CHAPTER 9

Fermented and Microbial Foods

9.1 INTRODUCTION

So far in this book we have been almost exclusively concerned with the negative roles that micro-organisms play in food. There is however a huge diversity of foods where microbial activity is an essential feature of their production. Some are listed in Table 9.1 and in this chapter we will describe a few of these in more detail and discuss some general features of food fermentation.

Almost without exception, fermented foods were discovered before mankind had any knowledge of micro-organisms other than as witness to the effects of their activity. It was simply an empirical observation that certain ways of storing food effected desirable changes in its characteristics (Table 9.2). Originally the most important of these changes must have been an improvement in the shelf-life and safety of a product, although these became less important in the industrialized world with the advent of alternative preservation methods such as canning, chilling and freezing. Modern technologies have in no way diminished the sensory appeal of fermented products however. This is clear from the way people rarely enthuse over grape juice or milk as some are prone to do over the vast array of wines and cheeses.

We now know that, in food fermentation, conditions of treatment and storage produce an environment in which certain types of organism can flourish and these have a benign effect on the food rather than spoiling it. The overwhelming majority of fermented foods is produced by the activity of lactic acid bacteria and fungi, principally yeasts but also, to a lesser extent, moulds. Both groups of organisms share a common ecological niche, being able to grow under conditions of low pH and reduced a_w, although only lactic acid bacteria and facultative yeasts will prosper under anaerobic conditions. As a consequence, they frequently

Table 9.1 *Some fermented foods*

Food	*Ingredients*	*Geographical Distribution*
Busa	Rice, millet, sugar	Turkey
Beer	Barley	Widespread
Cheese	Milk	Widespread
Chicha	Maize and others	S. America
Dawadawa	Locust beans	W. Africa
Gari	Cassava	Nigeria
Idli/dosa	Rice and black gram	India
Injera	Tef	Ethiopia
I-sushi	Fish	Japan
Kefir	Milk	Eastern Europe
Kenkey	Maize, sorghum	Ghana
Kimchi	Vegetables	Korea
Koko	Maize, sorghum	Ghana
Leavened bread	Wheat	Europe, N. America
Lambic beer	Barley	Belgium
Mahewu	Maize	S. Africa
Nam	Meat	Thailand
Ogi	Maize, sorghum, millet	Nigeria
Olives		Mediterranean Area
Palm wine	Palm sap	Widespread
Poi	Taro	Hawaii
Puto	Rice	Philippines
Salami	Meat	Widespread
Salt stock, cucumbers	Cucumbers	Europe, N. America
Sauerkraut	Cabbage	Europe, N. America
Sorghum beer	Sorghum	S. Africa
Sourdough bread	Wheat, rye	Europe, N. America
Soy sauce, miso	Soy beans	S.E. Asia
Tempeh	Soy beans	Indonesia
Tibi	Fruit	Mexico
Yoghurt	Milk	Widespread

Table 9.2 *Effects of food fermentation*

Raw material	*Stability*	*Safety*	*Nutritive Value*	*Acceptability*
Meats	++	+	−	(+)
Fish	++	+	−	(+)
Milks	++	+	(+)	(+)
Vegetables	+	(+)	−	(+)
Fruits	+	−	−	++
Legumes	−	(+)	(+)	+
Cereals	−	−	(+)	+

++ Definite improvement
+ Usually some improvement
(+) Some cases of improvement
− No improvement

occur together in fermented foods; in some cases members of both groups act in concert to produce a product while in others, one group plays the role of spoilage organisms. Some examples of these are presented in Table 9.3.

Table 9.3 *Yeasts and lactic acid bacteria in fermented foods*

Yeasts[1]	*Lactic acid bacteria*[2]	*Yeasts and lactic acid bacteria*
Modern European beers	Yoghurt	Sourdough bread
Bread	Sauerkraut	Kefir
Wine	Salami	Soy sauce
Cider	Cheese	African beers
		Lambic beer

[1] The presence of lactic acid bacteria in these foods is often associated with spoilage
[2] The presence of yeasts in these foods is often associated with spoilage

9.2 YEASTS

The yeasts are true fungi which have adopted an essentially single celled morphology reproducing asexually by budding or, in the case of *Schizosaccharomyces*, by fission. Although they have a simple morphology, it is probable that they are highly evolved specialists rather than primitive fungi. Their natural habitat is frequently in nutritionally rich environments such as the nectaries of plants, plant exudates, decaying fruits and the body fluids of animals. The yeasts frequently show complex nutritional requirements for vitamins and amino acids.

The yeast morphology has undoubtedly evolved several times for there are species with Ascomycete or Basidiomycete affinities and quite a number with no known sexual stage. Although a number of yeasts almost always occur as single celled organisms, quite a few can develop the filamentous structure of a typical mould. Indeed, there are a number of moulds which can take on a yeast morphology under certain conditions, usually in the presence of high nutrient, low oxygen and enhanced carbon dioxide concentrations.

A major taxonomic study of the yeasts by Kreger–van Rij (1984) describes about 500 species divided into 60 genera of which 33 are considered to be Ascomycetes, 10 Basidiomycetes and 17 Deuteromycetes. A number of yeasts, though certainly not all, are able to grow anaerobically using a fermentative metabolism to generate energy. The majority, if not all, of these fermentative yeasts grow more effectively aerobically and anaerobic growth usually imposes more fastidious nutritional requirements on them.

Although there is a large diversity of yeasts and yeast-like fungi, only a relatively small number are commonly associated with the production of fermented and microbial foods. They are all either ascomycetous yeasts or members of the imperfect genus *Candida*. *Saccharomyces cerevisiae* is the most frequently encountered yeast in fermented beverages and foods based on fruits and vegetables, an observation which is reflected in the existence of more than eighty synonyms and varieties for the species. All strains ferment glucose and many ferment other plant-associated

carbohydrates such as sucrose, maltose and raffinose but none can ferment the animal sugar lactose. In the tropics *Schizosaccharomyces pombe* is frequently the dominant yeast in the production of traditional fermented beverages where a natural fermentation is allowed to occur, especially those produced from cereals such as maize and millet. *Kluyveromyces marxianus* is able to hydrolyse lactose and ferment galactose. There are a number of varieties which had previously been recognized as separate species associated with a range of different fermented milk products. *K. marxianus* var. *marxianus* (= *K. fragilis*) is the perfect state of *Candida kefir* and has been isolated from eastern European fermented milks such as koumiss and kefir. *K. marxianus* var. *bulgaricus* has been isolated from yoghurt and *K. marxianus* var. *lactis* from buttermilk, Italian cheese and fermented milks from Manchuria.

Because of its ability to grow at low water activities in the presence of high concentrations of sugar or salt, *Zygosaccharomyces rouxii* is especially associated with the fermentation of plant products in which the addition of salt is an integral part of the process. Many strains of *Hansenula anomala* and *Debaryomyces hansenii* can also grow in fairly concentrated salt solutions and the latter is frequently isolated from brined meat products and fermented sausages.

Although able to ferment carbohydrates, yeasts such as *Pichia guilliermondii* and *Saccharomycopsis fibuligera* grow best as surface pellicles and have been isolated from a number of tropical fermented products. The latter is able to break down starch and is associated with 'chalky bread'. *Geotrichum candidum* is usually considered as a filamentous mould but it has a strong affinity with the ascomycetous yeasts and is frequently isolated as part of the surface flora of fermented milk products such as cheeses. It is important to realize that, although all these species of yeasts and yeast-like fungi are thought to play a positive role in the production of a diverse range of fermented foods, they also occur as spoilage organisms in other commodities where their biochemical activities are undesirable.

One of the most important yeasts associated with spoilage is *Zygosaccharomyces bailii*. It has the ability to grow at relatively low water activities and low pH, as well as being remarkably resistant to preservatives, such as sorbic, benzoic and ethanoic acids, sulfur dioxide and ethanol, commonly used to prevent microbial spoilage of fruit juices, fruit juice concentrates, fermented beverages, pickles and sauces. *Z. bailii* is strongly fermentative and spoilage of products stored in plastic packs and glass bottles can lead to explosion of the containers. The survival of a single cell in a product containing an appropriate nitrogen source and fermentable carbohydrate can result in spoilage, so pasteurization or membrane filtration before filling, followed by stringent hygiene to prevent post-treatment contamination, are essential.

The following dichotomous key indicates how the genera discussed above differ from each other:

1. Vegetative reproduction by cross-wall formation followed by fission — *Schizosaccharomyces*[*]
1. Vegetative reproduction by budding — 2

2. Ascospores not formed — *Candida*
2. Ascospores formed — 3

3. Nitrate assimilated — *Hansenula*
3. Nitrate not assimilated — 4

4. Abundant true mycelium as well as budding — 5
4. True mycelium scarce or absent — 6

5. Asci formed exclusively on the true hyphae — *Saccharomycopsis*
5. Asci not formed exclusively on the true hyphae — *Pichia*

6. Asci dehiscent — *Kluyveromyces*
6. Asci persistent — 7

7. No conjugation preceding ascus formation — *Saccharomyces*
7. Conjugation preceding ascus formation — 8

8. Ascospores warty or with ridges — *Debaryomyces*
8. Ascospores spherical and smooth — *Zygosaccharomyces*

[*]*Schizosaccharomyces* belongs to the Archiascomycetes

9.3 LACTIC ACID BACTERIA

The term lactic acid bacteria (LAB) has no strict taxonomic significance, although the LAB have been shown by serological techniques and 16S ribosomal RNA cataloguing to be phylogenetically related. They share a number of common features: they are Gram-positive, non-sporeforming rods or cocci; most are aerotolerant anaerobes which lack cytochromes and porphyrins and are therefore catalase- and oxidase-negative. Some do take up oxygen through the mediation of flavoprotein oxidases and this is used to produce hydrogen peroxide and/or to re-oxidize NADH produced during the dehydrogenation of sugars.

Cellular energy is derived from the fermentation of carbohydrate to produce principally lactic acid. To do this, they use one of two different

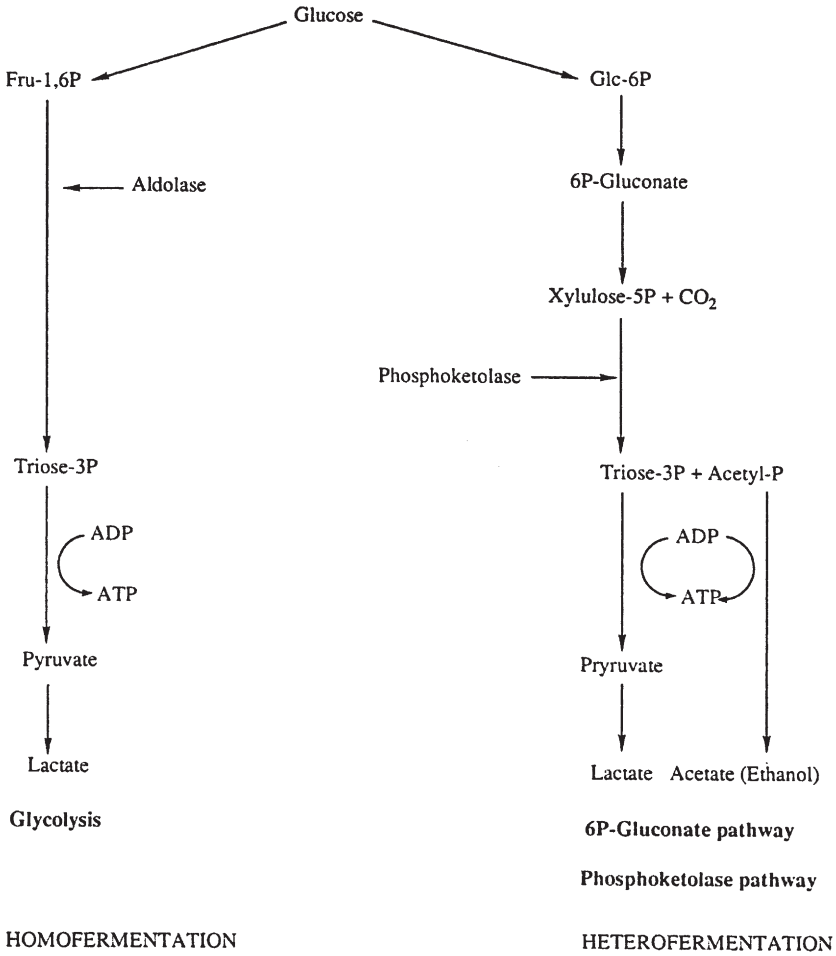


Figure 9.1 *The homo- and heterofermentation pathways*

pathways and this provides a useful diagnostic feature in their classification (Figure 9.1). Homofermenters produce lactate as virtually a single product from the fermentation of glucose. They follow the Emden–Meyerhof–Parnas (EMP) glycolytic pathway whereby the six-carbon molecule glucose is phosphorylated and isomerized before cleavage by the enzyme aldolase into glyceraldehyde-3-phosphate. This is then converted to pyruvate during which ATP is produced by substrate-level phosphorylation at two sites to give an overall yield of two molecules of ATP for every molecule of glucose fermented. In order to regenerate the NAD^+ consumed in the oxidation of glyceraldehyde-3-phosphate, pyruvate is reduced to lactate using NADH.

Heterofermenters produce roughly equimolar amounts of lactate, ethanol/acetate, and carbon dioxide from glucose. They lack aldolase and transform the hexose, glucose, into a pentose by a sequence

involving oxidation and decarboxylation. The pentose is cleaved into glyceraldehyde phosphate and acetyl phosphate by the enzyme phosphoketolase. The triose phosphate is converted into lactate by the same sequence of reactions as occurs in glycolysis to give two molecules of ATP. The fate of the acetyl phosphate depends on the electron acceptors available. In the absence of alternatives, acetyl phosphate fulfils this role and is reduced to ethanol while regenerating two molecules of NAD^+ from NADH. In the presence of oxygen, NAD^+ can be regenerated by NADH oxidases and peroxidases, leaving acetyl phosphate available for conversion to acetate. This provides another site for substrate level phosphorylation and increases the overall ATP yield of heterofermentation from one to two molecules ATP per molecule of glucose dissimilated. When this is possible, the increased yield of ATP is reflected in a faster growth rate and a higher molar growth yield. The same effect can be achieved with other electron acceptors, for example fructose which is reduced to mannitol.

Heterofermenters and homofermenters can be readily distinguished in the laboratory by the ability of heterofermenters to produce carbon dioxide in glucose-containing media.

The principal genera of the lactic acid bacteria are described in Table 9.4. *Lactobacillus* is recognized as being phylogenetically very heterogeneous and this is evidenced by the broad range of %GC values exhibited within the genus. Some non-acidoduric, heterofermentative lactobacilli have been reclassified in the new genus *Carnobacterium* and there is likely to be significant further refinement of the genus in the future. Currently the lactobacilli are subdivided into three groups: obligate homofermenters, facultative heterofermenters and obligate heterofermenters. The obligate homofermenters correspond roughly to the Thermobacterium group of the Orla–Jensen classification scheme and include species such as *Lb. acidophilus*, *Lb. delbrückii* and *Lb. helveticus*. They ferment hexoses almost exclusively to lactate but are unable to ferment pentoses. The facultative heterofermenters ferment hexoses via the EMP pathway to lactate but have an inducible phosphoketolase which allows them to ferment pentoses to

Table 9.4 *Principal genera of the lactic acid bacteria*

Genus	*Cell Morphology*	*Fermentation*	*Lactate isomer*	*DNA (mole %GC)*
Lactococcus	cocci in chains	homo	L	33–37
Leuconostoc	cocci	hetero	D	38–41
Pediococcus	cocci	homo	DL	34–42
Lactobacillus	rods	homo/hetero	DL, D, L	32–53
Streptococcus	cocci in chains	homo	L	40[a]

[a] *S. thermophilus*
(Other genera that are currently included in the lactic acid bacteria, *Carnobacterium*, *Enterococcus*, *Oenococcus*, *Vagococcus*, *Aerococcus*, *Tetragenococcus*, *Alloiococcus*, *Weissella*)

lactate and acetate. They include some species important in food fermentation such as *Lb. plantarum*, *Lb. casei*, and *Lb. sake*. Obligate heterofermenters which include *Lb. brevis*, *Lb. fermentum* and *Lb. kefir* use the phosphoketolase pathway for hexose fermentation.

Leuconostoc is treated as a separate genus on morphological grounds as its members are typically irregular cocci. This is not entirely satisfactory since the vexed question, 'When does a short rod become a coccus?' often arises; for example, *Lactobacillus confusus* was originally classed as a *Leuconostoc*. It is possible to distinguish leuconostocs from most heterofermentative lactobacilli by two phenetic characteristics: their production of only D-lactate and inability to produce ammonia from arginine.

The genus *Pediococcus* also includes species of importance in food fermentations such as *P. pentosaceus* and, until fairly recently, *P. halophilus* now in a genus of its own as *Tetragenococcus halophilus*.

Nucleic acid studies of the streptococci have shown that they comprise three distinct groups worthy of genus status. The enterococci now form the genus *Enterococcus* although the faecal strains of *S. bovis* and *S. equinus* which also react with the group D antisera used in Lancefield's classical serological classification scheme are not included. What were known as Lancefield's group N streptococci, the lactic or dairy streptococci, are now members of the genus *Lactococcus* and a number of these which were considered distinct *Streptococcus* species are now classified as subspecies of *Lactococcus lactis*. The yoghurt starter *Streptococcus thermophilus* does not possess the Group N antigen and remains in the genus *Streptococcus*.

Some authors also include *Bifidobacterium* among the lactic acid bacteria although this has less justification as they are quite distinct both phylogenetically and biochemically. For example, hexose fermentation by bifidobacteria follows neither the EMP glycolytic pathway nor the phosphoketolase pathway but produces a mixture of acetic and lactic acids.

9.4 ACTIVITIES OF LACTIC ACID BACTERIA IN FOODS

9.4.1 Antimicrobial Activity of Lactic Acid Bacteria

Lactic acid bacteria are often inhibitory to other micro-organisms and this is the basis of their ability to improve the keeping quality and safety of many food products. The principal factors which contribute to this inhibition are presented in Table 9.5. By far the most important are the production of lactic and acetic acids and the consequent decrease in pH. Just how organic acids and low pH inhibit microbial growth and survival is discussed in Section 3.2.2 and will not be repeated here.

Bacteriocins are bactericidal peptides or proteins which are usually active against species closely related to the producing organism.

Table 9.5 *Factors contributing to microbial inhibition by lactic acid bacteria*

Low pH
Organic acids
Bacteriocins
Hydrogen peroxide
Ethanol
Diacetyl
Nutrient depletion
Low redox potential

Production of bacteriocins by lactic acid bacteria has been extensively studied in recent years and a number have been described. Interest in them stems from the fact that they are produced by food-grade organisms and could therefore be regarded as 'natural' and hence more acceptable as food preservatives. A few promising candidates have been found but many others have a spectrum of activity which is too limited to be of any practical utility.

Nisin is the only bacteriocin to find practical application in the food industry to date. Produced by certain strains of *Lactococcus lactis*, it was first discovered when a nisin-producing strain caused problems in cheesemaking by inhibiting the other starter organisms present. Nisin is available commercially and has been used as a food preservative in the UK and some other countries since the early 1950s, though it was not approved for use in the United States until 1988. It differs from many other bacteriocins produced by lactic acid bacteria in having a relatively broad spectrum of activity against Gram-positive bacteria generally. In vegetative cells it acts by creating pores in the plasma membrane through which there is leakage of cytoplasmic components and a breakdown of the transmembrane potential. In Gram-negatives, the outer membrane acts as a barrier preventing nisin access to its site of action, thus making them resistant. Some Gram-negatives have been shown to become sensitive when their outer membrane has been damaged by thermal shock or by treatment with a chelating agent such as EDTA. Bacterial spores are particularly sensitive and the most important commercial applications of nisin have been to inhibit spore outgrowth in heat processed products, principally processed cheese and canned foods, but also, in some countries, in products such as clotted cream, dairy desserts, crumpets, pasteurized soups and pasteurized eggs. Nisin's value lies not just in the fact that it can improve shelf life by inhibiting spore outgrowth but its use can also permit milder heat processing regimes, allowing retention of heat sensitive properties in some products.

Nisin is an amphiphilic polypeptide containing 34 amino acids and is remarkably heat stable at acid pH. It belongs to a group of antibiotics known as lantibiotics, most of which are produced by non-lactic acid

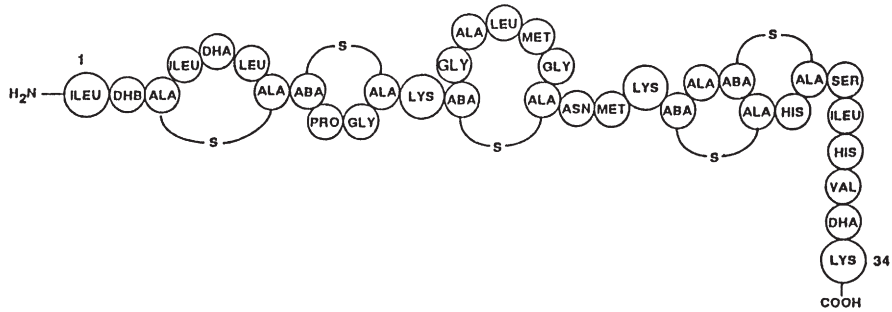


Figure 9.2 *Nisin* (ABA. *aminobutyric acid;* DHA. *dehydroalanine;* DHB, *dehydrobutyrine)*

bacteria and are characterized by the possession of unusual amino acids such as lanthionine (3,3′-thiodialanine) and β-methyl lanthionine (Figure 9.2). These are produced by a series of post translational modifications to a pre-propeptide which is then cleaved to remove a leader peptide.

Production of many bacteriocins appears to be a plasmid-encoded function but the gene coding for nisin has been cloned and sequenced from both chromosomal and plasmid DNA. Introduction of the ability to produce nisin into a chosen starter organism may prove useful in some fermented foods where competition from other Gram-positives needs to be controlled, although this is not desirable in cheesemaking where nisin production could inhibit the lactobacilli that contribute to cheese maturation.

Hydrogen peroxide is well known for its antimicrobial properties. Since lactic acid bacteria possess a number of flavoprotein oxidases but lack the degradative enzyme catalase, they produce hydrogen peroxide in the presence of oxygen. This will confer some competitive advantage as they have been shown to be less sensitive to its effects than some other bacteria. Accumulation of hydrogen peroxide has been demonstrated in some fermented foods but its effects are, in general, likely to be slight. Lactic acid fermentations are essentially anaerobic processes so hydrogen peroxide formation will be limited by the amount of oxygen dissolved in the substrate at the start of fermentation. It may be, however, that at this critical initial stage of a fermentation hydrogen peroxide production provides an important additional selective advantage. In milk, hydrogen peroxide is also known to potentiate the lactoperoxidase antimicrobial system (see Section 3.2.4).

Heterofermentative LAB produce ethanol, another well-established antimicrobial. It may make some contribution to the inhibition of competitors, although its concentration in lactic fermented products is generally low.

There are a number of other factors which may, like ethanol, give LAB a selective advantage in some situations. In most cases however

their contribution is likely to be negligible, particularly when compared to the ability of LAB to produce lactic acid in quantities up to around 100 millimolar and a pH in the range 3.5 to 4.5.

9.4.2 Health-promoting Effects of Lactic Acid Bacteria-Probiotics

Fermented foods have long had a reputation for being positively beneficial to human health in a way that ordinary foods are not. Ilya Metchnikoff, the Russian founder of the theory of phagocytic immunity, was an early advocate of this idea based on his theories on disharmonies in nature. He held that the human colon was one such disharmony since intestinal putrefaction by colonic bacteria produced toxins which shorten life. One solution to this which he advocated in his book 'The Prolongation of Life', published in 1908, was the consumption of substantial amounts of acidic foods, particularly yoghurt. He thought that the antimicrobial activity of the lactic acid bacteria in these products would inhibit intestinal bacteria in the same way they inhibit putrefaction in foods and attributed the apparent longevity of Bulgarian peasants to their consumption of yoghurt.

Since then a number of claims have been made for lactic acid bacteria, particularly in association with fermented milks (Table 9.6). So much so, that live cultures of lactic acid bacteria (and some others such as *Bifidobacterium* spp.) consumed in foods are frequently termed 'probiotics' (Greek: for life). Much of the evidence available on these putative benefits is however inadequate or contradictory at present, and many remain rather ill defined.

Several studies have shown improved nutritional value in grains as a result of lactic fermentation, principally through increasing the content of essential amino acids. Such improvements however may be of only marginal importance to populations with a varied and well balanced diet. It has also been reported that fermentation of plant products reduces levels of antinutritional factors which they may contain such as cyanogenic glycosides and phytic acid, although this effect is often the result of other aspects of the process such as soaking or crushing rather than microbial action. Some have claimed that fermentation of milks increases the bioavailability of minerals, although this is disputed.

Table 9.6 *Beneficial effects claimed for lactic acid bacteria*

Nutritional improvement of foods
Inhibition of enteric pathogens
Alleviation of diarrhoea/Constipation
Hypocholesterolaemic action
Anticancer activity
Simulation of the immune system

One area where there is good evidence for a beneficial effect is in the ability of fermented milks to alleviate the condition known as lactose intolerance. All human infants possess the enzyme lactase (β-galactosi-dase) which hydrolyses the milk sugar lactose into glucose and galactose which are then absorbed in the small intestine. In the absence of this enzyme when milk is consumed, the lactose is not digested but passes to the colon where it is attacked by the large resident population of lactose-fermenting organisms producing abdominal discomfort, flatulence and diarrhoea. Only people of north European origin and some isolated African and Indian communities maintain high levels of gut β-galactosidase throughout life. In most of the world's population it is lost during childhood and this precludes the consumption of milk and its associated nutritional benefits. If however lactase-deficient individuals take milk in a fermented form such as yoghurt, these adverse effects are less severe or absent. This is not simply a result of reduced levels of lactose in the product since many yoghurts are fortified with milk solids so that they have lactose contents equivalent to fresh milk. It appears to be due to the presence of β-galactosidase in viable starter organisms, as pasteurized yoghurts show no beneficial effect. In the gut, the ingested cells become more permeable in the presence of bile and this allows them to assist the body in the hydrolysis of lactose.

The protective role of the gut's microflora has been discussed already (Section 6.5) and there is evidence that ingested lactic acid bacteria can contribute to this. Yoghurt has been shown to have a strong inhibitory effect on the growth of coliform bacteria in the stomach and duodenum of piglets and studies of human infants with diarrhoea have shown that the duration of illness was shorter in those groups given yoghurt than in control groups. However, the usual starter organisms in yoghurt, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* are not bile tolerant and do not colonize the gut. They will persist in the alimentary tract and be shed in the stools only as long as they are being ingested, so that any improving effect is likely to be transient. Recently attention has focused on lactic acid bacteria such as *Lactobacillus acidophilus* and bifidobacteria such as *Bifidobacterium longum* which can colonize the gut and these organisms have been included in yoghurts and other fermented milks and some proprietory preparations. There is some evidence that such probiotic lactobacilli can shorten the duration of viral diarrhoea and may be useful in reducing antibiotic associated diarrhoea. Studies with traveller's diarrhoea however have given contradictory results.

Pathogen inhibition *in vivo* by LAB unable to colonize the gut must be by mechanisms broadly similar to those which apply *in vitro* (Section 9.4.1). With those organisms able to colonize the gut, the masking of potential attachment sites in the gut may also be involved.

Lactic acid bacteria have been reported to stimulate the immune system and various studies have described their ability to activate macrophages and lymphocytes, improve levels of immunoglobulin A (IgA) and the production of gamma interferon. These effects may contribute to a host's resistance to pathogens and to the antitumour activity noted for LAB, mainly *Lactobacillus acidophilus*, in some animal models. An additional or alternative possible mechanism proposed for the antitumour effect is the observed reduction in activity of enzymes such as β-glucuronidase, azoreductase and nitroreductase in faecal material when LAB are ingested. These enzymes, produced by components of the intestinal flora, can convert procarcinogens to carcinogens in the gut and their decreased activity is probably due to inhibition of the producing organisms by LAB.

A number of studies have indicated that probiotics may have a role in preventing and treating atopic diseases such as atopic eczema and asthma in children.

High levels of serum cholesterol are established as a predisposing factor for coronary heart disease. It has been suggested that consumption of fermented milks has a hypocholesterolaemic action and some have suggested a variety of mechanisms by which this can occur. The evidence is however weak and it has not proved possible to demonstrate this effect in a number of trials.

An alternative approach to the consumption of large numbers of probiotic bacteria is to encourage the growth of indigenous bifidobacteria and lactobacilli in the gut through consumption of prebiotics. These are defined as non-digestible food components that exert a beneficial effect on the consumer by selectively stimulating the growth and activity of certain bacteria in the colon. The most common prebiotics are polymeric forms of fructose such as inulin, a natural component of foods such as Jerusalem artichokes, leeks, onions and garlic.

9.4.3 The Malo-lactic Fermentation

LAB can decarboxylate L-malic acid to produce L-lactate in a reaction known as the malo-lactic fermentation (Figure 9.3). This process is particularly associated with wines, where malic acid can form up to half the total acid, and its effect is to reduce substantially a wine's acidity. It is particularly encouraged in wines from cool regions which tend to have a naturally high acidity and, although less desirable in wines from warmer regions, it is often promoted to provide bacteriological stability to the bottled product. It may also modify and improve the body and flavour of a wine.

A natural malo-lactic fermentation can be encouraged by refraining from sulfiting the new wine and leaving it on the yeast lees (sediment) for

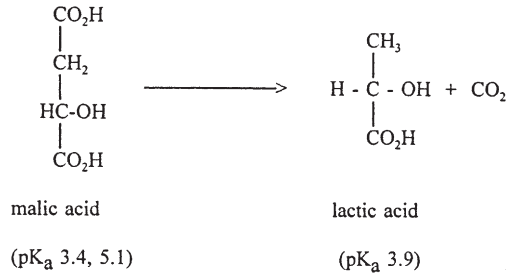


Figure 9.3 *The malo-lactic fermentation*

longer than usual. Commercial starter cultures are also available usually consisting of strains of *Oenococcus oeni*, formerly *Leuconostoc oenos*.

Until recently it was unclear how LAB derive any benefit from performing this reaction. Substrate-level phosphorylation does not occur (it is not therefore, strictly speaking, correct to call it a fermentaion) and the free energy of the decarboxylation reaction is low. It now seems that the reaction conserves energy through a proton motive force generated across the cell membrane by the transport of malate, lactate and protons.

9.5 FERMENTED MILKS

9.5.1 Yoghurt

Fermentation to extend the useful life of milk is probably as old as dairying itself. The first animals to be domesticated are thought to have been goats and sheep in the Near East in about 9000 BC. In the warm prevailing climate it is likely that their milks furnished the first fermented milks and only some time later, between 6100 and 5800 BC in Turkey or Macedonia, was the cow first domesticated.

Fermented milks which include yoghurt, buttermilk, sour cream, and kefir differ from cheese in that rennet is not used and the thickening produced is the result of acidification by lactic acid bacteria. Yoghurt whose name comes from the Turkish word 'Jugurt' is the most widely available fermented milk in the Western world today where its popularity derives more from its flavour and versatility than from its keeping properties.

It is made from milk, skimmed milk or fortified milk usually from cows but sometimes from other animals such as goats or sheep. The production process most commonly applied commercially is outlined in Figure 9.4.

The first prerequisite of any milk to be used in a fermentation process is that it should be free from antimicrobials. These could be antibiotic residues secreted in the milk as a result of mastitis chemotherapy or

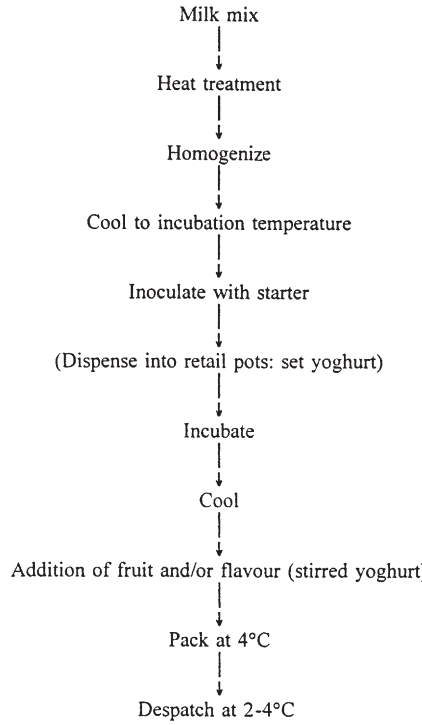


Figure 9.4 *Yoghurt production*

sanitizers carried into the milk as a result of inadequate equipment cleaning regimes at the farm or dairy. Inhibition of the starter culture would result not only in economic losses but could potentially allow pathogens to grow.

In commercial practice it is usual to supplement the solids content of the milk to enhance the final texture of the product. The SNF (solids not fat) content is increased to between 11 and 15%, compared with a level of around 8.5% in fresh milk. The simplest way of achieving this is by addition of skim- or whole-milk powder depending on whether a conventional or low-fat product is required. The properties of the product may also be improved and stabilized by the addition of small amounts of natural or modified gums which bind water and thicken the product.

If left to stand, the milk fat would separate out to form a cream layer. To prevent this, the milk is homogenized by passing it through a small orifice under pressure, typically 100–200 $kg\,cm^{-2}$ at 50–60 °C, to reduce the size of the fat globules to below 2 μm. This improves the product's stability, increases the milk's viscosity, and also makes it appear whiter as the number of light-reflecting centres is increased.

Before addition of the starter culture, the milk is heated at 80–90 °C for about 30 min. Being well in excess of the normal pasteurization

requirements for safety, this has a substantial lethal effect on the microflora. All but heat-resistant spores are eliminated so that the starter culture encounters little by way of competition. The heat process also improves the milk as a growth medium for the starter by inactivating immunoglobulins, expulsion of oxygen to produce a microaerophilic environment, and through the release of stimulatory levels of sulfydryl groups. Excessive heating can however lead to the production of inhibitory levels of these compounds. Heating also promotes interactions between whey or serum proteins and casein which increase the yoghurt viscosity, stabilize the gel and limit syneresis (separation of whey).

The heat-treated milk is cooled to the fermentation temperature of 40–43 °C which is a compromise between the optima of the two starter organisms *Strep. thermophilus* (39 °C) and *Lb. delbrueckii* subsp. *bulgaricus* (45 °C). The starter culture is added at a level of about 2% by volume to give an initial concentration of 10^6–10^7 cfu ml^{-1} composed of roughly equal numbers of the two organisms. The fermentation can be conducted in the retail pack to produce a firm, continuous coagulum, which is known as a set yoghurt, or in bulk tanks to produce a stirred yoghurt where the gel has been broken by mixing in other ingredients and by pumping into packs.

The fermentation takes about 4 h during which the starter bacteria ferment lactose to lactic acid decreasing the pH from its initial level of 6.3–6.5. The lactic acid helps solubilize calcium and phosphate ions which destabilize the complex of casein micelles and denatured whey proteins. When the pH reaches 4.6–4.7, the isoelectric point of the casein, the micelles aggregate to produce a continuous gel in which all the components are entrapped with little or no 'wheying-off'.

During fermentation growth of the streptococci is fastest in the early stages, but as the pH drops below 5.5 it slows and the lactobacilli tend to predominate. By the end of fermentation the product has a total acidity of 0.9–0.95% and the populations of the two starter organisms are roughly in balance again with levels in excess of 10^8 cfu ml^{-1}.

The relationship between the two starter organisms is one known as protoco-operation, that is to say they have a mutually favourable interaction but are not completely interdependent. Both will grow on their own in milk but will grow and acidify the product faster when present together. Growth of the streptococcus in milk is limited by the availability of peptides and free amino acids which are present in relatively low concentrations (≈ 50 mg kg^{-1}). The lactobacillus is slightly proteolytic and liberates small amounts of these, particularly valine, which stimulate streptococcal growth. In its turn the streptococcus produces formate, pyruvate and carbon dioxide all of which stimulate the lactobacillus. Formate is used in the biosynthesis of the purine base adenine, a component of RNA and DNA and *Lb. delbrueckii* subsp. *bulgaricus*

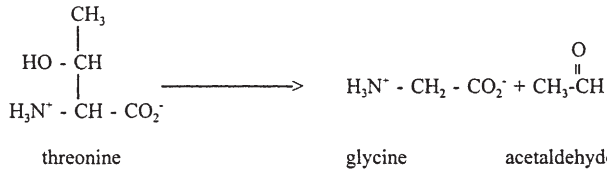


Figure 9.5 *The threonine aldolase reaction*

tends to grow poorly in milk with low levels of formate, forming elongated, multinucleate cells.

Acetaldehyde (ethanal) is the most important flavour volatile of yoghurt and should be present at 23–41 mg kg^{-1} (pH 4.2–4.4) to give the correct yoghurt flavour. Its accumulation is a consequence of the fact that both starter organisms lack an alcohol dehydrogenase which would otherwise reduce the acetaldehyde to ethanol. Both will produce acetaldehyde from the glucose portion of lactose *via* pyruvate and through the action of threonine aldolase. The latter activity (Figure 9.5) is more pronounced in the lactobacillus but in the streptococcus methionine has been shown to increase levels of acetaldehyde *via* threonine. Diacetyl, an important flavour compound in many dairy products, is present at very low levels (≈ 0.5 mg kg^{-1}) but is thought to make a contribution to the typical yoghurt flavour.

When the fermentation is complete the yoghurt is cooled to 15–20 °C before the addition of fruits and flavours and packaging. It is then cooled further to below 5 °C, under which conditions it will keep for around three weeks. Yoghurt is not usually pasteurized since chill storage will arrest the growth of the starter organisms. The acidity will however continue to increase slowly during storage.

Because of its high acidity and low pH (usually 3.8–4.2), yoghurt is an inhospitable medium for pathogens which will not grow and will not survive well. It is unusual therefore for yoghurt to be involved in outbreaks of foodborne illness, although the hazelnut yoghurt botulism outbreak in the UK in 1989 (see Section 7.5.5) is a notable exception. Yoghurts are spoiled by acidoduric organisms such as yeasts and moulds. Yeasts such as the lactose-fermenting *Kluyveromyces fragilis* and, in fruit-containing yoghurts, *Saccharomyces cerevisiae* are particularly important but the yeast-like fungus *Geotrichum* and surface growth of moulds such as *Mucor*, *Rhizopus*, *Aspergillus*, *Penicillium*, and *Alternaria* can also be a problem. Advisory guidelines for microbiological quality have suggested that satisfactory yoghurts should contain more than 10^8 cfu g^{-1} of the starter organisms, <1 coliform g^{-1}, <1 mould g^{-1} and <10 yeasts g^{-1} (fruit-containing yoghurts may contain up to 100 yeasts g^{-1} and remain of satisfactory quality).

9.5.2 Other Fermented Milks

The popularity of acidophilus milk is largely due to health-promoting effects which are claimed to stem from the ability of *Lactobacillus acidophilus* to colonize the gut. It is a thermophilic homofermenter but is slow fermenting and a poor competitor and is easily outgrown. As a result, the fermentation takes longer than for yoghurt and great care must be taken to avoid contamination. In the original process whole or skimmed milk was sterilized prior to fermentation by a Tyndallization process. This involved two heating stages of 90–95 °C for up to an hour separated by a holding period of 3–4 h to allow spore germination to occur. Nowadays the same effect can be achieved more swiftly and economically by UHT processing. The milk is then homogenized, cooled to the fermentation temperature of 37–40 °C and inoculated with 2–5% of starter culture. It can take as long as 24 h to produce the required acidity of about 0.7%, after which the product is cooled to 5 °C.

In addition to the extra care required in its production, acidophilus milk suffers from a number of other drawbacks. In particular, it lacks the sensory appeal of yoghurt, being restricted to a rather sour, acidic taste. Also, the *Lb. acidophilus* cells do not survive well in the acid product, dying out after about a week's storage at 5 °C. To avoid these problems, a non-fermented sweet acidophilus milk is produced in the United States where large numbers of *Lb. acidophilus* are simply added to pasteurized milk without incubation.

In an attempt to combine the supposed virtues of acidophilus milk with those of yoghurt, a number of 'bio-yoghurts' are now produced. These contain a mixture of organisms, those able to colonize the gut such as *Lb. acidophilus* and *Bifidobacterium* spp. with *Strep. thermophilus* to provide the characteristic yoghurt flavour. However, because of their poor survival at acid pH, it is likely that the strains used are chosen for their ability to survive in the product as much as for any benefit they may have *in vivo*.

Kefir and koumiss are distinctive fermented milks produced by a mixed lactic acid bacterial fermentation and an alcoholic yeast fermentation. Kefir is further distinguished by the fact that the microflora responsible is not dispersed uniformly throughout the milk but is added as discrete kefir 'grains'. These are in fact sheets composed largely of a strong polysaccharide material, kefiran, which folds upon itself to produce globular structures resembling cauliflower florets. The outside of the sheets is smooth and is populated by lactobacilli while the inner, rougher side of the sheet carries a mixed population of yeasts and lactic acid bacteria. A large variety of different organisms have been reported as being associated with the fermentation, probably reflecting the widespread and small-scale nature of production. The morphology of the

grain itself suggests that the lactic acid bacteria are responsible for its production and a capsular, homofermenter *Lactobacillus kefiranofaciens* has been shown to produce kefiran. A heterofermentative lactobacillus *Lb. kefir* is numerically very important in many grains and plays a key role in the fermentation, probably among other things contributing to the required effervescence in the product. Although less significant numerically, several yeasts have been reported including *Candida kefir*, *Saccharomyces cerevisiae* and *Sacc. exiguus*. The latter is particularly interesting because it was shown to utilize galactose preferentially in the presence of glucose and this may confer an advantage when growing in a mixed culture of organisms most of which will preferentially metabolize the glucose portion of lactose.

Kefir is produced commercially in a number of countries, most importantly in Russia and those states which comprised the old Soviet Union. In the mid-1980s production of kefir reached 12 million tonnes representing 80% of all dairy products, excluding soft cheese and sour cream. In commercial practice, milk for kefir production is homogenized and heated to 85–95 °C for between 3 and 10 min. It is cooled to 22 °C before addition of kefir grains at a level of up to 5%. The fermentation itself lasts for 8–12 h but is sometimes followed by slow cooling to around 8 °C over 10–12 h to allow for the required flavour development.

Kefir has an acidity of about 0.8% and an alcohol content which has been reported as varying between 0.01% and 1%. Ethanol levels tend to be lower in commercial products than domestically produced kefir and increase with the age of the product. In addition to the character imparted by the ethanol, lactic acid and carbon dioxide, acetaldehyde (ethanal) and diacetyl are also present as flavour components.

Koumiss is a fizzy, greyish white drink produced traditionally from mare's milk in eastern Europe and central Asia. It can have an acidity up to 1.4% and an ethanol content up to 2.5%. A mixed yeast/LAB flora is responsible for the fermentation comprising *Lb. delbrueckii* subsp. *bulgaricus* and a number of lactose fermenting yeasts. These are dispersed throughout the product and do not form discrete particles as in kefir. Cow's milk is a more convenient raw material to use nowadays and this is usually modified to resemble more closely the composition of mare's milk which has a lower fat content and higher carbohydrate levels.

Strictly speaking, buttermilk is the liquid which separates from cream during the churning of butter (see Chapter 5). However, to achieve a consistent quality product most buttermilk today is produced directly by the fermentation of skimmed or partially skimmed milk. Cultured buttermilk is an acidic refreshing drink with a distinctive buttery flavour. A mixture of starter organisms is required to produce these attributes; *Lactococcus lactis* produces most of the lactic acid, while the buttery

flavour is the result of diacetyl production by so-called flavour bacteria such as strains of *Lactococcus lactis* subsp. *lactis* and *Leuconostoc mesenteroides* subsp. *cremoris*.

Most bacteria produce diacetyl and acetoin from carbohydrate *via* pyruvate. However, because of the key role pyruvate plays as an electron acceptor in LAB, it cannot usually be spared for this purpose unless an additional source other than carbohydrate or an alternative electron acceptor is available. Citrate metabolism can provide this extra pyruvate and lead to the accumulation of diacetyl as indicated in Figure 9.6. Fresh milk contains citrate but levels decline during storage so that, for the

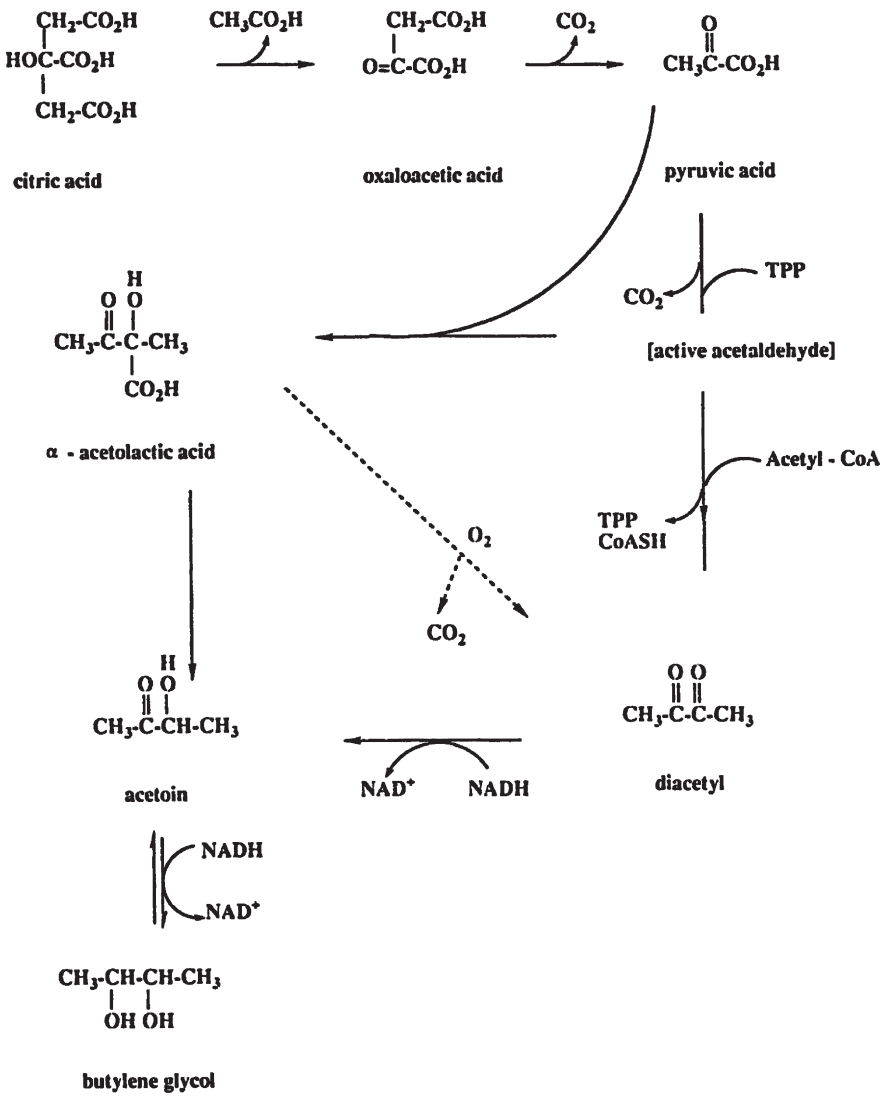


Figure 9.6 *Citrate fermentation. TPP,* thiamine pyrophosphate

production of cultured buttermilk, the milk is often supplemented with 0.1–0.2% sodium citrate to ensure good flavour development.

In the production process, pasteurized, homogenized milk is fermented at 22 °C for 12–16 h. The product contains 0.7–0.9% lactic acid and will keep for two weeks at 5 °C.

Another property of LAB valued in some fermented milks is their ability to produce a glycoprotein slime which provides a characteristic texture and viscosity to products such as Swedish *langfil* and Finnish *villi*. Like several other properties of LAB important in dairy fermentations such as the ability to ferment citrate, slime production is a plasmid-mediated characteristic and the ease with which this ability can be lost by the 'ropy' strains of *Lactococcus lactis* used in these fermentations can cause serious problems in commercial production.

9.6 CHEESE

Cheese can be defined as a consolidated curd of milk solids in which milk fat is entrapped by coagulated casein. Unlike fermented milks, the physical characteristics of cheese are far removed from those of milk. This is because protein coagulation proceeds to a greater extent as a result of the use of proteolytic enzymes and much of the water content of the milk separates and is removed in the form of whey. Typically the yield of cheese from milk is of the order of 10%.

Cheesemaking can be broken down into a number of relatively simple unit operations. Slight variations of these and the use of different milks combine to generate the huge range of cheeses available today; said to include 78 different types of blue cheese and 36 Camemberts alone. Classification of cheeses is made difficult by this diversity and the sometimes rather subtle distinctions between different types. Probably the most successful approach is one based on moisture content, with further subdivision depending on the milk type and the role of micro-organisms in cheese ripening (Table 9.7).

Cheese is a valuable means of conserving many of the nutrients in milk. In many people, it evokes a similar response to wine, playing an indispensible part in the gastronome's diet and prompting Brillat-Savarin (1755–1826) to coin the rather discomforting aphorism that 'Dessert without cheese is like a pretty woman with only one eye'. Despite this, the attraction of a well-ripened cheese eludes many people and it is sometimes hard to understand how something that can smell distinctly pedal can yield such wonderful flavours. This paradox was encapsulated by a poet, Leon-Paul Fargue, who described Camembert as 'the feet of God'.

Today cheesemaking is a major industry worldwide, producing something approaching 14 million tonnes per annum. Much is still practised

Table 9.7 *Cheese varieties and their classification*

Moisture Content	
50–80% *SOFT CHEESES*	
	Unripened Cottage, Quark, Cream, Mozzarella Ripened Camembert, Brie, Neufchatel (as made in France), Caciotta, Cooked Salt-cured or pickled Feta, Domiati
39–50% *SEMI SOFT CHEESES*	
	Ripened principally by internal mould growth Roquefort (milk from sheep), Stilton, Gorgonzola, Danish Blue Ripened by bacteria and surface micro-organisms Limburger, Brick, Trappist, Port Salut Ripened primarily by bacteria Bel Paesa, Pasta Filata, Provolone, Brick, Gouda, Edam
<39% *HARD CHEESES*	
	Without eyes, ripened by bacteria Cheddar, Caciocavallo With eyes, ripened by bacteria Emmental, Gruyère
<34% *VERY HARD CHEESES*	
	Asiago old, Parmesan, Romano, Grana

Based on USDA, 1978

on a relatively small scale and accounts for the rich diversity of cheeses still available. Large-scale industrialized production is increasingly important, however, and is dominated by one variety, Cheddar, which is now produced throughout the world, far removed from the small town in Somerset where it originated. Cheddar cheese is particularly valued for its smooth texture and good keeping qualities, although products sharing the name can vary dramatically in flavour. In what follows we will describe the basic steps in cheesemaking with particular reference to the manufacture of Cheddar cheese.

Cow's milk for cheese production must be free from antibiotics and sanitizing agents that might interfere with the fermentation. Although it is not compulsory, a heat treatment equivalent to pasteurization is usually applied at the start of processing. This helps to ensure a safe product and a reliable fermentation, although cheeses made from raw (unpasteurized) milk have been claimed to possess a better flavour. The milk is then cooled to the fermentation temperature which, in the case of Cheddar and other English cheeses such as Stilton, Leicester and Wensleydale, is 29–31 °C. The starter organisms used in most cheese-making

are described as mesophilic starters, strains of *Lactococcus lactis* and its subspecies. Thermophilic starters such as *Lactobacillus helveticus*, *Lb. casei*, *Lb. lactis*, *Lb. delbrueckii* subsp. *bulgaricus* and *Strep. thermophilus* are used in the production of cheeses like Emmental and Parmesan where a higher incubation temperature is employed.

The role of starter organisms in cheesemaking is both crucial and complex. Their central function is the fermentation of the milk sugar lactose to lactic acid. This and the resulting decrease in pH contribute to the shelf-life and safety of the cheese and gives a sharp, fresh flavour to the curd. The stability of the colloidal suspension of casein is also weakened and calcium is released from the casein micelles improving the action of chymosin. After the protein has been coagulated, the acid aids in moisture expulsion and curd shrinkage, processes which govern the final cheese texture.

There are two different systems for uptake and metabolism of lactose in LAB. In most lactobacilli and *Strep. thermophilus*, lactose is taken up by a specific permease and is then hydrolysed intracellularly by β-galactosidase. The glucose produced is fermented by the EMP pathway which the galactose also enters after conversion to glucose-6-phosphate by the Leloir pathway (Figure 9.7). Most lactococci and some lactobacilli such as *Lb. casei* take up lactose by a phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) which phosphorylates lactose as it is transported into the cell. The lactose phosphate is then hydrolysed by phospho-β-galactosidase to glucose, which enters the EMP pathway, and galactose-6-phosphate which is eventually converted to pyruvate via the tagatose-6-phosphate pathway. These pathways are of practical import in cheesemaking; in the lactococci, lactose utilization is an unstable, plasmid encoded characteristic and loss of these genes can

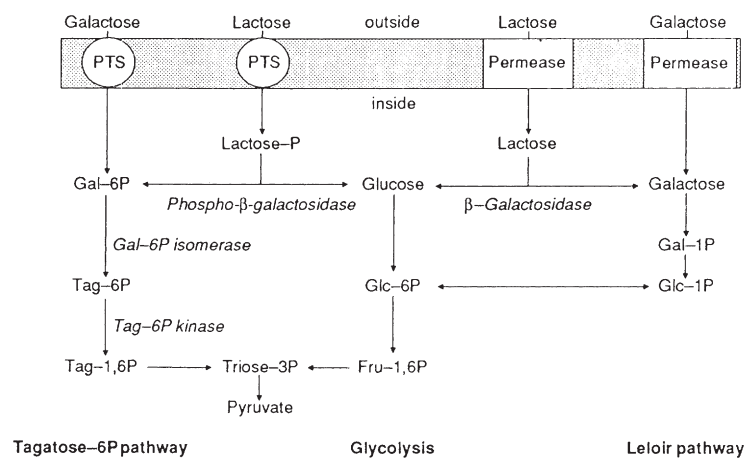


Figure 9.7 *Lactose uptake systems*

clearly have serious consequences for milk fermentation. Using transduction techniques, molecular biologists have produced strains of *Lactococcus lactis* in which this property has been stabilized by integration of the lactose utilization genes in the chromosome.

The thermophilic lactobacilli, which employ a lactose permease and β-galactosidase, metabolize the glucose produced preferentially, turning to galactose only when lactose becomes limiting. This can be a problem in some products. The accumulation of galactose can give rise to a brown discolouration during the heat processing of Mozzarella cheese. In Swiss cheeses such as Emmental, residual galactose can affect product flavour since propionic acid bacteria ferment it in preference to lactate. In doing so they produce a preponderance of acetic (ethanoic) acid which does not confer the usual nutty flavour associated with the equimolar concentrations of acetate and propionate produced by the *Propionibacterium* from lactate.

Lactic acid bacteria are nutritionally fastidious and require preformed nucleotides, vitamins, amino acids and peptides to support their growth. To grow to high cell densities and produce acid rapidly in milk, dairy starters must have proteolytic activity to overcome the limitation imposed by the low non-protein nitrogen pool in native milk. These systems are comprised of proteinases, associated with the surface of the bacterial cell wall, which can hydrolyse casein proteins. Peptidases in the cell wall degrade the oligopeptides produced down to a size that can be transported into the cell (4–5 amino acid residues) where they are further degraded and utilized. While this ability is essential to starter function, it also plays an important role in the development of cheese flavour during ripening or maturation (see below).

Citrate fermentation to diacetyl is required in some cheese varieties and starter cultures for these include species such as *Lactococcus lactis* subsp. *lactis* or *Leuconostoc cremoris*. Carbon dioxide is another product of this pathway and is important in producing the small eyes in Dutch cheese like Gouda or giving an open texture that will facilitate mould growth in blue-veined cheeses. In other cheese, such as Cheddar, this would be regarded as a textural defect.

To produce Cheddar cheese, starter culture is added at a level to give 10^6–10^7 cfu ml^{-1}. In the past these cultures were grown-up in the dairy from stock cultures or from freeze-dried preparations bought in from commercial suppliers. Nowadays frozen, concentrated cultures that are added directly to the cheese vat are increasingly used because of their ease of handling and the greater security they offer the cheesemaker. This applies particularly to the risk of bacteriophage inhibition of the fermentation which has been a major preoccupation of the cheesemaker since it was first identified in New Zealand in the 1930s. Problems of phage infection are not confined to cheesemaking but have also been encountered in the production of yoghurt and fermented meats.

A bacteriophage is a bacterial virus which in its virulent state infects the bacterial cell, multiplies within it, eventually causing the cell to burst (lysis). When this occurs during a cheese fermentation, acidification slows or even stops causing financial losses to the producer as well as an increased risk that pathogens might grow. An important source of phage in cheesemaking is thought to be the starter culture organisms themselves which carry within them lysogenic phages that can be induced into a virulent state. Problems occur particularly when starters contain a single strain or only a few strains and the same culture is reused over an extended period. During this time, phages specific to that organism build up in the plant and can be isolated from the whey and from environmental sources such as drains and the atmosphere, increasing the chance of fermentation failure. In the past, control of this problem has been based on the observation of rigorous hygiene in the dairy, the rotation of starter cultures with differing phage susceptibilities and propagation of starters in phage-inhibitory media which contain phosphate salts to chelate Ca^{2+} and Mg^{2+} required for successful phage adsorption to the bacterial cell. LAB possess their own resistance mechanisms to phage infection which include restriction/modification of non-host DNA, inhibition of phage adsorption by alteration or masking of specific receptors on the cell surface, and reduction of burst size (the number of phages released per infected cell). Most of these mechanisms appear to be plasmid encoded and this has opened the way for new strategies for phage control so that transconjugants with enhanced phage resistance are now available.

A time course for the production of Cheddar cheese showing pH changes and the timing of different process stages is shown in Figure 9.8. A good starter should produce around 0.2% acidity within an hour's incubation. It will multiply up to around 10^8–10^9 cfu g^{-1} in the curd producing an acidity of 0.6–0.7% before its growth is stopped by salting.

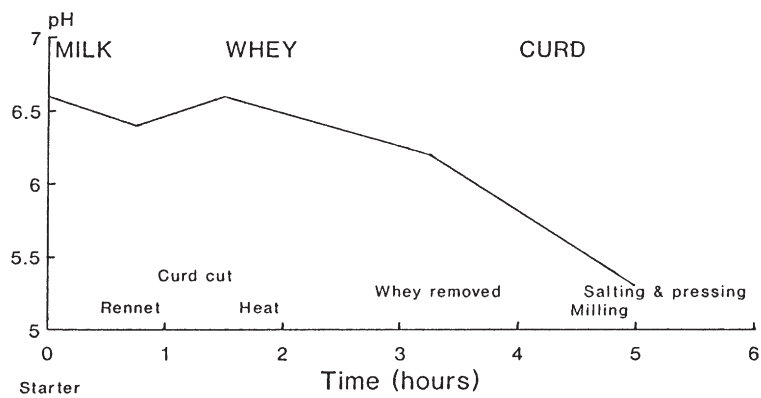


Figure 9.8 *pH changes during Cheddar cheese manufacture*

After about 45 min rennet is added. The time of renneting and the amount added are other important variables in cheesemaking which differ with cheese type. Rennet is a preparation from the fourth stomach or abomasom of suckling calves, lambs or goats. Its most important component is the proteolytic enzyme rennin or chymosin which cleaves *k*-casein, the protein responsible for the stability of the casein micelle, between phenylalanine 105 and methionine 106. This releases a 64 amino acid macropeptide into the whey leaving the hydrophobic *para-k*-casein attached to the micelle. Loss of the macro-peptide leads to the formation of cross-links between the micelles to form a network entrapping moisture and fat globules.

Authentic chymosin is produced as a slaughterhouse by-product but microbial rennets are available, produced from fungi such as *Mucor miehei*, *Mucor pusillus* and *Endothia parasitica*. These lack the specificity of animal rennet and have been associated with the production of bitter peptides in the cheese. Now however the genes for chymosin have been cloned into a number of organisms and nature-identical chymosin is available commercially, produced using the bacterium *E. coli* and yeasts.

After 30–45 min, coagulation of the milk is complete and the process of whey expulsion is started by cutting the curds into approximately 1 cm cubes. Whey expulsion is further assisted by the process known as scalding when the curds, heated to 38–42 °C, shrink and become firmer. The starter organisms are not inhibited by such temperatures and continue to produce acid which aids curd shrinkage. Cheeses produced using thermophilic starters can be scalded at higher temperatures without arresting acid development. When the acidity has reached the desired level (generally of the order of 0.25%), the whey is run off from the cheese vat.

It is at this stage that the process known as cheddaring occurs. The curd is formed into blocks which are piled up to compress and fuse the curds, expelling more whey. Nowadays, the traditional manual process is mechanized in a cheddaring tower.

At the end of cheddaring, the curd has a characteristic fibrous appearance resembling cooked chicken breast. The blocks of curd are then milled into small chips. This facilitates the even distribution of salt which, in Cheddar, is added at a level of between 1.5 and 2% w/w. The salted curd is formed into blocks which are then pressed to expel trapped air and whey.

Finally the cheese is ripened or matured at 10 °C to allow flavour development. During this stage, which can last up to five months to produce a mild Cheddar, the microflora is dominated by non-starter lactobacilli and a complex combination of bacterial and enzymic reactions give the cheese its characteristic flavour. In particular, proteases and peptidases from the starter culture continue to act, even though the

organism can no longer grow. With other proteases from the rennet, they release free amino acids (principally glutamic acid and leucine in Cheddar) and peptides which contribute to the cheese flavour. In some cases this can give rise to a flavour defect: casein proteins contain a high proportion of hydrophobic amino acid residues such as leucine, proline and phenylalanine and if they are degraded to produce peptides rich in hydrophobic residues, the cheese will have a bitter taste.

The lipolytic and proteolytic activities of moulds play an important role in the maturation of some cheeses. In blue cheeses such as Stilton, *Penicillium roquefortii* grows throughout the cheese. It can grow at reduced oxygen tensions, but aeration is improved by not pressing the curds and by piercing the blocks of curd with needles. *P. camembertii* is associated with surface-ripened soft cheeses such as Camembert and Brie.

The keeping qualities of cheese vary with the type but are always much superior to those of milk. This is principally the result of the reduced pH (around 5.0 in Cheddar), the low water activity produced by whey removal and the dissolution of salt in the remaining moisture. Under these conditions yeasts and moulds are the main organisms of concern. The latter are effectively controlled by traditional procedures to exclude air such as waxing or by modern refinements such as vacuum packing.

9.7 FERMENTED VEGETABLES

9.7.1 Sauerkraut and Kimchi

Most horticultural products can be preserved by a lactic acid fermentation. In the West the most important commercially are cabbage, cucumbers and olives, although smaller amounts of others such as carrots, cauliflower, celery, okra, onions, sweet and hot peppers, and green tomatoes are also fermented. In Korea fermented vegetables known as kimchi are an almost ubiquitous accompaniment to meals. More than 65 different types of kimchi have been identified on the basis of differences in raw materials and processing. Cabbages and radishes are the main substrates but garlic, peppers, onions and ginger are often also used. Surveys have shown its importance in the Korean diet, variously reporting kimchi to comprise 12.5% of the total daily food intake or a daily adult consumption of 50–100 g in summer increasing to 150–200 g in winter.

Sauerkraut production is thought to have been brought to Europe from China by the Tartars. Like a number of other traditional fermentations, the commercial process is technologically simple (Figure 9.9), but involves some interesting and complex chemistry and microbiology.

Usually where sauerkraut is produced commercially special cabbage cultivars are grown. These tend to have a higher solids content than normal and so minimize production of liquid waste during processing.

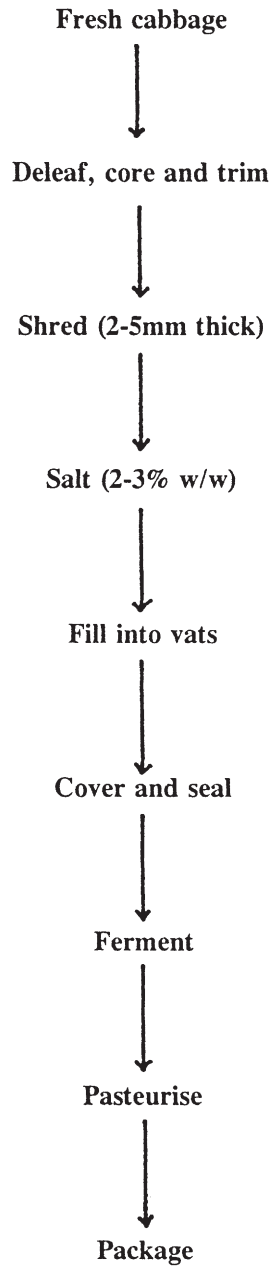


Figure 9.9 *Sauerkraut production*

The outer leaves are removed mechanically and the cabbages decored before cutting into shreds about 1 mm thick. The shredded cabbage is then salted and packed into vats for the fermentation stage.

The level of salting is critical to obtaining a satisfactory product, it must be within the range 2–3% w/w and is normally about 2.25%. Too

little salt (<2%) and the product softens unacceptably, too much salt (>3%) and the correct microbial sequence is not obtained. The salt serves a number of purposes:

(i) it extracts moisture from the shredded cabbage by osmosis to form the brine in which the fermentation will take place;
(ii) it helps to inhibit some of the natural microflora of the cabbage such as pseudomonads which would otherwise cause spoilage and helps to select for the lactic acid bacteria;
(iii) it helps maintain the crisp texture of the cabbage by withdrawing water and inhibiting endogenous pectolytic enzymes which cause the product to soften;
(iv) finally, salt contributes to the flavour of the product.

Traditionally, fermentation vats have been made of wood but nowadays are more often of concrete with a synthetic polymer lining to protect from attack by the acid brine. The tanks are sealed by covering the salted cabbage with plastic sheeting. They are then filled with brine to press the sheeting on to the cabbage expelling the entrapped air.

Although commercial starter cultures for sauerkraut fermentation are available, they are used less often than in other food fermentations. The time course of a typical sauerkraut fermentation is shown in Figure 9.10 and shows how strongly selective the process is. At the start, lactic acid bacteria (LAB) comprise only about 1% of the total microflora, but many of the non-lactics fail to grow and two days later LAB account for more than 90% of the total microflora. During this time, they produce sufficient acid to decrease the pH to below 4 further inhibiting the competing microflora. Underlying this overall dominance by LAB is a natural succession of different species which contribute to the characteristic flavour of sauerkraut. The fermentation is initiated by *Leuconostoc mesenteroides* which is among the less acid- and salt-tolerant LAB but grows fastest during these early stages. As a heterofermenter it produces CO_2 which replaces entrapped air and helps establish anaerobic conditions within the product and prevent the oxidation of vitamin C and loss of colour. Since fructose is present as an alternative electron acceptor, it also produces appreciable amounts of acetic (ethanoic) acid from acetyl-CoA which is a major contributor to sauerkraut flavour. Reduction of fructose leads to the accumulation of mannitol. As the pH drops due to acid production in a weakly buffered medium so the *Leuconostoc* is inhibited and replaced, first by heterofermentative lactobacilli, and then by more acid-tolerant homofermentative lactobacilli such as *Lactobacillus plantarum*. Acid accumulation continues in the form of lactic acid although the pH stabilizes somewhere around 3.8 (the pK_a of lactic acid). At the end of fermentation which can last from 4–8

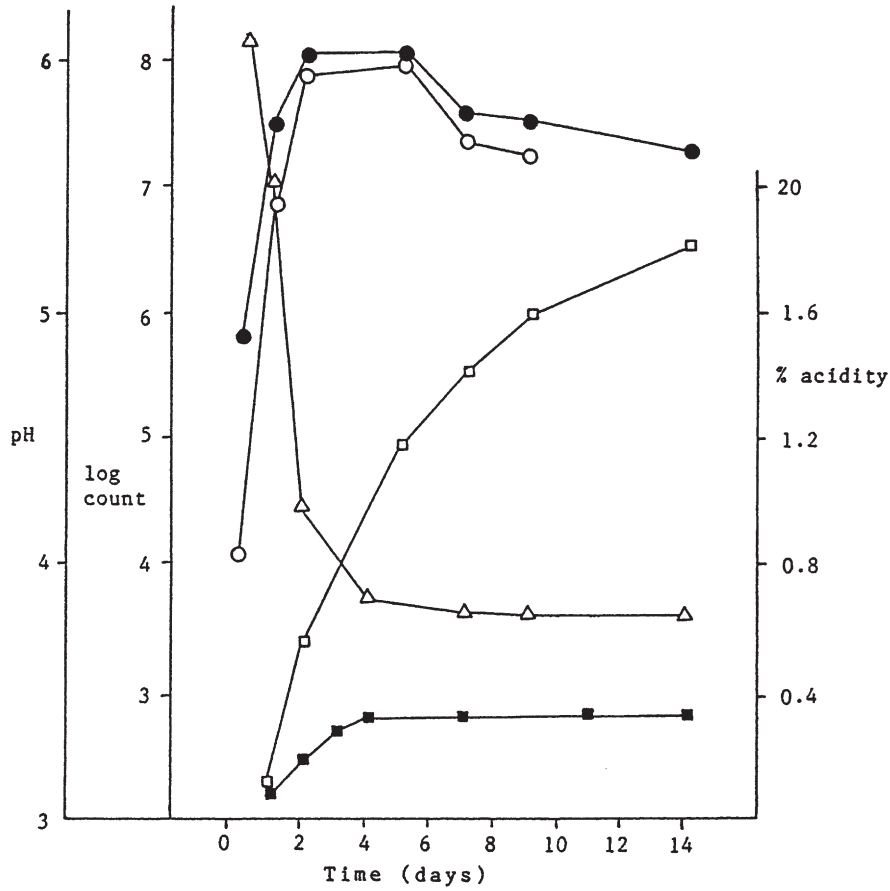


Figure 9.10 *Chemical and microbiological changes during sauerkraut production.* ● *Total bacterial count;* ○ *lactic acid bacterio;* △ *pH;* □ *total titratable acidity (principally lactic plus acetic);* ■ *volatile acidity (principally acetic);*
(Adapted from Stamer, 1974.)

weeks the total acidity of the product is 1.7–2.3%, expressed as lactic acid, with a ratio of volatile to nonvolatile acid of around 1 to 4.

Defects of sauerkraut arise mostly as a result of yeast and mould growth. These can produce off-odours, loss of acidity, a slimy, softened product as a result of pectolytic activity, or a pink discolouration due to the growth of the yeast *Rhodotorula*. In the early stages of fermentation, *Leuconostoc mesenteroides* fermenting sucrose will preferentially utilize fructose, polymerizing the glucose moieties to produce a dextran slime. This is however transient and is later degraded and utilized by other LAB.

In some brined and fermented vegetables nutrients are not particularly well conserved. These tend to employ high salt levels which draw nutrients and moisture from the product into a high-strength brine which is often discarded and replaced before consumption. This is not

the case with sauerkraut which uses a low-salt brine and is not desalted before use. As a result several vitamins are partially conserved, particularly ascorbic acid, vitamin C. Sauerkraut was used extensively as an anti-scorbutic (for the prevention of scurvy) in the Dutch navy in the 18th century and was also highly regarded in this respect by Captain Cook who ordered servings of a pound per man twice weekly during his voyage of 1772. Some losses of vitamin C will occur during processing, a 50% reduction was observed in the first five weeks of kimchi fermentation, but nutritional labels on commercial sauerkraut in the United States usually show an ascorbic acid content of 50% of the Recommended Daily Allowance per 100 g serving.

Kimchi is similar to sauerkraut in some respects since cabbage is a common ingredient and the level of salt used is low ($<3\%$). It differs principally in having a shorter fermentation time; the best taste is claimed after 3 days at 20 °C when the acidity is 0.6% and the pH around 4.2. Consequently *Leuconostoc mesenteroides* is the principal organism responsible for the fermentation and dominance of *Lactobacillus plantarum* is regarded as a defect which results in an excessively sour product.

9.7.2 Olives

Olives are native to the eastern Mediterranean region where they have been cultivated since at least 3000 BC. Today 98% of the world's hectarage of olives is in the Mediterranean region, most of this going to the manufacture of olive oil. Substantial quantities ($>600\,000$ tonnes annually) are also processed into table olives; some are preserved by a canning process similar to other foods but the production of most types includes a period of storage in brine during which a fermentation occurs contributing to the product's stability. Pickled olives in their various forms have a complex taste which often requires considerable application to acquire. In colder climes their consumption has a certain cachet summed up by the 19th century poet and philosopher Ralph Waldo Emerson who likened them to life at sea: exotic and distasteful.

In the production of Spanish-style green olives, which account for 38% of world production, the unripe fruits are first treated with lye (1.0–2.6% sodium hydroxide solution) to hydrolyse the glucoside oleuropein which imparts a bitter flavour and also inhibits lactic acid bacteria (see Section 3.2.4). This lasts for up to 10 h during which the lye penetrates flesh between a half and three quarters of the way to the stone. The lye is then washed off with water over several hours and the fruits placed into a brine. Initially this contains 5–6% salt but the level is increased in strength during the course of the fermentation up to around 8%. Because some of the natural sugars in the olives will have been removed during

the lye treatment and washing, fermentable sugar may be added to the brine.

Complex sequences of bacteria have been reported by different investigators but the most important species appears to be *Lactobacillus plantarum*. Several other LAB have been reported, including an early phase of growth by *Leuconostoc mesenteroides*. This, in particular, will depend upon the salt level used since *Leuc. mesenteroides* is not markedly salt tolerant. Essentially though, the decreasing pH, increasing acidity and the salt combine to eliminate the natural microflora dominated by Gram-negatives and replace it with one composed of lactic acid bacteria and some yeasts. The fermentation process lasts for several weeks and culminates in a product with a pH of 3.6–4.2 containing around 1% lactic acid. Starter cultures are available but rarely used at present, the most important measure taken to control the fermentation is to ensure that air is excluded from the fermenting product to prevent the growth of oxidative moulds and yeasts.

The traditional Greek-style product, natural black olives in brine, accounts for 31% of world production. Processing starts with ripe olives which are placed in a higher strength brine than Spanish-style olives, usually containing up to 10% w/v salt. Fermentation is very slow because the absence of a lye treatment means that oleuropein is still present and that nutrients diffuse slowly through the tough fruit skin. The microflora is usually dominated by yeasts of which a large number of different species have been isolated and identified including members of the genera *Saccharomyces*, *Hansenula*, *Candida*, *Torulopsis*, *Debaryomyces*, *Pichia*, *Kluyveromyces*, and *Cryptococcus*. Lactic acid bacteria may be significant if the salt content is low ($<$6–7%) but are generally a minor component. As a result, there is less acid production than in low-salt vegetable fermentations and the final product generally has a pH of 4.5–4.8 and a total acidity of 0.1–0.6% expressed as lactic acid. This is not sufficient to confer reasonable stability on the product so the salt content is usually increased to above 10% for storage.

9.7.3 Cucumbers

Lactic fermentation following pickling in a brine was once the only method for successful preservation of cucumbers. Since the 1940s, 'fresh pack' techniques have evolved which do not require a fermentation to confer stability. The first of these is based on direct acidification with vinegar or acetic acid followed by pasteurization while more recently direct acidification coupled with refrigerated storage has become increasingly popular. Today in the United States, where more than half a million tonnes of cucumbers are preserved each year, only about 40% are preserved by fermentation, approximately equal volumes are pasteurized

and the remainder are preserved by refrigeration. Despite this recent trend, fermentation does have a number of advantages over other methods.

(1) Fermented cucumbers have flavour and texture characteristics not possessed by the other products.
(2) Bulk fermentation techniques facilitate quick and easy processing in busy harvest seasons.
(3) Under these conditions, products can be stored in bulk until they are required for further processing, so that year-round working is possible.
(4) Fermentation is more economical with energy than techniques which require pasteurization or an efficient cold chain.

Cucumber fermentations can be divided into essentially two different types: high-salt, or salt stock, and low-salt fermentations. Salt stock cucumbers are fermented in a brine containing 5–8% salt until they are stabilized by conversion of all the fermentable sugars to organic acids and other products. *Lb. brevis*, *Lb. plantarum* and *Pediococcus pentosaceus* are most commonly isolated. At these levels of salt *Leuconostoc mesenteroides* does not play the same crucial role as in sauerkraut or kimchi production and at 8% salt it is often not even detected. During the first phase of the fermentation which lasts for 2–3 days the microflora contains a large diversity of bacteria, yeasts and moulds. The environment is selective for LAB and yeasts which increase while other organisms decrease. The fermentation process is not restricted to the surrounding brine but also occurs within the cucumbers as a result of organisms entering through stomata. Sometimes this can lead to defects in the product known as 'bloaters'. Carbon dioxide accumulates within the fruit and is unable to diffuse out, some of this gas production arises from endogenous respiration of the tissues but much is the result of microbial action such as the malo-lactic fermentation and the heterofermentation of sugars. In a controlled fermentation process which has been developed, measures taken to control this problem include the use of strongly homofermentative starter cultures containing *Lactobacillus plantarum* or *Pediococcus pentosaceus* and intermittent purging of CO_2 from the system by bubbling nitrogen through the fermentation.

Genuine dill pickles are fermented in a lower salt brine (3–5%) in the presence of dill (an umbellifer, *Anethum graveolens*) and spices. The fermentation resembles sauerkraut production in the sequence of lactic acid bacteria that develops though it is usually conducted at a slightly higher temperature, 20–26 °C compared with 18 °C for sauerkraut. The full curing process can take up to 8 weeks although active fermentation usually lasts for only 3–4 weeks. The product brine has a pH of 3.2–3.6

and contains 0.7–1.2% acidity (as lactic acid) but will include appreciable amounts of acetic (ethanoic) acid.

9.8 FERMENTED MEATS

Fermented sausages are sometimes claimed to have originated in the Mediterranean region, although traditional products in China and Southeast Asia suggest that they probably developed independently in several locations. Like cheesemaking, meat fermentation is a method for improving the keeping qualities of an otherwise highly perishable commodity. Key features in this are the combination of lactic fermentation with salting and drying which, in many cases, produces a product which is shelf-stable at ambient temperatures.

A further similarity to cheese is the bewildering variety of different types, 330 produced in Germany alone. In the United States fermented sausages are divided into two categories: dry, which have a moisture content of 35% or less, and semi-dry typically containing about 50% moisture. Spreadable fermented sausages, produced in Germany, such as Teewurst, and Mettwurst are not dried during production and in this respect are similar to the Thai product *nam*.

The ingredients of a European-style fermented sausage may comprise:

lean meat, 55–70%
fat, 25–40%
curing salts, 3%
fermentable carbohydrate, 0.4–2.0%
spices and flavouring, 0.5%
starter, acidulant, ascorbic acid, *etc*. 0.5%

Pork is most commonly used in southern Europe but elsewhere beef, mutton and turkey meat are also used. The meat should always be of high quality since the products are usually consumed without cooking and so are essentially a raw-meat product. Unlike fermented milk products, it is not possible to heat treat the meat before processing as this would destroy the sausage's textural characteristics, but some are given a final pasteurization to ensure safety.

The curing salts added are a similar mixture of sodium chloride and sodium nitrate and/or nitrite to that used in the production of cured meats such as ham and bacon. Here too, they contribute to the taste, colour, safety, stability and texture of the product.

Spices are added primarily for reasons of flavour but are known to have potentially important roles in retarding microbial spoilage and promoting lactic fermentation. The antimicrobial effect of spice components, which has already been discussed in Section 3.2.4, could help

inhibit the normal spoilage microflora of the meat. Spices have also been shown to stimulate the growth of lactic acid bacteria. This is a result of their manganese content, spice extracts or spices low in manganese do not have this stimulatory effect. Most aerobes have micromolar quantities of the enzyme superoxide dismutase (SOD) to scavenge the toxic superoxide anion radical produced by a one-electron reduction of molecular oxygen (see Section 3.2.3). Aerotolerant lactic acid bacteria do not have SOD but have developed an alternative protective mechanism based on the accumulation of millimolar concentrations of manganous (Mn^{2+}) ion.

$$2H^+ + O_2^{-\bullet} + Mn^{2+} \rightarrow H_2O_2 + Mn^{3+} \quad (9.1)$$

The Mn^{2+} is regenerated by a subsequent reduction step. Increasing the manganese content of the medium can stimulate LAB growth.

Other ingredients which may be included are glucono-δ-lactone which improves acidulation by slowly hydrolysing to produce gluconic acid, ascorbic acid to improve colour production and stability, and glucose to supplement the available fermentable sugar in the starting mix.

Ingredients are blended together in a bowlchopper at low temperature. When the ingredients have been blended together they are packed into casings of the appropriate diameter. Traditionally collagen from the gastrointestinal tract of animals has been used but nowadays fibrous cellulose and regenerated collagen produced from animal hides are more common. The packing material must have certain properties: it must adhere to the meat mix and must shrink with it during processing and must be permeable to moisture and smoke.

Fermented sausages are still often made by natural fermentations in which the selectivity of the starting mix determines which components of the heterogeneous microflora dominate. Starter cultures are however being increasingly used for the greater assurance of a satisfactory fermentation they provide. The principal components of commercial starters are lactic acid bacteria and nitrate-reducing bacteria. Some will also include yeasts and moulds such as *Debaryomyces hansenii* and *Candida famata*, and moulds, usually *Penicillium* spp. such as *P. nalgiovense*. LAB included in early starters were mainly *Lactobacillus plantarum*, *Pediococcus acidilactici* and *Ped. pentosaceus*, not necessarily those most important in the natural fermentation. Surveys of naturally fermented products demonstrated that the dominant LAB were psychrotrophic, facultatively heterofermentative lactobacilli (see page 317) that were slightly less acid tolerant than usual (minimum pH 3.9 compared with 3.7–3.8). Most of these are now assigned to the species *Lactobacillus sake* and *Lactobacillus curvatus* and strains of these have been incorporated into commercial starters.

Members of the Micrococcaceae such as *Micrococcus varians* and *Staphylococcus carnosus* are important with respect to the reduction of nitrate to nitrite although this activity has also been demonstrated in some lactobacilli. Their presence would not be required in nitrite-cured products.

Sausage fermentations are conducted at temperatures ranging from 15 °C up to in excess of 40 °C, depending on the starter used, and last for 20–60 h. The relative humidity is also controlled to ensure that a slow drying of the product commences. Acid production and the decrease of the pH to below 5.2 promote the coagulation of meat proteins and this aids moisture expulsion and development of the desired texture and flavour. It also contributes to the microbiological stability and safety of the product.

North American and northern European sausages are often smoked. This confers a characteristic flavour but phenolic components of the wood smoke also have important anti-oxidative and antimicrobial properties which improve shelf-life. Fungal growth may occur on the surface of unsmoked sausages providing a particular character to these products as a result of fungal lipolytic, proteolytic and antioxidative activity.

In the final drying stage which can last up to 6 weeks, the moisture content is reduced further by storage at low temperatures, 7–15 °C, and at low relative humidity (65–85%).

The combination of antimicrobial hurdles or barriers introduced during sausage fermentation is normally sufficient to ensure product safety. *Staphylococcus aureus* with its ability to tolerate reduced a_w and pH and grow anaerobically would seem well suited to growth in these products. Occasional outbreaks of *Staph. aureus* food poisoning have been reported from the United States where higher fermentation temparature are used. However, studies suggest that *Staph. aureus* does not compete well with the LAB present, particularly if the latter have a large numerical superiority as a result of starter addition. The risk is also reduced since enterotoxin production appears to be more susceptible than growth to inhibition by adverse conditions (see Section 7.14.2).

Numbers of *Salmonella* and other Enterobacteriaceae have been shown to fall throughout fermentation and drying. At present though, our knowledge of such processes is insufficient to allow us to predict this lethal effect reliably. It is therefore, most important that only good quality raw materials are used so that undue reliance is not placed upon these factors.

Outbreaks of Verotoxin producing *E. coli* in the United States associated with fermented meats highlighted this problem and prompted the US Food Safety and Inspection Service to recommend that the procedures used in the production of ready-to-eat fermented products should achieve a 5 $\log_{10}$ cfu g^{-1} reduction in pathogen numbers.

One way of achieving this reliably would be to introduce a heating step. Following an outbreak of salmonellosis in the UK associated with a salami stick product imported from Germany, the production process was changed to incorporate a final pasteurization step without adverse effects on sensory quality.

Nam, the Thai fermented sausage, differs in several respects from European fermented sausages. It is a low-fat product which is subjected to a short fermentation and is not dried. It is also wrapped in water-impermeable plastic material or, traditionally, banana leaves. As the fermentation proceeds and the pH drops the moisture is expelled but is trapped within the packaging giving the consumer an indication of the age of the product. It is not always stored chilled and its largely anecdotal association with food poisoning has prompted test marketing of irradiated *nam* in some areas of Thailand.

9.9 FERMENTED FISH

The term fermented fish is applied to two groups of product, mostly confined to East and Southeast Asia: the more widely known fish/salt formulations such as fish sauces and pastes, and fish/salt/carbohydrate blends. Strictly speaking, only in the latter case is the description 'fermented' fully justified. Microbial action in the production of fish sauces and pastes is slight if not insignificant and the term is being used in its looser, non-microbiological, sense to apply to any process where an organic material undergoes extensive transformation.

In many areas where they are produced, fish sauces and pastes are the main flavour principle in the local cuisine and provide a valuable balanced source of amino acids. The names of some fish sauces and pastes and their countries of origin are given in Table 9.8.

Fish sauces and pastes are usually made from a variety of small fish which are packed into tanks or jars with salt usually at a ratio of around three parts fish to one part salt. This is more than sufficient to saturate the aqueous phase, to produce an a_w below 0.75 and arrest the normal pattern of spoilage. The only organisms likely to be able to grow under such conditions are anaerobic extreme halophiles. Although there have been recent reports of isolations of organisms such as the proteolytic *Halobacterium salinarium* from fish sauce, their importance remains to be established since earlier work has shown that acceptable fish sauce could be made using fish sterilized by irradiation.

The production process can take up to 18 months or more, during which the fish autolyse, largely through the action of enzymes in the gut and head of the uneviscerated fish, to produce a brown salty liquid rich in amino acids, soluble peptides and nucleotides. Products in which autolysis is less extensive are described as fish pastes.

Table 9.8 *Fish sauces and pastes and their countries of origin*

	Name	
Country	*Sauce*	*Paste*
	Amber/brown liquid, salty taste, cheese-like aroma	Red/brown salty paste
Burma	*ngapi*	*nga-ngapi*
Indonesia	*ketjap-ikan*	*trassi-ikan* *trassi-udang* (shrimps)
Kampuchea	*nuoc-mam* *nuoc-mam-gau-ca* (livers only)	*prahoc* *mam-ruoac* (shrimps)
Laos	*nam-pla* (pa)	*padec*
Malaysia	*budu*	*belachan* (shrimps)
Philippines	*patis*	*bagoong*
Thailand	*nam-pla*	*kapi*
Vietnam	*nuoc-mam*	*man-ca* *man-tom* (shrimps)

Table 9.9 *Fermented fish/salt/carbohydrate products*

Country	*Products*
Japan	*I-shushi, e.g. ayu-sushi, funa-suchi tai-suchi,*
Kampuchea	*phaak, mam-chao*
Korea	*sikhae*
Laos	*som-kay-pa-eun, som-pa, mam-pa-kor, pa-chao, pa-khem som-pa-keng*
Malaysia	*pekasam, cencalok*
Philippines	*burong-isda, e.g. burong-ayungi, burong-dalag, burong-bangus*
Thailand	*pla-ra, pla-som, pla-chao, som-fak*

Authentic lactic-fermented fish products have to include as an ingredient an exogenous source of fermentable carbohydrate. Considerable variation in recipes has been noted but production is governed by two general principles: the higher the salt content of the product, the longer the production process takes but the better the product's keeping qualities; and the higher the level of added carbohydrate, the faster the fermentation and the more acidic the flavour.

Fish/salt/carbohydrate products (Table 9.9) are generally much less popular than the fish sauces and pastes and are produced on a smaller scale. Their production also tends to be more common away from the coast and to use freshwater fish. Though superficially their production appears similar to that of fermented meat sausages, they are quite distinctive.

In products such as *Burong-isda* (Philippines), *Pla-jao*, and *Pla-som* (Thailand) and *I-sushi* (Japan), cleaned fish flesh is dry salted with about 10–20% salt and left for a period of up to a day. The flesh is then usually removed from the brine that develops and may be subjected to further

moisture reduction by sun-drying for a short period. Lactic fermentation is then initiated by addition of carbohydrate. This is usually in the form of rice although traditional saccharifying agents (*koji*, Japan; *look-pang*, Thailand; *ang-kak*, Philippines) employing mould enzymes may be added. These accelerate the fermentation, since most LAB are not amylolytic, and also increases the total acid produced. For example, *Burong-isda* containing *ang-kak* has a lower pH (3.0–3.9) than that produced with rice alone (4.1–4.5). Garlic is often added along with the rice as a flavouring ingredient and this may play a similar role in directing the fermentation as spices do in fermented sausage production. Garlic is also a source of the fermentable carbohydrate inulin. The product is normally ready for consumption after about two weeks of fermentation when the microflora is dominated by yeasts and LAB which are present at levels around 10^7 cfu g^{-1} and 10^8 cfu g^{-1} respectively.

With the exception of *I-sushi*, these products are usually cooked before consumption and this along with the low pH generally guarantees safety. However, the small, very often domestic-scale, production can lead to extreme variations in a product's character and failure to obtain a satisfactory rapid fermentation in *I-sushi* has led to outbreaks of botulism in Japan caused by *C. botulinum* type E.

9.10 BEER

The popularity of products resulting from the conversion of sugars into ethanol by yeasts is almost universal and there is hardly a culture without its own indigenous alcoholic beverage. All that is required is a material that will furnish sufficient fermentable carbohydrate; a condition fulfilled by honey, cereals, root crops, palm saps and many fruits, pre-eminently grapes, but also, apples, pears, plums and others. The ethanol concentration achieved by fermentation is limited by the sugar content of the raw material and also by the ethanol tolerance of the yeast which is normally around 14% v/v. *Sake*, Section 9.12.2 below, is something of an exception. Potency can be increased by distillation of a fermented wash to produce spirits such as whisky, vodka, brandy, calvados and arrack, and ethanol partially purified by distillation can also be added back to a fermented product to give fortified wines such as port, sherry and madeira. Here, we will concentrate on a single product which has spread throughout the world and is now produced more widely than any other alcoholic drink: European-style beer.

Brewing is thought to have originated in Mesopotamia where it is said that as much as 40% of total cereal production was used for this purpose. Because of the relative complexity of the process, it is likely that beer was a later discovery than wine. The Romans were disinterested

and after tasting British ale in the 4th century the Emperor Julian was compelled to pen a little poem:

Who made you and from what
By the true Bacchus I know you not
He smells of nectar
But you smell of goat.

Clearly the unhopped ale of the time was not to his taste, but even today beer enjoys an inferior reputation to that of wine.

Barley is the principal cereal used in the production of beer, although other cereals are occasionally used and wheat beers such as Berliner Weisse and the Gueuze–Lambic beers of Belgium are notable exceptions. Africa has a number of traditional beers produced from local cereals such as sorghum or millet and some of these are produced on a substantial industrial scale. These however are the result of a mixed lactic/ethanolic fermentation and bear little resemblance to European-style beers.

One reason for barley's pre-eminence is that the grain retains the husk which affords protection during storage and transport and also acts as an aid to filtration during wort separation. The gelatinization temperature of malted barley starch is also low relative to that of other cereals (52–59 °C) and this enables the starch to be gelatinized (solubilized), prior to enzymic digestion, at temperatures which will not inactivate the starch degrading enzyme α-amylase. A further advantage is the presence in barley of substantial quantities of a second enzyme, β-amylase, which is essential for the rapid conversion of starch and dextrins to maltose.

Since the brewing yeast, *Saccharomyces cerevisiae*, is unable to ferment starch, the first stage in the production of any alcoholic beverage from starchy materials is conversion of the starch into fermentable sugars. Human ingenuity has come up with a number of ways of doing this. In the Oriental Technique, exemplified by products such as *sake*, mould enzyme preparations like *koji* are used, whereas the prevalent Western technique uses endogenous starch-degrading enzymes produced in the grain through the process of malting. A third technique used in some native cultures in South America is to use salivary amylase by chewing the substrate so that it becomes coated with saliva and then spitting it out to saccharify and ferment. This approach is not amenable to industrialization and is not, as far as we are aware, the basis of any large-scale commercial production of alcoholic beverages.

In malting, the grain is moistened by steeping in water and is then spread on to a malting floor and allowed to germinate. During germination, hydrolytic enzymes, produced in the aleurone layer surrounding the grain endosperm, attack the endosperm, mobilizing the nutrient and

energy reserves it contains for the growing barley plant. To encouraged this, maltsters sometimes add gibberellins, plant growth hormones which are the natural regulators of this process.

The development from a seed to a plant is arrested by kilning which reduces the moisture content of the malt to 3–5%. During kilning, some non-enzymic browning reactions occur between amino acids and sugars in the malt and these contribute to the final beer colour. Darker beers tend to include malts that have been kilned at higher temperatures to promote browning reactions.

Nowadays malts are usually bought in by brewers as one of their raw materials and the brewing process proper starts with its conversion into a liquid medium (wort) capable of supporting yeast growth: a step known as mashing (Figure 9.11).

The malt is ground to reduce the particle size and increase the rate of enzymic digestion and is then mixed with hot water. Water, known in brewers' parlance as liquor, is an important ingredient in brewing and the quality of the local water was one of the reasons for the development of traditional UK brewing centres such as Burton on Trent, London and Edinburgh. In particular, calcium content has a significant impact on the brewing process because calcium ions precipitate out as calcium phosphate during mashing. This decreases the wort pH from 6.0 to 5.4, nearer the optimum for a number of malt enzymes, and thus increases the yield of fermentable extract. Starchy adjuncts may be added during mashing to boost the fermentable sugar content of the wort.

There are two traditional systems of mashing: the British technique of infusion mashing where the mash is held in a single vessel at a constant temperature of around 65 °C, and the continental decoction system where the mash is heated through a range of temperatures by removing a portion, heating it, then adding it back. Nowadays a number of variations on these techniques are used so that the differences are less distinct.

In mashing, a number of enzymic activities contribute to the production of the clear liquid medium known as sweet wort. For instance, it requires two enzymes operating in concert to break down starch into maltose, a disaccharide of glucose fermentable by the brewing yeast. Barley starch is composed of two fractions: amylose (20–25%), a linear polymer of α-1,4-linked glucose units, and amylopectin (75–80%), a branched polymer containing linear chains of α-1,4-linked glucose units with branches introduced by occasional α-1,6-linkages. Alpha amylase hydrolyses α-1,4-linkages to produce a mixture of lower molecular weight dextrins while the exoenzyme, *β*-amylase, attacks dextrins at their non-reducing end, snipping off maltose units. Limit dextrins containing the α-1,6-linkages are left in the wort largely untouched unless the non-malt enzyme amyloglucosidase is added to the mash.

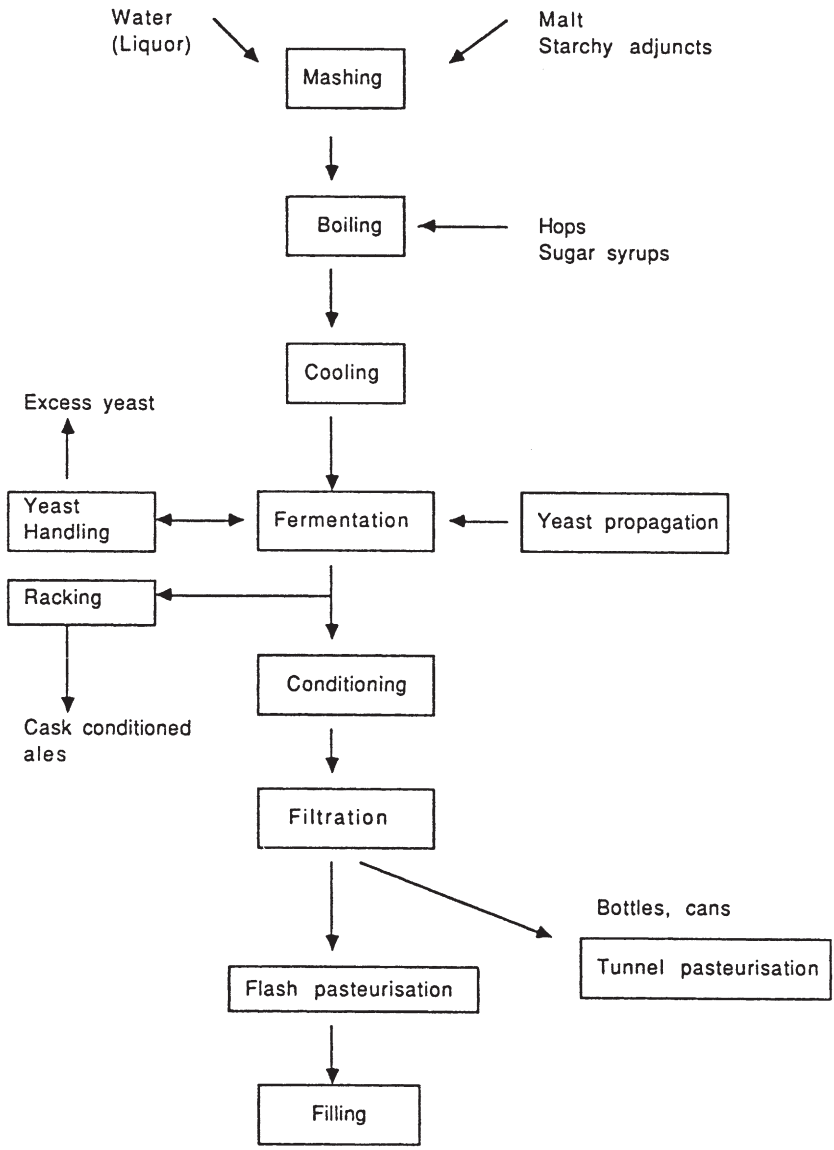


Figure 9.11 *The brewing process*

Proteinases solubilize malt proteins and supply yeast nutrients so that about 35–40% of malt protein is solubilized during mashing compared with 90–95% of the malt starch. Phosphatases release inorganic phosphate, which is important for yeast nutrition and for contributing to the buffering capacity of the wort. The activity of β-glucanases can also be useful in breaking down β-glucans which can cause subsequent handling problems with the beer.

After mashing, sweet wort is boiled. This stops the degradative processes by inactivating the malt enzymes. It also pasteurizes the wort, completes ionic interactions such as calcium phosphate precipitation, denatures and precipitates proteins and tannins which separate as a material known as hot break or trub and helps dissolve any sugars which may be added at this stage as an adjunct.

Hops are also added during boiling. These are the cones or strobili of the plant *Humulus lupulus* whose principal purpose is the bittering of the wort. The hop resin contains α-acids such as humulone and cohumulone which are only partially soluble in wort. During boiling they isomerize to isohumulones which are more soluble and more bitter than α-acids (Figure 9.12). Although hop resins have some antibacterial action, they play little part in assuring the bacteriological stability of beer as spoilage bacteria such as lactobacilli rapidly acquire a tolerance to them.

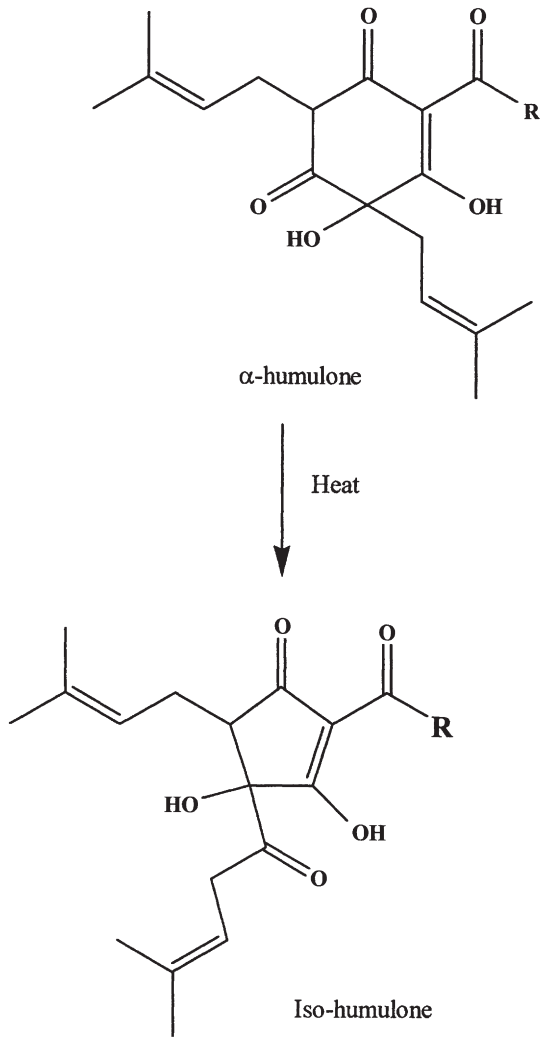
Wort boiling lasts for 1–2 h during which 5–15% of the volume is evaporated. Hop residues are then strained off, hot trub is removed in a whirlpool separator, and the hopped wort cooled to the fermentation temperature.

The yeasts used to brew ales and lagers are strains of *Saccharomyces cerevisiae*, known as *S. cerevisiae* var. *cerevisiae* and *S. cerevisiae* var. *carlsbergensis (uvarum)* respectively. The distinctions between the yeasts used in ale and lager brewing are slight. Traditionally, ale yeasts were regarded as top fermenters which formed a frothy yeast head on the surface of brewing beer and was skimmed off to provide yeasts for pitching (inoculating) subsequent batches, while lager yeasts were bottom fermenters which formed little surface head and were recovered from the bottom of the fermenter. Nowadays this is a less useful distinction as many ales are brewed by bottom fermentation.

The cardinal temperatures of the two organisms differ and this is reflected in the different temperatures used for lager fermentations (8–12 °C) and for ale fermentations (12–18 °C). They can also be distinguished by the ability of *S. cerevisiae* var. *carlsbergensis* to ferment the disaccharide melibiose, although this is of no practical import since the sugar does not occur in wort.

During fermentation the yeast converts fermentable carbohydrate to ethanol via the EMP pathway. Although this is an anaerobic process, a vigorous fermentation is often helped by aeration of the wort before pitching with yeast. This supplies oxygen, necessary for the synthesis of unsaturated fatty acid and sterol components of the yeast cell membrane, and may sometimes be repeated later in the fermentation.

A time course of a typical ale fermentation is shown in Figure 9.13. After an initial vigorous phase during which there is active yeast growth, ethanol production and a drop in pH as nitrogen is removed from the wort, there is a second phase of slower ethanol production in the absence of further yeast



$R = -CH_2.CH(CH_3)_2$ humulone

$R = -CH(CH_3)_2$ cohumulone

$R = -CH(CH_3).CH_2.CH_3$ adhumulone

Figure 9.12 *Isomerization of hop α- and β-acids*

growth. Overall the yeast population increases about six-fold during fermentation. This yeast can be recycled, usually after an acid wash to control bacterial contamination, but eventually its performance drops as viability declines and it is used in animal feed and the manufacture of yeast extract.

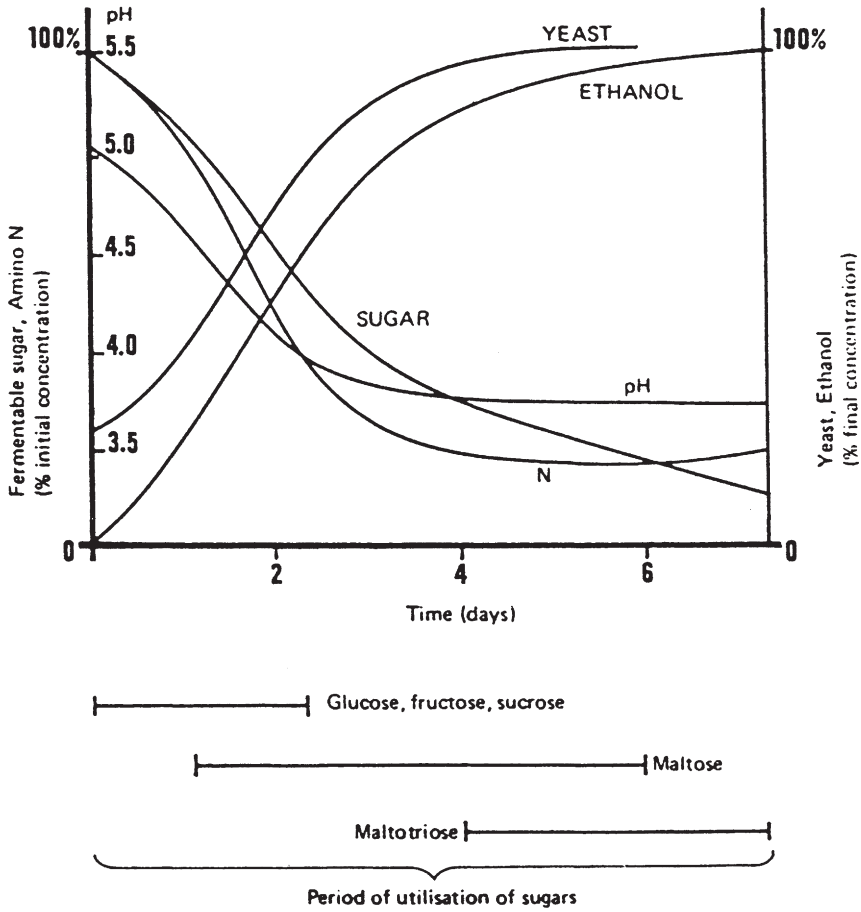


Figure 9.13 *Changes during the beer fermentation*
(Reproduced courtesy of the Institute of Brewing)

Lager fermentations take longer due to the lower temperature. The name lager originates from the German word for store and describes the period of secondary fermentation (storage) at low temperature which these products undergo to improve yeast settling, clarification and CO_2 dissolution. In the past, this could last for several months, but with modern techniques such as centrifugation and artificial carbonation it is less protracted and is now complete within one to two weeks.

Depending on the product, beer from the fermenter can be subjected to a variety of downstream processes. It may be run to casks where priming sugars are added to stimulate the secondary fermentation necessary in cask-conditioned beers, or it may be filtered prior to pasteurization and kegging. Bottled and canned beers usually undergo a combination of filtration and centrifugation before packing and pasteurization.

Table 9.10 *Possible taints in beer*

Taint	*Associated Flavours*	*Possible Cause*
Acetaldehyde	Apples, paint, grassy	Bacterial spoilage: Acetic acid bacteria/*Zymomonas*
Sulphur	Bad eggs, drains	Formed during fermentation, wild yeasts/*Zymomonas*
Cloves	Herbal phenolic	Bacterial spoilage/wild yeasts
Musty/fungal	Mouldy, stale, hessian	Mould or bacteria: Usually in water
Fruity	Estery, pineapples, solvent, bananas, pear drops	Wild yeast/*Brettanomyes*: Wort bacteria/*Enterobacter agglomerans*
Ethyl Acetate	Solvent-like	Wild yeast/*Hansenula anomala*
Diacetyl	Toffee, butterscotch, honey	Formed during fermentation/ lactic acid bacterial spoilage: *Lactobacillus*/*Pediococcus*
DMS	Sweetcorn, jammy	Bacterial spoilage: *Hafnia*/ *Obesumbacterium*. Wort bacteria
TCP Acetic Acid	Medicinal, antiseptic Sour, vinegar	Bacterial spoilage: Wort bacteria Bacterial spoilage: *Acetobacter*/*Gluconobacter*
Acidic	Sourness and creaminess Sourness and apples	Bacterial spoilage: *Lactobacillus Acetobacter*

Courtesy L. Hargreaves

Brewing is quite a robust process microbiologically due to a combination of factors such as low nutrient status, ethanol content and low pH and there is a limited range of micro-organisms of concern to the brewery microbiologist. Members of the Enterobacteriaceae such as *Obesumbacterium proteus*, *Klebsiella* and *Enterobacter* species are sensitive to the low pH and ethanol of beer but can grow in wort producing off-odours like dimethyl sulfide which can persist through to the final product. They also contribute to the production of nitrosamines by reducing wort nitrate to nitrite. *Obesumbacterium proteus* is particularly associated with top-fermenting yeasts but can be controlled by acid washing.

Acetic acid bacteria of the genera *Acetobacter* and *Gluconobacter* can be found throughout the brewery. As obligate aerobes they are particularly associated with cask-conditioned beer where they cause spoilage as a result of turbidity, ropiness and the oxidation of ethanol to ethanoic (acetic) acid. *Zymomonas mobilis* is an anaerobic, Gram negative rod which can ferment sugars to ethanol. It causes more problems in ale brewing where it grows in the primed beer producing turbidity and off-flavours.

Lactic acid bacteria of the genera *Lactobacillus* and *Pediococcus* can grow widely in the brewery environment and in beer where they produce acid, diacetyl, which gives beer a sweet butterscotch flavour, and

polymeric material known as rope. Yeasts that are not used for the fermentation can cause hazes and off-odours and are known as wild yeasts. Normally these are described as being *Saccharomyces* and non-*Saccharomyces*. *Saccharomyces* wild yeasts can be detected using a medium containing copper sulfate to inhibit the brewing yeast. Non-*Saccharomyces* yeasts such as *Pichia*, *Hansenula*, *Brettanomyces* and others can be detected with a medium containing lysine as the sole nitrogen source which *Saccharomyces* cannot utilize.

Some of the possible taints in beer and their causes are presented in Table 9.10.

9.11 VINEGAR

Vinegar is the product of a two-stage fermentation. In the first stage, yeasts convert sugars into ethanol anaerobically, while in the second ethanol is oxidized to acetic (ethanoic) acid aerobically by bacteria of the genera *Acetobacter* and *Gluconobacter*. This second process is a common mechanism of spoilage in alcoholic beverages and the discovery of vinegar was doubtless due to the observation that this product of spoilage could be put to some good use as a flavouring and preservative. The name vinegar is in fact derived from the French *vin aigre* for 'sour wine' and even today the most popular types of vinegar in a region usually reflect the local alcoholic beverage; for example, malt vinegar in the UK, wine vinegar in France, and rice vinegar in Japan.

In vinegar brewing, the alcoholic substrate, known as vinegar stock, is produced using the same or very similar processes to those used in alcoholic beverage production. Where differences occur they stem largely from the vinegar brewer's relative disinterest in the flavour of the intermediate and his concern to maximize conversion of sugar into ethanol. In the production of malt vinegar for example, hops are not used and the wort is not boiled so the activity of starch-degrading enzymes continues into the fermentation. Here we will concentrate on describing the second stage in the process, acetification.

Acetification, the oxidation of ethanol to acetic acid is performed by members of the genera *Acetobacter* and *Gluconobacter*. These are Gram-negative, catalase-positive, oxidase-negative, strictly aerobic bacteria. *Acetobacter* spp. are the better acid producers and are more common in commercial vinegar production, but their ability to oxidize acetic acid to carbon dioxide and water, a property which distinguishes them from *Gluconobacter*, can cause problems in some circumstances when the vinegar brewer will see his key component disappearing into the air as CO_2. Fortunately over-oxidation, as it is known, is repressed by ethanol and can be controlled by careful monitoring to ensure that ethanol is not completely exhausted during acetification. Most acetifications are run on

a semi-continuous basis; when acetification is nearly complete and acetic acid levels are typically around 10–14% w/v, a proportion of the fermenter's contents is removed and replaced with an equal volume of fresh alcoholic vinegar stock. Since a substantial amount of finished vinegar is retained in the fermenter, this conserves the culture and means that a relatively high level of acidity is maintained throughout the fermentation, protecting against contamination. It also protects against over-oxidation as it has been found that *Acetobacter europaeus*, a species commonly found in commercial vinegar fermenters, will not over-oxidize when the acetic acid concentration is more than 6%.

Many of the acetic acid bacteria associated with commercial acetification are difficult to culture on conventional solidified media, although some success has been enjoyed using a double-layer medium which provides colonies growing on the surface with a constant supply of ethanol and moisture from a lower, semi-solid layer. As a result, vinegar fermentations are usually initiated with seed or mother vinegar, an undefined culture obtained from previous fermentations. Depending on the type of acetification, the culture can be quite heterogeneous and *A. europaeus*, *A. hansenii*, *A. acidophilum*, *A. polyoxogenes*, and *A. pasteurianus* have all been isolated from high-acidity fermentations.

Oxidation of ethanol to acetic acid is the relatively simple pathway by which acetic acid bacteria derive their energy. It occurs in two steps mediated by an alcohol dehydrogenase and an aldehyde dehydrogenase (Figure 9.14). Both enzymes are associated with the cytoplasmic membrane and have pyrroloquinoline quinone (PQQ) as a coenzyme. PQQ acts as a hydrogen acceptor which then reduces a cytochrome *via* an intermediate quinone. The consequent electron transport establishes a proton motive force across the membrane which can be used to synthesize ATP.

Overall, acetification can be represented chemically by:

$$C_2H_5OH + O_2 = CH_3COOH + H_2O \qquad (9.2)$$

From the stoichiometry of the equation it can be calculated that 1 litre of ethanol should yield 1.036 kg of acetic acid and 0.313 kg of water. This leads to the approximate relationship that 1% v/v ethanol will give 1% w/v acetic acid, and this is used to predict the eventual acidity of a vinegar and to calculate fermentation efficiency. It implies that, in the absence of over-oxidation, evaporative losses and conversion to biomass, the sum of the concentration of ethanol (%v/v) and the concentration of acetic acid (%w/v), known as the total concentration or GK (German: Gesammte Konzentration) should remain constant throughout acetification. The GK yield is the GK of the final vinegar expressed as a percentage of the GK at the start of acetification.

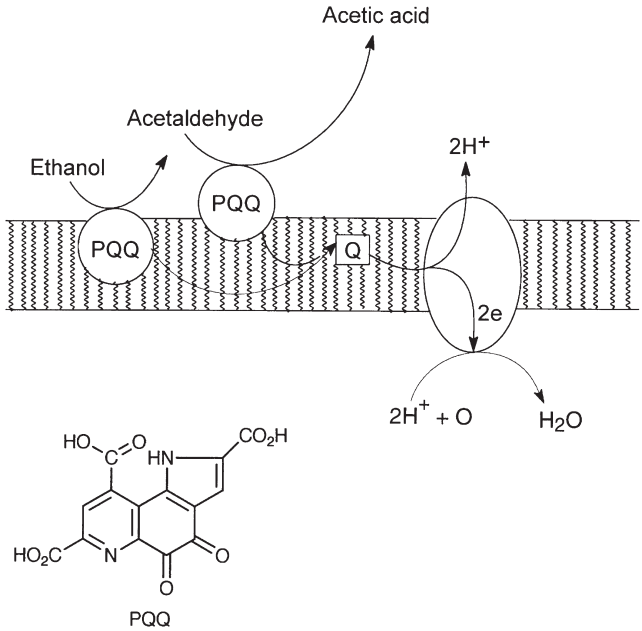


Figure 9.14 *Oxidation of ethanol by acetic acid bacteria*

There are a number of techniques for acetification which differ in the means by which the three interacting components, ethanol, bacteria and oxygen, are brought together. Surface culture techniques, where the bacteria form a surface film at the interface between the acetifying medium and air, are the simplest but can be applied with varying levels of sophistication. In the Orleans process, vinegar stock in partially filled casks drilled with air holes (Figure 9.15) is left to acetify until the acidity reaches the appropriate level determined by the initial GK value. At this point a proportion, typically one-third to two-thirds, is drawn off through the tap, replaced with fresh stock and the process restarted. The vinegar stock is usually added *via* a pipe passing through the top of the barrel and resting on the bottom. In this way the surface film of bacteria is not disturbed and the delays and losses that result from having to reform the film are avoided. Usually the time taken to complete one acetification cycle is of the order of 14 days.

Only a small proportion of the world's vinegar is produced by surface culture today, although it is claimed to produce the finest quality vinegar. More elaborate surface culture techniques based on series of trays have been described but these have received only very limited application.

The quick vinegar process derives its name from the faster rates of acetification achieved by increasing the area of active bacterial film and improving oxygen transfer to the acetifying stock. The acetic acid bacteria grow as a surface film on an inert support material packed into

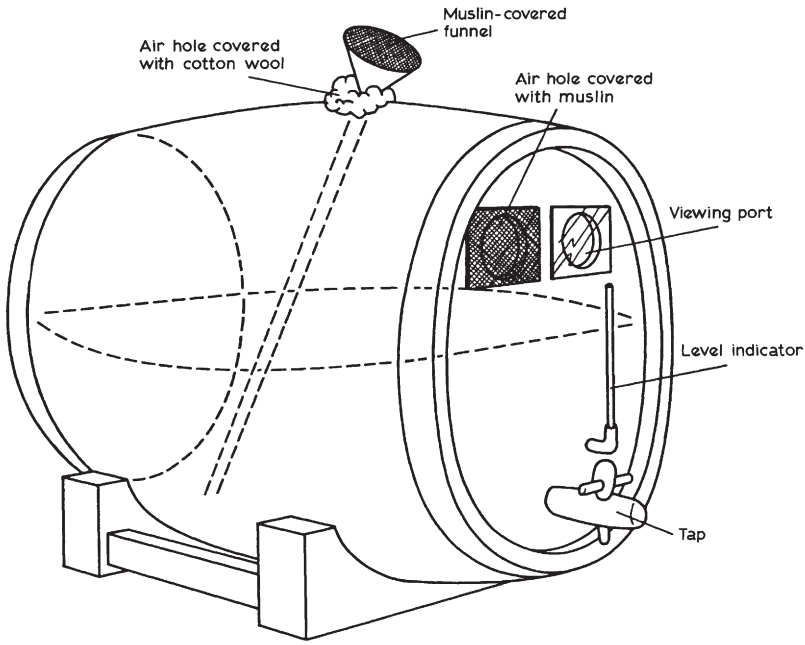


Figure 9.15 *The Orleans process of vinegar manufacture*

a false-bottomed vat. The acetifying stock is sprayed on to the surface of the packing material and trickles down against a counter-current of air which is either pumped through the bed or drawn up by the heat of reaction within it. The packing material normally consists of some lignocellulosic material such as birch twigs, vine twigs, rattan, wood wool, or sugarcane bagasse, although other materials such as coke have also been used. The vinegar stock is collected in a sump at the bottom of the vat and recirculated until the desired level of acidity is reached. The faster rate of reaction achieved means that the wash heats up during passage through the bed and, depending on the size of the fermenter, some cooling may be required.

The process is operated semicontinuously to maintain a high level of acidity throughout, and most of the biomass is retained within the packed bed. A well operated quick vinegar process fitted with temperature control and forced aeration can usually acetify a vinegar stock with a GK of 10 and an initial ethanol content of 3% in 4–5 days.

The fastest rates of acetification are achieved using submerged acetification in which acetic acid bacteria grow suspended in a medium which is oxygenated by sparging with air. The most commercially successful technique to have been developed is the Frings Acetator (Figure 9.16) which uses a patented self-priming aerator to achieve very efficient oxygen transfer.

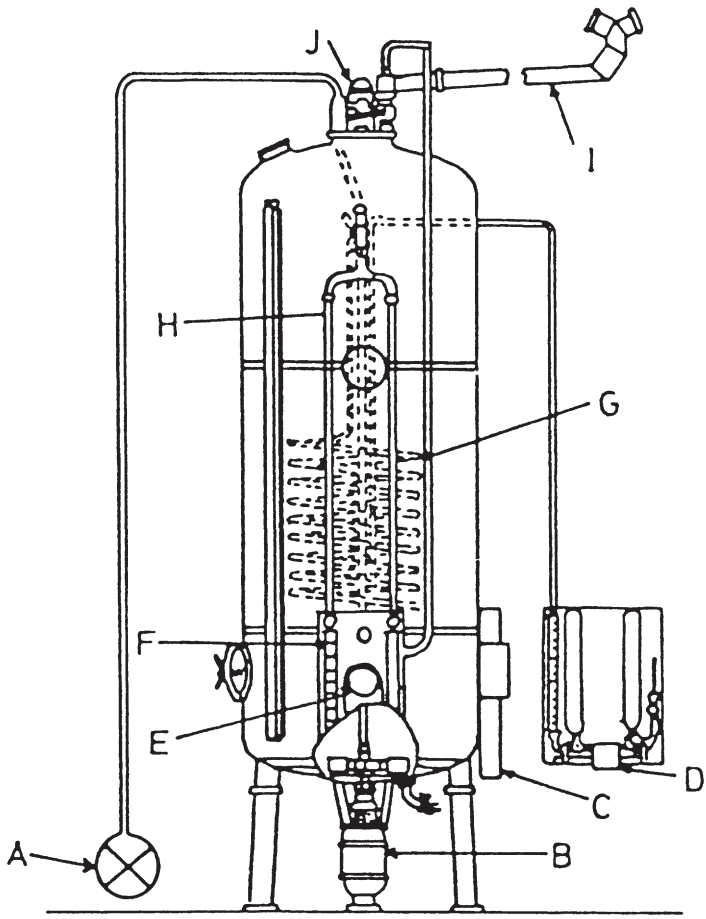


Figure 9.16 *The Frings Acetator. A, charging pump; B, aerator and motor; C, alkograph; D, cooling water value; E, thermostat controlling D; F, rotameter; G, cooling coil; H, air line; I, air exhaust line; J, defoamer*
Reproducted by kind permission of Heinrich Frings GmbH, Bonn

Submerged culture is very efficient and rapid, a semicontinuous run normally takes 24–48 h. It does however require far more careful control than simpler processes. The acetic acid bacteria are very susceptible to interruptions to the air supply, indicating that, in order to survive suspended in a medium with a pH of 2.5 and 10–14% acidity, the bacteria need a constant supply of energy from respiration. A stoppage of only one minute in a stock with a GK of 11.35 is enough to completely arrest acetification which will not resume when aeration is resumed.

Another possible cause of fermentation failure in submerged acetification is phage infection. The presence of bacteriophage particles has been demonstrated in disturbed vinegar fermentations both in submerged acetifiers and the quick vinegar process. The performance of

quick vinegar generators appears to be less affected as their acetification rate may slow but rarely stops. This is probably due to the greater heterogeneity of the culture present which allows organisms of different phage susceptibility to take over in the event of phage attack.

Where legal definitions of vinegar exist, it is specified as a fermentation product. 'Artificial vinegars' made by diluting and colouring acetic acid are thus excluded and, in the UK, have to be known rather laboriously as 'non-brewed condiment'. Although vinegar can be made up to 14% acidity, it is usually diluted down to an appropriate strength for bottling. The minimum acetic acid content is usually prescribed to be something between 4 and 6% w/v, but higher strength vinegars are available for pickling.

Though most often thought of in terms of its use as a condiment, vinegar is an important food ingredient. It is used as a preservative and flavouring agent in a large and expanding range of products such as mayonnaise, ketchups, sauces and pickles. In the United States only about 30% of the vinegar produced is sold as table vinegar, the rest being used in food processing.

The antimicrobial action of organic acids such as acetic acid has already been discussed (see Section 3.2.2) and the use of vinegar in a formulated product usually restricts the spoilage microflora to yeasts, moulds and lactobacilli. Vinegar preserves were one of the earliest areas where predictive models were developed (see Chapter 3). Work at what became the Leatherhead Food Research Association indicated that to achieve satisfactory preservation of a pickle or sauce a minimum of 3.6% acetic acid, calculated as a percentage of the volatile constituents, is necessary. That is to say:

$$\%\ \text{acetic acid on whole product} = 0.036 \times \%\ \text{volatile constituents} \qquad (9.3)$$

A different formula has been described specifically for sweet cucumber pickles:

$$\%\ \text{acetic acid on whole product} = (80 - \text{S})/20 \qquad (9.4)$$

where S is the % sucrose on the whole product.

More elaborate formulae have been produced which apply to emulsified and non-emulsified sauces. These are based largely on work conducted at the laboratories of Unilever in the Netherlands and are known as the CIMSCEE code, after the French acronym of the European Sauces Trade Association. The code consists of two formulae; one to determine the potential for spoilage by acetic acid tolerant yeasts, moulds and LAB, and another derived from inactivation rates of salmonella to assess microbiological safety. Each contains terms for salt, sugar, and acetic acid content and pH. If, when the relevant values are

substituted in the formulae, the result is higher than a specified value then this indicates that the product would be microbiologically stable or safe depending on the formula used.
The formula for safety is:

$$15.75(1-\alpha)(\%\text{ total acetic acid}) + 3.08(\%\text{ salt}) + (\%\text{ hexose}) + 0.5(\%\text{ disaccharide}) + 40(4.0 - \text{pH}) = \sum\nolimits_{s} \quad (9.5)$$

Where $(1-\alpha)$ is the proportion of undissociated acetic acid, α is the proportion dissociated, given by the expression:

$$pH = pK + \log[\alpha/(1-\alpha)] \quad (9.6)$$

where $pK = 4.76$.

Any sauce based on acetic acid with $\sum_s > 63$ is regarded as intrinsically safe, since viable numbers of *E. coli* in it will decline by more than 3 log cycles in less than 72 h at 20 °C. The formula for stability is:

$$15.75(1-\alpha)(\%\text{ total acetic acid}) + 3.08(\%\text{ salt}) + (\%\text{ hexose}) + 0.5(\%\text{ disaccharide}) = \sum \quad (9.7)$$

If $\sum > 63$, any sauce with this formulation should be microbiologically stable without refrigeration, even after opening.

The mould *Moniliella acetoabutans*, the micro-organism most resistant to acetic acid, would still grow in such products but can be controlled by good hygienic practices and pasteurization of ingredients containing vinegar. Experience in the pickle and sauce industry indicates that spoilage by *Moniliella* is rare.

Products meeting the CIMSCEE requirement for stability would have a relatively strong taste of acid or salt. If levels of these ingredients are reduced to produce a milder taste then some supplementary preservative measure would be necessary such as sorbate, a final pasteurization step or refrigerated storage.

9.12 MOULD FERMENTATIONS

Mould fermentations are an aspect of fermented foods that, up until now, we have mentioned only briefly. Failure to remedy this neglect would give a seriously unbalanced view for, in the East particularly, moulds play a key role in a number of food fermentations.

9.12.1 Tempeh

Tempeh is a traditional mould-fermented food in Indonesia, though it has also attracted interest in the Netherlands and United States. The most

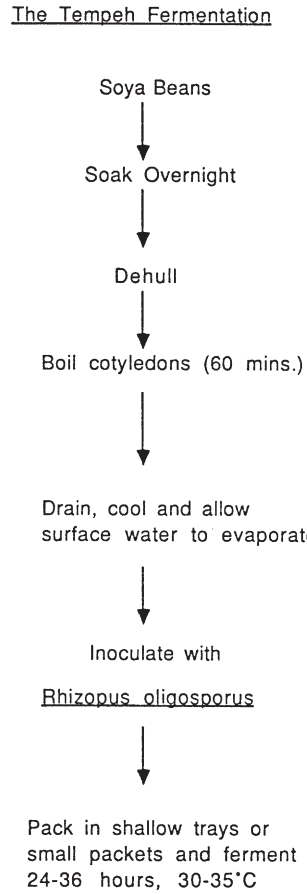


Figure 9.17 *Tempeh production*

popular type of tempeh is produced from soya beans and is also known as tempeh kedele. The process of tempeh production is outlined in Figure 9.17. Whole clean soya beans are soaked overnight in water to hydrate the beans. A bacterial fermentation occurs during this stage decreasing the pH to 4.5–5.3. The hydrated beans are dehulled and the moist cotyledons cooked; a process which pasteurizes the substrate, destroys the trypsin inhibitor and lectins contained in the bean and releases some of the nutrients required for fungal growth. After cooking, the beans are drained and may be pressed lightly to remove excess moisture before spreading into shallow bamboo trays and allowing to cool. Starter culture is added either by mixing some tempeh in with the cooked soya beans prior to packing in the trays or by sprinkling a spore inoculum, prepared by extended incubation of a piece of tempeh, on to the beans.

The fermentation is invariably a mixed culture of moulds, yeasts and bacteria but the most important component appears to be *Rhizopus*

oligosporus, although other *Rhizopus* and *Mucor* species are often isolated. Over two days incubation at ambient temperature (30–35 °C), the mycelium develops throughout the mass of beans knitting it together. During fermentation the pH rises to around 7, fungal proteases increase the free amino acid content of the product and lipases hydrolyse over a third of the neutral fat present to free fatty acids.

Unlike many fermented foods, tempeh production is not a means of improving the shelf-life of its raw material which is in any case inherently quite stable. Tempeh contains antioxidants which retard the development of rancidity but will keep for only one to two days as sporulation of the mould discolours the product and a rich ammoniacal odour develops as proteolysis proceeds.

Tempeh production does however improve the acceptability of an otherwise rather unappealing food. Fresh tempeh has a pleasant nutty odour and flavour and can be consumed in a variety of ways, usually after frying in oil.

In addition to improving acceptability, fermentation also improves the nutritional quality of soya beans. In part this stems from the reduction or removal of various anti-nutritional factors at different stages in the processing. Destruction of the trypsin inhibitor and lectins during cooking of the beans has already been mentioned and levels of phytic acid, which can interfere with mineral nutrition, are also reduced by about a third in the course of processing. The notorious ability of beans to produce flatulence is also regarded as an anti-nutritional property and flatulence-inducing oligosaccharides such as stachyose and raffinose are partially leached out of the beans during the soaking stage.

Despite the extensive proteolytic changes which occur during fermentation, studies have failed to show that the protein in tempeh is more easily digested. With the exception of thiamine which decreases, other vitamins increase to varying degrees during fermentation. Vitamin B_{12}, the anti-pernicious anaemia factor, shows the most marked increase and this is associated with the growth of the bacterium *Klebsiella pneumoniae* during fermentation. The usual source of this vitamin in the diet is animal products and it has been suggested that tempeh could be an important source of B_{12} for people subsisting on a largely vegetarian diet.

Tempeh can be made from a number of different plant materials including other legumes, cereals and agricultural by-products. One variety that has achieved some notoriety is tempeh bongkrek which is made in central Java using the presscake remaining after extraction of coconut oil. Tempeh bongkrek has been associated with occasional serious outbreaks of food poisoning due to the bacterium *Burkholderia cocovenenans* growing in the product and elaborating the toxins bongkrekic acid and

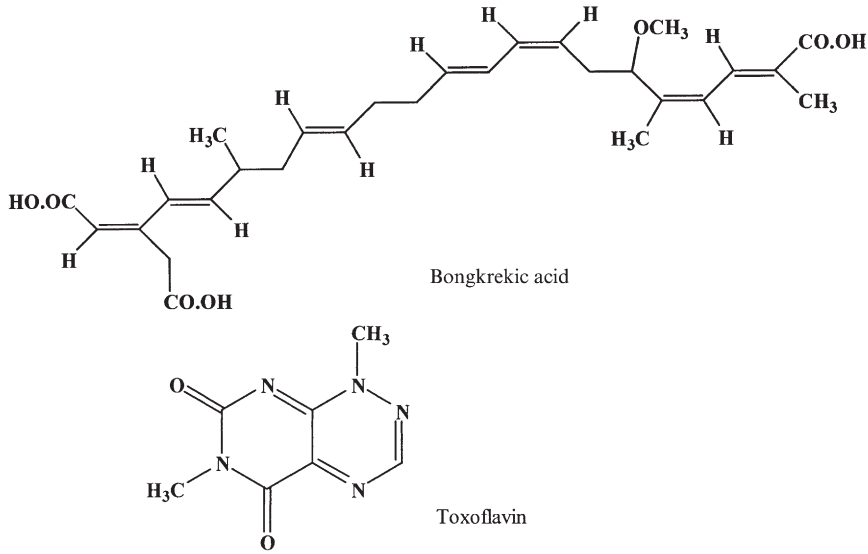


Figure 9.18 *Toxoflavin and bongkrekic acid*

toxoflavin (Figure 9.18). Since 1951, at least 1000 people are known to have died as a result of this intoxication and in 1988 the Indonesian Government prohibited the production of tempeh bongkrek.

Two factors are thought to give rise to this problem. Reduction or omission of an initial soaking of the presscake may fail to give a lactic fermentation sufficient to reduce the pH below 6, a level at which the bacterium cannot grow. Also, the fungal inoculum may be too small since it has been shown that *B. cocovenenans* cannot grow if *Rhizopus oligosporus* has more than a tenfold numerical superiority (estimated by plate counts).

Ontjom is a tempeh-like product produced in Indonesia from peanut presscake which normally has a fruity/mincemeat character. It can be produced using the tempeh mould but *Neurospora intermedia* is also often used. This mould has strong α-galactosidase activity which can further contribute to the reduction of flatulence-inducing oligosaccharides.

9.12.2 Soy Sauce and Rice Wine

Though they are markedly different in character, rice wine and soy sauce share sufficient common features in their production to warrant discussing them together. Both are representatives of products which involve mould activity in a two-stage fermentation process. The mould starter used is often known as *koji*, a Japanese term derived from the Chinese character for mouldy grains. In the *koji* stage, aerobic conditions allow moulds to grow on the substrate producing a range of hydrolytic

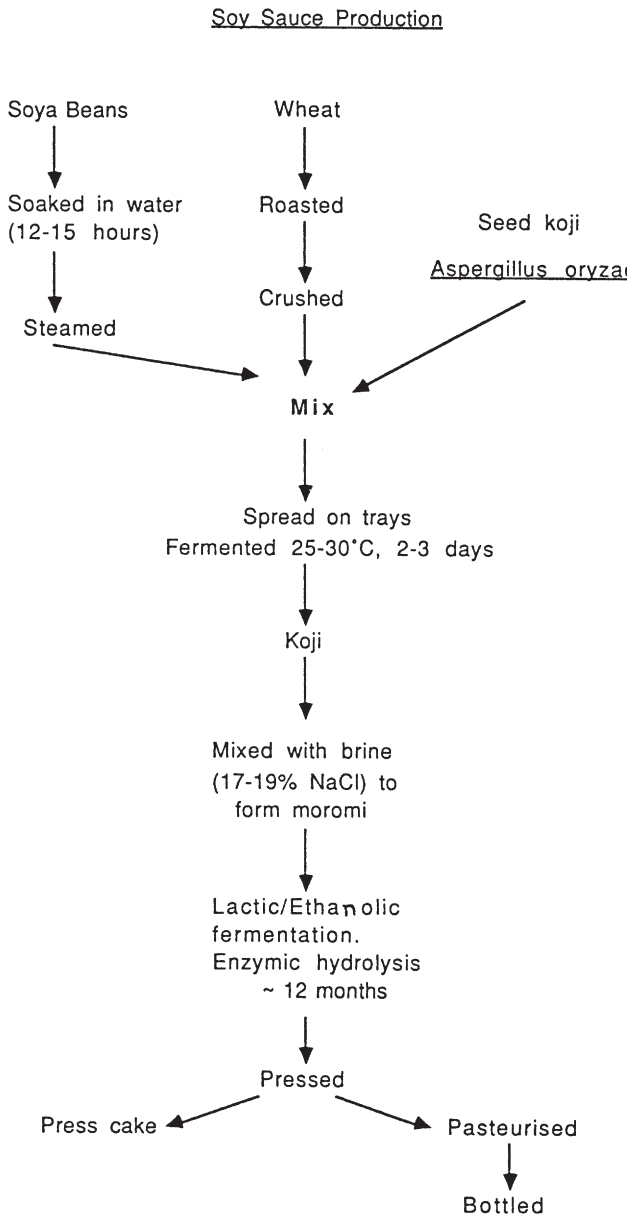


Figure 9.19 *Soy sauce production*

enzymes necessary for utilizing the macromolecular material present. In the case of soy sauce (Figure 9.19), soaked and cooked soya beans are mixed with roasted cracked wheat in about equal proportions and inoculated with *tane koji* or seed *koji*. This has been previously grown-up on a similar mixture of substrates and contains a mixture of strains of

Aspergillus oryzae. The moulds are then allowed to grow throughout the mass of material spread as layers about 5 cm deep for 2–3 days at 25–30 °C.

In the second, mash or *moromi* stage, conditions are made anaerobic so no further mould growth can occur. In soy sauce production, this is achieved by mixing the *koji* with an approximately equal volume of brine to give a final salt concentration of 17–20%. Although the moulds can no longer grow, the activity of a whole battery of hydrolytic enzymes continues breaking down proteins, polysaccharides and nucleic acids to produce a liquid rich in soluble nutrients. Yeasts and lactic acid bacteria dominate the microflora producing a number of flavour components and converting roughly half of the soluble sugars to lactic acid and ethanol so that the final soy sauce normally has a pH of 4.5–4.9 and ethanol and lactic acid contents of 2–3% and 1% respectively. The halophilic lactic acid bacterium *Tetragenococcus halophilus* (formerly *Pediococcus halophilus*) and the yeasts *Zygosaccharomyces rouxii* and *Torulopsis* have been identified as being important in this stage.

The *moromi* stage can be quite protracted, lasting up to a year or more, at the end of which the mash is pressed to remove the solid residues which may then be mixed with brine to undergo a second fermentation and produce a lower grade product. The liquid is pasteurized and filtered, possibly after a period of maturation, and then bottled.

Rather similar steps are involved in the production of soya bean pastes known as *miso* in Japan and *chiang* in China. These include up to 40% of a grain such as rice or barley, use dry salt rather than brine, and employ a shorter fermentation so the product has the consistency of a paste rather than a liquid.

In the brewing of the Japanese rice wine, *sake*, a *koji* prepared on steamed rice is used. Although the mould used is the same species as in soy sauce production, *Aspergillus oryzae*, the strains used in *sake* production are particularly noted for their ability to produce amylolytic enzymes. In the *moromi stage*, water is added along with strains of the yeast *Saccharomyces cerevisiae* specially adapted to the *sake* fermentation. During this stage amylolytic enzymes from the mould continue to break down the starch in the rice to produce fermentable sugars which are then converted to ethanol by the yeast. The high alcohol content of around 20% v/v achieved in such fermentations is thought to be due to a combination of factors. Particularly important is the slow rate of fermentation which results from the relatively low fermentation temperature (13–18 °C) and the slow release of fermentable sugars. The high solids content in the *moromi* is also thought to help in keeping the

Table 9.11 *Amylolytic mould preparations*

koji	Japan
look pang	Thailand
ang-kak	Philippines
ragi-tapai	Malaysia/Indonesia
nuruk	Korea
peh-yueh	China
bakhar	N. India

yeast in suspension and active at such high alcohol concentrations. At the end of fermentation which typically lasts for three weeks, the product is settled, filtered and blended before being pasteurized and bottled.

A number of other rice-based mould starters are used in the countries of East and Southeast Asia to fulfil a similar role to *koji* (Table 9.11). They are used to produce sweetened rice products which can be consumed fresh or added to other products (see Section 9.9) or can be used as a base for the production of rice wine and rice vinegar. The microbiological composition of these generally differs from that of *koji* and comprises primarily *Rhizopus* and *Mucor* species and amylolytic yeasts.

9.12.3 Mycoprotein

Products such as *tempeh* and *koji* contain a significant amount of mould biomass and a reasonable extension of this type of approach would be to grow up mycelium itself as a source of food. Of the many investigations into the growth of moulds on readily available substrates one has successfully emerged as a commercial product. Mycoprotein, marketed as Quorn, is essentially the mycelium of *Fusarium venenatum* (formerly *F. graminearum*) grown in continuous culture in a medium containing glucose, ammonium salts and a few growth factors. Advantages associated with the use of a filamentous organism are that it can be harvested by filtration and washing and can be readily textured to give the product an acceptable mouth-feel.

To be acceptable as a food for human consumption it is necessary to reduce the level of RNA, which is nearly 10% based on mycelial dry weight, to below the levels likely to lead to kidney-stone formation or gout. This is achieved by a mild heat treatment prior to filtration which activates the mould's RNAases and leads to a dramatic reduction of RNA to about 1% which is acceptable. The product has a useful protein content of 44% and is high in 'fibre' because of the cell walls of the filamentous fungal structure.

9.13 CONCLUSION

Here we have described a limited selection of fermented foods which we believe illustrates their diversity and importance as well as some of their general microbiological features. There is a large and growing literature on this topic and details of others among the plethora of fermented foods produced can be found in some of the references recommended as further reading in Chapter12.