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Review

Preservative agents in foods
Mode of action and microbial resistance mechanisms

S. Brul[a,*], P. Coote[b]

[a]*Unilever Research Division, Foods laboratories, Vlaardingen, The Netherlands*
[b]*Unilever (Colworth, UK), p/a P.O. Box 114, 3130 AC Vlaardingen, The Netherlands*

Abstract

Preservative agents are required to ensure that manufactured foods remain safe and unspoiled. In this review, we will discuss the mode of action of both chemical and biological (nature-derived) preservatives and the stress response mechanisms induced by these compounds in microorganisms of concern to the food industry. We will discuss the challenges that food manufacturers face with respect to the assurance of food safety and the prevention of spoilage. Following this, chemical preservatives will be discussed, in particular, weak organic acids such as sorbic and benzoic acid which are widely used in preservation. Furthermore, the mechanisms of microbial inactivation with hydrogen peroxide mediated systems and chelators such as citric acid and EDTA and their potential use in preservation will be covered. We will then address the potential of naturally occurring ''preservatives''. Of the antimicrobial compounds present in nature, first to be discussed will be the nonproteinaceous compounds often present in herbs and spices and we will speculate on the stress response(s) that microorganisms may elicit to these natural compounds. Next to be addressed will be compounds that attack cell walls and membranes, for example, peptides, proteins and lytic enzymes. In discussing the resistance mechanisms against membrane and wall perturbation, the extensive knowledge of stress responses against osmotic stress and temperature stress will be refered to. Finally, in the concluding paragraphs, options for combination preservation systems are evaluated.

Keywords: Microorganisms; Bacteria; Fungi; Safety; Spoilage; Toxicity; Hygiene; Natural antifungals; Synergy; Resistance; Inactivation; Lag-time; Modelling

1. Scope of the review

In the production of food it is crucial that proper measures are taken to ensure the safety and stability of the product during its whole shelf-life. In particular, modern consumer trends and food legislation have made the successful attainment of this objective much more of a challenge to the food industry. Firstly, consumers require more high quality, preservative-free, safe but mildly processed foods with extended shelf-life. For example, this may mean that foods have to be preserved at higher pH values and have to be treated at mild-pasteurisation rather than sterilisation temperatures. As acidity and sterilisation

*Corresponding author. Exploratory Research Foods Group, Unilever Research Laboratory Vlaardingen, Olivier van Noortlaan 120, 3133 AT Vlaardingen, South-Holland, The Netherlands. Tel.: +31-10-460-5161; fax: +31-10-460-5188.

E-mail address: stanley.brul@unilever.com (S. Brul)

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treatments are two crucial factors in the control of outgrowth of pathogenic spore-forming bacteria, such as *Clostridium botulinum*, addressing this consumer need calls for innovative approaches to ensure preservation of products (Gould, 1995; Schellekens, 1996; Peck, 1997). Secondly, legislation has restricted the use and permitted levels of some currently accepted preservatives in different foods. This has created problems for the industry because the susceptibility of some microorganisms to most currently used preservatives is falling. For example, recent work has identified that resistance to weak acid preservatives in spoilage yeast is mediated by a multidrug resistance protein (Piper et al., 1998).

In this review, we will compile the data available on the commonly used chemical preservatives and subsequently discuss emerging alternatives. We will present the current understanding of the mode of action of these compounds and the possibilities for microbial resistance development. This will lead the discussion of the options that are now emerging for new preservation regimes where combinations of various sublethal treatments provide the required protection of the food and consumer against pathogenic or spoilage organisms. In this review, we will refer to microbial stress responses against common physico–chemical preservation factors such as heat, water activity (a_w), and pH without detailed discussion of how these factors may be used in their own right in preservation strategies. The reader is referred to extensive reviews on the molecular aspects of these topics by Kultz and Burg (1998) and Banuett (1998), and for original scientific papers on the more applied aspects by McMeekin et al. (1997) and Buncic and Avery (1998).

An extensive discussion of physical alternatives to heat treatments falls outside the scope of this paper, but occasionally we will touch upon a few options (see also Wouters and Smelt, 1997; Raso et al., 1998; Ritz et al., 1998; Smelt, 1998; Calderón-Miranda et al., 1999).

2. Chemical preservative agents

2.1. Weak-organic acids

2.1.1. Mode of action

The most common classical preservative agents are the weak organic acids, for example acetic, lactic, benzoic and sorbic acid. These molecules inhibit the outgrowth of both bacterial and fungal cells and sorbic acid is also reported to inhibit the germination and outgrowth of bacterial spores (Sofos and Busta, 1981; Blocher and Busta, 1985).

In solution, weak acid preservatives exist in a pH-dependent equilibrium between the undissociated and dissociated state. Preservatives have optimal inhibitory activity at low pH because this favours the uncharged, undissociated state of the molecule which is freely permeable across the plasma membrane and is thus able to enter the cell. Therefore, the inhibitory action is classically believed to be due to the compound crossing the plasma membrane in the undissociated state. Subsequently, upon encountering the higher pH inside the cell, the molecule will dissociate resulting in the release of charged anions and protons which cannot cross the plasma membrane. In conclusion, the preservative molecule diffuses into the cell until equilibrium is reached in accordance with the pH gradient across the membrane resulting in the accumulation of anions and protons inside the cell (Booth and Kroll, 1989). Therefore, inhibition of growth by weak acid preservatives has been proposed to be due to a number of actions including, membrane disruption (Freese et al., 1973 and more recently, Stratford and Anslow, 1998; Bracey et al., 1998), inhibition of essential metabolic reactions (Krebs et al., 1983), stress on intracellular pH homeostasis (Salmond et al., 1984; Cole and Keenan, 1987; Bracey et al., 1998) and the accumulation of toxic anions (Eklund, 1985). In yeasts, it has also been proposed that the actual inhibitory action of weak acid preservatives could be due to the induction of an energetically expensive stress response that attempts to restore homeostasis and results in the reduction of available energy pools for growth and other essential metabolic functions (Holyoak et al., 1996; Bracey et al., 1998).

2.1.2. Resistance mechanisms

Microbial resistance to weak organic acids can involve various mechanisms. For bacteria, significant knowledge exists on their intrinsic, noninducible resistance mechanisms against these compounds (reviewed in Russel, 1991). Gram-positive bacteria do not possess an outer membrane and the exclusion limit of the cell wall of vegetative cells of *Bacillus megaterium* has even been calculated to be as high as 30 000 D (Lambert, 1983). Hence preservatives

can easily enter these cells and their intrinsic resistance is relatively low. In gram-negative bacteria, resistance mechanisms are more complicated since these organisms possess an inner and an outer membrane. The latter membrane has a clear role in modulating the accessibility of a cell to preservatives and other small molecules (Nikaido and Vaara, 1985; Vaara, 1992; Helander et al., 1997); the lipopolysaccharide layer is of crucial importance in this respect (Kabara, 1983 and for a more recent update Helander et al., 1996). In some cases microorganisms are able to degrade the added preservatives by making use of specific enzymes. An example of this is the degradation of methyl *para*(4)-hydroxybenzoate by *Pseudomonas aeruginosa* (Hugo and Foster, 1964). Recently, inducible resistance mechanisms in microorganisms have been more extensively studied. For *Salmonella typhimurium*, it is known that cells encounter many potential stress factors in their ''natural'' habitat, e.g., the extremely low pH in the stomach or the presence of large amounts of partially hydrophobic weak organic acids in the intestine. Indeed, this organism is known to possess an acid tolerance response which consists of a complex defence system that allows cells to survive pH values as low as pH 3 (Park et al., 1996). Interestingly enough, this stress response also conveys cellular resistance against the weak acids butyric, acetic and propionic acid (Baik et al., 1996). Furthermore, in a recent study with *Escherichia coli* O157:H7, resistance towards benzoic acid was observed upon induction of an acid tolerance response with a strong acid at pH 2.0 (Lin et al., 1996).

Also, certain gram-positive bacteria, e.g., *Listeria monocytogenes*, are known to induce an acid tolerance response at a challenge pH of 3 after prior exposure to a mild-acid at pH 5.0 (Davis et al., 1996). No data are currently available on whether this response gives protection to weak organic acids commonly used in the food industry.

In fungi, similar resistance mechanisms may be found. The enzymatic degradation of sorbic acid to pentadien by certain fungal species is well documented (Samson et al., 1995). The resistance of spoilage yeasts to weak organic acid preservatives has been extensively studied and is known to depend on the H^+-pumping P-type membrane ATPase (Holyoak et al., 1996). Studies by Kubo and Lee (1998) have shown that compounds that inhibit the plasma membrane H^+-ATPase synergistically enhance the activity of sorbic acid. Work by Piper et al. (1997) showed that the long term stress response of yeasts to weak organic acids also involves the induction of an integral membrane protein, Hsp30, which downregulates the increased activity of the membrane ATPase. It was deduced from these observations that Hsp30 was acting as a molecular ''switch'' downregulating the activity of the membrane ATPase in order to conserve cellular energy pools which would otherwise be consumed by the enzyme attempting to restore homeostasis (Braley and Piper, 1997). Studies by Henriques et al. (1997) have shown that *Saccharomyces cerevisiae* is able to actively extrude [C-14] labelled benzoic acid, suggesting that there is an efflux system, presumably membrane localised, that removes accumulated anions from inside the cell. Supporting this, data gathered by Piper et al. (1998) has demonstrated the existence of a multidrug resistance pump, the ATP binding cassette transporter, Pdr12, that actively extrudes preservative anions from the cell. Our current understanding of the mechanisms that are involved in weak acid resistance in yeasts are summarised in Fig. 1. As indicated on the diagram, simply pumping preservative anions out of the cell could create a futile cycle where the anions reassociate at the lower external pH and reenter the cell. However, this assumes that any rate of diffusion across the plasma membrane remains the same and that the cell makes no effort to alter membrane composition or structure to reduce the access of the toxic compound. In fact recent studies by Loureiro-Dias reported at the 19th International Specialised Symposium on Yeast (1998, Braga, Portugal) have shown that adapted yeasts reduce the diffusion coefficient of preservatives across the plasma membrane such that passage of weak acids into the cell is reduced. Therefore, efflux of protons and anions by the H^+-ATPase and Pdr12 respectively would not create a futile cycle if there is a concurrent reduction in the ability of the compounds to diffuse across the cell membrane and enter the cytosol.

2.2. Hydrogen peroxide

2.2.1. Mode of action and use

The lactoperoxidase system, found in milk, has profound antimicrobial effects against both bacteria and fungi (discussed in Reiter and Harnulv, 1984; Russel, 1991; de Wit and vanHooydonk, 1996). The

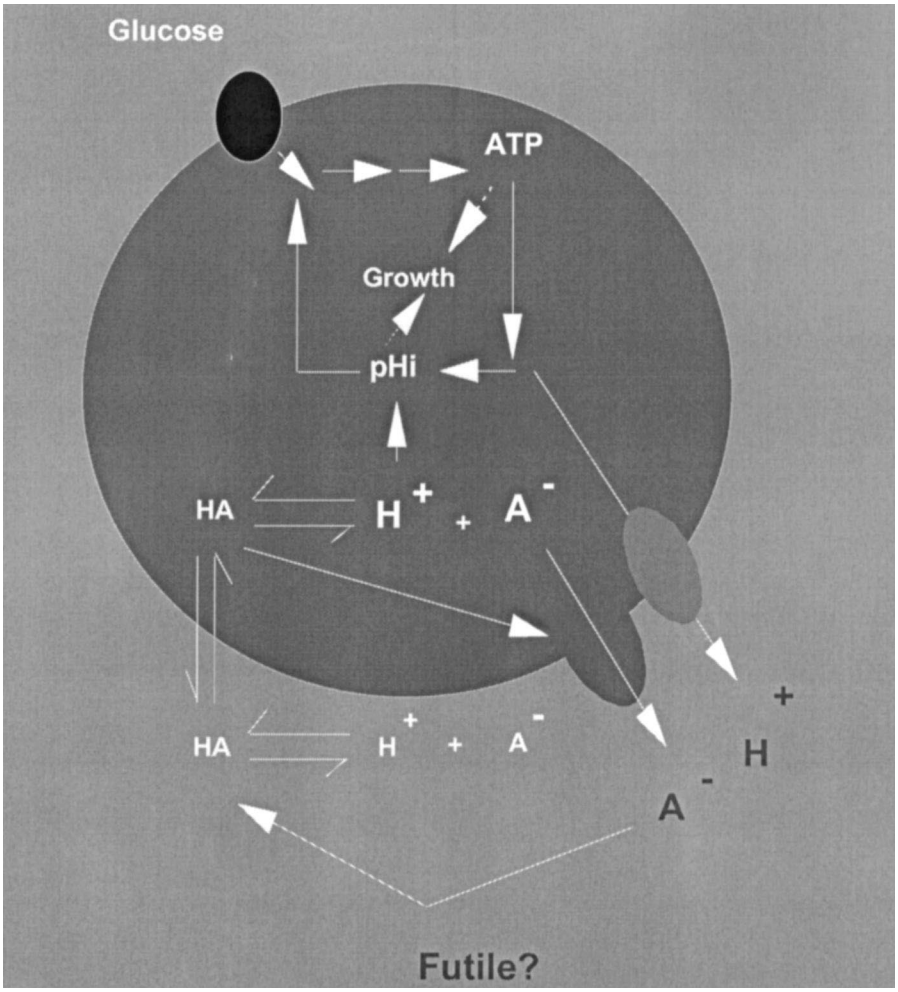

Fig. 1. A schematic diagram of the stress response of a yeast cell challenged with weak organic acids (Piper et al., 1998). Shown are, a glucose transporter, the membrane located Pdr12 multidrug resistance pump active against anions of acetic, sorbic and benzoic acid, and the plasma membrane P-type H^+-ATPase.

system requires hydrogen peroxide and thiocyanate for optimal activity and is thus primarily active against microorganisms producing H_2O_2. Alternatively, hydrogen peroxide can be added to the foods that are to be preserved. Under suitable experimental conditions, the reaction generates a short lived singlet oxygen species which is extremely biocidal (for recent studies see Tatsozawa et al., 1998). Furthermore, during incomplete reduction of molecular oxygen, the superoxide radical is generated. H_2O_2 may lead together with the superoxide radical and trace amounts of transition metal ions [e.g., Fe(II)] in the so called Fenton reaction to the formation of the extremely biocidal hydroxyl radical (see, e.g., Luo et al., 1994). A wide range of both gram-negative bacteria (Borch et al., 1989; Wray and McLaren, 1987) and gram-positive bacteria (Oram and Reiter, 1966; Siragusa and Johnson, 1989) are inhibited by the lactoperoxidase system. However, studies have shown that gram-negative bacteria were generally found to be more sensitive to lactoperoxidase mediated (food) preservation than gram-positive species (see Marshal and Reoter, 1980; de Wit and van-Hooydonk, 1996).

Hydrogen peroxide on its own is also known to be bactericidal depending on the concentrations applied

and on environmental factors such as pH and temperature (see Cords and Dychdala, 1993; Juven and Pierson, 1996). Temperature is also an extremely important parameter in determining the sporicidal efficacy of hydrogen peroxide. H_2O_2 was found to be weakly sporicidal at room temperatures but very potent at higher temperatures (Toledo, 1975; see also Block, 1991). While the mechanism by which hydrogen peroxide kills spores is not known, killing of vegetative bacteria (Ananthaswamy and Eisenstark, 1977; Imlay and Linn, 1988) and fungi (Frankenberg et al., 1993) is known to involve DNA damage.

In the USA, the regulatory authorities allow the direct addition of hydrogen peroxide to food products such as raw milk for the preparation of certain cheese variants; whey intended for use in modified whey preparation; corn starch and dried eggs; and finally, for the decontamination of packaging materials (reviewed in Toledo, 1975; Busta and Foegeding, 1983; Russel, 1991; Juven and Pierson, 1996). Several other procedures where H_2O_2 is used as a preservative have been reported, such as fruit and vegetable disinfection (Falik et al., 1994) and raisin decontamination (Simmons et al., 1995).

2.2.2. Resistance mechanisms

Bacteria and fungi protect themselves against hydrogen peroxide in various ways. Some of the most ubiquitous systems include glutathione with associated enzymes and catalase (de Wit and van-Hooydonk, 1996). Indeed when yeast cells are treated with low concentrations of hydrogen peroxide a number of adaptive systems are activated which will protect against a subsequent stress with higher H_2O_2 concentrations. Table 1 summarises the primary antioxidant defences of yeasts (Moradas-Ferreira et al., 1996). Interestingly, Li et al. (1998) recently demonstrated for the first time that the signal transduction pathways that mediate the oxidative stress response and the osmostress response communicate via the Sln1 protein, a putative membrane receptor (see also Krems et al., 1995).

While yeasts have a whole array of defence responses against oxidative stress (Table 1), most bacteria rely on the action of catalase to degrade toxic levels of hydrogen peroxide. However, damage may still occur because H_2O_2 has a very high diffusion rate into the cell and endogenous catalase in *E. coli* cells is not active enough to protect individual cells against the toxic compound. However, in high density populations, catalase positive cells produce enough enzyme to protect most of the population against killing by hydrogen peroxide (Ma and Eaton, 1992).

The resistance of bacterial spores to the killing action of hydrogen peroxide basically stems from the presence of the small, acid-soluble proteins of the α/β type that are synthesised in the developing spore

Table 1
The primary antioxidant defences of yeasts (essentially taken from Moradas-Ferreira et al., 1996)

Gene (product)	Function
SOD1 (CuZnSOD; cytoplasmic) SOD2 (MnSOD; mitochondrial)	Dismutation of superoxide anion
CTA1 (catalase A; peroxisomal) CTT1 (catalase T; cytoplasmic)	Decomposition of hydrogen peroxide
CCP (cytochrome *c* peroxidase)	Reduction of hydrogen peroxide
GSH (γ-glutamyl-cysteine synthetase)	Production of glutathione which is key to reduction of protein disulfide, scavenging of free radicals, etc.
GLR1 (glutathione reductase)	Reduction of oxidised glutathione
ZWF (glucose-6-phosphate dehydrogenase)	Reduction of $NADP^+$ to NADPH
CUP1 (metallothionein)	Binding of Cu, prevention of the Fenton reaction; scavenging superoxide and hydroxyl radicals
TRX2 (thioredoxin) TPX (thioredoxin peroxidase)	Reduction of protein disulphide reduction of hydrogen peroxide and alkyl hydroperoxides
SPE2 (S-adenosylmethionine decarboxylase)	Mediates polyamine biosynthesis which protects lipids from oxidation

and protect the DNA in the dormant spore against damage (Setlow, 1992; Bagyan et al., 1998). Spore coats of *Bacillus* are generally not implicated in H_2O_2 resistance (Setlow and Setlow, 1993), while they are known to be important, for example in *Clostridium bifermentans* survival of hydrogen peroxide stress (Bayliss and Waites, 1976).

2.3. Chelators

Chelators that can be used as food additives include the naturally occurring acid, citric acid, and the disodium and calcium salts of ethylenediaminetetraacetic acid (EDTA) (Russel, 1991). EDTA is known to potentiate the effect of weak acid preservatives against gram-negative bacteria (see Hart, 1984), while citric acid inhibits growth of proteolytic *C. botulinum* due to its Ca^{2+} chelating activity (Graham and Lund, 1986).

Helander et al. (1997) discussed the role of chelators as permeabilising agents of the outer membrane of gram-negative bacteria. Indeed, exposure to citric acid is well known to potentiate the effect of monolaurin against gram-negative bacteria (Shibasaki and Kato, 1978).

With regard to fungi, Brul et al. (1997a) have shown that both EDTA and 1,10-*o*-phenanthroline inhibit yeast growth presumably through an inhibitory effect on the biogenesis of a normal cell wall. The chelators mediated this effect through zinc binding rather than through calcium chelation. Finally, chelators may play a role in resistance against H_2O_2 mediated killing. It is likely that reactive radicals are formed at cellular sites that are also accessible to the chelators (see Marquis et al., 1994).

3. Naturally occurring preservatives

3.1. Small organic biomolecules

3.1.1. Use and mode of action

Next to the weak organic acids, H_2O_2 and certain chelators, a few other antimicrobial compounds are permitted by the regulatory authorities for inclusion in foods. In fact, some of these compounds are naturally present in spices, for example, oregano, thyme, dill, clove and their essential oils. These compounds are generally hydrophobic and can have membrane perturbing or even membrane rupturing characteristics (see, e.g., Helander et al., 1998). However, the concentrations at which the latter occurs, and thus cell death is achieved, are way above tolerable taste thresholds. Hence, in practice, most of the above mentioned compounds are used at concentrations that may have only growth inhibitory effects on microorganisms.

An extensive overview of the antimicrobial (and antioxidant) properties of spices can be found in Hirasa and Takemasa (1998). An interesting account of the antimicrobial function of spices in food preparation is given by Billing and Sherman (1998) who state that the principal reason spices are used is to enhance palatability. However, the ultimate reason is most likely that spices help cleanse foods of unwanted microorganisms and thereby contribute to health. Indeed, recent work by Samy et al. (1998) identified extracts from Indian medicinal plants that showed antibacterial activity against gram-negative bacteria at levels of around 1 mM. Whatever the reason spices are used, it is clear that essential oils from plants such as basil, cumin, caraway and coriander have inhibitory effects on organisms such as, *Aeromonas hydrophila*, *Pseudomonas fluorescence* and *Staphylococcus aureus* (Wan et al., 1998; Fricke et al., 1998). Tests in real food products have been described by Hao et al. (1998) who looked at the efficacy of plant extracts in inhibiting *A. hydrophila* and *L. monocytogenes* in refrigerated, cooked poultry. Delaquis and Mazza (1995) described the antimicrobial properties of isothiocyanates derived from onion and garlic. Considerable amounts of allylisothiocyanates and methylisothiocyanates have been used in the agricultural industry (pesticides) while their direct application in foods is hampered by the intense sensory attributes of these compounds. For isothiocyanates, it has been hypothesised that their antimicrobial activity is related to inactivation of extracellular enzymes through oxidative cleavage of disulphide bonds. Also, the formation of the reactive thiocyanate radical was proposed to mediate the antimicrobial effect (see Delaquis and Mazza, 1995). Helander et al. (1998) tested the effect of carvacrol, (+)-carvone, thymol and *trans*-cinnamaldehyde on *E. coli* O157:H7 and *S. typhimurium*. They noted that carvacrol and thymol decreased the intracellular ATP content of *E. coli* cells while simultaneously the

extracellular ATP increased. This was taken to indicate a disruptive action of the compounds on the plasma membrane. In a study on quantitative structure activity relationships for the effect of benzoic acid, cinnamic acid and benzaldehydes, it was found that a lipophilicity parameter as measured by reversed-phase HPLC was a very significant term in describing the anti-*L. monocytogenes* activity of these compounds (Ramos-Nino et al., 1996). However, it should be noted that the behaviour in real foods can be dramatically different from in vitro model predictions. Ramos-Nino et al. (1996) showed that when the quantitative models described above were applied to foods with a high protein or lipid content their anti-*Listeria* predictive power turned out to be very poor.

3.1.2. Resistance mechanisms

Not much is known about the resistance mechanisms of microorganisms against these naturally occurring antimicrobial compounds. Most naturally occurring small organic antimicrobial biomolecules are hydrophobic and contain aromatic structures similar to the ones found in some solvents (e.g., toluene) and classical preservatives (e.g., benzoic acid). Fig. 2 shows some of the most ubiquitous naturally occurring small organic antimicrobial biomolecules. Recently Ramos et al. (1998) described

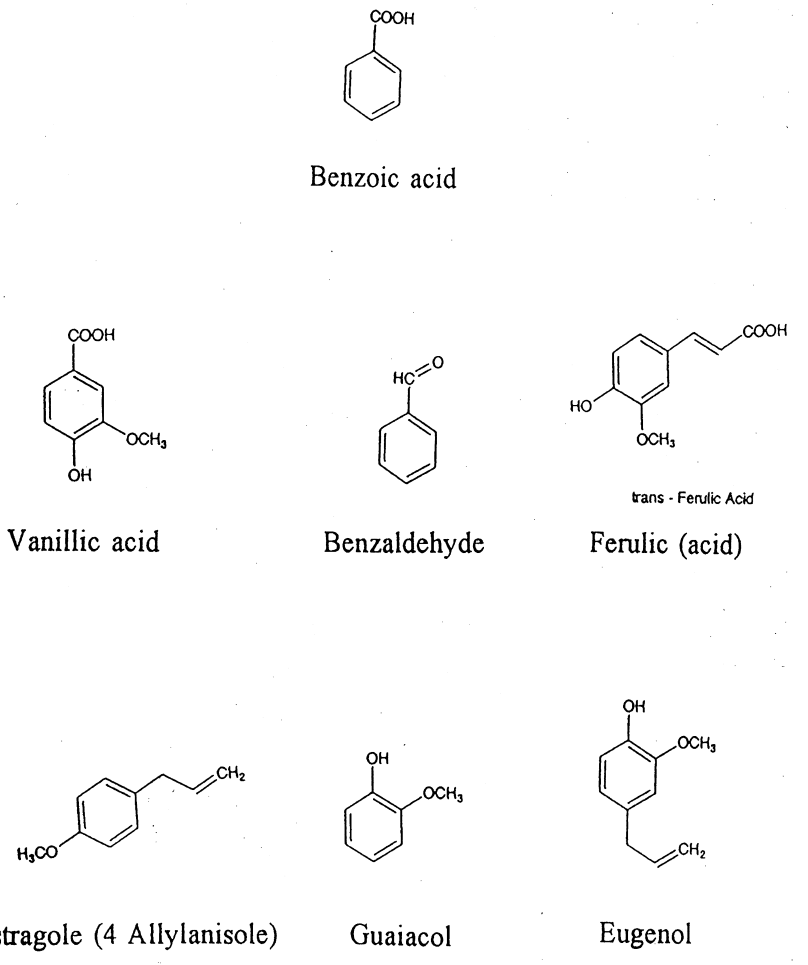

Fig. 2. The structural formula of some of the most ubiquitous naturally occurring small organic antimicrobial biomolecules as compared to the classical preservative benzoic acid.

efflux pumps in *Pseudomonas putida* that are involved in toluene tolerance but do not confer enhanced resistance to benzene. Indeed, in gram-negative bacteria, the known repertoire of multidrug resistance proteins includes pumps for neutral compounds (EmrAB) as well as pumps for amphipathic cations and anions (AcrAB) (Lomovskaya and Lewis, 1992; Ma et al., 1993; see also the recent review by de Bont, 1998). In the gram-positive bacterium *Staph. aureus* the NorA multidrug resistance pump is responsible for the extrusion of both synthetic amphipathic cationic compounds such as ethidium bromide, the antiseptics chlorhexidine and benzalkonium chloride and plant-derived antimicrobials such as berberine and palmatine (Hsieh et al., 1998).

3.2. Membrane perturbing proteins and peptides

3.2.1. Mode of action

Plants contain membrane perturbing proteins, termed defensins, that protect them from microbial infection (see Broekaert et al., 1995; Anzlovar et al., 1998; Segura et al., 1999). The inhibitory action of most naturally occurring antimicrobial proteins and peptides is thought to be due to membrane disruption. The exact mode of action of antimicrobial peptides from plants is not known. Some are structurally similar to lipid transfer proteins (Cammue et al., 1995; Nielsen et al., 1996). Many other peptides show significant homology to amphipathic antimicrobial peptides from insects and amphibians that are well known to have membrane lytic activity. The latter are potent inhibitors of fungal plant pathogens (see Cavallarin et al., 1998). From these observations, it is often speculated that the pore-forming capabilities of this class of peptides may be crucial for their mode of action (see Matsuzaki, 1998 for a recent account of this field of research).

The specificity of many of these peptides for microorganisms and not for the cells of higher eukaryotes is, in the case of prokaryotes, proposed to be due to the high content of anionic phospholipids such as phosphatidylglycerol, phosphatidylserine and phosphatidic acid present in bacterial membranes (reviewed in Matsuzaki, 1998).

The specificity of amphipathic antimicrobials peptides for fungal membranes may be due to the presence of ergosterol (De Lucca et al., 1998), while similar to the situation in bacteria, a transmembrane inside-negative potential facilitates the membrane perturbing action of the peptides (see Fleury et al., 1998). Recently, data gathered by de Nobel and coworkers showed that incubation of yeast cells with a (synthetic) amphipathic antimicrobial peptide is more lethal in cells that lack components of the PKC1 pathway, i.e., cells that do not respond normally to a hypoosmolarity stress induced membrane perturbation (de Nobel et al., unpublished observations). This corroborates the notion that in fungi as well, amphipathic antimicrobial peptides exhibit an effect on the plasma membrane.

The only antimicrobial peptides that are currently used as food preservatives are nisin and related compounds such as pediocin, which are secreted by lactic acid bacteria (Hansen, 1994; Montville and Chen, 1998).

3.2.2. Resistance mechanisms

Resistance development against antimicrobial proteins and peptides can occur at various levels. The first is secretion of proteolytic enzymes by the target microorganism. It has been speculated that resistance of *Bacillus* spp. to the bacteriocin nisin is mediated at least partially by the secretion of an enzyme that degrades this antibiotic (discussed in Hansen, 1994). Recent data have shown that the extracytoplasmic protease, OmpT, is key to the resistance development of *E. coli* against the antimicrobial peptide protamine (Stumpe et al., 1998). Also, fungi are known to secrete large amounts of proteases (Pavlukova et al., 1998) such that one can easily envisage similar mechanisms of peptide resistance as discussed above for bacteria.

Other mechanisms of resistance may include the prevention of uptake of the antimicrobial peptide by the cells. Dielbandhoesing et al. (1998) have shown that the cell wall proteins, Cwp1 and 2, in bakers yeast are involved in the constitutive resistance of *Sac. cerevisiae* against the antimicrobial action of nisin and a synthetic amphipathic antimicrobial peptide.

Finally, cells may alter their membrane composition as a response to the stress imposed upon exposure to a membrane active antimicrobial peptide. Indeed Verheul et al. (1997a) showed that a nisin resistant strain of *L. monocytogenes* Scott A showed a different phospholipid membrane com-

position compared to the nisin sensitive parent. The levels of diphosphatidylglycerol were markedly lower in the nisin resistant strain. Also, combinations of cell wall and membrane alterations may occur (Crandall and Montville, 1998). These authors observed that a nisin resistant strain of the parent *L. monocytogenes* ATCC 700302 showed a marked increase in zwitterionic phosphatidylethanolamine and lowering of anionic phosphatidylglycerol and cardiolipin. Simultaneously, the nisin resistant strain showed altered sensitivity to wall perturbing compounds such as lytic enzymes.

3.3. Cell wall perturbation

3.3.1. Mode of action

This brings us to the final class of antimicrobials, the cell wall perturbing biomolecules. Microbial cell walls are both characteristic and crucial cell survival. Most of these structures do not occur in man and hence form ideal targets for microbial inactivation. Perturbing the wall structure of bacteria is the principal inhibitory action of many commercial antibiotics commonly produced by the pharmaceutical industry (see a recent review on the action of penicillin against staphylococci by Giesbrecht et al., 1998). Several proteins involved in the binding of penicillin have been identified, some of which, for example Pbp2, are involved in the polymerisation and insertion of new glycan chains into bacterial cell walls. When these proteins are inhibited, cells swell and ultimately die (Vinella et al., 1993).

Enzymes that degrade bacterial walls from the outside, such as lysozyme, have been applied in the preservation of foods (see e.g. Gould, 1995; Fuglsang et al., 1995). Lysozyme hydrolyses the β1,4-glucosidic linkages in sugar polymers such as N-acetylmuramic acid and N-acetylglucosamine. Inhibitory activity of the enzyme is greatest against gram-positive bacteria as these microorganisms lack an outer membrane. The combination of lysozyme hydrolysing the bacterial cell wall, nisin perturbing the membrane and the chelator citrate is very effective against the gram-positive bacteria *L. monocytogenes* and *Listeria innocua* (Jones et al., 1990; ter Steeg, 1993). Gram-negative bacteria can be sensitised to the action of lysozyme by adding EDTA (discussed in Helander et al., 1997).

For fungal cells, extensive studies have been carried out with the aim of developing more natural crop protection systems. The basic biochemical structure and chemical linkages present in fungal cell walls has only recently been described (see Brul et al., 1997b and Kapteyn et al., 1999a,b for a recent update). Fig. 3 gives a representation of the fungal wall. In the literature, significant data are available on plant lytic enzymes that degrade the β1,3-glucan, the β1,6-glucan and the chitin polymers in the fungal cell wall (Sela-Buurlage, 1996). Furthermore, many antifungal enzymes of microbial origin exist, including those originating from the biocontrol fungus, *Trichoderma harzianum* (discussed in Fuglsang et al., 1995, Lora et al., 1995; Bom et al., 1998). Individual enzyme activities have been characterised and in some cases purified (Sivan and Chet, 1989; Lora et al., 1995; Bom et al., 1998).

Using wall lytic enzymes for the purpose of food preservation is not a route without obstacles (see also Fuglsang et al., 1995). First of all, enzyme activity may be lost in the food matrix. Secondly, production costs are generally still too high to guarantee effective application possibilities in bulk food products. Finally, modern biotechnology will often in their ''natural'' habitathave to be used to introduce the enzymes into foods. Consumer acceptance of these techniques is clearly an issue that should be taken most seriously in this context (see, e.g., Hoban, 1997).

3.3.2. Resistance mechanisms

Next to problems of consumer acceptance, if these compounds are ever to be successfully applied in foods, the potential issue of resistance development will have to be addressed. Various recent reviews have appeared on how yeast cells deal with outside ''attack'' on their cell wall by either enzymes or environmental factors such as temperature, pH and a_w (see Gustin et al., 1998). Yeast cells activate a sequence of enzymatic reactions that transmit the detection of cell wall problems to the nucleus. The output of this is increased chitin synthesis, enhanced expression of an alternative β1,3-glucan synthase (FKS2), and enhanced incorporation of at least one cell wall protein, Cwp1p (see Kapteyn et al., 1999a and references therein). Fig. 4 gives a schematic impression of the basic principles of this stress response in the spoilage yeast *Sac. cerevisiae*. As can be seen, the pathway is believed to be activated via

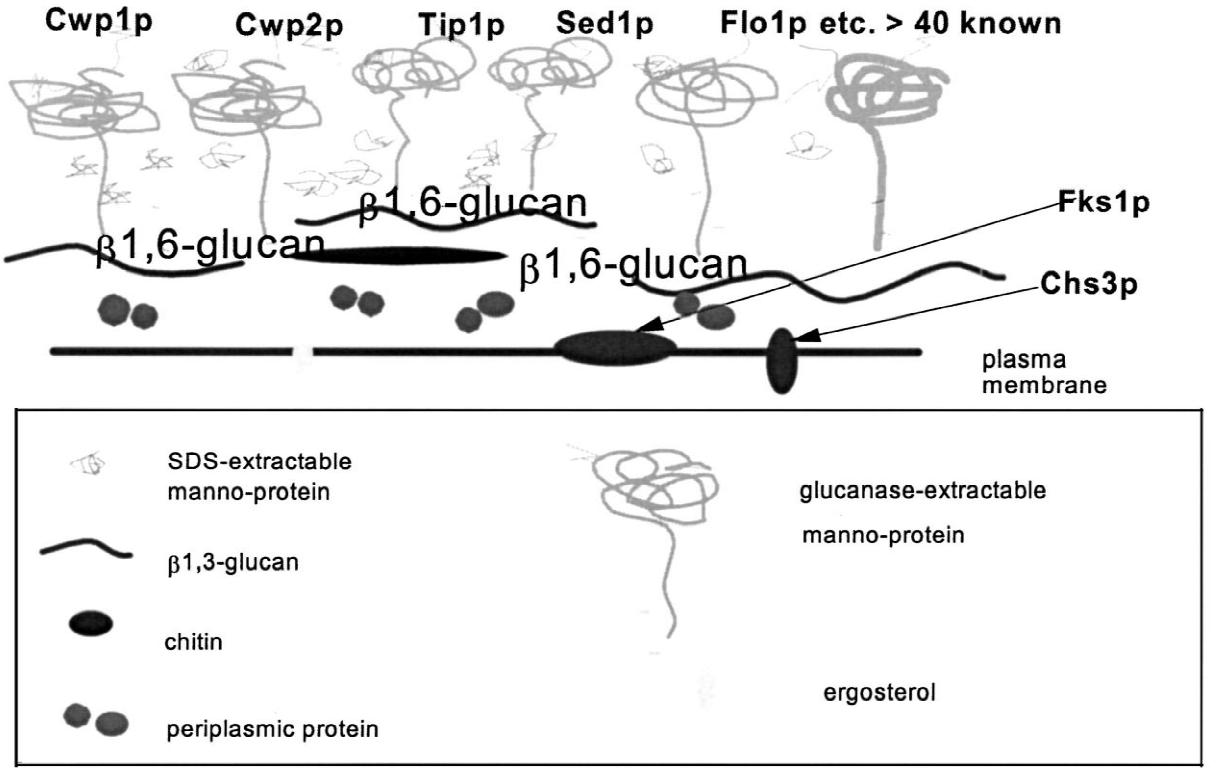

Fig. 3. Schematic overview of the cell wall of *Saccharomyces cerevisiae*. For more details, see Kapteyn et al. (1999a,b). The filamentous fungi from the Ascomycetes are proposed to build their cell wall along similar lines as *Sac. cerevisiae*. Cwp1, Cell wall protein 1; Cwp2, Cell wall protein 2; Tip1, Temperature induced protein 1; Sed1, multicopy suppressor of *erd2*; Flo1, Flocculin 1; Fks1, glucan synthase 1; Chs3, Chitin synthase 3. β1,6-Glucan is a central sugar polymer module that links most of the cell wall proteins to β1,3-glucan and chitin. Note, however, that there is also a minor class of glucanase extractable cell wall proteins (PIR proteins) that do not use the β1,6-glucan coupling module (Kapteyn et al., 1999b). The chemical nature of the link between chitin and β1,3-glucan and chitin and β1,6-glucan is known (see Table 1 in Kapteyn et al., 1999a).

the action of plasma membrane localised sensor proteins belonging to the Wsc family. Currently four members of this family are known, Wsc1p, Wsc2p, Wsc3p and Wcs4p (Verna et al., 1997). The proteins can, at least partially, substitute each other's function, as the phenotype of a *wcs1,2,3Δ* mutant is much stronger than the phenotype of each of the single mutants (Verna et al., 1997). Interestingly, the triple knock-out mutant has a similar phenotype to a *pkc1Δ* strain. Whether the Wsc proteins are the stress sensors themselves, or are needed in supporting the signal transduction process, remains to be elucidated. It is clear that the extracellular part of the Wsc proteins and the enzymes involved in cross-linking of wall components constitute excellent targets for inactivation of the stress response and thus enhanced efficacy of preservation regimes based on mild-temperature treatment, hypoosmotic treatment or enzymatic breakdown of the fungal cell wall.

Another relevant regulatory system depends on the activity of the small GTP-binding protein Rho1p. This protein binds to and is required for the activity of Pkc1p in vivo (Kamada et al., 1996). However, it should be noted that Rho1p regulates many more proteins than just Pkc1p and that Pkc1p regulates more than just the cell wall integrity/repair pathway.

Further interactions of the PKC1 pathway with other pathways exist. In yeasts, the Ca^{2+} and calmodulin regulated phosphatase calcineurin plays an important role in the stress response to NaCl, and in

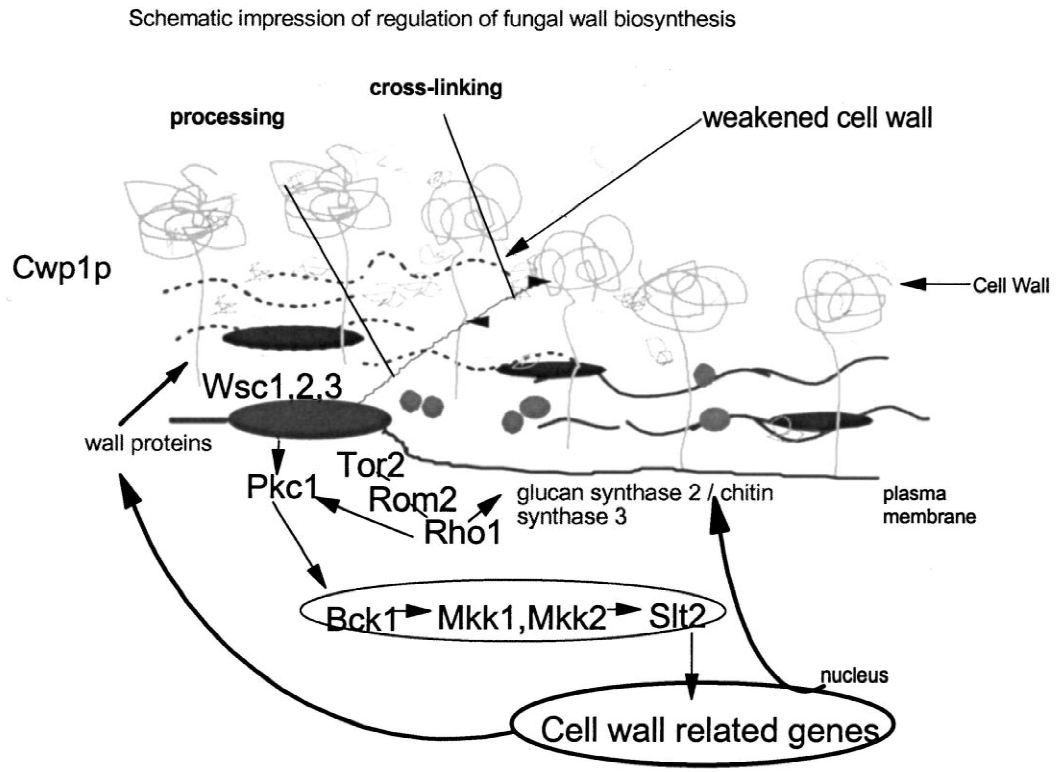

Fig. 4. Cell wall compensation mechanism induced by wall weakening treatments. Speculative scheme of events (see also Verna et al., 1997; Gustin et al., 1998). Local dissolution of the cell wall is proposed to result in membrane stretch, which activates the Wsc1, 2 or 3 protein. This step would subsequently result in at least part of the necessary activation reaction of the Pkc1 protein. The external stimuli leading to the proposed scheme of events can be nutrients, high temperature, low osmolarity, pheromone, and the presence of wall degrading enzyme systems (de Nobel, personal communication). Activation of the PKC1 pathway would then result in enhanced transcription of at least CWP1 and the gene coding for glucan synthase 2. At the same time, a regulatory mechanism is activated that enhances the synthesis of chitin up to more than tenfold.

the stress response against wall perturbation (see Eng et al., 1994). Recent experiments by Zhao et al. (1998) and de Nobel et al. (unpublished observations) showed that a temperature stress, continuous growth at 37°C or a short heat shock of 50°C, induces the gene encoding the stress protein β1,3-glucan synthase 2 (FKS2) both through the PKC1 route and the calcineurin pathway. Preliminary data by de Nobel et al. (unpublished observations) in very recent studies suggest that similar mechanisms are operative in cells that are stressed with a cocktail of wall lytic enzymes consisting of subinhibitory levels of β1,3, β1,6-glucanase and chitinase. Increasing the extracellular calcium chloride concentration partially rescues the temperature sensitivity of a *Δpkc1* strain whereas an equivalent concentration of $MgCl_2$ had little effect (Levin and Barlett-Heubusch, 1992).

Bacteria possess two basic mechanisms of membrane mediated signal transduction (see Fig. 5). Firstly, similar to the situation previously discussed for fungi, an extracellular signal is transformed via a transmembrane signal transfer protein into an intracellular signalling event. In an alternative process the signalling molecule enters the cell via passive membrane diffusion or active uptake via a transporter. This area was recently reviewed by Hellingwerf et al. (1998). Changes at the level of the cell wall or membrane induced by either lytic enzymes, membrane actives or changes in the physico–chemical parameters of the culture media are presumably

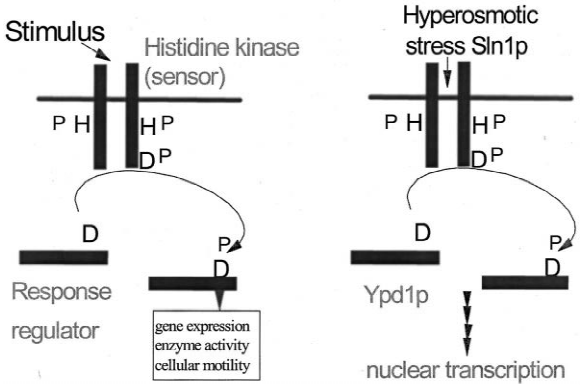

Fig. 5. Bacterial two component signal transduction systems in comparison to the yeast MAP kinase signal transduction systems (exemplified by the high osmolarity Sln1p branch). In bacteria, a sensor protein, which in most cases is an integral membrane protein recognises and binds a periplasmic signalling protein. Alternatively, in the case of a physical stimulus, one may speculate that the sensor protein responds to a change in the membrane environment analogous to, e.g., the Wsc and Sln proteins in yeast. Phosphoryl groups are transferred via a histidine based phospho-relay system to the receiver domain of the cytoplasmic regulator protein. The effector domain of the latter mediates either a change in gene expression, enzyme activity or cellular motility (through flagellar rotation) as a response reaction.

perceived through the former system. However, detailed molecular data are still scarce. Only on the output reactions towards osmotic stress and cold stress do we have molecular details (see Csonka, 1989; Verheul et al., 1997b).

4. Combination preservation

Food preservation is carried out during food processing in an attempt to maintain raw material quality, physico–chemical properties and functionality whilst providing safe products that have a low spoilage potential. This is mainly achieved through purposely designed processing that varies from one product to the next. Hence, in preservation processes, which form about a third of the unit operations used in food processing (Farkas, 1977), the general aim is to employ combination processes where, for example, a mild heat stress and a low concentration of preservatives are combined in order to fulfill all of the above listed objectives. Additionally, there is currently significant interest in using alternative physical treatments such as ultra high pressure (UHP) or pulsed electric fields (PEF) to replace the classical heat treatment. Progress in these areas has been discussed in this issue by Barbosa et al.. UHP presumably ''denatures'' microbial cell wall proteins such that access to the rest of, e.g., the fungal wall and membrane is greatly facilitated (Rommens, A., Brul, S., Verrips, C.T., in preparation; Brul, 1999). Indeed combination preservation treatments are often advocated and where possible patented (Knorr, 1998; Brul et al., 1997c). Combinations of preservation treatments allow the required level of protection to be achieved while at the same time retaining the organoleptic qualities of the product such as, colour, flavour, texture and nutritional value.

The potential use of some of the novel, ''natural'' preservatives, discussed previously in this review, in combinations with physical treatments (i.e., mild-heat, UHP, PEF), has not been extensively evaluated and may lead to the development of novel mild preservation regimes tailored to the organoleptic quality needs of individual products.

5. Concluding remarks

Scientifically, the most urgent problem is that there is still little understanding of the effectiveness of the use of classical preservatives and naturally occurring antimicrobial biomolecules (biological, ''natural'' preservatives) in conjunction with other common components of food preservation systems. This lack of understanding encompasses whether microorganisms die, survive, adapt or grow and what the physiological and molecular mechanisms are within cells that result in these phenotypes. For example, which signal transduction systems are involved and which stress proteins are induced? How are these systems linked? How much cellular energy (ATP) is involved in each of the systems? Ultimately, the amount of available energy will determine the extent to which a given microbial cell can have various stress response pathways activated. Stress responses leading to adaptation are energetically expensive and a microbial cell must strive to maintain a balance between the induction of energy-consuming mechanisms used to restore homeostasis and retaining sufficient energy to continue general housekeeping functions that allow growth (see, e.g., ter Steeg et al., 1995 for an example relevant to food

microbiology). The link between macroscopic bioenergetic parameters (growth-rate, yield) and microscopic bioenergetic parameters (substrate utilisation, ATP levels, ATP/ADP ratios, intracellular redox balance), and the molecular reactions in stress response mechanisms is a field of research that is only just emerging.

Indeed, the large number of signalling systems that operate in parallel both in pro- and eukaryotes in response to preservatives and preservation regimes give the molecular interconnections in cells the characteristics of *neural* networks. The links between the different branches of these networks need to be understood. This understanding can now be rapidly advanced through the advent of modern biotechnological tools (for example genomics and proteomics). These techniques allow for a rapid assessment of the total response of cells towards an external environmental stimulus. It will be our job as researchers to identify which of these responses is crucial, important, present or irrelevant under a particular circumstance. In this way, we should ultimately reach a situation where we can attempt to build ''whole cell'', mechanistic understanding of microbial adaptation, growth and survival. The inclusion of this data into mathematical models describing microbial growth and death would represent a significant advance compared to the empirical, descriptive models of microbial growth, with limited predictive capability, that are currently used in the food industry (see Zwietering et al., 1994; Cuppers et al., 1997).

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