vomiting, nausea, stomach cramps, diarrhoea and headache. There is usually no fever. These symptoms last 2 to 3 days and recovery is complete. Mortality is very low.

b) Diagnosis= The diagnosis can be made by tracing ***S. aureus*** in the suspect food product. If high numbers are formed, there is a strong suspicion that SE was produced. This can also be demonstrated by means of serological methods. If the food product was heated after growth and toxin production, causing death of ***S. aureus*** without destruction of the thermoresistant SE, the thermoresistant DNase or thermonuclease (TNase) can be traced, and the SE-production becomes evident.

c) Immunity= Sensitivity to food poisoning by staphylococci can differ considerably from one individual to another.

E. ***Sources of contamination***

The major sources of contamination are human beings and animals. ***Staphylococcus aureus*** can be found in the nose (50 %) and on hands (5-30 %) of healthy persons. They also occur in hair, eyes, throat, and intestines. Human beings and animals spread ***S. aureus*** in the air and contaminate clothing and equipment. This way food products are contaminated. Contamination of raw food products has few risks because of the

antagonistic activities of the initial flora. Possibility of growth and toxin production by ***S. aureus*** is larger in heated and cured food products than in raw foods because of the reduced or even totally disappeared antagonistic activity of the accompanying microflora. Food intoxication caused by ***S. aureus*** is mostly due to bad preparation techniques in kitchen at home and in industrial kitchens. They especially occur in prepared food products that are heated first and then contaminated with ***S. aureus***. By slow and insufficient cooling large amounts of ***S. aureus*** will develop, with SE production in the food. Heating causes cell death of ***S. aureus*** whilst thermoresistant SE will survive and may cause food intoxication. These bad methods of preparation may also occur on food industry. The most sensitive groups of food products are the heated food products that are contaminated with ***S. aureus*** because of further processing and that are cooled to slowly or not cooled at all.

F. ***Physiological properties of S. aureus***

S. aureus is capable of producing a series of other important physiological metabolites. First the enterotoxin producing strains produce a coagulase. However, not all coagulase-positive strains produce enterotoxin. Under the influence of coagulase, the soluble fibrinogen present in blood plasma is converted into the insoluble fibrin (coagulation). In addition to this fibrinolysin is released. This enzyme liquefies fibrine. As mentioned above the enterotoxin producing strains are able to produce a thermostable extracellular nuclease during their growth, the so called deoxyribonuclease (DNase), a phosphodiesterase that cleaves extracellular nucleic acids and that can be easily shown. There is a good correlation between the thermostable DNase or TNase and toxin production. However, this correlation does not always exist. Consequently TNase can be traced in suspected reheated foods in which the ***S. aureus*** cells are killed. Presence of TNase is always an indicator for growth of ***S. aureus***. Afterwards SE can be traced. It needs to be mentioned however that ***S. epidermis*** and many micrococci also produce a nuclease. However, this nuclease is thermolabile. Some ***Streptococcus faecalis*** strains produce TNase with optimal activity at pH=6.7; whereas the optimal activity of staphylococci TNase is at pH = ± 9.

Another enzyme produced by ***S. aureus*** is hyaluronidase. This enzyme affects hyaluronic acid, which is an essential element of the connective tissue. The influence of the enzyme lowers the viscosity of the connective tissue, and as a result the staphylococci spread in the connective tissue.

G. ***Measures to prevent S. aureus intoxication***

a) Prevent contamination by respecting the rules of personal and general hygiene (cleaning and disinfection).

b) Inhibit multiplication by storage under refrigeration or by reducing pH.

c) Kill ***S. aureus*** by heating or by irradiation with g-rays.

5.1.2. *CLOSTRIDIUM PERFRINGENS*

A. ***Morphology and growth***

C. perfringens is a Gram-positive non motile spore-forming (sub terminal) rod, that only occurs in couples or in chains. There are 6 serological types, namely A,B,C,D,E and F. Types A and C may lead to food intoxication ; but type A is the major causative agent. ***C. perfringens*** type A is responsible both for "gas gangrene" as for food poisoning. However, there is a substantial difference between strains causing "gas gangrene" and strains causing food intoxications (table 29).

TABLE 29. *C. perfringens* type A strains

"Gas-gangrene"	***Food intoxication***
High a-toxin production (a)	Low a-toxin production
Always Q-toxin production (b)	Seldom Q-toxin production
Always k-toxin production (c)	Variable k-toxin production
Heat-sensitive spores	Thermoresistant spores (6hrs/100°C)

(a) a-toxin is a phospholipase with haemolytic (b) and lecithinase activity.

Breakdown of lecithine leads to the formation of Stearyldiglyceride and Phosphorylcholine

(b) Q-toxin has a haemolytical activity (b).

(c) k-toxin is a collagenase that breaks down connective tissue.

The optimal growth temperature of ***C. perfringens*** is 46°C. Min. and max. temperatures for growth are 15°C and 55°C respectively.

The pH-range for growth is between pH = 5 and pH = 9. The min. a_w for growth of spores and growth of vegetative cells is between 0.97 and 0.95. Although ***C. perfringens*** isn't as strickly anaerobic as ***C. botulinum***, growth will still be determined by the Eh of the growth medium. The max. Eh for growth depends on the strain and on other factors such as pH, temperature and composition of the food product, e.g. max. Eh varies from +310 mV at pH = 7.7 to +230 mV at pH = 6.0. Optimum Eh for growth is -200 mV. Growth of active, growing cultures is not inhibited by the presence of oxygen, implicating that ***C. perfringens*** is capable of growth in suitable foods that are not anaerobically packed.

B. ***C.perfringens enterotoxin (CPE)***

CPE is a protein with a molecular weight of 36.000. It contains 19 amino acids, and the following ones occurs in the highest concentration : aspartic acid, serine, leucine and glutamic acid. CPE is inactivated by pronase and protease (produced by ***B. subtilis***) but not by chymotrypsin, papain, bromelain or carboxypeptidase. CPE is thermolabile with D_{60} = 4 min. The minimal lethal dose (MLD) for mice (intravenous) amounts to 2.000 mg N. The toxin activity is situated between pH = 1.0 and 12.0 with an optimum between 5.0 and 9.0.

CPE has an antigen structure, which means that a specific antibody can be formed, that will not react with other toxins produced by ***C. perfringens*** type A. CPE usually is produced in the intestines, seldom in food products. This is explained by the fact that CPE production takes place at the moment of sporulation. However sporulation in food products and in laboratory media is extremely difficult, if not impossible whereas ***C. perfringens*** sporulates easily in the intestine.

The mechanism of food intoxication caused by ***C. perfringens*** occurs as follows:

1. consumption of food contaminated with large numbers of ***C. perfringens*** cells ;
2. multiplication and sporulation in the intestine ;
3. CPE-production ;
4. release of CPE after lysis of the cells ;
5. diarrhoea.

C. ***Conditions for CPE intoxication***

1) The food product needs to be contaminated with heat-resistant spores of ***C. perfringens*** type A.

2) The spores have to survive the heat process and the food product has to be insufficiently chilled and not stored under refrigeration, so that spores are able to germinate, resulting in growth of vegetative cells.

3) The food product has to be consumed after insufficient heating, and this way high numbers of ***C. perfringens*** end up in the intestine, where they may cause food intoxication according to the mechanism mentioned above.

D. ***Disease***

a) Symptoms= After an incubation period of 8 to 24 hrs., the following symptoms occur : diarrhoea, stomach cramps (usually without vomiting). The disease may be lethal for weak persons.

b) Diagnosis= The diagnosis can be made by isolating ***C. perfringens*** from the suspect food product and mainly from the faeces of the patient. If both isolated strains appear to be similar it may be concluded that, the intoxications have been

caused by the isolated ***C. perfringens***.

c) Immunity= *In vitro*, the CPE will be neutralized by the specific antitoxin. However, *in vivo* the CPE will be neutralized but not in the intestine in the bloodstream.

E. ***Contamination sources***

Human beings are a major source of contamination of food products with ***C. perfringens***. Human feces always contain ***C. perfringens*** and 2 to 30 % of the faeces contain heat-resistant strains. Heat-resistant strains were isolated from faeces of pigs, cattle, chicken, sheep and rats. Muscles and viscera of animals that suffered from stress could be contaminated with ***C. perfringens***. Hence the importance of 24 to 48 hrs of rest prior to slaughter. Soil and dust are also important sources of contamination. Flies, on the other hand, only play a minor role.

F. ***Measures to prevent C.perfringens intoxication***

a) Chill heated food products rapidly and efficiently

b) Store heated products above 70°C

c) Reheat the remains of a meal

d) Personal and general hygiene.

5.1.3. *CLOSTRIDIUM BOTULINUM*

A. ***Morphology and growth***

Clostridium botulinum is a Gram-positive motile (peritrichous flagella), spore-forming (sub-terminal) rod, that occurs alone, in couples or in chains. The strains of ***C. botulinum*** are classified into 7 serological types, deducted from the different antigen structures of the formed toxins. Following serotypes are recognized : A,B,C,D,E,F and G. Types A,B,E,F and G cause disease in humans. In general ***C. botulinum*** grows in a temperature range between 10°C and 48°C. The optimum temperature for growth is 35°C. The minimum temperature for germination is 10°C. Exceptions are ***Clostridium botulinum*** type E, the spores of which germinate and grow at min. temperature of 3.3°C. Non-proteolytic strains of type B are also psychrotrophic and are able to grow at temperatures > 3.3°C. The min. pH for growth is 4.6. However, type E is less acid sensitive than the other types, which presents a risk for fish marinades, since type E mainly occurs in fish. Min. a_w values for growth and toxin production are summarized in table 30.

TABLE 30. Min. a_w values for growth and toxin-production of *C. botulinum*

	Type	***Min. a_w***
Growth of vegetative cells	A B E	0.93-0.94 0.93-0.94 0.965
Sporulation and toxin-production	A B E	0.95 0.94 0.97

Clostridium botulinum is a strictly anaerobic bacterium and will consequently grow at low Eh (< +150 mV). Spores of ***Clostridium botulinum*** are heat-resistant. This resistance differs, depending on the type. For example, it has been determined in vitro that spores of type A resist 20 minutes heating at 110°C and spores of type B survive 30 minutes heating at 80°C.

B. ***Botulinal toxins***

a) Stability

Botulinal toxins consist of two components, one of them being toxic. The toxic component is a neurotoxin. The non-toxic component protects the toxic component from inactivation. At pH > 7.5 the toxin dissociates into 2 components. Since in most food products pH < 7.0, the botulinal toxin will be present in its most stable form. In potable water, on the other hand, pH is usual above 7, so dissociation occurs and the toxic component loses spontaneously its biological activity. Moreover, there are no substances that influence toxicity in food products. Salts and acid pH do not influence stability. Yet, botulinal toxins are thermolabile. Heating at 80°C during 10 minutes is sufficient to inactivate the botulinal toxins present in food products. A similar effect is obtained at 86°C during 1 minute.

b) Characteristics

The botulinal neurotoxin is neutralized by type-specific antitoxins. However, there are cross-reactions between D and E, C and D. There are no cross-reactions between A,B and E. The minimal lethal dose (MLD) is the amount of toxin causing death of 20 g weighing mouse in 24 hrs ; e.g. 1 mg botulinal toxin type E is 4.800.000 MLD for guinea pigs, 31.000.000 MLD for mice and 10.000 MLD for human beings.

C. ***Conditions for botulism***

1. The food product has to be contaminated with spores of ***C. botulinum*** type A,B,E, F or G.
2. Spores have to be able to germinate, so that vegetative cells will multiply and produce toxin.
3. The food product has to be heated insufficiently to inactivate toxin.

4. The food has to be absorbed.

For canned foods, spores have to survive because of insufficient heating.

Moreover, the storage conditions have to be favourable for germination, multiplication and toxin production.

D. ***Disease***

a) Symptoms= There is an incubation period of 12 to 36 hours. The first phase of the disease is characterized by gastrointestinal symptoms such as nausea, vomiting and diarrhea. This results from resorption of toxin in the gastrointestinal tract. After botulinal toxins are absorbed into the bloodstream, they enter into the peripheral nervous system where they affect nerves. Hence, in the second stage of the disease, paralysis phenomena occur : paralysis of muscles, double vision and finally, respiratory failure and death.

b) Diagnosis= ***C. botulinum*** is traced in the suspect food product. This can be done both in vivo (mice) as in vitro (immunological tests).

c) Immunity= Active immunity after consumption of a food product contaminated with botulinal toxin, cannot be obtained, because the dose of toxin causing clinical phenomena is to low to stimulate antitoxin production. Botulinal toxoid can be administered to persons who came regularly in contact with botulinal toxin. The toxoid is obtained by treating the toxin with chloroform, and has a high antigenic activity. Immunity for ca 10 years can be obtained by vaccination. In addition to this, antitoxin can be administered to persons who have consumed foods contaminated with botulinal toxin and who do not show the symptoms yet. The antibotulinal toxin only neutralises the toxin that occurs freely.

E. ***Sources of contamination***

C. botulinum mainly occurs in the soil and this way it ends up in food products (especially vegetables) and feed. ***C. botulinum*** type E mainly occurs in seawater and thus in fish. Meat is contaminated by faecal contamination during slaughter. Mainly type B, pathogenic to human beings, is found regularly in meat.

F. ***Measures to prevent botulism***

a) Canned food products need to be sufficiently sterilised.

b) Swollen cans have to be removed.

c) Change in colour, odour or flavour may not occur.

d) Consumption of heated foods that are stored at high temperatures (room t°) must be refused.

e) Suspect canned foods have to be boiled for 15 minutes.

Special attention should be paid to smoked vacuum-packed fish. Fish can be smoked at low temperatures (28°C) or at high temperatures (70°C). ***C. botulinum*** spores will survive this process. After the smoking process, the fish has to be packed rapidly and stored under refrigeration (< 3°C). The same measures apply for prepared chilled meals with extended shelf-life.

5.1.4. *BACILLUS CEREUS*

A. ***Morphology and growth***

B. cereus is a Gram-positive, motile, spore-forming (central, ellipsoid) rod with a granular internal structure. The optimum temperature for growth is between 28°C and 35°C with min. t° = 40°C and max. t° = 50°C. ***B. cereus*** grows between pH = 4.9 and pH = 9.3. The min. a_w for growth = 0.90, which corresponds to 15 % NaCl. Spores are thermoresistant. The D_{100}-value of spores in skimmed milk amounts to 2.7-3.1 minutes, in slightly acid food products (pH > 4.8) to 5 minutes and in phosphate buffer (pH = 7), to 8 minutes. Spores germinate at 50°C (heat shock).

B. ***Enterotoxins***

The ***B. cereus*** enterotoxin is a protein with an antigenic structure, that is sensitive to trypsin and pronase. It is stable in a pH-range between 5 and 10. Furthermore, the enterotoxin is thermolabile and is inactivated if heated at 56°C during 5 minutes.

C. ***Conditions for intoxication caused by B. cereus***

1. The food product has to be contaminated with spores of ***B. cereus***.
2. The food product must have been insufficiently heated.
3. The food product has to be a suitable substrate for germination of spores, growth and toxin production.
4. The time/temperature combination has to be appropriate.
5. The food product (containing enterotoxin) needs to be absorbed.

D. ***Disease***

a) Symptoms= Intoxication can be divided into 2 groups, depending on the symptoms, "diarrhoea syndrome" food poisoning and "Emetic syndrome" food poisoning. Intoxication is not lethal and recovery is quick and complete. The major characteristics of/and differences between the two types are shown in table 31.

TABLE 31. The 2 types of symptoms of *B. cereus* intoxication

	Type 1	***Type 2***
Incubation	8-16 hrs	1-5 hrs
Diarrhoea	general	regularly
Vomiting	occasionally	general
Duration	12-14 hrs	6-24 hrs
Food product	meat, soup, sauces, vegetables, puddings	fried rice

b) Diagnosis= Intoxication can be determined by tracing ***B. cereus*** in suspect food.

c) Immunity= Little or no immunity can be obtained, depending on the individual.

E. ***Sources of contamination***

B. cereus is a frequently occurring microorganism that is mainly found in air, soil, water and all kinds of waste matter. Food products are contaminated via these sources.

F. ***Physiological characteristics***

B. cereus produces a number of extracellular metabolites such as protease, β-lactamase, specific peptide antibiotics a phospholipase and a haemolysine. The last ones are 2 metabolites that exercise a joint action comparable to that exercised by the α-toxin, produced by ***C. perfringens***.

G. ***Measures to prevent B. cereus intoxication***

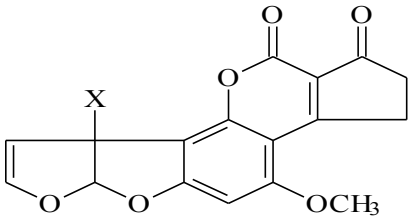
Since ***B. cereus*** commonly occurs in nature it is evident that it sometimes is found in foods. Small numbers are not harmful, but germination of spores and multiplication of vegetative cells should be prevented. In practice, this means that foods have to be consumed immediately after heating, or otherwise they have to be cooled rapidly to $t° < 10°C$.

5.1.5. Mycotoxins

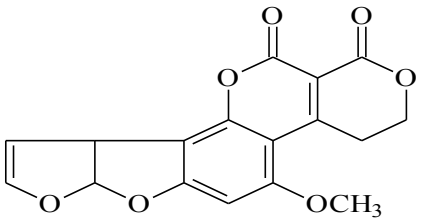
Numerous moulds are capable of producing toxic metabolites in food products. The major group is the aflatoxin-group, produced by some strains of ***Aspergillus flavus*** and ***Aspergillus parasiticus***.

A. ***Chemical characteristics***

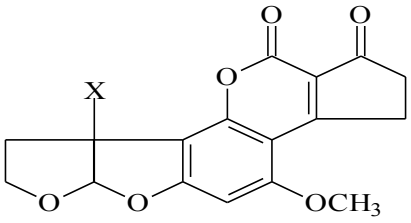
Aflatoxins are coumarin derivatives. Four important components are formed : aflatoxin B_1 and aflatoxin B_2 (blue fluorescent) and G_1 and G_2 (green fluorescent). The structure of these metabolites is as follows :



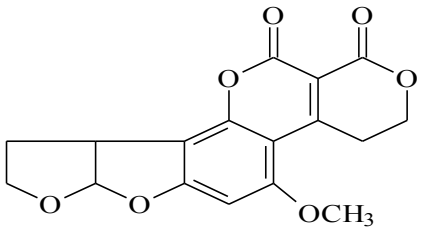
B_1: X = H
M_1: X = OH



G_1



B_2: X = H
M_2: X = OH



G_2

The aflotoxins B_1 and B_2 are converted by hydroxylation in milk to M_1 and M_2.

Aflatoxins are soluble in methanol and chloroform and a slight water-solubility.

B. ***Substrate***

Each food product on which ***Aspergillus flavus*** and ***Aspergillus parasiticus*** can grow and produce toxin is suitable substrate. Especially dry food products (still having a_w > 0.85) are qualified. One strain can only form 2 or 3 aflatoxins, at least one of them being B_1. The most sensitive food products are cereals and nuts. In milk B_1 and B_2, that may be present in feed as a result of mould growth, are converted into M_1 and M_2.

C. ***Biological activity***

The LD_{50}-values of aflatoxins are summarised in table 32.

TABLE 32. LD_{50}-values of aflatoxins

Type	***Ld_{50} in mg/kg body weight***
B_1 and M_1	0,36
G_1	0,79
B_2 and M_2	1,8
G_2	3,45

Aflatoxins are carcinogenic. They are hepatoxic and hepacarcinogenic. The pathological implications for human beings are characterised by 1) bleedings of kidneys, lungs, intestines and 2) a chronic stage during which hepatomes and sarcomers are formed.

D. ***Other mycotoxins***

Numerous moulds occurring in food products are capable of producing mycotoxins (see table 33).

TABLE 33. Mycotoxin producing moulds in food products

Toxin	Microorganism	Food product
Zearalenone	Fusarium roseum Fusarium tricinctum	Cereals (maize)
Ochratoxin A	Aspergillus ochraceus Penicillium viridicatum	Cereals, beans, nuts, poultry, pork meat
Citrinin	Penicillium citrinum Penicillium viridicatum	Cereals
Penicillic acid	Penicillium spp. Aspergillus spp.	Cereals, beans
Patulin	Penicillium expansum Penicillium urticae	Apples, cider, applejuice
Sterigmatocystin	Aspergillus versicolor Aspergillus viridulans Aspergillus rugulosus Bipolaris spp.	Wheat, green coffeebeans
Luteoskyrin	Penicillium islandicum	Rice
Trichoticene	Fusarium Trichothecium	Maize
A.T.A. (alimentary toxic aleukia)	Various moulds	Cereals
Roquefortine	Penicillium spp.	Bleu cheeses

E. ***Measures to prevent mycotoxicosis***

a) Prevent fungi growth and toxin production by dry storage and use of fungicides.

b) Detect moulds and toxins in foods.

c) Destroy present mycotoxins if possible. This is usually hard to accomplish

because of the high resistance to various physical and chemical treatments.

Aflatoxins do not have a antigenic structure, and as a result the body does not form antibodies will not be produced.

5.2. FOOD INFECTIONS

A bacterial food infection refers to food-borne illnesses caused by the entrance of bacteria into the body through ingestion of contaminated foods and the reaction of the body to their presence of their metabolites.

5.2.1. *SALMONELLA*

The genus ***Salmonella*** belongs to the family of ***Enterobacteriaceae***. So far, approximately 2000 serotypes have been isolated and identified, based on the antigenic properties of ***Salmonella*** spp. ***Salmonella*** can have 4 types of antigens. First there are the O-antigens (somatic or cell bound) which are thermostable. There are 64 O-antigens known, designated by Arabic figures. Then, there are the H-antigens (flagellar bound) which are thermolabile. H-antigens are of 2 types : phase 1 consists of 44 specific antigens, designated with small in letters, and phase 2 consist of 7 specific antigens, designated by arabic figures. There are also the K-antigens (capsular antigen) and the Vi (Virulence antigens) that is linked to the capsule.

A. ***Morphology and growth***

Salmonella is a Gram-negative, motile (peritrichous flagella) non-spore-forming rod. The optimum temperature for growth is between 35° and 37°C with min. and max. temperatures 5°C and 47°C respectively. Salmonellae are heat-sensitive and are killed if heated at 66°C during 12 minutes. However, salmonellae survive freezing and chilling temperatures. ***Salmonella*** grows in a pH range of 4.5 to 9.0 with optimum pH from 6.5 to 7.0 but below pH = 4 and above pH = 9.0 ***Salmonella*** will die off. Min. a_w for growth is 0.95 but ***Salmonella*** survives in dry foods. ***Salmonella*** is an aerobic or facultative anaerobic bacterium. The species name of ***Salmonella*** refers to the city or place where it first was isolated (***S. newport***, ***S. panama***, ***S. dublin***) or to the disease it has caused (***S. typhi***, ***S. typhimurium***, ***S. enteritidis***) or to the host (***S. pullorum***, ***S. gallinarum***).

B. ***Infection***

The possibility of infection due to consumption of contaminated food depends on the resistance of the consumer, the ineffectiveness of the strain and the number of ingested cells. The number of cells that needs to be ingested in order to cause infection is determined by the type of ***Salmonella*** with a specific ineffectiveness e.g. an uptake of 1.25×10^5 ***S. bareilly*** suffices to cause disease symptoms, whereas this

only amounts to 10^{10} cells for ***S. pullorum***.

C. ***Conditions for infection***

1. The food product has to be contaminated with living ***Salmonella*** cells.
2. The food has to be a suitable substrate for growth, in order to attain the infectious dose.
3. Enough time must be allowed and the temperature must be favourable for growth.
4. The amount of ingested food must be sufficient to cause infection. Also the acid pH of the gastric juice, that has a bactericidal effect (amongst others against Salmonella) has to be taken into account.

D. ***Disease***

a) Symptoms= Salmonellosis is characterised by in three types of symptoms : gastro-enteritis, typhoid fever and a localised infection. Gastro-enteritis is caused by numerous ***Salmonella*** spp. and is characterised by an incubation period of 8 to 72 hrs. Symptoms are bloody, slimy diarrhoea, abdominal pain, nausea and vomiting (during the first few days) and fever (38-39°C). Typhoid fever occurs as a result of sepsis by ***S. typhi*** and is characterised by an incubation period of 3 to 28 days. The initial symptoms of the disease are fever, combined with malaise and headache. The fever rises (39-40.5°C) and high temperatures will hold for a few days. Subsequently, the fever drops, and typhoid symptoms appear : abdominal pain, general pain and weakening, anorexia and delirium. Some serotypes, like ***S. paratyphi*** A, B and C and also ***S. enteriditis*** and ***S. choleraе-suis*** may cause paratyphoid fever. This is characterised by gastro-enteritis followed by sepsis. Local infections may occur in various tissues, such as appendicitis, peritonitis, local ulceration, pneumonia, meningitis, infection of the urinary tract, et al.

b) Diagnosis= In case of gastro-enteritis, ***Salmonella*** is traced in the faeces, whilst in case of typhoid fever, ***Salmonella*** is traced in the blood. In a further stage, both blood and faeces are examined. In case of paratyphoid fever, ***Salmonella*** is usually traced in the blood, and sometimes in the faeces. 10^6 to 10^9 salmonellae/g faeces may be present during the acute stage of diarrhoea. An infected person may still be carrier of ***Salmonella*** for 2 to 4 weeks after his recovery. Hence, it is important to control people working in food industry regularly.

c) Immunity= Immunity can be obtained by vaccination (dead cells).

E. ***Sources of contamination***

Human beings and animals are the major sources of contamination and may contaminate each other. Human and animal faeces will contaminate various types of

vegetables via liquid manure, and oysters and mussels via waste water that ends up in estuaries. Duck eggs may be contaminated with ***Salmonella*** via the oviduct ; the shell of hen's eggs may also be contaminated with ***Salmonella*** bacteria. If an omelette is made of such eggs, ***Salmonella*** may be present in the omelette. Food products may be contaminated with ***Salmonella*** via vermin and insects.

Meat and meat products, poultry, dairy products, fish and especially shrimps and frog legs are risk-bearing products.

F. ***Measures to prevent salmonellosis***

a) Avoid food products being contaminated with ***Salmonella*** bacteria. This can be obtained mainly by respecting the general rules of hygiene.

b) Check raw materials and ingredients regularly.

c) Destroy ***Salmonella*** by pasteurisation, sterilisation and g-irradiation.

d) Store food products under refrigeration to prevent growth of ***Salmonella***.

5.2.2. SHIGELLA

The genus ***Shigella*** belongs to the family ***Enterobacteriaceae***, and consists of four species, each of which consists of a distinct serogroup : Group A consists of 10 types of ***S. dysenteriae*** ; group B consists of 6 types of ***S. flexneri*** ; group C consists of 14 types of ***S. boydii*** and finally group D that consists of 1 type of ***S. sonnei***.

A. ***Morphology and growth***

Shigella is a Gram-negative, nonmotile, non-spore-forming rod. The optimum growth temperature is 37°C, and min. and max. temperatures are 10° and 40° respectively. ***Shigella*** is heat-sensitive, which means that common pasteurisation temperatures are sufficiently high to kill the bacterium. ***Shigella*** grows in a relatively narrow pH-range (6.6-8.8) with optimum pH = 7.8. The min. a_w for growth is 0.95 and growth occurs both under aerobic as under anaerobic conditions. ***Shigella*** has a limited life in food products, depending on the internal and external factors.

B. ***Toxins***

If ***Shigella*** ends up in the intestine (colon) it will grow, and produce toxin. A thermostable endotoxin (an O-antigen) is formed. This toxin is enterotrophic and has a glucido-lipido protein structure. Another toxin that is formed is a neurotrophic, thermolabile toxin, which has a protein structure.

C. ***Conditions for Shigellosis***

Conditions for shigellosis are the same as those for salmonellosis.

D. ***Disease***

a) Symptoms= The infection is characterised by an incubation period of an average 4 days (1-7 days). This bacillary dysentery is characterised by severe symptoms such as stomach-ache diarrhoea and high fever, followed by typical intoxication phenomena, such as bloody slimy stools and loss of weight.

b) Diagnosis= Diagnosis can be made by tracing ***Shigella*** in faeces rapidly.

c) Immunity= As only a very small immunity can be obtained, vaccino- and serotherapy cannot be applied.

E. ***Sources of contamination***

Shigella is not a saprophyte and consequently cannot live long in food products. Survival depends on temperature : it is relatively long at -20°C, and rather short at 0°C. At 5° to 10°C, the period of survival is relatively long, whilst it shortens at 20°C to 30°C. The major source of contamination are human beings, who may be carrier of ***Shigella*** either as a pathogen or as a commensal even up to three months after the symptoms of dysentery disappeared.

F. ***Measures to prevent shigellosis***

The measures to prevent shigellosis are similar to those to prevent salmonellosis.

5.2.3. *ESCHERICHIA COLI*

The genus ***Escherichia*** is also classified in the family ***Enterobacteriaceae***.

Escherichia coli is a lactose-fermenting faecal microorganism.

A. ***Morphology and growth***

E. coli is a Gram-negative, motile (peritrichous) or sometimes non-motile, non-spore-forming rod. ***E. coli*** grows between 10°C and 50°C with optimal growth at 37°C. Unlike other coliforms, faecal coli grow well at 44°C. This selective property is used to assess ***E. coli*** in food products. Compared to other Enterobacteriaceae, ***E. coli*** has also a certain thermoresistance. This property is applied to control pasteurisation of certain food products, such as dairy products and meat products. Survival of ***E. coli*** indicates insufficient pasteurisation. ***E. coli*** is extremely tolerant of pH. Min. a_w is 0.95 and ***E. coli*** is a facultative anaerobic bacterium. Resistance to phenols, dyes and bile salts is typical. Consequently, these components are frequently used in selective media for isolation and enumerating of ***E. coli***.

B. ***Toxins and other metabolites***

a) Toxins: ***E. coli*** produces the following toxins in the intestine :

- an exotoxin which is thermolabile and causes paralysis ;
- an endotoxin which is thermostable and affects the intestinal tract.

b) Other metabolites: ***E. coli*** produces colilysine. This is a haemolysine that affects horse red blood cells. In addition to this colilysines may be formed, and they have mainly an antagonistic effect on ***Salmonella*** and ***Shigella*** in the intestine, and as a result the intestinal flora is controlled. ***E. coli*** is also capable of producing lactamase which will inhibit the effect of lactam antibiotics.

C. ***Conditions***

The conditions are similar to those required for infection caused by ***Salmonella***, but only clearly defined serotypes of ***E. coli*** are pathogenic.

D. ***Disease***

a) Symptoms= Infection caused by ***E. coli*** is characterised by a short incubation period (2-14 hrs) and the disease is characterised by stomach-ache, headache, nausea, diarrhoea and fever. Diarrhoea only occurs among babies and children, and exceptionally among adults if the colonic mucosal tissue had already been damaged by another infection. Adults may develop an extraintestinal infection (urinary tract infection).

b) Diagnosis= ***E. coli*** can be traced in a sample of the suspect food product and in a sample of faeces of the patient. If similar serological types, known as pathogens, are found in both samples, it will be an infection caused by ***E. coli***.

c) Immunity= Immunity cannot be obtained, and hence vaccino- and serotherapy cannot be applied.

E. ***Sources of contamination***

E. coli occurs as commensal in men and animals and can mainly be found in faeces. It ends up in nature via faeces and consequently it also occurs in water and food products. Thus, presence of ***E. coli*** always indicates faecal contamination.

F. ***Measures to prevent E. coli infection***

Similar measures as those to prevent salmonellosis are required.

5.2.4. *VIBRIO CHOLERAE*

A. ***Morphology***

V. cholerae is a Gram-negative, comma-like, motile (polar), non-spore-forming bacterium. The optimum temperature for growth is 30°C-35°C with min. and max. temperatures 15°C and 42°C respectively. ***V. cholerae*** is heat sensitive and is killed

if heated at 55°C for 15 minutes. Growth occurs in a pH-range of 6.5 to 9.6 with an optimum growth at slightly alkaline pH (7.6-8.6). Min. a_w is 0.95, but ***V. cholerae*** is extremely sensitive to dryness. ***V. cholerae*** is an aerobic or facultative anaerobic bacteria.

B. ***Toxin***

The vegetative cells multiply in the intestinal tract and produce toxin. This toxin is an endotoxin with protein-structure (MW = 84.000). It is thermolabile and is killed if heated at 56°C. Endotoxin consists of 2 components. The first component A has a MW of 28.000, is biologically active and causes hypothermia (cold fever). The other component B has a MW of 56.000 and its toxicity is based on interaction between specific receptors on the cell membrane of eukaryotes, such as the cells of the intestine wall. This causes cholera.

C. ***Conditions for cholera infection***

The conditions required for cholera infection are similar to those required to cause salmonellosis.

D. ***Disease***

a) Symptoms= Cholera is characterised by an incubation period of an average 2 days (1-5 days). The symptoms are : vomiting, liquid diarrhoea, resulting in dehydration. The consequences of dehydration are : muscle cramps, loss of weight (half the body weight in 24 hrs) and anuria. Cholera can be lethal (after 2 to 4 days).

b) Diagnosis= The diagnosis can be made by tracing ***V. cholerae*** in the patient's faeces.

c) Immunity= Immunity can be obtained by vaccinating death cells (vaccinotherapy). Each year 2 injections are administered. Antibiotics cannot be administered curatively, because of the fact that, whilst the symptoms occur, the cells are lysed before the endotoxins that caused cholera are released. Preventively, a vaccine and sulphonamides are administered simultaneously.

E. ***Sources of contamination***

The major source of contamination is water, and subsequently numerous food products can also be contaminated.

F. ***Measures to prevent cholera***

Cholera can be prevented if the same precautions are taken as the ones taken to prevent salmonellosis.

5.2.5. *VIBRIO PARAHAEMOLYTICUS*

A. ***Morphology and growth***

V. parahaemolyticus is a Gram-negative, straight or curved, motile, non-spore-forming, rod. Petrichous flagella are formed on solid substrates, and in liquid substrates solar flagella are formed. ***V. parahaemolyticus*** grows between 4°C and 42°C with optimum temperature between 30°C to 35°C. If heated at 60°C for 15 minutes, ***V. parahaemolyticus*** is killed. Growth occurs in a pH-range of 5.6 to 9.6 with optimal growth at pH = 7.6 to 8.6. ***V. parahaemolyticus*** is acid sensitive. ***V. parahaemolyticus*** is a facultative anaerobic, halophilic bacterium that grows in a medium containing 1 to 8 % salt ; with optimum growth in a medium containing 2 to 4 % salt. Hence, min. a_w for growth amounts to 0.94.

B. ***Toxicity***

V. parahaemolyticus strains, isolated from patients are haemolytic, whereas strains isolated from fish and water are non-haemolytic. This phenomenon is termed the Kanagawa phenomenon. Kanagawa-positive strains cause infections. All ***V. parahaemolyticus*** strains produce haemolysines that can be divided into 4 groups:

1. Thermolabile haemolysine, produced by all strains and not inactivated by trypsin, and not toxic to mice.

2. Thermostable haemolysine, occurring in the lipid fraction of all strains.

3. Haemolysines, occurring in the supernatant of liquid cultures of some strains, and associated with phospholipase activity.

4. A haemolytic fraction that is only present in the supernatant of cultures of Kanagawa-positive strains but not in that of Kanagawa negative strains, causes gastro-enteritis and has an haemolytic effect on human blood (but not on horse blood).

Kanagawa positive strains produce 2 toxins: toxin a and toxin a'. They have different chemical structures and some different immunological properties. Nevertheless they have similar haemolytic activities and similar toxicity in laboratory animals.

C. ***Conditions for V. parahaemolyticus infection***

Similar conditions as those to cause salmonellosis are required.

D. ***Disease***

a) Symptoms= Infection caused by ***V. parahaemolyticus*** is characterised by a relatively short incubation period of an average 12 hrs (2-48 hrs). Symptoms are : stomach-ache, diarrhoea, nausea and vomiting. Mortality is low.

b) Diagnosis= ***V. parahaemolyticus*** is traced both in the suspect food product as in

the faeces.

c) Immunity= Immunity cannot be obtained.

E. ***Sources of contamination***

The major sources of contamination are seawater, sediment and plankton, mainly of warm coastal waters. This way fish and fish products (slightly salted) are contaminated.

F. ***Measures to prevent V. parahaemolyticus infections***

The measures are similar to those taken to prevent salmonellosis.

5.2.6. *CAMPYLOBACTER*

Two species of ***Campylobacter*** spp. may cause food infections: ***Campylobacter jejuni*** and ***Campylobacter coli*** (to a smaller degree).

A. ***Morphology and growth***

Campylobacter is a small non-spore-forming, Gram-negative rod, curved or spiral shaped bacterium. Unlike other ***Vibrio*** types, ***Campylobacter*** is strictly microaerophilic and will therefore grow at reduced oxygen level. Growth occurs optimally in an environment consisting of 5 % oxygen, 10 % carbon dioxide and 85 % nitrogen. Another important property is the narrow temperature range for growth. The temperature for growth of most ***C. jejuni*** strains ranges between 32°C and 45°C, with optimal growth at 42°C and 45°C. HTST treatment is sufficient to kill ***C. jejuni***. Optimal growth occurs at pH=6 and pH=8. ***C. jejuni*** is acid-sensitive and dies at pH=5. ***C. jejuni*** is rather sensitive to drying. Various factors play a role by the inactivation of ***C. jejuni*** during drying and storage in a dry environment. The major factors are temperature, R.H. of the surroundings and the medium in which the microorganism occurs. For example, cell death occurs more rapidly if dried at 25°C than at 4°C. Best survival is at R.H. = 14 % and the lowest at R.H. = 59 %. In practice, this means that ***C. jejuni*** is rather sensitive to drying and storage at room temperatures, whereas ***C. jejuni*** can survive at refrigerated temperatures and an appropriate humidity, as it is the case with chilling of poultry carcasses. ***C. jejuni*** survives better if dried in Brucella broth than dried in skimmed milk.

B. ***Disease***

The incubation period ranges from 2 to 11 days. The major symptoms are : stomach-ache, diarrhoea, fever, headache, qualm and delirium. Symptoms may last 40 to 72 hrs, but sometimes the disease may last several weeks. Death is exceptional. The ***C. jejuni*** cells can remain for 4 to 7 weeks in the patient's intestines after the

acute stage of the infection. Some people may still be carrier of ***C. jejuni*** up to one year after the symptoms had disappeared. The mechanism of pathogenesis has not been elucidated completely yet. There are usually polymorphic nuclear leukocytes in the faeces of the infected persons, which could indicate the invasive character of the disease.

C. ***Sources of contamination***

The major sources of contamination are animals. ***C. jejuni*** and ***C. coli*** may occur as commensals in the intestines of domestic animals such as poultry, cattle, pigs, sheep, dogs and cats.

Campylobacter is also found in the secretions of healthy rodents. In developed countries, human beings are definitely no important sources of contamination, but in developing countries human beings can play a role in transmitting ***Campylobacter***.

Water and soil, contaminated with animal faeces containing ***Campylobacter***, can also be a source of contamination although survival in such substrates is relatively low, depending on temperature (the lower, the longer the survival).

D. ***Measures to prevent Campylobacter infection***

The measures are similar to those taken to prevent salmonellosis.

5.2.7. *LISTERIA MONOCYTOGENES*

A. ***Morphology and growth***

L. monocytogenes is a small, short Gram-positive non-spore-forming rod, that occurs both among human beings and animals as in the environment. ***L. monocytogenes*** has a temperature growth range of -0.1°C and 44°C, with an optimum at 30°C to 37°C. ***L. monocytogenes*** survives deep-freeze temperatures but nevertheless the cells are sublethally damaged. ***L. monocytogenes*** appears to be more heat-resistant than many other non-spore-forming spoilage organisms. Various ***L. monocytogenes*** strains are capable of growth in 10 % NaCl-solutions (a_w = 0.94) and of survival in 20 % NaCl-solutions (a_w = 0.88). Minimum pH for growth of ***L. monocytogenes*** is determined by the type of acid it is acidified with. In media acidified with HCl min. pH for growth is 4.39, but in cabbage juices, whose pH is controlled by lactic acid, min. pH for growth is 4.80. This results from the fact that some organic acids have antimicrobial activity on top of their pH-reducing effect. In practice, this means that min. pH for growth differs, depending on type and acid concentration applied to obtain the desired pH.

L. monocytogenes is facultative anaerobic and can therefore grow under aerobic and anaerobic conditions.

B. ***Infection***

The number of ***L. monocytogenes*** cells required to cause listeriosis is still unknown. However, this should be examined, because in certain groups of food products presence of low numbers of ***L. monocytogenes*** have to be tolerated, because total absence of ***L. monocytogenes*** is not realistic, e.g. in raw food products, raw fermented meat products and accidentally recontaminated foods.

C. ***Conditions for infection***

Listeriosis may be caused to human beings if the following conditions are fulfilled.

1. The food product needs to be contaminated with living ***L. monocytogenes*** cells.
2. The infective dose has to be present or the medium has to be a suitable substrate so that the infective dose can be obtained.
3. The period has to be long enough and temperature needs to be favourable for growth.
4. The amount of consumed food has to be high enough to cause infection.

D. ***Disease***

L. monocytogenes is a facultative, intracellular pathogen. The bacterium enters the epithelial cells of the intestine and subsequently they migrate to the liver and the spleen. Macrophages in the blood do not destroy the bacteria, so that they not only survive but also multiply by the formation of haemolysine. By formation of actine the bacterium migrates from one macrophage to another and this way the bacterium withdraws from the immune system.

It is assumed that human beings obtains immunity after uptake of the bacterium, except for babies and immunocompromised persons.

Bacteria will enter epithelial cells of the inestine and are subsequently taken up by macrophages. In humans this process may be accompanied by transitory flulike symptoms that may include general malaise, diarrhoea and mild fever. Virulent ***L. monocytogenes*** strains are capable of multiplying in macrophages, disrupting these cells and consequently end up in the bloodstream (septicaemia). In this stage the organisms have access to other parts of the human body which may involve the central nervous system, the heat, the eyes, and the foetus of pregnant women. The stage of pregnancy when invasion occurs determines the outcome of the disease : abortion, stillbirth or prenatal sepsis, resulting in death within a week. The infected baby may also be born without disease symptoms and subsequently develop acute meningitis within 3 weeks.

The high risk groups are clearly defined. In one of two thirds of the cases the

disease occurs during pregnancy or during the prenatal period. Elderly persons and liver patients and in general the immunocompromised (e.g. alcohol- and drug addicts) belong to the high-risk group. Also AIDS patients and cancer patients who get chemotherapy are more sensitive to listeriosis. Mortality of persons in the high risk groups amount to 30 % or more.

E. ***Sources of contamination***

It has been demonstrated 4.2 to 58 % of the samples of raw milk were contaminated with ***Listeria*** spp. and 1.3 to 45 % were contaminated with ***L. monocytogenes***. ***Listeria*** mainly ends up in milk via the faeces. It may also be secreted in the milk of cows contaminated with ***Listeria***.

The main source of contamination of cheeses is post-contamination as a result of lack of hygiene, mainly in the ripening rooms.

Raw meat and poultry may be contaminated with ***L. monocytogenes***, and consequently meat products may also be contaminated with ***L. monocytogenes***.

Presence of ***L. monocytogenes*** in heated food products indicates insufficient pasteurisation and/or post-contamination as result of insufficient general and/or personal hygiene.

Vegetables are usually contaminated by animal faeces, contaminated with ***L. monocytogenes***. This is mainly important for lettuce and cabbages, since those vegetables are stored under refrigeration for a relatively long period, and then eaten raw.

Listeria has been isolated from deep-frozen fish and shellfish: 46% of the positive samples were contaminated with ***L. innocua*** and 28% with ***L. monocytogenes***. Data concerning the degree of contamination of seawater are not available at the moment. Whether ***Listeria*** ends up in those products in a natural way or during processing has not been elucidated yet.

F. ***Measures to prevent listeriosis***

Listeriosis can mainly be prevented by avoiding food products to be contaminated with ***L. monocytogenes*** bacteria. This can be obtained if the general rules of hygiene are respected, and "Good Manufacturing Practice" is pursued.

In practice, following measures should be taken :

a) use of recycled products possibly contaminated with animal or human waste must be avoided during production of food products ;

b) food processing plants must be situated in a bacteriologically clean environment ;

c) all equipment used to transport raw materials to the processing plant must be properly cleaned and disinfected ;

d) food processing plants must be designed to prevent the entrance of animals, birds, insects and dust ;

e) finished products must be separated from raw products to avoid cross-contamination ;

f) food processing plants must have a comprehensive quality control program that addresses not only processing parameters but also all aspects of product environment control, including the control of personnel.

Certain areas in the plant should receive special attention :

a) floor and walls should receive regular sanitising, with special attention to floor drains ;

b) air conditioners have the potential to create aerosols that may contain ***Listeria*** ;

c) elevated cold surfaces may provide contamination through condense dripping onto food contact surfaces or into the product ;

d) used water or even starter cultures used in the manufacturing process of fermented food products should be routinely examined ;

e) disassembly and thoroughly cleaning and disinfecting tubes, valves and pumps are also required.

If ***L. monocytogenes*** bacteria are present, they can be killed by an efficient heat treatment or by irradiation of the food product with gamma-rays.

5.2.8. LESS FREQUENTLY OCCURING FOOD INFECTIONS

Other bacteria may sporadically cause food infections, e.g. ***Yersinia enterocolitica***, ***Arizona***, ***Proteus***, ***Providencia***, ***Klebsiella***, ***Enterobacter*** and ***Pseudomonas aeruginosa***.

5.2.9. VIRAL FOOD INFECTIONS

Food infections may also be caused by viruses. The following groups play here a role: enteroviruses, reoviruses, parvoviruses, adenoviruses and hepatitis A virus.

A. ***Enteroviruses***

Enteroviruses are found in the intestines of human beings where they multiply and, consequently they can be found in large numbers in faeces. As a result of faecal

contamination these viruses may end up in food products. If these food products are consumed raw (vegetables) or insufficiently heated (minced meat), infection may occur. For example, polioviruses may cause infantile paralysis. There are three serological types. Polio-vaccination is given prevently. Consackieviruses will damage the respiratory tract. Group A consists of over 20 serotypes and group B has 6 serotypes. Vaccination and chemotherapy are without success. Echoviruses affect also the respiratory tract. This group has 30 serotypes and again vaccination and chemotherapy fail.

B. ***Reoviruses***

Reoviruses are viruses of the bovine type and can contaminate human beings via beef and this way cause gastro-enteritis.

C. ***Parvoviruses***

Parvoviruses cause gastro-enteritis to human beings.

D. ***Adenoviruses***

Adenoviruses cause infections of the respiratory tract and of the eye membrane, and this way they can end up in food products. Eating raw or insufficiently heated contaminated food products may cause gastro-enteritis.

E. ***Hepatitis A virus***

A person infected with hepatitis A may cause faecal or oral contamination of food products. By consuming such contaminated product, hepatitis may occur after an incubation period of 15 to 50 days (an average of 28 days). Hepatitis is an inflammation of the liver, characterised by fever, swollen liver and sometimes yellow skin colour. Symptoms disappear after 2 to 4 weeks. After this period the infected person is no longer carrier of the virus. An infected person is a potential carrier from 7 days prior to the appearance of the symptoms.