CHAPTER 3

Factors Affecting the Growth and Survival of Micro-organisms in Foods

3.1 MICROBIAL GROWTH

Microbial growth is an autocatalytic process: no growth will occur without the presence of at least one viable cell and the rate of growth will increase with the amount of viable biomass present. This can be represented mathematically by the expression:

$$dx/dt = \mu x \tag{3.1}$$

where dx/dt is the rate of change of biomass, or numbers x with time t, and μ is a constant known as the specific growth rate.

The same exponential growth rule applies to filamentous fungi which grow by hyphal extension and branching since the rate of branching normally increases with hyphal length.

Integration of Equation (3.1) gives:

$$x = x_0 e^{\mu t} \tag{3.2}$$

or, taking natural logarithms and re-arranging:

$$\ln(x/x_0) = \mu t \tag{3.3}$$

where x_0 is the biomass present when $t = 0$.

The doubling or generation time of an organism τ can be obtained by substituting $x = 2x_0$ in Equation 3.3. Thus:

$$\tau = \ln 2/\mu = 0.693/\mu \tag{3.4}$$

An alternative way of representing exponential growth in terms of the doubling time is:

$$x = x_0 2^{t/\tau} \tag{3.5}$$

This can be simply illustrated by considering the case of a bacterial cell dividing by fission to produce two daughter cells. In time τ, a single cell will divide to produce two cells; after a further doubling time has elapsed four cells will be present; after another, eight, and so on. Thus, the rate of increase as well as the total cell number is doubling with every doubling time that passes.

If, however, we perform the experiment measuring microbial numbers with time and then plot log x against time, we obtain the curve shown as Figure 3.1 in which exponential growth occurs for only a part of the time.

A simple analysis of this curve can distinguish three major phases. In the first, the lag-phase, there is no apparent growth while the inoculum adjusts to the new environment, synthesizes the enzymes required for its exploitation and repairs any lesions resulting from earlier injury, *e.g.* freezing, drying, heating. The exponential or logarithmic phase which follows is characterized by an increase in cell numbers following the simple growth law equation. Accordingly, the slope of this portion of the curve will equal the organism's specific growth rate μ, which itself will depend on a variety of factors (see below). Finally, changes in the medium as a result of exponential growth bring this phase to an end

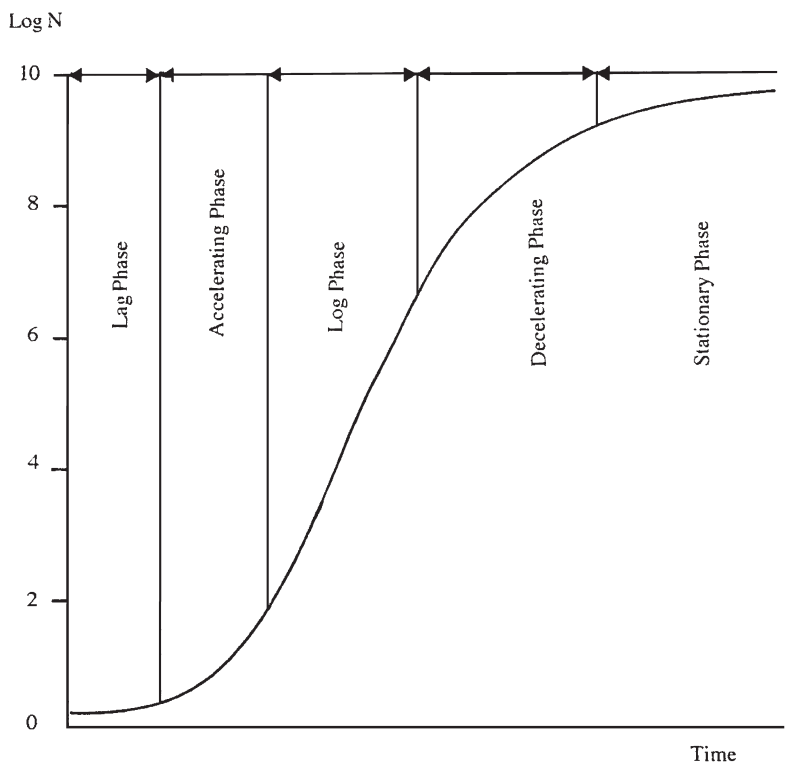


Figure 3.1 *The microbial growth curve*

as key nutrients become depleted, or inhibitory metabolites accumulate, and the culture moves into the stationary phase.

One way of representing this overall process mathematically is to modify the basic growth Equation (3.1) so that the growth rate decreases as the population density increases. An equation that does this and gives us a closer approximation to the observed microbial growth curve is the logistic equation:

$$dx/dt = (\mu_m - \mu_m x/K)x \tag{3.6}$$

where K is the carrying capacity of the environment (the stationary phase population) and μ_m, the maximum specific growth rate. As x increases and approaches K, the growth rate falls to zero. Or, in its integrated form:

$$x = Kc/(c + e^{-\mu_m t}) \tag{3.7}$$

where $c = x_0/(K - x_0)$.

The significance of exponential growth for food processing hardly needs emphasizing. A single bacterium with a doubling time of 20 minutes ($\mu = 2.1 hr^{-1}$) growing in a food, or pockets of food trapped in equipment, can produce a population of greater than 10^7 cells in the course of an 8-hour working day. It is therefore, a prime concern of the food microbiologist to understand what influences microbial growth in foods with a view to controlling it.

The situation is complicated by the fact that the microflora is unlikely to consist of a single pure culture. In the course of growth, harvesting/slaughter, processing and storage, food is subject to contamination from a range of sources (Chapter 2). Some of the micro-organisms introduced will be unable to grow under the conditions prevailing, while others will grow together in what is known as an association, the composition of which will change with time.

The factors that affect microbial growth in foods, and consequently the associations that develop, also determine the nature of spoilage and any health risks posed. For convenience they can be divided into four groups along the lines suggested more than 50 years ago in a seminal review by Mossel and Ingram (Table 3.1) – physico-chemical properties of the food itself (intrinsic factors); conditions of the storage environment (extrinsic factors); properties and interactions of the micro-organisms present (implicit factors); and processing factors. This last group of factors (subsumed under intrinsic properties by Mossel and Ingram) usually exert their effect in one of two ways: either they change an intrinsic or extrinsic property, for example slicing a product will damage antimicrobial structures and increase nutrient availability and redox potential, or they eliminate a proportion of the product microflora as would occur in washing, pasteurization or irradiation.

Table 3.1 *Factors affecting the development of microbial associations in food*

Intrinsic Factors
Nutrients
pH and buffering capacity
Redox potential
Water activity
Antimicrobial constituents
Antimicrobial structures
Environmental factors
Relative humidity
Temperature
Gaseous atmosphere
Implicit factors
Specific growth rate
Mutualism
Antagonism
Commensalism
Processing factors
Slicing
Washing
Packing
Irradiation
Pasteurization

The microbiological effects of different processing factors applied in the food industry will be discussed as they arise elsewhere in the text, principally in Chapter 4.

Although it is often convenient to examine the factors affecting microbial growth individually, some interact strongly, as in the relationships between relative humidity and water activity a_w, and gaseous atmosphere and redox potential. For this reason, in the following discussion, we have not been over zealous in discussing individual factors in complete isolation.

3.2 INTRINSIC FACTORS (SUBSTRATE LIMITATIONS)

3.2.1 Nutrient Content

Like us, micro-organisms can use foods as a source of nutrients and energy. From them, they derive the chemical elements that constitute microbial biomass, those molecules essential for growth that the organism cannot synthesize, and a substrate that can be used as an energy source. The widespread use of food products such as meat or casein digests (peptone and tryptone), meat infusions, tomato juice, malt

extract, sugar and starch in microbiological media bears eloquent testimony to their suitability for this purpose.

The inability of an organism to utilize a major component of a food material will limit its growth and put it at a competitive disadvantage compared with those that can. Thus, the ability to synthesize amylolytic (starch degrading) enzymes will favour the growth of an organism on cereals and other farinaceous products. The addition of fruits containing sucrose and other sugars to yoghurt increases the range of carbohydrates available and allows the development of a more diverse spoilage microflora of yeasts.

The concentration of key nutrients can, to some extent, determine the rate of microbial growth. The relationship between the two, known as the Monod equation, is mathematically identical to the Michaelis–Menten equation of enzyme kinetics, reflecting the dependence of microbial growth on rate-limiting enzyme reactions:

$$\mu = \frac{\mu_m S}{S + K_s} \tag{3.8}$$

where μ is the specific growth rate; μ_m the maximum specific growth rate; S the concentration of limiting nutrient; and K_s the saturation constant.

When $S \gg K_s$, a micro-organism will grow at a rate approaching its maximum, but as S falls to values approaching K_s, so too will the growth rate. Values for K_s have been measured experimentally for a range of organisms and nutrients; generally they are extremely low, of the order of 10^{-5} M for carbon and energy sources, suggesting that in most cases, nutrient scarcity is unlikely to be rate-limiting. Exceptions occur in some foods, particularly highly structured ones where local microenvironments may be deficient in essential nutrients, or where nutrient limitation is used as a defence against microbial infection, for example the white of the hen's egg (Section 3.2.4).

3.2.2 pH and Buffering Capacity

As measured with the glass electrode, pH is equal to the negative logarithm of the hydrogen ion activity. Activity is proportional to concentration and the proportionality constant, the activity coefficient, approaches unity as the solution becomes more dilute. Thus:

$$\mathrm{pH} = -\log(a_{\mathrm{H}}) = \log 1/(a_{\mathrm{H}}) \approx \log 1/[\mathrm{H}^+] \tag{3.9}$$

where (a_{H}) is the hydrogen ion activity and $[\mathrm{H}^+]$ the hydrogen ion concentration.

For aqueous solutions, pH 7 corresponds to neutrality (since $[\mathrm{H}^+][\mathrm{OH}^-] = 10^{-14}$ for water), pH values below 7 are acidic and those above 7 indicate an alkaline environment. It is worth remembering that

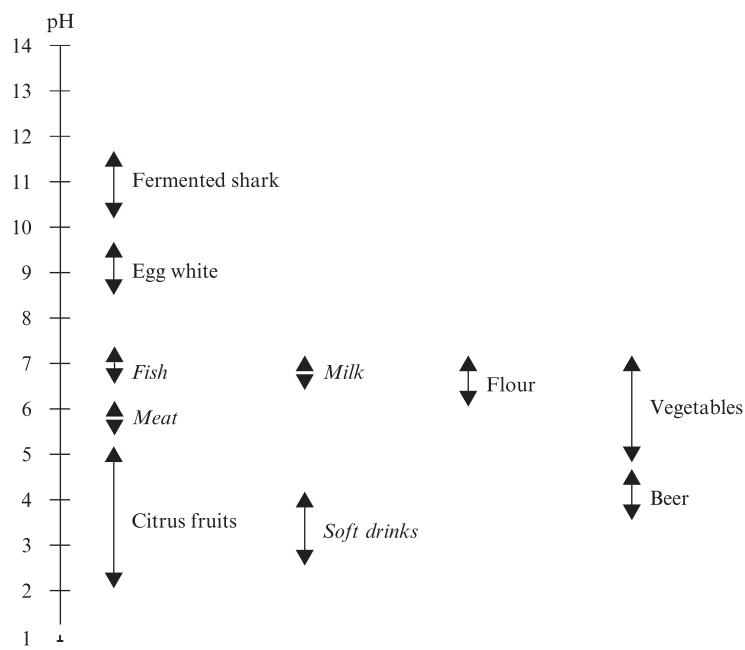
since pH is a logarithmic scale differences in pH of 1, 2 and 3 units correspond to 10-, 100- and 1000-fold differences in the hydrogen ion concentration.

The acidity or alkalinity of an environment has a profound effect on the activity and stability of macromolecules such as enzymes, so it is not surprising that the growth and metabolism of micro-organisms are influenced by pH. Plotting microbial growth rate against pH produces an approximately symmetrical bell-shaped curve spanning 2–5 pH units, with a maximum rate exhibited over a range of 1–2 units.

In general, bacteria grow fastest in the pH range 6.0–8.0, yeasts 4.5–6.0 and filamentous fungi 3.5–4.0. As with all generalizations there are exceptions, particularly among those bacteria that produce quantities of acids as a result of their energy-yielding metabolism. Examples important in food microbiology are the lactobacilli and acetic acid bacteria with optima usually between pH 5.0 and 6.0.

Most foods are at least slightly acidic, since materials with an alkaline pH generally have a rather unpleasant taste (Table 3.2). Egg white, where the pH increases to around 9.2 as CO_2 is lost from the egg after laying, is a commonplace exception to this. A somewhat more esoteric example, which many would take as convincing evidence of the inedibility of

Table 3.2 *Approximate pH ranges of some common food commodities*



alkaline foods, is fermented shark, produced in Iceland and known as hakar, which has a pH of 10–12.

The acidity of a product can have important implications for its microbial ecology and the rate and character of its spoilage. For example, plant products classed as vegetables generally have a moderately acid pH and soft-rot producing bacteria such as *Pectobacterium carotovorum* and pseudomonads play a significant role in their spoilage. In fruits, however, a lower pH prevents bacterial growth and spoilage is dominated by yeasts and moulds.

As a rule, fish spoil more rapidly than meat under chill conditions. The pH of post-rigor mammalian muscle, around 5.6, is lower than that of fish (6.2–6.5) and this contributes to the longer storage life of meat. The pH-sensitive genus *Shewanella* (formerly *Alteromonas*) plays a significant role in fish spoilage but has not been reported in normal meat ($pH < 6.0$). Those fish that have a naturally low pH such as halibut ($pH \approx 5.6$) have better keeping qualities than other fish.

The ability of low pH to restrict microbial growth has been deliberately employed since the earliest times in the preservation of foods with acetic and lactic acids (see Chapters 4 and 9).

With the exception of those soft drinks that contain phosphoric acid, most foods owe their acidity to the presence of weak organic acids. These do not dissociate completely into protons and conjugate base in solution but establish an equilibrium:

$$HA \rightleftharpoons H^+ + A^- \tag{3.10}$$

The equilibrium constant for this process, K_a, is given by

$$K_a = \frac{[H^+][A^-]}{[HA]} \tag{3.11}$$

where [ ] denotes concentration.

This expression can be rearranged:

$$\frac{1}{[H^+]} = \frac{1}{K_a}\frac{[A^-]}{[HA]} \tag{3.12}$$

If we take logarithms to the base 10 we get:

$$pH = pK_a + \log\frac{[A^-]}{[HA]} \tag{3.13}$$

Equation (3.13) is known as the Henderson–Hasselbalch equation and describes the relationship between the pH of a solution, the strength of the acid present and its degree of dissociation. When the pH is equal to an acid's pK_a, then half of the acid present will be undissociated. If the pH is increased then dissociation of the acid will increase as well, so that

Table 3.3 *pK_a values of some common food acids*

Acid	*p*K_a
Acetic (ethanoic)	4.75
Propionic	4.87
Lactic	3.86
Sorbic	4.75
Citric	3.14, 4.77, 6.39
Benzoic	4.19
Parabens	8.5
Phosphoric	2.12, 7.12, 12.67
Carbonic	6.37, 10.25
Nitrous	3.37
Sulfurous	1.81, 6.91

when $pH = pK_a + 1$ there will be 10 times as much dissociated acid as undissociated. Similarly as the pH is decreased below the pK_a the proportion of undissociated acid increases. Table 3.3 presents a list of some common food-associated acids and their pK_a values.

This partial dissociation of weak acids, such as acetic acid, plays an important part in their ability to inhibit microbial growth. It is well established that, although addition of strong acids has a more profound effect on pH *pro rata*, they are less inhibitory than weak lipophilic acids at the same pH. This is because microbial inhibition by weak acids is not solely due to the creation of a high extracellular proton concentration, but is also directly related to the concentration of undissociated acid.

Many essential cell functions such as ATP synthesis in bacteria, active transport of nutrients and cytoplasmic regulation occur at the cell membrane and are dependent on potential energy stored in the membrane in the form of a proton motive force. This force is an electrochemical potential produced by the active translocation of protons from the cell interior to the external environment. Unlike protons and other charged molecules, undissociated lipophilic acid molecules can pass freely through the membrane; in doing so they pass from an external environment of low pH where the equilibrium favours the undissociated molecule to the high pH of the cytoplasm (around 7.5 in neutrophiles). At this higher pH, the equilibrium shifts in favour of the dissociated molecule, so the acid ionizes producing protons which will tend to acidify the cytoplasm and break down the pH component of the proton motive force. The cell will try to maintain its internal pH by neutralizing or expelling the protons leaking in but this will slow growth as it diverts energy from growth-related functions. If the external pH is sufficiently low and the extracellular concentration of acid high, the burden on the cell becomes too great, the cytoplasmic pH drops to a level where growth is no longer possible and the cell eventually dies (Figure 3.2).

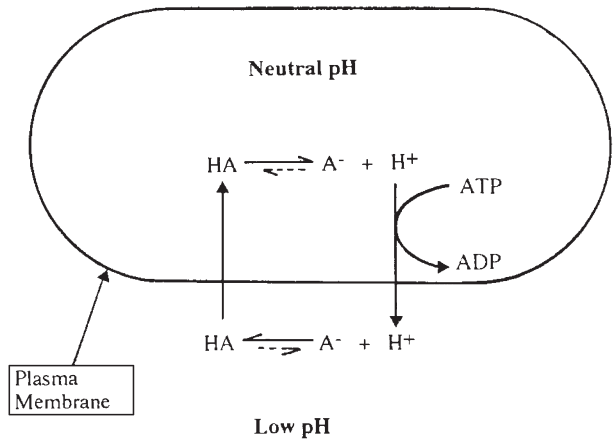


Figure 3.2 *Microbial inhibition by weak organic acids*

3.2.3 Redox Potential, E_h

An oxidation–reduction (redox) reaction occurs as the result of a transfer of electrons between atoms or molecules. In the equation below, this is represented in its most general form to include the many redox reactions which also involve protons and have the overall effect of transferring hydrogen atoms.

$$[\text{Oxidant}] + \text{H}^+ + ne \rightleftharpoons [\text{Reductant}] \tag{3.14}$$

where n is the number of electrons, e, transferred.

In living cells an ordered sequence of both electron and hydrogen transfer reactions is an essential feature of the electron transport chain and energy generation by oxidative phosphorylation.

The tendency of a medium to accept or donate electrons, to oxidize or reduce, is termed its redox potential (E_h) and is measured against an external reference by an inert metal electrode, usually platinum, immersed in the medium. If the balance of the various redox couples present favours the oxidized state then there will be a tendency to accept electrons from the electrode creating a positive potential which signifies an oxidizing environment. If the balance is reversed, the sample will tend to donate electrons to the electrode which will then register a negative potential – a reducing environment. The redox potential we measure in a food is the result of several factors summarized in Table 3.4.

The tendency of an atom or molecule to accept or donate electrons is expressed as its standard redox potential, E_0'. A large positive E_0' indicates that the oxidized species of the couple is a strong oxidizing agent and the reduced form only weakly reducing. A large negative E_0' indicates the reverse. Some redox couples typically encountered in food

Table 3.4 *Factors influencing the measured E_h of foods*

Redox couples present
Ratio of oxidant to reductant
pH
Poising capacity
Availability of oxygen (physical state, packing)
Microbial activity

Table 3.5 *Some important redox couples and their standard redox potential*

Couple	*$E_0(mV)$*
1/2 O_2/H_2O	+820
Fe^{3+}/Fe^{2+}	+760
Cytochrome C ox/red	+250
Dehydroascorbic acid/ascorbic acid	+80
Methylene blue ox/red	+11
Pyruvate/lactate	−190
Glutathione oxid./Glutathione red.	−230
NAD^+/NADH	−320

materials and their E_0' values are shown in Table 3.5. The measured E_h will also be influenced by the relative proportions of oxidized and reduced species present. This relationship for a single couple is expressed by the Nernst equation:

$$E_h = E_0' + \frac{RT}{nF} \ln \frac{[\text{Oxidant}][H^+]}{[\text{Reductant}]} \tag{3.15}$$

where E_h and E_0' are both measured at pH 7; R is the gas constant; T, the absolute temperature; n, the number of electrons transferred in the process and F is the Faraday constant.

Thus, if there is a preponderance of the oxidant over its corresponding reductant, then this will tend to increase the redox potential and the oxidizing nature of the medium.

With the notable exception of oxygen, most of the couples present in foods, *e.g.* glutathione and cysteine in meats, and to a lesser extent, ascorbic acid and reducing sugars in plant products, would on their own tend to establish reducing conditions. From the Nernst equation, it is clear that the hydrogen ion concentration will affect the E_h, and for every unit decrease in the pH the E_h increases by 58 mV. The high positive E_h values registered by fruit juices (see Table 3.6) are largely a reflection of their low pH.

As redox conditions change there will be some resistance to change in a food's redox potential, known as poising. This is analogous to buffering of a medium against pH changes and is, like buffering, a 'capacity'

Table 3.6 *Redox potentials of some food materials*

	E (mV)	*pH*
Raw meat (post-rigor)	−200	5.7
Raw minced meat	+225	5.9
Cooked sausages and canned meats	−20 to −150	*Ca.* 6.5
Wheat (whole grain)	−320 to −360	6.0
Barley (ground grain)	+225	7.0
Potato tuber	*Ca.* −150	*Ca.* 6.0
Spinach	+74	6.2
Pear	+436	4.2
Grape	+409	3.9
Lemon	+383	2.2

effect dependent on, and increasing with, the concentration of the couple. Also, like buffering, poising is greatest when the two components of a redox couple are present in equal amounts.

Oxygen, which is present in the air at a level of around 21%, is usually the most influential redox couple in food systems. It has a high E_0' and is a powerful oxidizing agent; if sufficient air is present in a food, a high positive potential will result and most other redox couples present will, if allowed to equilibrate, be largely in the oxidized state. Hence the intrinsic factor of redox potential is inextricably linked with the extrinsic factor of storage atmosphere. Increasing the access of air to a food material by chopping, grinding, or mincing will increase its E_h. This can be seen by comparing the values recorded for raw meat and minced meat, and for whole grain and ground grain in Table 3.6. Similarly, exclusion of air as in vacuum packing or canning will reduce the E_h.

Microbial growth in a food reduces its E_h. This is usually ascribed to a combination of oxygen depletion and the production of reducing compounds such as hydrogen by the micro-organisms. Oxygen depletion appears to be the principal mechanism; as the oxygen content of the medium decreases, so the redox potential declines from a value of around 400 mV at air saturation by about 60 mV for each tenfold reduction in the partial pressure of oxygen.

The decrease in E_h as a result of microbial activity is the basis of some long-established rapid tests applied to foods, particularly dairy products. Redox dyes such as methylene blue or resazurin are sometimes used to indicate changes in E_h which are correlated with microbial levels. Methylene blue is also used to determine the proportion of viable cells in the yeast used in brewing. A cell suspension stained with methylene blue is examined under the microscope and viable cells with a reducing cytoplasm appear colourless. Non-viable cells fail to reduce the dye and appear blue.

Redox potential exerts an important elective effect on the microflora of a food. Although microbial growth can occur over a wide spectrum of

redox potential, individual micro-organisms are conveniently classified into one of several physiological groups on the basis of the redox range over which they can grow and their response to oxygen.

Obligate or strict aerobes are those organisms that are respiratory, generating most of their energy from oxidative phosphorylation using oxygen as the terminal electron acceptor in the process. Consequently they have a requirement for oxygen and a high E_h and will predominate at food surfaces exposed to air or where air is readily available. For example, pseudomonads, such as *Pseudomonas fluorescens*, which grows at an E_h of +100 to +500 mV, and other oxidative Gram-negative rods produce slime and off-odours at meat surfaces. *Bacillus subtilis* (E_h −100 to +135 mV) produces rope in the open texture of bread and *Acetobacter* species growing on the surface of alcoholic beverages, oxidize ethanol to acetic acid to produce either spoilage or vinegar.

Obligate anaerobes tend only to grow at low or negative redox potentials and often require oxygen to be absent. Anaerobic metabolism gives the organism a lower yield of utilizable energy than aerobic respiration, so a reducing environment that minimizes the loss of valuable reducing power from the microbial cell is favoured. The presence or absence of oxygen can naturally affect this, but for many anaerobes, oxygen exerts a specific toxic effect of its own. For example, it has been observed that *Clostridium acetobutylicum* can grow at an E_h as high as +370 mV maintained by ferricyanide, but would not grow at +110 mV in an aerated culture. This effect is linked to the inability of obligate or aero-intolerant anaerobes to scavenge and destroy toxic products of molecular oxygen such as hydrogen peroxide and, more importantly, the superoxide anion radical ($O_2^{-\bullet}$) produced by a one-electron reduction of molecular oxygen. They lack the enzymes catalase and superoxide dismutase, which catalyse the breakdown of these species as outlined below.

$$2\,O_2^{-\bullet} + 2H \underset{\text{superoxide dismutase}}{\longrightarrow} H_2O_2 + O_2 \tag{3.16}$$

$$2\,H_2O_2 \underset{\text{catalase}}{\longrightarrow} 2\,H_2O_2 + O_2 \tag{3.17}$$

Obligate anaerobes, such as clostridia, are of great importance in food microbiology. They have the potential to grow wherever conditions are anaerobic such as deep in meat tissues and stews, in vacuum packs and canned foods causing spoilage and, in the case of *C. botulinum*, the major public health concern: botulism.

Aerotolerant anaerobes are incapable of aerobic respiration, but can nevertheless grow in the presence of air. Many lactic acid bacteria fall into this category; they can only generate energy by fermentation and lack both catalase and superoxide dismutase, but are able to grow in the

presence of oxygen because they have a mechanism for destroying superoxide based on the accumulation of millimolar concentrations of manganese.

3.2.4 Antimicrobial Barriers and Constituents

All foods were at some stage part of living organisms and, as such, have been equipped through the course of evolution with ways in which potentially damaging microbial infections might be prevented or at least limited.

The first of these is the integument: a physical barrier to infection such as the skin, shell, husk or rind of a product. It is usually composed of macromolecules relatively resistant to degradation and provides an inhospitable environment for micro-organisms by having a low water activity, a shortage of readily available nutrients and, often, antimicrobial compounds such as short chain fatty acids (on animal skin) or essential oils (on plant surfaces).

The value of these physical barriers can be clearly seen when they are breached in some way. Physical damage to the integument allows microbial invasion of the underlying nutrient-rich tissues and it is a commonplace observation that damaged fruits and vegetables deteriorate more rapidly than entire products, and that this process is initiated at the site of injury. Consequently it is important to the farmer and food processor that harvesting and transport maintain these barriers intact as far as possible.

As a second line of defence, the product tissues may contain antimicrobial components, the local concentration of which often increases as a result of physical damage. In plants, injury can rupture storage cells containing essential oils or may bring together an enzyme and substrate which were separated in the intact tissue. The latter occurs in plants such as mustard, horseradish, watercress, cabbage and other brassicas to produce antimicrobial isothiocyanates (mustard oils) and in *Allium* species (garlic, onions and leeks) to produce thiosulfinates such as allicin (Figure 3.3). A class of antimicrobials known collectively as phytoalexins are produced by many plants in response to microbial invasion, for example the antifungal compound phaseollin produced in green beans.

Many natural constituents of plant tissues such as pigments, alkaloids and resins have antimicrobial properties, but limited practical use is made of these. Benzoic and sorbic acids found in cranberries and mountain ash berries respectively are notable exceptions that are used in their pure forms as food preservatives. Considerable attention has been directed to the antimicrobial properties of those plants used as herbs and spices to flavour food (Table 3.7). Analysis of their volatile flavour and odour fractions, known as essential oils, has frequently

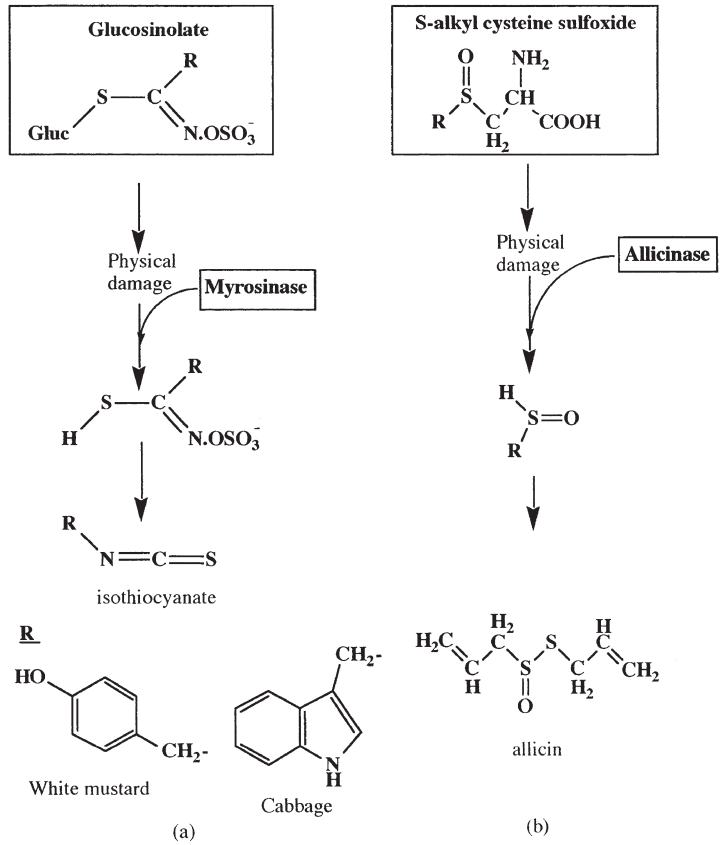


Figure 3.3 *Production of plant antimicrobials as a result of physical damage: (a) isothiocyanate and (b) allicin*

Table 3.7 *Plants used for flavouring food that also possess antimicrobial activity*

Achiote	Dill	Oregano
Allspice (pimento)	Elecampane	Paprika
Almond (bitter)	Fennel	Parsley
Angelica	Garlic	Pennyroyal
Basil (sweet)	Ginger	Pepper
Bay (laurel)	Lemon	Peppermint
Bergamot	Liquorice	Rosemary
Calmus	Lime	Sage
Cananga	Mace	Sassafras
Caraway	Mandarin	Spearmint
Cardamon	Marjoram	Star anise
Celery	Musky bugle	Tarragon (estragon)
Cinnamon	Mustard	Thyme
Citronella	Nutmeg	Turmeric
Clove	Onion	Verbena
Coriander	Orange	Wintergreen

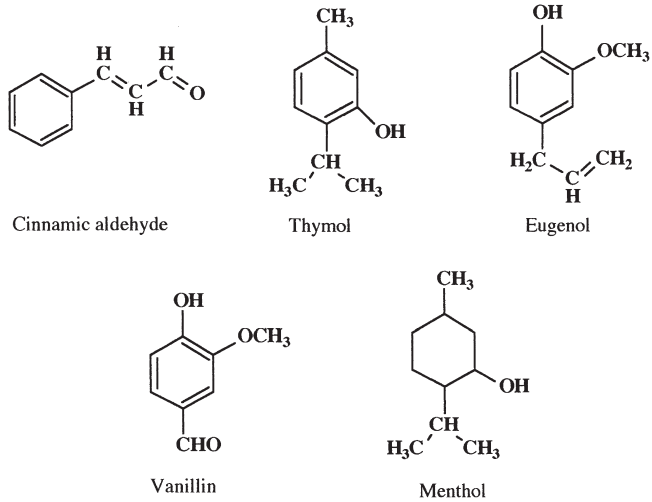


Figure 3.4 *Essential oil components with antimicrobial activity*

identified compounds such as allicin in garlic, eugenol from allspice (pimento), cloves and cinnamon, thymol from thyme and oregano, and cinnamic aldehyde from cinnamon and cassia which have significant antimicrobial activity (Figure 3.4). As a consequence, herbs and spices may contribute to the microbiological stability of foods in which they are used. It has, for example, been claimed that inclusion of cinnamon in raisin bread retards mould spoilage. Usually, however, their role in preservation is likely to be minor and, in some cases, they can be a source of microbial contamination leading to spoilage or public health problems. Outbreaks of botulism associated with crushed garlic in oil and home canned peppers demonstrate that even in relatively high concentrations plant antimicrobials are not a complete guarantee of safety.

Antimicrobial components differ in their spectrum of activity and potency, they are present at varying concentrations in the natural product, and are frequently at levels too low to have any effect. Hops and their extracts are ubiquitous ingredients in beer. Humulones contained in the hop resin and isomers produced during processing, impart the characteristic bitterness of the product but have also been shown to possess activity against the common beer spoilage organisms, lactic acid bacteria. When first introduced into brewing, hops probably contributed to microbiological stability, but this is less likely nowadays with the relatively low hopping rates used. In fact the ability of lactic acid bacteria to acquire resistance to hop resins means that the brewery environment probably acts as a very efficient natural enrichment culture for humulone-tolerant bacteria, thus negating any beneficial effects.

A rather different example of the importance of plant antimicrobials is provided by oleuropein, the bitter principle of green olives. In the

production of Spanish-style green olives, it is removed by an alkali extraction process, primarily for reasons of flavour. However oleuropein and its aglycone are also thought to be inhibitory to lactic acid bacteria; if not removed at this early stage, they would prevent the necessary fermentation occurring subsequently.

Animal products too, have a range of non-specific antimicrobial constituents. Probably the supreme example of this is the white or albumen of the hen's egg which possesses a whole battery of inhibitory components. Many of the same or similar factors can also be found in milk where they are present in lower concentrations and are thus less effective.

Both products contain the enzyme lysozyme which catalyses the hydrolysis of glycosidic linkages in peptidoglycan, the structural polymer responsible for the strength and rigidity of the bacterial cell wall. Destruction or weakening of this layer causes the cell to rupture (lyse) under osmotic pressure. Lysozyme is most active against Gram-positive bacteria, where the peptidoglycan is more readily accessible, but it can also kill Gram-negatives if their protective outer membrane is damaged in some way.

Other components limit microbial growth by restricting the availability of key nutrients. Ovotransferrin in egg white and lactoferrin in milk are proteins that scavenge iron from the medium. Iron is an essential nutrient for all bacteria and many have evolved means of overcoming iron limitation by producing their own iron-binding compounds known as siderophores.

In addition, egg white has powerful cofactor-binding proteins such as avidin and ovoflavoprotein which sequester biotin and riboflavin restricting the growth of those bacteria for which they are essential nutrients, see Table 3.8.

Table 3.8 *Antimicrobials in hen's egg albumen and milk*

Albumen	*Milk*
Nutrient Status	
High pH	Moderate pH
Low levels of available nitrogen	High levels of protein, carbohydrate and fat
Antimicrobials	
Ovotransferrin (conalbumin) (12% of solids)	Lactoferrin
Lysozyme (3.5% of solids)	Lysozyme
Avidin – (0.05% of solids)	–
Ovoflavoprotein –(0.8% of solids)	–
Ovomucoid & ovoinhibitor – (protease inhibitors) (11% of solids)	–
	Lactoperoxidase (30 mg l^{-1})
	Immunoglobulin (300 mg l^{-1})

Milk also has the capacity to generate antimicrobials in the presence of hydrogen peroxide. The enzyme lactoperoxidase constitutes about 0.5% of whey proteins and catalyses the oxidation of thiocyanate by hydrogen peroxide. Thiocyanate is naturally present in milk and its level can be boosted by consumption of brassicas which are rich in thiocyanate precursors. Hydrogen peroxide can be generated by endogenous enzyme activity or by the aerobic metabolism of lactic acid bacteria. The reaction produces short lived oxidation products such as hypothiocyanate which can kill Gram negative bacteria and inhibit Gram positives, possibly by damaging the bacterial cytoplasmic membrane (Figure 3.5).

3.2.5 Water Activity

Water is a remarkable compound. Considered as a hydride of oxygen (H_2O) it has quite exceptional properties when compared with the hydrides of neighbouring elements in the periodic table such as ammonia (NH_3), methane (CH_4), hydrogen sulfide (H_2S), and hydrofluoric acid (HF), see Table 3.9. Life as we know it is totally dependent on the presence of water in its liquid state. The reactions which take place in the cytoplasm do so in an aqueous environment and the cytoplasm is surrounded by a membrane which is generally permeable to water molecules which may pass freely from the cytoplasm to the environment and from the environment to the cytoplasm. This dynamic two way flow

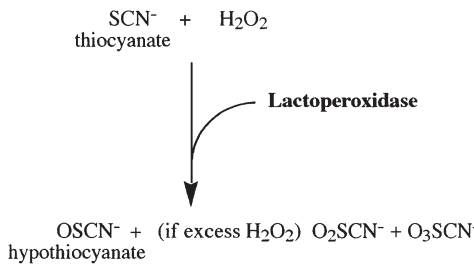


Figure 3.5 *The lactoperoxidase system*

Table 3.9 *The boiling points (°C) of hydrides of elements surrounding oxygen in the periodic table*

B_2H_6	CH_4	NH_3	H_2O	HF
−92.5	−161.7	−33	100	19.4
	SiH_4	PH_3	H_2S	HCl
	−112	−87	−60	−85
			H_2Se	
			−42	

of water molecules is normally in a steady state and a living organism will only be stressed if there is a net flow out of the cytoplasm, leading to plasmolysis, or a net flow into the cell leading to rupture of the membrane, and the latter is normally prevented by the presence of a cell wall in the bacteria and fungi.

In our everyday lives we think of water as existing in its liquid state between its freezing point (0 °C) and boiling point (100 °C) and we might expect that this would limit the minimum and maximum temperatures at which growth could possibly occur. But, of course, the freezing point of water can be depressed by the presence of solutes and there are a number of micro-organisms which can actively grow at subzero temperatures because their cytoplasm contains one or more compounds, such as a polyol, which act as an antifreeze. Similarly the boiling point of water can be elevated by increased hydrostatic pressure and, in nature, very high pressures exist at the bottom of the deep oceans. Under these circumstances the temperature of liquid water may be well above 100 °C and the relatively recent exploration of submarine volcanic vents has uncovered some remarkable bacteria which can indeed grow at such high temperatures.

Although the cytoplasm must be in the liquid phase for active growth (and it is important not to confuse growth and survival, for many microorganisms can survive but not grow when their cytoplasm has been completely dried), water in the environment of the living organism may be present, not only in the liquid phase as pure water or a solution, but also in the atmosphere in the gaseous phase, or associated with what would be described macroscopically as the solid phase (Figure 3.6).

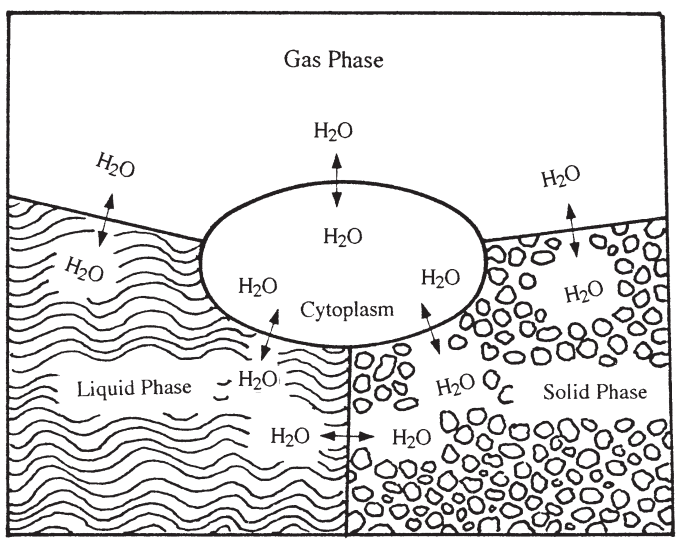


Figure 3.6 *A cell in equilibrium with liquid, solid and gaseous phases, each of these being in equilibrium with each other*

A useful parameter which helps us to understand the movement of water from the environment to the cytoplasm or from the cytoplasm to the environment is water activity, a_w. The water activity of a substrate is most conveniently defined as the ratio of the partial pressure of water in the atmosphere in equilibrium with the substrate, P, compared with the partial pressure of the atmosphere in equilibrium with pure water at the same temperature, P_0. This is numerically equal to the equilibrium relative humidity (ERH) expressed as a fraction rather than as a percentage:

$$a_w = \frac{P}{P_0} = \frac{1}{100}\text{ERH} \tag{3.18}$$

This has important implications for the storage of low a_w foods (see Section 3.3.1).

In 1886 François Marie Raoult described the behaviour of an ideal solution by an equation which has since then been known as Raoult's law:

$$P_A = X_A P_{A_0} \tag{3.19}$$

where P_A is the partial vapour pressure of A above a solution, in which X_A is the mole fraction of the solvent A, and PA_0 is the vapour pressure of pure liquid A at the same temperature. If the solvent A is water then Equations (3.18) and (3.19) can be combined to give:

$$a_w = X_{\text{water}} \tag{3.20}$$

Thus for an aqueous solution the water activity is approximately given by the ratio of the number of moles of water to the total number of moles (*i.e.* water + solute), *i.e.*:

$$a_w = N_w/(N_w + N_s) \tag{3.21}$$

It should be noted that water activity is a colligative property, that is to say it depends on the number of molecules or ions present in solution, rather than their size. Thus a compound like sodium chloride, which dissociates into two ions in solution, is more effective at reducing the water activity than a compound like sucrose on a mole-to-mole basis.

Physical chemists would prefer to work with the chemical potential of water (μ_w), which is a complex parameter made up of a reference state, a water activity term, a pressure term and a gravitational term:

$$\mu_w = \mu_w^* + RT \ln a_w + V_m P + mgh \tag{3.22}$$

which can be rearranged to give a new parameter, ψ, known as the water potential having the same dimensions as pressure:

$$\psi = \frac{\mu_w - \mu_w^*}{V_m} = \frac{RT}{V_m} \ln a_w + P + \frac{mgh}{V_m} \tag{3.23}$$

For situations associated with everyday life on the surface of the Earth it is possible to ignore the pressure and gravity terms and a good approximation of the relationship between the water potential and water activity is given by Equation (3.24):

$$\psi = \frac{RT}{V_m} \ln a_w \tag{3.24}$$

where R (the gas constant) $= 0.08205\,\text{dm}^3\ \text{atm}\,\text{K}^{-1}\ \text{mol}^{-1}$; and V_m (the molar volume of water) $= 0.018\,\text{dm}^3\ \text{mol}^{-1}$.

Thus at 25 °C (298 °K) a water activity of 0.9 would correspond to a water potential of −143 atm or −14.5 MPa.

Water potential may contain both an osmotic component, associated with the effect of solutes in solution, and a matric component, associated with the interaction of water molecules with surfaces, which can be clearly demonstrated by the rise of water in a capillary tube. The latter might be particularly important in discussions about the availability of water in a complex matrix such as cake.

A parameter related to water activity is osmotic pressure which can be thought of as the force per unit area required to stop the net flow of water molecules from a region of high to one of low water activity. Cytoplasm is an aqueous solution and so must have a lower water activity than pure water; thus a micro-organism in an environment of pure water will experience a net flow of water molecules into the cytoplasm. If it cannot control this it will increase in size and burst. Bacteria, fungi and algae cope by having a rigid strong wall capable of withstanding the osmotic pressure of the cytoplasm which may be as high as 30 atm (*ca.* 3 MPa) in a Gram-positive bacterium or as little as 5 atm (*ca.* 0.5 MPa) in a Gram-negative species. Freshwater protozoa, on the other hand, cope with the net flow of water into the cell by actively excreting it out again with a contractile vacuole.

As water activity is decreased, or osmotic pressure is increased, in the environment it is essential that the water activity of the cytoplasm is even lower, or its osmotic pressure even higher. This is achieved by the production of increasing concentrations of solutes which must not interfere with cytoplasmic function. They are thus known as compatible solutes and include such compounds as the polyols glycerol, arabitol and mannitol in the fungi and amino acids or amino acid derivatives in the bacteria.

With a reduction of water activity in their environment the number of groups of micro-organisms capable of active growth decreases (Table 3.10). The exact range of water activities allowing growth is influenced by other physico-chemical and nutritional conditions but Figure 3.7 illustrates the range for a number of individual species of micro-organisms

Table 3.10 *Minimum water activities at which active growth can occur*

Group of micro-organism	*Minimum a_w*
Most Gram-negative bacteria	0.97
Most Gram-positive bacteria	0.90
Most yeasts	0.88
Most filamentous fungi	0.80
Halophilic bacteria	0.75
Xerophilic fungi	0.61

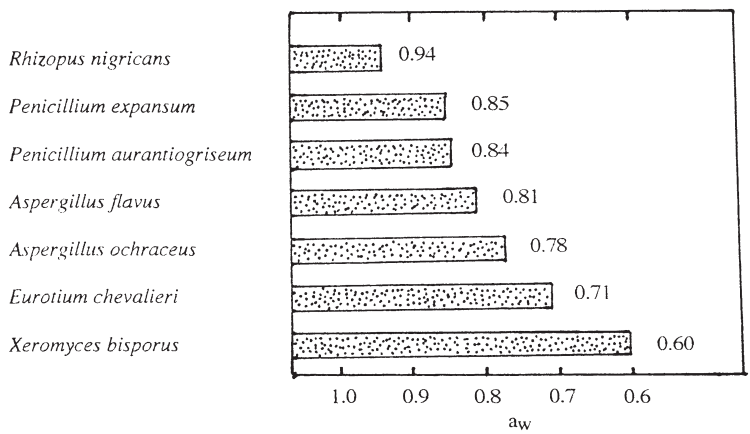


Figure 3.7 *Range of a_w values allowing growth of a number of species of micro-organisms*

and Figure 3.8 demonstrates the interaction between temperature and water activity for *Aspergillus flavus* and *Penicillium expansum*.

Figure 3.9 shows the range of a_w values associated with a number of different food commodities. Because low water activities are associated with three distinct types of food three terms are used to describe the micro-organisms especially associated with these foods:

(i) *halotolerant* – able to grow in the presence of high concentrations of salt
(ii) *osmotolerant* – able to grow in the presence of high concentrations of unionized organic compounds such as sugars
(iii) *xerotolerant* – able to grow on dry foods.

These terms do not describe rigidly exclusive groups of micro-organisms but are useful in the context of studies of particular food commodities. Some micro-organisms actually grow better at reduced a_w and may be described as halophilic, osmophilic or xerophilic, indeed the halobacteria are obligately halophilic and cannot grow in the absence of high

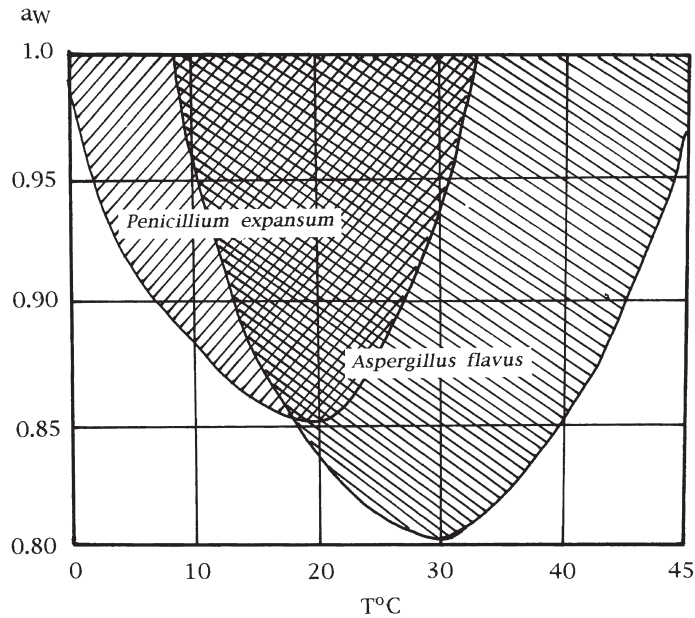


Figure 3.8 *Temperature water activity combinations allowing the growth of* Aspergillus flavus *and* Penicillium expansum

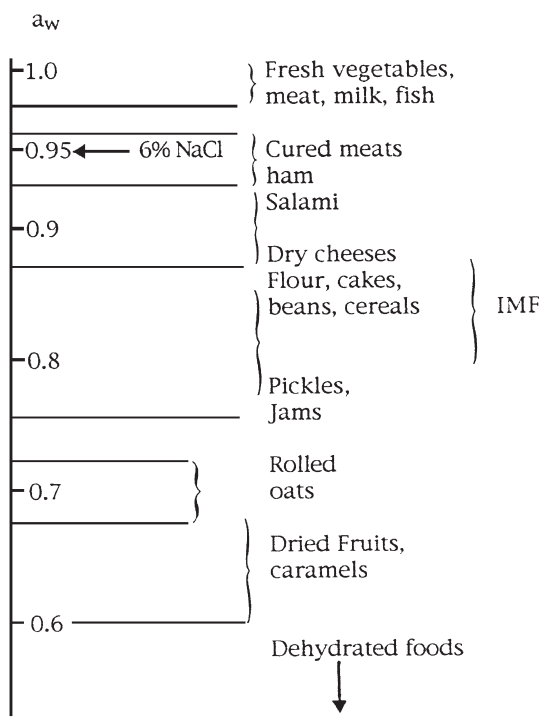


Figure 3.9 *Range of a_w values associated with a number of food commodities*

concentrations of salt. This group of bacteria, which includes such genera as *Halobacterium* and *Halococcus*, belong to the Archaebacteria and accumulate potassium chloride as their compatible solute. They are obligately halophilic because the integrity of their outer wall depends on a high concentration of sodium chloride in their environment. They are usually associated with salt lakes or salt pans where solar salt is being made and may cause the proteolytic spoilage of dried, salted fish.

The limiting value of water activity for the growth of any micro-organism is about 0.6 and below this value the spoilage of foods is not microbiological but may be due to insect damage or chemical reactions such as oxidation. At a water activity of 0.6, corresponding to a water potential of −68 MPa, the cytoplasm would need to contain very high concentrations of an appropriate compatible solute and it is probable that macromolecules such as DNA would no longer function properly and active growth must cease. However, it is important to note that, even if active growth is impossible, survival may still occur and many micro-organisms can survive at very low water activities and are frequently stored in culture collections in this form.

It is a relatively simple matter to determine the water content of a food commodity by drying to constant weight under defined conditions. The water content, however, may not give a good indication of how available that water is, *i.e.* what the water activity is, unless the relationship between these two properties has been established. Thus, oil-rich nuts with a water content of 4–9%, protein rich legumes with 9–13% water content and sucrose rich dried fruits with a water content of 18–25% could all have the same water activity of about 0.7 and would thus be acceptably stable to spoilage by most micro-organisms.

The relationship between water activity and water content is very sensitive to temperature and may seem to depend on whether water is being added or removed from a substrate. An example of a water sorption isotherm is shown in Figure 3.10. In this example the material has been allowed to equilibrate effectively at a known water activity before measuring the water content but Figure 3.11 demonstrates the differences which may be observed depending on whether a given water content is achieved by adding water to a dry commodity or removing it from a wet commodity. The same water content seems to be associated with a higher a_w in the former case than in the latter. This hysteresis phenomenon is a reflection of the long time that it may take for water to equilibrate with the constituents of a complex food matrix.

The measurement of water activity can thus be achieved by measuring the water content if the shape of the isotherm has been determined. Water activity can be measured by measuring the equilibrium relative humidity of the atmosphere in contact with the sample. This can be done by the dew point method or with a hair hygrometer. There are a number

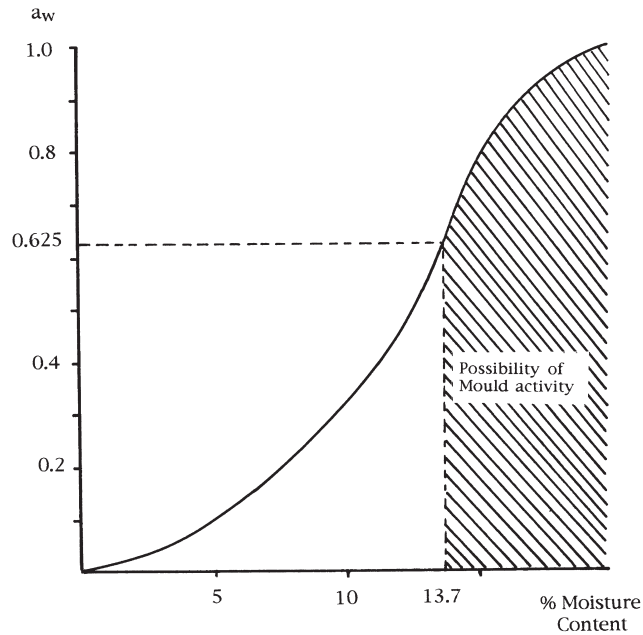


Figure 3.10 *Water sorption isotherm for wheat at 25 °C*

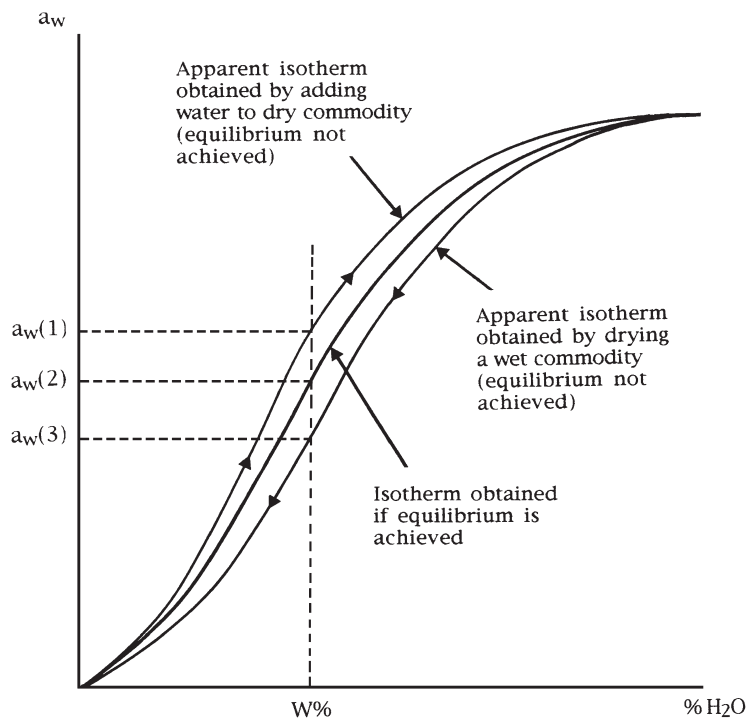
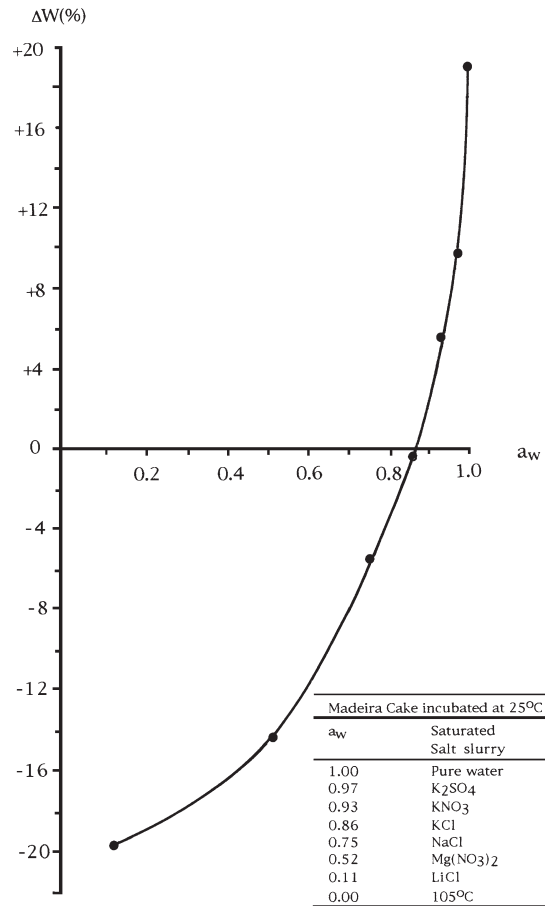


Figure 3.11 *Hysteresis associated with the relationship between apparent water activity and water content*

of instruments which measure relative humidity through its effect on the electrical properties, such as conductivity or resistivity, of materials. Thus the resistance of lithium chloride, or the capacitance of anodized aluminium, changes with changes of relative humidity.

A method known as the Landrock–Proctor method depends on gravimetrically measuring changes in water content of samples of the material after equilibration with atmospheres of known relative humidity which can be obtained using saturated solutions of a number of inorganic salts. If the sample has a lower a_w than the atmosphere then it will gain weight, if it has a higher a_w then it will lose weight. By carrying out measurements of weight change over a range of relative humidities it is possible to extrapolate to the relative humidity which would cause no weight loss and thus corresponds to the a_w of the sample. Figure 3.12 shows the result of such an experiment with samples of Madeira cake and Table 3.11 shows the water activities of a variety of saturated salt



Madeira Cake incubated at 25°C at a range of aw

a_w	Saturated Salt slurry	ΔW(%)
1.00	Pure water	+18.92
0.97	K_2SO_4	+9.75
0.93	KNO_3	+5.53
0.86	KCl	-0.26
0.75	NaCl	-5.58
0.52	$Mg(NO_3)_2$	-14.30
0.11	LiCl	-19.52
0.00	105°C	-22.90

Figure 3.12 *Weight changes of samples of madeira cake at different ERH values*

Table 3.11 *Water activities of saturated salts solution at 25 °C*

Salt	a_w
Lithium chloride	0.11
Zinc nitrate	0.31
Magnesium chloride	0.33
Potassium carbonate	0.43
Magnesium nitrate	0.52
Sodium bromide	0.57
Lithium acetate	0.68
Sodium chloride	0.75
Potassium chloride	0.86
Potassium nitrate	0.93
Pure water	1.00

solutions at 25 °C. Some of these salt solutions have large temperature coefficients and so the temperature needs to be very carefully controlled.

3.3 EXTRINSIC FACTORS (ENVIRONMENTAL LIMITATIONS)

3.3.1 Relative Humidity

It has already been seen in Section 3.2.5. that relative humidity and water activity are interrelated, thus relative humidity is essentially a measure of the water activity of the gas phase. When food commodities having a low water activity are stored in an atmosphere of high relative humidity water will transfer from the gas phase to the food. It may take a very long time for the bulk of the commodity to increase in water activity, but condensation may occur on surfaces giving rise to localized regions of high water activity. It is in such regions that propagules which have remained viable, but unable to grow, may now germinate and grow. Once micro-organisms have started to grow and become physiologically active they usually produce water as an end product of respiration. Thus they increase the water activity of their own immediate environment so that eventually micro-organisms requiring a high a_w are able to grow and spoil a food which was initially considered to be microbiologically stable.

Such a situation can occur in grain silos or in tanks in which concentrates and syrups are stored. Another problem in large-scale storage units such as grain silos occurs because the relative humidity of air is very sensitive to temperature. If one side of a silo heats up during the day due to exposure to the sun then the relative humidity on that side is reduced and there is a net migration of water molecules from the cooler side to re-equilibrate the relative humidity. When that same side cools down again the relative humidity increases and, although water molecules migrate back again, the temporary increase in relative humidity may be sufficient

to cause local condensation onto the grain with a localized increase in a_w sufficient to allow germination of fungal spores and subsequent spoilage of the grain. This type of phenomenon can often account for localized caking of grain which had apparently been stored at a 'safe' water content.

The storage of fresh fruit and vegetables requires very careful control of relative humidity. If it is too low then many vegetables will lose water and become flaccid. If it is too high then condensation may occur and microbial spoilage may be initiated.

3.3.2 Temperature

Microbial growth can occur over a temperature range from about −8 °C up to 100 °C at atmospheric pressure. The most important requirement is that water should be present in the liquid state and thus available to support growth (see Section 3.2.5). No single organism is capable of growth over the whole of this range; bacteria are normally limited to a temperature span of around 35 °C and moulds rather less, about 30 °C.

A graph showing the variation of growth rate with temperature illustrates several important features of this relationship (Figure 3.13). Firstly, each organism exhibits a minimum, optimum and maximum temperature at which growth can occur. These are known as cardinal temperatures and are, to a large extent, characteristic of an organism, although they are influenced by other environmental factors such as nutrient availability,

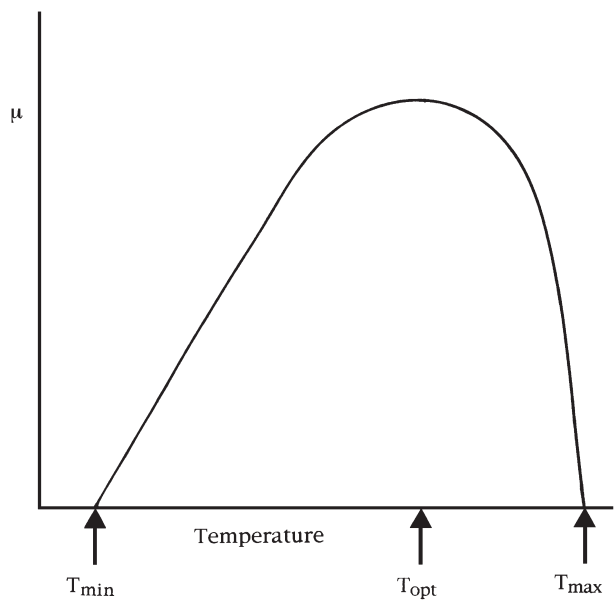


Figure 3.13 *Effect of temperature on growth rate*

Table 3.12 *Cardinal temperatures for microbial growth*

	Temperature (°C)		
Group	*Minimum*	*Optimum*	*Maximum*
Thermophiles	40–45	55–75	60–90
Mesophiles	5–15	30–40	40–47
Psychrophiles (obligate psychrophiles)	−5 to +5	12–15	15–20
Psychrotrophs (facultative psychrophiles)	−5 to +5	25–30	30–35

(Adapted from ICMSF 1980)

pH and a_w. Micro-organisms can be classified into several physiological groups based on their cardinal temperatures. This is a useful, if rather arbitrary, convention, since the distribution of micro-organisms through the growth temperature range is continuous. To take account of this and the effect of other factors, it is more appropriate to define cardinal temperatures as ranges rather than single values (Table 3.12).

In food microbiology mesophilic and psychrotrophic organisms are generally of greatest importance. Mesophiles, with temperature optima around 37 °C, are frequently of human or animal origin and include many of the more common foodborne pathogens such as *Salmonella*, *Staphylococcus aureus* and *Clostridium perfringens*.

As a rule mesophiles grow more quickly at their optima than psychrotrophs and so spoilage of perishable products stored in the mesophilic growth range is more rapid than spoilage under chill conditions. Because of the different groups of organisms involved, it can also be different in character.

Among the organisms capable of growth at low temperatures, two groups can be distinguished: the true or strict psychrophiles ('cold loving') have optima of 12–15 °C and will not grow above about 20 °C. As a result of this sensitivity to quite moderate temperatures, psychrophiles are largely confined to polar regions and the marine environment. Psychrotrophs or facultative psychrophiles will grow down to the same low temperatures as strict psychrophiles but have higher optimum and maximum growth temperatures. This tolerance of a wider range of temperatures means that psychrotrophs are found in a more diverse range of habitats and consequently are of greater importance in the spoilage of chilled foods.

Thermophiles are generally of far less importance in food microbiology, although thermophilic spore formers such as certain *Bacillus* and *Clostridium* species do pose problems in a restricted number of situations (see Chapter 4).

Another feature evident from Figure 3.13 is that the curve is asymmetric – growth declines more rapidly above the optimum temperature than below it. As the temperature is decreased from the optimum the

growth rate slows, partly as a result of the slowing of enzymic reactions within the cell. If this were the complete explanation however, then the change in growth rate with temperature below the optimum might be expected to follow the Arrhenius Law which describes the relationship between the rate of a chemical reaction and the temperature. The fact that this is not observed in practice is, on reflection, hardly surprising since microbial growth results from the activity of a network of interacting and interregulating reactions and represents a far higher order of complexity than simple individual reactions.

A most important contribution to the slowing and eventual cessation of microbial growth at low temperatures is now considered to be changes in membrane structure that affect the uptake and supply of nutrients to enzyme systems within the cell. It has been shown that many micro-organisms respond to growth at lower temperatures by increasing the proportion of unsaturated and/or shorter chain fatty acids in their membranes and that psychrotrophs generally have higher levels of these acids than mesophiles. Increasing the degree of unsaturation or decreasing the carbon chain length of a fatty acid decreases its melting point so that membranes containing these will remain fluid and hence functional at lower temperatures.

As the temperature increases above the optimum, the growth rate declines much more sharply as a result of the irreversible denaturation of proteins and the thermal breakdown of the cell's plasma membrane. At temperatures above the maximum for growth, these changes are sufficient to kill the organism – the rate at which this occurs increasing with increasing temperature. The kinetics of this process and its importance in food preservation are discussed in Chapter 4.

3.3.3 Gaseous Atmosphere

Oxygen comprises 21% of the earth's atmosphere and is the most important gas in contact with food under normal circumstances. Its presence and its influence on redox potential are important determinants of the microbial associations that develop and their rate of growth. Since this topic has already been discussed in some detail under redox potential (Section 3.2.3), this section will be confined to the microbiological effects of other gases commonly encountered in food processing.

The inhibitory effect of carbon dioxide (CO_2) on microbial growth is applied in modified-atmosphere packing of food and is an advantageous consequence of its use at elevated pressures (hyperbaric) in carbonated mineral waters and soft drinks.

Carbon dioxide is not uniform in its effect on micro-organisms. Moulds and oxidative Gram-negative bacteria are most sensitive and the Gram-positive bacteria, particularly the lactobacilli, tend to be most

resistant. Some yeasts such as *Brettanomyces* spp. also show considerable tolerance of high CO_2 levels and dominate the spoilage microflora of carbonated beverages. Growth inhibition is usually greater under aerobic conditions than anaerobic and the inhibitory effect increases with decrease of temperature, presumably due to the increased solubility of CO_2 at lower temperatures. Some micro-organisms are killed by prolonged exposure to CO_2 but usually its effect is bacteriostatic.

The mechanism of CO_2 inhibition is a combination of several processes whose precise individual contributions are yet to be determined. One factor often identified is the effect of CO_2 on pH. Carbon dioxide dissolves in water to produce carbonic acid which partially dissociates into bicarbonate anions and protons. Carbonic acid is a weak dibasic acid (pK_a 6.37 and 10.25); in an unbuffered solution it can produce an appreciable drop in pH, distilled water in equilibrium with the CO_2 in the normal atmosphere will have a pH of about 5, but the effect will be less pronounced in buffered food media so that equilibration of milk with 1 atmosphere pCO_2 decreased the pH from 6.6 to 6.0. Probably of more importance than its effect on the growth medium is the ability of CO_2 to act in the same way as weak organic acids (see Section 3.2.2), penetrating the plasma membrane and acidifying the cell's interior.

Other contributory factors are thought to include changes in the physical properties of the plasma membrane adversely affecting solute transport; inhibition of key enzymes, particularly those involving carboxylation /decarboxylation reactions in which CO_2 is a reactant; and reaction with protein amino groups causing changes in their properties and activity.

3.4 IMPLICIT FACTORS

A third set of factors that are important in determining the nature of microbial associations found in foods are described as *implicit factors* – properties of the organisms themselves, how they respond to their environment and interact with one another.

At its simplest, an organism's specific growth rate can determine its importance in a food's microflora; those with the highest specific growth rate are likely to dominate over time. This will of course depend upon the conditions prevailing; many moulds can grow perfectly well on fresh foods such as meat, but they grow more slowly than bacteria and are therefore out-competed. In foods where the faster growing bacteria are inhibited by factors such as reduced pH or a_w, moulds assume an important role in spoilage. Alternatively, two organisms may have similar maximum specific growth rates but differ in their affinity (K_s) for a growth limiting substrate (see Equation 3.8). If the level of that substrate is sufficiently low that it becomes limiting, then the organism with the lower K_s (higher affinity) will outgrow the other.

In Sections 3.2 and 3.3 we described how microbial growth and survival are influenced by a number of factors and how micro-organ-isms respond to changes in some of these. This response does however depend on the physiological state of the organism. Exponential phase cells are almost always killed more easily by heat, low pH or antimicrobials than stationary phase cells and often the faster their growth rate the more readily they are killed. This makes sense intuitively; the consequences of a car crash are invariably more serious the faster the car is travelling at the time. At higher growth rates, where cell activity is greater and more finely balanced, the damage caused by a slight jolt to the system will be more severe than the same perturbation in cells growing very slowly or not at all. The precise mechanism leading to cell death is almost certainly very complex. One proposal is that lethal damage is largely a result of an oxidative burst, the production of large numbers of damaging free radicals within the cell in response to the physical or chemical stress applied. This would mean that cell death is in fact a function of the organism's response to a stress rather than a direct effect of the stress itself.

A cell's sensitivity to potentially lethal treatments can also be affected by its previous history. Generally, some form of pre-adaptation will decrease the damaging effect of adverse conditions. Growth or holding organisms such as *Salmonella* at higher temperatures has been shown to increase their heat resistance. Pre-exposure to moderately low pH can increase an organism's subsequent resistance to a more severe acid challenge. Growth at progressively lower temperatures can reduce the minimum temperature at which an organism would otherwise grow.

Some reaction to stress can be apparent very soon after exposure as existing enzymes and membrane proteins sense and react to the change. Other responses occur more slowly since they involve gene transcription and the production of proteins. The most extensively studied of this type of response is the production of heat shock proteins; proteins produced following exposure to elevated temperatures and which protect the cell from heat damage. Some heat shock proteins, described as chaperones or chaperonins, interact with unfolded or partially unfolded proteins and assist them in reaching their correct conformation. Chaperonins are present in normal cells but obviously far more will be needed during processes such as heating which increase the rate at which cellular proteins denature.

Heat shock proteins are encoded by genes which have a specific sigma factor, sigma 32 also known as RpoH, for transcription. Sigma factors are proteins which bind to DNA-dependent RNA polymerase, the enzyme which transcribes DNA into messenger RNA. When bound to the polymerase they confer specificity for certain classes of promoter on the DNA and thus help determine which regions of the genome are

transcribed. Another alternative sigma factor RpoS, also known as the stationary phase sigma factor, has been identified in a number of Gram-negative bacteria and a similar regulon sigma B operates in Gram-positive bacteria. RpoS is produced in cells throughout growth but is rapidly degraded in exponential phase cells. As growth slows at the end of exponential phase it accumulates and directs the transcription of a battery of genes associated with the stationary phase, many of which are protective.

It is now clear that RpoS is a general stress response regulator and also accumulates in response to environmental stresses such as low pH and osmotic stress. Since the RpoS response confers resistance to a range of stresses, exposure to one factor such as low pH can confer increased resistance to other stresses such as heat. Of equal concern is the observation that RpoS also plays a role in regulating expression of genes associated with virulence in some food borne pathogens and that virulence factors expressed as the cells enter stationary phase can also be induced by stress. The implications of this for food microbiology are considerable, for not only do they suggest that stresses micro-organisms encounter during food processing may increase resistance to other stresses, but that they could also increase the virulence of any pathogens present.

Until now we have dealt with micro-organisms largely as isolated individuals and have not considered any effects they might have on each other. Cell to cell communication has however been shown to play a part in the induction of stress responses. Molecules such as acylhomoserine lactones and proteins secreted by cells in response to a stress have been shown to produce a stress response in others, implying that cells in the vicinity which have not necessarily been directly exposed to the stress may also increase in resistance.

Ecologists have identified a number of different ways in which organisms can interact and several of these can be seen in the microbial ecology of food systems. Mutualism, when growth of one organism stimulates the growth of another, is well illustrated by the interaction of the starter cultures in yoghurt fermentation (see Section 9.5.1). Similar stimulatory effects can be seen in spoilage associations or in sequences of spoilage organisms seen when growth of one organism paves the way for others. For example, a grain's water activity may be sufficiently low to prevent the growth of all but a few fungi, once these begin to grow however water produced by their respiration increases the local water activity allowing less xerophilic moulds to take over. Alternatively, one organism might increase the availability of nutrients to others by degrading a food component such as starch or protein into more readily assimilable compounds. Some micro-organisms may remove an inhibitory component and thereby permit the growth of others. This last example has had

safety implications in mould-ripened cheeses where mould growth increases the pH allowing less acid tolerant organisms such as *Listeria monocytogenes* to grow.

Alternatively, micro-organisms may be antagonistic towards one another producing inhibitory compounds or sequestering essential nutrients such as iron. The best practical examples of this in food microbiology are the lactic fermented foods which are discussed in some detail in Chapter 9.

3.5 PREDICTIVE FOOD MICROBIOLOGY

Understanding how different properties of a food, its environment and its history can influence the microflora that develops on storage is an important first step towards being able to make predictions concerning shelf-life, spoilage and safety. The food industry is continually creating new microbial habitats, either by design in developing new products and reformulating traditional ones, or by chance, as a result of variations in the composition of raw materials or in a production process. To be able to predict microbial behaviour in each new situation and determine its consequences for food safety and quality, we must first describe accurately the food environment and then determine how this will affect microbial growth and survival.

Characterization of a habitat in terms of its chemical and physical properties is generally straightforward, although problems can arise if a property is not uniformly distributed throughout the product. This can be a particular problem with solid foods, for example the local salt concentration may vary considerably within a ham or a block of cheese and we have seen in Section 3.3.1 how water can migrate through a mass of food.

A considerable amount of data is available on how factors such as pH, a_w, and temperature affect the growth and survival of microorganisms and some of this was described in Sections 3.2 and 3.3. Much of this information was however acquired when only one or two factors had been changed and all the others were optimal or near-optimal. In many foods a very different situation applies, micro-organisms experience a whole battery of sub-optimal factors which collectively determine the food's characteristics as a medium for microbial growth.

Leistner described this situation as the 'hurdle effect', where each inhibitory factor can be visualized as a hurdle contributing to a food's overall stability and safety (Figure 3.14). The analogy of a hurdle race, while vivid, has been criticized on the grounds that it can lead to the misapprehension that micro-organisms confront each hurdle in turn. In reality they are usually faced with the aggregate effect of all the barriers at once and it is this which determines whether the organisms can grow

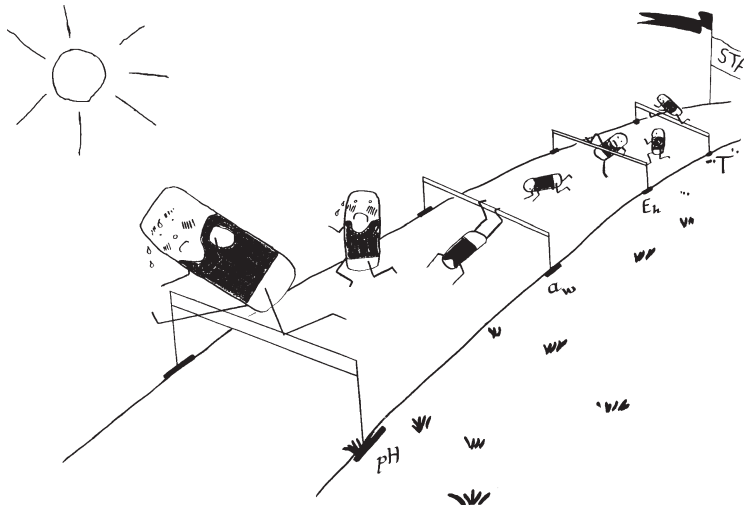


Figure 3.14 *The hurdle effect*

or how fast they can grow. Perhaps a better way to visualise it is as a protective wall; each adverse condition contributes one or more layers of bricks to the wall. The overall height of the wall will determine which organisms are able to climb it and how fast they will grow once they have done so. Scientific description of this multi-factorial technique for preserving foods may be relatively recent but the concept has been applied empirically since antiquity in numerous traditional products such as cheese, cured meats, smoked fish and fruit preserves, all of which rely on a number of contributing factors for their stability and safety.

When confronted with this situation there are three basic approaches to predicting the fate of particular organisms. The first is to seek an expert judgement, based on the individual expertise of a food microbiologist and their interpretation of the published literature. While this can be useful qualitatively, it rarely provides reliable quantitative data.

To its credit, the food industry has generally not placed too great a reliance on this sort of approach but has resorted to the challenge trial. In this, the organism of concern is inoculated into the food material and its fate followed through simulated conditions of processing, storage, distribution, temperature abuse, or whatever is required. Though it provides reliable data, the challenge trial is expensive, time consuming and labour intensive to perform properly. It also has extremely limited predictive value since its predictions hold only for the precise set of conditions tested. Any change in formulation or conditions of processing or storage will invalidate the predictions and necessitate a fresh challenge trial under the new set of conditions.

The third and increasingly popular approach is the use of mathematical models. A model is simply an object or concept that is used to

represent something else and a mathematical model is one constructed using mathematical concepts such as constants, variables, functions, equations, *etc*.

Mathematical models are not entirely new to food microbiology having been used with great success since the 1920s for predicting the probability of *Clostridium botulinum* spores surviving a particular heat process and enabling the design of heat processes for low acid canned foods with an acceptable safety margin (see Section 4.1.5).

The log-linear *C. botulinum* model is an inactivation model, describing microbial survival, but models predicting the potential for microbial growth to occur under a range of conditions can also be constructed. These are generally more complex but their development has been facilitated by the availability and accessability of powerful modern computers.

There are four essential steps in developing a model.

(1) *Planning*. This requires a clear definition of the problem:

   (i) are we interested in spoilage or safety, which organisms are our main concern?
   (ii) what is the appropriate response or dependent variable, *e.g*. growth rate, toxin production, time to spoilage?
   (iii) what are the relevant explanatory or independent variables, *e.g*. temperature, pH, a_w?

(2) *Data collection*. The response variable identified in the planning stage is measured for various levels of the explanatory variables. These should cover the full range in which we may be interested since the predictive value of the model is limited to situations where unknown values can be interpolated. Extrapolation into areas where there are no data points will not yield valid predictions.
(3) *Model fitting*. Different models which relate the response variable to the explanatory variables are tested to see how well they fit the experimental data.
(4) *Model validation*. The model is evaluated using experimental data not used in building the model.

A number of different types of model are commonly used. Probabilistic models give a quantitative assessment of the chance that a particular microbiological event will occur within a given time and are most suited to situations where the hazard is severe. The event most often described in such models is the probability of toxin formation (*i.e*. growth) by *C. botulinum*. The work was initially prompted by the

perceived need to reduce nitrite levels in cured meats such as hams and to assess quantitatively the relative importance of factors contributing to their safety.

In the original work, the probability of toxin production, p, (the proportion of samples containing toxin within each treatment combination) was fitted to a logistic model to describe the relationship between the probability of toxin production and the level of factors/variables present (Figure 3.15). Any factor which tends to decrease μ in Figure 3.15 reduces the probability of toxin production. Of the different factors included in the model, it can be seen that nitrite, incubation temperature and salt are more important in preventing toxin production than the others and that they are acting independently; there is no evidence of synergistic interactions between them.

Here the logistic equation is being used simply as a regression equation, a common practice in modelling situations where there are two possible outcomes to an event, *e.g.* pass/fail, toxin production/no toxin production. Its use in this context should not be confused with its use to represent the microbial growth curve (Section 3.1).

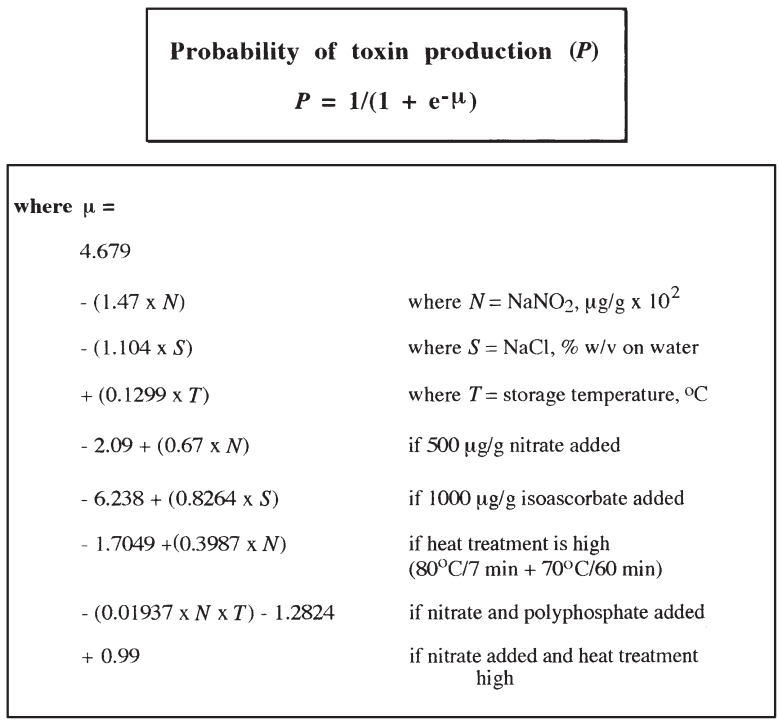


Figure 3.15 *Logistic model for probability of toxin production by* Clostridium botulinum *types A and B in pasteurized pork slurry in the pH range 5.5 to 6.3*

One disadvantage of probabilistic models is that they do not give us much information about the rate at which changes occur. Models that predict times to a particular event such as growth to a certain level or detectable toxin production are termed response surface models. One such model for the growth of *Yersinia enterocolitica* at sub-optimal pH and temperature is described by the equation:

$$\begin{aligned}\text{LTG} = {} & 423.8 - 2.54(T) - 10.97(\text{pH}) + 0.0041(T)^2 + 0.52(\text{pH})^2 \\ & + 0.0129(\text{pH})(T)^*\end{aligned} \tag{3.25}$$

where LTG is the natural logarithm of the time for a 100-fold increase in numbers, T is temperature, pH is pH with acetic acid as acidulant. Terms marked with an asterisk have an insignificant contribution at the 5% confidence level.

Such models are derived by analysing the data for growth under different (known) conditions for a least squares fit to a quadratic equation. For many, the practical implications of equations are not immediately obvious and a graphical representation as a three dimensional response surface has more impact (Figure 3.16).

Although the model is simply a fitted curve and is not based on any assumptions about microbial growth, an interesting consequence of the *Yersinia* model is that the cross-product term is not significant. This means that the two preservative factors, temperature and pH appear to be acting independently; a fact that is also apparent from the graph where there is little or no curvature in the response surface.

Kinetic models take parameters which describe how fast a micro-organism will grow, such as duration of lag phase and generation time, and model these as the response variable for different conditions of pH, temperature, a_w, *etc*. This is described as a second level growth model since it is used to predict lag phase and generation time for a given set of conditions. The predictions are then used in a primary level growth model, one which describes the microbial growth curve, to predict the effect of the chosen conditions on how microbial numbers change over time. This approach is more precise than response surface methods since individual parts of the growth curve may respond differently to changing conditions.

In building the model, experimental values for lag phase and growth rate are derived by fitting microbial count data to a mathematical function, the primary model, which describes the microbial growth curve. Some have used the logistic equation for this, but more commonly the Gompertz equation was used:

$$y = a\exp[-\exp(b - ct)] \tag{3.26}$$

where y is bacterial concentration, a, b and c are constants, and t is time.

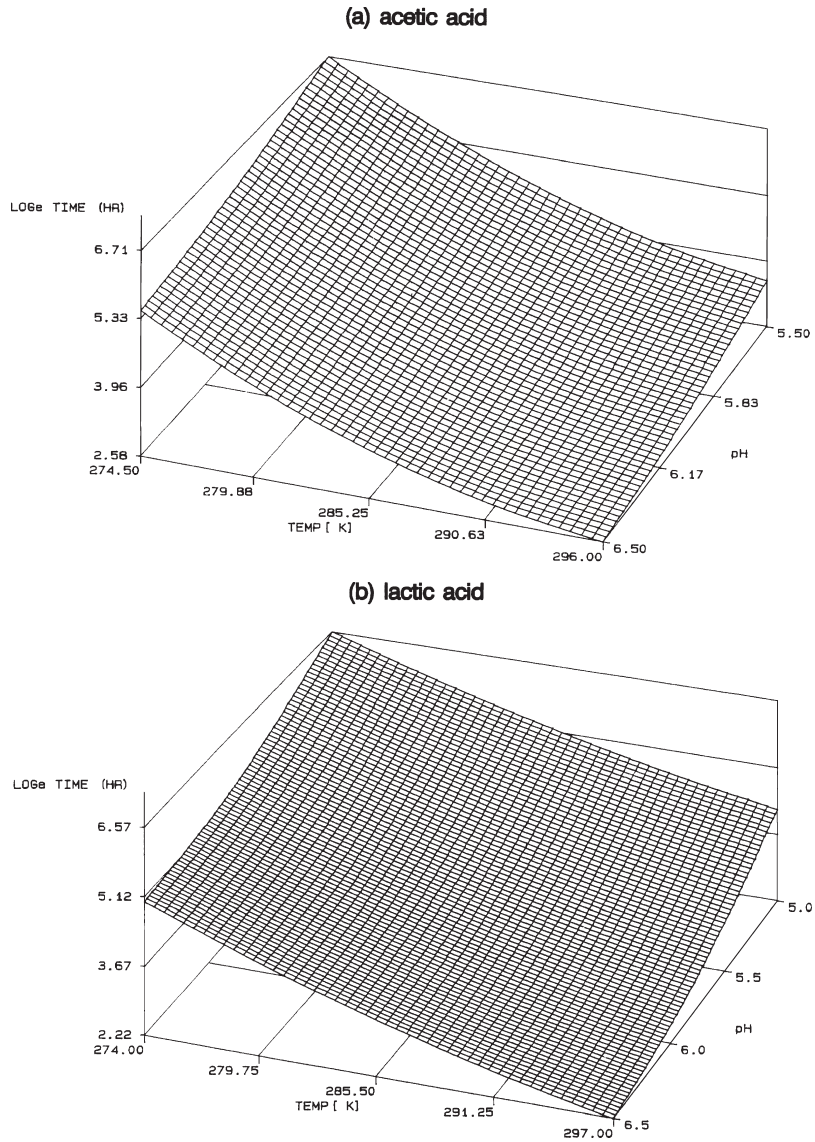


Figure 3.16 *Response surface plot describing the combined effect of temperature and pH on the time for a 2 $\log_{10}$ cycle growth increase of* Yersinia enterocolitica. *(a) Acetic acid and (b) lactic acid*
(C. Little)

This equation was originally developed in the 19th century to describe mortality as a function of age and is used for actuarial purposes, but was found to give a good fit to microbial growth data. More recently the Baranyi equation has found favour and, although considerably more complex, has the advantage of having been developed specifically to describe microbial growth in foods. Once a large number of such values

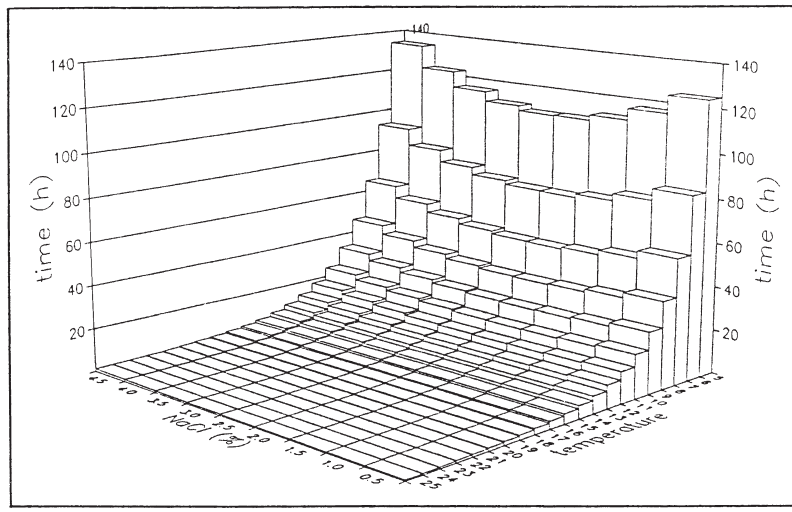


Figure 3.17 *3-D graph showing predicted generation time of salmonellae at fixed pH* (Reprinted from Food Technology International 1990 with permission from Sterling Publications Ltd)

of lag phase and growth rate have been obtained under a variety of environmental conditions, their variation with factors such as temperature, salt, pH, *etc.* can be modelled (the secondary model) using response surface techniques to give a polynomial equation, usually of degree 2 or 3, *i.e.* a quadratic or cubic polynomial (Figure 3.17). This is the approach used in two software packages, Food Micromodel developed in the UK as a result of a Ministry of Agriculture funded research programme, and the United States Department of Agriculture Pathogen Modeling Programme. Food Micromodel has now been superseded by ComBase Predictor based on the same data set and available at http://www.combase.cc/. The latest version of the Pathogen Modelling Programme is available at http://ars.usda.gov/main/site_main.htm?modecode=19353000. ComBase is a collaborative project involving researchers in the UK, USA, Australia and elsewhere which is combining the databases of numerous groups with a view to producing an integrated set of models to be known as ComBase-PMP: Combined Database and Predictive Microbiology Programme. This is due to be available in 2007.

Some models have started off as attempts to model the effect of temperature on microbial growth and have been refined to incorporate other factors such as pH and a_w. The classical Arrhenius equation relates the rate constant (k) of a chemical reaction to absolute temperature T:

$$k = A \exp(-E/RT) \tag{3.27}$$

where E is the activation energy, A is the collision factor and R is the universal gas constant.

If we assume that microbial growth is governed by a single rate limiting enzyme, then we can interpret k as the specific growth rate constant and E as a temperature characteristic. If this is the case and A and E are constant with temperature, then a plot of $\ln k$ against $1/T$ (the absolute temperature) would give a straight line. In fact a concave downward curve is obtained indicating that the activation energy E increases with decreasing temperature.

To improve the fit with observed behaviour, the basic equation has been modified by Davey to include a quadratic term:

$$\ln k = C_0 + C_1/T + C_2/T^2 \tag{3.28}$$

This can be further modified to include other parameters affecting k such as pH and a_w.For example:

$$\ln k = C_0 + C_1/T + C_2/T^2 + C_3 a_w + C_4 a_w^2 \tag{3.29}$$

The Schoolfield equation is another variation of the Arrhenius model where additional terms have been added to the basic equation to account for the effects of high-and low-temperature inactivation on growth rate. Terms describing the effect of a_w and pH can also be incorporated here to give a considerably more complex equation.

An alternative, rather simpler, approach which has met with some success is the square root model to describe growth at sub-optimal temperatures:

$$\sqrt{k} = b(T - T_{\min}) \tag{3.30}$$

where k is the rate of growth, T the absolute temperature (K), and $T_{\min}$ is a conceptual minimum temperature of no physiological significance since it is usually below the freezing point of microbiological media.

Application of this expression to describe microbial growth was first described by Ratkowsky, although it is now recognized as a special form of the Bělehrádek power function originally described nearly 70 years ago.

A plot of $\sqrt{k}$ against T should give a straight line with an intercept on the T axis at $T_{\min}$ and this has been observed and reported by a number of authors monitoring growth in both laboratory media and foods (Figure 3.18).

To include the effects of other constraints on growth the square root equation has been extended separately to give similar equations including an a_w term and a pH term.

$$\sqrt{k} = c\sqrt{(a_w - a_{w_{\min}})}(T - T_{\min}) \tag{3.31}$$

and

$$\sqrt{k} = d\sqrt{(\mathrm{pH} - \mathrm{pH}_{\min})}(T - T_{\min}) \tag{3.32}$$

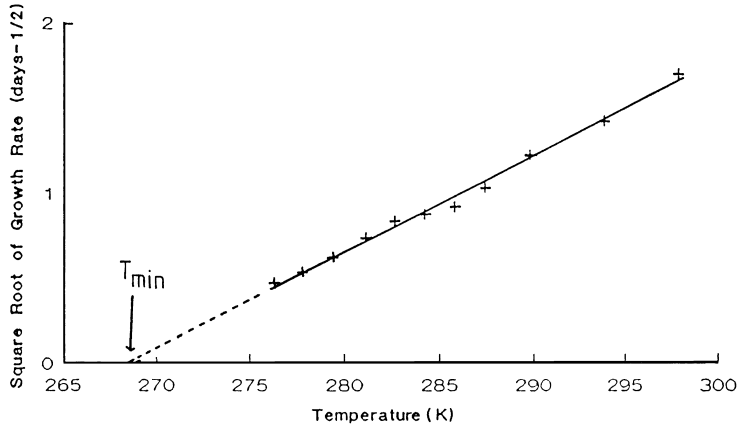


Figure 3.18 *Growth data for* Yersinia enterocolitica *plotted according to the Ratkowsky square root model*

The fact that $T_{\min}$ is not affected by a_w or by pH over the ranges tested indicate that these factors act independently of temperature. These two models have been combined to describe the growth of *Listeria monocytogenes* at sub-optimal pH, a_w and temperature using an equation of the form:

$$\sqrt{k} = e\sqrt{(a_w - a_{w_{\min}})(\mathrm{pH} - \mathrm{pH}_{\min})}(T - T_{\min}) \tag{3.33}$$

Mathematical models of growth are not simply tools for use in development laboratories. For instance, by being able to predict accurately the response of microbial growth rate to temperature, the effect of a fluctuating temperature environment on microbial numbers throughout a distribution chain can be predicted. The value of the technique is illustrated by Figure 3.19 where what might appear as slightly different temperature histories between depot and supermarket can have a dramatic effect on microbial numbers.

Time–temperature function integrators are available which integrate the temperature history of a batch of product and express it as time at some reference temperature. If the temperature of the product remains at the reference temperature, say 0 °C, then they run as clocks recording real time. If the temperature fluctuates, then they speed up or slow down depending on whether the temperature deviates above or below the reference temperature. The relationship between rate and temperature used is the same as that between microbial growth rate and temperature. So quality loss as a result of microbial growth in a fluctuating temperature environment can be known with some accuracy and without the need for microbiological testing.

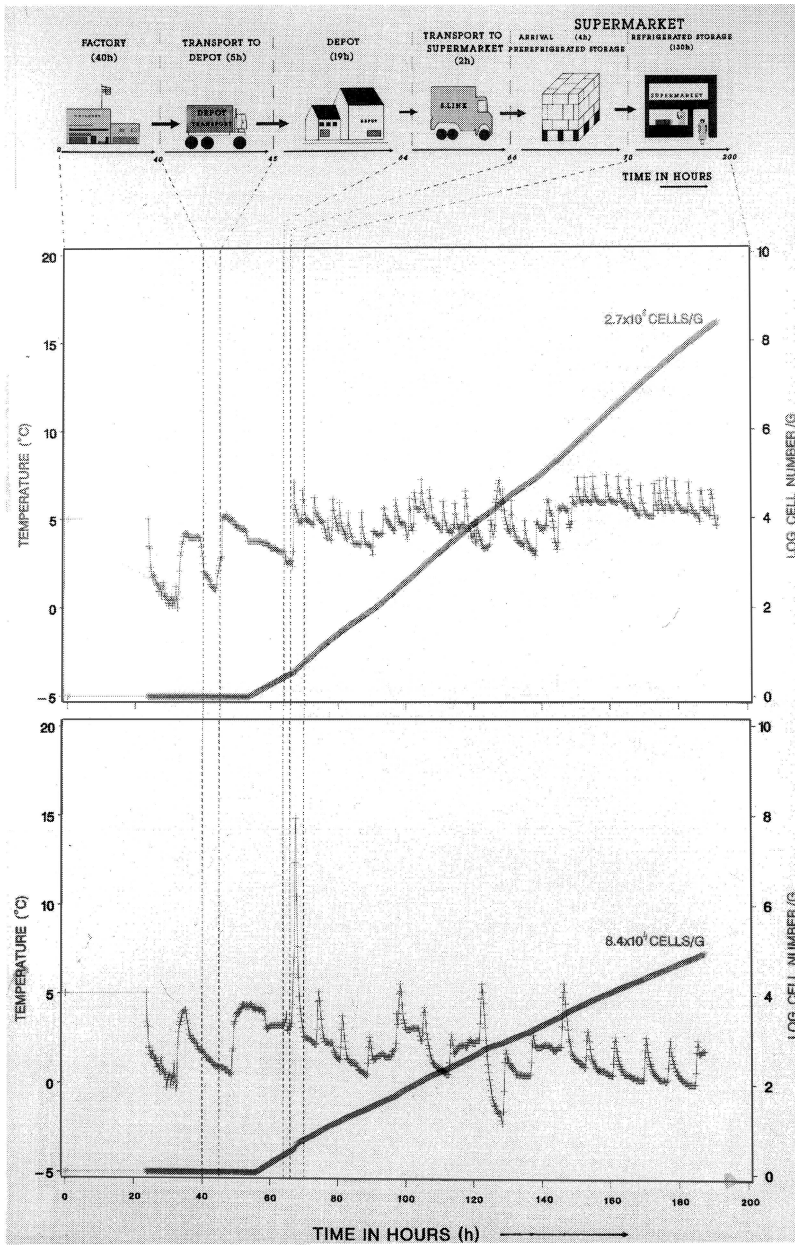


Figure 3.19 *Predicted growth of spoilage organisms during two chill distribution chains* (C. Adair, Unilever Research)

Mathematical models may play a part in the development of computer-based expert systems in food microbiology. The expert systems would provide advice and interpretation of results provided by mathematical models in the same way as human experts would but by

embodying their expertise in the form of rules a computer can apply. One expert system, developed at the UK's Flour Milling and Baking Research Association, predicts the mould-free shelf life of bakery products. Here a_w and temperature are the principal determinants of shelf-life and previous storage trials have shown that, at a given temperature, there is a linear relationship between the logarithm of the mould-free shelf-life and the a_w, expressed as equilibrium relative humidity (ERH). For example, at 27 °C:

$$\log_{10} \text{mould-free shelf-life} = 6.42 - (0.0647 \times \text{ERH}) \tag{3.34}$$

The user is led through a series of screen menus, to choose a product type, and input the ingredients, their relative amounts, the weight loss during processing and the storage temperature. The programme then calculates the ERH of the product and uses the appropriate isotherm to calculate the mould-free shelf-life.