Bactericidal action of carvacrol towards the food pathogen *Bacillus cereus*

A case study of a novel approach to mild food preservation

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Proefschrift

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op gezag van de rector magnificus,
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Stellingen
behorend bij het proefschrift
"Bactericidal action of carvacrol towards the food pathogen *B. cereus*"
Annemieke Ultee, 2 juni 2000, 13.30 uur

1. In studies naar de gevoeligheid van sporen voor antimicrobiële stoffen, is de invloed van de kweekomstandigheden van sporen minstens zo belangrijk als de invloed van de stof zelf.

2. Het RIVM-rapport over de aantallen voedselinfecties- en vergiftigingen in Nederland moet met voorzichtheid benaderd worden, daar uitsluitend de bij de Inspectie Gezondheidsbescherming (IGB) geregistreerde gevallen zijn vermeld en hierdoor het werkelijk aantal patiënten onderschat wordt.
   (Registratie van voedselinfecties en -vergiftigingen onderzocht door GGD's en Regionale Inspecties Gezondheidsbescherming/ Keuringsdiensten van waren, 1997, RIVM, 216851002)

3. Gezien het feit dat sporen pas een gevaar vormen wanneer ze zich ontwikkelen tot vegetatieve cellen, is het relevanter om te zoeken naar een conserveermiddel dat vegetatieve cellen doodt, dan te zoeken naar een stof die (ook) sporen doodt.

4. Bij onderzoek naar de toepassing van probiotica, moeten behalve de gunstige gezondheidsbevorderende effecten ook de eventueel aanwezige schadelijke effecten nader bekeken worden.
   (ASM News, november 1999, februari 2000)

5. Indien de toelatingsprocedure voor nieuwe conserveermiddelen niet wordt versneld, blijft het minder aantrekkelijk voor producenten om naar deze nieuwe middelen te zoeken.

6. Goede voorlichting kan weerstand tegen nieuwe conserveringstechnieken bij consumenten wegnemen.

7. Het is opvallend dat de inwoners van Athene, een stad waar je door de smogvorming nauwelijks adem kunt halen en de mensen de koelvloeistof van koelmachines (vol met PCB's en dioxine) op straat laten weglopen, tijdens de dioxinecrisis direct alle Belgische en Nederlandse producten uit de schappen haalden.
   (Food Management, 1999, nr. 9)

8. Het valt nog te bezien of de mens de bacterie de baas is, of de bacterie de mens.

9. Die Einheit Deutschlands war verbunden mit vielen Träumen und Visionen etwas Besseres zu schaffen. Letztlich war es die uneingeschränkte Übernahme des westdeutschen Systems im Osten Deutschlands, mit allen Vorzügen aber auch Schwächen.

10. Groen Links kan beter een kleine partij blijven, zodat politieke belangen geen grotere rol gaan spelen dan milieubelangen.

11. Hoe meer de wereld een dorp wordt, des te groter wordt de behoefte aan eigen, nationale of zelfs regionale producten
(*NRC Handelsblad, 10-1-00*).

12. Het gedrag van ouders gaat meer lijken op dat van een koekoek: de zorg en aandacht voor de kinderen wordt steeds vaker uitbesteed.

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Om een promotieonderzoek tot een goed einde te brengen, heb je hulp van anderen nodig. Ook ik heb mogen ervaren dat mensen mij hebben willen helpen. Tips bij het opzetten van experimenten, het lezen van manuscripten, monsters nemen, rijst koken of in het weekend een keertje aanenten. Ik ben iedereen die mij op een of andere manier geholpen heeft, heel dankbaar. Graag wil ik enkele mensen noemen:

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Introduction

INTRODUCTION

Bacillus cereus is an important causal agent of outbreaks of food poisoning in Europe and North America. The risk of outgrowth of *B. cereus* can be minimised by using one or a combination of different preservation methods. Instead of using traditional methods such as heating or addition of chemical preservatives, a more natural approach to inhibit growth of *B. cereus* can be taken: the use of natural antimicrobial compounds present in plants, such as herbs and spices.

This chapter describes some characteristics of *B. cereus*, the formation and germination of spores, food poisoning caused by *B. cereus* and the use of natural antimicrobial systems to enhance the safety of foods. Finally, carvacrol, an antimicrobial compound present in oreganum and thyme, will be discussed in more detail.

BACILLUS CEREUS

General characteristics of *Bacillus cereus*

B. cereus is a Gram positive, facultative anaerobic, rod shaped and spore forming pathogenic flagellae bacterium. Cells are motile due to the presence of flagellae and are 1.0 - 1.2 µm in diameter and 3.0 - 5.0 µm in length. Growth was reported between 5 and 50°C, with an optimum range of 28-35°C. Generation times are 18-27 min at 35°C in laboratory media and 26-57 min in boiled rice at 30°C (41). Minimal water activity (a_w) required for growth is 0.95 and growth occurs over a pH range of approximately 4.9 to 9.3 (58). *B. cereus* is commonly found in soil and water and can be isolated from a variety of food products such as rice, milk and other dairy products, cereals, meat, vegetables and soup. Spices are often contaminated with spores of *B. cereus*. After rehydration of the spices, the bacterial spores can germinate and start to grow and proliferate in the food products (28). *B. cereus* is sometimes involved in outbreaks of food poisoning. Strains of *B. cereus* causing food poisoning, produce one or more of the extracellular enzymes and toxins: lecithinase, proteases, ß-lactamase, cereolysin (mouse lethal toxin, hemolysin I), emetic enterotoxin and diarrhoeagenic enterotoxin. Only the last two toxins are associated with foodborne gastro-enteritis syndromes (32). *B. cereus* is unable to metabolise mannitol and this feature is used to isolate and enumerate *B. cereus* from different sources. Isolation of *B. cereus* from a food product is made possible by incubation in *B. cereus* selective medium containing mannitol as only carbon source. Addition of a pH indicator, e.g. bromothymol blue, makes it possible to make a distinction between strains which can metabolise mannitol and strains which can not. Metabolisation of mannitol decreases the pH-value and the colour will turn yellow. *B. cereus* will ferment aminoacids, proteins and peptides instead of mannitol. Consequently, the pH will increase and the colour turn blue.

Spores

Vegetative cells of *B. cereus* can form spores. Spores are resistant to heat, desiccation, radiation, oxidising agents and many chemicals, due to their special structure and the absence of active metabolism. Sporulation is a response to slowed growth or starvation and starts with an unequal cell division in the vegetative cell. Two compartments are formed, a smaller forespore compartment and a larger mother cell compartment. During sporulation, two cells with their own complete genome develop. The smaller prespore is engulfed by the mother cell in a process containing several different steps. The final step of sporulation is called maturation. The morphology of the spore does not change very much anymore, however, resistance, dormancy and germinability develop. Finally, the mature spore is released by lysis of the mother cell, however, this stage can occur very late (58).

The formation and characteristics of spores are influenced by temperature, pH, aeration, presence of minerals, carbon and nitrogen source, etc. The optimal temperature and pH of sporulation are respectively 25-30°C and 7 (8). Sporulation is an irreversible process which occurs normally after the exponential growth phase, when the generation time increases (8). During the process of sporulation a large amount of dipicolinic acid (DPA) and divalent cations are accumulated. Germination can occur over a temperature range of 8 to 30°C. Since the spore is formed within the mother cell, it is called an endospore (8, 20, 58).

A spore which is released from the mother cell at the end of sporulation, is biochemically and physiologically different from the vegetative cell. As a result of many physiological changes during sporulation, spores can survive extremely long periods in the absence of exogenous nutrients. They can remain viable for several decades without germination. The low water content probably plays an important role in maintaining this physiological state (57, 58, 68).

The size of the spores varies between 0.5 - 3 μm length and 0.5 - 1.3 μm width and the spores can be made visible by phase contrast microscopy (68). Spores obtained from strains associated with food poisoning are hydrophobic and can easily attach to food surfaces (58). They can be inactivated by ionising radiation, ultraviolet radiation and chemical compounds, such as H_2O_2, formaldehyde, ethylene oxide, propylene oxide, alcohols, Hg, Pb, quaternairy ammonium compounds, etc. (40). In contrast to these treatments, spores can survive mild heat treatment often applied during food preparation. As a consequence, spores will germinate and proliferate if the food product is stored too long at ambient temperatures (27).

Spore structure

The spore is biochemically and physiologically different from the vegetative cell. Fig. 1 shows the structure of a dormant spore, consisting of several layers. This structure differs significantly from that of a vegetative cell. The different layers will be shortly described here.

The **protoplast** or **core** is similar to the vegetative protoplast. Most components of the cytoplasm of bacteria are present in the core, such as DNA, RNA, ribosomes, enzymes, etc.

Spores contain tRNA and rRNA, however, they lack mRNA. Most of the commonly found high-energy cofactors normally present in growing cells (ATP, NADH and NADPH) are absent in the spore core, although the corresponding low-energy forms of these compounds (AMP, ADP, NAD^+ and $NADP^+$) are present. In addition, a considerable amount of 3-phosphoglycerate, an intermediate of glycolysis, is found in the core, together with a large amount of divalent cations, predominantly Ca^{2+}, but also Mg^{2+} and Mn^{2+}. Further, some spore specific compounds, such as dipicolinic acid (DPA) and by the developing spore synthesised small acid soluble proteins (SASP) are also detected in the core. DPA is accumulated to concentrations higher than 20% of the dry weight of the spore. SASPs (10-20% of core protein) protect the DNA from damage. The water content of the core is extremely low. This low water content, the presence of SASPs and the absence of metabolic activity probably play a role in spore dormancy and spore resistance to many agents. In contrast to the low water content of the core, other parts of the spore have a more normal water content (8, 58).

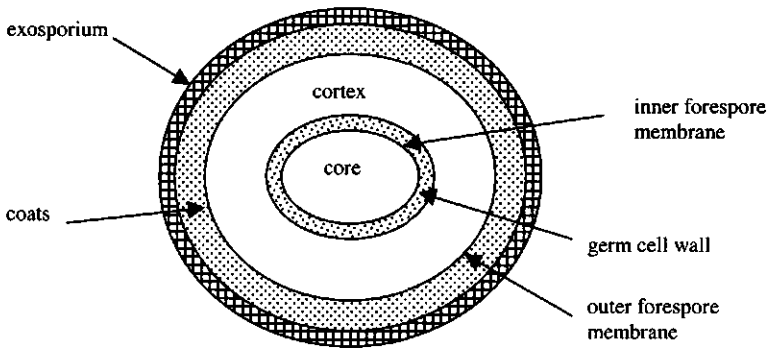

Figure 1: Structure of a dormant spore (not precisely to scale) (58)

The core of the spore is surrounded by the plasma membrane (inner forespore membrane) (58). This **inner forespore membrane** is a complete membrane and appears to be a very strong permeability barrier to hydrophilic molecules, charged molecules and to most molecules of a molecular weight of >150 Da. The phospholipid content is similar to that of growing cells. (57, 58). The structure of the next layer, the **germ cell wall**, is similar to the cell wall in vegetative cells. It is largely composed of peptidoglycan (PG). Together with the cortex this layer determines cell shape and resists the turgor pressure within the cell. The **cortex** is rich in PG and consequently important for the heat resistance of the spore. This structure is responsible for the dehydration of the spore core and consequently the resistance of the spore to several treatments. The precise mechanism of cortex synthesis is not known, however, the required gene products are made in the mother cell.

The **outer forespore membrane** plays a large role in the extreme impermeability of the spore

to small molecules. The protein composition of this membrane is different from the inner forespore membrane. The form and firmness of the spore is mainly caused by the **coats** (under, inner and outer coat). They are rich in proteins and protect the cortex from attack by lytic enzymes. They also provide an initial barrier to chemicals. However, they do not play a role in the heat or radiation resistance of the spore. These layers occupy about 50% of the spore volume (18) and are composed of protein and inorganic phosphate with minor amounts of complex carbohydrates and lipids. The **exosporium** too protects the cell against enzymes and chemicals. It is a loosely fitting structure and easily removed from the spore The structure of this layer is not very well characterised, but the protein content of the exosporium is very high (80%) (58, 68, 81).

Germination

In spite of the spore's extreme dormancy, the spore can rapidly return to active metabolism if the appropriate stimulus is given (58). This process is called germination and is a succession of different processes: activation, triggering and outgrowth. The whole process is not very well understood. There are still some uncertainties, however, a short overview of the germination will be given here.

Activation is a reversible process and not always required for spore germination. Different circumstances lead to the activation: sublethal heating, low pH (1.0 – 1.5), reducing and oxidising compounds, ageing (8, 58, 68). Although the process is far from clear, it is known that heat activation releases a small amount of the spore's DPA (58).

Triggering involves the irreversible, degradative biochemical reactions that transform the highly resistant and metabolically dormant spore into a more sensitive and metabolically active cell. The first events are release of protons and some divalent cations. This is followed by excretion of DPA, loss of spore refractility and cortex degradation. The spore excretes up to 30% of its dry weight and the core increases its water content to that of the vegetative cell. Increase of the water content restores enzyme activity in the core. Also core proteins and SASP are degraded and RNA and protein synthesis are initiated. These changes in the spore are responsible for the loss of resistance towards heat, radiation and chemical compounds. The process of triggering can take place in the absence of exogenous nutrients and is initiated by different compounds such as nucleosides, amino acids, sugars, salts, DPA, long chain alkylamines, inorganic salts. These compounds are not metabolised (8, 58, 68).

Outgrowth of germinated spores starts if sufficient nutrients are present. If not, sporeforming starts again immediately. Germinated spores will not continue to grow if the water activity is too low (8, 68). As most processes during germination are supported by endogenous reserves, exogenous sources (carbon, nitrogen, amino acids, etc) are required for outgrowth. During this process, the spore regains the ability to synthesise amino acids, nucleotides and other small molecules. The volume of the outgrowing spore increases and consequently requiring

synthesis of membrane and cell wall components (58). Outgrowth takes place under conditions that can support cell growth. After outgrowth in the food product becomes spoiled or toxins are formed (8). Different factors contribute to outgrowth of *B. cereus*, e.g. inadequate cooling, inadequate heating, preparation of food far in advance and lack of hygienic circumstances (33).

Foodborne illness

Foodborne illnesses are caused by different pathogens. The percentage of outbreaks of food poisoning and food infection and the corresponding agents in the Netherlands reported at the Dutch Inspection of Health Protection (IGB) in 1995-1997 are shown in Table 1. Although these data are retrieved from only the incidents reported at the IGB, they show that the highest percentage of cases of food poisoning or -infection was caused by *B. cereus*. The total number of foodborne outbreaks of *B. cereus* is underestimated in literature. This is probably due to relatively mildness and short duration of the illness (26).

Table 1: Reported incidents of food poisoning and –infection at Dutch Inspection of Health Protection (IGB) in 1995-1997 (numbers represent % of total reported incidents at IGB) (19).

Agents	1995	1996	1997
Campylobacter	0.5	0.0	0.2
Salmonella	0.9	1.7	1.2
Yersinia	0.1	0.1	0.1
Escherichia coli	0.4	0.3	0.5
Staphylococcus. aureus	0.4	1.3	1.3
Bacillus cereus	2.6	2.7	2.0
Clostridium perfringens	0.4	0.9	1.6
Other	10.6	7.6	11.3
Two or more agents	8.3	10.8	6.5
Unknown	75.8	74.6	75.3

B. cereus is known to be responsible for two types of foodborne illness, associated with the production of two different toxins. The first syndrome, mainly causing diarrhoea and therefore called diarrhoeal syndrome, is associated with products such as meat products, soups, vegetables, puddings and sauces, milk and other dairy products. This illness is similar to that caused by *Clostridium perfringens*. Another syndrome, the emetic, is mainly associated with consumption of rice, pasta and noodles, resulting in vomiting and similar to *Staphylococcus aureus* food poisoning (31, 33). Certain strains may produce either the enterotoxin or the emetic toxin, dependent on the food in which growth occurs (41).

The characteristics of both types of illness are shown Table 2. Besides these foodborne toxins, *B. cereus* also forms phopholipase C, hemolysins and mouse lethal toxins (31, 33).

Diarrhoeal enterotoxin

The diarrhoeal enterotoxin is an extracellular protein with a molecular weight of approximately 50 000. Its structure is not yet certain, but it is composed of at least two components and produced in the exponential phase of growth. The maximum amount of toxin is found early in the stationary phase (14, 24, 32, 33). Some studies show this toxin consists of two major and one minor components. Separation of these components leads to a loss of the biological activity. The activity could be restored upon reconstitution of the components (31).

Table 2: Characteristics of the two types of disease caused by *B. cereus* (26, 33).

Characteristics	diarrhoeal	Emetic
type of toxin	protein	cyclic peptide
stability	unstable	stable
infective dose (cells)	10^5-10^7 (total)	10^5-10^8/g
toxin produced	small intestine of host	preformed in food
incubation period	8 - 16 h	0.5 - 5 h
duration of illness	12 - 24 h	6 - 24 h
symptoms	abdominal pain, watery diarrhoea, occasionally nausea	nausea, vomiting, malaise, sometimes diarrhoea
food most frequently implicated	meat products, soups, vegetables, puddings, sauces, dairy products	fried and cooked rice, pasta, pastry, noodles

The diarrhoeal enterotoxin is an unstable protein. Activity is lost when kept for 5 min at 56°C or reduced when kept for 1-2 days at 32°C. Proteases such as trypsin and pronase inactivate the toxin (14). Between pH 5 and 10 the enterotoxin is stable, but at other pH values it is rapidly inactivated (31). The toxin is probably formed in the small intestine. If the toxin should have been preformed in the food, it should be inactivated by the low pH in the stomach and not cause food poisoning.

The levels of toxin produced are dependent on the growth medium. Addition of starch to the growth medium increases the production. Low concentrations of glucose (up to 10 g/l) support toxin production, however, higher concentrations (> 50 g/l) completely suppressed toxin production (70)

Although the exact mechanism of action is still unclear, the diarrhoeal toxin stimulates the adenylate cyclase-cyclic AMP system. This system synthesises cyclic AMP from ATP. Cyclic AMP is a very stable compound and acts inside human cells by degrading the storage of fluids

in the cell. This could contribute to the accumulation of fluid in the intestines and consequently appearance of diarrhoea (33, 69).

Two test kits are commercially available for the detection of the diarrhoeal enterotoxin: a reversed passive latex agglutination kit (RPLA) and the enzyme-linked immunosorbent assay (ELISA). These two kits detect different non-toxic proteins (2). The findings of Day *et al.* (17) suggest the ELISA test is a more reliable detection kit than the RPLA kit for the detection of *B. cereus* diarrhoeal toxin.

Emetic toxin

Most incidents of emetic outbreaks have been associated with rice (41). The emetic toxin is an extracellular compound with a molecular weight of 1.2 kDa (26). In contrast with the diarrhoeal toxin, the emetic toxin is extremely stable to heat (retains its activity after 90 min at 126°C), pH (stable for 2 hours or more at pH 2 and pH 11) and resistant to trypsin and pepsin (14). Another name for this toxin is cereulide and it consists of a ring structure of three repeats of four amino and/or oxy acids: D-*O*-Leu-D-Ala-L-*O*-Val-L-Val]$_3$ (dodecadepsipeptide) (26). The optimal temperature for production in rice is 25-32°C (41). Hughes *et al.* (29) developed a HEp 2 cell system for the detection of the emetic toxin. This method is based on the fact that culture filtrates from different *B. cereus* strains produced vacuoles in HEp 2 cells. Other tests involve oral challenge of monkeys. However, no commercial test kit is available (60).

NATURAL ANTIMICROBIALS FROM PLANTS

From the previous paragraphs it can be concluded that *B. cereus* is a serious hazard in foods. In order to inhibit outgrowth of *B. cereus* and prevent food poisoning, different traditional preservation systems such as heating or the addition of chemicals are being used. The disadvantage of heating is its large impact on the organoleptic and nutritional properties of the food product. Although chemicals can be very effective as preservative agents, consumers are asking for high quality products which are additive free, natural, safe, fresh and to which low amounts of salt, sugar or fat are added. The use of natural antimicrobial systems (mild preservation) could serve this demand. Examples are animal constituents (lysozyme, lactoferrin, lactoperoxidase), animal induced systems (antibodies), microorganims (lactic acid bacteria), plant constituents (antimicrobial compounds from herbs and spices), etc. These systems could be used in combination with e.g. low pH, low a_w, low O_2, mild heat, pressure treatment, low temperature, raised CO_2 (25). As has been reviewed, compounds from plants possess antimicrobial activity and some of them were found to be very effective towards different microorganims (52, 59, 63, 65).

Essential oils

The antimicrobial activity of compounds from plants can be explained by the fact that plants have developed a range of strategies for defence against their enemies: bacteria, fungi, insects, animals. An important strategy is a chemical defence in the form of secondary plant metabolites with antimicrobial activity (80). Secondary plant metabolites are natural compounds that are not strictly necessary for growth or reproduction. Essential oils belong to this group and are defined as complex mixtures of compounds, being a group of odorous principles, soluble in alcohol and to a limited extent in water, consisting of mixtures of esters, ethers, phenols, aldehydes, ketones and terpenes. The antimicrobial compounds in plant materials are commonly found in the essential oil fraction. The phenolic compounds showed to possess the strongest antimicrobial activity. The composition of essential oil fractions is dependent on the plant species, the chemotypes, geographical location of the growing plant and environmental factors such as soil, microclimate and altitude (6, 50). Essential oils can be extracted from plant materials such as spices, herbs, berries and the roots and stems of plants by steam distillation, dry and vacuum distillation, solvent extraction or supercritical extraction. They are mainly responsible for the characteristic aroma and flavour of the plant they are extracted from (50, 66, 83).

Although herbs and spices are often applied in food products to enhance the taste or flavour, nowadays they are receiving more attention as potential natural antimicrobials for the preservation of food products due to their strong antimicrobial and antioxidative activity (9).

Antimicrobial properties

The strong antimicrobial activity of compounds of essential oils has been described extensively in literature for both bacteria and fungi as target organisms (1, 4-7, 10, 12, 13, 21, 22, 30, 35-38, 42, 43, 47, 48, 67, 71, 74, 77, 78). However, all these studies describe the efficacy of the compounds towards specific microorganims. None of them are dealing with the mechanism of action.

Table 3 shows MIC-values of some components of essential oils for different pathogens. Carvacrol and thymol seem to be the most effective, carvone and cuminaldehyde do not show any activity towards these pathogenic bacteria. However, experiments have shown the latter are more effective towards fungi and yeast, specifically when they are administered via the gas phase (51, 64, 65).

Table 3: MIC-values of thymol, cuminaldehyde, cinnamaldehyde, carvacrol and carvone for different pathogens in Brain Heart Infusion (BHI) broth at 30°C (76).

Target organisms (strain)	MIC-value (mM) th	cu	ci	cl	cn
Listeria monocytogenes (LCDC 81-861)	1.50	>3	5.00	1.88	>3
Bacillus cereus (F46-26-90)	0.75	>3	1.50	0.75	>3
Aeromonas hydrophila (DSM 30187)	0.56	>3	1.25	0.47	>3
Yersinia enterocolitica (DSM 4780)	0.56	>3	3.75	1.88	>3
Salmonella typhimurium (DSM 554)	0.75	>3	3.00	0.94	>3
Staphylococcus aureus (ATCC 6538)	2.25	>3	>3.75	3.00	>3

th = thymol (oreganum, thyme, savory, sage)
cu = cuminaldehyde (cumin)
ci = cinnamaldehyde (cinnamon)
cl = carvacrol (oreganum, thyme)
cn = carvone (caraway, dill seeds)

Little is know about the mechanisms of toxicity of essential oils. Some compounds are bactericidal (fungicidal), although other compounds exhibit only bacteristatic (fungistatic) activity. Gram-positive bacteria have a cytoplasmic membrane which is surrounded by a thick rigid cell wall. In addition to the cytoplasmic membrane, Gram-negative bacteria have an outer membrane which consists of phospholipids and lipopolysaccharides (LPS). The outer membrane shows very low permeability towards hydrophobic compounds, most probably due to the presence of lipophilic LPS.

The cytoplasmic membrane of bacteria consists of a phospholipid bilayer. This bilayer forms a matrix in which enzymes and transport proteins (carriers) are embedded. Before compounds can enter the cell, they have to pass the cytoplasmic membrane. Uncharged and neutral molecules were found to be more antimicrobial than charged molecules (11). This is caused by the very low permeability of the cytoplasmic membrane for polar and charged molecules. Apolar or hydrophobic compounds can easily penetrate the membrane by diffusion (61). A factor which partly determines the toxicity of a compound is the phase partition coefficient (log P) (82). Due to the hydrophobicity of the essential oils and their constituents, they have a high preference to partition into biological lipid bilayers. The degree of accumulation into the

bilayer depends on the lipophilicity of the compound and the fluidity of the membrane. Accumulation of these compounds into biological membranes enhances their availability to the cell and therefore may affect cell vitality (63). In general, essential oils exert antibacterial activity by 1) interfering with the phospholipid bilayer of the membrane and thereby increasing the permeability and loss of cellular constituents; 2) impairing enzyme systems (61, 3) inactivating or destroying genetic material (16, 39, 61). The different factors can be directly or indirectly dependent on each other (61, 74). However, it has to be kept in mind that essential oils consist of a mixture of compounds from different chemical classes. Therefore, it is rather difficult to describe a single mechanism by which they act as antimicrobial agents.

Very few studies describe the relation between the chemical structure of the compound and its antimicrobial activity. The number of double bonds in terpenes or the introduction of a ketone radical does not seem to play a large role (56). In general, the presence of an aromatic ring containing a functional group is important for the antimicrobial activity (21). The introduction of a hydroxyl group directly bonded to a cyclohexane or benzene ring results in an increase of the antibacterial activity. This can be illustrated with the comparison of antibacterial activity of *p*-cymene (a biosynthetic precursor of thymol and carvacrol), thymol and carvacrol (Fig. 2). *p*-Cymene exhibits almost no antimicrobial activity, whereas thymol and carvacrol are much more effective (56). The hydroxyl group is quite reactive and can easily form hydrogen bonds with active sites of enzymes. But also the inductive effect of the isopropyl group and the aromaticity can contribute to the antimicrobial activity (21).

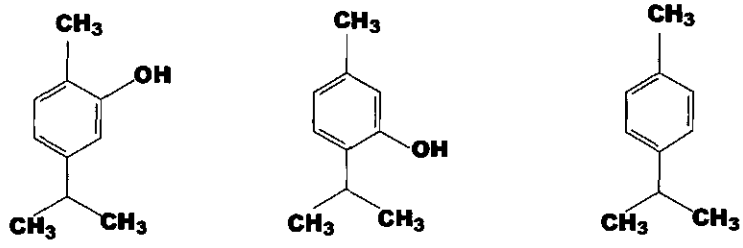

Figure 2: Chemical structure of carvacrol (left), thymol (centre) and *p*-cymene (right).

Antioxidative properties

Oxidation reactions occur when foods are exposed to air and they result in the production of undesirable flavours, rancid odours, discoloration and other forms of spoilage. During oxidation of lipids, peroxyde radicals are produced. These peroxyde radicals are very reactive and can cause a chain reaction. Several antioxidants are used as additives in fats and oils and in food processing. They react with the peroxyde radicals by forming a hydroperoxide molecule and a free radical of the antioxidant. These compounds are relatively stable and do not initiate a chain autooxidation. The most commonly used antioxidants are tocopherols,

ascorbic acid and synthetic compounds such as BHA (butylated hydroxyanisole) and BHT (butylated hydroxytoluene). The synthetic antioxidants are very effective and stable, but they may have undesirable effects on enzymes of human organs. This makes it necessary to look for new, natural, antioxidants. Almost all plants, microorganims, fungi and even animal tissues contain antioxidants of various types. The majority of natural antioxidants are phenolic compounds (55). Herbs and spices such as rosemary, sage, pepper, ginger, thyme, clove, allspice, oregano, etc. do have antioxidative properties (49, 55). Tsimidou *et al.* (75) showed that 1% (w/w) oreganum retards the oxidation of oil extracted from mackerel. Carvacrol and thyme, present in oreganum, showed very high antioxidant activity (49). The antioxidative activity of some compounds compared to the synthetic compound BHA is show in Fig. 3. All the compounds retarded oxidation, whereas cinnamaldehyde, thymol and carvacrol were the most effective.

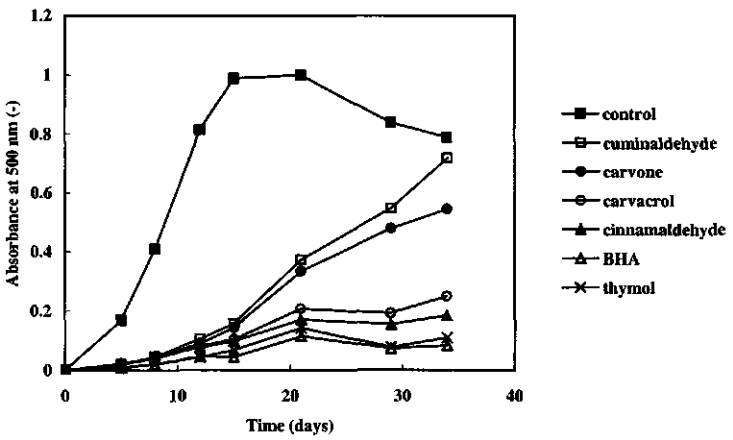

Figure 3: Antioxidative properties of BHA (butylhydroxylamide), cuminaldehyde, carvone, carvacrol, thymol and cinnamaldehyde. Absorbance A_{500} is a measure for the amount of oxidised linoleic acid (76).

Essential oils or their components are very effective as antimicrobial or antioxidative agent *in vitro*, however, the effectiveness is decreased when applied *in situ*. This is probably due to components of the food matrix, such as fats, proteins, carbohydrates or other additives, which immobilise and/or inactivate the essential oil components (11, 63). Partition of essential oil components into the lipid phase reduces their effective concentration in the aqueous phase where the microorganims proliferate (54, 71). During processing of the product, a considerable percentage of the antimicrobial compound can be lost due to evaporation or chemical reactions, such as oxidation or hydrolysation. Before applying a compound on a food product the effect of the food composition, pH, temperature, way of storage and the nature of microorganim(s) has to be determined (71) However, also a synergistic activity with food compounds could be possible (38).

Another problem concerning the application of essential oils and/or their components as additive, is the strong influence on the taste and flavour of a food product at the effective concentration (83). This problem can be reduced by a combination of different preservation systems, called "Hurdle Technology". This principle was described by Leistner *et al.* (45). Due to the presence of several preservative factors (so-called hurdles), the (pathogenic) microorganims in the product will not be able to grow as they can not overcome all the hurdles present. These hurdles can be mild temperature, pH, water activity (a_w), redox potential, other preservatives, but also gas packing, biopreservation, bacteriocins, ultra high pressure, edible coatings, pulse electric field, etc. As a result of the presence of other (mild) hurdles, the actual concentrations of an antimicrobial compound can be reduced and consequently a smaller impact on the sensoric and nutritive properties of the product are expected. In industrialised countries Hurdle Technology is currently of particular interest for minimal processing and has proved to be very successful (46).

Carvacrol

Carvacrol (Table 4) is a compound present in the essential oil fraction of mainly oreganum (51-84.5%), thyme (45-60%) and savory (26-41%) (3, 10, 53, 79). As described in Table 4, carvacrol is very poorly soluble in water. Fig. 4 shows the actual concentration in water at room temperature as a function of the carvacrol concentration added. From these data it can be concluded that carvacrol is soluble in water till approximately 10 mM. Carvacrol is applied as a flavouring compound in baked goods (15.7 ppm), soft candy (21.4 ppm), non-alcoholic beverages (28.5 ppm/0.18 mM) and chewing gum (8.4 ppm) (23). Due to its strong taste and flavour application of carvacrol is limited to a certain concentration in a certain product.

The antimicrobial properties of carvacrol have been studied on different microorganims e.g. *Fusarium* spp., *Aspergillus* spp., *Rhizobium leguminosarum, Escherichia coli, Salmonella typhimurium, Listeria monocytogenes, Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus subtilis, Rhodobacter sphaeroides* and *Vibrio vulnificus* (15, 34, 36, 37, 39, 62, 73, 74). Although carvacrol is very active as an antimicrobial compound, not much is known about the mechanism of action.

Legislation

Before natural antimicrobials can be used in food products they have to pass legislatory tests because "natural" is not the same as "safe". Even natural compounds can be toxic or carcinogenic (11). Application of carvacrol as a flavouring compound is permitted in the USA (Code of Federal Regulations (CFR)) if the minimum quantity required to produce its intended effect is used and, otherwise, used in accordance with all the principles of good manufacturing practice. The Flavor and Extract Manufacturers' Association's (FEMA) expert panel has

recognised carvacrol as GRAS (Generally Recognised As Safe) (23). In general, herbs and spices and several of their antimicrobial constituents do have the GRAS status. However, the legislative viewpoint on natural antimicrobials is that they are new food additives or are applied for new purposes and consequently require a non-toxicity record, despite their possible GRAS status. A solution would be the application of the whole spice or herb that contains the desired active ingredient, because this still may be regarded as the most natural way of application (63). However, addition of the pure compound makes it possible to reach higher concentrations and consequently a higher activity, than when the whole herb or spice is added.

Table 4: Main characteristics of the natural compound carvacrol (23, 24, 72)

Carvacrol	
Other names	2-*p*-cymenol
	2-hydroxy-*p*-cymene
	isopropyl-*o*-cresol
	isothymol
	2-methyl-5-isopropyl phenol
formula	$C_{10}H_{14}O$
molecular weight	150.22 g/mol
melting point	0-1°C
boiling point	236-237°C
specific gravity	0.9743 g/cm^3 (20°C)
solubility	water: slightly soluble
	ethanol: soluble
	ethyl ether: soluble
	acetone: very soluble
appearance	colourless to yellow liquid
organoleptic characteristics	characteristic pungent, warm odour
toxicity	LD_{50} (rabbits) = 100 mg/kg

OBJECTIVE OF THIS STUDY

As was described in this chapter, there are potential advantages of the use of natural antimicrobial systems for the preservation of foods. Although the antimicrobial spectrum has been described for many compounds, the mode of action in still rather unclear. This thesis describes the antimicrobial action of carvacrol towards the pathogen *B. cereus*. Different aspects of the use of carvacrol to reduce the risk of food poisoning by *B. cereus* are studied.

In **Chapter 2** the bactericidal activity of carvacrol towards *B. cereus* was investigated under different growth conditions. As growth and exposure temperature and pH are important factor

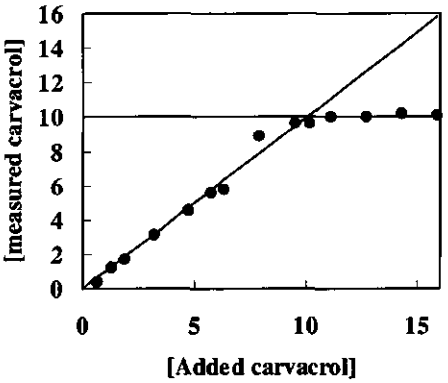

Figure 4: Solubility of carvacrol in water at room temperature (44).

considering the sensitivity of pathogens to antimicrobial compounds, the influence of these factors was studied in more detail. **Chapter 3** describes the mode of action of carvacrol. The hydrophobic compound carvacrol interacts with the cytoplasmic membrane of *B. cereus* and therefore affects some bioenergetic parameters. A disturbance of the membrane integrity will affect ATP pools and the membrane potential. To investigate leakage of essential ions, H^+ and K^+ concentrations were measured. **Chapter 4** gives an overview of the adaptation of *B. cereus* to non-lethal concentrations of carvacrol. In Chapter 3 an effect of carvacrol on the cytoplasmic membrane was observed. It is expected that carvacrol interacts with the lipid acyl chains of the phospholipids in the membrane. Adaptation to carvacrol will result in changes in the membrane properties. Therefore, the phase transition temperature was measured as a function of the carvacrol concentration in the growth medium. *B. cereus* will probably try to reduce the actual concentration of carvacrol in the membrane, by changing its phase transition temperature and consequently the membrane fluidity. A common observed way to adapt to different circumstances is by changing the fatty acid and head group composition of the membrane. These factors were studied to acquire more knowledge about the adaptation mechanism of *B. cereus* to carvacrol.

As carvacrol is very effective as an inhibitor of growth of *B. cereus* in laboratory media, this compound was studied for its activity in a food product. The antimicrobial activity of carvacrol on rice is described in **Chapter 5**. To reduce the effective carvacrol concentration, carvacrol was combined with cymene and/or soya sauce, Finally, the influence of carvacrol on the toxin production is reported in **Chapter 6**. As toxin production is the most important factor considering food poisoning by *B. cereus*, it is of great interest when carvacrol not only inhibits growth, but also inhibits toxin production. This was studied at carvacrol concentrations which did not affect the viable count of *B. cereus*.

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2

Bactericidal activity of carvacrol towards the foodborne pathogen *Bacillus cereus*

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Bactericidal activity of carvacrol towards the foodborne pathogen *Bacillus cereus*

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ABSTRACT

Carvacrol, a natural plant constituent occurring in oregano and thyme, was investigated for its bactericidal effect, towards the foodborne pathogen *Bacillus cereus*. Carvacrol showed a dose-related growth inhibition of *B. cereus*. At concentrations of 0.75 mM and above, total inhibition of the growth was observed. Below this concentration, carvacrol extended the lag-phase, reduced the specific growth rate and reduced the maximum population density. Incubation for 40 minutes in the presence of 0.75 to 3 mM carvacrol decreased the number of viable cells of *B. cereus* exponentially. Spores were found to be approximately 2.3 fold less sensitive to carvacrol than vegetative cells. *B. cereus* cells showed reduced susceptibility towards carvacrol at pH 7.0 compared to different values between pH 4.5 and 8.5. The culture and exposure temperatures had a significant influence on the survival of vegetative cells. The highest death rate of cells was observed at an exposure temperature of 30°C. Membrane fluidity was found to be an important factor influencing the bactericidal activity of carvacrol.

INTRODUCTION

B. cereus is a spore-forming foodborne pathogen often associated with meat products, soup, rice, milk and other dairy products. Between 1 and 20% of the total outbreaks of food infections in the world is caused by *B. cereus* (17). Growth of vegetative cells occurs within the temperature range of 10-50°C, with an optimum between 28 and 35°C. However, psychrotrophic variants of *B. cereus*, capable to grow at temperatures below 5°C have been identified (7, 19). Vegetative cells of *B. cereus* are easily inactivated by heating. Spores however can survive such treatment and after subsequent germination cause food spoilage (7). Therefore it is of great importance that additional preservative measures are taken that suppress outgrowth of *B. cereus* and inactivate spores. The present study evaluated whether antimicrobial compounds from herbs and spices are suitable candidates for such additional preservation measures.

Herbs and spices have been known for their antimicrobial and antioxidant activity since antiquity. Both activities are often associated with the essential oil fraction, which is mainly composed of different types of terpenes and phenols. The safe use of herbs or spices and the components contained in them has lead to their current status of being food-grade or Generally Recognised As Safe (GRAS) food ingredients. The interest in plant derived compounds is growing markedly due to the potential beneficial role that these compounds can play in food processing and the general desire to use more natural systems for food preservation. The use of antimicrobial plant compounds in food preservation has developed rather empirically. Consequently, their antimicrobial effect has rarely been studied on the cellular level. One of the essential oil components of which the antifungal and antibacterial effects have been studied is carvacrol, the major component in the essential oil fraction of

oregano (60-74% carvacrol) and thyme (45% carvacrol) (2, 18). This compound has been tested towards different microorganisms, e.g. *Fusarium* spp., *Aspergillus* spp., *Rhizobium leguminosarum, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium, Staphylococcus aureus, Bacillus subtilis, Vibrio vulnificus, Rhodobacter sphaeroides.* All these test showed carvacrol inhibited the growth of these strains (5, 12-14, 16, 21, 23-24). The effect of some herbs/spices or essential oil preparations towards the foodborne pathogen *B. cereus* has been partly investigated. These results show that many herbs and spices can inhibit the growth of *B. cereus*. Carvacrol inhibits the growth of *B. cereus*, even at low concentrations (4, 11, 15) . Whereas such studies indicate an antimicrobial effect of carvacrol, the influences of exposure temperature and duration as well as pH of the food medium are not well known. These factors are very important regarding application.

This study describes the effect of carvacrol on vegetative cells and spores of *B. cereus* in relation to carvacrol concentration, exposure time, temperature and medium pH. The mode of action of carvacrol towards the cell membrane has been studied and the correlation between its antibacterial effect and cell membrane fluidity has been evaluated.

MATERIALS AND METHODS

Bacterial strain and growth conditions

B. cereus IFR-NL94-25 (obtained from the Institute of Food Research, Norwich, UK) was used in all experiments. Cells were grown in Brain Heart Infusion (BHI) medium (Oxoid, Basingstoke, UK) supplemented with 0.5% (w/v) glucose (initial pH 6.7). Cell cultures were maintained at -80°C in 15% glycerol as a cryoprotectant.

Determination of antibacterial activity

To determine an effect of carvacrol on the growth of *B. cereus*, cells were cultivated at 30°C, washed twice in BHI and incubated at 8°C in BHI supplemented with different concentrations (0 to 3.75 mM) of carvacrol in microtiterplates. The starting optical density at 660 nm (OD_{660}) of the cell suspension was set at 0.02 (light path 1 cm). The OD_{660} was measured at different time intervals until a constant reading was observed.

Monitoring viability

Vegetative cells of *B. cereus* were harvested by centrifugation, washed twice in a 50 mM potassium-HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid) buffer (pH 7.0), containing 1 mM $MgSO_4$ and diluted to $OD_{660} \pm 0.1$. Suspensions of 20 ml were kept at a constant temperature (8° or 30°C). Carvacrol was added to give a final concentration between 0.5 and 3 mM. Samples were taken at different time intervals during exposure (maximum exposure time 40 min) and immediately diluted (10^2 to10^5 fold) in peptone-physiological salt

solution (1 g/l peptone and 8.5 g/l NaCl) to quench the influence of carvacrol. Serial dilutions were plated on BHI-agar plates and incubated for 24 hours at 30°C.

Spores were obtained by harvesting cells in the late stationary phase. The presence of spores was confirmed by light microscopy.

Influence of medium pH on antibacterial activity

Cells of an overnight culture of *B. cereus* were harvested by centrifugation and washed twice in a polybuffer. This buffer consisted of 10 mM Tris (Tris(hydroxymethyl)-aminomethane), 10 mM HEPES and 10 mM MES (2-(*N*-morpholino)ethanesulfonic acid). The desired pH-values were obtained by the addition of KOH or HCl. The amount of K^+ was made equal for all buffers with KCl to ensure that the antimicrobial activity determined was not affected by the potassium concentration. Finally, 1 mM $MgSO_4$ was added to all buffers. The viability of the cells was analysed at 30°C both in the absence and presence of 1.25 mM carvacrol for a period of 40 min, as described above.

Determination of phase transition temperature

The temperature dependent vibrational frequency of CH_2-stretches in lipids of whole *B. cereus* cells was measured with Fourier transformed infrared spectroscopy (FTIR) as an indication of membrane fluidity. FTIR measurements were carried out using a Perkin-Elmer (Beaconsfield, UK) series 1760 FTIR (detector: EXT MCT; beamsplitter: KBr; resolution: 4; apodization: weak; velocity: 2). Exponentially growing cells were harvested and washed twice in HEPES-buffer (pH 7.0). The pellet was placed between two CaF_2 windows. The sample was cooled to -50°C and subsequently heated (1.5°C/min) to 70°C. The spectra were recorded after every stepwise increase in temperature. The temperature control in this instrument was realised using a liquid nitrogen cooled cell equipped with a resistance heater (6).

Chemicals

Purified carvacrol was obtained from Fluka Chemie AG (Buchs, Switzerland). A stock solutions (1 M) was made in 95% ethanol. The final ethanol concentration in the experiments was always kept below 2% ethanol (v/v).

Growth data processing

To evaluate the bactericidal effect of carvacrol under different circumstances, the logarithm of the viable count was plotted against time. After calculating the slope (k) of the straight lines obtained, the $t_{1/2}$-value (time needed to kill 50% of the cells) was calculated using the following equation:

$$t_{1/2} = - \ln 2 / k \text{ (min)}$$

RESULTS

Antibacterial activity

To determine the effect of carvacrol on the growth of *B. cereus*, cells were cultured at 8°C in the presence of different concentrations of carvacrol (Fig. 1A). Carvacrol showed a dose-related inhibition of *B. cereus*. At 0.75 mM carvacrol, full suppression of growth was observed. Below this concentration carvacrol extended the lag phase and reduced both the specific growth rate and the maximum population density (Fig. 1B).

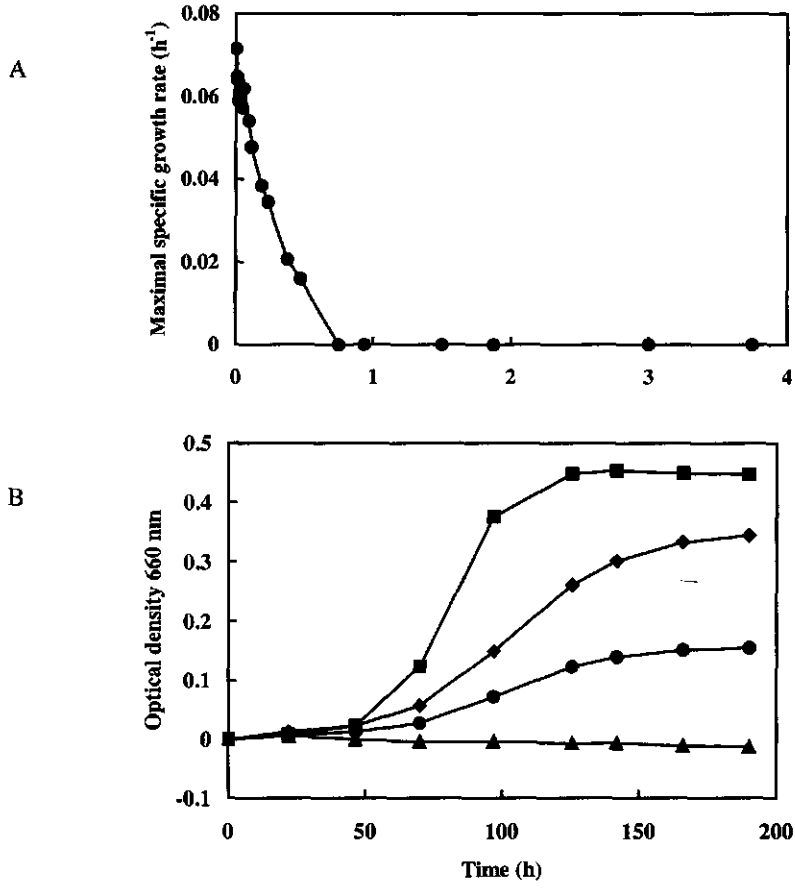

Figure 1. A: Maximal specific growth rate of *B. cereus* cultured in the presence of different concentrations of carvacrol. Cells were grown in BHI at 8°C. B: Growth of *B. cereus* (8°C) in the presence of different concentrations of carvacrol : 0 mM (■), 0.19 mM (◆), 0.23 mM (●) and 0.75 mM (and higher up to 3 mM) (▲). All data represent mean values of triplicate measurements.

Bactericidal or bacteriostatic activity.

To investigate whether carvacrol acts either as a bactericidal or as a bacteriostatic agent, washed cells were incubated in the presence of increasing concentrations of carvacrol. Samples were taken at regular time intervals during exposure and plated on BHI-agar plates to monitor the viable counts (Fig. 2). For all carvacrol concentrations tested, a log-linear relationship was observed between the time of exposure to carvacrol and the viable count of *B. cereus*, indicating that exposure to carvacrol resulted in an exponential decrease in the number of viable cells. *B. cereus* was found to be able to survive such a treatment at concentrations below 0.75 mM . The death rate and maximum level of the viable count was dependent on the carvacrol concentration. At 0.75 mM no growth but also no significant reduction of the initial viable count was observed ("bacteristatic effect"), while at 1.0 mM a very small reduction of the viable count was found. At 1.25 mM a very significant decline in viable count was found ("bactericidal effect"). Higher concentrations of carvacrol increased the death rate proportionally. A more than 10^4 fold reduction of the viable count was monitored within 40 seconds after the addition of 3 mM carvacrol (data not shown). These results show that carvacrol is bactericidal against *B. cereus* cells when present at 1 mM or higher, while 0.75 mM is the minimal inhibitory concentration (MIC) level.

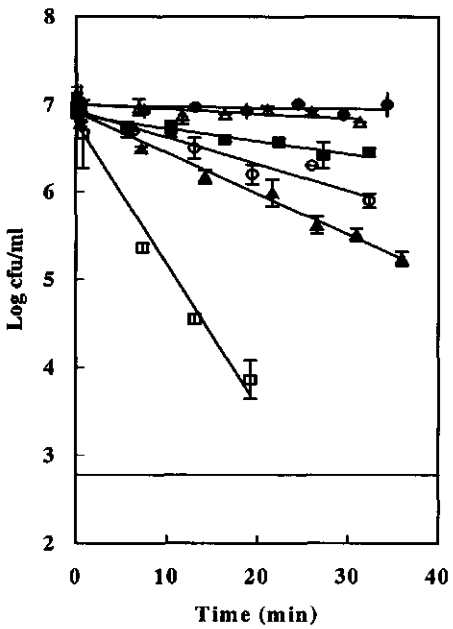

Figure 2: Viable count of *B. cereus* (log cfu/ml) at different time intervals after the addition of carvacrol. Cells were grown in BHI, washed and maintained in HEPES-buffer at pH 7.0. Growth and exposure temperature was 8°C. Carvacrol concentrations tested were: 0.75 mM (●), 1.0 mM (△), 1.25 mM (■), 1.5 mM (○), 1.75 mM (▲) and 2.0 mM (□).
—— = detection limit ($6.3 \cdot 10^2$ cfu/ml).
The data represent mean values of triplicate measurements. Error bars are indicated.

Spores versus vegetative cells

The difference in sensitivity between vegetative cells and spores was studied by exposing both cell types to carvacrol at 8°C (Fig. 3). At 1.75 mM carvacrol, hardly any effect on the

viability of spores was observed, while a sharp decrease in the number of viable vegetative lower then with vegetative cells under identical conditions. These results indicate that spores are approximately 2.3 fold less sensitive to carvacrol than vegetative cells. Viable counts of both vegetative cells and spores were reduced more than 10^4 fold within 40 seconds at carvacrol concentrations higher than 3 mM (data not shown).

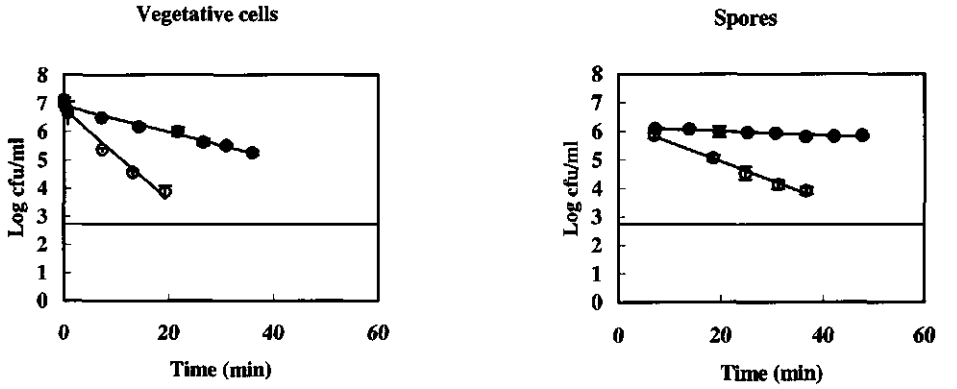

Figure 3: Viable count of vegetative cells and spores of *B. cereus* exposed to carvacrol at 1.75 mM (●) and 2 mM (○). Cells were grown in BHI, washed and maintained in HEPES-buffer at pH 7.0. Growth and exposure at 8°C. The data represent mean values of triplicate measurements. —— = detection limit ($6.3 \cdot 10^2$ cfu/ml)

Influence of pH on antibacterial activity

To investigate the effect of the pH of the buffer on the bactericidal effect of carvacrol, *B. cereus* cells were exposed to 1.25 mM carvacrol at 30°C and at different pH-values (Fig. 4). The $t_{1/2}$-values presented in Fig. 4 were corrected for the reduction of the viable count in the absence of carvacrol. At a pH 4.5 a 10^4 fold reduction of the viable count was observed in less than 50 sec (data not shown). *B. cereus* cells showed a reduced susceptibility towards carvacrol at pH 7.0. The death rate was increased again at higher pH-values.

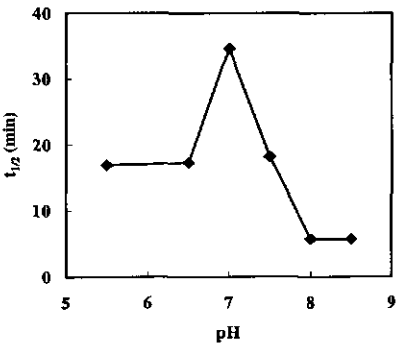

Figure 4: Effect of pH on death rate of *B. cereus* cells. Cells were grown in BHI, washed and exposed to carvacrol (1.25 mM) at different pH-values. Growth and exposure at 30°C. Data represent mean values of triplicate measurements.

Effect of temperature

To determine the effect of culture conditions and temperature on the antibacterial activity of carvacrol, *B. cereus* cells were first cultured at 8 or 30°C and subsequently exposed to carvacrol (0.5 - 3 mM) at both 8 or 30°C for a maximum duration of 40 minutes (Table 1). Cells cultured at 30°C and subsequently exposed to carvacrol were found to exhibit the highest death rate at 30°C. Exposure of these cells to carvacrol at 8°C resulted in a sharp decrease (about 11 fold at 1.5 mM carvacrol) of the death rate. When cells were grown at 8°C, the highest death rates were again observed at an exposure temperature of 30°C: about 4 fold higher compared exposure to 1.5 mM carvacrol at 8°C.

Table 1: Influence of the carvacrol concentration on $t_{1/2}$-values (min) of *B. cereus* at different growth and exposure temperatures. The data represent mean values of triplicate measurements.

	Growth temperature			
	8°C		30°C	
	Exposure at		Exposure at	
Concentration	8°C	30°C	8°C	30°C
0.5	-*	-	-	347
0.75	693	173	-	-
1.0	139	43	99	53
1.25	46	7	-	27
1.5	22	6	69	6.2
1.75	15	0.5	13	0.9
2.0	4.3	0.2	2.6	<0.1

-* = not determined

Phase transition temperature

To investigate a correlation between membrane fluidity and sensitivity towards carvacrol, the wavenumber of the CH_2-stretch of fatty acids in *B. cereus* cells was determined (Fig. 5). An increase in the wavenumber was observed at increasing temperature. This indicates that the membranes change their fluidity from a gel to a fluid crystalline state during an increase of the temperature. The melting temperature of the lipids was approximately 7.9°C for cells grown at 8°C and 31°C for cells grown at 30°C. Cells grown at 8°C have a higher fluidity at any temperature than cells grown at 30°C. The difference in fluidity between cells grown at respectively 8° and 30°C seems to be at maximum around 20°C (Fig. 5). The observed $t_{1/2}$-values at an exposure temperature of 20°C in the presence of 1.5 mM carvacrol were 14 and 46 min, for growth at 8 and 30°C, respectively. This difference in $t_{1/2}$-values is not at a maximum as can been seen in Table 1.

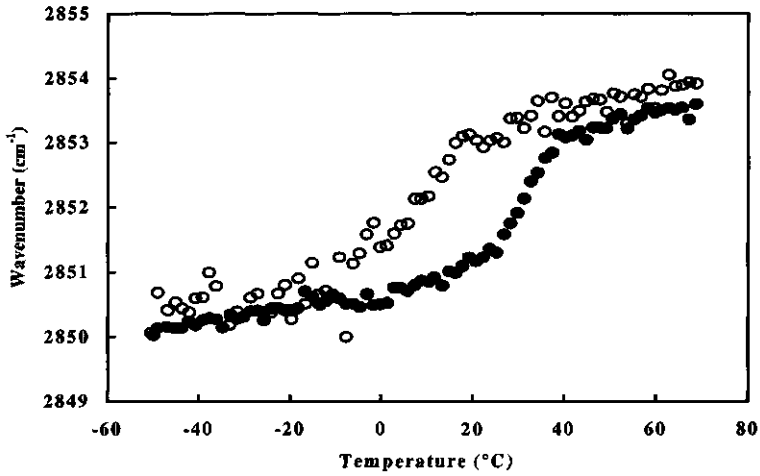

Figure 5: Phase transition temperature of *B. cereus* cultured in BHI at 8°C (○) and 30°C (●). Wavenumber of the CH_2-symmetric stretches in the lipids were measured using Fourier Transformed Infrared Spectroscopy (FTIR) at various temperatures.

DISCUSSION

This study describes the antibacterial effect of carvacrol towards the foodborne pathogen *B. cereus*. The antibacterial activity of carvacrol was found to be dependent on the concentration of carvacrol as well as on exposure time, pH and temperature. Sikkema *et al.*, (20) found that the accumulation of lipophilic compounds in the cell membrane (tested in liposomes prepared from *E. coli* phospholipids) is proportional to the concentration in the aqueous phase and the membrane-aqueous phase partition coefficient. When increasing the carvacrol concentration, more of the compound is expected to dissolve in the membrane and more damage of the membranes appears. The MIC-value of carvacrol for growth inhibition of *B. cereus* was found to be 0.75 mM. Below this concentration, *B. cereus* was able to partly recover from the inhibition by carvacrol. A change in the composition of the cell membrane as a result of altered partition characteristics of the lipophilic compound (carvacrol) may have effected the sensitivity (8). In addition, detoxification of carvacrol by cells of *B. cereus* may offer an explanation for this observation. At concentrations higher than 1.0 mM, the cells could not overcome the presence of carvacrol and the viable count diminished proportionally to the carvacrol concentration.

Spores of *B. cereus* were found to be less sensitive to carvacrol than vegetative cells. This is not unexpected, since spores are resistant to many chemicals due to the firm layer (coating and cortex) around the cells (9). However, at 3 mM carvacrol, a very short exposure time (40 sec) sufficed to reduce spore viability more than 10^4 fold.

The pH of the medium in which the cells were exposed to carvacrol had a strong influence on

the bactericidal activity. The lowest sensitivity was observed at pH 7.0. Sensitivities recorded at pH 5.5 and 8.0 were 2 and 6 fold higher, respectively. This agrees with the observation of Thompson (23), who found that carvacrol is more fungicidal against *Aspergillus* species at pH 4 and 8 than at pH 6. At high pH-values, carvacrol will partially dissociate due to which its hydrophobicity decreases. Consequently, the inhibitory effect should decrease at higher pH-values. However, the pK_a of phenols is approximately 10 and in the pH-range tested, the concentration of undissociated carvacrol will not have changed significantly and, thus, the observed influence of pH on the bactericidal activity cannot be explained by dissociation of the phenolic proton from the hydroxyl group of carvacrol.
With regard to the possible cellular target of carvacrol, it is important to consider the very hydrophobic nature of this compound. Due to this, it may interact with the phospholipid bilayer of the cell membrane, causing increased permeability and loss of cellular constituents (20, 24). In addition, the impairment of several enzyme systems, including those involved in energy production and synthesis of structural components, has been reported (3, 10, 16, 25).

The differences in sensitivity of *B. cereus* towards carvacrol observed at different growth and exposure temperatures may be explained by the influence of the culture and/or exposure temperature on the lipid composition of acyl lipids in the membranes. It has been known for many years that the lipid composition of membranes depends on the incubation temperature (1). In bacteria containing *iso*- and *anteiso*-branched fatty acids (e.g. *B. cereus*), the adaptation to variations in the environmental temperature involves changes in (1) the type of branching in the methyl end of the fatty acid, (2) fatty acid chain length or (3) degree of fatty acid unsaturation. One of the most common changes observed in fatty acid branching in response to a low temperature, is an increase in the proportion of the lower-melting *anteiso*-branched acids and a concomitant decrease in the higher-melting, *iso*-branched acids. This increase in fatty acid unsaturation and decrease in fatty acid chain length lowers the gel to liquid crystalline phase transition temperature and increases the passive permeability of the membrane (22). This may also explain why cells are more sensitive at an exposure temperature of 30°C than at 8°C. At 30°C carvacrol migrates more easily into the membrane of *B. cereus* because of its higher fluidity as compared to cells at 8°C. The resulting relatively higher carvacrol concentration in the membrane at 30°C may cause a higher death rate.
The highest death rates of *B. cereus* in the presence of carvacrol were observed when cells were cultured at 8°C and subsequently exposed at 30°C. When these cells were exposed to carvacrol at 8°C they had a lower wavenumber than cells exposed at 30°C, corresponding to a lower membrane fluidity and higher membrane fluidity respectively (physical process) (Fig. 5). Growth at 8°C probably leads to changes in the lipid composition (chemical process) necessary to maintain an adequate proportion of the liquid-crystalline lipid and an ideal physical state of cellular membranes. It has been postulated that the proportion of the lower-

melting lipids increases on decreasing the growth temperature (22). If these cells are transferred to 30°C, the membrane becomes more fluid, because of the higher temperature (physical process). When the fluidity increases, more carvacrol can dissolve in the membranes (changing partition coefficient) and carvacrol accumulates to higher concentrations, which finally leads to more damage of the membranes.

The phase transition temperature of *B. cereus* cultures pre-grown at different temperatures was found to be almost equal to the growth temperature, indicating that the membrane composition is adjusted to the actual growth temperature. These changes in the lipid composition of the cell membrane are essential for the maintenance of the membrane integrity between the gel and liquid-crystalline state.

Considering the membrane fluidity, the largest difference in sensitivity could be expected at an exposure temperature of 20°C (Fig. 5). At this temperature, the differences in membrane fluidity between 8°C and 30°C cultured cells were at a maximum. However, no larger difference in death rate was observed at 20°C compared to exposure at 8°C or 30°C.

In conclusion, carvacrol at 0.75 mM or above is bactericidal against the foodborne pathogen *B. cereus*. Cells of *B. cereus* grown and exposed at 8°C are less sensitive than cells grown and exposed at 30°C, although the membrane fluidity is the same in both cases. These results show that the membrane fluidity is an important factor affecting the sensitivity of *B. cereus* towards carvacrol, but probably not the only factor. The changes in the cellular membrane (lipid membrane composition, leakage of essential components) have to be studied in more detail to obtain more insight in the mechanisms underlying the bactericidal effect of carvacrol towards *B. cereus*.

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3

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ABSTRACT

Carvacrol, a naturally occurring compound, mainly present in the essential oil fraction of oregano and thyme, was studied for its effect on bioenergetic parameters of vegetative cells of the foodborne pathogen *Bacillus cereus*. Incubation for 30 minutes in the presence of 1 to 3 mM carvacrol reduced the viable cell numbers exponentially. Carvacrol (2 mM) significantly depleted the intracellular ATP pool to values close to zero within 7 min. No proportional increase of the extracellular ATP pool was observed. Depletion of the internal ATP pool was associated with a change of the membrane potential ($\Delta\psi$). At concentrations of 0.01 mM carvacrol and above, a significant reduction of $\Delta\psi$ was observed, leading to full dissipation of $\Delta\psi$ at concentrations of 0.15 mM and higher. Finally, an increase of the permeability of the cytoplasmic membrane for protons and potassium ions was observed (at 0.25 and 1 mM carvacrol, respectively). From this study, it could be concluded that carvacrol interacts with the membranes of *B. cereus* by changing its permeability for cations such as H^+ and K^+. The dissipation of ion gradients leads to impairment of essential processes in the cell and finally to cell death.

INTRODUCTION

Bacillus cereus is a spore-forming foodborne pathogen often associated with food products such as meat, vegetables, soup, rice, milk and other dairy products. Between 1 and 20% of the total number of outbreaks of food infection in the world is caused by *B. cereus* (19). Growth of vegetative cells usually occurs within the temperature range of 10 to 50°C, with an optimum between 28 and 35°C. However, psychrotrophic variants of *B. cereus*, capable of growing at temperatures below 5°C, have been identified (6, 22). Although vegetative cells of *B. cereus* can easily be inactivated by heating, spores are considerably more resistant and can cause food spoilage after subsequent germination (6).

Mild preservation technologies are becoming more important in modern food industries. As a consequence, spore forming microorganisms are likely to proliferate and hence become a serious food safety risk. Mild processes are often combined to obtain safe products with improved organoleptic quality. A novel way to reduce the proliferation of microorganisms is the use of essential oils. The antifungal and antibacterial effects of these components on different microorganisms have been described in several studies (5, 14, 16-18, 26-29). Among the diverse group of chemical components in essential oils, carvacrol exerts a distinct antimicrobial action. Carvacrol is the major component of the essential oil fraction of oregano (60-74% carvacrol) and thyme (45% carvacrol) (1, 20). In practice, carvacrol is added to different products, e.g. baked goods (15.75 ppm), non-alcoholic beverages (28.54 ppm; 0.18 mM), chewing gum (8.42 ppm), etc. (8). However, not much is known about the mechanisms of action of this compound. A better knowledge of the mode of action is

important regarding application in food systems. Recently, we (29) showed the antimicrobial effect of carvacrol on *B. cereus*. Hydrophobic compounds such as carvacrol are likely to have an influence on biological membranes. The cytoplasmic membrane of bacteria has two principal functions: 1) barrier function and energy transduction, which allows the membrane to form ion gradients that can be used to drive various processes and 2) formation of a matrix for membrane embedded proteins (such as the membrane integrated F_0-complex of ATP-synthase) (12, 24).

In the present study, changes in the energy transducing processes of *B. cereus* caused by carvacrol were studied in more detail. The effect of carvacrol on the intracellular ATP pool, the membrane potential, the pH-gradient across the cytoplasmic membrane and the potassium gradient was evaluated.

MATERIALS AND METHODS

Bacterial strain and growth conditions

B. cereus IFR-NL94-25 (obtained from the Institute of Food Research, Norwich, UK) was used throughout this study. Cells were grown in Brain Heart Infusion (BHI) medium (Oxoid) supplemented with 0.5% (w/v) glucose (initial pH 6.7) at 30°C. Cell cultures were maintained at -80°C in 15% glycerol as a cryoprotectant.

Chemicals

Purified carvacrol was obtained from Fluka Chemie AG (Buchs, Switzerland). A stock solution (1 M) was made in 95% ethanol. The final ethanol concentration in the experiments was always kept below 2% ethanol (v/v).

Monitoring viability

Vegetative cells of *B. cereus* were harvested by centrifugation, washed twice in a 50 mM potassium-HEPES (N-2-hydroxyethylpeperazine-N-2-ethanesulfonic acid) buffer (pH 7.0), containing 1 mM $MgSO_4$ and diluted to an Optical Density at 660 nm (OD_{660}) of 0.1 (light path 1 cm). Suspensions of 20 ml were kept at 20°C. Carvacrol was added to a final concentration between 1 and 2 mM. Samples were taken every 5 minutes during exposure (maximum exposure time 40 min) and immediately diluted (10^2 to 10^5 fold) in peptone-physiological salt solution (1 g/l peptone and 8.5 g/l NaCl) to quench the influence of carvacrol. Serial dilutions were plated on BHI-agar plates and incubated for 24 hours at 30°C.

Determination of intra- and extracellular ATP concentration

Cells of an overnight culture were washed three times in a 25 mM potassium phosphate buffer (pH 7) and a suspension was prepared with an OD_{660} of 1 (light path 1 cm). The

experiment was started by adding glucose to a final concentration of 0.5% (w/v). Samples of 200 µl were taken at every 2 minutes and added to Eppendorf tubes, containing 200 µl of a mixture of silicon oil (AR200:AR20=2:1) (Wacker Chemicals, Munich, Germany) on top of 100 µl TCA-EDTA buffer (10% trichloroacetic acid and 2 mM ethylenediaminetetraacetic acid) and centrifuged directly (5 min 12000 x *g*). The extracellular (upper layer) and the intracellular (lower layer) ATP concentrations were measured using a 1243-107 ATP Assay Kit (Bio-Orbit, Turku, Finland). Luminescence was recorded with a model 1250 luminometer (Bio-Orbit, Turku, Finland).

Influence of carvacrol on the membrane potential ($\Delta\psi$)

Cells of an overnight culture were washed twice in a 50 mM potassium-HEPES buffer (pH 7.0), containing 1 mM $MgSO_4$. The cell pellet was diluted until an OD_{660} (light path 1 cm) of 10 was reached. Exactly 30 µl of the cell suspension was diluted in 2 ml buffer, containing 5 µM 3,3-dipropylthiacarbocyanine ($DiSC_3(5)$) (Molecular Probes, Leiden, The Netherlands). The membrane potential ($\Delta\psi$) was monitored with a Perkin Elmer LS 50B spectrofluorometer at 20°C (excitation wavelength 643 nm; emission wavelength 666 nm). Following equilibration, 15 mM glucose was added to energise the cells. After a constant reading had been reached, 1 nM nigericin was added to diminish the pH-gradient across the cytoplasmic membrane. After a steady fluorescence reading was reached, different concentrations of carvacrol (0.01 - 2 mM) were added. Valinomycin (1 nM) was added as a control.

Intracellular pH measurements

The determination of the intracellular pH was based on the method described by Breeuwer *et al.* (3). Cells of an overnight culture were harvested, washed three times in a 50 mM HEPES-buffer (pH 7.0) and diluted to an OD_{660} (light path 1 cm) of 1. Subsequently, cells were incubated in the presence of 1.5 µM carboxyfluorescein diacetate succinimidyl ester (cFDASE) for 10 minutes at 30°C. cFDASE is hydrolyzed to cFSD (carboxyfluorescein succinimidyl ester) in the cell and subsequently conjugated to aliphatic amines. After washing with 50 mM potassium phosphate buffer (pH 5.81), cells were incubated with 10 mM glucose for 30 minutes at 30°C to eliminate non conjugated cFSE. In addition, cells were washed twice, resuspended in 50 mM potassium phosphate and kept on ice until further use. The analysis was started by adding 30 µl of the cell suspension in a quartz cuvette containing 3 ml of a 50 mM potassium phosphate buffer (pH 5.81), placed in a cuvette holder of a spectrofluorometer (Perkin-Elmer LS 50B) and stirred continuously. Fluorescence intensities were measured at excitation wavelengths of 490 nm (pH sensitive) and 440 nm (pH insensitive) by rapidly altering the monochromator between both wavelengths. The emission was determined at 525 nm and the excitation and emission slit widths were set on 5 and

10 nm respectively. The intracellular pH was calculated from the ratio of the emission at 490 and 440 nm excitation. A calibration curve was determined in buffers with pH-values ranging from pH 3 to 10. Buffers contained 50 mM glycine, 50 mM citric acid, 50 mM $Na_2HPO_4.2H_2O$ and 50 mM KCl. pH-values were adjusted with NaOH or HCl. The pH_{in} and pH_{out} (intracellular and extracellular pH, respectively) were equilibrated by addition of 1 µM valinomycin and 1 µM nigericin.

Determination of intra- and extracellular amount of potassium

Exponentially growing cells (overnight culture) were harvested and washed twice in a 50 mM sodium-HEPES buffer (pH 7.0). Cells were concentrated till an OD_{660} of 1 (light path 1 cm) was reached. The extraction of potassium from the cells was carried out as described for the ATP determination. The potassium concentration was measured with a Flame Photometer (Jenway, Felsted, England) after diluting the samples in distilled water. Values were compared with a standard calibration curve of KCl.

Protein determination

The determination of the amount of protein in the cells of *B. cereus* was carried out according to work of Lowry *et al.* (21) with bovine serum albumin as a standard.

RESULTS

Viability of *B. cereus* in the presence of carvacrol

Carvacrol inhibits the growth of *B. cereus* effectively. Incubation and exposure temperatures significantly affect the death rate of *B. cereus* (29). The viability of *B. cereus* cells, cultured at 30°C was determined under conditions (pH 7, 20°C) which were used throughout this study.

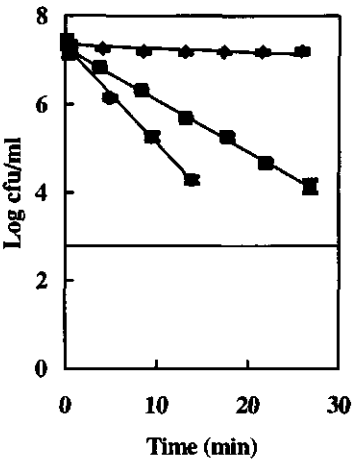

Figure 1: Viable count of *B. cereus* (log cfu/ml) at different time intervals after the addition of carvacrol. Cells were cultured in BHI (30°C), washed and maintained in HEPES-buffer at pH 7.0 (20°C). Carvacrol concentrations tested were: 1 mM (◆), 1.25 mM (■) and 1.5 mM (●). The data represent mean values of triplicate measurements. Error bars are indicated. —— represents the detection limit ($6.3 \cdot 10^2$ cfu/ml).

Samples were taken every 5 minutes and plated on BHI plates to monitor the viable count (Fig. 1). A log-linear relationship was found between the time of exposure and the viable count of *B. cereus*. At 1 mM carvacrol almost no reduction of the viable count was observed after 30 min, while 1.25 mM and 1.5 mM carvacrol resulted in a clear reduction of the viable counts. Therefore it can be concluded that carvacrol is bactericidal towards *B. cereus* cells at 20°C when present at concentrations above 1 mM.

Effect of carvacrol on ATP pools

The bactericidal activity may lie in the disruption of the membrane integrity, since carvacrol is a lipophilic compound preferentially partitioning in this cell compartment. Cytoplasmic membrane disruption is expected to have a large impact on the membrane associated energy transducing system. Therefore the effect of carvacrol on the intra- and extracellular ATP pools was studied. Addition of 1 mM carvacrol decreased the intracellular amount of ATP to values close to zero within 14 minutes (Fig. 2A). No increase of the extracellular ATP pool was observed. Similar results were obtained with the addition of 2 mM carvacrol (Fig. 2B). The intracellular ATP pool was reduced to zero within 10 min. A small increase of the extracellular ATP pool was observed, although this was not proportional to the decrease of the intracellular ATP pool.

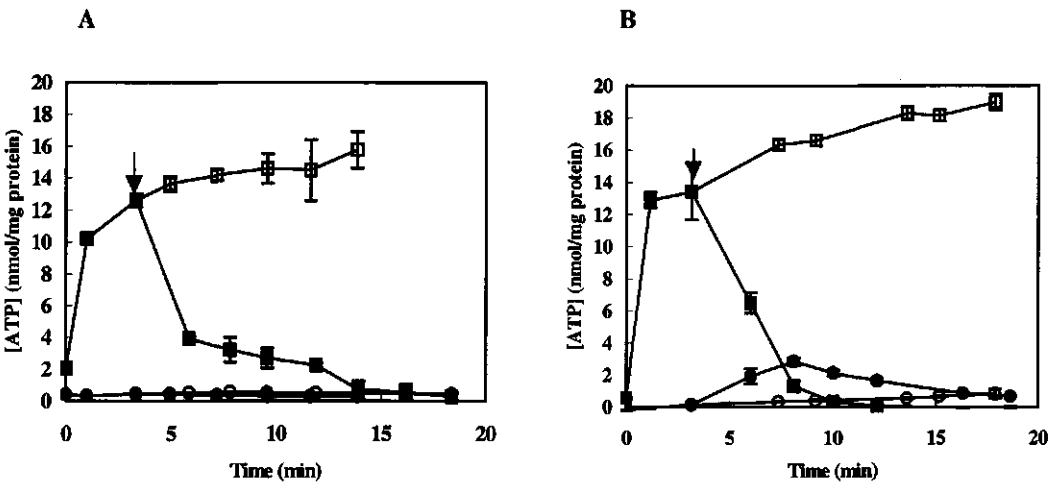

Figure 2: Intracellular (■/□) and extracellular (●/○) ATP-pools of glucose energised vegetative cells of *B* cereus in the absence (open symbols) or presence (closed symbols) of 1 mM (A) or 2 mM (B) carvacrol of glucose energised vegetative cells of *B. cereus*. Carvacrol was added at t = 4 min (indicated by arrow). Values represent the mean of duplicate measurements. Error bars are indicated.

Influence of carvacrol on membrane potential ($\Delta\Psi$)

Depletion of the internal ATP pool by carvacrol may be associated with a reduced ATP synthesis. Therefore, we investigated the effect of carvacrol on the membrane potential, the driving force for ATP synthesis. Changes in the membrane potential can be visualised by changes in the fluorescence of a potentiometric dye. *B. cereus* cells were incubated in the presence of $DiSC_3(5)$. After the addition of glucose and nigericin, carvacrol was added (Fig. 3). Carvacrol reduced the membrane potential if present at 0.01 mM or higher. Increased concentrations caused a higher rate of reduction (0.01 units/s and 0.25 units/s at 0.01 and 0.5 mM, respectively) and a lower steady state membrane potential was reached. At concentrations higher than 0.15 mM a complete dissipation of the membrane potential was observed.

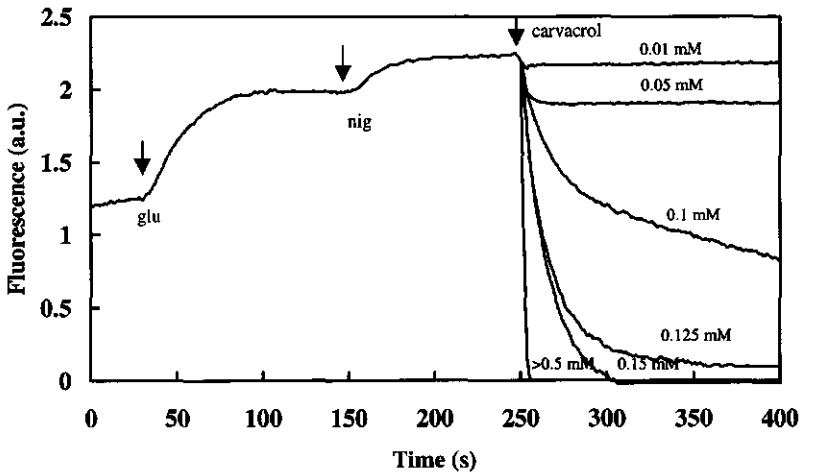

Figure 3: Effect of carvacrol on the membrane potential of *B. cereus* in the presence of glucose (glu), nigericin (nig) and different concentrations of carvacrol. Exponentially growing cells were washed and maintained in HEPES-buffer at pH 7.0 (20°C). Carvacrol was added at t = 250 s. The membrane potential was monitored using the fluorescent probe $DiSC_3(5)$ (arbitrary units).

Effect of carvacrol on intracellular pH

Depletion of intracellular ATP and dissipation of $\Delta\psi$ by carvacrol suggest effects on ion gradients across the cellular membrane. Therefore the pH_{in} was investigated in more detail. To rule out other essential gradients apart from proton gradients, all measurements were carried out in the presence of valinomycin. The pH_{in} of *B. cereus*, cultured in BHI at pH 6.7 and washed in HEPES-buffer (pH 5.81), was approximately 7.1. Addition of glucose and valinomycin did not affect the pH_{in}. As expected, nigericin dissipated the pH-gradient across the membrane (data not shown). Exposure of the cells to carvacrol (Fig. 4) decreased pH_{in}. In the presence of 0.25 mM carvacrol, the pH-gradient across the cell membrane was reduced to

1 unit. A further reduction of the ΔpH to 0.5 units was observed with 0.5 mM carvacrol. No effect on the ΔpH was observed at concentrations below 0.25 mM. Complete dissipation of the pH-gradient was reached in the presence of 1 mM carvacrol or higher.

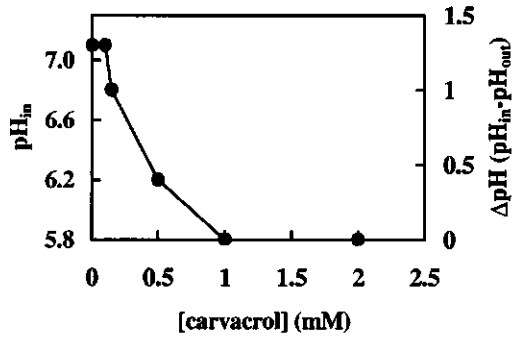

Figure 4: Effect of carvacrol on the pH_{in} and ΔpH of vegetative cells of *B. cereus*. Cells were cultured in BHI, washed and incubated in a 50 mM potassium phosphate buffer (pH 5.8) (see materials and methods).

Influence of carvacrol on potassium efflux

Carvacrol affects the intracellular ATP concentrations and the transmembrane electrical potential and dissipates the ΔpH. Subsequently, the effect of carvacrol on the permeability of the membrane towards potassium ions was investigated.

Addition of glucose (to energise the cells) at t=0 caused an approximately 100% increase of the intracellular potassium pools (K^+_{in}) (Fig. 5). Extracellular pools (K^+_{out}) decreased from 0.98 µmol/l to 0.67 µmol/l. Addition of 1 mM carvacrol after 5 minutes of incubation rapidly decreased the intracellular amount of K^+. After 9 minutes of incubation the intracellular amount of K^+ was reduced from 12 µmol/mg cell protein (at t = 5 min) to 0.99 µmol/mg cell protein and the extracellular K^+ was raised from 0.67 µmol/l after 5 minutes to 1.14 µmol/l at 9 minutes of incubation. The total amount of potassium (K^+_{in} + K^+_{out}) remained constant throughout the experiment.

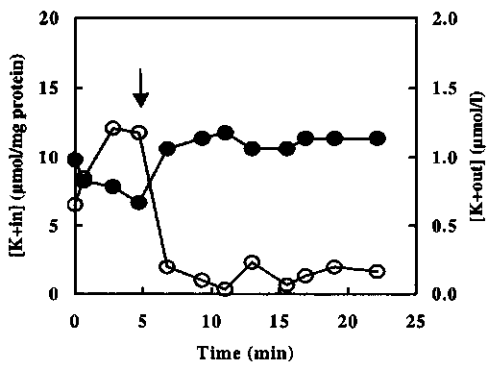

Figure 5: Changes in intracellular (K^+_{in}) (○) and extracellular (K^+_{out}) (●) potassium pools of *B. cereus* cells during exposure to 1 mM carvacrol. Cells were cultured in BHI, washed and maintained in Na-HEPES-buffer (pH 7.0). Carvacrol was added at t = 5 min.

DISCUSSION

This study describes the effect of carvacrol on cells of the foodborne pathogen *B. cereus*. Carvacrol acts as a bactericidal compound, with its activity being dependent on the concentration and the time of exposure. Sikkema *et al.* (23) found that the accumulation of lipophilic compounds in the cell membrane (tested in liposomes prepared from *E. coli* lipids) is proportional to the concentration in the aqueous phase and the membrane aqueous phase partition coefficient. Enomoto *et al.* (7) observed a decrease of $\Delta\psi$ in liposomes during exposure to some fragrance compounds. These hydrophobic compounds dissolve in the membrane and their activity was closely correlated with the membrane fluidity. Based on these studies it is expected that more carvacrol dissolves in the membrane at higher concentrations. Our study has shown that exposure to carvacrol leads to a decrease of the ATP_{in} concentration. No proportional increase of the ATP_{out} was observed. Therefore it is concluded that carvacrol does not enhance the permeability of the membrane for ATP. Consequently depletion of the internal ATP pool results from a reduced rate of ATP synthesis or increased ATP hydrolysis. A depletion of the ATP pool upon the addition of a lipophilic component has been observed in different studies (13, 15). In contrast to the present study, Helander *et al.* (11) observed a leakage of ATP from cells which were exposed to carvacrol (2 mM). However, this study was carried out with Gram negative bacteria which have a different cell envelope.

The observation that already low concentrations carvacrol (> 0.01 mM) cause a decrease of the membrane potential, suggests that the membrane becomes more permeable for protons. This conclusion is supported by the observation that exposure of cells to carvacrol also causes dissipation of the proton gradient across the membrane. In accordance with these results, Sikkema *et al.* (25) showed an increased proton permeability of liposomal membranes during exposure to tetralin. Similarly, Cartwright *et al.* (4) described dissipation of the ΔpH in the presence of ethanol, due to an increased influx of protons.

Analysis of the intracellular and extracellular potassium pools revealed an increased permeability of the cell membrane for K^+ upon exposure to carvacrol. K^+ is the major cytoplasmic cation of growing bacterial cells, involved in several key functions of bacterial cells. This ion plays a role in the activation of cytoplasmic enzymes, the maintenance of turgor pressure and possibly in the regulation of the cytoplasmic pH (2). Different studies showed that an efflux of potassium ions is a first indication of membrane damage in bacteria (10, 24, 30). $\Delta\psi$ depends mainly on the potassium concentration in the cell (2). Heipieper *et al.* (9) showed a significant excretion of K^+ to the external environment during exposure of *Pseudomonas putida* P8 to phenol. Gradients of solutes across the cytoplasmic membrane which use H^+ as the coupling ion, can also be affected by a dissipation of the proton motive force.

Although there was no immediate effect of carvacrol on the viability at concentrations of

1 mM and lower, clear effects on different bioenergetic parameters have been observed. Cells can probably cope with very low concentrations of carvacrol. Not only reduction of ATP synthesis by a dissipation of the proton motive force, but also other (secondary) effects of carvacrol may result in the bactericidal or bacteriostatic action. For example, an inhibition of several enzymes due to leakage of essential ions, loss of turgor pressure, influence on DNA synthesis, reduced metabolic activities and other processes in the cell can be a cause of the decreased viability during exposure to carvacrol. A loss of membrane integrity due to disturbance of hydrophobic interactions between lipids and proteins is often an important factor when considering the activity of toxic compounds (24). It can be concluded that the hydrophobic compound carvacrol interacts with the membranes of *B. cereus* by changing its permeability for cations such as H^+ and K^+. The dissipation of ion-gradients leads to impairment of essential processes in the cell and finally to cell death.

This study shows that carvacrol has biological effects at concentrations which are relevant for flavouring of foods (e.g. non-alcoholic beverages (0.18 mM/28.54 ppm), baked goods (15.75 ppm)) (8). To products associated with outbreaks of *B. cereus* (e.g. rice, pasta, soup), carvacrol could be applied both as an antimicrobial and as a flavouring compound.

ACKNOWLEDGEMENTS

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Adaptation of the foodborne pathogen *Bacillus cereus* to carvacrol

This paper is submitted for publication as:
Adaptation of the foodborne pathogen *Bacillus cereus* to carvacrol
Ultee, A., E.P.W. Kets, M. Alberda, F.A. Hoekstra and E.J. Smid
Archives of Microbiology.

ABSTRACT

Carvacrol, a natural antimicrobial compound present in the essential oil fraction of oreganum and thyme, is bactericidal towards *B. cereus*. A decrease of the sensitivity of *B. cereus* towards carvacrol was observed after growth in the presence of non-lethal carvacrol concentrations. This adaptation was studied in more detail. Measurement of the membrane fluidity showed cells adapted to 0.4 mM carvacrol (30°C) have a lower membrane fluidity than non-adapted cells. Adaptation to 0.4 mM carvacrol increased the phase transition temperature of the lipid bilayer (T_m) from 20.5°C to 28.3°C. The addition of carvacrol to cell suspensions of adapted *B. cereus* cells decreased T_m again to 19.5°C, approximately the same value as for the non-adapted cells in the absence of carvacrol. During adaptation changes in the fatty acid composition and head group composition were observed. From this study, it could be concluded that *B. cereus* adapts to carvacrol when present at non-lethal concentrations in the growth medium by lowering its membrane fluidity, caused by changes in the fatty acid and head-group composition.

INTRODUCTION

The use of natural antimicrobial systems for the preservation of foods is increasing. An example of a natural preservative agent is carvacrol. Carvacrol is present in the essential oil fraction of oreganum (60 - 74% carvacrol) and thyme (45% carvacrol) (1, 16). Several studies showed the inhibition of growth of different microorganisms by carvacrol (5, 11, 13-15, 25, 27-29). Recently, we described the inhibition of *B. cereus* by carvacrol in more detail. Addition of carvacrol to vegetative *B. cereus* cells results in a dose-related extension of the lag-phase and a lower final population density. Above approximately 1 mM (dependent on temperature) carvacrol decreases the viability exponentially. Carvacrol is a hydrophobic compound and is likely to dissolve in the hydrophobic domain of the cytoplasmic membrane, between the lipid acyl chains. Exposure of *B. cereus* to carvacrol leads to a leakage of potassium ions, a decrease of the intracellular pH, a collapse of the membrane potential and inhibition of ATP synthesis. Finally, these events lead to cell death (29, 30).

In general, three physical phases of bacterial membranes can be distinguished: a gel phase bilayer (ordered lipid chains), a liquid-crystalline phase bilayer (disordered lipid chains) and a hexagonal-II structure (20, 26). For optimal biological functionality of membranes, they are maintained in the fluid liquid-crystalline state (9). Biological membranes are involved in maintaining the integrity of the cell by providing a selective permeable barrier and protective environment for hydrolytic enzymes and toxins. The gel to liquid-crystalline phase transition temperature (T_m) of the major membrane constituents primarily influences the flexibility and stability of the membrane (26). Toxic compounds can have an effect on these membrane properties. To withstand harmful effects of cyto-toxic compounds, bacterial cells possess

several adaptation mechanisms. Two different mechanisms compensate for the accumulation of toxic hydrophobic compounds in the membrane: 1) keeping an adequate proportion of the liquid-crystalline lipid in the membrane (restoration of membrane fluidity) and 2) the maintenance of a proper balance between bilayer and non-bilayer promoting phospholipids (keeping membrane integrity) (4, 22, 31).

The most often observed adaptations in bacterial cells is a change in the fatty acid composition of the cytoplasmic membrane (3, 4, 7-10, 18, 21, 24): (1) to avoid a deleterious effects of the toxins on the membrane and/or (2) to reduce the concentration of the toxins in the membrane (31). The fatty acid (moieties of the membrane phospholipids) participate in the regulation of the activity of membrane-bound enzymes. *B. cereus* contains *iso*- and *anteiso*-branched fatty acids and the mode of adaptation will have an effect on the type of branching, chain length and the degree of fatty acid unsaturation.

Another important mechanism involved in adaptation of the cell membrane to the presence of lipophilic compounds, is an alteration of the phospholipid head group composition of the membrane (4, 9, 12, 31). The polar head groups are important for the barrier function of membranes (31).

Before applying carvacrol in food systems to prevent outgrowth of *B. cereus*, it is important to retrieve more information about possible adaptation of *B. cereus* towards this compound. In addition for a study of the effect of incubation of *B. cereus* in the presence of carvacrol on the viability, different membrane properties such as the membrane fluidity, phase transition temperature (T_m), fatty acid composition and lipid head group composition were investigated in the presence of this compound.

MATERIALS AND METHODS

Bacterial strain and growth conditions

Bacillus cereus IFR-NL94-25 (obtained from the Institute of Food Research, Norwich, UK) was used in the experiments. Cells were grown in Brain Heart Infusion (BHI) medium (Oxoid) supplemented with 0.5% (w/v) glucose (initial pH 6.7). Cell cultures were maintained at -80°C in 15% glycerol as a cryoprotectant.

In all experiments, an overnight culture of *B. cereus* was 100 times diluted in fresh BHI + 0.5% glucose to which 0 – 0.4 mM carvacrol was added (30°C). The culture was incubated approximately 4 hours at 30°C. Finally, cells were harvested at OD_{660}= 0.1 (light path 1 cm) and washed in buffers (dependent on assay).

Monitoring viability

Cells were cultured in the presence of 0 to 0.4 mM carvacrol, harvested by centrifugation (3000 *x g*) and washed twice in a 50 mM potassium-HEPES (*N*-2-hydroxyethylpiperazine-*N*-

2-ethanesulfonic acid) buffer (pH 7.0), supplemented with 1 mM $MgSO_4$. Diluted cell suspensions (20 ml, OD_{660} = 0.1 (light path 1 cm)) were kept at 20°C until further use. Carvacrol was added to give a final concentration of respectively 1.25 mM and 1.75 mM. Samples were taken at different time intervals during exposure (maximum exposure time 40 min) and immediately diluted (10^2 to10^5 fold) in peptone-physiological salt solution (1 g/l peptone and 8.5 g/l NaCl) to quench the influence of carvacrol. Serial dilutions were plated on BHI-agar plates and incubated for 24 hours at 30°C.

Inactivation kinetics

To evaluate the bactericidal effect of carvacrol under different circumstances, the logarithm of the viable count was plotted against time. After calculating the slope (k) of the straight lines obtained, the $t_{1/2}$-value (time needed to kill 50% of the cells) was calculated using the following equation:

$$t_{1/2} = - \ln 2 / k \quad \text{(min)}$$

Determination carvacrol concentration

To investigate the possibility of bioconversion of carvacrol by *B. cereus*, the concentration of carvacrol was monitored for 74 hours during incubation in BHI + 0.5% glucose (30°C). Samples (1 ml) were taken from the suspension and centrifuged (5 min 13000 *x g*). The supernatant (S_1) was removed, the pellet resuspended in 1 ml deionised water and centrifuged (5 min 13000 *x g*). The second obtained supernatant was added to S_1 and further analysed on HPLC using a Nova Pak C18 column (250 * 4 mm, Waters Corporation, Milford, USA), eluted with acetonitril:water:2% tetraethylammoniumhydroxide:2M citric acid (100:100:1:1) as mobile phase. Peaks were analysed spectroscopically at 283 nm using a UV-VIS detector (Waters Corporation, Milford, USA).

Determination of phase transition temperature

The CH_2-stretching vibration band (2854-2850 cm^{-1}) in whole *B. cereus* cells was measured by Fourier Transform Infrared Microspectroscopy (FTIR) as an indication of membrane fluidity. IR spectra were recorded using a Perkin-Elmer 1725 Fourier transformed IR spectrometer (Perkin-Elmer, Beaconsfield, Buckinghamshire, UK) equipped with a liquid nitrogen-cooled mercury/cadmium/telluride detector and Perkin-Elmer microscope. Exponentially growing cells, incubated in the presence of carvacrol (0 - 0.4 mM) were harvested by centrifugation and washed four times in 25 mM potassium phosphate buffer (pH 7.0). The pellet was placed between two CaF_2 windows (13 mm diameter) and fitted in a liquid nitrogen-cooled, temperature-controlled brass cell. The sample was cooled to -40°C and subsequently heated (1.5°C/min) to 90°C. The spectra were recorded every minute (32).

Lipid extraction

Cultures (2.4 l) of exponentially growing cells of *B. cereus* were exposed to 0 (30°C and 37°C) and 0.4 mM (30°C) carvacrol, centrifuged (10 min 6000 x *g*) and washed twice in 50 mM potassium phosphate buffer (pH 7). Lipids were extracted with chloroform-methanol-water (10:5:4) as described by Bligh and Dyer (2) and detailed by Galinski *et al.* (6).

Determination fatty acid composition

Fatty acid methyl esters were prepared by incubating lipid extracts for 15 minutes at 95°C in boron tri-fluoride-methanol (19). The fatty acid methyl esters were extracted with hexane. The analysis of the fatty acids was performed by gas chromatography with a capillary column model CP-Sil 88 (50 m x 0.25 mm, film thickness 0.20 μm), using a temperature program of 4 min 160°C, 4°C/min to 220°C (1 min) and a flame ionisation detector. The instrument used was a CP-9000 gas chromatograph (Chrompack-Packard, Middelburg, The Netherlands). The fatty acids were identified with the aid of standards. The relative amounts of the fatty acids were determined from peak areas of the methyl esters with a Chromatopac C-R6A integrator (Shimadzu, Kyoto, Japan).

Determination head group composition

The head group composition of the lipid extracts was determined by one-dimensional thin layer chromatography (TLC) on pre-coated silica gel 60 plates (Merck, Darmstadt, Germany) using chloroform-methanol-acetic acid-water (85:15:10:3.5, by volume) as developing solvent. Separated lipids were identified by comparison of their retention times with standards (Sigma) and by their staining behaviour with specific spray reagents: iodine vapour (general staining), concentrated sulphuric acid (phospholipids), α-naphtol (0.5 g in 100 ml methanol-water (1:1)) (glycolipids) and ninhydrin (0.1% in ethanol) (aminolipids). Plates were heated at 100°C until maximal staining intensity of the glycolipids and phospholipids was observed.

Chemicals

Purified carvacrol was obtained from Fluka Chemie AG (Buchs, Switzerland). A stock solution (1 M) was made in 95% ethanol. The final ethanol concentration in the experiments was always kept below 2% ethanol (v/v). Lipid standards were obtained from Sigma-Aldrich BV (Zwijndrecht, The Netherlands).

RESULTS

Viability at non-lethal carvacrol concentrations

To investigate the effect of incubation in the presence of different carvacrol concentrations on the viability of *B. cereus*, cells were incubated in the presence of 0 - 0.4 mM carvacrol. These concentrations were shown to be inhibitory to growth, but not lethal (29). After reaching an OD_{660} of 0.1, cells were harvested by centrifugation, washed and exposed to respectively 1.25 and 1.75 mM carvacrol. Samples were taken at regular time intervals during exposure and plated on BHI-agar plates to monitor the viable counts. $t_{1/2}$-Values were calculated from the viable counts (Fig. 1). Cells incubated in the presence of carvacrol were less sensitive to subsequent exposure to carvacrol than cells incubated in the absence of carvacrol. This decrease of sensitivity was concentration dependent. After incubation of *B. cereus* on non-lethal concentrations of carvacrol, an increase of the obtained $t_{1/2}$-values was observed. Incubation in the presence of 0.4 mM carvacrol resulted in 2.6 and 4.9 times higher $t_{1/2}$-values when exposed to respectively 1.25 and 1.75 mM carvacrol compared to incubation in the absence of carvacrol. It is clear from these results that *B. cereus* cells can to a certain extent adapt to carvacrol when incubated on non-lethal concentrations of this compound.

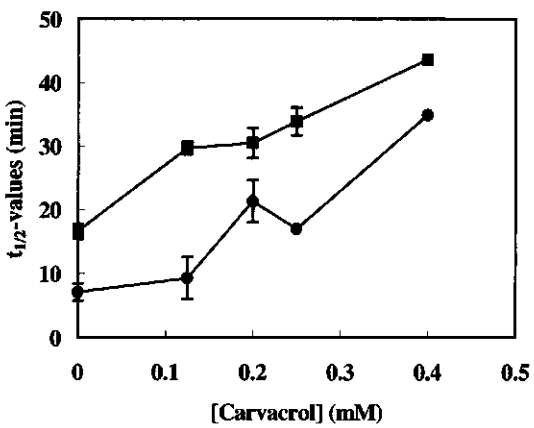

Figure 1: $t_{1/2}$-Values (min) after incubation of *B. cereus* in BHI supplemented with 0 – 0.4 mM carvacrol. Cells were harvested, washed and exposed to 1.25 (■) or 1.75 mM (●) carvacrol. $t_{1/2}$-value = time needed to kill 50% of the cells. The data represent mean values of triplicate measurements. Error bars are indicated.

Bioconversion of carvacrol

To investigate the possibility of bioconversion of carvacrol by *B. cereus*, the carvacrol concentration in the culture broth was monitored during growth. The carvacrol concentrations did not change significantly during the incubation of *B. cereus* in the broth (data not shown). This indicates that carvacrol is not metabolised by the organism or evaporated from the culture broth.

Phase transition temperature

In order to obtain more knowledge about adaptation mechanisms of *B. cereus* exposed to carvacrol, the effect of this compound on the membrane fluidity was studied. Cells were grown in the presence of 0 to 0.4 mM carvacrol at 30°C, washed and analysed by the FTIR to determine the CH_2-stretching vibration of the fatty acids (Fig. 2). An increase of the wavenumber and a concomitant increase of the membrane fluidity was observed at increasing temperature. The membrane fluidity changed from a gel to a liquid-crystalline state. Addition of 0.4 mM carvacrol to the growth medium decreased the membrane fluidity (a lower wavenumber was observed) between -14 and 40°C. Growth of *B. cereus* at 37°C in the absence of carvacrol also resulted in a decrease of the membrane fluidity compared to growth at 30°. However, the effect at 37°C was more pronounced than incubation in the presence of 0.4 mM carvacrol at 30°C.

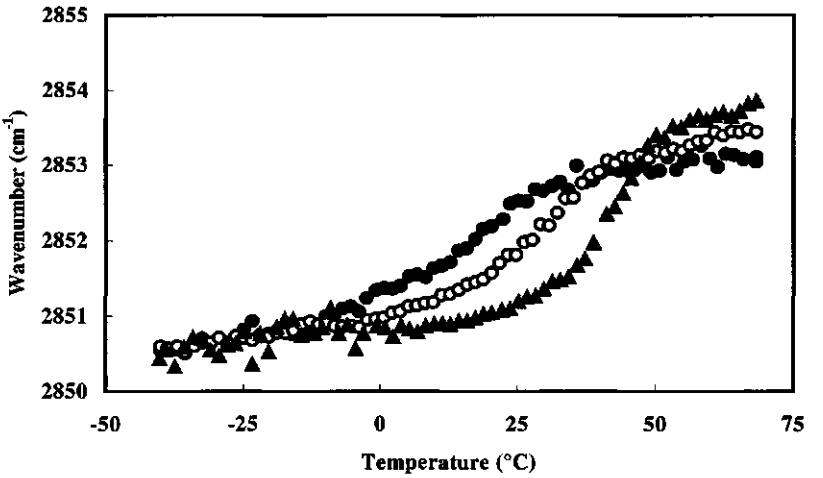

Figure 2: Wavenumber of CH_2-stretch of membrane lipids of cells adapted to 0.4 mM carvacrol (30°C) (○) and non-adapted (30°C (●) and 37°C (▲)) cells. Wavenumber of the CH_2-symmetric stretches were measured using Fourier-transformed infrared spectroscopy at various temperatures.

Calculation of the phase transition temperature (T_m) (Fig. 3A) showed an increase of T_m at increasing carvacrol concentrations in the growth medium. Between 0 and 0.15 mM carvacrol a small increase (3°C) of T_m was observed. A larger increase (9.6°C) was investigated when the carvacrol concentration was raised to 0.2 mM. A further increase of the carvacrol concentration in the growth medium to 0.4 mM did not result in a further increase of T_m. Addition of carvacrol to cells adapted to 0.4 mM carvacrol (Fig. 3B), resulted in a reduction of the T_m-value from 28.2°C to 19.4°C, the later being approximately the same value as was determined for non-adapted cells in the absence of carvacrol. Addition of 0.4 mM carvacrol

to non-adapted cells decreased the T_m-value from 20.5°C to 12.6°C.

From these results it can be concluded that growth in the presence of carvacrol increases the phase transition temperature of *B. cereus*. Addition of carvacrol to the cells after harvesting decreases the phase transition temperature.

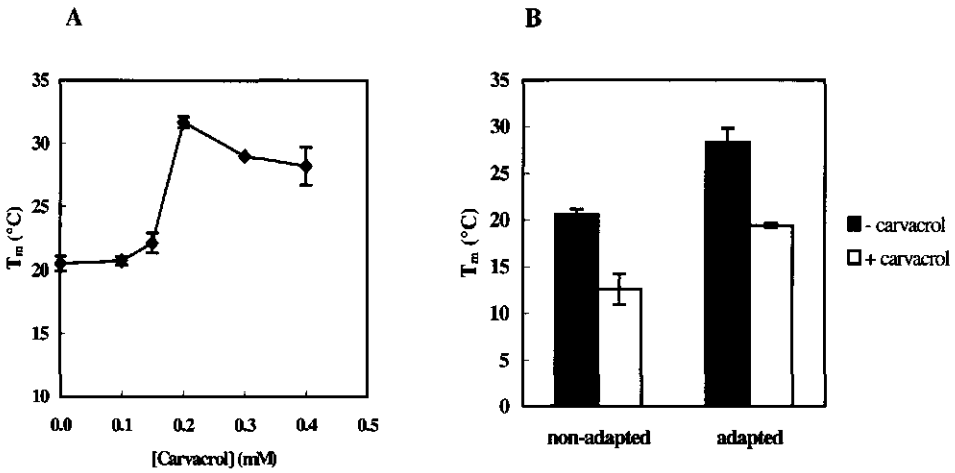

Figure 3: Phase transition temperature (T_m) of *B. cereus* A) in the absence of carvacrol after growth in the presence of non-lethal concentrations of carvacrol. B) in the absence or presence of 0.4 mM carvacrol. Cells were harvested, washed and the phase transition temperature was calculated from FTIR-spectra. Data represent mean values of triplicate (0 and 0.4 mM) and duplicate measurements. Error bars are indicated.

Fatty acid composition

The membrane fluidity is dependent on the fatty acid composition of the membrane. After discovering a decrease of the membrane fluidity in adapted cells compared to non-adapted cells, fatty acids were extracted and analysed. A change in the fatty acid composition might explain the change in the membrane fluidity and consequently the T_m-value. Many lipids were present, only the changes in lipid composition compared to the control (incubated in the absence of carvacrol) are shown (Fig. 4). Incubation in the presence of carvacrol resulted in an increase of the relative amounts of *iso*-$C_{13:0}$, $C_{14:0}$ and *iso*-$C_{15:0}$ fatty acids. At the same time a reduction of *cis*-$C_{16:1}$ and $C_{18:0}$ fatty acids was observed.

The membrane fluidity of the cells grown at 37°C was increased compared to growth at 30°C. To determine if this increase was caused by similar changes in fatty acid composition as was observed in adapted cells, the fatty acid composition of cells grown at 37°C was also determined. The observed changes were not similar to the changes observed in the adapted cells at 30°C. A decrease of the *iso*-branched fatty acids was observed and a very large increase of *cis*-$C_{16:1}$ fatty acids. The relative amounts of $C_{14:0}$ fatty acids increased and of $C_{18:0}$ fatty acids decreased.

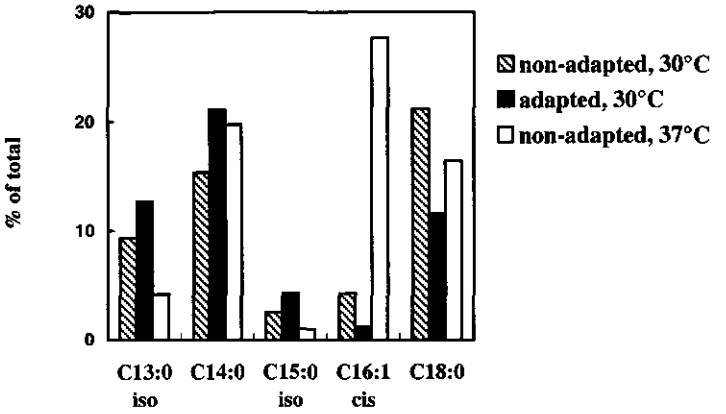

Figure 4: Differences in lipid composition of non-adapted and adapted cells. Cells were grown in BHI + 0.5 % glucose with resp. 0 (30°C and 37°C) and 0.4 mM (30°C) carvacrol.

Head group composition

Besides a change in fatty acid composition, a change in the head group composition of the membrane lipids may explain the observed decrease in sensitivity of *B. cereus* to carvacrol. After extraction of the lipids from cell suspensions, the head groups of the membrane lipids were analysed by TLC. The extracts retrieved from incubation on 0 and 0.4 mM carvacrol both contained only phospholipids, some of them giving a positive reaction with the amino group staining ninhydrin. No glycolipids were present in both extracts. The extracts retrieved from incubation on carvacrol were found to have some additional phospholipids (R_f-values of 0.91 and 0.94) and lacked one phospholipid, which was present in the membranes of the non-adapted cells (R_f-value of 0.93). Spraying with different reagents showed these lipids were phospholipids without aminogroups. No obvious differences in relative amounts of PE, DPG and PG were observed. In conclusion, during adaptation to carvacrol a change in the head group composition was observed.

DISCUSSION

This study describes the effect of carvacrol on cells of *B. cereus*, when added to the growth medium at non-lethal concentrations. Although carvacrol has bactericidal activity, *B. cereus* is still able to grow at concentrations up to 0.4 mM (29, 30). Addition of carvacrol to the growth medium at levels up to 0.4 mM decreased the sensitivity of *B. cereus* to bactericidal concentrations of carvacrol. Therefore, it can be concluded that cells of *B. cereus* can adapt to carvacrol at 30°C when carvacrol is present at non-lethal concentrations in the growth medium. Carvacrol is a hydrophobic compound and is expected to dissolve in the cytoplasmic

membrane with the hydroxyl group close to the polar head groups and the other part of the molecule between the apolar fatty acid chains. Sikkema *et al.* (23) found that the accumulation of lipophilic compounds in the cell membrane (tested with liposomes prepared from *E. coli* phospholipids) is proportional to the concentrations in the aqueous phase and the membrane-aqueous phase partition coefficient. When the concentration of carvacrol increases, more of this compound is expected to dissolve in the membrane and more damage of the membranes appears (30). Measurement of the carvacrol concentration in the medium indicated that *B. cereus* does not metabolise carvacrol. Accumulation of carvacrol in the membrane will have an effect on the interactions in the lipid bilayer. The Van der Waals interactions between lipid acyl chains will be affected by the partitioning of a compound in the membrane. The disturbance of these lipid interactions will have an effect on the membrane fluidity (31). It seems likely that adaptation of *B. cereus* to carvacrol is caused by changes in the membrane to counteract this effect of carvacrol.

Determination of the membrane fluidity showed that adapted cells have a lower membrane fluidity than non-adapted cells. This could be some kind of defence mechanism exhibited by the cell. At a lower membrane fluidity, less carvacrol can dissolve in the membrane and therefore the cells are exposed to relatively lower carvacrol concentrations and can better resist the carvacrol present in the external environment. As we described earlier (30), carvacrol increases the permeability of the cytoplasmic membrane for protons and potassium ions. A decrease of the membrane fluidity decreases the passive permeability of the membrane (26).

Interestingly, the addition of carvacrol to the growth medium resulted in a similar, but smaller, effect on the membrane fluidity as an increase of the growth temperature to 37°C. Cells adapt by lowering their membrane fluidity and therefore increase the phase transition temperature (T_m) from 20.6°C (30°C growth temperature) to 42°C (37°C growth temperature) thereby optimising membrane functions.

An increase of T_m (from 20.5 to 31.7°) was retrieved at carvacrol concentrations up to 0.2 mM in the growth medium. No further increase was noticed with increasing concentrations.

During washing in a buffer, carvacrol is extracted from the membranes of the adapted cells. Subsequent addition of carvacrol to washed adapted cells, resulted in a decreased T_m-value compared to adapted cells without carvacrol in their membranes (resp. 19.4°C and 28.2°C). As a result of the addition of carvacrol to non-adapted cells, the T_m-value was decreased to 12.6°C. From these results it is clear that the actual membrane fluidity increases in the presence of carvacrol in the membrane. This can be explained by the interaction of carvacrol molecules with the fatty acids in the membrane. Carvacrol spaces the fatty acid and therefore the fluidity of the membrane increases. Interestingly, addition of carvacrol to adapted cells resulted in approximately the same phase transition temperature as of non-adapted cells in the

absence of carvacrol.

A change in the membrane fluidity is often caused by a change in the lipid composition of the membranes. These changes are made to keep an adequate proportion of liquid-crystalline lipid in the membrane and to maintain the bilayer phase. This is important to withstand disruption of membrane organisation and impairment of the selective permeability (22).

Ingram *et al.* (8) found that growth of *E. coli* was inhibited by lipophilic agents, such as alcohols. No growth was observed until the fatty acid composition of the cells was changed. Heipieper *et al.* (7) described the adaptation of *Pseudomonas putida* S12 to ethanol and toluene. Changes in the fatty acid composition were observed as an adaptation mechanisms to these compounds. A study of the changes in the lipid composition of the membranes of cells of *B. cereus* incubated in the presence of 0.4 mM carvacrol shows an increase of *iso*-$C_{13:0}$ and *iso*-$C_{15:0}$ fatty acids. Higher melting *iso*-fatty acids have a decreasing effect on the membrane fluidity compared to the lower melting *anteiso*-fatty acids (26). Furthermore a decrease of the unsaturated fatty acid *cis*-$C_{16:1}$ was observed. A decrease of unsaturated fatty acids generally leads to a lower membrane fluidity (26). In contrast to these results which could explain the observed change in membrane fluidity during incubation in the presence of carvacrol, the percentage of long chain fatty acids ($C_{18:0}$) decreased and of the short chain fatty acids ($C_{14:0}$ and *iso*-$C_{13:0}$) increased in the presence of carvacrol. This observation is not in line with the observed decrease of the membrane fluidity and can be interpreted as an antagonistic effect with respect to the chemo-physical properties of the membrane. Suutari *et al.* (26) described changes in fatty acid composition on lowering temperature. During temperature decrease also some antagonistic effects were observed. However, the overall effect of the changes in lipid composition on the membrane fluidity is determined by the sum of the effect of every single lipid on the membrane fluidity. A small change of a certain lipid could have a larger impact than a larger change of another lipid and can thus outweigh the other effect. To be more sure about the influence of changes in lipid composition on the membrane fluidity, the magnitude of every single lipid on the membrane fluidity has to be determined. The observed changes at 37°C growth were not similar to growth in the presence of carvacrol. However, cells seem to be able to change their mode of regulation depending on growth conditions (26). The large increase of *cis*-$C_{16:1}$ fatty acids compared to growth at 30°C was not expected, due to the fluidising effect of this fatty acid. The higher melting *iso*-fatty acids decreased and could not explain the observed change in membrane fluidity. The increase of the short chain fatty acid $C_{14:0}$ and the decrease of the long chain fatty acid $C_{18:0}$ are probably the main factors determining the membrane fluidity at 37°C.

Beside changes in the lipid composition of the membranes, changes in the head group composition of membranes have been described in studies with adapted cells (4, 9, 31). The data of this study show the production of additional phospholipids in cells growing in the presence of carvacrol.

No glycolipids were found in both the adapted and non-adapted cells. Earlier research showed the absence of glycolipids in *B. cereus*, specially in psychrotrophic species (17, 22). The production of additional phospholipids could be an explanation for the observed adaptation to carvacrol. Bygraves (4) described the changes in head group composition in solute tolerant food spoilage organisms. These changes were a result of changes in the osmolarity of the medium. Ingram (9) showed that addition of different organic solvents and food additives results in a change of the phospholipid composition of the membranes.

The interaction of compounds with the cytoplasmic membrane could also have an influence on membrane embedded enzymes. The activity is strongly influenced by the fatty acid and headgroup composition (8, 31).

From the obtained results it can be concluded that the addition of carvacrol to cells of *B. cereus* results in an increase of the membrane fluidity. The cells can adapt to carvacrol when present at non-lethal concentrations in the growth medium. They respond by lowering their membrane fluidity, caused by changes in their fatty acid and head-group composition.

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5

Antimicrobial activity of carvacrol towards *Bacillus cereus* on rice.

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Antimicrobial activity of carvacrol towards *Bacillus cereus* on rice
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ABSTRACT

The antimicrobial activity of carvacrol, a compound present in the essential oil fraction of oreganum and thyme, towards the foodborne pathogen *B. cereus* on rice was studied. Carvacrol showed a dose-related inhibition of growth of the pathogen. Concentrations of 0.15 mg/g and higher inhibited the growth and the extent of inhibition depended on the initial inoculum size. To decrease the input of carvacrol on the taste and flavour of the product, a combined treatment with the structure analogue cymene was tested. Due to the smell and taste of carvacrol at high concentrations, carvacrol was combined with cymene, a natural antimicrobial compound with a similar structure. A synergistic effect was observed when 0.30 mg/g carvacrol was combined with 0.27 mg/g cymene. Finally it was demonstrated that also a common taste enhancer like soya sauce increased the antimicrobial action of carvacrol towards *B. cereus*. The antimicrobial activity of carvacrol with cymene or soya sauce was influenced by the addition of NaCl.

INTRODUCTION

Bacillus cereus is a spore-forming pathogen often associated with foodborne illnesses, caused by the production of toxins. Two forms of foodborne illness can be distinguished. The first one, a diarrhoeal syndrome, is caused by a labile enterotoxin and is associated with meat, pasta, vegetable dishes, milk, desserts. The second form is a rapid emetic syndrome, caused by a stable emetic toxin. Outbreaks of the emetic syndrome are mostly associated with cooked rice, but cases with other products such as vegetable dishes, dairy products and poultry have been reported (9, 17, 19, 23, 28). *B. cereus* was the most common reported cause of food infection or -intoxication in the Netherlands in 1997. A total of 14 outbreaks of *B. cereus* was reported (18). Vegetative cells of *B. cereus* can easily be inactivated by heating. However, spores can survive this treatment and cause, after subsequent germination, food intoxication.

There is currently a large interest in the use of natural antibacterial compounds, such as plant extracts, herbs and spices, for mild preservation of foods. They have a characteristic flavour and sometimes possess antioxidant activity and antimicrobial activity (22). An example of a compound present in the essential oil fraction of oreganum and thyme, is carvacrol (1, 14). This compound has been characterised as an inhibitor of growth of different pathogens (5, 10-13, 21, 24, 25#47). Recently, we demonstrated the bactericidal activity of carvacrol towards *B. cereus* (26). Carvacrol affects the membrane integrity of vegetative cells of *B. cereus*. Exposure to carvacrol makes the membrane permeable for potassium ions and protons. This results in a decrease of the internal pH and a dissipation of the membrane potential. Consequently the synthesis of ATP will be inhibited. These processes finally lead to cell death (27).

Due to its flavour, application of carvacrol is limited to certain products. Application of

carvacrol as a flavouring agent is known in products such as non-alcoholic beverages (28.54 ppm), soft candy (21.43 ppm), chewing gum (8.42 ppm), baked goods (15.75 ppm) (6). The objective of this study was to evaluate the antimicrobial activity of carvacrol towards *B. cereus* when applied on rice. A close association of *B. cereus* with rice preparations has been well documented (4, 7, 20, 23, 28). It is important to keep concentrations of carvacrol as low as possible, to avoid an influence on the taste and smell of rice. Therefore carvacrol is used in combination with other compounds. Cymene, a compound with a very similar structure as carvacrol, was tested both *in vitro* and *in situ*. Together with cymene, soya sauce and NaCl, two ingredients often applied on rice products, were studied to investigate their influence on the antimicrobial activity of carvacrol. Finally, combined effects of cymene, soya sauce and NaCl were determined.

MATERIALS AND METHODS

Bacterial strain and growth conditions

Bacillus cereus IFR-NL94-25 (obtained from the Institute of Food Research, Norwich, UK) was used throughout this study. Cells were grown overnight in Brain Heart Infusion (BHI) medium (Oxoid®) supplemented with 0.5% (w/v) glucose (initial pH 6.7) and washed with sterile destilled water. Cell cultures were maintained at -80°C in 15% glycerol as a cryoprotectant.

Preparation rice and monitoring viability in situ

Rice (Kwaliteitsmerk®) was obtained from a local supermarket, cooked for 8 minutes in water (500 g/l) and kept for 15 minutes (to "steam out"). The antimicrobial compounds and bacterial cells (10^4/g) were added to 200 gram rice. During incubation (8°C), samples (10 g) were taken at regular time intervals, ten times diluted in peptone physiological salt (1 g/l peptone and 8.5 g/l NaCl) and mixed in a Stomacher® bag. Serial dilutions were plated on *Bacillus cereus* selective agar (Oxoid®). Plates were enumerated after an incubation period of 24 hours at 37°C, followed by 24 hours at 20°C.

Monitoring viability in vitro

Vegetative cells of *B. cereus* were harvested by centrifugation, washed twice in a 50 mM potassium-*N*-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES) buffer (pH 7), containing 1 mM $MgSO_4$, and diluted to OD_{660} = 0.1 (light path 1 cm). The cell suspension (20 ml) was kept at 8°C during the course of the experiment. Carvacrol, cymene or a combination of both were added to give a final concentration between 0.25 and 6 mM. Samples were taken at regular time intervals during exposure (maximum exposure time 40 min) and immediately diluted (10^2 - to 10^5-fold) in peptone physiological salt solution to

quench the influence of the antimicrobial compounds. Serial dilutions were plated on BHI agar plates and incubated for 24 hours at 30°C.

Growth data processing

To evaluate the bactericidal effect of carvacrol under different circumstances, the logarithm of the viable count was plotted against time. After calculating the slope (k) of the straight lines obtained, the $t_{1/2}$-values (time needed to kill 50% of the cells) was calculated using the following equation:

$$t_{1/2} = - \ln 2/k \text{ (min)}$$

Chemicals

Purified carvacrol and cymene were obtained from Fluka Chemie AG® (Buchs, Switzerland). Stock solutions of carvacrol and cymene were made in 95% ethanol. Soya sauce (Conimex BV, Baarn, The Netherlands) was obtained from a local supermarket.

RESULTS

Effect of carvacrol on growth

To determine the influence of carvacrol on the growth of *B. cereus* on rice, cooked rice was inoculated with vegetative cells (10^4/g) in the presence of different carvacrol concentrations (8°C). Growth of vegetative cells of *B. cereus* on rice was observed (Fig. 1A). Carvacrol showed a dose related inhibition of the growth of *B. cereus*. Addition of 0.19 mg/g carvacrol inhibited the growth rate of *B. cereus* slightly and reduced the final population density by approximately 1 log unit. Full suppression of growth and even a decline in the number of viable cells were observed after the addition of 0.38 mg/g carvacrol. Concentrations of 0.53 mg/g carvacrol and higher caused a reduction of the viable count to levels below the detection limit ($6.3 \cdot 10^2$ cfu/g) within 7 days. No *B. cereus* cells were detected on non-inoculated cooked rice throughout the experiment.

To investigate the influence of the inoculum size on the activity of carvacrol, the experiment was repeated with an initial population size of $6.3 \cdot 10^5$/g. Higher concentrations of carvacrol were needed to reach the same effect (Fig. 1B) as when a starting inoculum of 10^4/g was used (Fig. 1A). At concentrations up to 0.30 mg/g growth could still occur. At 0.45 mg/g growth was suppressed and the viable count of *B. cereus* ($1.6 \cdot 10^5$/g) remained constant during the experiment. Higher concentrations of carvacrol (≥0.60 mg/g) gave a continuous reduction of the viable count. Cells could still be detected after 15 days. The initial population was shown to be an important factor considering the antimicrobial activity of carvacrol.

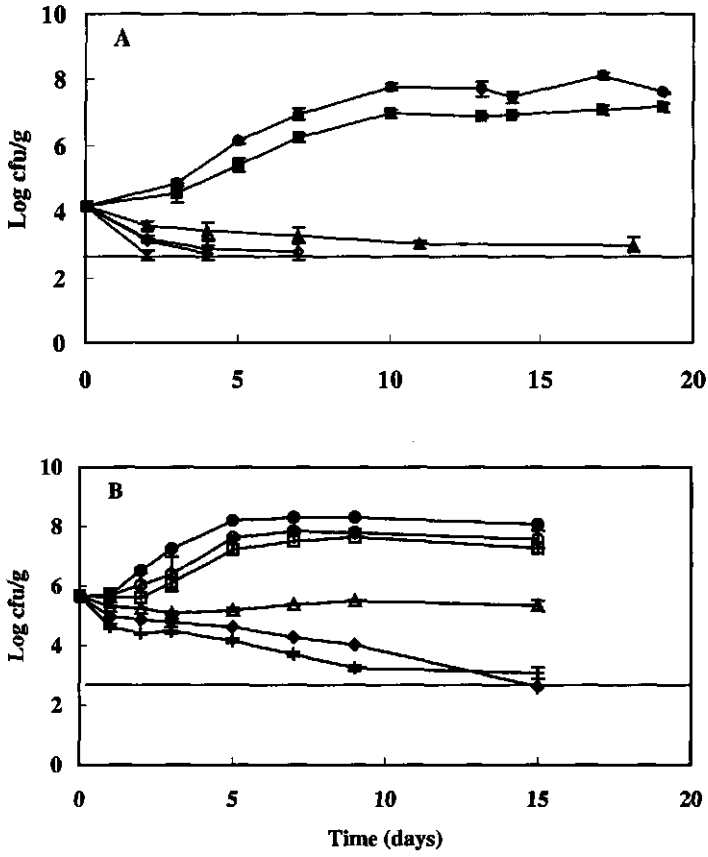

Figure 1: Influence of carvacrol on growth of *B. cereus* on rice (8°) at a starting population of 10^4 cfu/g (A) or $6.3 \cdot 10^5$ cfu/g (B). Carvacrol concentrations tested were: 0 mg/g (●), 0.15 mg/g (○), 0.19 mg/g (■), 0.30 mg/g (□), 0.38 mg/g (▲), 0.45 mg/g (Δ), 0.53 mg/g (✧), 0.60 mg/g (◆), 0.68 mg/g (x) and 0.75 mg/g (+). The data represent mean values of triplicate measurements. Error bars are indicated. Carvacrol (1 ml) was added from a stock solution. —— = detection limit ($6.3 \cdot 10^2$ cfu/g).

Combination of carvacrol and cymene

Although carvacrol could completely inhibit the growth of *B. cereus* on rice, an effect on sensoric attributes like the taste and smell of the product could become a problem at high concentrations. Therefore, carvacrol was combined with cymene, a compound with a similar structure. Testing the two compounds on washed vegetative cells of *B. cereus* in HEPES-buffer, an effect on the viability was observed (Table 1).

Cymene alone was not very effective as an antimicrobial compound at low concentrations. When the concentration was raised to 6 mM, a bactericidal effect was observed ($t_{1/2}$ = 2.8 min). Carvacrol was found to be bactericidal at concentrations above 1 mM. A combination of carvacrol and cymene was much more effective. Interestingly, a synergistic

effect was observed with the combination of 0.5 mM carvacrol and 0.25 mM cymene. The viable count was reduced to 50% of the initial viable count within 48 min. An increase of the concentrations of both compounds resulted in a higher death rate. Addition of 1 mM carvacrol and 2 mM cymene decreased the viable count to 50% in less than 1 min.

After the observation of a synergistic activity between carvacrol and cymene *in vitro*, both compounds were tested on rice (Fig. 2). Incubation in the presence of 0.30 mg/g carvacrol hardly affected the growth rate of *B. cereus* (Fig. 2a). Only the final population density was found to be approximately 1 log unit lower compared to incubation in the absence of carvacrol. Addition of cymene (0.27 mg/g) to rice did not alter the growth rate or the final population density of *B. cereus*. The combination of carvacrol and cymene was, like *in vitro*, much more effective. A synergistic activity between both compounds was observed. No viable cells could be detected after incubation for more than 7 days at 8°C.

Table 1: Influence of different concentrations of carvacrol and cymene on the $t_{1/2}$-value (min) of *B. cereus* (8°C) in HEPES-buffer (pH 7).

		concentration carvacrol (mM)						
		0	0.5	0.75	1	1.5	1.75	2.0
concentration cymene (mM)	0	-*	-	-	>100	69	13	2.6
	0.25	-	48	-	-	-	-	-
	0.5	>100	8.9	-	-	-	-	-
	0.75	-	-	<1.5	-	-	-	-
	1	-	4.6	-	<0.9	-	-	-
	2	-	-	-	<1.0	-	-	-
	6	2.8	-	-	-	-	-	-

-* = not determined

$t_{1/2}$ -value (min) is time needed to kill 50% of the cells (see Materials and Methods). The data represent mean values of triplicate measurements.

Influence of food ingredients on activity of carvacrol

Application of carvacrol together with other food ingredients to rice could alter the antimicrobial activity due to interaction between compounds. Therefore, soya sauce, a compound which is often applied to rice, was added to rice, to investigate the effect on the activity of carvacrol. Incubation of rice with soya sauce (10 µl/g) did not result in a significant change of the viable count of *B. cereus* (Fig. 2b). Interestingly, during incubation of rice in the presence of the combination of carvacrol and soya sauce, a rapid decrease of the viable count was observed. No viable cells of *B. cereus* could be detected anymore after 7 days incubation. Sorbate, an antifungal compound added to soya sauce, did not have any effect on

the growth of *B. cereus* on rice when added alone or in combination with carvacrol (data not shown).

A combination of carvacrol, cymene and soya sauce did not result in a larger suppression of growth compared to the addition of a combination of carvacrol and cymene or soya sauce (Fig. 2c). Cymene and soya sauce seem to antagonise each others effect on outgrowth of *B. cereus* on rice when combined with carvacrol. Finally, we investigated the effect of NaCl (1.25 g/l) on the *in situ* antimicrobial action of carvacrol combined with cymene or soya sauce. NaCl did not have any influence on the growth of *B. cereus* on rice (Fig. 3). Addition of carvacrol, cymene or soya sauce alone or in combination with each other did not have any significant effect on the growth rate of *B. cereus* in the presence of NaCl. NaCl antagonised the antimicrobial effect of the different compounds.

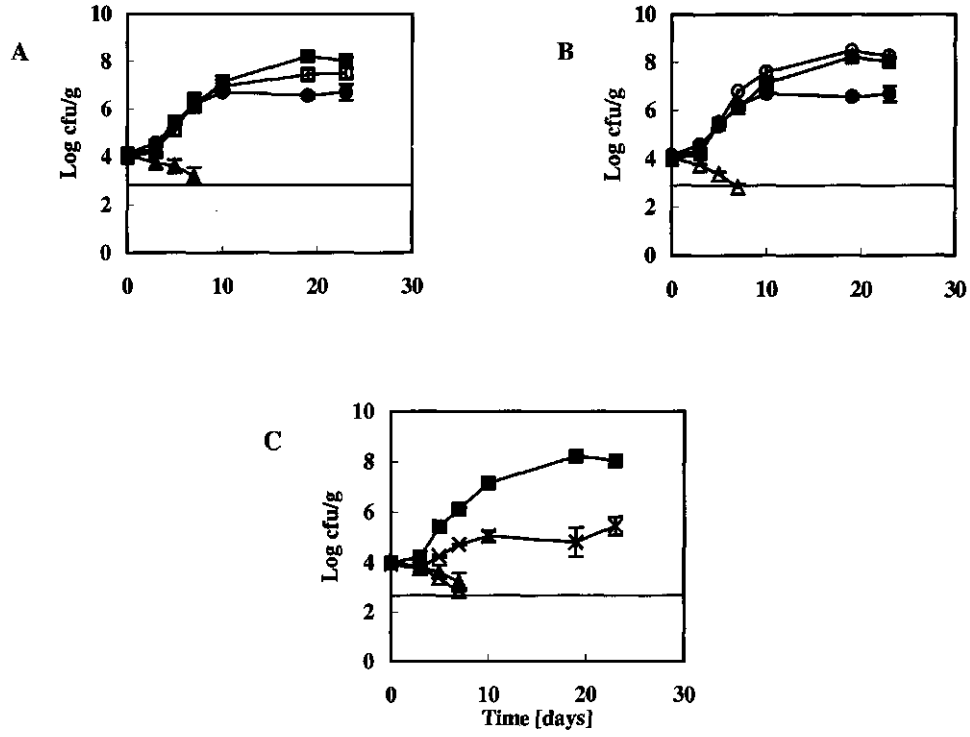

Figure 2: Influence of carvacrol on the growth of *B. cereus* (8°C) on rice in the presence of cymene (2a), soya sauce (2b) and a combination of cymene and soya sauce (2c). control (■), 0.30 mg/g carvacrol (●), 0.27 mg/g cymene (□), 0.30 mg/g carvacrol + 0.27 mg/g cymene (▲), 10µl/g soya sauce (○), 0.30 mg/g carvacrol + 10µl/g soya sauce (Δ) and 0.30 mg/g carvacrol + 0.27 mg/g cymene + 10 µl/g soya sauce (X). —— = detection limit ($6.3 \cdot 10^2$ cfu/g). The data represent mean values of triplicate measurements. Error bars are indicated. Carvacrol (1 ml) and cymene (1 ml) were added from a stock solution, 2 ml soya sauce was added.

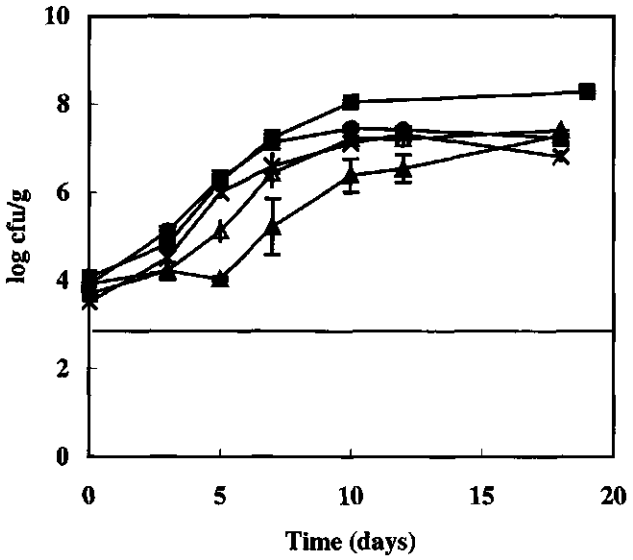

Figure 3: Effect of NaCl on the activity of (0.30 mg/g) carvacrol in the presence of respectively cymene (0.27 mg/g), soya sauce (10µl/g) and a combination of cymene and soya sauce on rice (8°C). NaCl (■), NaCl + carvacrol (●), NaCl + carvacrol + cymene (▲), NaCl + carvacrol + soya sauce (Δ), NaCl + carvacrol + cymene + soya sauce (X). —— = detection limit ($6.3 \cdot 10^2$ cfu/g). The data represent mean values of triplicate measurements. Error bars are indicated.

DISCUSSION

This study describes the effect of the plant essential oil compound carvacrol on growth of *B. cereus* on rice. Inhibition of growth occurs at concentrations of 0.15 mg/g or higher, depending on the initial inoculum size. Although a clear antimicrobial activity of carvacrol on rice was observed, high concentrations could have an influence on the taste and smell of rice. The use of hurdle technology to reduce concentrations and to use the advantages of combinations of preservation systems could be a solution for this problem. The principles of hurdle technology were described by Leistner (15). Microorganisms are sometimes unable to overcome the combination of several preservative factors, such as low temperature, pH, redox potential, a_w and the addition of preservatives (so-called hurdles). These hurdles result in a microbial stable and safe product and in the meantime a control of the sensory quality. Therefore, carvacrol was combined with another natural antimicrobial agent, namely cymene. This combination increased the antimicrobial activity of carvacrol, a synergistic activity between the two compounds was observed. This synergistic activity was also evident when carvacrol and cymene were applied on rice. The mechanisms of the action are yet unclear, although the two compounds have almost the same structure. Cymene lacks the hydroxyl group which is present in carvacrol. Addition of cymene alone does not show an inhibiting

effect on the growth of *B. cereus*. Because cymene is, like carvacrol, a hydrophobic compound, it is expected to partition preferentially in the membranes of the target cells. This may facilitate the uptake of carvacrol in the lipid bilayer of the cytoplasmic membrane.

Food ingredients can have an influence on the activity of antimicrobial compounds like carvacrol (3, 8, 10, 11, 22). Soya sauce was tested to obtain more information about the influence of this food ingredient on the antimicrobial activity of carvacrol. Soya sauce itself did not show any antimicrobial activity. Beuchat *et al.* (2) described the stimulation of growth of *B. cereus* by 5 – 10% soya sauce in rice flour medium. However, the concentration of soya sauce in our study was 1%. Interestingly, soya sauce enhances the antimicrobial activity of carvacrol. Soya sauce (a fermented product) is a complex mixture of different compounds. It is not clear which compound in soya sauce is responsible for this synergistic activity. Sorbate, present in soya sauce, did not have any effect on the growth of *B. cereus*. Possibly one or more compounds in soya sauce facilitate the accumulation of carvacrol in the cytoplasmic membrane of *B. cereus*.

Unexpectedly, the synergistic effects of carvacrol with respectively cymene and soya sauce were diminished by a combination of the three compounds. This suggests an interaction between cymene and a component is soya sauce.

The synergistic effect of carvacrol with respectively cymene and soya sauce was antagonised by NaCl. This is important information regarding application in food products. Food products are mostly composed of different compounds, which could have an influence on the activity of carvacrol alone or in combination with other compounds. Liu *et al.* (16) showed an enhancing effect of salt on the antimicrobial activity of essential oils. However they did not test the effect of salt on the antimicrobial activity of carvacrol. Kivanç *et al.* (12) studied the effect of the combination of 0.05 mg/g carvacrol with 20 mg/g salt on the growth of *B. cereus*. They did not find any antimicrobial activity. These results are difficult to compare with our study. Firstly because in our study we used a higher (0.38 mg/g) carvacrol concentration and a lower salt concentration (0.25 mg/g salt, assuming that all the salt added during cooking was absorbed by the rice). Secondly, the strains in the study of Kivanç *et al.* were grown on agar plates and our study describes the growth on rice.

This study shows that growth of *B. cereus* on rice is inhibited by the carvacrol. The carvacrol concentration which is effective with respect to its antimicrobial activity can be reduced by the addition of cymene or soya sauce. Together with a low temperature (8°C), the problem of outgrowth of *B. cereus* on rice is reduced to a minimum.

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6

Influence of carvacrol on growth and toxin production by *Bacillus cereus*

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Influence of carvacrol on growth and toxin production by *Bacillus cereus*

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ABSTRACT

The natural antimicrobial compound carvacrol was investigated for its effect on the diarrhoeal toxin production by *Bacillus cereus*. Carvacrol (0 - 0.06 mg/ml) reduced the viable count and the maximal specific growth rate (μ_{max}) of *B. cereus* in BHI broth. Total amount of protein was not affected by carvacrol. However, a sharp decrease (80%) of the toxin production was observed in the presence of 0.06 mg/ml carvacrol. Carvacrol also inhibited toxin production in soup, but approximately 50 fold higher concentrations were needed to reach the same effect as in broth. From this study it can be concluded that carvacrol can be added to food products at doses below the MIC-value (0.11 mg/ml), thereby reducing the risk of toxin production by *B. cereus* and increasing the safety of the product.

INTRODUCTION

Bacillus cereus is a motile, spore forming, facultative anaerobe and Gram positive rod. Some strains have the ability to grow at low temperatures and can be regarded as psychrotrophics with a minimal growth temperature of 4°C (6). In 1997, *B. cereus* was rated as the number one causing food poisoning in The Netherlands. However, the number of cases reported is probably an underestimation as a consequence of the short durance and relatively mildness of the illness (5, 6). *B. cereus* is associated with two kinds of foodborne illnesses: a diarrhoeal and emetic type, caused by two distinct toxins (7). Strains causing the diarrhoeal type outbreaks produce a labile enterotoxin, which is easily inactivated by heat, low pH and proteases. Main symptoms of this illness are abdominal pain and diarrhoea, similar to the symptoms of the food intoxication caused by *Clostridium perfringens*. Foods mostly implicated with *B. cereus* contamination are meat products, soups, vegetables, puddings, sauces, milk and other dairy products. The infective dose is 10^5-10^7 cells and production of the toxin probably occurs in the small intestine of the host. Incubation time is 8 to 16 hours and the duration of the illness is 12 to 24 hours (6). A second illness, the emetic type, mainly causes vomiting due to the production of the heat stable emetic toxin. Products involved mainly are rice, pasta and noodles.

Several food preservation systems such as heating, refrigeration and addition of antimicrobial compounds can be used to reduce the risk of outbreaks of *B. cereus* food poisoning. A novel way to preserve foods is the use of plant essential oils. The antifungal and antibacterial effects of these volatile oils towards different microorganisms have been described in several studies (4, 8-11, 15, 19-21). An example of an antimicrobial compound present in the essential oil fraction of oreganum and thyme is carvacrol. In earlier studies, we demonstrated the bactericidal action of carvacrol towards *B. cereus*. In addition, we showed the interaction of this compound with the cytoplasmic membrane by changing its permeability for protons and potassium ions (21, 22). Since toxin is an essential factor in outbreaks of food poisoning, it is important to know the effect of antimicrobial compounds on the toxin production. This is the

first paper describing the effect of carvacrol on the toxin production by *B. cereus* in both broth medium and mushroom soup.

MATERIALS AND METHODS

Bacterial strain and growth conditions

Pre-incubation *Bacillus cereus* IFR-NL94-25 (obtained from the Institute of Food Research, Norwich, UK) was used in all experiments. Cells were grown in Brain Heart Infusion (BHI) medium (Oxoid) supplemented with 0.5% (w/v) glucose (initial pH 6.7). Cell cultures were maintained at -80°C in 15% glycerol as a cryoprotectant.

Growth in BHI To determine growth of *B. cereus* in BHI, an overnight culture of *B. cereus* was incubated (17°C) in BHI, supplemented with 0 to 0.06 mg/ml carvacrol in a microtiterplate. The starting Optical Density at 660 nm (OD_{660}) (light path 1 cm) of the cell suspension was set at 0.02. The OD_{660} was measured at different time intervals until a constant reading was observed. For toxin measurement, an overnight culture of *B. cereus* was diluted 200 times in fresh BHI + 0.5% glucose to which 0 - 0.06 mg/ml carvacrol was added. Toxin production was determined after incubation for 24 hours at 17°C.

Growth in soup Different (sterile) soups were bought at a local supermarket. Mushroom soup, goulash soup, vegetable soup and chicken soup (Uno® soup, Unox®, Rotterdam, The Netherlands) were ready to eat. Bouillon (Libra, IJsselstein, The Netherlands) and chicken bouillon (Knorr Best Foods Benelux®, Hilversum, The Netherlands) were prepared by dissolving one tablet in 500 ml water and heating until the tablet was suspended completely. After cooling to room temperature (when necessary) carvacrol (0 – 3 mg/ml) was added to 50 ml soup. Vegetative cells of *B. cereus* were washed in water, diluted to an OD_{660} (light path 1 cm) of 0.025. The soups were inoculated with 250 µl cell suspension and incubated 5 days (17°C).

Determination viable counts

Viable counts were determined by plate counting. Samples of 1 ml were taken and directly diluted in peptone physiological salt (1 g/l peptone and 8.5 g/l NaCl). Serial dilutions were plated on BHI-agar plates (BHI samples) (Oxoid) or *Bacillus cereus* selective agar (soup samples) (Oxoid) and incubated for 24 hours at respectively 30 and 37°C. The *Bacillus cereus* selective agar plates were incubated another 24 hours at room temperature before they were enumerated (following the instructions provided by the manufacturer).

Determination toxin production

Analysis of the excreted toxin production was carried out using an enzyme linked immunosorbent assay (ELISA) developed by Tecra (Roseville, NSW, Australia) for the

detection of *B. cereus* diarrhoeal enterotoxin. Measurement of the toxin production was performed as specified by the instructions provided by the manufacturer. Toxin quantities were defined as extinction at 405 nm. An uninoculated sample was used as a negative control, an enterotoxin positive control was provided with the kit.

Protein determination

The determination of the amount of protein in the cells of *B. cereus* was carried out according to Lowry *et al.* (12) using bovine serum albumin as a standard.

Chemicals

Purified carvacrol was obtained from Fluka Chemie AG (Buchs, Switzerland). A stock solution (1 M) was made in 95% ethanol. The final ethanol concentration in the experiments was always kept below 2% ethanol (v/v).

RESULTS

Toxin production in BHI

B. cereus is a pathogen which causes food poisoning by the production of a diarrhoeal toxin or emetic toxin. To investigate an effect of carvacrol on the diarrhoeal enterotoxin production, cells were incubated in BHI in the presence of 0 to 0.06 mg/ml carvacrol. Toxin production was measured after 24 hours incubation (17°C). Toxin quantities were determined as a function of the carvacrol concentration and were corrected for the amount of cell protein. An increase of the carvacrol concentrations from 0 to 0.015 mg/ml, reduced the toxin production with 21% to 79% (Fig. 1A) . Toxin production is dramatically inhibited (to 24% of the control) when 0.06 mg/ml carvacrol was added. At the same time a significant reduction viable counts reduced in the presence of 0.06 mg/ml carvacrol to approximately 27% of the control (no carvacrol added) (Fig. 1B). In addition, no significant differences in total amount of cell protein in the culture were measured (Fig. 1C). However, addition of 0.06 mg/ml carvacrol reduced μ_{max} from 0.48 (absence of carvacrol) to 0.24 h^{-1} (Fig 1D). The stationary phase was reached after approximately 12 hours (0 - 0.045 mg/ml) and 13 hours (0.06 mg/ml) (data not shown), indicating that the period of the stationary phase was similar for all objects.

Growth in soup

To find a suitable product to measure toxin production by *B. cereus*, different soups were inoculated with vegetative cells (17°C). Samples were taken after 24 hours incubation, diluted and plated on *B. cereus* agar plates (Fig. 2A). There were no significant differences in the

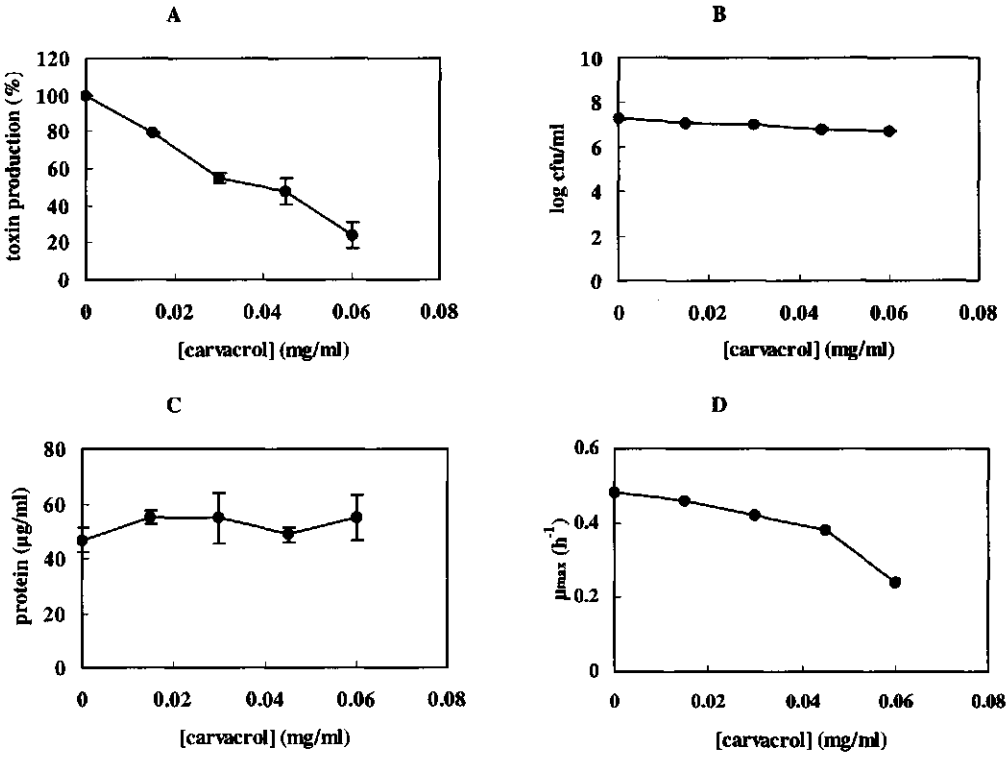

Figure 1: Effect of carvacrol on different parameters of *B. cereus* after 24 hours growth in Brain Heart Infusion (BHI) (17°C), data represent mean values of duplicate measurements and error bars are indicated:
A: Toxin production as percentage of control (no carvacrol added (= 100%)). Data corrected for total amount of protein in cell.
B: Viable count of *B. cereus* (log cfu/ml)
C: Total amount of protein in sample (µg/ml).
D: Maximal specific growth rate (h^{-1})

viable counts at the start of the experiment ($2.5*10^5$ cfu/ml). Growth occurred in all soups, except vegetable soup and goulash soup. Viable counts in these two soups were reduced to levels below the detection limit (10^3 cfu/ml) after 24 hours. The highest viable counts were observed in mushroom soup. To determine if the pH of the soups plays a role in the capacityof *B. cereus* to grow, pH values were measured (Fig. 2B). No growth and even a decline of the viable counts occurred in soups which had an initial pH below 5. In chicken bouillon, no decrease of pH was observed. The pH values after 24 hours in bouillon, chicken soup and mushroom soup were all around 5. These results indicate that no growth occurs below pH 5.

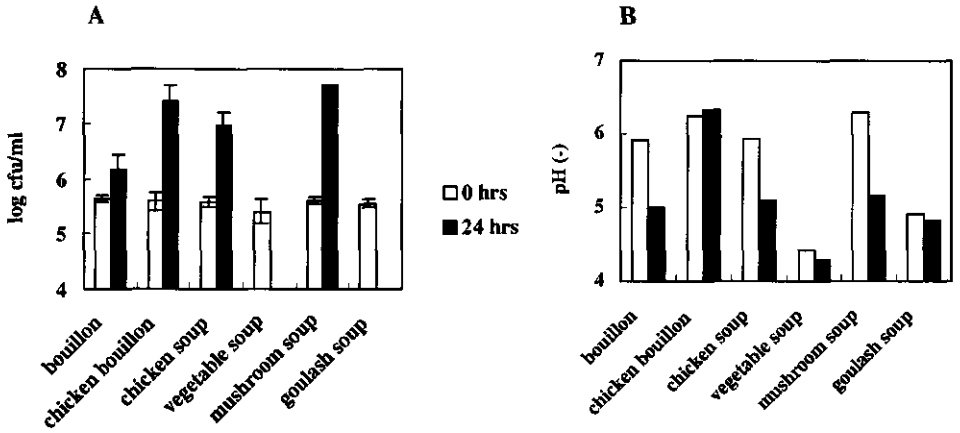

Figure 2: Viable counts (A) of *B. cereus* and pH values (B) in different soups after 0 or 24 hours incubation at 17°C. Data represent mean values of triplicate measurements. Error bars are indicated.

Toxin production in soup

B. cereus was inoculated in mushroom soup in the presence of 0 to 3.0 mg/ml carvacrol. No significant effect of carvacrol on the growth of *B. cereus* was observed after 5 days incubation. The obtained viable counts were 10^7 cfu/ml for all samples (Fig. 3). Only the addition of 3.0 mg/ml resulted in a 10 fold lower viable count (10^6 cfu/ml). Toxin was produced after 5 days of incubation (17°C). Addition of 0.5 mg/ml carvacrol did not result in a significant decrease of the toxin production (Fig. 3). However, when the carvacrol concentration was raised to concentrations of 0.9 mg/ml and above, toxin production decreased dramatically. At 3.0 mg/ml no toxin was detected anymore (below detection limit). Carvacrol inhibits toxin production in both BHI and mushroom soup at concentrations which do not have a significant effect on the viable counts of *B. cereus*. Approximately 50 fold higher concentrations were needed to reach the same effect in soup as in BHI.

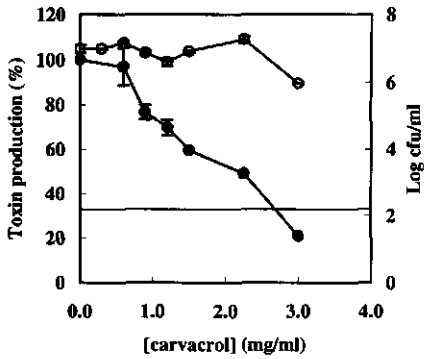

Figure 3: Influence of carvacrol on the toxin production (●) by *B. cereus* in mushroom soup after 5 days incubation (17°C) as percentage of control (no carvacrol added (=100%)) and on the viable count (○).
—— = detection limit (35%). Data represent mean values of duplicate measurements. Error bars are indicated.

DISCUSSION

Carvacrol is a natural antimicrobial compound present in oreganum and thyme. Previously, we described the bactericidal effect of carvacrol towards *B. cereus* in BHI at 8°C. The observed Minimal Inhibitory Concentrations (MIC-value) at this temperature was 0.11 mg/ml (21). Addition of carvacrol to rice (0.38 mg/g) inoculated with *B. cereus* resulted in a clear reduction of the viable count of this strain (23). Application of carvacrol to food products is limited to certain concentrations due to its influence on the taste and flavour of the product. However, the main problem of contamination of food products with *B. cereus* is the toxin production by this pathogen. It is of great interest to determine if the toxin production could be inhibited at concentrations at which growth of *B. cereus* can still occur. This study evaluates the influence of carvacrol on toxin production by *B. cereus* at 17°C. It was found that carvacrol reduced the viable count of *B. cereus* in BHI to the same extent as toxin production. However, the total amount of protein remained constant. This indicates that the toal amount of cells of *B. cereus* is not affected, however the proportion of viable cells after the experimental incubation period is reduced by the presence of carvacrol. An increase of the carvacrol concentration to 0.06 mg/ml in BHI decreased the toxin production to 28% toxin of the control (no carvacrol present). This observation shows that carvacrol could be effective in food products at concentrations lower than the MIC-value.

Paster *et al.* (13) showed a reduction of aflatoxin B1 production by *o*-coumaric acid and caffeic acid at concentrations which did not influence the final yield in mycelial dry weight of *Aspergillus niger*. A comparable effect was described by Bullerman (3). Cinnamon reduced aflatoxin production by *Aspergillus parasiticus* more than final mycelial weight. Tassou *et al.* (18) observed a similar effect when a phenolic extract of olives was added to a culture of *Staphylococcus aureus*. In all these studies toxin production is more affected than growth yield. However, Buchanan *et al.* (2) showed this is not valid for all (antimicrobial) compounds. They observed that thymol inhibited aflatoxin production by *Aspergillus parasiticus* to a smaller extent than growth (mycelium wet weight). It seems that this phenomenon is dependent on the strain and antimicrobial compound.

The mechanism of inhibition of toxin production in the cell is still unclear. It could be at the level of gene regulation, transcription or translation or by affecting transport and excretion of the toxin by carvacrol (17). There are different possible hypotheses that explain the effect of carvacrol on the toxin production. Toxin production starts at the beginning of the stationary phase. As a consequence of the lower amount of viable cells in BHI after growth in the presence of carvacrol, less toxin is produced. This phenomenon is concentration dependent. The presence of proteases in the external environment (due to lysis of the cells), could also affect the toxin concentration, due to the sensitivity of the diarrhoeal enterotoxin for proteases.

A second hypothesis is based on current knowledge of the mechanism of action of carvacrol

(22). The diarrhoeal toxin is produced in the cell and excreted. The excretion is most probably an active process and therefore energy dependent. Carvacrol makes the membrane permeable for K^+ and H^+ and consequently inhibits ATP synthesis by dissipating the proton motive force. Based on this we hypothesize that during exposure to carvacrol, the driving force for optimal secretion of the toxin (ATP or proton motive force) will not be sufficient, resulting in accumulation of the toxin inside the cell. Consequently, the intracellular toxin might inhibit its own synthesis (feedback inhibition). However, it is also possible that, as a result of a lower specific growth rate, toxin synthesis is directly inhibited, although toxin synthesis starts at the beginning of the stationary phase. Production of the toxin will cost metabolic energy and in the presence of carvacrol the cell will use the limited amount of metabolic energy for maintaining its viability and not for toxin production. Further studies, where the intracellular toxin concentration is examined during exposure to carvacrol, could give more insight in the mechanism.

Growth of *B. cereus* was observed in different soups, dependent on the pH of the medium. No growth occurred in soups when the pH was below approximately 5. This phenomenon has been described earlier (14, 17). However, the lack of essential nutrients could also play a role. While good growth was observed in mushroom soup, toxin production was detected in this medium at carvacrol concentrations up to 3.0 mg/ml. In contrast to growth in BHI, carvacrol inhibited toxin production in mushroom soup at concentrations which did not influence the viable count significantly. These results support the second hypothesis about the mechanism of action of toxin inhibition. Approximately 50 times higher concentrations were needed to reach the same effect in soup as in BHI. An interaction of carvacrol with components in mushroom soup most likely explains this observation. Fats, proteins, carbohydrates or other constituents can immobilise or inactivate carvacrol (1, 16). Soups contain more fat (30 mg/ml) than BHI. Carvacrol is a hydrophobic compound with a preference to dissolve in the lipid fraction of the soup. Consequently, the effective concentration of carvacrol in the aqueous phase where *B. cereus* tends to proliferate will be reduced. Therefore, the effective concentration of carvacrol necessary to inhibit toxin production will be higher in soup than in BHI. We recently described the antimicrobial activity of carvacrol on rice (23). The effective carvacrol concentration in the food product could be reduced by a combination of carvacrol with cymene or soya sauce as a result of the synergistic effect between carvacrol and these compounds. Possibly, these combinations also inhibit toxin production by *B. cereus*.

In conclusion, carvacrol inhibits toxin production of *B. cereus* in both BHI and mushroom soup at concentrations which do not affect the viable count of *B. cereus*. This interesting observation can be exploited for food preservation purposes. Carvacrol can be added to food products at doses below the MIC-value, thereby reducing the risk of toxin production by *B. cereus* and increasing the safety of the products. In the same time, the low doses do not affect the flavour and taste of the products.

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7

Discussion

The risk of food infections, food intoxications and microbial spoilage made it necessary to develop preservation systems long ago. The most significant reduction in risk would be achieved if the pathogens and spoilage organisms would be excluded from the food product completely. This situation can not always be reached as raw materials are often contaminated with microorganims or products are processed in open processing lines. The large number of antimicrobial systems we have available nowadays (e.g. heating, freezing, addition of chemicals), makes it possible to produce safe food products. However, consumers have a desire for high quality foods, that are not extremely processed and have a fresher, more natural taste (3, 5). The use of compounds from plants, like herbs and spices, as preservatives could serve this demand. They have been used in foods for a long time as flavour enhancers and have a future as natural antimicrobial systems as well. Billing *et al.* (1) found a positive correlation between the average temperature in a country and the amount of different herbs and spices used for the preperation of foods. This indicates that more herbs and spices are used when the risk of food poisoning and intoxication increases. Although we are probably not fully aware of this development, it seems that herbs and spices can contribute to safer food products.

Different studies addressed the antimicrobial activity of herbs, spices or its essential oils towards both (pathogenic) bacteria and fungi. However, the mechanism of antimicrobial action has not been given much attention. Due to the high hydrophobicity of these compounds an effect on the cytoplasmic membrane is expected, but not studied extensively. This thesis is a case study of different aspects of carvacrol when used in mild preservation. Carvacrol is present in the essential oil fraction of oreganum and thyme and possesses strong antimicrobial activity towards different microorganims. The mechanism of the bactericidal activity towards *Bacillus cereus* was studied in detail. Furthermore, carvacrol was applied in rice. Finally, the effect of carvacrol on the toxin production was investigated.

Mechanism of action

The bactericidal activity of carvacrol depends on different factors such as concentration, exposure time, pH and growth and exposure temperature (Chapter 2). At concentrations which do not affect the viable count, an inhibition of growth was observed, resulting in an extension of the lag-phase, a decreased maximal specific growth rate and a lower final population density. An effect of carvacrol on the cytoplasmic membrane was observed during exposure of *B. cereus* to carvacrol (Chapter 3). It was demonstrated that carvacrol makes the membrane permeable for cations such as K^+ and H^+, what results in a decrease of the membrane potential ($\Delta\psi$) and pH-gradient (ΔpH) across the cytoplasmic membrane. This leads to impairment of essential processes in the cell and finally to cell death. One process which is inhibited is ATP synthesis, but also other processes e.g. inhibition of enzymes due to loss of essential ions, loss of turgor pressure, influence on DNA activity or reduced metabolic

activity could result in cell death caused by carvacrol.
Although carvacrol is lethal for *B. cereus* at concentrations above 1 mM (20°C), this bacterium can cope with low concentrations of carvacrol. Below 1 mM (20°C) an effect on the membrane potential was observed and growth was inhibited, however the viability was not affected.

Measurement of the phase transition temperature (T_m) of the membrane lipids of *B. cereus* showed that carvacrol increases the membrane fluidity of the cytoplasmic membrane (Chapter 4). Being a hydrophobic compound, carvacrol most likely interacts with the (hydrophobic) lipid acyl chains of the fatty acids in the bilayer, thereby disordering the bilayer of the membrane. Consequently, the structural integrity of the membrane will be lost. A higher membrane fluidity will be the result of spacing of the lipid acid chains by carvacrol. A presentation of the molecular structure of phosphatidylethanolamine with two $C_{18:0}$ fatty acids is drawn in Fig. 1.

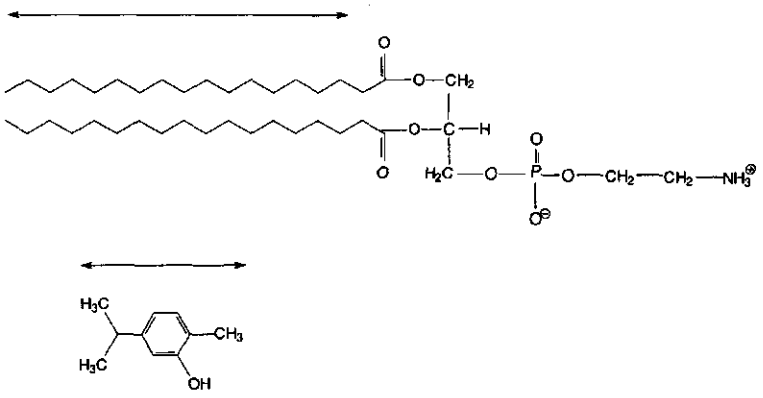

Figure 1: Structural formulae of phosphatidylethanolamine (top structure), containing $C_{18:0}$ fatty acids, and carvacrol (bottom structure). The various parts are not drawn precisely to scale.

The part of the cytoplasmic membrane where carvacrol is expected to accumulate (indicated by the arrow at the top structure) is 20 Å in length. The length of one carvacrol molecule is approximately 8.9 Å. These calculated values show carvacrol could fit well between these lipid acyl chains. *B. cereus* also contains shorter fatty acids, such as C_{14} (≈14.96 Å) up to C_{17} (≈18.74 Å). However, even these shorter chains will be long enough for carvacrol to be lodged between them. Theoretically, the hydroxyl group of carvacrol can form H-bridges with the ketone group (C=O) at the fatty acid chain. A loss of membrane integrity due to disturbance of hydrophobic interactions between lipids and proteins could be an important factor considering the influence of carvacrol on the membranes. A more thermodynamic approach could give more information about the effect of carvacrol in the membrane. In

addition, measurement of the water/membrane partition coefficient of carvacrol could give more quantitative data about its preference to accumulate in the cytoplasmic membrane.

Valinomycin is a K^+/H^+ ionophore. Exposure of cells to valinomycin results in a decrease of $\Delta\psi$ due to leakage of K^+. Valinomycin facilitates K^+ transport across the membrane by forming a hydrophilic cation complex with K^+ . This promotes the diffusion into the hydrophobic region through the hydrophilic part of the membrane. Subsequently, valinomycin is transformed to a hydrophobic complex which can cross the membrane. The complex moves physically through the membrane lipids, carrying K^+ and returns in the protonated form. Valinomycin specifically drains the cell of K^+. Growth ceases because of the requirement for K^+ in the cell (4). Although this is a plausible explanation, the structure of valinomycin differs from the structure of carvacrol. It is not likely that carvacrol can form complexes with K^+. The most acceptable hypothesis at this stage of knowledge about the mode of action of carvacrol in relation to its structure, is that carvacrol forms pathways in the membrane, by spacing the fatty acid chains of the phospholipids. Consequently, ions can pass the hydrophobic part of the cytoplasmic membrane through these pathways.

The use of spin probes in combination with Electronic Spin Resonance Spectroscopy will make it possible to gain more insight in the location and mode of action of carvacrol as this is not yet fully understood. When the exact position of carvacrol in the membrane is known, it will be easier to elucidate its mechanism of bactericidal activity on a molecular level.

Besides its effect on growth of several pathogens, carvacrol inhibits the diarrhoeal toxin production by *B. cereus* at concentrations which do permit growth of the strain. As described in Chapter 6, this could be a result of the lower amount of viable cells. However, this can also be caused by accumulation of the toxin in the cell or a direct inhibition of the toxin production by carvacrol, both due to insufficient ATP for biosynthesis. As a result of the decrease of the proton motive force across the membrane, ATP synthesis is inhibited. The cell will consume the limited amount of ATP for maintenance purposes and not to produce or excrete the diarrhoeal toxin. Based on the results described in Chapter 6, it can be concluded that carvacrol can be added to a product at concentrations below the MIC-value, thereby reducing the risk of toxin production by *B. cereus*. Carvacrol will have less influence on the taste and flavour of the product at low doses. However, further studies where the toxin concentration inside the cell is measured have to be carried out to give more insight in the mechanism of inhibition of toxin production.

Caution has to be taken, because *B. cereus* can adapt to a certain extend to carvacrol (Chapter 4). Addition of carvacrol to the growth medium increases the T_m-value of the membrane lipids and as a consequence a lower membrane fluidity is observed. At a lower membrane fluidity less carvacrol can dissolve in the membrane. This effect of carvacrol on the membrane fluidity is a physiological effect, additional studies on a genetic level will provide new information on the molecular and physiological responses of *B. cereus* exposed to stress.

Not much is known about the possibility of the cells to become resistant. Some preliminary experiments have not demonstrated a development of resistance of *B. cereus* to carvacrol.
In addition to the effect of carvacrol on vegetative cells, it will be interesting to study the effect of carvacrol on spores in more detail since sensitivity of spores to carvacrol was observed (Chapter 1). However, this sensitivity was found to be dependent on the growth condition of the spores (not described in this thesis) and therefore, more information about the influence of the growth medium is required.

Application in food products

Chapter 5 describes the application of carvacrol in rice as a natural antimicrobial agent. Carvacrol was found to be very effective as an inhibitor of growth of *B. cereus* or toxin production by this food pathogen. However, the minimal concentration necessary to inhibit growth of *B. cereus* in rice, is higher than in laboratory media (0.38 mg/g and 0.11 mg/g respectively). In general, it can be stated that components in the food matrix, such as fats, proteins, carbohydrates or other additives, can immobilise or inactivate antimicrobial compounds (2, 12). Specially fats can be a problem considering the activity of hydrophobic antimicrobial compounds. Carvacrol will have a preference to dissolve in the lipid phase. Consequently, the effective concentration in the aqueous phase where microorganims tend to proliferate will be reduced.
At the concentration of carvacrol that completely inhibits growth of *B. cereus* in rice, an effect on the flavour is expected and this could influence the sensoric properties of the product. Addition of carvacrol to fresh products such as cabbage, endive, lettuce, etc. ("living products") will result in browning of the product as a consequence of the cytotoxic activity of carvacrol. Therefore, application of carvacrol is limited to cooked or prepared foods, but could play an important role as preservative agent in these products.

The main role of carvacrol in future would therefore be in a combination system where a reduced level of carvacrol can be applied. The combination with the phenolic compound cymene or the taste enhancer soya sauce is described in Chapter 5. Strong synergistic activity between carvacrol and cymene or soya sauce was observed. Also other compounds could be suitable to be combined with carvacrol. An example is the antimicrobial compound nisin. Synergy between carvacrol and nisin was observed when applied to *B. cereus* and *Listeria monocytogenes* (11).
Carvacrol could also be used in combination with physical techniques which have an effect on the outgrowth of organisms. Pulsed Electric Field (PEF), Ultra High Pressure (UHP) or mild heat treatment are new preservation technologies which were found to be effective by reducing the viability of several pathogens. Pol *et al.* (10) described the synergistic action between nisin and PEF towards *B. cereus*. Since a synergistic action between carvacrol and

nisin exists, a combination of the three preservative factors could be even more effective. The combination of carvacrol and mild heat treatment to prevent outgrowth of *L. monocytogenes* was studied by Karatzas *et al.* (6). This combination was much more effective than the use of carvacrol or mild heat alone. UHP could be an interesting method to combine with carvacrol as well. Research is currently being done to obtain more information about the combined action.

The above mentioned combinations of mild preservation techniques and methods are examples of Hurdle Technology. This principle was described by Leistner *et al.* (7, 8). A combination of existing and novel preservation techniques (so-called hurdles) are used in order to obtain mild, but reliable preservation effects. Examples of other hurdles are temperature, modified atmosphere packaging, biopreservation, a_w, pH, redoxpotential, edible coatings, other preservatives, etc. These hurdles, when properly combined, can keep spoilage or pathogenic microorganims under control. Hurdle Technology as a concept has proven to be very successful to achieve microbial stability and safety, but also to stabilise the sensoric, nutritive and economic properties of food products (8).

Many studies report the antimicrobial activity of carvacrol using laboratory media, too few studies have been undertaken using foods. Before carvacrol can be applied in a specified product, more research dealing with the antimicrobial effect in a product and the interaction with compounds present, is required. The net effect of a number of interactive factors can be calculated using predictive modelling studies (3). Not described in this thesis, but an important observation is that carvacrol was also found to be effective towards different fungi, when applied in the gas phase. Therefore, carvacrol could also be used to products contaminated with fungi (e.g. bread).

Since carvacrol is a natural compound extracted from herbs and spices and used as a flavour enhancer, too much concern with the consumers is not expected. However, before a natural antimicrobial compound can be used as a preservative factor in a food system, it needs to be produced economically on a large scale, not cause unacceptable organoleptic changes and it must be toxicological safe (3). The application of carvacrol will be limited due to legislative regulations. Carvacrol is Generally Recognised As Safe (GRAS-status). Existing food legislation in most countries would not favour the use of natural antimicrobial compounds purified from their natural source. Purification will bring these chemicals into the same category as synthetic chemical compounds. The legislative viewpoint is that these compounds are new food additives or applied for new purposes and thus require a non-toxicity record, despite their GRAS-status (12). Any antimicrobial extract or purified compound from a natural source will have to pass many toxicological test. The new preservative systems should be non-toxic to test animals and humans, based on several studies. It is also important that the

antimicrobial compound is metabolised or excreted by the body. The compound or its breakdown products should also not result in accumulation of residues in body tissues (2). Tests which give enough data to accept carvacrol as a preservative are expensive and time consuming. This makes it not attractive for industries to look for new natural antimicrobial systems. Therefore the most favourable application of carvacrol would be the inclusion of oreganum or thyme, both containing high percentages of carvacrol in their essential oil fraction. However, the activity of the pure compound will be much higher, since higher concentrations can be reached than when the respective herb or spice is added.
In addition to the antimicrobial activity, phenolic compounds such as carvacrol, often show antioxidant activity. There are some indications that these compounds could exhibit a protective effect against liver cirrhosis, emphysema and arteriosclerotic heart disease and cancer (9). To profit from these (possible) advantages of carvacrol, the procedure of obtaining permission to use it as a new natural antimicrobial compound, should be accelerated.

In conclusion, carvacrol could play an important role in the future as a natural antimicrobial compound to inhibit outgrowth of several pathogens, particularly in additive or synergistic combination with other compounds or preservation techniques. Since even spores were found to be sensitive to exposure to carvacrol, the expectations for carvacrol as a natural antimicrobial compound in the future are high.

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SUMMARY

A new trend in food preservation is the use of mild preservation systems, instead of more severe techniques such as heating, freezing or addition of chemical preservatives. Carvacrol, a phenolic compound present in the essential oil fraction of oreganum and thyme, is known for its antimicrobial activity since ancient times. This thesis describes a study of the antimicrobial activity of carvacrol towards the foodborne pathogen *B. cereus*. Carvacrol shows a dose-related inhibition of growth of *B. cereus*. Concentrations of 0.75 mM and higher inhibit growth completely at 8°C. Below 0.75 mM, carvacrol extends the lag-phase and reduces the specific growth rate as well as the final population density. Exposure to 0.75-3 mM carvacrol decreases the number of viable cells of *B. cereus* exponentially. Spores are approximately two fold more resistant towards carvacrol than vegetative cells.

The incubation and exposure temperature have a significant influence on the sensitivity of *B. cereus* to carvacrol. An increase of the growth temperature from 8°C to 30°C decreases the fluidity of the membrane of vegetative cells and as a consequence, *B. cereus* becomes less sensitive to carvacrol. The change in membrane fluidity is probably the result of a higher percentage of lower melting lipids in the membranes at 8°C (chemical process) as an adaptation to lower temperature. Cells need to maintain an adequate proportion of the liquid-crystalline lipid in the membrane, as this is the ideal physical state of the membrane. On the other hand, an increase of the exposure temperature from 8 to 30°C, reduces the viability again. This can be explained by an increase of the membrane fluidity at a higher temperature as a result of melting of the lipids (physical process). At a higher membrane fluidity, relatively more carvacrol can dissolve in the membrane and the cells will be exposed to relatively higher concentrations than at a lower membrane fluidity.

Not only the temperature plays a role in the activity of carvacrol, also pH is an important factor. The sensitivity of *B. cereus* to carvacrol is reduced at pH 7, compared to other pH-values between pH 4.5 and 8.5.

Carvacrol interacts with the cytoplasmic membrane by changing its permeability for cations such as K^+ and H^+. Consequently, the dissipation of the membrane potential ($\Delta\psi$) and ΔpH leads to inhibition of essential processes in the cell, such as ATP synthesis, and finally to cell death. At carvacrol concentrations as low as 0.15 mM, $\Delta\psi$ is completely dissipated, however the viable count of *B. cereus* is not affected.

Vegetative cells of *B. cereus* can adapt to carvacrol when the compound is present at concentrations below the MIC-value. Compared to non-adapted cells, lower concentrations of carvacrol are needed to obtain the same reduction in viable count of adapted cells. Adapted cells were found to have a lower membrane fluidity, caused by a change in the fatty acid composition and head group composition of the phospholipids in the cytoplasmic membrane. Adaptation to 0.4 mM carvacrol increases the phase transition temperature of the lipid bilayer (T_m) from 20.5°C to 28.3°C. Addition of carvacrol to cell suspensions of adapted *B. cereus*

cells decreases T_m again to 19.5°C, approximately the same value as was found for non-adapted cells in the absence of carvacrol.

Incubation of cooked rice in the presence of different carvacrol concentrations results in a dose-related reduction of the viable count of *B. cereus*. Concentrations of 0.15 mg/g and above, reduce the viable count, leading to full suppression of growth at 0.38 mg/g. The influence of carvacrol on the viable count is dependent on the initial inoculum size. Although carvacrol is an effective inhibitor of growth of *B. cereus* in rice, it could affect the flavour and taste of the product at concentrations where full suppression of growth is observed. However, strong synergistic activity is observed when carvacrol is combined with the biosynthetic precursor cymene or the flavour enhancer soya sauce. This makes it possible to use lower carvacrol concentrations and consequently a smaller influence on the sensoric properties of the rice is expected.

Besides its influence on the viability of vegetative cells, carvacrol also shows inhibition of diarrhoeal toxin production by *B. cereus* at concentrations below the MIC-value. Addition of 0.06 mg/ml carvacrol to the growth medium, inhibits the toxin to 21% of the control (no carvacrol added). The inhibition correlates with the reduction of the viable count of *B. cereus* in the presence of carvacrol. At the same time, the total amount of cells did not change. In mushroom soup, also an inhibition of the toxin production was observed, however, the viable count did not change. This effect on the toxin production is most probably caused by a lack of sufficient metabolic energy, since carvacrol affects ATP synthesis. The cell will use its low levels of ATP to maintain its viability, rather than using it for toxin production or excretion. It could also be possible that the decreased toxin synthesis in BHI was the result of the lower amount of viable cells. The inhibition of toxin production at carvacrol concentrations which do permit growth of *B. cereus*, reduces the risk of food intoxication by this pathogen.

In conclusion, carvacrol may play an important role in future as a natural antimicrobial compound. However, its application will most probably be in combination with other natural antimicrobial systems.

SAMENVATTING

Om de groei van pathogene micro-organismen in levensmiddelen te remmen of om deze te inactiveren, bestaan verschillende methoden, zoals verhitten, vriezen, toevoegen van chemische componenten, etc. Hoewel toepassing van deze technieken het risico van groei van pathogenen sterk vermindert, hebben consumenten steeds meer voorkeur voor producten die lang houdbaar zijn, er natuurlijk en vers uit zien en waar geen chemische conserveermiddelen aan toegevoegd zijn. Om op deze vraag in te spelen, worden er nieuwe natuurlijke antimicrobiële conserveringstechnieken ontwikkeld, zoals gerichte toepassing van kruiden, specerijen of secundaire plantenmetabolieten. Een voorbeeld is carvacrol, een component die aanwezig is in de etherische oliefractie van oregano en tijm. Deze stof remt de groei van de voedselpathogene bacterie *Bacillus cereus*. Bij lagere concentraties dan de MIC-waarde (Minimal Inhibiting Concentration) wordt de lag-fase verlengd, neemt de specifieke groeisnelheid van de bacterie af en is de uiteindelijke populatiedichtheid lager. Boven 0.75 mM of 1 mM (bij resp. 8°C en 30°C) heeft carvacrol een dodende werking voor *B. cereus*. Behalve de concentratie, spelen zowel de groei- als de blootstellingstemperatuur hierbij een belangrijke rol. Verhoging van de groeitemperatuur van 8°C naar 30°C verlaagt de gevoeligheid van *B. cereus* voor carvacrol. Wanneer echter de blootstellingstemperatuur wordt verhoogd van 8°C naar 30°C, neemt deze gevoeligheid weer toe. De vloeibaarheid van de membraan is hierbij een belangrijke factor. Bij een lagere membraanvloeibaarheid kan minder carvacrol de membraan binnendringen en worden de cellen blootgesteld aan relatief lagere carvacrol concentraties. Naast de temperatuur is de pH ook van invloed op de activiteit van carvacrol. *B. cereus* is het minst gevoelig voor carvacrol bij neutrale zuurgraad (pH 7).

De werking van carvacrol berust op het vermogen van deze stof de membraan van *B. cereus* permeabel te maken voor H^+ en K^+. Deze studie heeft aangetoond dat blootstelling aan carvacrol leidt tot lekkage van K^+ de cel uit en H^+ de cel in. Het gevolg is o.a. een verlaging van de membraanpotentiaal ($\Delta\psi$). Concentraties die nog geen effect hebben op de vitaliteit van de cellen, blijken wel een invloed op $\Delta\psi$ te hebben. Boven 0.15 mM wordt $\Delta\psi$ volledig opgeheven. Als gevolg van de verplaatsing van H^+-ionen, daalt de pH gradiënt. Deze verandering van de proton motive force leidt tot een verlaging van de ATP synthese activiteit. Het gevolg is een verlaagde intracellulaire ATP concentratie. ATP is van vitaal belang voor de cel en een afname van ATP in de cel kan leiden tot een afname van de levensvatbaarheid van de cellen.

Groei van *B. cereus* in aanwezigheid van niet dodelijke carvacrol concentraties resulteert in een lagere gevoeligheid van *B. cereus* voor carvacrol. Deze adaptatie wordt veroorzaakt door een lagere membraanvloeibaarheid als gevolg van een verhoging van de smelttemperatuur van de lipiden in de membraan (phase transition temperature T_m). Bij deze temperatuur gaan de lipiden in de membraan over van een geordende gel fase naar een minder geordende vloeibare kristallijn fase. Een verhoging van T_m zal resulteren in lagere carvacrol

concentraties in de membraan en hierdoor een lagere gevoeligheid van *B. cereus* voor carvacrol. De aanwezigheid van 0.4 mM carvacrol in een exponentieel groeiende cultuur van *B. cereus* cellen, verhoogt T_m van 20.5°C (afwezigheid van carvacrol in groeimedium) tot 28.3°C. Het toevoegen van carvacrol aan een niet-groeiende celsuspensie van geadapteerde *B. cereus* cellen, verlaagt T_m opnieuw naar 19.5°C, ongeveer dezelfde waarde als voor niet-geadapteerde cellen in de afwezigheid van carvacrol. Een verandering in de vetzuursamenstelling en kopgroepsamenstelling van de membraanlipiden van *B. cereus* verklaart mogelijk de waargenomen adaptatie aan carvacrol.

Het toevoegen van concentraties van 0.15 mg/g carvacrol of hoger aan gekookte rijst leidt tot een verlaging van het aantal levensvatbare cellen van *B. cereus* op de rijst. Door de invloed van carvacrol op de smaak van levensmiddelen is een combinatie met andere (mengsels van) componenten de meest waarschijnlijke toepassing van carvacrol. Een synergistisch effect tussen carvacrol en cymeen (een intermediair in de biosynthese van carvacrol) is waargenomen op gekookte rijst. Dit effect is ook aanwezig wanneer carvacrol wordt gecombineerd met de smaakversterker ketjap.

Behalve antimicrobiële activiteit, remt carvacrol de diarree veroorzakende enterotoxine productie door *B. cereus*. Deze eigenschap van carvacrol wordt al waargenomen bij doseringen die groei van *B. cereus* toelaten. Een verhoging van de carvacrol concentratie naar 0.06 mg/ml veroorzaakt een verlaging van de toxineproductie in groeimedium (Brain Heart Infusion Medium) tot 21% van de controle (geen carvacrol toegevoegd). Deze verlaging correleert met een verlaging van het aantal levende cellen (hoewel het totale aantal cellen gelijk gebleven is). In champignonsoep werd ook een remming van de toxine productie waargenomen, hoewel deze afname niet gepaard ging met een afname van het aantal levende cellen. De oorzaak van deze remming van de toxineproductie is waarschijnlijk de invloed van carvacrol op de proton motive force, gevolgd door een remming van de ATP synthese. Door het tekort aan metabole energie zal de cel deze energie zo efficiënt mogelijk gebruiken voor het in stand houden van de vitaliteit van de cellen en niet gebruiken voor de productie of excretie van het enterotoxine. Daarnaast is het ook mogelijk dat door een afname van het totale aantal levende cellen, zoals waargenomen in BHI, minder toxine geproduceerd wordt.

Op grond van de resultaten beschreven in dit proefschrift kan geconcludeerd worden dat carvacrol in de toekomst toegepast zou kunnen worden als natuurlijk conserveermiddel, naar verwachting in combinatie met andere milde conserveringstechnieken.

CURRICULUM VITAE

Annemieke Ultee werd geboren op 15 november 1971 in Eindhoven. In 1990 behaalde zij aan het Pius X-College in Bladel haar VWO-diploma. In datzelfde jaar begon zij aan de Landbouwuniversiteit in Wageningen (LUW) met de studie Levensmiddelentechnologie. Haar eerste afstudeervak (voor de sectie Levensmiddelenchemie van de LUW) voerde ze uit bij het ATO-DLO in Wageningen. Hierna heeft ze zeven maanden stage gelopen in Engeland (University of Nottingham). Een tweede afstudeervak werd uitgevoerd bij de sectie Levensmiddelenmicrobiologie en -hygiëne aan de LUW. In maart 1996 studeerde zij *cum laude* af. Per 15 september 1996 werd zij aangesteld als wetenschappelijk onderzoeker bij het ATO in Wageningen en verrichtte het onderzoek dat leidde tot dit proefschrift. Het onderzoek werd financieel ondersteund door de Commission of the European Union (FAIR CT 96-1066). Vanaf 1 september 1999 is zij als post-doc werkzaam bij Wageningen Centre for Food Sciences (WCFS) en gedetacheerd bij het ATO. De afronding van de experimenten voor dit proefschrift werd gefinancieerd door WCFS.

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