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NEW SIGNALLING MOLECULES IN SOME FOODBORNE BACTERIA

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Introduction

Secondary metabolites

Secondary metabolites are microbial and plant products that are non-essential for growth and reproduction of the organisms that produce them. Each secondary metabolite is formed by a limited number of species and is encoded by sets of dispensable genes (Martín *et al*., 2000). These compounds are synthesized at the end of the exponential growth phase and their formation is highly influenced by the growth conditions, especially by the composition of the culture medium (Omura *et al*.,2001). Understanding of the biosynthesis of secondary metabolites and the molecular genetics of the producer strains have advanced considerably in the past two decades. The genes for the biosynthesis of secondary metabolites are usually organized in clusters on their producer strain (Martín and Liras, 1989; Keller and Hohn, 1997). These clusters include, in addition to the genes that encode the biosynthetic enzymes and regulatory proteins, genes for resistance to the toxic action of secondary metabolites (e.g. antibiotics and toxins) and genes for secretion of these metabolites. Most secondary metabolites serve as communication signals between the producer organism and other living beings, such as plants, animals or other microorganisms, which share the same habitat. Examples of these secondary metabolites include antibiotics, mycotoxins, plant growth factors, fungal elicitors and host plant and animal defensis (Davies,1990; Vining, 1992; Demain, 1993). Other secondary metabolites serve as quorum-sensing signals that trigger the differentiation of the cells or the production of pathogenicity determinants in the population (Kaiser and Losick, 1993). For this purpose, secondary metabolites are secreted to the extracellular medium to interact with other organisms.

1.1 Why do microbes make secondary products?

Why do microbes make secondary products? That question has been the subject of

intense debate for many decades. There are two extreme opinions. Some argue that most secondary metabolites play no role in increasing the fitness of an organism. The opposite view, now widely held, is that every secondary metabolite is made because it possesses, or did possess at some stage in evolution, a biological activity that endows the producer with increased fitness. These opposing views can be reconciled by recognizing that, because of the principles governing molecular interactions, potent biological activity is a rare property for any molecule to possess. Consequently, in order for an organism to evolve the rare potent, biologically active molecule, a great many chemical structures have to be generated, most of which will possess no useful biological activity. Thus, the two sides of the debate about the role and evolution of secondary metabolism can be accommodated within the view that the possession of secondary metabolism can enhance fitness, but that many products of secondary metabolism will not enhance the fitness of the producer. There have indeed been many discussions of the role of secondary metabolites in microbes (Stone and Williams, 1992; Vining, 1992b; Demain, 1995), yet the study and exploitation of secondary metabolites has progressed despite this lack of agreement as to why some microbes possess such chemical diversity. The fact that some secondary metabolites possess such potent biological activity is now widely regarded as being indicative of their purpose. However, sceptics of this viewpoint point to the fact that the very great majority of secondary metabolites have not been shown to benefit the producer. It is contended that the finding that a few secondary products possess very potent biological activity, but that the majority do not, is not contradictory but predictable on the basis that potent, specific biological activity is a rare property for a molecule to possess (Jones and Firn, 1991). The strict structural requirements that must be fulfilled in order for a low-molecular-weight chemical to bind tightly to a target protein must have been a very important evolutionary constraint in organisms that developed a secondary metabolism. For an organism to gain fitness by producing a potent biologically active chemical, it can be postulated that the possession of metabolic traits that enhance the likelihood of producing and

retaining chemical diversity would have been highly advantageous. This model for the evolution of secondary metabolism not only explains why very potent, biologically active molecules are made by some organisms, but also explains why many secondary metabolites possess unimpressive biological activity. As in the case of the immune system in animals (Lodish *et al.,* 1999), the possession of the overall machinery is crucial, but most substances made by that machinery confer no advantage to the producer.

1.2 Big effect from small changes

The biosynthesis of secondary metabolites follows the usual metabolic pathways. The enzymes that bring up a desired compound are determined by the corresponding mRNA which itself is based on the complementary DNA. One result of this multistep information flowchart is the possibility to manipulate the desired biosynthesis at different steps (figure 1). On the DNA level, for example, mutagenesis or combinatorial biosynthesis offers an easy possibility to generate new enzymatic activities resulting in modified products, (Kennedy and Hutchinson, 1999; Reynolds, 1998) whereas precursor-directed biosynthesis and mutasynthesis act in vivo using the lack of specificity of some biosynthetic enzymes to introduce different precursors into the target molecule (Thiericke, 1993). Finally, biotransformation and derivatization deal with chemical or biological modifications of the intermediates or end products of a given biosynthesis (Oikawa, 1988).

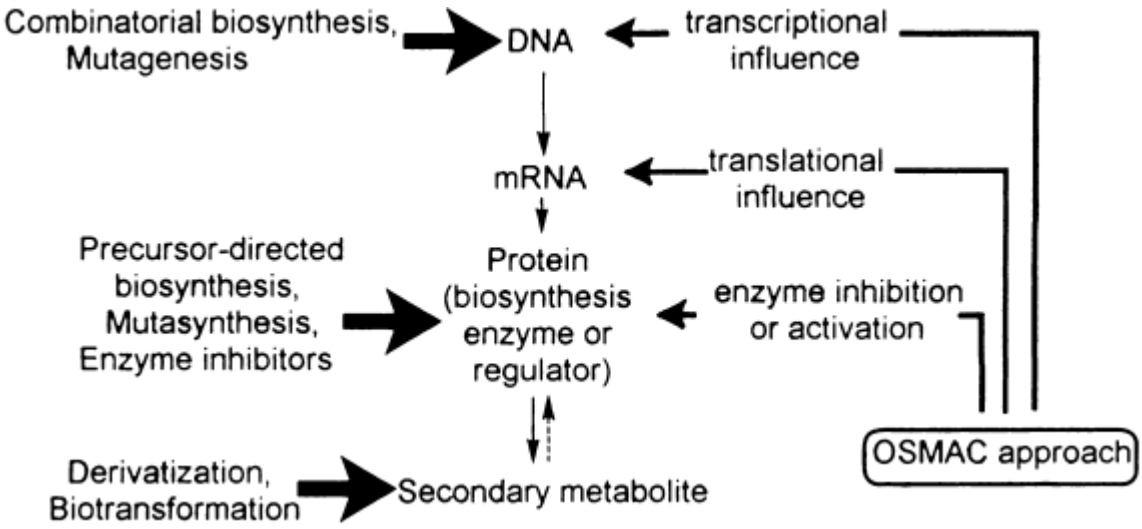

Figure 1. Possible ways to influence the biosynthesis of secondary metabolites.

It is well known that media composition can have a great impact on the production of microbial products. High glucose, phosphate, or ammonium concentrations are generally regarded as repressors of secondary metabolism, and several examples of the production of secondary metabolites in media with low contents of these components are described in the literature (Masuma *et al.,* 1983; Omura and Iwai, 1982). Contrary to these observations, high phosphate concentrations might induce the production of selected metabolites(Aoki *et al.*,1976; Gotoh *et al.*,1982; Shimada *et al..*,1986). Even usual amino acids are described as potential inducers of secondary metabolites; this underlines once more the random character of finding the optimized production media (Troost *et al.*, 1980; Zahner *et al.*,1982). In general, variation of cultivation parameters to induce the production of formerly unknown compounds is a very similar but even more random approach to the improvement of fermentations to obtain maximum production titers of desired compounds(Bushell, 1988; Strobel *et al.*, 1999; Waites *et al.*, 2001; Stansburyin, 2000). It is used the systematic alteration of easy accessible cultivation parameters (for example, media composition, pH value, temperature, addition of enzyme inhibitors, oxygen supply, culture vessel), probably the most simple and natural approach to increase the number of secondary metabolites from one single organism (Bethe, 1994). In theory every single

biosynthesis step can be influenced either at the transcriptional, the translational, or the enzyme level; this would possibly result in a vast number of permutations of new natural products (figure 1). In nature, where a different environment results in a different transcriptome, proteome, and finally a different metabolome which allows an organism to survive, one can speculate that different secondary metabolites might be the result of these special requirements (Firn *et al.*, 2000). These can be as simple as the production of siderophores after iron deficiency, but one can speculate further about the role of secondary metabolites in even more complex situations (for example, signaling, communication, predators) (Plaga *et al.*, 1998). Due to our lack of knowledge of the complex biosynthetic and regulative crosstalk in a single cell and between cells, all levels of secondary metabolite biosynthesis can be influenced by this random approach imitating natural environmental changes. We have termed this way of releasing nature's chemical diversity the 'OSMAC (One Strain-Many Compounds) approach', and it resulted from the observation that very small changes in the cultivation conditions can completely shift the metabolic profile of various microorganisms. Furthermore, the biosynthetic pathways that are prerequisite to this diversity will be discussed.

1.3 Substrate specificity: different rules in primary and secondary metabolism

When a new enzyme variants arises by mutation to extend metabolism, it usually differs from the wild type in terms of its substrate specificity and not the type of chemical catalysis it can conduct (Petsko *et al*., 1993). New enzyme variants that arise with a broad substrate specificity will be more likely to carry out a new transformation than new variants with a very narrow substrate specificity, simply because the range of substrates available to the broad-specificity variant will be larger. Thus, it seems probable, but not inevitable, that most new enzymes will possess a broad substrate specificity, and high specificity will more usually be gained by subsequent selection. Selection to reduce the range of substrates acted upon will

only occur if increased benefits or reduced costs result from improving selectivity. Judging by the fact that most, but not all, enzymes involved in primary metabolism are highly substrate specific, the benefits that accrue from increasing specificity may be very significant in primary metabolism. However, in secondary metabolism, in which some of the benefits may only accrue spasmodically and where new threats are ever present, the selection pressures would be expected to be different from those operating on primary metabolism. Selection pressures to increase substrate specificity may not exist, quite the contrary. By retaining a broad substrate specificity, the generation and maintenance of chemical diversity may be enhanced, as illustrated in figure 2.

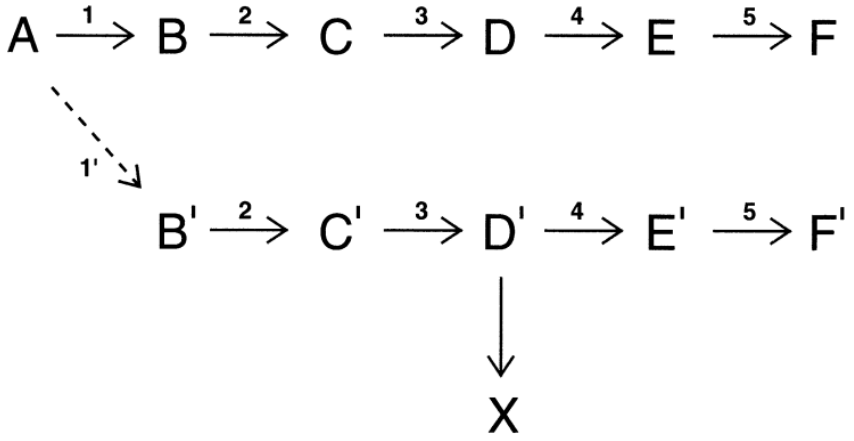

Figure 2. The increased generation of chemical diversity after a mutational event if a broad substrate tolerance is available. The addition of one new enzyme 1' results in six new products.

In this model, a substrate A is converted by a series of enzymes into five other compounds, with each conversion being carried out by a unique enzyme. Suppose a mutation gives rise to a new variant of the organism, which produces a compound B', which is structurally similar to B. If the enzymes in the pathway B → F now act on B', new compounds C', D', E' and F' will arise. The addition of one new enzyme (1') has resulted in the production of five new compounds. If any of these compounds possesses beneficial biomolecular activity and if the costs incurred are sustainable,

the new variant may be advantaged during selection. The best available evidence for this model to explain secondary product diversity comes from a study of terpene biosynthesis in plants (one can justify using evidence from plant secondary metabolism because the basic principles governing the evolution of biomolecular activity are molecular, and the type of organism or the type of product should not negate these principles). A mutant of spearmint produced a mix of monoterpenes that were characteristic of peppermint (Croteau *et al*., 1991). A single gene mutation caused the spearmint to lose several compounds and to gain several more. The changes were caused by the mutant hydroxylation enzyme adding a hydroxyl to a 3-position in a cyclohexene ring (B' in figure 2) instead of the wild-type 6-hydroxylation (B in figure 2). The subsequent substrate-tolerant enzymes in the pathway accepted the new substrates to give the new products. Furthermore, the generation of chemical diversity will beget further diversity. Thus, in figure 2, X is shown as being formed from D by some enzyme not in the A → F pathway. This is similar to the reported appearance of a new, unexpected product in the spearmint. A microbial example of this concept is illustrated by the finding that the addition of a gene coding for phytoene desaturase from *Erwinia* into *Rhodobacter* resulted in the production of a number of new carotenoids (Garcia-Asua *et al*., 1998). This could be an example of a "gene saving device", which Cerda-Olmedo (1994) suggested was needed to explain how so few genes could produce such large chemical diversity in some microbes. More recent evidence for such inherent biosynthetic flexibility in microorganisms comes from a study of polyketide synthases (PKS) (Hutchinson, 1999; Shen *et al*., 1999). The flexibility of the PKS pathway derives from an impressive substrate tolerance (Byford *et al*., 1997). This tolerance not only allows each unit of the modular pathway to accept a wide range of substrates, but it also allows the substitution or elimination of individual modules to give another layer of chemical diversity generation. The biochemical flexibility of the PKS pathway not only helps to explain the existence of the > 3000 polyketides known in nature, but also provides a rational basis for further attempts to manipulate the PKS pathway

genetically to generate new chemical diversity. The possibility of creating thousands, if not millions, of 'new' (at least to humans) polyketides (McDaniel *et al*., 1999) can be seen not to be fortuitous but an inherent trait predicted by the principles discussed. Studies of a microbial peptide synthase also showed a relaxed substrate specificity, which was considered to contribute to the generation of chemical diversity (Baldwin *et al*., 1994). Furthermore, it was postulated that the use that the use of enzymes that produce more than one product, or the incorporation of non-enzymic reactions into secondary metabolic pathways, would be advantageous in terms of generating and retaining chemical diversity at low cost. A microbial example of this concept is illustrated by isopenicillin N synthase from *Cephalosporium*, which has the ability to convert one substrate into six different β-lactam products (Baldwin *et al*., 1984).

1.4 Secretion systems for secondary metabolites

Considerable progress has been made regarding the characterization of secretion systems for primary metabolites, such as the amino acid lysine in several bacteria (Vrljic, et al.1999). This has led to the recognition of a new family of membrane proteins involved in secretion of amino acids that are different to the classical amino acid permeases (import systems) (Vrljic, et al., 1996; Bellmann et al., 2001)

Knowledge of the systems involved in the secretion of secondary metabolites has also advanced in the past two decades. Transmembrane proteins encoded by genes located in the clusters of antibiotic biosynthesis genes have been cloned and their role in antibiotic secretion and antibiotic resistance is becoming clearer. These proteins include efflux systems for secretion of industrially important antibiotics, such as penicillins and cephalosporins, and several other secondary metabolites.

These ‘antibiotic pumps’ belong to the multiple drug resistance (MDR) protein class. Knowledge of the specificity of these exportation systems in the antibiotic-producing organisms is of great interest because of their basic and industrial relevance. This information could also help us to understand the role of the vast array of MDR

proteins in the detoxification of chemical compounds. In this review, we will discuss those examples of secondary metabolite secretion systems that are the best characterized to date (Table 1).

Table 1. Examples of bacterial and fungal transporters located in the clusters of biosynthesis of secondary metabolites.

Transporters	**Gene**	**Microorganism**	**Secreted product**
ABC	*atrD*	*Aspergillus nidulans*	Penicillin
	carA	*Streptomycs thermotolerans*	Carbomycin
	dirB	Streptomyces *peucetius*	Daunorubicin
	nocH	*Nocardia uniformis*	Nocardicin A
	mtrA, mtrB	*Streptomyces argillaceus*	Mithramycin
	oleB, oleC	*Streptomyces antibioticus*	Oleandomycin
	orf7, orf8, orf10	*Lysobacter lactamgenus*	Cephabacin
	pimA, pimB	*Streptomyces natalensis*	Pimaricin

Transporters	**Gene**	**Microorganism**	**Secreted product**
	srmB	*Streptomyces ambophaciens*	Spiramycin
	tlrC	*Streptomyces fradiae*	Tylosin
MFS	*actII-orf2, actII-orf3, actVA*	*Streptomyces coelicolor*	Actinorhodin
	cefT, cefT3	*Acremonium chrysogenum*	Cephalosporin
	CFP	*Cercospora kikuchii*	Cercosporin
	cmcT	*Streptomyces clavuligerus*	Cephamycin C
	cmcT	*Amycolatopsis lactamdurans*	Cephamycin C
	entC	*Streptomyces maritimus*	Enterocin
	frnF	*Streptomyces roseofulvum*	Frenolicin
	lmrA	*Streptomyces lincolnensis*	Lincomycin
	mmr	*Streptomyces coelicolor*	Methylenomycin

Transporters	Gene	Microorganism	Secreted product
	otrB	*Streptomyces rimosus*	Tetracyclin
	ptr	*Streptomyces pristinaespiralis*	Pristinamycin
	pur8	*Streptomyces alboniger*	Puromycin
	tcmA	*Streptomyces glaucescens*	Tetracenomycin
	thnJ	*Streptomyces cattleya*	Thienamycin
	toxA	*Cochliobolus carbonum*	HC-toxin
DME	*pecM*	*Erwinia chrysanthemi*	Indigoidine

Recent genome-sequence data, in addition to classical biochemical and molecular genetic studies, have revealed that most living organisms have multidrug transporters(Marger et al., 1993; Dean et al., 1995; Kuan et al.,1995). These membrane proteins recognize a wide variety of structurally different compounds and actively extrude them from the cytoplasm into the outer medium (Ambudkar et al.,1999; Putman et al., 2000; Georgiev, 2000; Neyfakh, 2002; Paulsen, 2003). These transporters are structurally diverse and belong to one of four different protein superfamilies: ATP-binding cassette (ABC) transporters; major facilitator

superfamily (MFS); small multidrug resistance (SMR); and resistance nodulation determinants (RNDs) (Paulsen et al., 1996; Sorbo et al., 2000). On the basis of bioenergetic criteria, multidrug transporters can be divided into two major classes: the primary active transporters, which include the ABC transporters that require ATP hydrolysis as an energy source, and secondary multidrug transporters (MFS, SMR and RND), which utilize the transmembrane electrochemical gradient of protons or sodium ions to drive the extrusion of drugs from the cell. Whereas the ABC transporters are, in general, multicomponent proteins that are capable of transporting both small molecules and macromolecules in response to ATP hydrolysis, the MFS transporters are single polypeptide secondary carriers that are only capable of transporting small solutes in response to chemiosmotic ion gradients. Antibiotic resistance in several antibiotic-producing *Streptomyces* is mediated by ABC transporters. The ABC transporters are a large family of membrane-associated export and import systems (Hyde et al., 1990; Higgins et al., 1992). Most ABC transporters contain four membrane-associated domains: two hydrophobic and two hydrophilic. The hydrophilic component is presumed to bind ATP and to couple its hydrolysis to the transport process. The two hydrophilic domains share a highly conserved amino acid region of about 200 residues. This region represents the ATP-binding domain and has two characteristic nucleotide sequences, known as Walker A and B motifs. They participate in the secretion of many different molecules from cells, including sugars, amino acids, oligopeptides and ions. Some of the most important transporters of this family, because of their clinical implications, are the eukaryotic multidrug resistance proteins, which are responsible for the generation of multiresistance to chemotherapeutic drugs (Paulsen and Skurray, 1993).

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1.5 Biomolecular activity and the evolution of secondary metabolism

Screening programmes provide ample evidence that, for any biological target, most chemicals, whether synthetic or naturally occurring, are inactive unless tested at high

concentrations (Firn and Jones, 1996). For example, when 400 000 microbial cultures were screened over a 10 year period, only three useable antibiotics were discovered (Fleming *et al*., 1982). However, the relevance of this evidence to discussions about the evolution of secondary metabolism has been challenged by Berenbaum and Zangerl (1996), who contended that the low frequency of activity found in screening trials was simply the result of using inappropriate screening methodologies. They argue that, if the "correct" targets were used, a very high frequency of biological activity would be found. Why is there such disagreement on such a fundamental issue? The crux of the disagreement seems to lie with the definition of the term "biological activity". Only by defining what biological activity means in terms of the evolution of secondary metabolism will it be possible to advance the debate. Biological activity studied at a molecular level *in vitro* can have a different meaning to biological activity studied at a whole organism level. At the molecular level, there is ample evidence that specific biological activity against a defined molecular target is a rare property for a molecule to possess that is why high-throughput screening protocols capable of assessing the biological activity of 100 000 chemicals per day have been developed, and it is why chemical libraries with in excess of 1 million compounds are commercially available for drug screening. The experience of several decades of large screening programmes is now underpinned by a secure conceptual understanding. Ligand-binding studies reveal that high-affinity, reversible, non-covalent interactions between a ligand and a protein only occur when the ligand has exactly the right molecular configuration to interact with the complex three-dimensional structure of the protein (Lodish *et al*., 1999). It is proposed that this type of biological activity should be given the term "biomolecular activity", and it should be defined as the ability of a molecule to interact with a biologically functional molecule such that its biological function is changed significantly. There is overwhelming experimental evidence that, at low concentrations ($< 10^{-5}$ M), any one chemical has a very low probability of showing biomolecular activity against any one target protein (Firn and Jones, 1996). However, it is predictable that the frequency of

molecules possessing biological activity will be higher if activity is assessed by targeting an organism instead of a protein. An organism contains thousands of potential protein targets; hence, if one were screening for a somewhat non-specific effect (performance or survival) on an unadapted organism, it is predictable that a higher frequency of activity will be found than in a screen based on biomolecular activity. Further aggregation will occur if the chemical is tested against many diverse species. Furthermore, if the concentration of every chemical being tested against an organism is increased, the laws of mass action predict that the frequency of finding any effect will increase further. Thus, the low probability of finding potent biomolecular activity against a specific molecular target at a low concentration is entirely consistent with the view that a higher frequency of less specific activity might be found if a very wide range of unadapted organisms is screened using a high concentration of each chemical (Berenbaum and Zangerl, 1996). However, where in this continuum between the extreme definitions of biological activity (potent biomolecular activity against a specific target versus low-potency 'toxicity' against any organism) is selection operating in terms of the chemical interactions between organisms? In evolutionary terms, the only target organisms that matter are those that have had an opportunity to interact with the producer organism. An effect produced in any other organism cannot act as a focus for selection. That restriction substantially reduces the number of possible chemical–target organism combinations (Firn and Jones, 1996). Similarly, in evolutionary terms, the only concentration that matters is that which a target organism would receive under normal circumstances physiological effects shown only at concentrations that are above those achievable in the natural environment cannot be of selective significance. In our opinion, the most common evolutionary scenario for selection operating on specific parts of the secondary metabolism will have involved few rather than many target organisms. Furthermore, we consider that selection will have favoured organisms that can produce effective chemicals at low cost, and that will favour the selection of organisms capable of producing highly potent chemicals. High potency results from a

strong ligand–protein interaction, and that is necessarily dependent on a very specific ligand structure fitting a precise target site on the protein, hence giving rise to a very specific biomolecular effect (Lodish *et al*., 1999). These considerations suggest that the constraints that apply to the evolution of 'biomolecular activity' will have been important in the evolution of secondary metabolism.

Volatile metabolites

Microbial interactions via infochemicals are fundamental to the development of spatial distribution and activity variations in ecosystems. Microorganisms produce a wide range of infochemicals, frequently secondary metabolites, most of which are soluble and many volatile. Volatile organic compounds (VOC) profiles produced by microorganisms are consistent, relating to cultural conditions, environment and inputs, and so to population and function dynamics. VOC-mediated interactions can result in functional responses by the organisms involved that result in selective advantage to some community members. Positive, negative or neutral interactions can occur between a very wide range of bacteria and fungi. These effects include both stimulation and inhibition of growth, by 40 and 60%, respectively, and enzyme production. These effects are usually transient, e.g. removal of an antagonist is followed by complete recovery. Up- and down-regulation of gene expression, by mRNA and protein profiling has been demonstrated. VOCs have played an important role during the evolution of microorganisms in the context of their communities.

2.1 Species specific

Chemical control mechanisms are common in biological systems. Many chemically mediated interactions have been reported in the biosphere, e.g. in the insect world, and between plants and mammals. The compounds involved in these interactions are termed 'infochemicals'. Frequently, changes in microbial process rates cannot be explained by corresponding changes in inputs and the environment. It is possible that such phenomena result from infochemical mediated interactions in the microbial facet of the biosphere. VOCs are ideal candidates for this role. Individual microbial species produce a reproducible profile of VOCs. There are many reports of a consistency of production in response to consistent environmental parameters such as nutrient availability and temperature (Tronsmo and Dennis 1978; Zechman and Labows 1985; Giudici et al. 1990; Fiddaman and Rossall 1994;Wheatley et al. 1997; Bruce et al. 2000). Variations in microbial growth conditions result in changes in both the types and amounts of VOCs produced. Such changes of input can be apparently quite small. For example changing only the specific amino acid, L-phenylalanine, L-arginine or glutamine, used in a growth medium, but maintaining the same C:N ratio and other cultural conditions, resulted in significant, reproducible changes in VOC output by *Trichoderma* spp. being used as antagonists (Bruce et al. 2000). Similarly, growth of the target cultures of *Neolentinus lepidus*, *Gloeophyllum trabeum* and *Coriolus versicolor* was also affected in significantly different ways, with growth being inhibited by between 20 and 60%, depending on the microbial couplet and amino-acid used in the antagonist's medium. Products from growth with L-arginine were the most suppressive against all the fungi and L-phenylalanine the least. Principal component analyses showed that aldehyde and ketone volatile products were associated with the greatest inhibition of these basidiomycetes and that the use of different amino acids consistently resulted in the production of a different suite of VOCs by the *Trichoderma* isolates. Perhaps surprisingly using a combination of all three amino acids together in the same substrate produced a different catalogue

of VOCs to that from the sum of each individually. Similarly, when different more complex media were used differences in the VOC outputs of *T. pseudokoningii* and *T. viride* (Table 2) were also reported (Wheatley *et al*. 1997).

Number	Compound	Number	Compound
1	Ethanol	24	2-Methyl- I -butanol
2	2-Methyl-pentane	25	I-Pentanol
3	Propanal	26	Hexanal
4	2-Propanone	27	p-Xylene
5	Isopropyl alcohol	28	1-Hexanol
6	4-Penten-2-ol	29	2-Heptanone
7	Hexane	30	Heptanal
8	5-Methyl-5-hexem-3-ol	31	2,2,4,6,6-Pentamethyl-3-heptene
9	Acetonitrile	32	Limonene
10	3-Methyl-propanol	33	Formic acid heptyl ester
11	I-Propanol	34	2-Octen-l-ol
12	Ethyl ester acetic acid	35	6-Methyl-5-hepten-2-one
13	2,4,6-Trimethyl-1 -nonene	36	2-Octanone
14	3-Methyl-hexane	37	Octane
15	Butanal	38	Benzaldehyde
16	2-Butanone	39	2-Propyl-l-pentanol
17	Heptane	40	Nonanal
18	2-Methyl-l-propanol	41	Acetic acid, 2-ethylester
19	Methyl-cyclohexane	42	Decanal
20	1-Butanol	43	Benzothiazole
21	Octane	44	Caryophyllene
22	2-Propenylidene-cyclobutene	45	Pentadecane
23	3-Methyl-1-butanol		

Table 2. The list of compounds identified in the headspace samples of *Trichoderma* spp. (Wheatley et al. 1997).

In this case biplot analyses of the VOCs produced by each isolate on the different media again showed a species specific consistency of output and also identified five 'candidate' chemicals that might be capable of affecting the growth rate of the basideomycetes (Figure 3). These were 2-propanone, 2-methyl-1-butanol, heptanal, and octanal and decanal, respectively (Wheatley et al. 1997). These and many other microbial products have been collected and identified from both cultures and soil

atmospheres, (Table 2) and related to community structure and function in relation to inputs of different nitrogen and carbon sources (Wheatley et al. 1996).

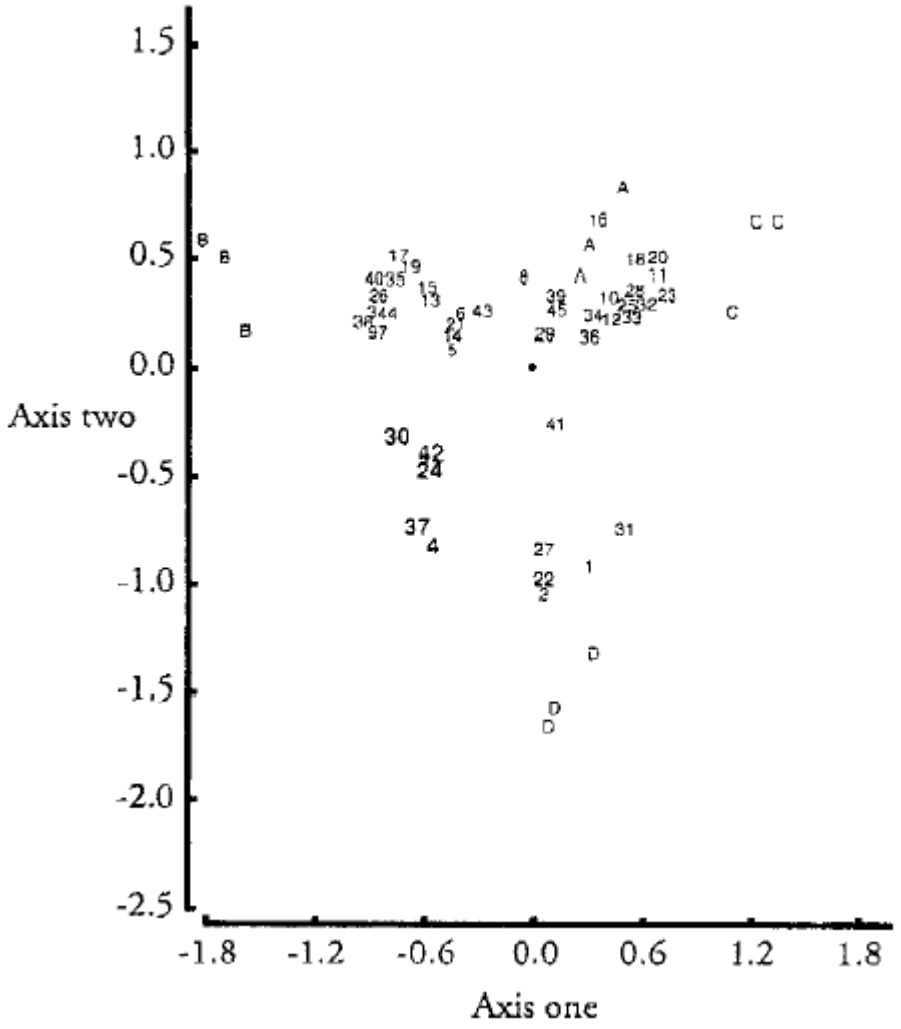


Figure 3. Principal component analysis biplot separating VOCs produced by *T.aureoviride* grown on low nutrient media containing phenylalanine (LNM-B); arginine (LNM-C); glutamine (LNM-D) and all three amino acids (LNM-A). Note: the map position of each VOC indicates its importance in the separation of the four media types. (Wheatley et al. 1997).

2.2 VOCs as infochemicals

VOCs are ideal as infochemicals because of the ability to be effective over a wide range of scales. Their spheres of influence will extend from proximal interactions, due to aqueous diffusion, to greater distances via 'atmospheric' diffusion through the

tortuous connectivity of such as soil pore structures and even into the open troposphere. In this way activity in the rhizoplane can be relayed over distance to the bulk soil. Diurnal patterns of water movement in, and mass flow of water down the soil profile will also move these volatile compounds rapidly around the system. There are many situations in which communication between microorganisms would be to the advantage of at least some of the parties involved. The evolution of organisms to a state were the opportunist can simply switch on in response to some advantage such as substrate availability, rather that having to continually drain its energy resources by maintaining a constant state of readiness, would obviously be of great competitive advantage. The substrate-dependent variation in VOC production will result in variations in microbial, and consequently system response. A rapid response to such intermittent production of substrates would be advantageous, as rapid aquiral would prevent competitors from using such substrates and from occupying any desired environmental niches. The effectiveness of other more active exploratory organisms, such as pathogenic fungi, will be enhanced as the organism will be able to follow a chemical gradient to a potential host rather than simply randomly spreading in opportunist hope. Also, VOCs produced by one organism could enhance its status by affecting the physiology of other competitor organisms causing them to function at a slight disadvantage.

2.3 Specific microbial interactions

Four fungi selected to be representative of a range of habitats, *T. viride*, a common soil saprophyte, *Phanaerochaete magnoliae*, a pathogen of beech trees, *Phytophthora cryptogea* a plant pathogen with a wide host range and *Gaeumannomyces graminis* var. *tritici*, a specific pathogen of wheat, were challenged by a number of randomly selected soil bacteria. This showed that VOC-mediated positive, negative or neutral interactions occur between a very wide range of soil bacteria and fungi (Mackie and Wheatley 1998). These responses were species specific, with each fungus responding

uniquely to the products of each of the bacterial cultures. The four numbered bacteria illustrate the differing range of effects specific bacterial isolates had on the different fungal isolates (Mackie and Wheatley 1998). All the bacterial isolates either significantly stimulated or inhibited the growth rate of at least one of the fungal species. Some fungal growth rates were inhibited, by up to 60%, and others stimulated by up to 35% ($P<0.05$). No one bacterial isolate was effective against all of the fungi. The majority, 54%, of the bacterial isolates inhibited the growth rate of some fungi but stimulated others. Many bacteria, 42%, could only inhibit growth, but none were solely stimulatory ($P<0.05$). Growth of some inhibited fungi only resumed when the fungus was placed onto fresh medium. Similarly, cores taken from the growing margins of cultures did not grow ($P<0.05$) when placed onto medium that had previously been exposed to the bacterial cultures. Similarly, an investigation of the effects of a random selection of 250 bacterial soil isolates, showed both significant stimulation and inhibition, of up to 40 and 60%, respectively, of the radial growth of fourteen biotypes of *Microdochium nivale*. The two most efficacious bacteria were preliminarily identified as an *Enterobacteriaceae* and a *Pseudomonas/Burholderi* type, and subsequently as *Citrobacter freundii*, and a strain of *Pseudomonas fluorescens.*

Alcohols:	**Ketones:**
Ethanol	Propane-2-one
Propan-1-ol	Butan-2-one
Butan-1-ol	Pentan-2-one
Butan-2-ol	Pentan-3-one
2-Methyl propan-1-ol	4-Methyl pentan-2-one
2-Methyl butan-1-ol	5-Methyl heptan-2-one
	3-Hydroxy butan-2-one
Aldehydes:	**Aromatics:**
2-Methyl-butan-1-al	Benzene
3-Methyl-butan-1-al	Ethyl Benzene
	Dimethyl benzene
Sulphides:	Trimethyl benzene
Dimethyl sulphide	Benzaldehyde
Dimethyl disulphide	
Dimethyl trisulphide	
2-Methyl propylsulphide	**Ethyl esters:**
	Acetic acid
Methyl esters:	Butanoic acid
2-Methyl butanoic acid	2-Methyl propanoic acid
3-Methyl-butanoic acid	2-Methyl-butanoic acid
	3-Methyl-butanoic acid
Butyl esters:	
Acetic acid	

Table 3. Volatile organic compounds detected in the headspace of aerobically and anaerobically incubated soils.

Similarly, Alstrom (2001) reported that all of the 21 strains of soil bacteria, isolated from oil seed rape roots, tested suppressed the pathogen *Verticillium dahliae* in both direct and indirect ways. Again, *Enterobacteriaceae* were prevalent amongst the interactive bacteria. Nine were *Enterobacteriaceae*, one being further identified as *Serratia proteamaculans*. Three species of interactive *Pseudomonads* were also

identified; two were strains of *Pseudomonas putida*, together with *Pseudomonas acidovorans* and *Pseudomonas chlororaphis*. Others identified as interactive included *Stenotrophomonas* sp. and *Alcaligenes* sp.
isolates. Some of the bacteria prevented symptom development in field rape plants. In a study of the interactions between mycorrhizal fungi and other soil organisms, Fitter and Garbaye (1994) stated that bacteria play an important role in promoting mycorrhizal formation in the soil. Azcon-Aguiler et al. (1986) reported that both germination and hyphal growth of the AM fungus *Glomus mosseae* were enhanced in the presence of rhizosphere bacteria and postulated that the organic products of soil bacteria may be responsible for these interactions (Azcon-Aguiler & Barea
1985).

2.4 Mechanisms

Bacterial VOCs affect both fungal mycelial growth and enzyme activity. There are several reports of the effects of VOCs on enzymes, both directly and indirectly. Laccase activity in *Phanaerochaete magnoliae* ceased completely on exposure to all of the bacterial isolates and was significantly reduced in *T. viride.* Tyrosinase activity in *Phanaerochaete magnoliae* was increased, inhibited or not affected, depending on the bacterium to which it was exposed, but activity in *T. viride* was not affected by any of the bacterial isolates used (Mackie and Wheatley 1998). Any effects on the limitation of mycelial growth may be the result of interference with enzyme production rather than some inhibition of enzyme activity. Preliminary studies on fungi at the molecular level have shown up and down-regulation of gene expression on exposure to VOCs. Similarly protein, synthesis in *Serpula lacrymans* was affected by the volatile secondary metabolites of *T. aureoviride* and *T. viride*, in a parallel way to the effects on mycelial growth (Humphris et al. 2002). It has been clearly demonstrated that VOC-mediated positive, negative or neutral interactions occur between a very wide range of bacteria and fungi. Indeed, it appears that interactions

are so widespread that it is probable that all microorganisms can have an effect on some other member of the microbial community. VOC mediated microbial interactions have also been shown to be species-specific, consistent and responsive to the environment, essential requirements for a signaling system. Such interactions are subtle, being neither fatal nor necessarily inhibitory, and usually reversible. Growth rates and enzyme activity levels may be modified and gene expression can be up or down regulated. So it is probable that microbially produced VOCs have played an important role during the evolution of microorganisms in the context of their interactions, and community, population and functional dynamics. Such interactions will have resulted in functional responses by the organisms involved that have given selective advantage to some community members and coincidental disadvantage to others. The interactions between individual target fungi and a selection of soil bacteria appear to reflect previous associations. In the relationships between the soil inhabiting plant pathogens *Phytophthora cryptogea* and *Gaeumannomyces graminis* and soil bacteria it appears that the presence of active bacteria in the rhizosphere prompts the pathogen to develop. So, the pathogen exploits the opportunities presented by the presence of a host plant only when investment in growth is liable to be profitable. Conversely, there are also a significant number of interactions in which the pathogens are significantly inhibited and so association by the plant with these particular bacteria would be to its positive benefit. Relationships between *T. viride* and *Phanaerochaete magnoliae* and soil bacteria are virtually all disadvantageous to the fungi. However, the ecological niches of these require consideration. In soil the saprophyte *T. viride* will be in direct competition for resources with soil bacteria. So, it will be to the latter's advantage to protect its good fortune in acquiring a carbon source, normally the limiting factor in a soil system, by reducing the effectiveness of any potential competitors to reach that source. Contrastingly, *Phanaerochaete magnoliae* is not normally in contact with soil organisms in its tree environment. Hence, the couplet pattern in which it is very strongly affected by the vast majority of

the soil organisms may reflect this fact that *Phanaerochaete magnoliae* has no history of competition with these organisms.

In conclusion, volatile organic compounds, microbial secondary metabolites, play an important role in the functional development of systems by contributing to the evolution of links between community members, which then use competition and detection to advantage. Such knowledge enlarges our understanding of the interlinking of processes, possibly at different trophic levels, in the whole biosphere.

Quorum sensing in bacteria

Quorum sensing is the regulation of gene expression in response to fluctuations in cell-population density. Quorum sensing bacteria produce and release chemical signal molecules called autoinducers that increase in concentration as a function of cell density. The detection of a minimal threshold stimulatory concentration of an autoinducer leads to an alteration in gene expression. Gram-positive and Gram-negative bacteria use quorum sensing communication circuits to regulate a diverse array of physiological activities. These processes include symbiosis, virulence, competence, conjugation, antibiotic production, motility, sporulation, and biofilm formation. In general, Gram-negative bacteria use acylated homoserine lactones as autoinducers, and Gram-positive bacteria use processed oligo-peptides to communicate. Recent advances in the field indicate that cell-cell communication via autoinducers occurs both within and between bacterial species. Furthermore, there is mounting data suggesting that bacterial autoinducers elicit specific responses from host organisms. Although the nature of the chemical signals, the signal relay mechanisms, and the target genes controlled by bacterial quorum sensing systems differ, in every case the ability to communicate with one another allows bacteria to coordinate the gene expression, and therefore the behavior, of the entire community. Presumably, this process bestows upon bacteria some of the qualities of higher organisms. The evolution of quorum sensing systems in bacteria could, therefore, have been one of the early steps in the development of multicellularity.

3.1 Intraspecies communication

Bacteria have evolved elaborate means to communicate with each other, both within

and between species. Intraspecies communication is far and away the best characterized, simply due to the ease of working with pure cultures of bacteria. From this work, it has been shown that signaling pheromones in gram-positive bacteria are generally peptides, while the vast majority of such pheromones in gram-negative bacteria are small molecules, such as N-acyl homoserine lactones. These signaling pheromones accumulate with increasing cell density, triggering signaling events when a "quorum" is reached; hence the name "quorum sensing" (QS) to describe this phenomenon (Fuqua et al.,1994). The general paradigm is that peptides in gram-positive bacteria signal through receptor-histidine kinases (RHKs) embedded in the membrane, (Inouye and Dutta, 2003) while small molecules can diffuse across the cytoplasmic membrane in gram-negative bacteria to bind to regulatory proteins within the cell to trigger transcriptional changes. However, there are already exceptions to this paradigm, as will be discussed in this review, and it is also most likely the case that many peptides and small molecules exist and signal through membrane-bound or cytoplasmic receptors in all types of bacteria and as of yet remain undiscovered. This is supported by genomic data indicating the presence of putative signaling peptides and transporters in gram-negative bacteria (Michiels *et al*., 2001) and the characterization of small signaling molecules, known as γ-butyrolactones, that appear to function in a cell density-dependent manner to elicit antibiotic production in the gram-positive genus *Streptomyces* (Takano et al., 2001). Further study of signaling mechanisms in *Streptomyces* is of particular importance given the fact that strains in this genus produce thousands of bioactive natural products, many of which are important in medicine and agriculture. The complete genome sequences of *Streptomyces coelicolor* and *Streptomyces avermitilis* were recently published, which should greatly aid further efforts to characterize signaling in these bacteria(Ikeda *et al*.,2003).

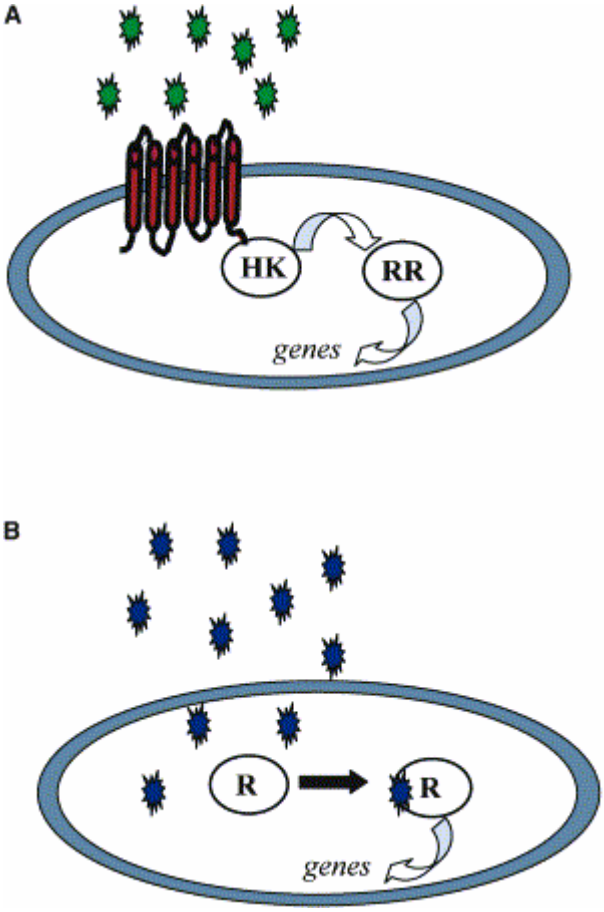


Figure 4. Schematic of Chemical Signaling in Bacteria(A) Peptide signaling through receptor-histidine kinases (RHKs) in gram-positive bacteria. The extracellular signaling molecules, shown as stars, bind to the sensor domain of the RHK, triggering activation via phosphorylation or dephosphorylation of the HK domain. A classic phosphorelay to or from the response regulator (RR) ensues, which controls gene expression at the level of transcription. The sensor domain of RHKs contains a variable number of transmembrane helices, with 6–8 TM helices as the standard for peptide binding.(B) Small molecule signaling through intracellular receptors in gram-negative bacteria. An intracellular receptor protein, labeled R, is stabilized upon binding the diffusible or actively transported signaling molecules (shown as stars). This receptor protein then binds to DNA and modulates gene expression.

3.2 QS in Gram positive bacteria

Gram-positive bacteria also regulate a variety of processes in response to increasing cell-population density. However, in contrast to Gram-negative bacteria, which use HSL autoinducers, Gram-positive bacteria employ secreted peptides as autoinducers for quorum sensing. In general, the peptide is secreted via a dedicated ATP-binding cassette (ABC) transporter. Again, in contrast to the widespread use of LuxR-type proteins as autoinducer sensors by Gram-negative bacteria, Gram-positive bacteria use two-component adaptive response proteins for detection of the autoinducers. The signaling mechanism is a phosphorylation -dephosphorylation cascade (Bassler, 1999; Kleerebezem et al., 1997; Lazazzera et al., 1998). A general model for quorum sensing in Gram-positive bacteria is shown in figure 5 . In brief, secreted peptide autoinducers increase in concentration as a function of the cell-population density. Two-component sensor kinases are the detectors for the secreted peptide signals. Interaction with the peptide ligand initiates a series of phosphoryl events that culminate in the phosphorylation of a cognate response regulator protein. Phosphorylation of the response regulator activates it, allowing it to bind DNA and alter the transcription of the quorum sensing–controlled target gene(s). Several Gram-positive quorum sensing systems have been extensively studied. Here we describe the model systems controlling competence in *Streptococcus pneumoniae*, competence and sporulation in *Bacillus subtilis*, and virulence in *Staphylococcus aureus*. As described above for Gram-negative quorum sensing bacteria, in Gram-positive bacteria the fundamental signaling mechanism is conserved, but differences in regulation/timing of the systems have apparently arisen to heighten the effectiveness of the signal transduction process for a given environment.

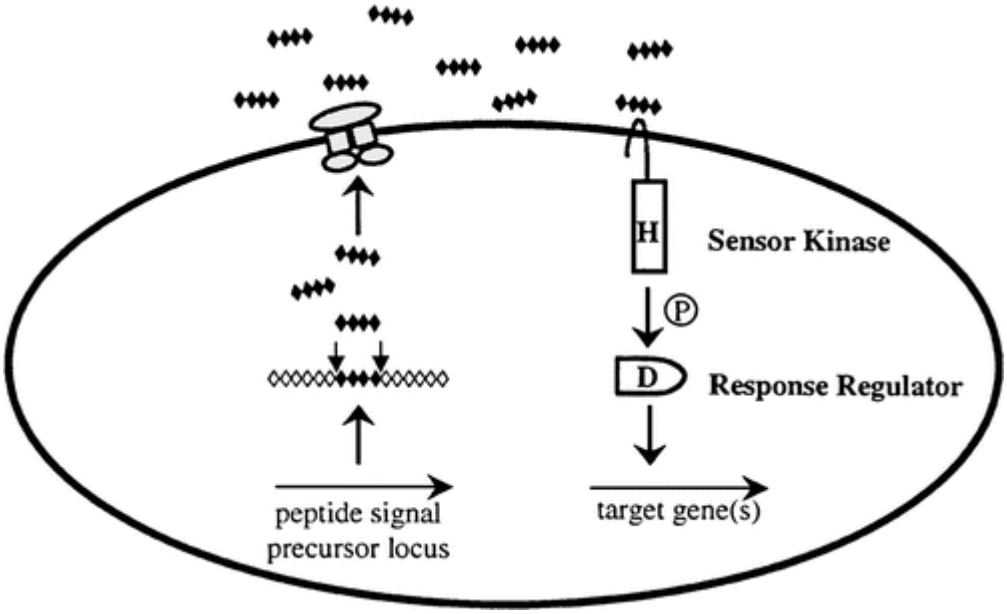


Figure 5. A general model for peptide-mediated quorum sensing in Gram-positive bacteria. In Gram-positive bacteria, a peptide signal precursor locus is translated into a precursor protein (black and white diamonds) that is cleaved (arrows) to produce the processed peptide autoinducer signal (black diamonds). Generally, the peptide signal is transported out of the cell via an ABC transporter (gray protein complex). When the extracellular concentration of the peptide signal accumulates to the minimal stimulatory level, a histidine sensor kinase protein of a two-component signaling system detects it. The sensor kinase autophosphorylates on a conserved histidine residue (H), and subsequently, the phosphoryl group is transferred to a cognate response regulator protein. The response regulator is phosphorylated on a conserved aspartate residue (D). The phosphorylated response regulator activates the transcription of target gene(s). The oval represents a bacterial cell. The "P" in the circle represents the phosphorylation cascade. Note that the lengths of the precursor and processed peptides are not meant to signify any specific number of amino acid residues.

There are at least 17 putative two-component signaling systems in the genome of the gram-positive bacterial pathogen *Staphylococcus aureus*, all of which play some role in cell-cell or cell-environment communication (. Many functions in *S. aureus*, including virulence, are controlled by at least one of these two-component systems, known as the accessory gene regulator (*agr*) operon (reviewed in Bassler et al., 1994; Bassler, 1994; Beck von Bodman 1995). As *S. aureus* cells grow, a small (<10 amino acid) extracellular peptide, known as the autoinducing peptide (AIP), is secreted and

accumulates. This AIP is derived from processing of the propeptide, AgrD, by the putative processing enzyme, AgrB. Upon reaching a threshold concentration in the tens of nanomolar range, the AIP binds to and triggers activation of the receptor-histidine kinase, AgrC. This activation results in increased transcription of the unique regulator, RNAIII, ultimately leading to increased secretion of virulence and other accessory factors and downregulation of various surface proteins. This signaling process is but one example of density-dependent or quorum-sensing systems widespread in bacteria (Figure 5 and Table)

Table 4. Some Bacterial Processes Controlled by Quorum Sensing

Biological Processes	Bacterial Species	Signaling Molecules	Inhibitors
virulence/competence	*Streptococcus pneumoniae*	CSP, BlpC*	RHK inhibitors
virulence	*Enterococcus faecalis*	GBAP, $CylL_s''$	
virulence	*Staphylococcus aureus*	AIP	TrAIP-II and other AIP analogs
competence	*Bacillus subtilis*	ComX	
bacteriocin production	*Lactococcus lactis*	Nisin	
bioluminescence	*Vibrio harveyi*	HSLs, AI-2	
virulence	*Vibrio cholerae*	CAI-1, AI-2	
biofilms/virulence	*Pseudomonas aeruginosa*	HSLs	Furanones, modified HSLs
conjugation	*Agrobacterium*	HSLs	
plant infection	*Bradyrhizobium japonicum*	Bradyoxetin	

Gram-negative bacteria are in the last five rows.

The sequence of the AIPs is highly variable, resulting in at least four specificity groups of strains within *S. aureus* and many more (>25) in other staphylococci (Cao et al., 1989; Chernin et al.,1998). A group is defined as the collection of strains that produce the same AIP. The *agrB*, *D*, and *C* regions vary in concert to maintain the specificity of AIP processing and function, and this specificity results in four different receptors for the AIPs in *S. aureus*, designated AgrC-I, -II, -III, and -IV, reflecting the group that expresses them. Remarkably, there is extensive cross-communication at the level of ligand-mediated signaling, as most AIPs activate their cognate receptor while inhibiting activation of nonnative receptors (Choi and Greenberg, 1991). This inhibition is a form of bacterial interference that does not result in growth inhibition but rather in the block of accessory gene functions, presumably resulting in an advantage for the strain producing the most abundant

and/or most potent AIP.

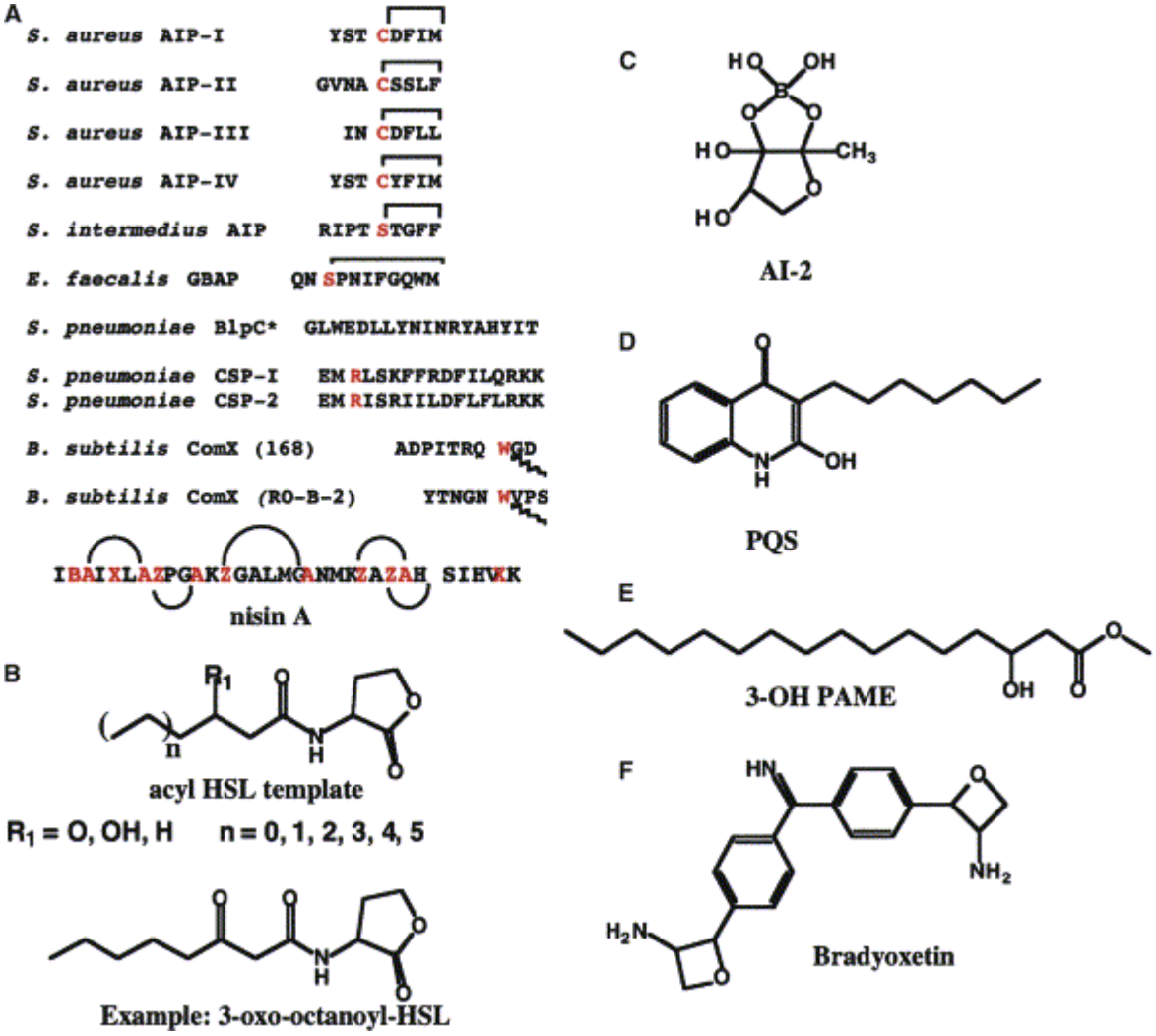


Figure 6. Chemical Composition of Bacterial Signaling Molecules.(A) Signaling peptides in gram-positive bacteria. Conserved residues that are posttranslationally modified and/or are critically important for agonist activity are marked in red. The connectivities for cyclization in the AIPs are shown with semicircles or lines. For nisin A, the lanthionine bridges are indicated by semicircles. B, dehydrobutyric acid (Dhb); X, dehydroalanine (Dha); Z, aminobutyric acid (Abu). The lipid modifications, which are different from each other in composition (see main text), on the tryptophan of *B. subtilis* AIPs are marked with a squiggly line.(B) Acyl-HSLs in gram-negative bacteria. A generic structure depicting some of the possible HSLs is shown, although this is by no means comprehensive, and all of the possible combinations have not yet been isolated. An example from *Agrobacterium tumefaciens* is shown for clarity. Furthermore, some HSLs contain an unsaturated double bond in their acyl chain, and the acyl chains of virtually all HSLs have an even number of carbons regardless of chain length as a necessity of their metabolic synthesis.(C–F) (C), AI-2 has been shown to trigger bioluminescene and virulence in *V. harveyi* and *V. cholerae*,

respectively; (D), PQS (*Pseudomonas* quinolone signal), 2-heptyl-3-hydroxyl-4-quinolone; (E), 3-OH PAME (3-hydroxypalmitic acid methyl ester); (F), bradyoxetin.

The *S. aureus* AIPs are 7–9 residues in length, depending on the group, and contain a thiolactone ring structure in which the α-carboxyl group at the C terminus is linked to the sulfhydryl group of a cysteine, which is always the fifth amino acid from the C terminus of the peptide (Figure 6) (Choi and Greenberg, 1992). Note, the AIP from *S. intermedius* has recently been shown to contain a lactone ring rather than the more usual thiolactone constraint (Christie, 1997). A combination of chemical synthesis, genetics, and structural and biological analysis has been used to study the structure-activity relationships within the AIPs and the RHK, AgrC (Cubo et al., 1992; Davies et al., 1998 ; Davis et al., 1995 ; Dawson et al., 1931 ; de Kievit et al., 2000 ; Dessaux et al., 1992; Dong 2000). This integrated approach has revealed some of the structural features important for the activation and inhibition activities of the AIPs (Figure 6) and has paved the way to the rational design of global inhibitors of *S. aureus* virulence (see below). A particularly remarkable finding relates to the effects of changing the thiolactone linkage within the 16-atom membered macrocycle of the AIP. Lactam analogs of AIP-I and AIP-II are potent cross-group inhibitors, but activate receptors within their group only at very high concentrations. NMR analysis of the AIP-II lactam analog revealed dramatic differences in the backbone chemical shifts of residues within the ring (to roughly the same extent as linearizing the peptide), whereas the chemical shifts of the tail residues were essentially unaffected. This points to the structural independence of the exocyclic (i.e., tail region) and endocyclic (i.e., within the macrocyle) regions of the molecule. Perhaps more importantly, these studies strongly suggest that the molecular recognition mechanisms underlying the competitive receptor-agonist and receptor-antagonist interactions are different; modification of the thiolactone moiety dramatically affects the structure of the macrocycle, yet this perturbation results only in loss of agonist activity.

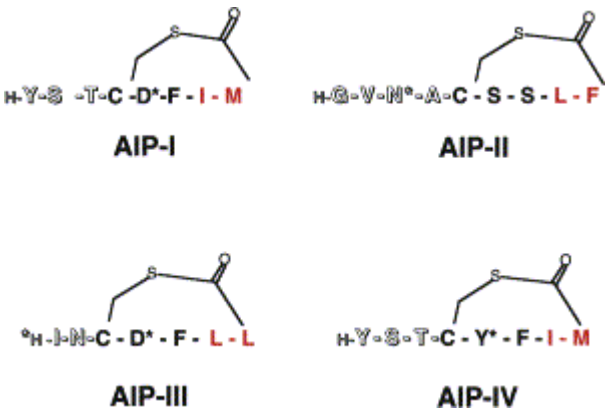


Figure 7. Composition and Key Determinants of the *S. aureus* AIPsStandard single-letter codes for amino acids are indicated. The sulfur atom of the cysteine and the carbonyl contributed from the C-terminal amino acid are shown in a thioester linkage, which closes the macrocycle. Exocyclic (tail) residues are represented by outlined and shaded text. Residues that are critical for receptor activation are marked with an asterisk. The N terminus of AIP-III is marked with an asterisk to reflect the fact that additional amino acids on the N terminus abolish receptor activation. The two C-terminal amino acids, highlighted in red, are conserved in terms of hydrophobicity in all staphylococcal AIPs characterized to date.

Based on the above studies, we now have a basic understanding of the mechanisms underlying agonism and antagonism of AgrC by native AIPs. However, our understanding of how AIP binding leads to presumed AgrC autophosphorylation is still in its infancy. The biosynthetic mechanism by which the AgrD propeptide is converted into the mature AIP is equally poorly understood. There is good evidence that the integral membrane protein, AgrB, is responsible for the posttranslational modification of AgrD and possibly the secretion of mature AIP (Dunny et al., 1978; Dworkin, 1973). For processing to occur, the propeptide must be cleaved internally in two locations, along with cyclization to form the thioester linkage. It is tempting to speculate that the cleavage of the C-terminal portion of the AIP from within the propeptide could occur through the formation of a acyl-enzyme intermediate, which would then be primed for nucleophilic attack by the sulfhydryl of the cysteine in the AIP, thus causing cyclization via thioester formation. However, the mechanistic details of this fascinating biotransformation remain to be elucidated, including how

the respective enzymes faithfully process staphylococcal AIPs that vary in length from 7–9 amino acids, where this length difference is entirely determined by the varying N-terminal cleavage sites within the corresponding AIP propeptides. Given the detailed understanding that has emerged concerning AIP-induced signaling in *S. aureus*, along with the naturally occurring cross-inhibition that has been characterized, it is only logical that efforts would be undertaken to develop inhibitors of this signaling, with an eye toward the development of novel antiinfectives. Substantial progress has been made toward this goal, which will be discussed later in this review in a separate section focusing on inhibitors of quorum sensing in general.

3.2.1 *Virulence control in Enterococcus faecalis*

There are at least nine putative two-component systems found in the genome of *Enterococcus faecalis*, some of which represent potential therapeutic targets (Dworkin and Kaiser, 1985). Analogous to the *agr* system in *S. aureus*, there exists one similar autoregulated two-component system in the bacterial pathogen *E. faecalis* known as the *E. faecalis* regulator (*fsr*) (Eberhard et al., 1981). This locus includes a receptor-histidine kinase, FsrC, a response regulator, FsrA, and a putative AgrB-like processing enzyme, FsrB. It has been shown that all three genes in the *fsr* operon are important for the production of virulence factors, such as gelatinase and a serine protease, and that mutation of these genes results in attenuated virulence in a mouse peritonitis model (Eber et al., 1999) and a relatively new *C. elegans* killing model (Eberl et al., 1996). In contrast with the *agr* system, where the AIP is processed from a dedicated propeptide AgrD, the *E. faecalis* AIP (also referred to as GBAP) is likely derived from the C terminus of the putative processing enzyme, FsrB (Engebrecht et al., 1983). However, there is ~19% sequence identity between FsrB and *S. aureus* AgrB-I-IV, and the propeptides in both systems are cleaved internally to release AIPs with new N and C termini. Furthermore, both AIPs contain a cyclic structure formed from the condensation of the α-carboxyl group of the peptide with a nucleophilic side chain situated on an amino acid located N-terminal to this in the AIP. It is likely that

this cyclization is mediated by their respective processing enzymes, AgrB and FsrB. For the one characterized *E. faecalis* AIP, the nucleophile corresponds to the hydroxyl group on a serine residue nine amino acids away from the AIP C terminus, thus forming a lactone peptide. The use of lactone peptides for bacterial cell-cell communication is further supported by the recent discovery of a *S. intermedius* lactone AIP. To date, no inhibitors of *E. faecalis* AIP-induced signaling have been reported. However, further structure-activity relationship studies of the *E. faecalis* AIP will most likely reveal key residues that are important for receptor activation but do not affect receptor binding. Such AIP analogs would constitute competitive antagonists, much like what has been developed in the *S. aureus agr* system, and thus might have therapeutic utility.

3.2.2 *Antimicrobial peptide production by autoinducer-mediated quorum sensing in lactic acid bacteria*

Many lactic acid bacteria (LAB) produce ribosomally synthesized antimicrobial peptides (AMPs) usually referred to as bacteriocins. These peptides are diverse in terms of structure, mode of action, spectrum of antimicrobial activity and potency. Because of their antimicrobial properties, the peptides are of relevance for the food and pharmaceutical industries, and therefore their production has been investigated. In recent years, several research laboratories have reported examples of LAB in which the production of AMPs is an inducible phenotype dependent on the presence of 'inducing peptides' in the culture supernatant (Kleerebezem and Quadri 2001). These examples contrast the more commonly found situation, where AMPs are apparently produced in a constitutive fashion and without the need for AIs. The first inducible systems were identified by serendipity. For example, production of AMPs by *Carnobacterium piscicola* was observed to be mysteriously lost from time to time. The explanation for this phenomenon was not as simple as it was first thought to be, that is, the head of the laboratory, who decided to work at the bench that summer, inoculated the wrong strain. Rather, the AMP− phenotype was eventually correlated

with small-size inocula utilized to start the cultures and the lack of AIs in the culture supernatants (Saucier et al. 1995, 1997; Quadri et al. 1997a,b; Franz et al. 2000a,b; Kleerebezem and Quadri 2001; Kleerebezem et al. 2001). Inducible production of the AMP nisin A by strains of *Lactococcus lactis* was also discovered by chance. It was observed that a 4-bp deletion in the *nisA* gene not only abrogated nisin A production, but also suppressed the transcription of the mutant allele *_nisA* (Kuipers *et al*. 1993). It was subsequently discovered that addition of nisin A to the culture supernatant of the mutant restored *_nisA* transcription, indicating that nisin A had AI activity (Kuipers et al.1995; Dodd et al. 1996; Van Kraaij et al. 1997). Today, 7 years after the first reported example of AI-mediated induction of AMPs in LAB, it is widely recognized that not only many LAB, but also other Gram-positive bacteria have evolved mechanisms to control production of AMPs via a phenomenon called quorum sensing (de Vos et al. 1997; Dunny & Leonard 1997). Quorum sensing, in its broadest definition, is a cell–cell communication strategy that enables unicellular organisms to behave in a multicellular manner by allowing population-wide synchronized behavioural responses as a function of cell density. All quorum sensing systems utilize AIs, however of different chemical natures, as communication signals (Fuqua et al. 1996, 2001; Dunny & Leonard 1997; Fuqua & Greenberg 1998; Kleerebezem et al. 1997a). In addition to the production of AMPs, examples of behavioural responses modulated by quorum sensing are production of antibiotics and toxins, sporulation and cell differentiation, development of genetic competence, bioluminescence, conjugative plasmid transfer, biofilm formation, and virulence response (for review see Dunny & Winans 1999).

3.3 QS in Gram-negative bacteria

Many gram-negative bacteria use acylhomoserine lactones (acyl-HSLs) as intercellular signals in density-dependent gene regulation (reviewed in (Fuqua et al., 2002; Whitehead et al., 2001). The first acyl-HSL, *N*-(3-oxohexanyoyl)-L-

homoserine lactone, was identified in the marine luminescent bacterium *Vibrio fischeri* in 1981 (Eberhard et al., 1981). Since that time, numerous bacteria, including *Pseudomonas aeruginosa, Agrobacterium tumefaciens, Rhizobium leguminosarum*, and *Rhodobacter sphaeroides*, have been shown to produce a wide range of acyl-HSLs, all differing in the length of the acyl moiety and in the degree of oxidation at the C3 position. Acyl-HSLs are known to signal through a protein known as LuxR (or its homologs) and are produced by an enzyme known as LuxI (or its homologs). LuxR contains two domains: the N-terminal region contains conserved residues known to be required for acyl-HSL binding, and the C-terminal region of the protein contains a predicted helix-turn-helix motif that has been implicated in DNA binding. It has been surmised that density-dependent accumulation of acyl-HSLs from basal LuxI-mediated production leads to increased binding of acyl HSLs to the N-terminal domain of already formed LuxR, thus relieving an autoinhibited conformation of the protein (reviewed in (Fuqua et al., 2001). However, recent structural studies on a LuxR homolog, TraR, from *Agrobacterium tumefaciens* have shown that the pheromone, at least for TraR, is deeply embedded in a hydrophobic cavity with virtually no solvent contact (Zhang et al., 2002; Vannini et al., 2002). Indeed, there is evidence that TraR is stabilized toward cellular proteolysis by binding to the pheromone(Zhu et al., 1999; Zhu et al., 2001), suggesting that the pheromone might indirectly affect gene transcription by stabilizing functional TraR dimers. It remains to be seen whether or not this mechanism of pheromone-induced protein stabilization holds true for other LuxR homologs, especially given the fact that it appears that some LuxR-related proteins bind DNA in the absence of acyl-HSLs (Von Bodman et al., 2003). Acyl-HSLs are produced by the LuxI family of synthases from the substrates acylated acyl carrier protein (acyl-ACP) and S-adenosyl-L-methionine (SAM). The enzymology of acyl-HSL synthesis has been investigated extensively, culminating most recently with the crystal structure of the LuxI homolog, EsaI (Watson et al., 2002). This study revealed structural similarities between EsaI and *N*-acetyltransferases, including a common phosphopantetheine binding fold as the

catalytic core. The structure provides support for a sequential ordered reaction (Schaefer et al., 1996) in which the acyl chain of the acyl-ACP, which is presented as a thioester of the ACP phosphopantetheine prosthetic group, is attacked by the nucleophilic amine of SAM. This is followed by lactonization, which occurs by intramolecular nucleophilic attack on the γ carbon of SAM by its carboxylate oxygen to produce the homoserine lactone product (fFigure 8). Furthermore, as acyl-HSLs produced by different bacterial species vary both in the length of the acyl chain as well as in the degree of oxidation at the C3 position, the structure suggests that such differences can be accommodated by coordinated sequence differences in and near the binding pocket, much like what is seen in HSL binding by LuxR homologs. Lastly, there are other groups of HSL biosynthetic enzymes that appear to have no significant homology to the LuxI enzyme, although they appear to catalyze HSL synthesis from the same substrates, at least for the LuxM type of enzymes.

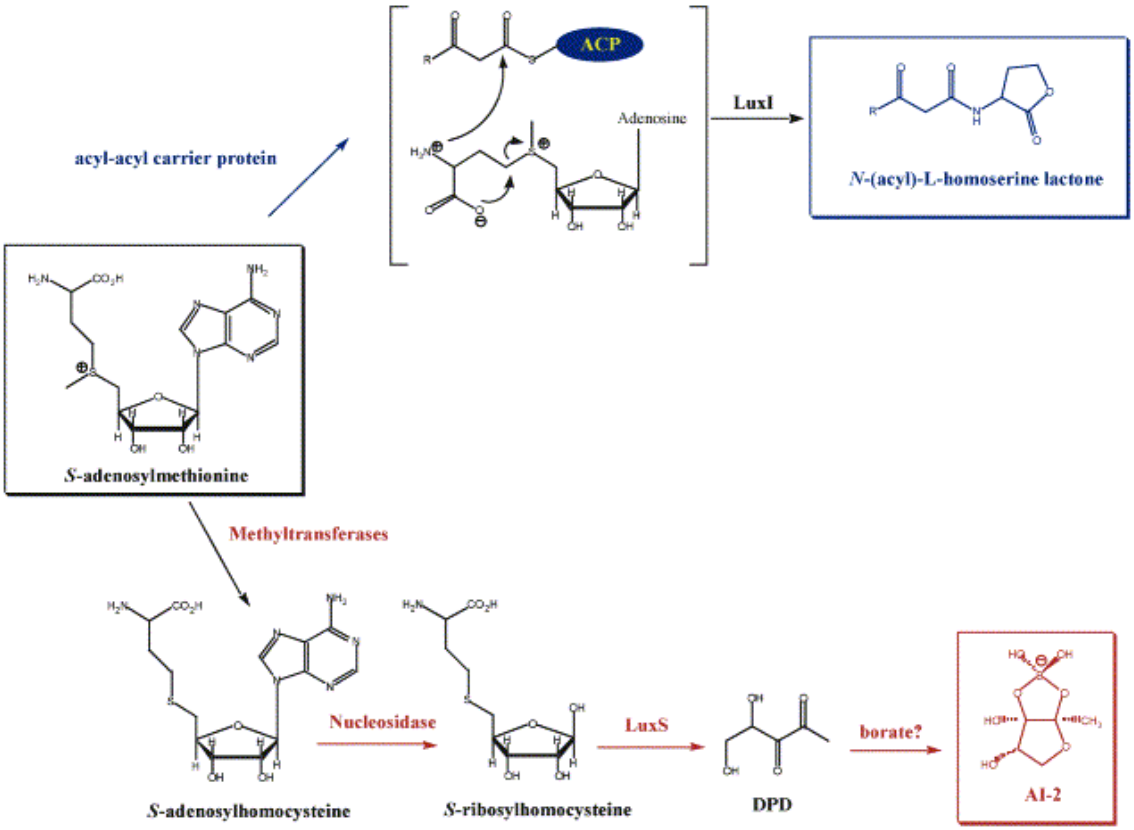


Figure 8. Biosynthesis of *N*-(Acyl)-L-Homoserine Lactones and AI-2, a Furanosyl Borate DiesterBoth signaling molecules are derived from *S*-adenosylmethionine. The synthase enzymes and cosubstrates involved in the ASL and AI-2 pathways are

indicated in blue and red, respectively. The mechanistic details of these transformations are still poorly understood, although structures of LuxI and LuxS enzymes have recently been determined (see main text). DPD, 4,5-dihydroxy-2,3-pentadione.

In recent years, many investigators have begun to focus on quorum sensing in the opportunistic human pathogen *Pseudomonas aeruginosa* due to its role in a variety of human illnesses, including infections in immunocompromised patients suffering from AIDS, cystic fibrosis (CF), severe burn wounds, or other ailments (reviewed in Smith et al., 2003) and references therein). *P. aeruginosa* produces and secretes multiple extracellular virulence factors, including proteases, hemolysins, exotoxin A, exoenzyme S, and pyocyanin, all of which can cause extensive tissue damage in humans and other mammals. *P. aeruginosa* produces at least two quorum-sensing acyl-HSLs, *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL) and *N*-butyryl-L-homoserine lactone (BHL), which signal through the LuxR homologs LasR and RhlR, respectively. Signaling through these quorum-sensing circuits potentially coordinates the expression of hundreds of genes during *P. aeruginosa* growth, as deduced from transcriptome analysis (Schuster et al., 2003; Wagner et al.,2003). Abundant evidence indicates that mutation of these quorum-sensing circuits results in virulence attenuation in burn, respiratory infection, and other animal models of human disease. Similarly, the role of quorum sensing in *P. aeruginosa* infection of CF patients is also well established, including in the regulation of biofilm formation (Singh et al., 2000). It is worth noting that there are other potential acyl-HSLs in *P. aeruginosa* (Shaw et al., 1997), although it is not known what the functions of these putative molecules might be. Given the serious nature of bacterial infections, including those caused by gram-negative bacteria and particularly *P. aeruginosa*, the acyl-HSL based quorum-sensing circuitry has become an important target for drug discovery efforts.

3.3.1 *The Vibrio fischeri LuxI/LuxR bioluminescence system*

The most intensely studied quorum sensing system is that of the bioluminescent marine bacterium *V. fischeri*. This bacterium lives in symbiotic association with a number of eukaryotic hosts. In each case the host has developed a specialized light organ that is inhabited by a pure culture of a specific strain of *V. fischeri* at very high cell density. In these symbiotic associations the eukaryotic host supplies *V. fischeri* with a nutrient-rich environment in which to live. The role of *V. fischeri* is to provide the host with light (for review see Ruby EG., 1996; Ruby and McFall-Ngai, 1992). Each eukaryotic host uses the light provided by the bacteria for a specific purpose. For example, in the squid *Euprymna scolopes–V. fischeri* association, the squid has evolved an antipredation strategy in which it counter-illuminates itself using the light from *V. fischeri*. Counter-illumination enables the squid to avoid casting a shadow beneath it on bright clear nights when the light from the moon and stars penetrates the seawater (Visick and McFall-Ngai, 2000). In contrast, the fish *Monocentris japonicus* uses the light produced by *V. fischeri* to attract a mate. In this case two luminescent regions exist on the fish that are apparently seductive to fish of the opposite sex. Other uses for the *V. fischeri* light, such as warding off predators and attracting prey, have also been documented (Nealson and Hastings, 1979). Regardless of the purpose for which the eukaryotic host has adapted the light, the regulation of light production by *V. fischeri* in the specialized light organs is identical. Light emission is tightly correlated with the cell-population density of the bacterial culture in the organ, and this phenomenon is controlled by quorum sensing. In the light organ the *V. fischeri* culture grows to extremely high cell densities, reaching 10^{11} cells per ml (Nyholm and McFall-Ngai, 1998). As the *V. fischeri* culture grows, it produces and releases an autoinducer hormone into the extracellular environment, and the hormone is trapped inside the light organ with the bacteria. The specialized eukaryotic light organ is the only niche in which the autoinducer molecule is predicted to accumulate to any significant concentration and thus act as a signal. Accumulation of the autoinducer is assumed to communicate to the bacteria that they are "inside" the host as opposed to

"outside" in the seawater. Detection of the autoinducer by *V. fischeri* elicits a signaling cascade that culminates in the emission of light (Engebrecht et al., 1983). Thus, the quorum sensing system of *V. fischeri* has evolved to specifically enable the bacteria to produce light only under conditions in which there is a positive selective advantage for the light. As mentioned above, the luciferase enzymes required for light production in *V. fischeri* are encoded by *luxCDABE*, which exists as part of the *luxICDABE* operon (Lee et al., 1993). Two regulatory proteins called LuxI and LuxR comprise the quorum sensing apparatus. LuxI is the autoinducer synthase enzyme, and it acts in the production of an HSL, *N*-(3-oxohexanoyl)-homoserine lactone . LuxR functions both to bind the autoinducer and to activate transcription of the *luxICDABE* operon (Stevens et al.,1994; Stevens et al., 1999;). Specifically, at low cell densities, the *luxICDABE* operon is transcribed at a low basal level. Therefore, a low level of autoinducer is produced (via *luxI*), and because the genes encoding luciferase are located directly downstream of the *luxI* gene, only a low level of light is produced. The HSL autoinducer is freely diffusible across the cell membrane, so the concentration of autoinducer in the extracellular environment is the same as the intracellular concentration of the autoinducer (Kaplan and Greenberg, 1985). As the *V. fischeri* culture grows, autoinducer accumulates to a threshold level (~1–10 μg/ml) that is sufficient for detection and binding by the cytoplasmic LuxR protein (Eberhard et al., 1981). Interaction of LuxR with the autoinducer unmasks the LuxR DNA binding domain, allowing LuxR to bind the *luxICDABE* promoter and activate its transcription (Hanzelka and Greenberg, 1995). This action results in an exponential increase in both autoinducer production and light emission. The LuxR-HSL complex also acts to negatively regulate expression of *luxR*. This negative feedback loop is a compensatory mechanism that decreases *luxICDABE* expression in response to the positive feedback circuit (Engebrecht et al., 1983).

3.3.2 *Quorum sensing in Salmonella enterica*

Quorum sensing in *Salmonella* has been an elusive topic for a long time. However, in the past 8 years, several research groups have demonstrated that these bacteria use several quorum-sensing systems, such as: the *luxS*/AI-2, AI-3/epinephrine/norepinephrine, indole, and the LuxR homolog SdiA to achieve intercellular signaling. The majority of these signaling systems are involved in interspecies communication, and the AI-3/epinephrine/norepinephrine signaling system is also involved in interkingdom communication. *Salmonella* resides in the human intestine, which is the largest and most complex environment in the mammalian host. The observation that these bacteria evolved quorum-sensing systems primarily involved in interspecies communication may constitute an adaptation to this environment. The gastrointestinal tract harbors a high density and diversity of bacterial cells, with the majority of the flora residing in the colon (10^{11}–10^{12} bacterial cells/ml). Given the enormous number and diversity of bacteria inhabiting the gastrointestinal environment, it should not be surprising that the members of this community communicate amongst themselves and with the host itself to coordinate a variety of adaptive processes. Furthermore, it has been previously reported (Surette and Bassler, 1998) that *S. typhimurium* and *E. coli* strains produce a signalling activity that stimulates *lux* expression in *V. harveyi*, and the signalling molecule acts exclusively through the *V. harveyi* quorum-sensing system 2. The characteristic quorum-sensing behaviour of *V. harveyi* is shown in the control experiment (closed circles). Immediately after dilution into fresh medium, the light emitted per cell by *V. harveyi* drops rapidly over 1000-fold. At a critical cell density, which corresponds to the accumulation of a critical concentration of endogenously produced autoinducer (AI-2) in the medium, the luminescence per cell increases exponentially, ≈3 orders of magnitude, to again reach the predilution level. Addition of 10% cell-free culture fluid prepared from *V. harveyi* BB152 (AI-1^{-}, AI-2^{+}) caused the reporter strain to maintain a high level of light output after dilution

(open circles). The increased light output is due to the *V. harveyi* BB170 cells responding to the presence of AI-2 in the cell-free culture fluids prepared from *V. harveyi* strain BB152 (Bassler *et al*., 1993). Similarly, addition of cell-free culture fluid from *S. typhimurium* LT2 grown in LB + 0.5% glucose induced luminescence in the reporter strain ≈800-fold over the control level (solid squares). No activity similar to *V. harveyi* AI-1 was produced by *S. typhimurium* LT2 under these conditions, and there is no AI-1 or AI-2 activity in LB + 0.5% glucose (Surette and Bassler, 1998). The observations reported on the regulation of signal production and degradation by *S. typhimurium* LT2 implicate a role for quorum sensing in pathogenesis of *Salmonella*. The conditions favouring signal production (nutrient rich, high osmolarity and low pH) are those likely to be encountered upon the first interaction of an enteric pathogen with its host. Conditions favouring degradation of the signal (nutrient poor, low osmolarity) are those most probably encountered as the pathogen exits the host. The initial colonization of the host may be a concerted effort between a population of cells coordinated through this cell–cell signalling system. Other cues, that have not yet tested, could also regulate quorum sensing in *S. typhimurium*. These may represent independent or overlapping signalling pathways involved in pathogenesis. It has been isolated *S. typhimurium* mutants to test these hypotheses. Finally, *Salmonella* pathogenesis is a dynamic process of interaction between the host and metabolically active bacteria. Consistent with a role for quorum sensing in pathogenesis, evidence suggests that this quorum-sensing system is not functioning during stationary phase. Furthermore, it has benn shown that the signalling molecule is not produced during stationary phase, and existing signal is degraded. Perhaps quorum sensing is critical for *S. typhimurium* to undergo the transition between a host-associated and a free-living existence.

3.4 Halogenated furanones inhibit quorum sensing

Many Gram-negative bacterial species employ *N*-acyl-L-homoserine lactones (AHLs) to control the synthesis of products that facilitate interactions with the surrounding environment, including interactions with eukaryotic host species (reviewed by Eberl, 1999; Kievit & Iglewski, 2000). AHL dependent gene expression has been suggested to constitute a mechanism by which bacteria can alter their behaviour in response to cell density and is thus commonly referred to as quorum sensing (reviewed by Swift *et al.*, 1999).

The diverse range of AHL regulated phenotypes includes the production of degradative extracellular enzymes by *Pseudomonas aeruginosa* and *Erwinia carotovora* (Jones *et al.*, 1993), bioluminescence in *Vibrio fischeri* (Sitnikov *et al.*, 1995) and *Vibrio harveyi* (Bassler *et al.*, 1993), plasmid transfer in *Agrobacterium tumefaciens* (Piper *et al.*, 1993), antibiotic production in *E. carotovora* (Bainton *et al.*, 1992), and more complex phenotypes such as surface motility in *Serratia liquefaciens* (Eberl *et al.*, 1999) and development of biofilm architecture in *P. aeruginosa* (Davies *et al.*, 1998)

AHLs are synthesized by homologues from either the AinS or LuxI family of AHL synthases and mediate transcription of various target genes through an interaction with, in most cases, a homologue of the LuxR protein of *V. fischeri* (reviewed by Fuqua *et al.*, 1996). AHLs show variation in the length, degree of saturation and adjoining substitutions of the acyl chain (reviewed by Fuqua & Eberhard, 1999). These structural variations account for the different responses elicited by different AHLs in quorum sensing assays (McClean *et al.*, 1997 ; Zhu *et al.*, 1998). The molecular mechanism by which AHLs trigger the transcriptional activation of target promoters via an interaction with LuxR homologues remains to be fully elucidated (see Discussion), but appears to involve AHL binding to and induction of conformational changes in the regulatory protein which lead to multimerization and DNA binding (Choi & Greenberg, 1992 ; Qin *et al.*, 2000 ; Welch *et al.*, 2000).

Other cellular components are involved in the expression of AHL-regulated genes including the cAMP receptor protein (Nealson *et al.*, 1972), the H-NS protein (Ulitzur *et al.*, 1997), and the molecular chaperones GroES and GroEL (Adar *et al.*, 1992).

The discovery of quorum sensing has afforded a novel opportunity to control unwanted microbial activity without the use of growth inhibitory agents such as antibiotics, preservatives and disinfectants that select for resistant organisms. A means of interfering with AHL-mediated gene expression not only has potential in a number of applied contexts, including the treatment of lung infections in cystic fibrosis patients, but would also constitute an evolutionary advantage for plant and animal species under selective pressure from quorum sensing pathogens.

Gram-negative bacteria engage in AHL-dependent phytopathogenic (Barras *et al.*, 1994 ; Zhang *et al.*, 1993) and phytosymbiotic (Rodelas *et al.*, 1999) relationships with terrestrial plants. Whilst there are no known examples of such relationships in the marine environment, marine plants are at once rich in secondary metabolite chemistry and, in the absence of more advanced immune systems, prone to disease (Correa, 1996 ; Fenical, 1997). For these reasons marine plants are likely candidates for the evolution of AHL antagonist activity (Kjelleberg & Steinberg, 2001). The marine macroalga *Delisea pulchra* produces a range of lactones, known more specifically as halogenated furanones, which inhibit quorum sensing (Givskov *et al.*, 1996 ; Manefield *et al.*, 1999 ; Rasmussen *et al.*, 2000). It has previously been proposed that the production of the halogenated furanones in specialized cells, which migrate to the surface of the alga to release the compounds (Dworjanyn *et al.*, 1999), is likely to have evolved in response to the negative impacts of AHL-dependent colonization of its surfaces by marine bacterial species (Givskov *et al.*, 1996 ; Kjelleberg *et al.*, 1997 ; Kjelleberg & Steinberg, 2001).

It has been demonstrated that halogenated furanones have inhibitory effects in a variety of biological assays designed to measure AHL-mediated gene expression

(Givskov *et al.*, 1996). Such inhibition was found to be partially relieved by increasing AHL concentrations in the bioasssays, indicative of competition for a regulatory function (Manefield *et al.*, 1999). Furthermore, halogenated furanones were found to have activity in an *in vivo* ligand-binding assay employed to monitor the displacement of AHLs from the LuxR protein (Manefield *et al.*, 1999). These results have been in accordance with a model in which halogenated furanones compete with AHLs for a common binding site on LuxR and LuxR homologues. In this study we tested directly for an interaction between a halogenated furanone and the LuxR protein, and discovered that the furanones inhibit AHL-mediated gene expression through accelerated degradation of the transcriptional activator.

From the few biochemical studies that have been performed on the function of LuxR-type regulatory proteins, a model of how AHLs cause transcription of specific genes is beginning to emerge (Zhu & Winans, 2001; Welch *et al.*, 2000; Qin *et al.*, 2000; Zhu & Winans, 1999). *In vitro* investigations with the CarR protein of *E. carotovora* and the TraR protein of *A. tumefaciens*, including DNA bandshift, fluorescence quenching and tryptic digestion experiments, have revealed that cognate AHLs directly interact with and induce conformational changes in these regulatory proteins (Zhu & Winans, 2001; Welch *et al.*, 2000). What remains uncertain is the effect these conformational changes have on the behaviour of the protein. Qin *et al.* (2000) have proposed a model in which the TraR protein is monomeric and membrane associated in the absence of 3-oxo-C8-HSL but dimeric, cytoplasmic and capable of transcriptional activation in its presence. Zhu & Winans (2001) have recently presented another model in which the TraR protein is monomeric and vulnerable to proteolysis in the absence of 3-oxo-C6-HSL but dimeric, resistant to proteolysis and capable of transcriptional activation in its presence. These models both draw attention to the relevance of the cytoplasmic concentration of TraR in the activation of target promoters. Studies have been cariied out to further define the molecular mechanism by which halogenated furanones inhibit the AHL-mediated transcriptional activation of target genes. It has been unable to detect the formation of a stable complex

between a tritiated halogenated furanone and the LuxR protein overproduced in *E. coli*. However demonstration that the cytoplasmic concentration of the LuxR protein is decreased in the presence of halogenated furanones. In the light of both these results and the demonstration that 3-oxo-C8-HSL protects the TraR protein in *E. coli* from proteolytic digestion (Zhu & Winans, 2001) it is suggested that halogenated furanones interact with the LuxR protein but that this interaction causes conformational changes that enlist the furanone–LuxR complex into rapid proteolytic degradation. This model is consistent both with the observed effects of furanones on the formation of the AHL–LuxR complex (Manefield *et al.*, 1999) and with the inability to detect a long-lived furanone–LuxR complex. Is the loss of LuxR the result of proteolytic degradation? The effect of furanones on the LuxR concentration was comparable in a wild-type *E. coli* strain and a *clpP* and a *lon E. coli* strain. Zhu & Winans (2001) found that a substantial change in the rate of TraR degradation in *E. coli* required the simultaneous crippling of both the *clp* and *lon* proteases. We found no significant change in the inhibition index for compound 30 in the different strains. This indicated that not even the Clp and Lon proteases in concert could severely affect the LuxR stability. This however, does not rule out the possibility that other proteases could be involved in the proteolytic turnover of LuxR. The possibility that the LuxR protein becomes compartmentalized in a manner analogous to the proposal of Qin *et al*. (2000) in the presence of the furanones is unlikely given that whole cells (i.e. including membranes) were used in the Western procedure employed here. It is not reproducibly detect an increase in LuxR stability in the presence of 3-oxo-C6-HSL as was demonstrated by Zhu & Winans (2001) for the TraR protein in the presence of 3-oxo-C8-HSL. Experiments however 3-oxo-C6-HSL was always added after the cessation of stimulation of the *luxR* promoter (i.e. after removal of IPTG). Zhu & Winans (2001) found that the AHL-induced protection of TraR was dependent on the presence of 3-oxo-C8-HSL during synthesis of the protein and that addition of 3-oxo-C8-HSL to *E. coli* cells already harbouring the TraR monomer did not afford the protein any protection against proteolysis. Our observations with LuxR are

therefore not inconsistent with those of Zhu & Winans (2001). The ability of 3-oxo-C6-HSL to protect the LuxR protein from furanone-induced degradation was dependent on the addition of the AHL before the furanone. This result suggests that, unlike 3-oxo-C8-HSL and TraR, 3-oxo-C6-HSL binds mature LuxR and that in this state the halogenated furanones are less able to compete for the AHL binding site. It is possible that 3-oxo-C6-HSL is binding and protecting low levels of freshly translated LuxR from persistent *luxR* mRNA transcript. However, the addition of chloramphenicol to block translation after cessation of transcription did not affect LuxR levels, indicating that residual translation was not occurring. Either way it is clear that 3-oxo-C6-HSL can protect the LuxR protein from furanone-induced degradation when present before the furanone, but is unable to rescue the protein if the furanone is present first and has already initiated degradation. The superior activity of compounds lacking a carbon chain extending from the furan ring structure in the control of both P_{luxI}–*gfp*(ASV) expression and LuxR concentration was somewhat unexpected because the homoserine lactone ring without the acyl chain has been shown not to interact with the LuxR homologue CarR (Welch *et al.*, 2000). The significance of this will remain unresolved until more structural information regarding LuxR homologues and their AHL binding sites is available. AHLs are required for the expression of Gram-negative bacterial phenotypes involved in many cases in an interaction with a higher organism. Amongst some of the best studied examples, including elastase production in *P. aeruginosa*, pectate lyase production in *E. carotovora* and conjugation of the plant-tumour-inducing Ti plasmid of *A. tumefaciens*, are behaviours with central roles in the success of bacterial infections of medical, agricultural and therefore economic significance. This investigation has demonstrated that halogenated furanones produced naturally by the marine alga *D. pulchra* can modulate the cellular concentration of the LuxR protein responsible for the reception of, and response to, AHLs. While studies are directed at the effects of furanones on the LuxR protein of *V. fischeri*, the findings highlight the potential for the use of halogenated furanones in the control of unwanted bacterial activity.

Additionally these results lend support to a model of AHL function in which the metabolite regulates the steady state concentration of LuxR homologues by shielding the regulator from proteolytic degradation.

Objectives

Quorum sensing is a mechanism by which diverse microrganisms can control specific processes in response to polulation density. Intercellular communication is accomplished through the production, release, and detection of small signalling molecules called autoinducers. Typically, Gram-negative bacteria use acylated-homoserine-lactones as autoinducers, whereas Gram-positive bacteria use modified oligopeptides. Unlike these autoinducers, which are specific to a particular species of bacteria, a recently discovered autoinducer (AI-2) is proposed by a large number of bacterial species. AI-2 has been proposed to serve as a universal signal for inter-species-communication. Except for same speciesm, the chemical identity of AI-2 has, however , proved elusive.

What characterises a quorum sensing system or, more generally, cell-to-cell communication, and separates it from other signal response mechanisms? Winzer et al. (2002) tried to define a number of criteria to individualize them:

- The production of "cell to cell signal molecule" (CCSM) occurs during specific stages of growth, under certain physiological conditions, or in response to changes in the environment.
- The CCSM accumulates extracellularly and is recognised by a specific receptor.
- Accumulation of the CCSM generates a concerted response, once a critical threshold concentration has been reached.
- The cellular response extends beyond physiological changes required to metabolise or detoxify the CCSM.

In this work, following the above criteria, I investigate a possible release of signalling molecules in same Gram-positive i.e *Lactobacillus helveticus Lactobacillus plantarum, Lactobacillus paraplantarum, Lactobacillus sanfranciscensis*, *Enterococcus faecalis* and in Gram-negative species, i.e *Salmonella enteritidis*.

Furthermore, the effects of exposure to their own species or to different species was

carried out to investigate on intraspecific and interspecific activity.

Materials and Methods

Bacterial strains and culture conditions

*Lactobacillus helveticu*s CNBL 1156, obtained from the collection of the Istituto di Microbiologia, Universita` Cattolica del Sacro Cuore, Piacenza (Italy), *Lactobacillus sanfranciscensis* 77ST, 20196, 274, CB1, BB12, *Lactobacillus plantarum* 14917, *Lactobacillus paraplantarum* 4DE, from the collection of the Dipartimento di Scienze degli Alimenti, Università di Teramo (Italy), were grown in MRS broth under anaerobic conditions at 45°C (Anaerocult A; Merck, Darmstadt, Germany). *Salmonella* Enterica strain 155, from the collection of the Dipartimento di Scienze degli Alimenti, Università di Bologna (Italy), and *Enterococcus faecalis* strain ORG1F from the collection of the Istituto di Microbiologia, Universita` Cattolica del Sacro Cuore, Piacenza (Italy), were routinely grown in Brain Heart Infusion (Oxoid, BHI) at 37°C under aerobic conditions. *Chromobacterium violaceum* 30191, obtained from DSMZ GmbH (Braunschweig, Germany) was grown on 0.5% yeast extract and 1% tryptone. For each experiment a fresh over night culture (27°C without shaking) was used.

Preparation of conditioned media and exposure to stress condition

The media used for the preparation of the CMs and/or for stress exposure were: 1) whey obtained from a preparation of Parmigiano Reggiano cheese and sterilized by filtration as previously reported (Guerzoni, Lanciotti & Cocconcelli, 2001) for *Lactobacillus helveticu*s; 2) wheat flour hydrolysed (WFH) broth (Gobbetti, Corsetti & Rossi, 1994) for *Lactobacillus sanfranciscensis*, *Lactobacillus plantarum* and *Lactobacillus paraplantarum*; 3) BHI for *Salmonella* Enterica and *Enterococcus faecalis.*

Each microbial species, grown overnight as preculture in the above reported media, was resuspended in fresh media (i.e., whey for *Lactobacillus helveticus*, WFH for *Lactobacillus sanfranciscensis, Lactobacillus plantarum* and *Lactobacillus paraplantarum*, and BHI for *Salmonella* Enterica and *Enterococcus faecalis*) and

grown overnight at their optimal temperatures. The cells were then centrifuged for 10 min (10000 x *g* at 4°C), filtered (0.22 μm) and supernatants were used as conditioned media (CMs) and for stress exposure.

Overnight cells of each microbial species were resuspended at concentrations ≥ 8.0±0.3 log CFU/ml in the various CMs, obtained as above described, modified or not with the addition of H_2O_2 (0.017%), sucrose (40% w/v) for *L. sanfranciscensis, Lactobacillus plantaruman* and *Lactobacillus paraplantarum,* NaCl (0.5M) for *Lactobacillus helveticus* and *Enterococcus faecalis* or chemical compounds, ie. hexanal, alpha-angelica lactone, isovaleric acid (300 ppm). Hexanal and alpha-angelica lactone have been chosen on the basis of previous researches evidencing their antimicrobial activity, while isovaleric acid is a metabolite frequently released under stress conditions by lactobacilli (Guerzoni, Vernocchi, Ndagijimana, Gianotti, & Lanciotti, 2006). After 2 h of exposure at the optimal temperature suspensions were centrifuged and analysed with gaschromatography–mass spectrometry-solid phase microextraction (GC–MS/SPME) in comparison to the original CMs. The data reported are the mean of four replicates.

Effect of the CMs from low density and high density cultures of Salmonella Enterica on the growth dynamics of fresh cells of the same species

Salmonella Enterica was cultured in BHI at 37°C overnight. Cells were collected by centrifugation, washed twice in sterile saline solution and inoculated (3-4 log CFU/ml) in CMs of *Salmonella* Enterica whose growth had been interrupted when cells attained a level of about 7 log CFU/ml (low density) or 8 log CFU/ml (high density). The growth dynamics were analysed on the basis of plate counts onto agarized BHI incubated at 37°C.

GC-MS/SPME analysis of volatile compounds

A divinylbenzene-carboxen-polydimethylsiloxane-coated fiber (65 μm) and a manual SPME holder (Supelco Inc., Bellefonte, PA) were used in this study after

preconditioning according to the manufacturer's instruction manual. Before each headspace sampling, the fiber was exposed to the GC inlet for 5 min for thermal desorption at 250°C in a blank run. Five milliliters of the sample was placed into 10-ml vials, and the vials were sealed. The samples were then equilibrated for 15 min at 65°C. The SPME fiber was exposed to each sample for 40 min, and finally, the fiber was inserted into the injection port of the GC for 5 min of sample desorption.

GC-MS analyses were carried out using an Agilent 6890 gas chromatograph (Agilent Technologies, Palo Alto, CA) coupled to an Agilent 5970 mass selective detector operating in electron impact mode (ionization voltage, 70 eV). A Chrompack CP-Wax 52 CB capillary column (50-m length, 0.32-mm internal diameter) was used (Chrompack, Middelburg, The Netherlands). The temperature program was 50°C for 2 min, then programmed to increase to 65°C at 1°C/min and finally to 220°C at 5°C/min, which was maintained for 22 min. Injector, interface, and ion source temperatures were 250, 250, and 230°C, respectively. Injections were performed in splitless mode, and helium (1 ml/min) was used as the carrier gas. Compounds were identified by use of available mass spectra databases (NIST/EPA/NIH version 1998 and Wiley version 1996) as well as by MS data in the literature (Bonini, Iavarone, Trogolo & Poultron, 1980).

Scanning Electron Microscopy (SEM) observations

The analysis has been carried out by a Philips 505 (Hillsboro, Oregon, USA) scanning electron microscope. 2 ml of cultured cells of the different samples have been collected by centrifugation at 8000 rpm x 10min at 4°C (Bekman Coulter) and washed with cacodilate buffer solution. Each sample have been fixed with glutaraldehyde (Taab), 2.5% (v/v), at 4°C for 1 h and then post-fixed with 1% v/v of osmium tetraoxide (OsO_4) (Taab) at 4°C for 1h.

Samples have been dehydrated using 4 water-acetone solution (50, 70, 95 and 100%) and subsequently critical point dried treatment using CO_2 (Emitech Critical Point Dried K850 Ashford, Kent; UK). The dehydrated samples have been then fixed on

aluminium supports with silver paint thinners and golden-plated.

Statistical analysis

The intensities of fragment ions of furanones detected by GC-MS/SPME analysis were used for Principal Component Analysis (PCA) and cluster analysis (CA) taking the Euclidean distance as metric. All computations for basic and multivariate statistics were performed with Statistica, 6.0 (StatSoft, Inc., USA)

Evaluation of autolysins by renaturing SDS-PAGE (Zymogram)

***L. helveticus* CNBL 1156**: an overnight culture (OD_{600} = 1,2) of *L. helveticus* CNBL 1156 was grown in whey medium in anaerobiosis at 45°C and the cells were recovered by centrifugation (at 2500 g for 10 min at 4°C). The cells were resuspended for 100 min, at a concentration of 8 ± 0.4 log CFU/ml, in the various CMs collected at different times during incubation in whey and added with NaCl or H_2O_2 as above described .

After 100 min of incubation at 45°C the samples for renaturing polyacrilamide gel electrophoresis were drawn.

***Enterococcus faecalis* and *S.* Enterica 155**: overnight cells were resuspended at concentration ≥ 8.0±0.5 log CFU/ml in the various media where were exposed to different stress condition: oxidative, H_2O_2 0.017% (v/v); chemical, hexanal 300ppm; pH 4.8 using 0.1M lactic acid; combination of oxidative and pH stress (stress conditions were chosen in relation of furanones amount released detected by SPME analysis). After 2 hours of incubation at 37°C the samples for zymogram.

Zymogram: to analyse cell wall hydrolase activity, 2 ml of the cell suspensions in the various CMs were harvested, centrifuged (2500g x 10 min at 4°C) and the cells were resuspended in 100 µl of Laemmli buffer (62.5mM Tris-HCl, pH6.8, containing 10% glycerol, 2% SDS, and 5% 2- mercaptethanol). The suspension was mixed gently, heated for 3 min at 100°C and placed on ice for 5 min, centrifuged (7,000 rpm

x 10min) and loaded onto an SDS-14% (wt/vol) polyacrylamide gel containing 0.2% (wt/vol) lyophilized *Micrococcus lysodeikticus* cells (Sigma, St. Louis, Mo.). After electrophoresis (1h, 50V and 5h at 250V) proteins were renatured to detect lytic activity the gels were incubated with gentle shaking in 0.05 M Tris-HCl buffer (pH 6.8) containing 1,5 % (vol/vol) Triton X-100 over night at 37°C. The renaturation of the protein in the samples and visualisation of clear zones in the gel was enhanced by staining the gels in 0.1% (w/v) methylene blue in 0.01% (w/v) potassium hydroxyde. Equivalence of loading between lanes was assessed by Coomassie Blue staining of SDS-PAGE gels run in parallel.

Bioassay with Chromobacterium violaceum using different CMs

C. violaceum 30191 served as indicator organism for quorum sensing bioassay by quantifying violacein synthesis. The organism was grown on 0.5% yeast extract and 1% tryptone. For each experiment a fresh overnight culture (27°C) was used and inoculated in fresh media modified or not. Experiments were conducted in 96 well flat bottom plastic microplates. *C. violaceum* was inoculated in 100µL (2±0.3 log CFU/ml) of fresh media or in fresh media modified with 50µL or 10µL (50% or 10% of final volume) of conditioned media derived from cells of *S.* Enterica and *E. faecalis* grown in various stress condition (as previously described). To test toxicity effects of stress condition on growing of *C. violaceum*, as control, cells were grown in presence of the same amount of medium stress-modified where no cells of *S.* Enterica and *E. faecalis* were grown. After 24h of incubation at 27°C, the formation of violacein was evident. The plates were then dried at 60°C until all medium had evaporated (overnight). The violacein was resolubilized by adding 200µL of DMSO to each well and the plates incubated on a lab shaker for 2h. The absorbance of each well-contents was measured with an UV-visible spectrophotometer (UV-1601, Shimadzu) at a fixed wavelength of 590nm. Each measurement was done in triplicate.

Results and discussion

GC-MS/SPME analysis of *Lactobacillus helveticus, Salmonella enteritidis and Enterococcus* faecalis supernatants after the exposure to the various stress combinations.

The effects of the exposure of *L. helveticus* (8 ± 0.3 log CFU/ml) to different stress combinations in whey according to an experimental design at 45°C , reported in Table 1, were evaluated after 100 minutes on the basis of metabolites release in comparison with the control. The SPME analyses of the three repetitions of the various combinations after a 100 minutes of stress exposure showed, in addition to its usually metabolites, the release of three medium chain fatty acids, hexanoic, octanoic and decanoic acids, identified on the basis of the comparison of their mass spectra and retention times with those of pure standards (figure 1). Moreover two peaks having retention times of 45.4 minutes and 45.9 minutes from now on called respectively furanone A and furanone B were detected (in accordance with described by Ndagijimana *et al*., 2006). These peaks were present in traces in the controls (figue 2). The data relative to the concentration of the two furanones and the medium chain FAs (mean of three repetitions) after 100 minutes of exposure to the stress conditions modulated according to the experimental design, excluding those relative to temperature effects, were analyzed in order to obtain polynomial equations describing the effects of the independent variables, as individual or quadratic terms, and of their interactive effect on the concentration of the molecules detected by GC-MS/SPME. A significant relationship was obtained only between furanone B concentration and the individual and quadratic term of H_2O_2 according to the equation: [furanone B] = $0.354[H_2O_2]-0.058[H_2O_2]^2$ (regression coefficient = 0.897, F-value = 30.808, standard error = 0.24).

The SPME analysis of the filtrates of a culture in BHI of *Salmonella enteritidis* indicated that also this species released, when the cells attained a level of about 8-8.5 log CFU/ml (late exponential phase-beginning of stationary phase), two molecules having retention times (45.4±0.2min and 45.9±0.2min) and MS profiles similar to

those of the furanones A and B already observed in the filtrates of the above described strain (Fig.3). In particular, when fresh cells were inoculated at low level (about 2 log CFU/ml) furanone A was detected at low concentration since cells began the exponential phase, however a pronounced increment of its amount was observed when concentration cells attained 8-8.5 log CFU/ml (late exponential phase). Otherwise, the furanone B was not detected till concentration cells was about 6 log CFU/ml achieving maximum level during late exponential phase in accordance with the evolution of the furanone A. While cells of *S.enteritidis* reached the stationary phase both furanones showed a drastically reduction probably due to their enzymatic degradation conversion.

Fresh cells of *S. enteritidis* (8-8.5 log CFU/ml), harvested from an overnight culture in BHI, were suspended in BHI containing different stress combination as described in table 2. The exposure of late exponential phase cells to different stress conditions (Table 2) gave rise, in many cases, to an enhanced accumulation of the medium chain fatty acids (hexanoic, octanoic and decanoic acids) and of the two furanones respect to those released during no stress growing condition as shown in figure 4 and 5. In particular, major modifications on the volatile compounds detectable by SPME considered were found when hexanal (300ppm), lactic acid (pH 4.8), hydrogen peroxide (H_2O_2 0.017% v/v) and combination of lactic acid and H_2O_2 were tested as stress conditions.

In addition an immediate release of furanones A and B was observed when fresh cells (8-8.5 log CFU/ml), harvested from an overnight culture in BHI, were suspended in the CMs of the same strain. Their levels were further enhanced when the fresh cells were suspended in CMs obtained inoculating 8 log CFU/ml in BHI under stress condition, H_2O_2 (0.017% v/v) or lactic acid addition (pH 4.8) (Figure 6).

With the purpose to evaluate if other Gram-positive species, during late exponential phase under stress conditions are able to release similar medium chain fatty acids and furanones to those identified in *L. helveticus* and *S. entertidis*, cells of *Enterococcus faecalis* were submitted to chemical-physical stresses and analyzed by SPME-GC-

MS. Also in this case, two molecules, having similar retention times and similar mass profiles, when grown up to the beginning of the stationary phase (8.5-9 log CFU/ml) were released in concomitance with hexanoic, octanoic and decanoic acids (figures 7 and 8). Likewise in the above mentioned studies, hexanal, lactic acid and hydrogen peroxide were identified as stress conditions able to stimulate higher furanones and fatty acids release.

The dependence of the furanones A and B on oxidative stress, the contemporaneous release of medium chain fatty acids (FAs) and the already reported formation of epoxides of linoleic acid in *L. helveticus* following oxidative stress (Guerzoni *et al.,* 2001) suggest that epoxidated or hydroxylated C18 chain membrane fatty acids may be precursors of furanones A and B. It is known that in many organisms reactive oxygen species such as H_2O_2 and superoxide ion are produced under many physiological conditions. They can oxidize unsaturated acyl chains integrated in membrane phospholipids. 2(5H) furanones can be subsequently produced throughout a sequence of shortening by β-oxidation and lactonization reactions. Peroxidation reactions are in fact the first step in the generation of plant signal compounds such as jasmonic acid (Spiteller, 2002).

Comparison of the mass spectral data of the two molecules identified as 2(5H)-furanones

The comparison of the mass fragmentation profiles of the molecules called furanones A and B produced by the various strains under the different conditions indicated that some fragments, relevant for the identification of the structure and the inclusion into the 2(5H)-furanones family, such as 41, 57, 69, 97, 123 m/z (table 4), were shared, although with different intensity, by all the species under the different conditions. However, some fragments appeared to be species related. The two peaks having retention times of 45.4 minutes and 45.9 minutes from now on called respectively A

and B were detected. The spectral data, reporting the mass of the characteristic ions and their intensities in the brackets, for compound A and B are shown in table 4 and figures 10 and 11. On the basis of the comparison of these spectral data with literature concerning MS fragmentation patterns (Bonini *et al*., 1980), it was possible to assume that both molecules are 3-hydroxy-2(5H)-furanones having an α-hydroxy-γ-lactone configuration. The chemical structures shown in figure 9 can be proposed for the furanone A and furanone B. The electron impact MS fragmentation patterns of furanones A and B evidenced that these molecules were characterised by different compositions and configurations of the substituent R. However the definitive identification of the two substituents (R) is still in progress. On the basis of the analysis of the mass spectral data, molecular weights of about 143 and 180 can be proposed respectively for furanone A and furanone B.

A novel approach, based on the principal component analysis of the MS fragmentation patterns of furanone A and B, was adopted in order to assess the existence of structural differences and possibly to group the profiles in relation to the species, growth media and stress condition. This analysis included also the spectral data of the furanone A and B previously identified detected in *L. helveticu*s, *L. sanfranciscensis, L. plantarum, L. paraplantarum, S. enteritidis and E. faecalis* filtrates. The figure 12 shows the projection on the PCA axes 1 and 2 of the fragmentation ions of furanone A. It accounts for 148 conditions (4 repetitions per strain and condition). More than 72% of the variability of the data are expressed with a projection on the axes 1 and 2, the axis 1 contributing to the majority of the variability. As indicated by the figure 12 the major part of the spectral data are grouped together excepted for *Lactobacillus sanfranciscensis* CB1 (under osmotic stress and control) and *Lactobacillus sanfranciscensis* BB12 (under oxidative stress and control) which were totally separated from all the other spectral data. The PCA analysis of furanone B fragmentation profiles gave rise to an alignment of almost all the strains along the component 2 (figure 13). The analysis accounted for 86% of the variability. However, in order to better evidence the data, a dendrogram of Euclidean

distances of the profiles of the 37 combinations strain/condition (4 repetitions) is reported in figure 14. The major part of combinations, i.e. 34 out of 37, presented a similarity higher than 75%. Within this large group 2 subgroups could be identified: one including *Salmonella enteritidis* and *Enterococcus faecalis* and the other including the various *Lactobacillus* spp. grown in WFH. It is interesting to observe that *Salmonella enteritidis* profiles could be further subdivided in relation to the medium, i.e. BHI or whey. On the other hand the stress to which the cells had been exposed did not affect the B profile with the exception of *Lactobacillus helveticus*. The furanone B fragmentation profiles of the combinations of these species showed a distance of 70% from the principal group, while the distance of the control corresponded to 100%.

This type of analysis, which allows the identification of the most significant mass fragments, demonstrated that a part from a few exception, furanone A has the same molecular mass and configuration in all the species and strains considered and it is independent on medium and stress applied. On the other hand, on the basis of the PCA anlysis dendrogram of Euclidean distances, the fragmentation profiles of furanone B of the major part of the species and strains could be grouped together and were differentiated from those of *L. helveticus*. However, the PCA analysis as well as the distance analysis do not attribute a hierarchic value to the various mass fragments and can result in an undervaluation of the key fragments and a overvaluation of less important ones. In fact, all the B profiles detected for the conditions analysed can be included in the family of 2(5H)-furanones.

Bioactivity of the Salmonella Enterica CMs containing the two furanones

While furanone A seems to present the same chemical configuration in all the species taken into consideration with only a few exception, the spectral data of furanone B produced by *Salmonella* Enterica, *Enterococcus faecalis L. plantarum, L.*

paraplantarum differentiated from those of *L. helveticus* according to the PCA and the dendrogram shown in figure 13 and 14. The biological activity of the cell free CMs of *L.helveticus* containing furanones A and B on fresh cells of same species has been previously shown (Ndagijimana et al., 2006). Therefore, in consideration of the structural differences of furanone B shown by the major part of the species with respect to *L. halveticus*, the biological activity of the CMs of *S. enteritidis* was evaluated. In particular their effects on growth rate and extent on cells of the same species were observed. The CMs obtained from the experiment described in figure 6 but collected in two different phases, i.e., when the cell level reached 7-7.5 log CFU/ml (low density culture) and 8-8.5 log CFU/ml (high density culture), were added (50% v/v) to fresh BHI inoculated with *Salmonella* Enterica (3-4 log CFU/ml).

The comparison of the growth dynamics suggests that the addition of CMs obtained from high density cultures, containing furanones A and B, played an inhibiting effect on growth rate and maximum cell load attained (figure 15). On the contrary, the grow rate and extent were not significantly reduced when low density cultures, containing undetectable levels of furanone A and B, were used. The specific activity of free cell supernatants of high density culture confirm that the release of active molecules and specifically of furanones A and B was a cell-density dependent phenomenon.

Cell morphological changes associated with exposure

Fresh cells of *L. helveticus* were suspended (to obtain a cell concentration of about 8 log CFU/ml) in CMs of the combinations 12, 13 and 14 of the experimental design containing different levels of the furanones A, B and medium chain fatty acids (table 1) and in whey added with commercial furanones HEMFi 7 μM or HEMF 7 μM. Autolysis phenomena were observed with the three CMs, as shown by the figure 17 relative to CM12. In particular, irregular lesions and cell debris were observed. Moreover the cells presented an heterogenous lenght (1.5-5.0 μm). A total of 250

cells in 5 micrographs per condition were individually measured. The percentage of cells having a lenght ≤ 1.5 μm was 38% when fresh cells were exposed to CM12 (figure 18). This percentage was 35 and 33 when the cells were exposed respectively to the CMs 13 and 14. On the other hand the proportion of cells having a lenght ≤ 1.5 μm was about 7% in the control (cells suspended in fresh whey) (figure 16). 41% of very short cells (lenght ≤ 1.5 μm) were observed when fresh cells were exposed for 100 minutes to 7μM HEMFi (5-ethyl-3-hydroxy-4-methyl-2(5H)-furanone) in whey. Moreover this 2(5H)-furanone as well as HEMF 7μM, gave rise to anomalous cell formation as shown in figure 19 relative to HEMF. These morphological anomalies presented different shapes and occurred in about 8% of cells. Also the exposure to hexanoic, octanoic and decanoic acids (4 μM) gave rise to shorter cell occurrence.

In order to asses if morphological changes were induced in gram-negative bacteria by stress conditions, fresh cells of *S. enteritidis* were suspended in BHI containing hexanal (300ppm) or H_2O_2 (0.017% v/v) pH 4.8 (with lactic acid), conditions chosen in relation of amount of furanones and medium chain fatty acids detected in the previous experiments. In presence of hexanal, S. enteritidis cells showed an interconnecting mesh between cells (figure 21). The figure shows clearly the formation of thin aggregative fimbriae very similar to those studied by Austin *et al*., (1998) responsible for biofilm formation in *E. enteritidis*. Indeed, *S. enteritidis* enteropathogens produce a variety of potentially adherent fimbrial types able to adhere to inert surfaces or to form thick cell aggregates. The combination of H_2O_2 and lactic acid used as stress condition stimulated in *S. enteritidis* a cellular deformations gave rise to a hanging between cells; presenting an fimbrial formation as well as previously described, even if less evident (figure 22). The number of the irregular cells were quite high if compared with control (cells suspended in BHI; figure 19) showing almost 80% of cells merged each other against 15% checked in control. Furthermore, to investigate in interspecific activity (between gram-negative and gram-positive) of furanones released by *L. helveticus*, fresh cells of *S. enteritidis* were suspended in cell free CMs of combination 12 of experimental design (table 1)

at two different ratio, 20% (v/v) or 80% (v/v), in BHI. In the first case (20% CM 12 in BHI), not significant morphological changes were showed (figure 23). On the other hand, when the ratio of CM12 was increased differences in morphology of cells were found (figure 24). In particular, the cells after 2 hours of exposure showed a pronounced reduction of the length becoming shorter. The percentage of 200 cells having a lenght ≤ 1.0 μm was 58% when fresh cells of *S. enteridis* were exposed to CM12 (ratio 80 v/v in BHI), the percentage of the cells shorter than 1.0 μm was 15% when CM12 ratio was 20%, while the proportion of cells having a lenght ≤ 1.0 μm was about 8% in the control (cells suspended in fresh BHI).
The same experiment was carried out exposing fresh cells of *Enterococcus faecalis* to different stress conditions (pH and H_2O_2 or hexanal) in accordance to those investigated for *S. enteritidis* morphological changes. In these cases (figures 26 and 27), significant differences were not observed between control and stressed cells, neither when fresh cells were exposed to cell free CMs of the same strain (figure 28). Indeed, only few (less than 5%) cells showed abnormal morphologies.

The effect of the stress conditions on furanones and medium chain FAs release and at the same time on cell morphology suggests that the morphological changes, and particularly the appearance of short cells, can be associated with the phenomena involved in the programmed death of a population induced by pheromones (Lewis, 2000). In fact *L. helveticus*, *S. enteritidis* and *E. faecalis* cells respond in concert to the stress releasing furanones and FAs. These molecules can promote agglutination, biofilm formation or morphology changes giving rise to shorter or anomalous cells.

Evaluation of cell wall lytic activity against Micrococcus lysodeikticus cells in renaturated SDS-PAGE (zymogram)

The bioactivity of the molecules, and namely furanones released in the medium was evaluated on the basis of their ability to induce autolysins in fresh cells of the same

strain. Moreover the effect of commercial furanones having similar molecular configuration and FAs was assessed (figure 29). Fresh cells of *L. helveticus* were transferred from a late exponential phase culture to: fresh whey containing 0.017% of H_2O_2; fresh whey added with commercial furanones such as HEMF 1ppm and HEMFi 1ppm in combination with NaCl (0.5 M) and lactic acid (pH4.4). HEMFi was chosen for its spectral similarity with furanones A and B.

In a second experiment (table 4), cells of *L. helveticus* were transferred from a late exponential phase culture to: fresh whey containing 0.3mM, 2.6mM and 5mM of H_2O_2 (respectively condition II, III, and IV) and into CMs of cells exposed to conditions II, III and IV.

Likewise, in order to assess if bioactivity of the molecules released by *S. enteritidis* and *E. faecalis* similar experiment were performed using as sensor *M. lysodeikticus.* Cells of both strains were incubated (8 log CFU/ml) in different media containing H_2O_2 (0.017%, v/v), combination of lactic acid (pH 4.8) and H_2O_2 (0.017%, v/v), hexanal (300ppm) and in the free cell CMs of late exponential phase of the same strain. The biological effects were evaluated on the basis of zymogram technique performed by renaturing SDS-PAGE gel containing *M. lysodeikticus* cells as a substrate. The zymogram revealed translucent bands corresponding to the lytic activities whose number and intensity depended on conditions.

L. helveticus. The first band having a molecular mass of about 31 kDa was present in the control and in all the conditions (figure 29 and table 4) except for conditions containing higher level of H_2O_2 (2.6 and 5 mM). A band corresponding to a molecular mass of about 63 kDa was observed when cells were exposed to the commercial 2(5H)-furanone HEMFi but the same band did not occur in presence of the 3(2H)-furanone HEMF. Only when the CMs II and III were taken into contact with fresh cells, a band of 43 kDa appeared. Moreover a band of about 45 kDa, whose intensity decreased with H_2O_2 concetration, was observed when the cells were taken into contact (100 minutes) with CMs II and III. In fact the exposure to CM II, III and IV gave rise to a significant viability decrease (table 4). It has been reported

that the autolysin A having a molecular mass of 41 kDa was associated with viable cells but as soon as the cells died the autolysin A disappeared (Valence, 1995). On the other hand a band of 63 kDa was observed when HEMFi was exposed to fresh cells. Commercial 2(3H)-furanones such as HEMF did not display any additional effect on autolytic activity with respect to the control.

S. enteritidis. No cell wall lytic activity was detected in the conditions tested (lines 1 to 5 in figure 30).

E. faecalis. In the control and in all the conditions tested for autolytic activity in *E. faecalis*, a clear lytic zones corresponding to a molecular mass of about 94 kDa against the opaque cell wall background was clearly observed (lines 6 to 10 in figure 30). The intensity of this band (94kDa) in lines 8 (late lag phase fresh cells expososed to H_2O_2 0.017%, pH 4.8) and 9 (late lag phase fresh cells expososed to hexanal 300ppm) was higher if compared to the line 6 (control: late lag phase fresh cells grown in BHI). At the contrary lines 7 (late lag phase fresh cells exposed to H_2O_2 0.017%) and 10 (fresh cells expososed to CM of same strain not exposed to stress) showed lower intensity. This latter band is probably due to the best known autolisyn present in *E. faecalis*, and it is regarded as a muramidase. Beliveau *et al.*,(1991) and Chu *et al.*, (1992) already described two different muramidases: 1) a muramidase-1 having a molecular weight 130 kDa (latent form), that can be converted into an active form (87 kDa) via the action of a variety of proteinases, including trypsin, and 2) a muramidase-2 which has a 125 kDa and a 75 kDa active form. Pfeffer et al. (2006) reported the occurrence, under the same conditions, of five lytic bands. It is possible that the different bands are the result of the breakdown of higher molecular weight autolysins.

A second band, not present in the control, and not corresponding to an apparent weight of 64 kDa was observed in lines 8, 9 and 10. This second band (64 kDa) have not been characterized yet and does not seem to be one of the two forms associated with the main muramidase. The most interesting aspect of these results is that both band are released within two hours of exposure to the various stress conditions. The

band 94 kDa was released in control in late exponential phase, moreover the band at 64 kDa was released after two hours of exposure to hexanal, combined pH-H_2O_2, and when fresh cells were exposed to spent medium of the same strain. The comparison of these latter results with the level of furanones A and B (figure 8) pointed out the relation of this second band to the higher amount of furanones present in the medium. In particular, it is interesting to observe that hexanal showed the higher concentration of furanone B.

Bioassay with *Chromobacterium violaceum* for the identification of quorum signal antagonists

To evaluate a possible presence of quorum sensing inhibition/interference (QSI) in cell free CMs derived from cells of *S. enteritidis* and *E. faecalis* exposed to different stress conditions, a simple method based on pigmentation inhibition in *Chromobacterium violaceum* 30191 was performed. Fresh culture of *C. violaceum* was suspended at two different concentration, 50% or 10%, into CMs obtained exposing above mentioned strains to different stress conditions with the purpose of observing the relation between possible signalling molecules released in CMs and violacein formation. In fact, this indicator bacteria regulate pigment production by N-hexanoyl-HSL (C_6-HSL) QS and is readily inhibited by acyl-homoserine-lactones (AHLs) analogues and other antagonist as autoinducer-2 (AI-2) (Mc Clean *et al*., 1997; Mc Clean *et al*., 2004; Martinelli *et al*., 2004). In both cases, CMs from fresh cells of *S.enteritidis* and *E. faecalis* exposed to hexanal (300ppm), oxidative (H_2O_2 0.017%) and combination of lactic acid (pH 4.8) and oxidative (H_2O_2 0.017%) stresses, showed to have a significant inhibition effects (figure 31 and 32 and respective tables 5 and 6) when present al 50 % v/v concentrations. Instead when lower amount of CMs, 10 % v/v, were tested not absorbance differences were evident. Moreover when CM (medium from unstressed culture) and CM-II (medium from cultures exposed to their own CMs) were tested, violecein production appeared

not to be inhibited. In both cases, no inhibition effect could be explained because violacein production is inhibited at narrow specific concentration of inhibent as described by Martinelli (2004).

Conclusions

A large number of different bacterial metabolites can be released in culture media during growing, supernatants (conditioned media), theorically, could contain molecules which have capacity to act as signal molecules in cell-to-cell comunication. The aim of this work was to investigate in two new molecules having 2(5H)-furanone configuration found to be released by some Gram-positive and Gram-negative bacteria.

They were released in the late exponential-stationary phase in different media by *Lactobacillus helveticus Lactobacillus plantarum, Lactobacillus paraplantarum, Lactobacillus sanfranciscensis* and *Enterococcus faecalis* and a Gram-negative species, i.e *Salmonella enteritidis*. Apparently, these molecules do not belong to the three well-defined classes of molecules that serve as the paradigms for chemical signaling bacteria: oligopeptides, acylhomoserine lactones and the LuxS/autoinducer-2 (AI-2) class (Keller & Surette, 2006). In fact, although the complete identification of the 2(5H)-furanones released by the various species, as well as their biosynthesis, requires further investigation, their origin from 4,5-dihydroxy-2,3-pemtanedione, which is the precursor of the well-known and widespread 3(2H)-furanones (acyl homoserine lactones), does not seem realistic on a chemical point of view. The spectral data of all the pairs of furanones identified shared the key fragments to include them in the 2(5H)-furanones family. However, some differences were observed in the MS fragmentation profiles. In particular, the use of PCA analysis indicated that the furanone A profiles of *Salmonella* Enterica, *Lactobacillus helveticus*, *Lactobacillus plantarum, Lactobacillus paraplantarum, Lactobacillus sanfranciscensis* and *Enterococcus faecalis* can be grouped in one unique cluster with only few exceptions. This type of analysis, which allows the identification of the most significant mass fragments demonstrating that a part from a few exception, furanone A has the same molecular mass and configuration in all the species. On the other hand, on the basis of the PCA anlysis dendrogram of Euclidean distances, the fragmentation profiles of furanone B of the major part of the species and strains could be grouped together and were differentiated from those of *L. helveticus*.

Quantitative detection of the two furanones evidenced that in the species considered the release of furanones A and B is shifted. In particular, the maximum excretion of furanone A was earlier and it disappeared overtime. On the other hand, B accumulation decreased later respect to furanone A or in same cases, i.e *Lactobacillus helveticus*, continued to increase overtime. This different dynamics can suggest that furanone B is chemically deriving from, or related to, the furanone A.

As reported by Winzer, Hardie and Williams (2002) all the cell-to-cell signal molecules are metabolized or degraded by enzymes whose activities depend on the microbial species. Therefore, the homogeneity of the furanone A and the major diversity of the furanone B could be due to diverse metabolism or degradation patterns.

Concerning the bioactivity of the 2(5H)-furanones, the species considered associated or triggered morphological changes when were exposed to stress conditions able to stimulate in concomitance high level of furanones A and B. Furthermore, it was also observed that when fresh cells were exposed to their own CMs, or to the cell free CMs of *L. helveticus*, morphological and autolysins production was spurred demonstrating the ability to induce a strong intra and inter-specific activity.

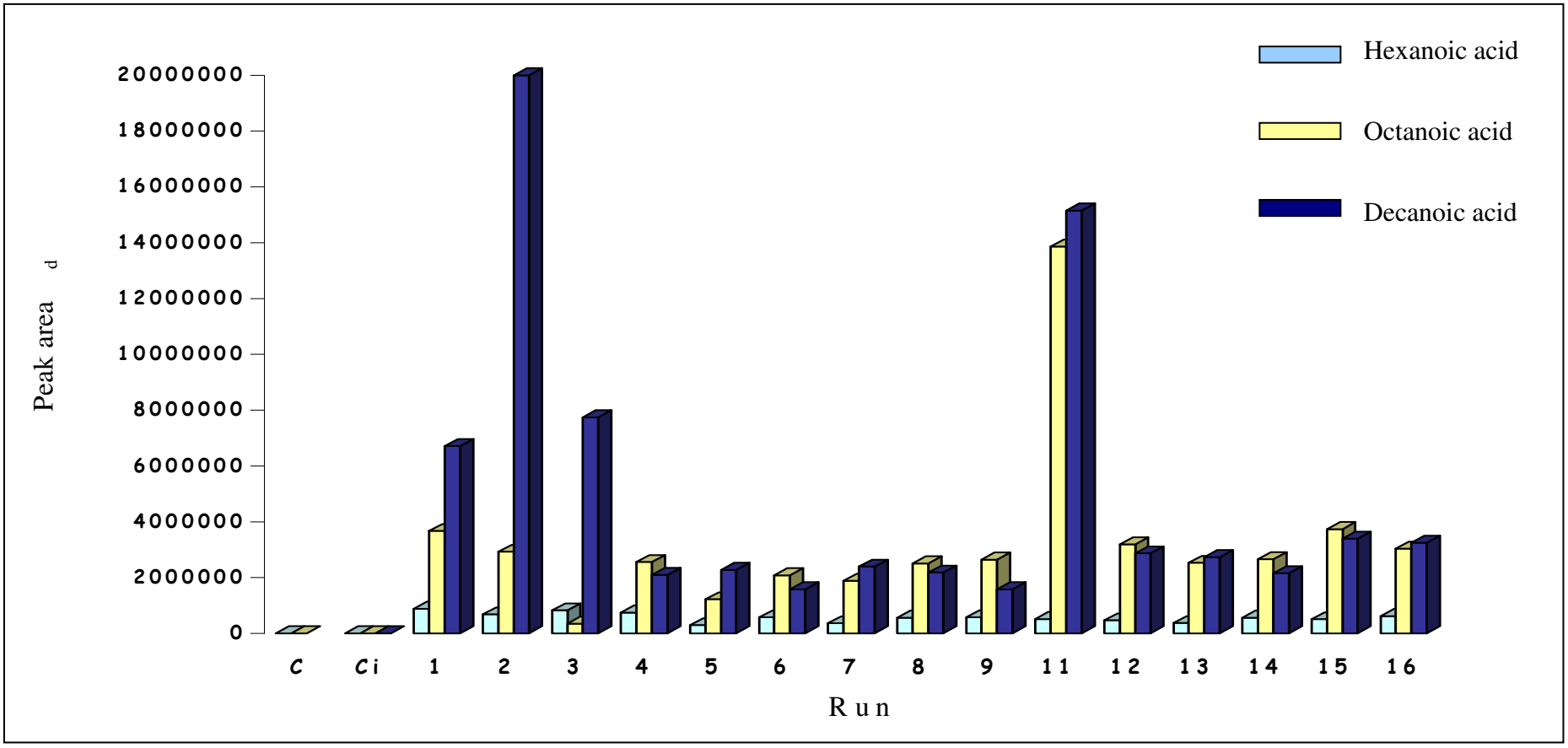
Compared with the many reports about the isolation of furanones from microorganisms and their potential applications (Kjelleberg *et al*.,2003; Bassler *et al*., 2003) there are only a few reports regarding natural furanones substituted at positions 2 and 5. Some of these compounds proved to be active in the quorum sensing system of *Chromobacterium violaceum* (Grossman et al., 2003). In the conditions tested, high content of furanones, in particular B was able to interfere with violacein formation. Although, under no conditions was observed violacein increase due to possible effect of predominance of AHLs. The structural similarity of 2(5H)-furanones and AHLs, which are 2(3H)-furanones notably produced by *Salmonella* and other gram-negative bacteria, suggested that the former ones mimic AHLs. In fact, de Nys et al. (1999) hypothesized that the high molecular weight 2(5H)-furanone produced by the red alga *Delisea pulchra* cross talk and interferer with

AHLs system of gram-negative bacteria.

Final proof of the role of the 2(5H)-furanones requires the use of chemically defined molecules. In this phase of the research only small amounts of A and B furanones were released which allowed neither a determination of the absolute configuration nor a more detailed investigation of the full biological properties. Therefore, given the growing interest in the antimicrobial activities of furanones of diverse origins and their biological effects observed also at low concentrations, the identification of the precursors of the 2(5H)-furanones and of the genes controlling their synthesis in Gram-positive and Gram-negative bacteria is of recognisable importance.

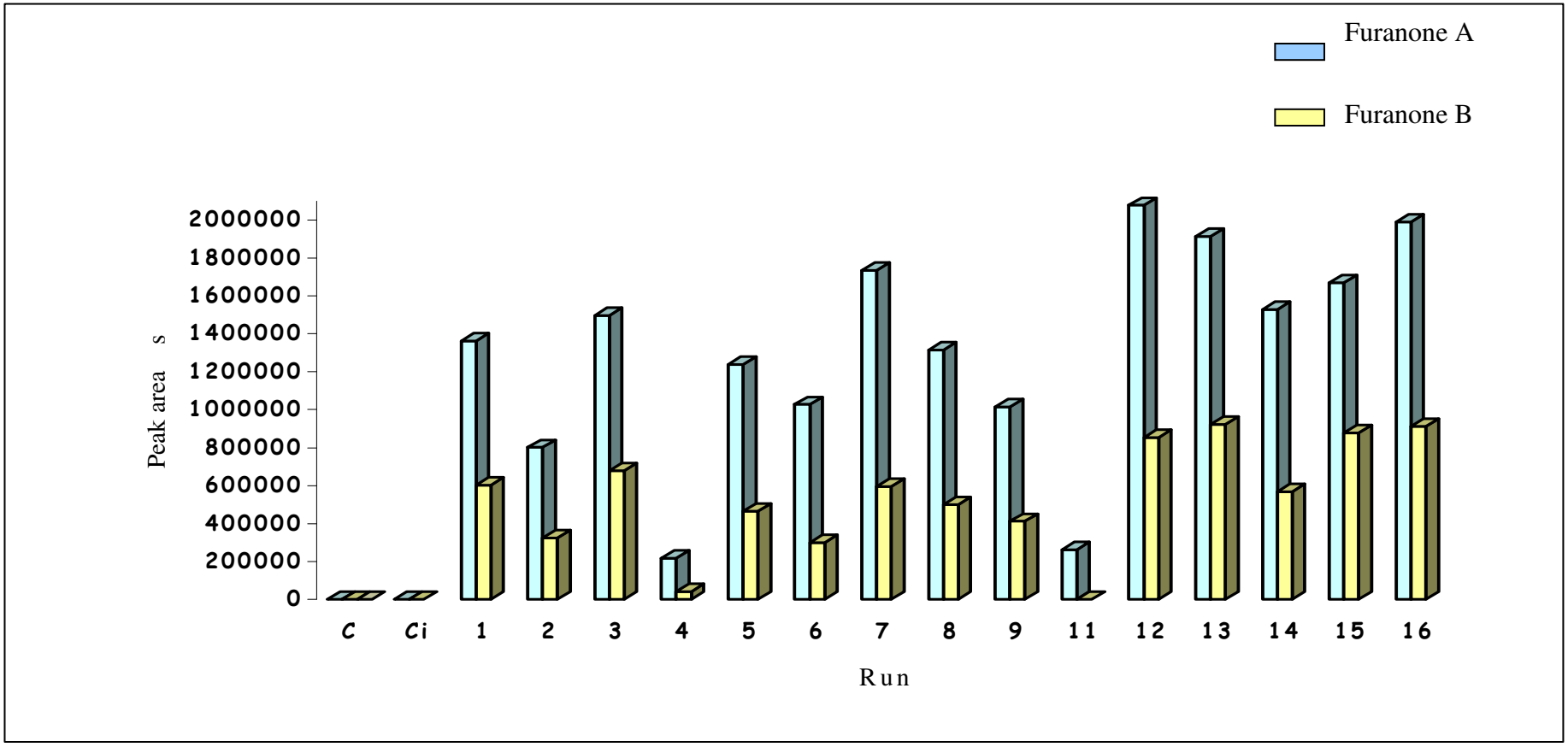
Tables and Figures

Figure 1– Levels (as peak chromatographic area) of volatile fatty acid (hexanoic, octanoic and decanoic acid) released by *Lactobacillus helveticus* after 2hours of exposure to different stress combination (Table 1)



C: whey; Ci: inoculated whey not modified; condition 9=10=17

Figura 2- Levels (as peak chromatographic area) of furanones A and B released by *Lactobacillus helveticus* after 2hours of exposure to different stress combination (Table 1)



C: whey; Ci: inoculated whey not modified; condition 9=10=17

Table 1 - Levels of the sublethal factors used for the experimental design

Stress combination	H_2O_2 %	NaCl M	pH
1	0.005	0.3	3.8
2	0.013	0.3	3.8
3	0.005	0.7	3.8
4	0.013	0.7	3.8
5	0.005	0.3	5.0
6	0.013	0.3	5.0
7	0.005	0.7	5.0
8	0.013	0.7	5.0
9	0.009	0.5	4.4
10	0.009	0.5	4.4
11	0.001	0.5	4.4
12	0.017	0.5	4.4
13	0.009	0.1	4.4
14	0.009	0.9	4.4
15	0.009	0.5	3.2
16	0.009	0.5	5.6
17	0.009	0.5	4.4

Figure3 – Levels (as peak chromatographic area) furanone A and B released by *Salmonella enteritidis* during growing in BHI medium.

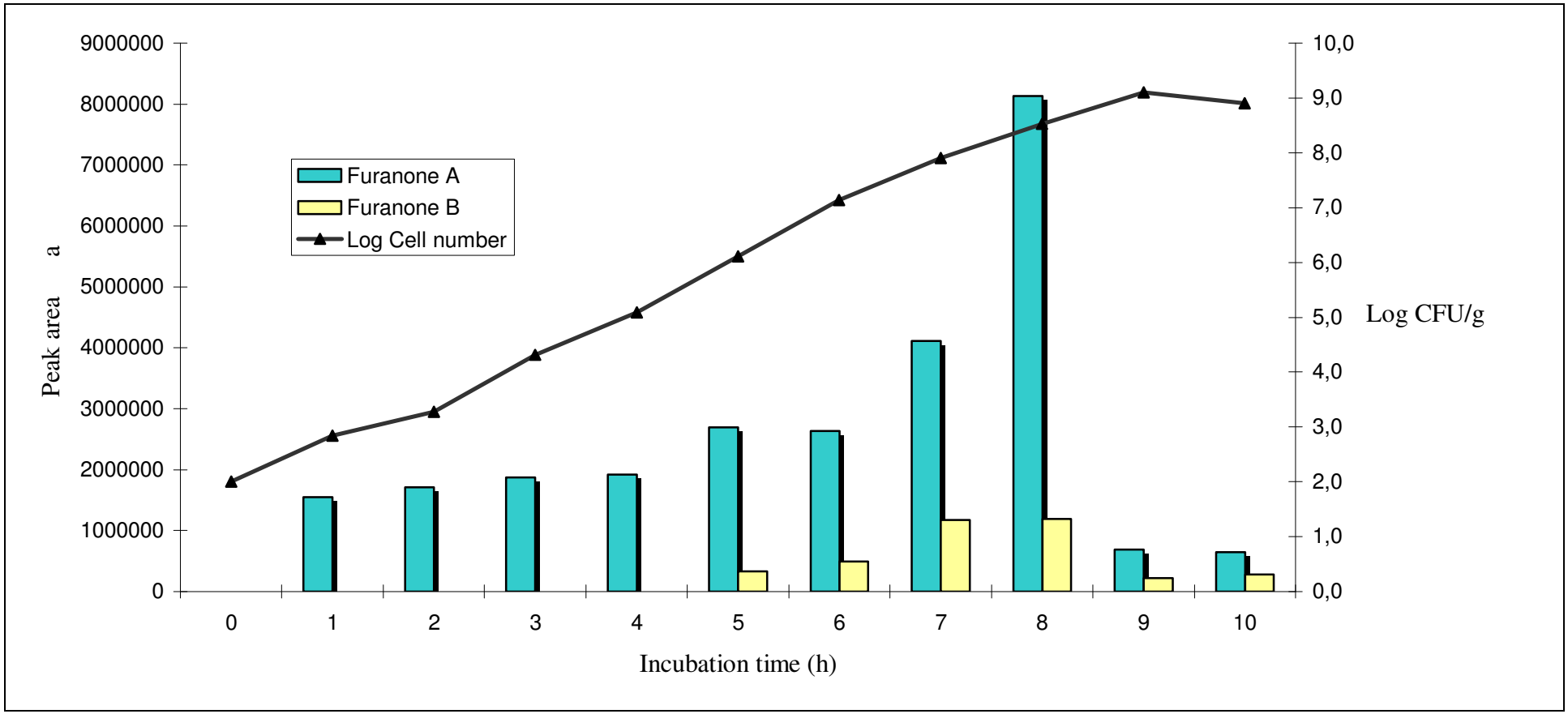
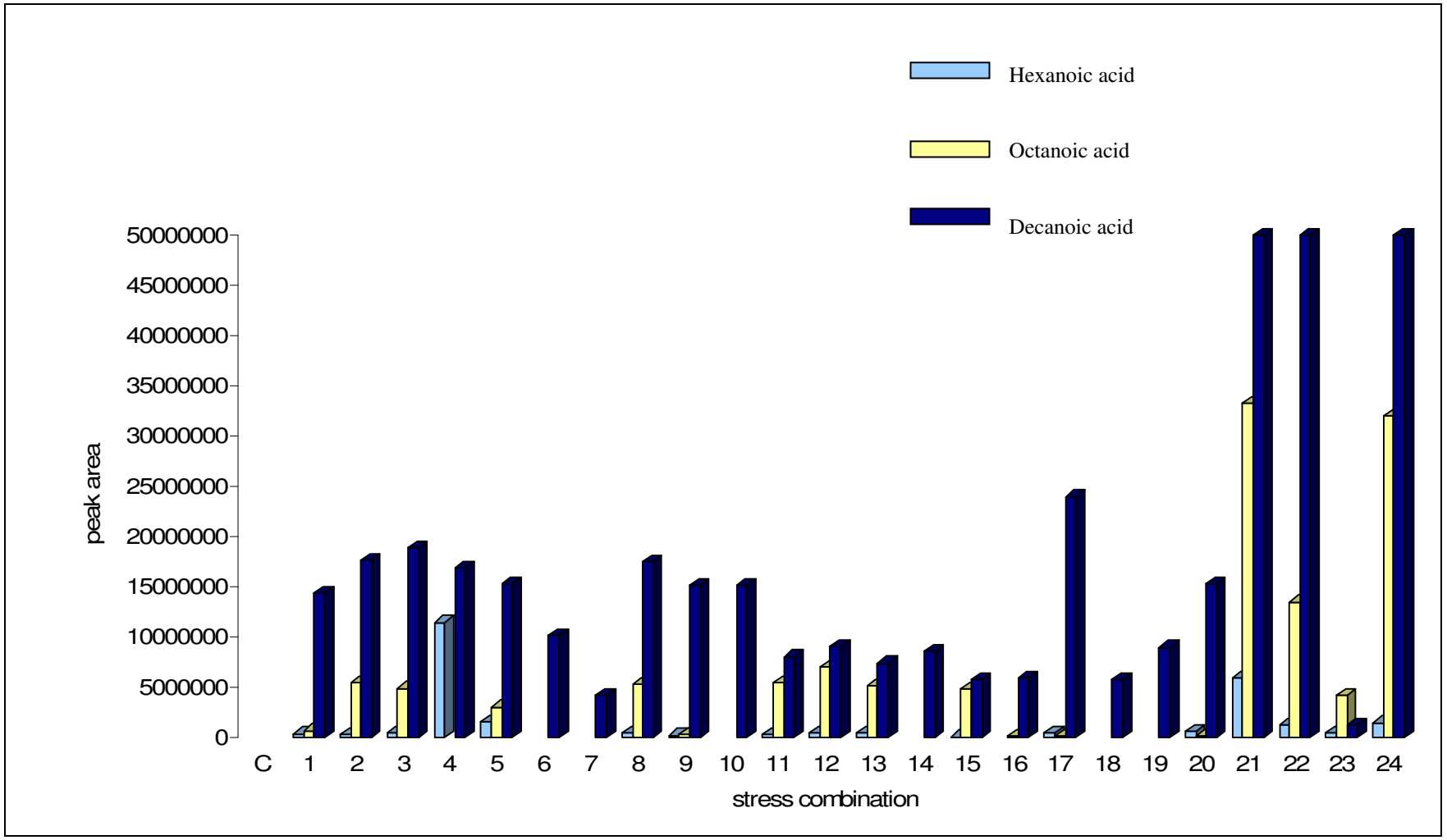
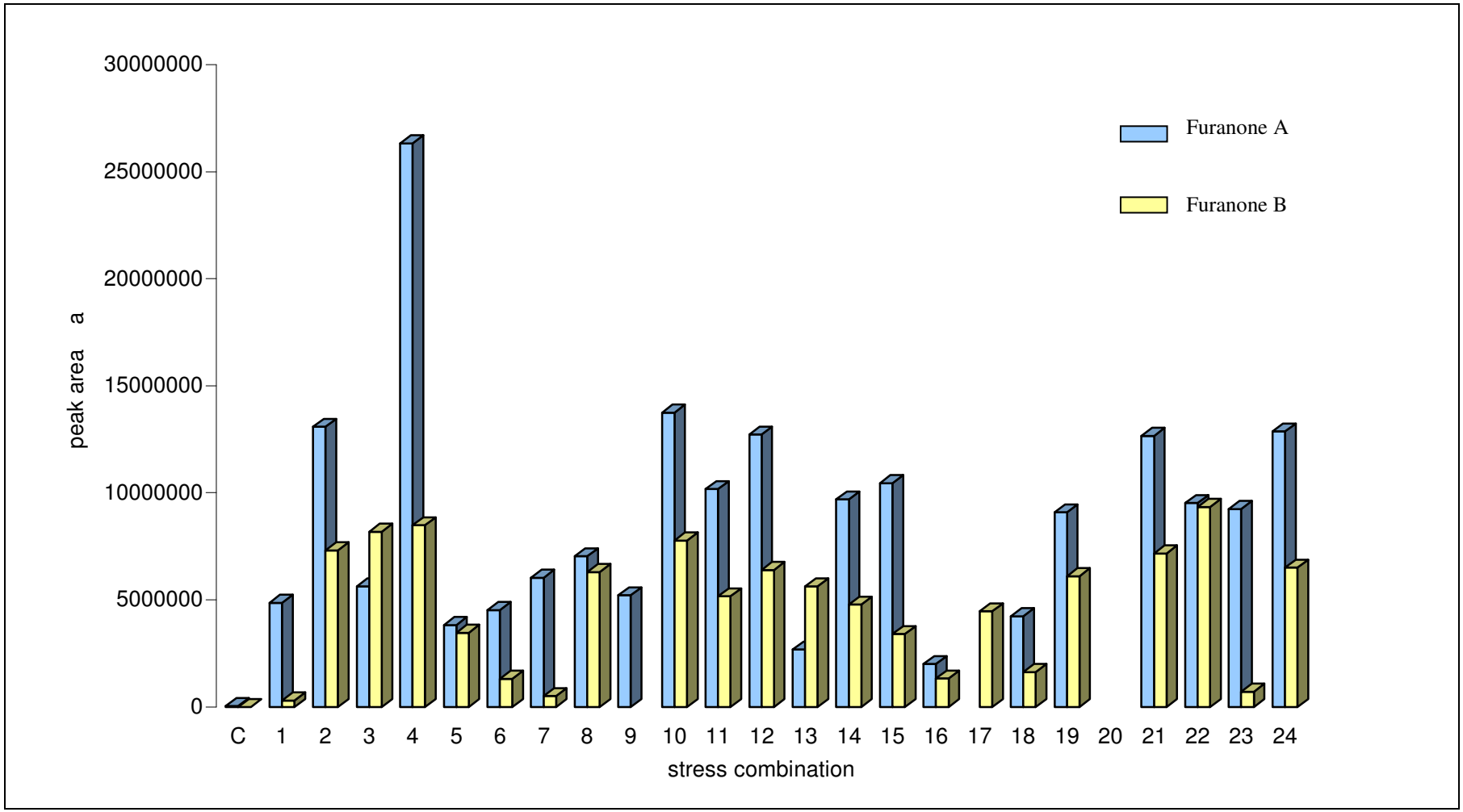


Figure 4 – Levels (as peak chromatographic area) of volatile fatty acid (hexanoic, octanoic and decanoic acid) released by *Salmonella enteritidis* after 2hours of exposure to different stress combination (Table 2)



C: BHI

Figure 5 - Levels (as peak chromatographic area) of furanones A and B released by *Salmonella enteritidis* after 2hours of exposure to different stress combination (Table 2)



C: BHI

Table 2 – List of compounds utilized to evaluate the release of volatile metabolite by *Salmonella enteritidis* under stress condition. The concentration of the compounds utilized was 300ppm except for otherwise specified.

Stress Combination		Stress Combination	
1	**2,5- dimethyl-4-hydroxy-3(2H)-furanone**	**13**	**Monomiristic acid**
2	**2-ethyl-4-hydroxy-5-methyl-3(2H)-furanone**	**14**	**Monolaurate acid**
3	**5-ethyl-3-hydroxy-4-methyl-2(5H)-furanone**	**15**	**Miristic acid**
4	**Hexanal**	**16**	**Lauric acid**
5	**Trans-2-hexanal**	**17**	**Capric acid**
6	**Tetronic acid**	**18**	**Monolaurate acid**
7	**4-hydroxy-5-methyl-3(2H)-furanone**	**19**	**Monostearate acid**
8	**3-methyl-2(5H)-furanone**	**20**	**Monoleate acid**
9	**R(+)-5-hydroxymethyl-2(5H)-furanone**	**21**	**pH4.8 (lactic acid)**
10	**Alphaangelicalactone**	**22**	**H_2O_2 0.017%**
11	**Monolinoleate acid**	**23**	**Aw 0.96 (NaCl)**
12	**Monolinoleanate acid**	**24**	**pH 4.8 (lactic acid) H_2O_2 0.017%**

Figure 6: Extent (as peak chromatographic area) of furanones A and B released by fresh cells of *Salmonella* enteritidis suspended (8 log CFU/ml) in the CMs of the same strain grown in BHI (control) and BHI modified with H_2O_2 or lactic acid addition.

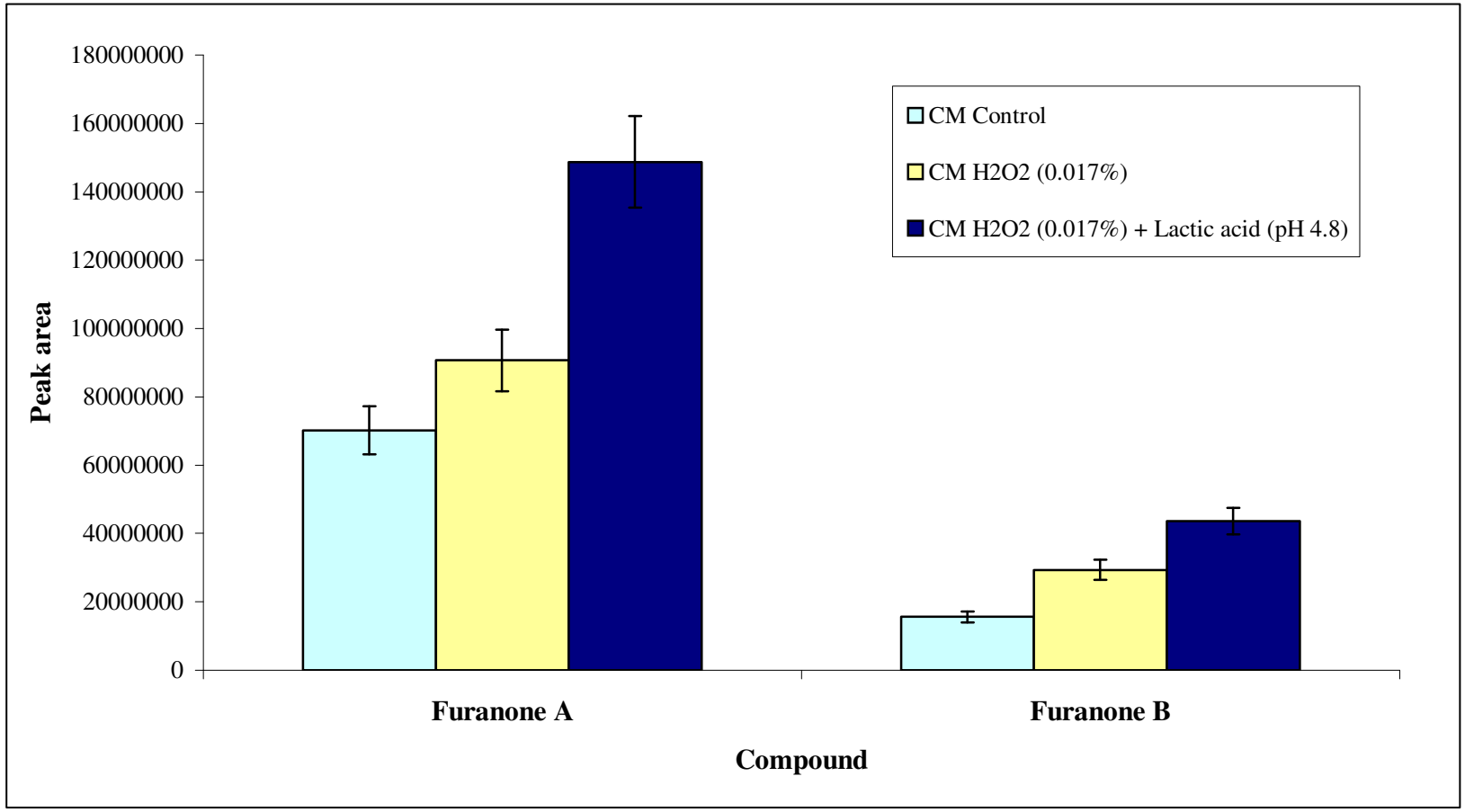
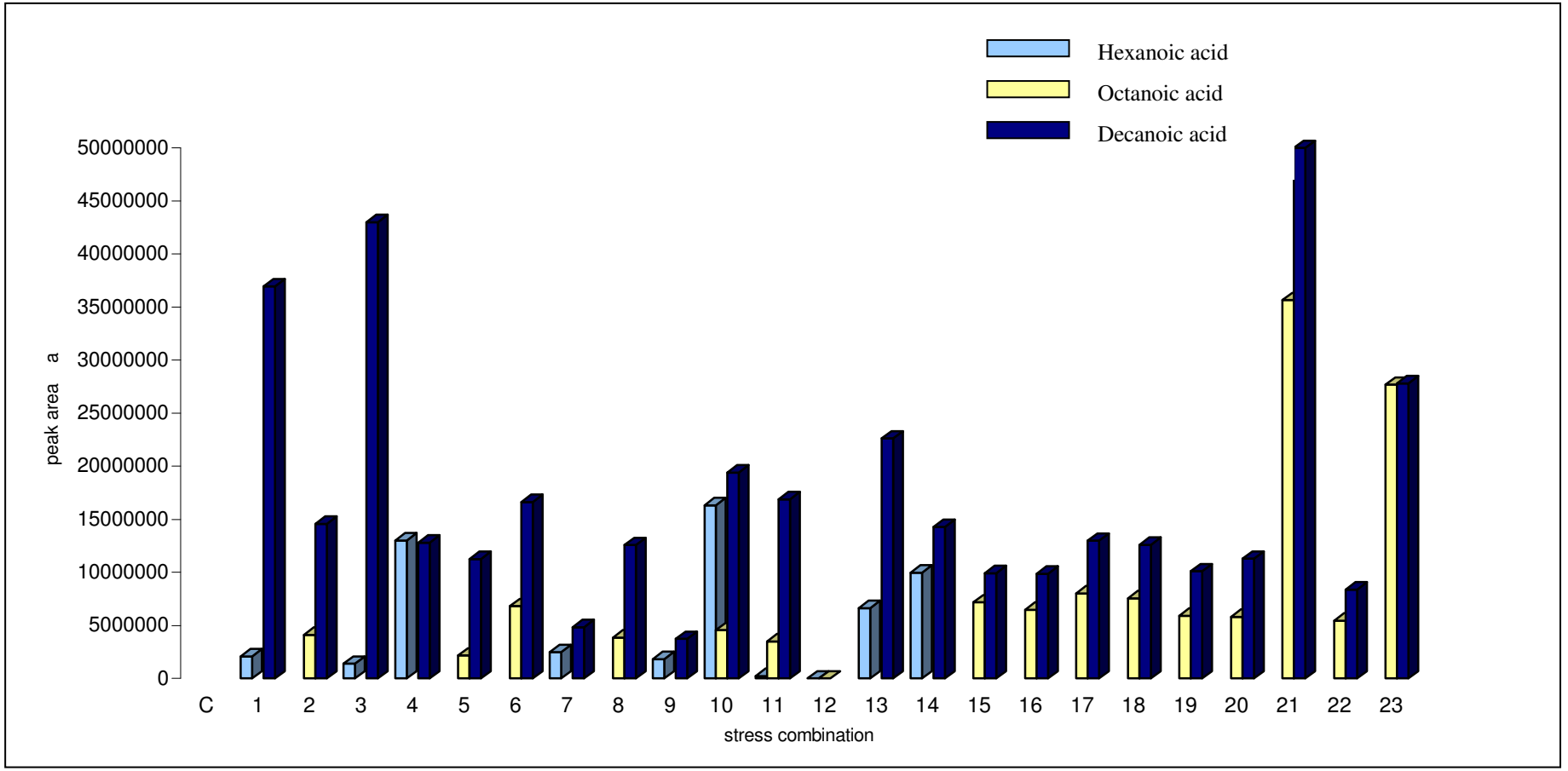
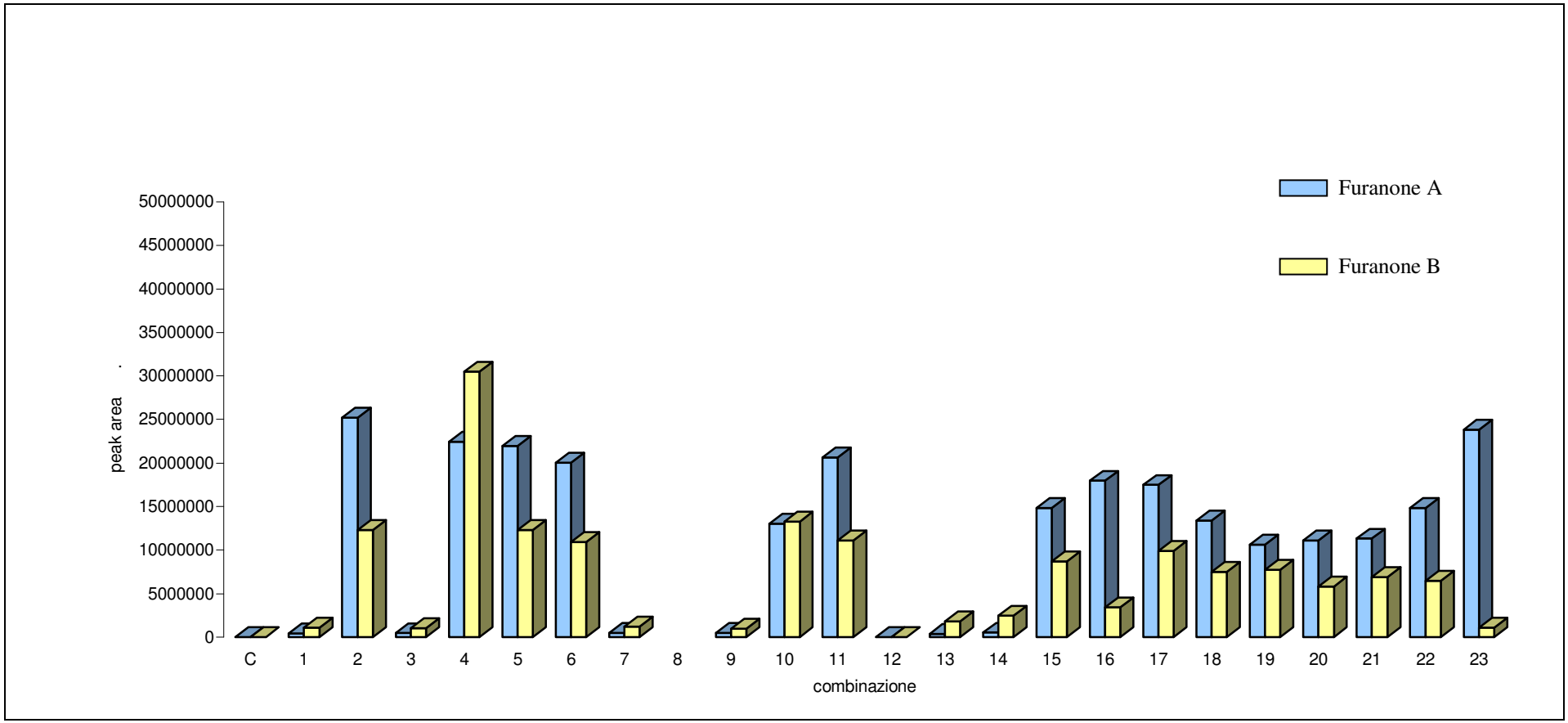


Figure 7 – Levels (as peak chromatographic area) of volatile fatty acid (hexanoic, octanoic and decanoic acid) released by *Enterococcus faecalis* after 2hours of exposure to different stress combination (Table 3)



C: BHI

Figura 8- Levels (as peak chromatographic area) of furanones A and B released by *Enterococcus faecalis* after 2hours of exposure to different stress combination (Table 3)



C: BHI

Table 3 – List of compounds utilized to evaluate the release of volatile metabolite by *Enterococcus faecalis* under stress condition. The concentration of the compounds utilized was 300ppm except for otherwise specified.

Stress Combination		Stress Combination	
1	2,5- dimethyl-4-hydroxy-3(2H)-furanone	13	Monomiristic acid
2	2-ethyl-4-hydroxy-5-methyl-3(2H)-furanone	14	Monolaurate acid
3	5-ethyl-3-hydroxy-4-methyl-2(5H)-furanone	15	Miristic acid
4	Hexanal	16	Lauric acid
5	Trans-2-hexanal	17	Capric acid
6	Tetronic acid	18	Monolaurate acid
7	4-hydroxy-5-methyl-3(2H)-furanone	19	Monostearate acid
8	3-methyl-2(5H)-furanone	20	Monoleate acid
9	R(+)-5-hydroxymethyl-2(5H)-furanone	21	pH4.8 (lactic acid)
10	Alphaangelicalactone	22	Aw 0.96 (NaCl)
11	Monolinoleate acid	23	H_2O_2 0.017%
12	Monolinoleanate acid		

Table 4– Mass fragmentation of the compound A and B

Compound A	41[a] (39)[b], 57 (80), 67 (28), 69 (25), 79 (18), 85 (8), 97 (100), 99 (73), 109 (40), 123 (7), 143 (9)
Compound B	43 [a] (28)[b], 57 (47), 69 (11), 83 (17), 97 (100), 111 (10), 123 (16), 137 (10), 151 (4), 165 (4), 180 (10)

[a] m/z

[b] realative intensity

Figure 9 – Molecular structure hypothesized for compound A and B

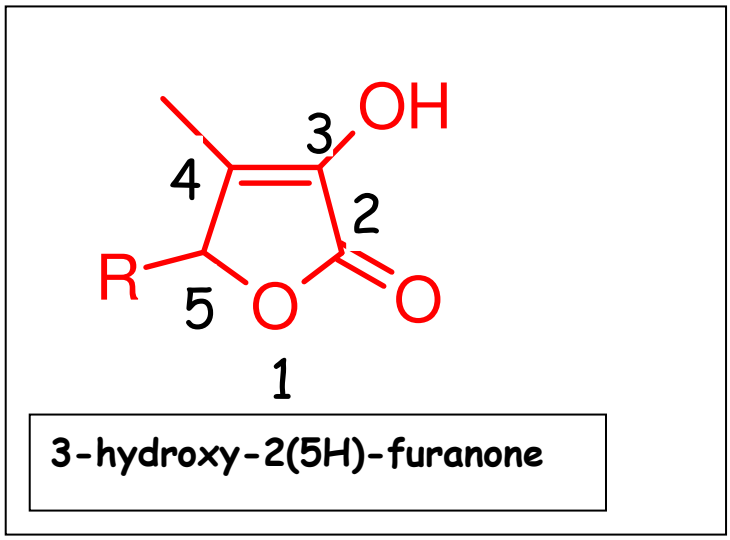


Figure 10 – Mass fragmentation pattern of the unknown molecule A (RT=45.4)

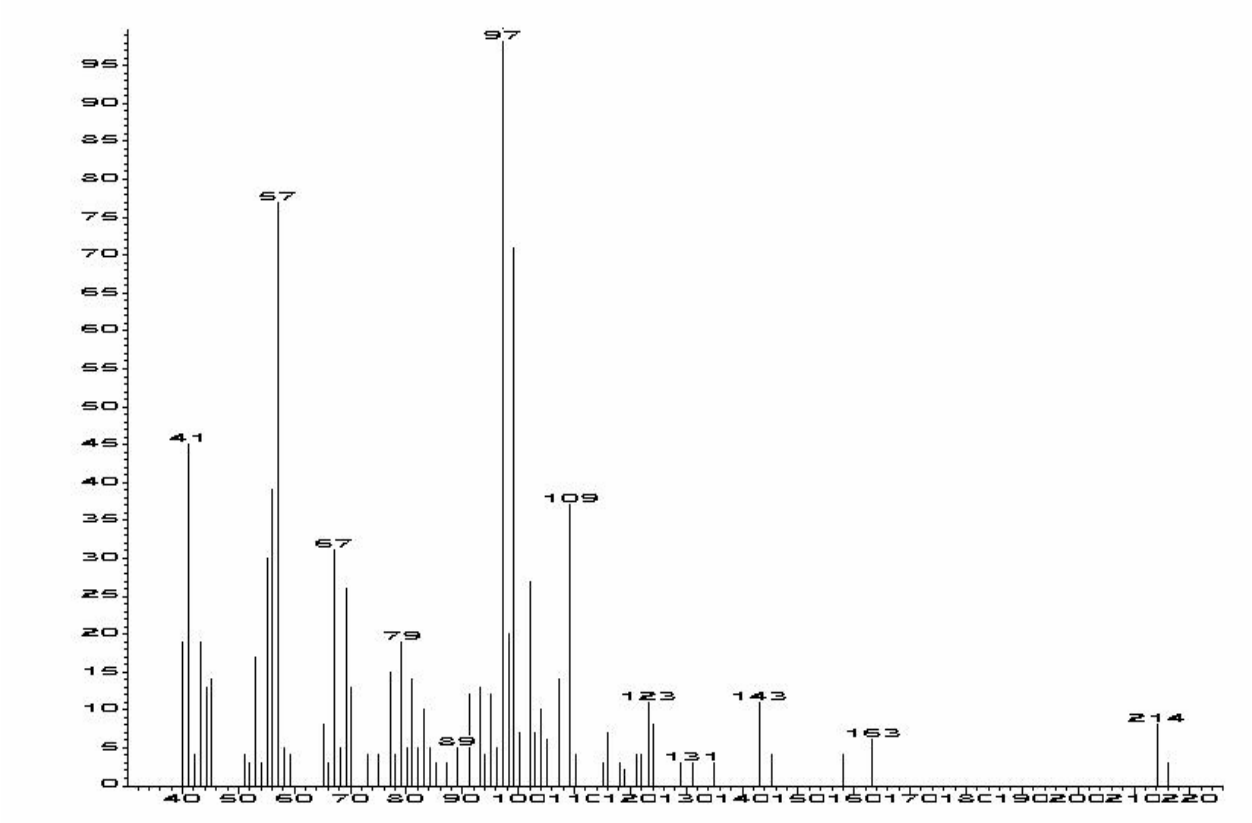


Figure 11 – Mass fragmentation pattern of the unknown molecule B (RT=45.9)

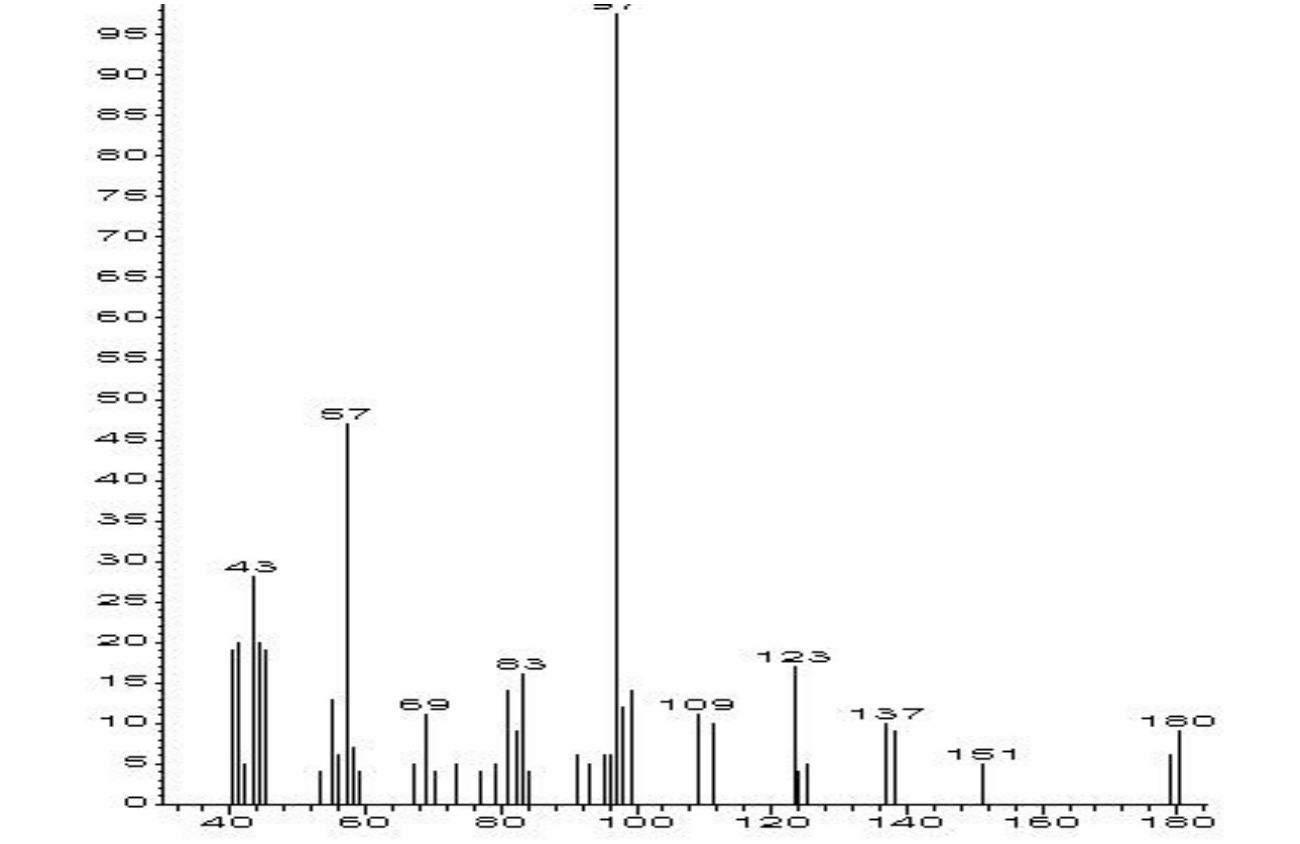


Figure 12 – Projection of PCA on axes 1 and 2 of the mass fragmentation profiles of the furanone A of all the combinations strain/medium/condition (37 combination per 4 repetition).

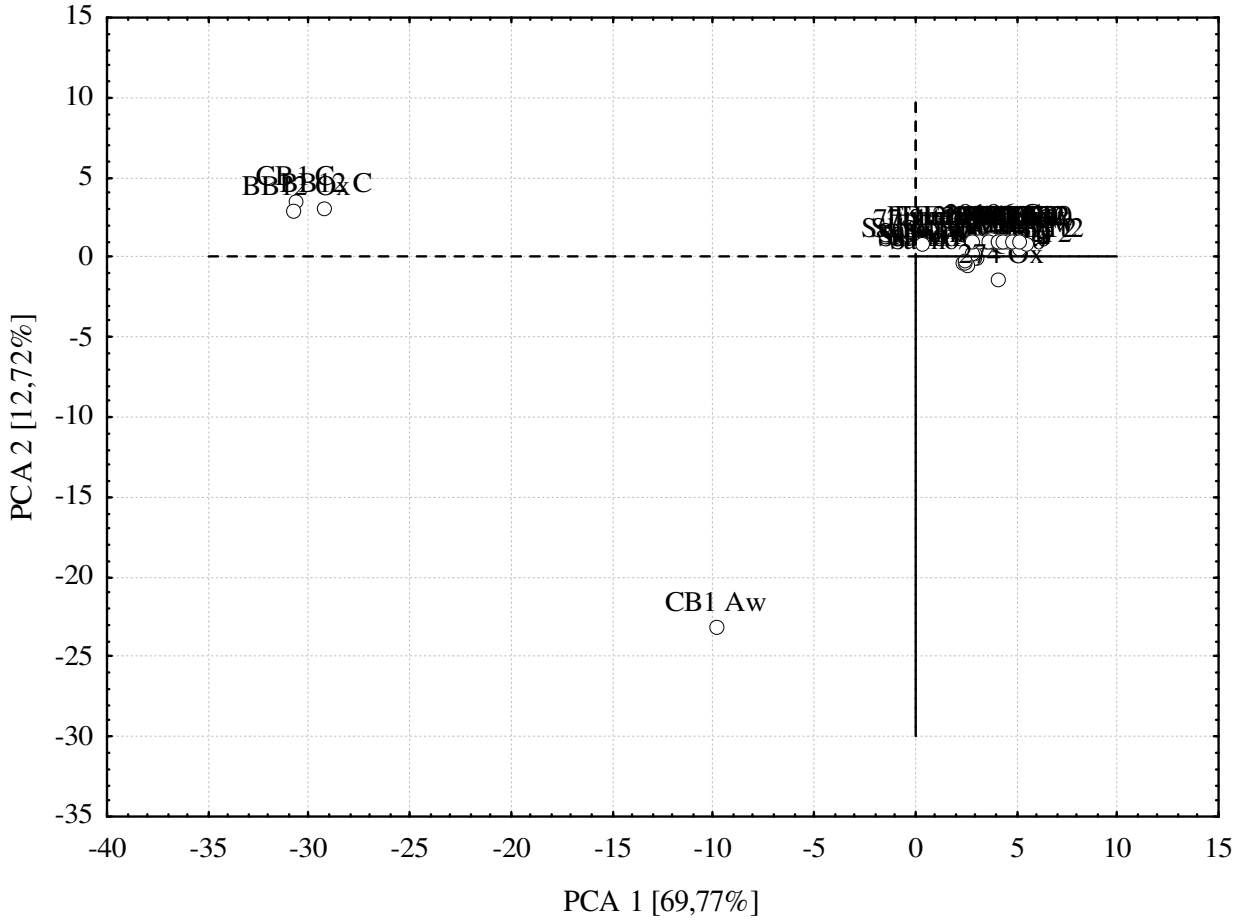


Figure 13 – Projection of PCA on axes 1 and 2 of the mass fragmentation profiles of the furanone B of all the combinations strain/medium/condition (37 combination per 4 repetition).

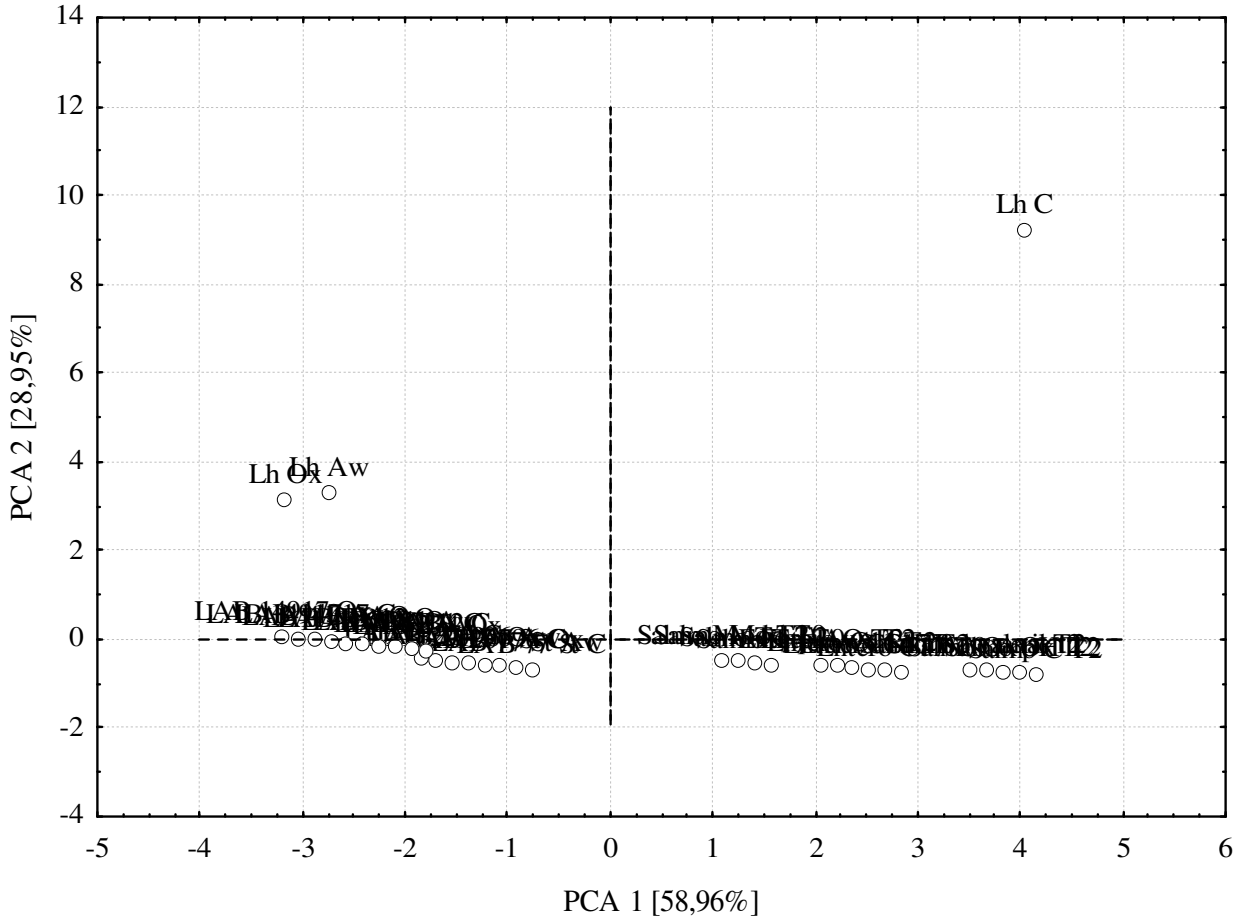


Figure 14 – Dendrogram of Euclidean distances among the mass spectral profiles of furanone B in the different combinations strain/medium/condition (37 combination).

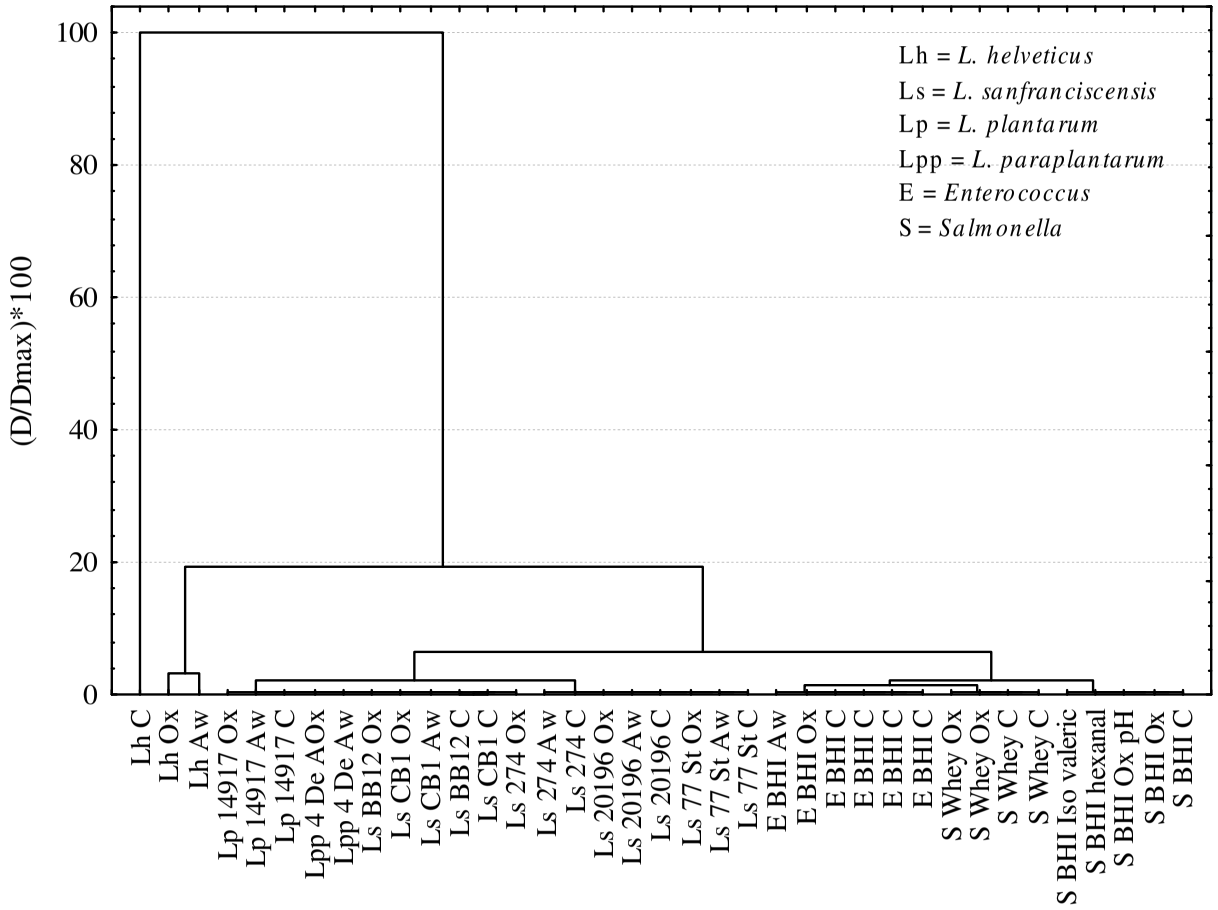
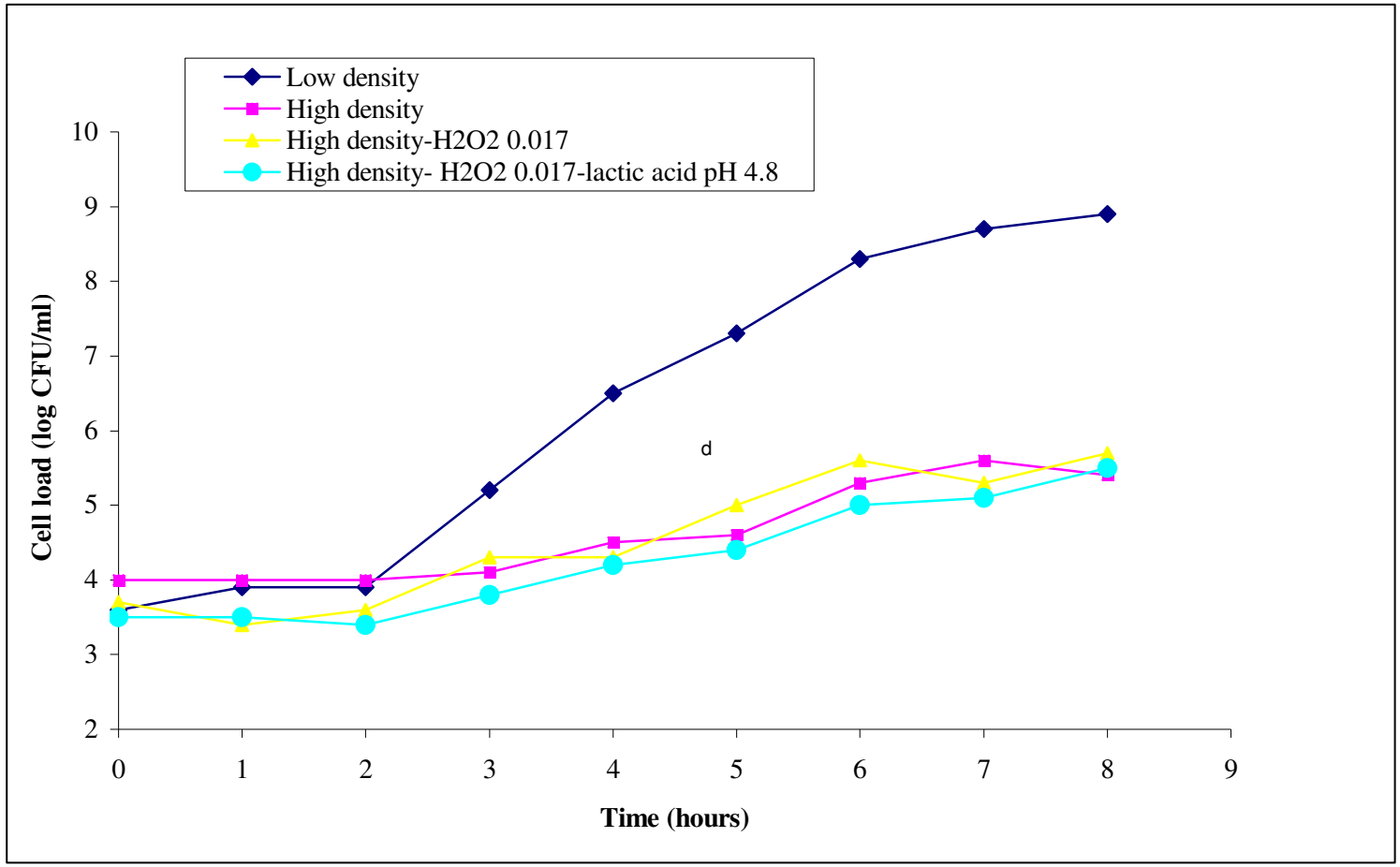


Figure 15: Effect of the CMs (additioned to 50% v/v to fresh BHI) from high density cultures in BHI (purple ■) or BHI with H_2O_2 0.017% (yallow ▲) or BHI with combination of H_2O_2 (0.017% v/v) and lactic acid (pH 4.8) (blue ∗) and from low density culture in BHI (dark blue ♦) on growth dynamics of *Salmonella enteritidis*.



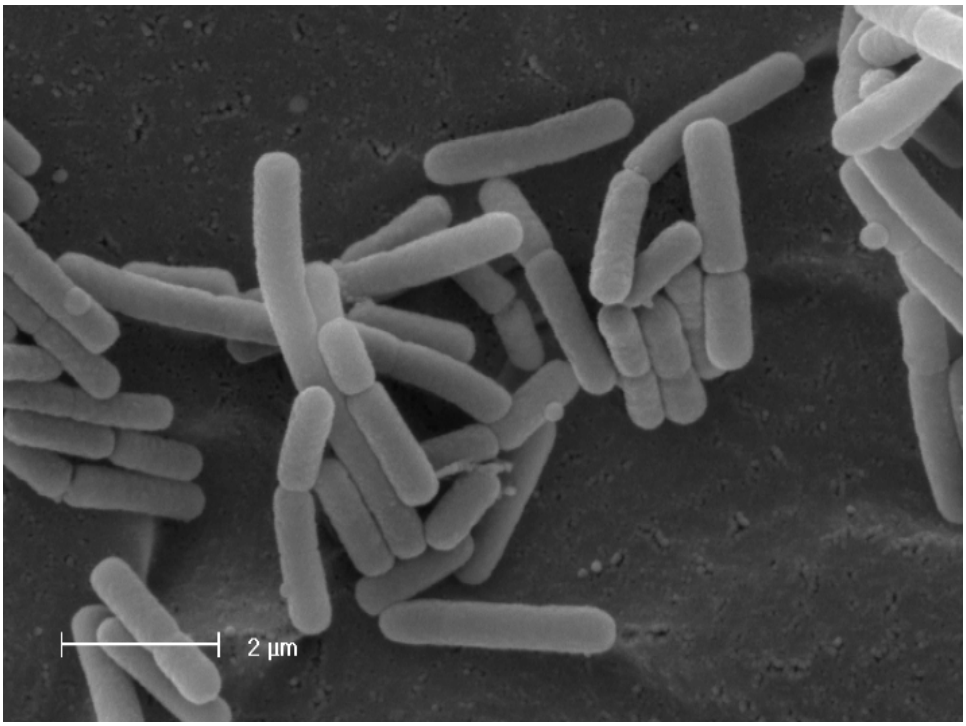


Figure 16– SEM micrograph of fresh cells of *L. helveticus Lactobacillus* grown in optimal conditions.

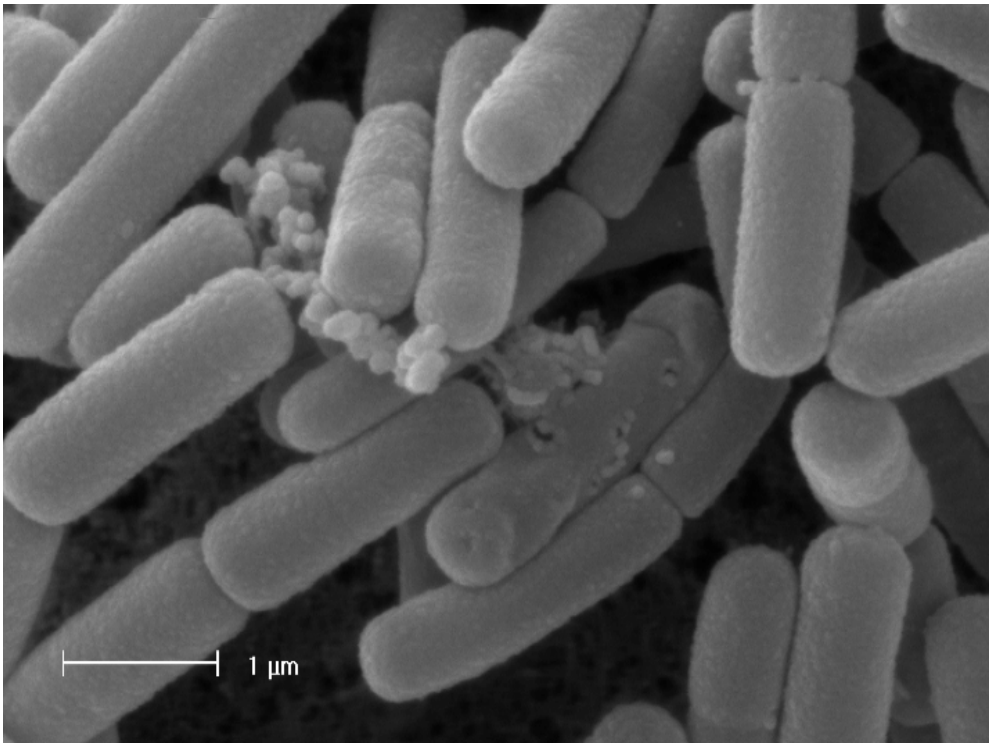


Figure 17– SEM micrograph of the fresh cells of *L. helveticus* exposed for 100 minutes to cell free CM 12 of the experimental design.

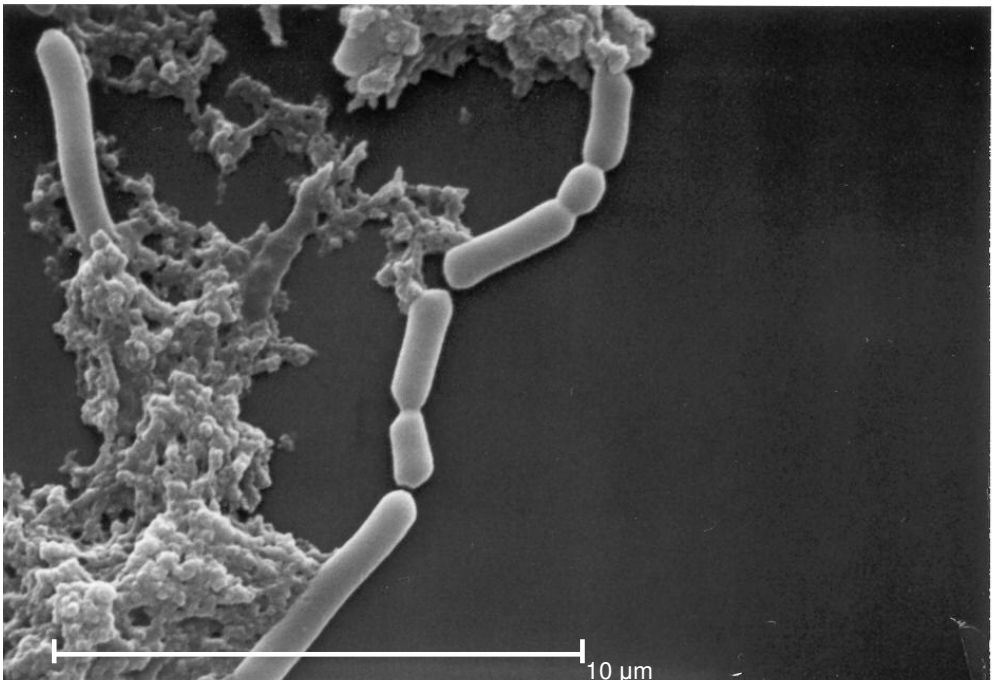


Figure 18– SEM micrograph of the fresh cells of *L. helveticus* exposed for 100 minutes to cell free CM 12 of the experimental.

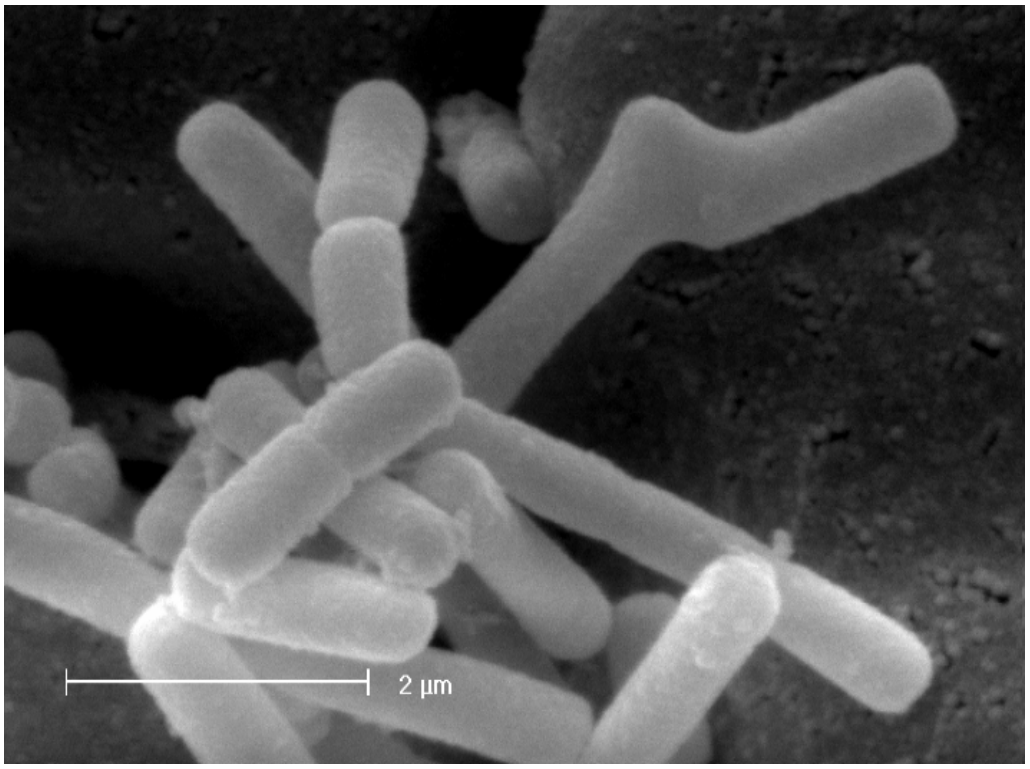


Figure 19- SEM micrograph of the fresh cells of *L. helveticus* exposed for 100 minutes to commercial HEMFi 7 µM in whey.

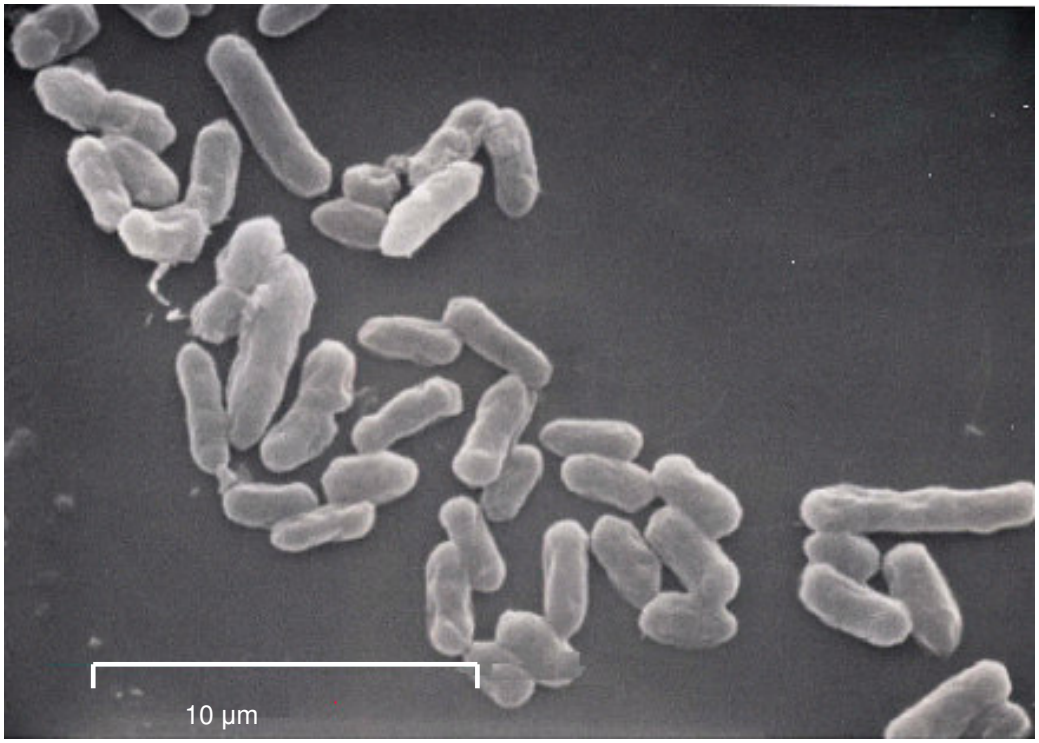


Figure 20- SEM micrograph of the fresh cells of *Salmonella enteritidis* grown in optimal conditions.

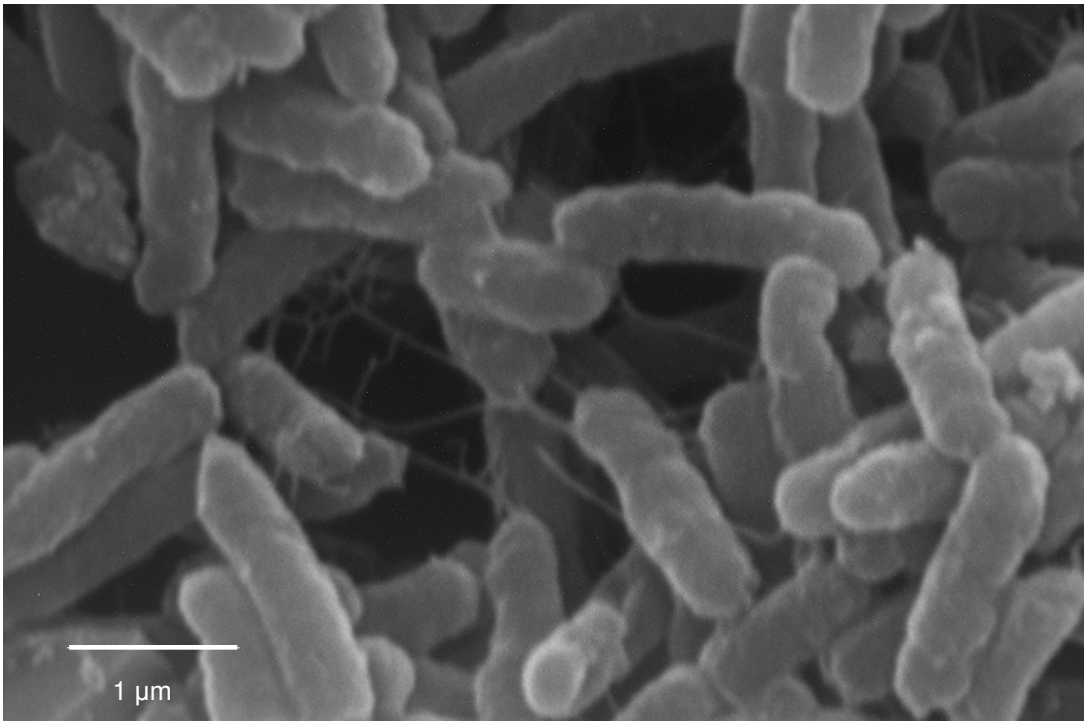


Figure 21- SEM micrograph of the fresh cells of *Salmonella enteritidis* exposed for 2 hours to hexanal (300ppm) in BHI.

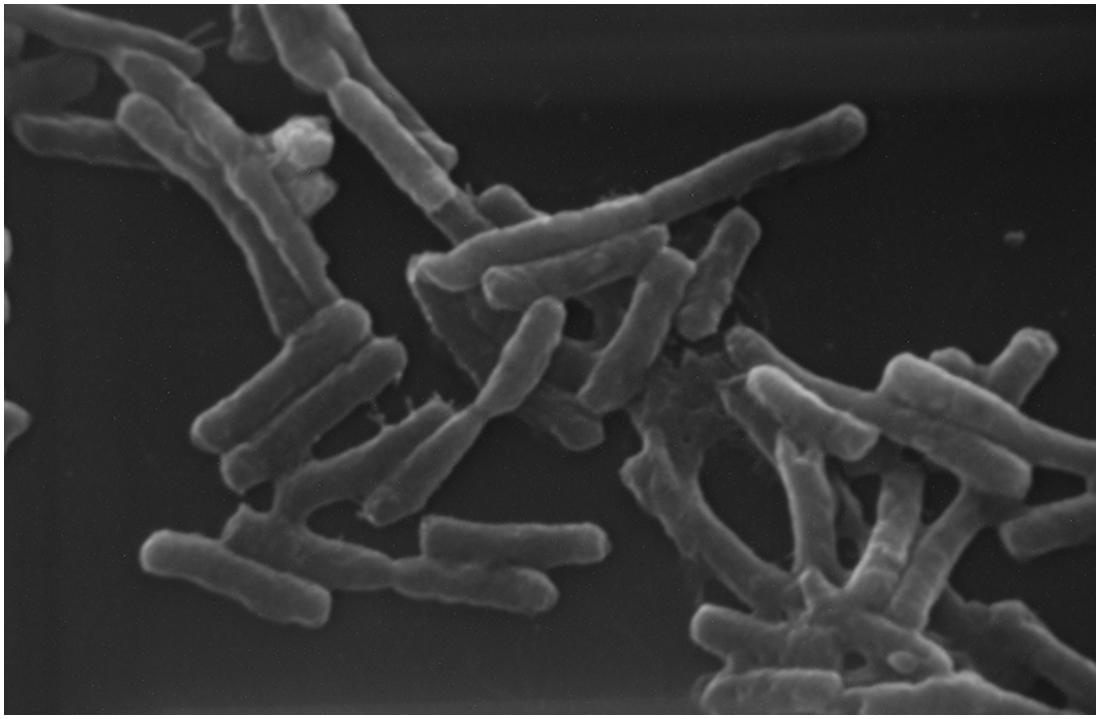


Figure 22- SEM micrograph of the fresh cells of *Salmonella enteritidis* exposed for 2 hours to H_2O_2 (0.017% v/v), pH 4.8 in BHI.

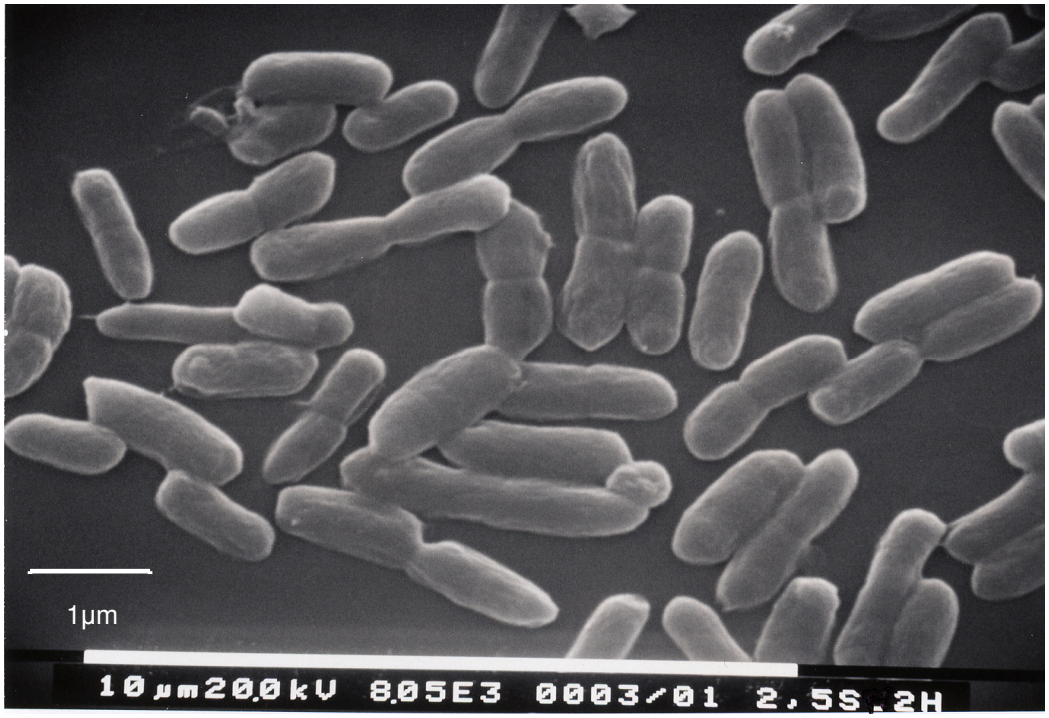


Figure 23- SEM micrograph of the fresh cells of *Salmonella enteritidis* exposed for 2 hours to cell free CM 12 of *L. helveticus* (20% v/v) of the experimental design.

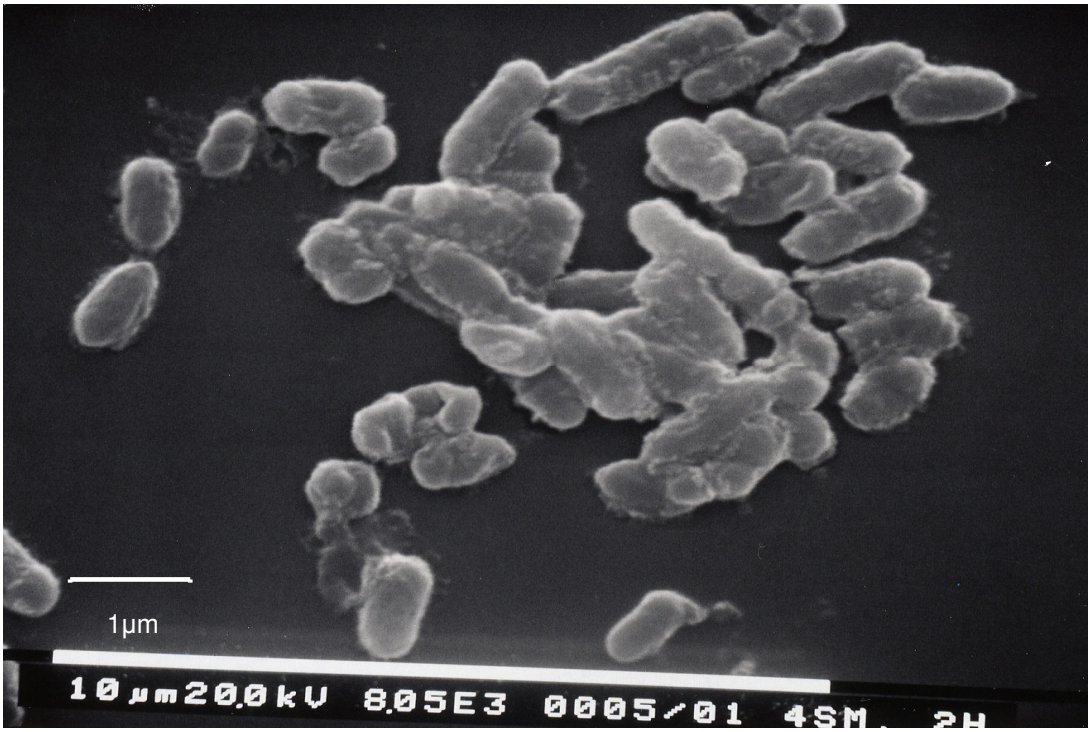


Figure 24- SEM micrograph of the fresh cells of *Salmonella enteritidis* exposed for 2 hours to cell free CM 12 of *L. helveticus* (80 % v/v) of the experimental design.

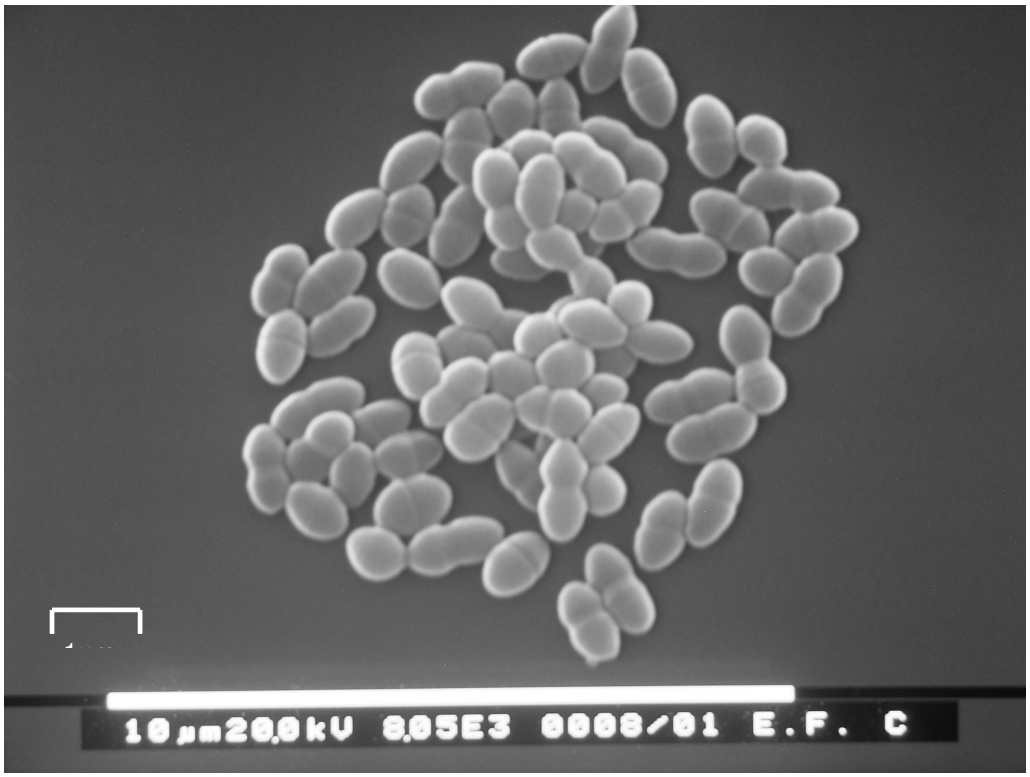


Figure 25- SEM micrograph of the fresh cells of *Enterococcus faecalis* grown in optimal conditions.

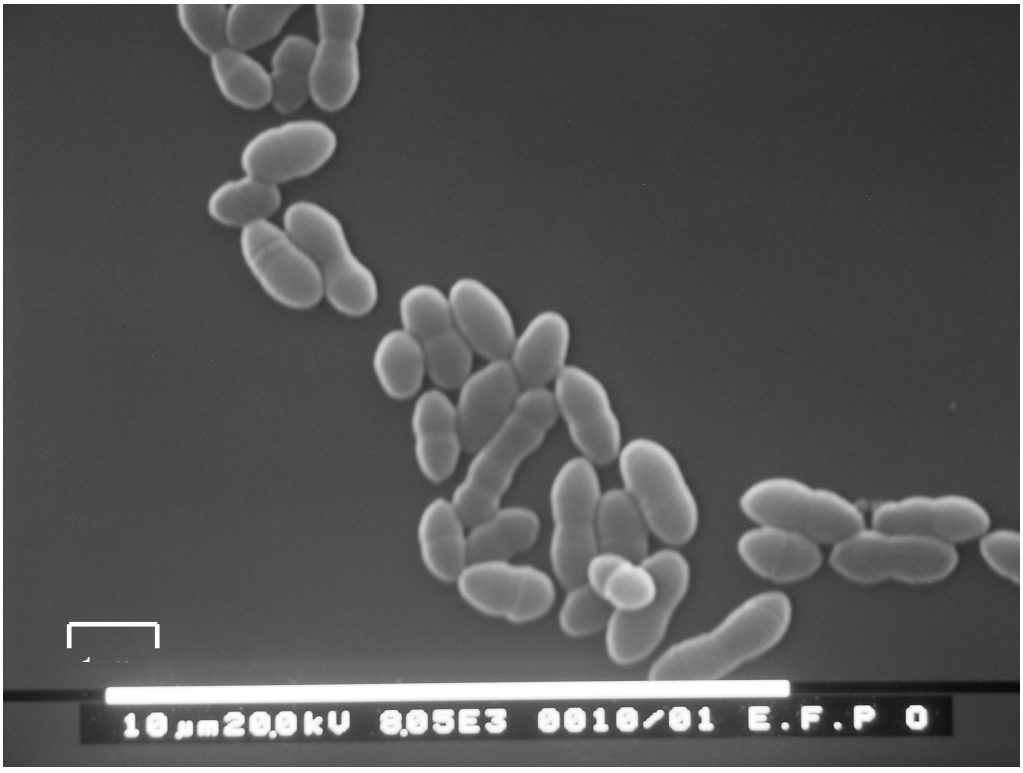


Figure 26- SEM micrograph of the fresh cells of *Enterococcus faecalis* exposed for 2 hours to H_2O_2 (0.017% v/v), pH 4.8 in BHI.

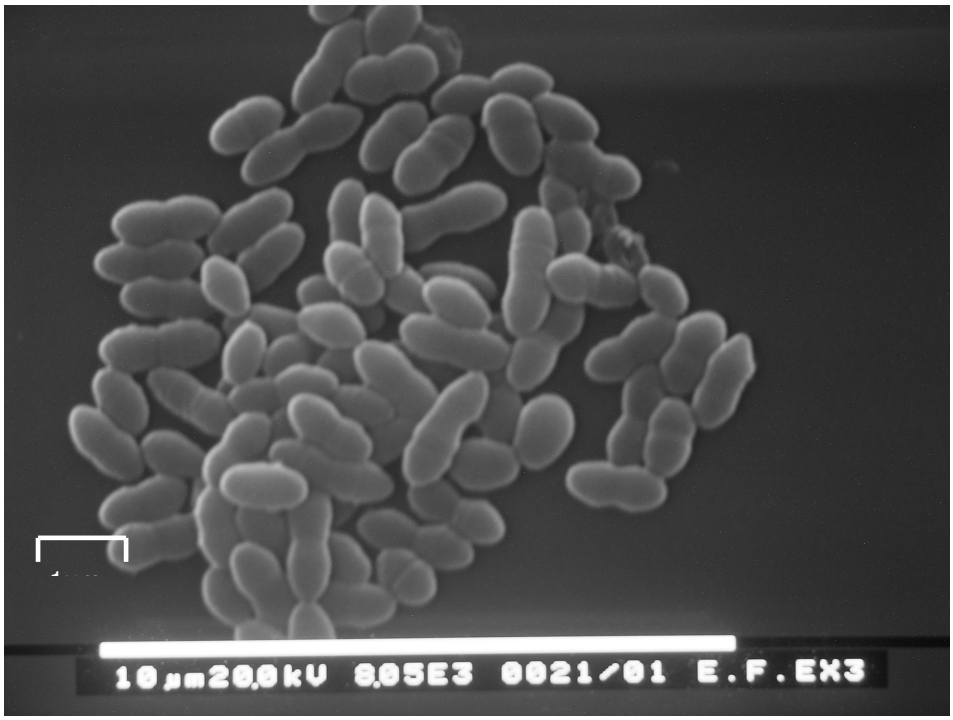


Figure 27- SEM micrograph of the fresh cells of *Enterococcus faecalis* exposed for 2 hours to hexanal (300ppm) in BHI.

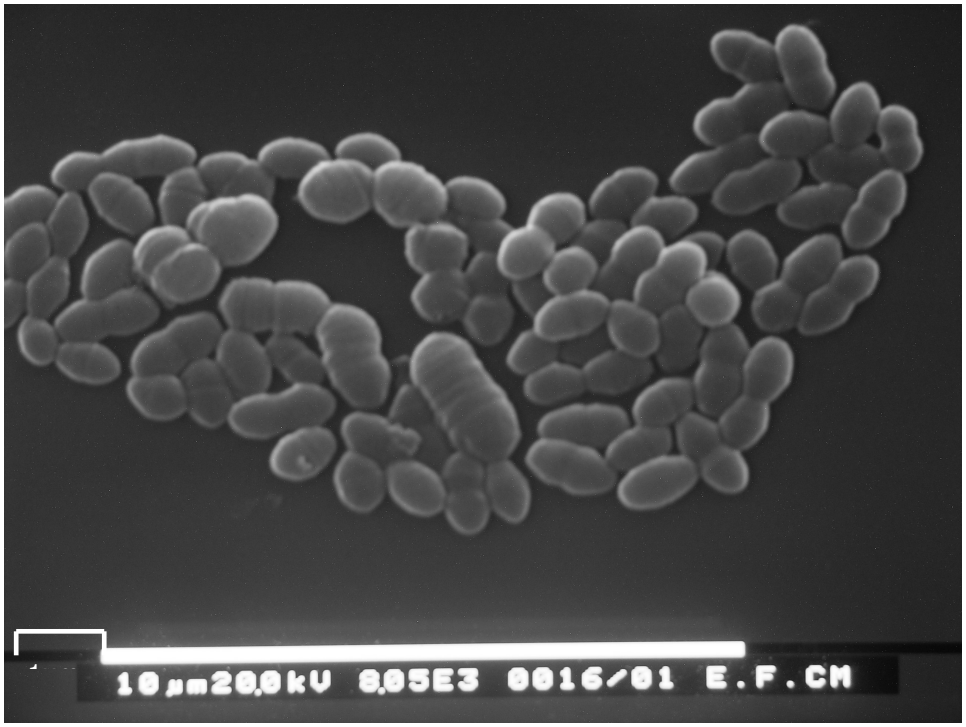
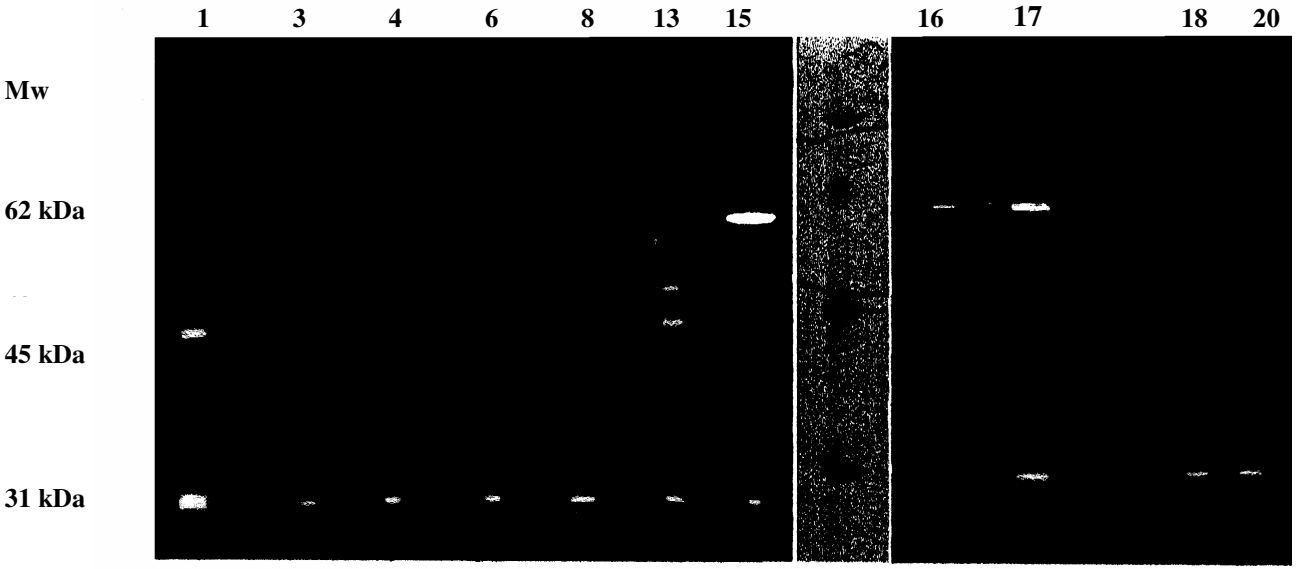


Figure 28- SEM micrograph of the fresh cells of *Enterococcus faecalis* exposed for 2 hours to cell free CM of the same cells.

Figure 29- Cell wall lytic activity of *Lactobacillus. helveticus* exposed to different stress conditions against Micrococcus lysodeikticus cells in renaturated SDS-PAGE (zymogram).



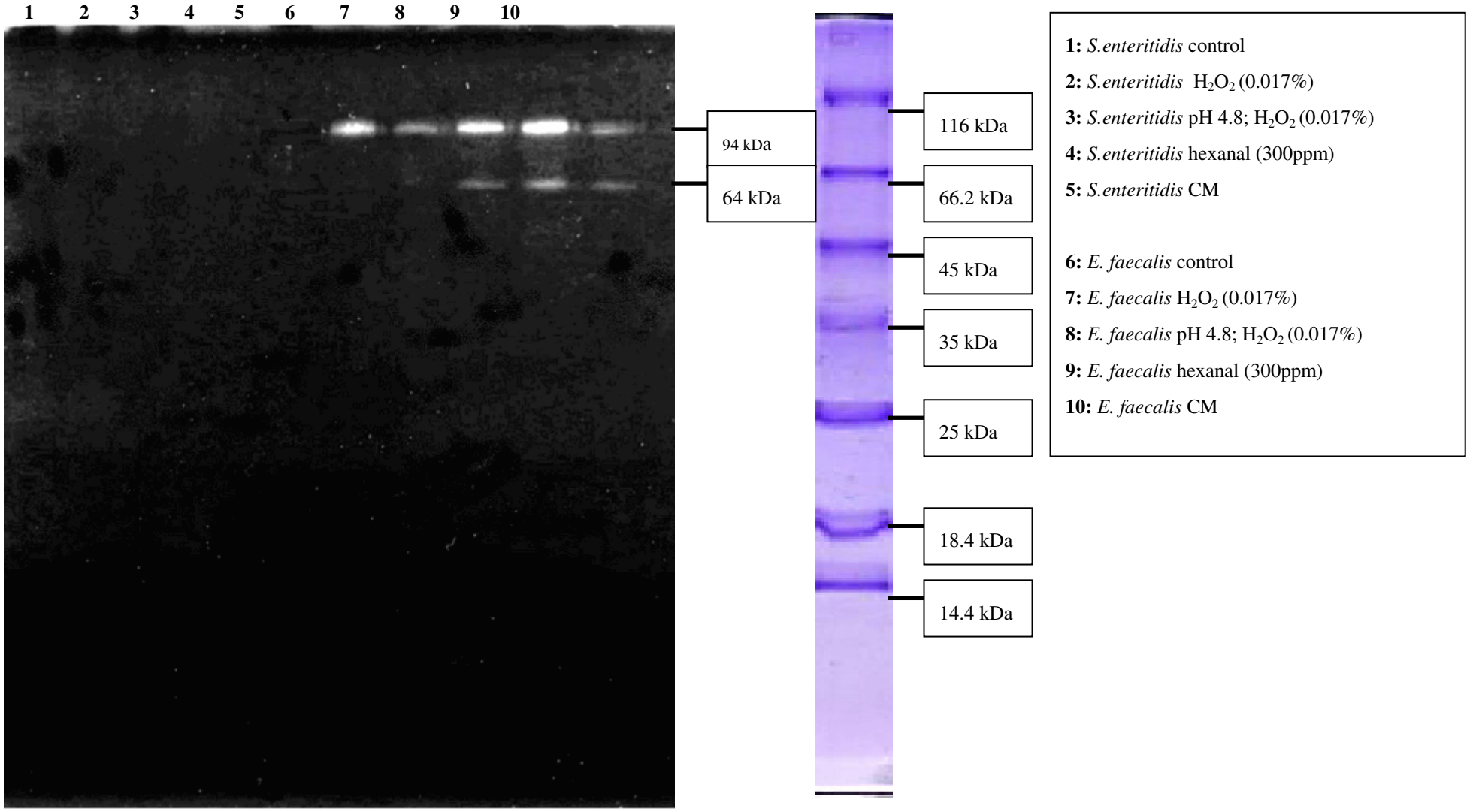
1: Control
3: H_2O_2 (0.017%)
4: HEMF 1ppm
6: HEMF 1ppm + H_2O_2 (0.017%)
8: Filtrate whey
13: HEMF 1ppm + NaCl 0.5M
15: HEMFI 1ppm + NaCl 0.5M
16: HEMFI 1ppm + H_2O_2 (0.017%)
17: HEMFI 1ppm
18: HEMF 1ppm + H_2O_2 (0.017%) + NaCl 0.5M pH 4.4
20: HEMFI 1ppm +H_2O_2 (0.017%) + NaCl 0.5M pH 4.4

Table 4. Intensity of the lytic bands of *L. helveticus* after 100 minutes of exposure to oxidative stress, CMs of cells exposed to oxidative stress and to commercial furanones or mixtures of fatty acids (values are expressed as relative units, RU)

Condition	31 kDa	43 kDa	45 kDa	63 kDa	Total	Viability (log CFU/ml)
Whey (control) (I)	82.7±4.9	n.d*	n.d	n.d	82.7	7.6±0.3
Whey H_2O_2 0.3mM (II)	43.7±2.6	n.d	n.d	n.d	43.7	7.4±0.3
Whey H_2O_2 2.6mM (III)	n.d	n.d	n.d	n.d	n.d	7.4±0.3
Whey H_2O_2 5mM (IV)	n.d	n.d	n.d	n.d	n.d	6.8±0.3
HEMF 7µM	39.8±2.4	n.d	n.d	n.d	39.8	6.8±0.3
HEMFi 7µM	74.8±4.5	n.d	n.d	101.7±6.1	176.5	6.6±0.3
CM I	35.2±1.6	n.d	n.d	n.d	35.2	7.1±0.3
CM II	41.1±2.5	54.9±3.3	31.1±1.9	n.d	127.1	4.3±0.1
CM III	77.0±4.6	54.0±3.2	20.9±1.2	n.d	151.9	4.5±0.1
CM IV	79.1±4.7	0	n.d	n.d	79.1	4.4±0.1
FAs 4µM	53.5±3.2	n.d	n.d	n.d	53.5	6.2±0.2

*not detectable

Figure 30- Cell wall lytic activity of Salmonella enteritidis and Enterococcus faecalis exposed to different stress conditions against Micrococcus lysodeikticus cells in renaturated SDS-PAGE (zymogram).



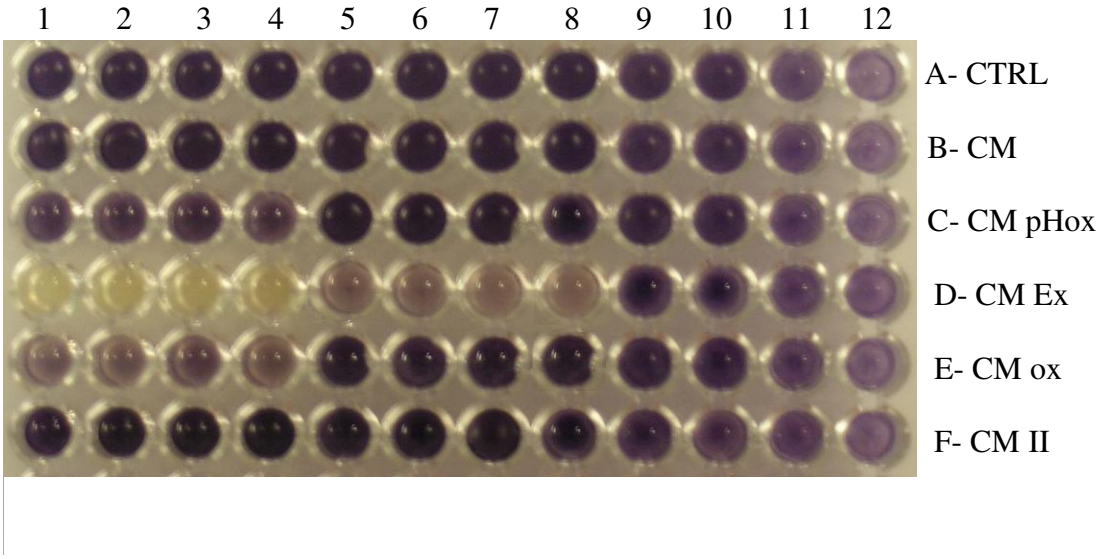


Figure 31- Bioassay with *Chromobacterium violaceum* for the identification of quorum signal antagonists in cell free CMs from *Salmonella enteritidis* exposed to different stresses. Columns 1-4 = 50% of CMs concentrations; columns 5-8 = 50% of SMs concentration; column 9-10 = 10% of CMs concentrations; columns 11-12 = 10% of SMs concentration. Row A = control; row B = CM from unstressed culture (named CM); row C = CM from pH-oxidative stressed culture (named CM-pHOx); row D = CM from hexanal stressed culture (named CM-Ex); row E = CM from oxidative stressed culture (named CM-Ox); row F = CMs from cultures exposed to their own CM (named CM-II).

	50% (v/v) of CM (1-4)	**50% (v/v) of SM (5-8)**	**10% (v/v) of CM (9-10)**	**10% (v/v) of SM (11-12)**
CTRL (A)	2.5 ± 0.3	2.4 ± 0.2	1.8 ± 0.3	1.8 ±0.2
CM (B)	2.2 ± 0.4	2.4 ± 0.2	1.9 ± 0.5	1.9 ± 0.4
CM-pHOx (C)	1.6 ± 0.5	2.2 ± 0.3	1.9 ± 0.3	1.8 ± 0.2
CM-Ex (D)	0.2 ± 0.4	1.5 ± 0.2	1.8 ± 0.2	1.8 ± 0.3
CM-Ox (E)	1.4 ± 0.3	2.0 ± 0.3	1.9 ± 0.4	1.8 ± 0.3
CM-II (F)	2.5 ± 0.3	2.3 ± 0.3	1.8 ± 0.2	1.8 ± 0.2

Table 5- Inhibition effects on violacein production by C. violaceum by different free cell CM from *Salmonella enteritidis* exposed to different stresses on spectrophotometric absorbance (590 nm).

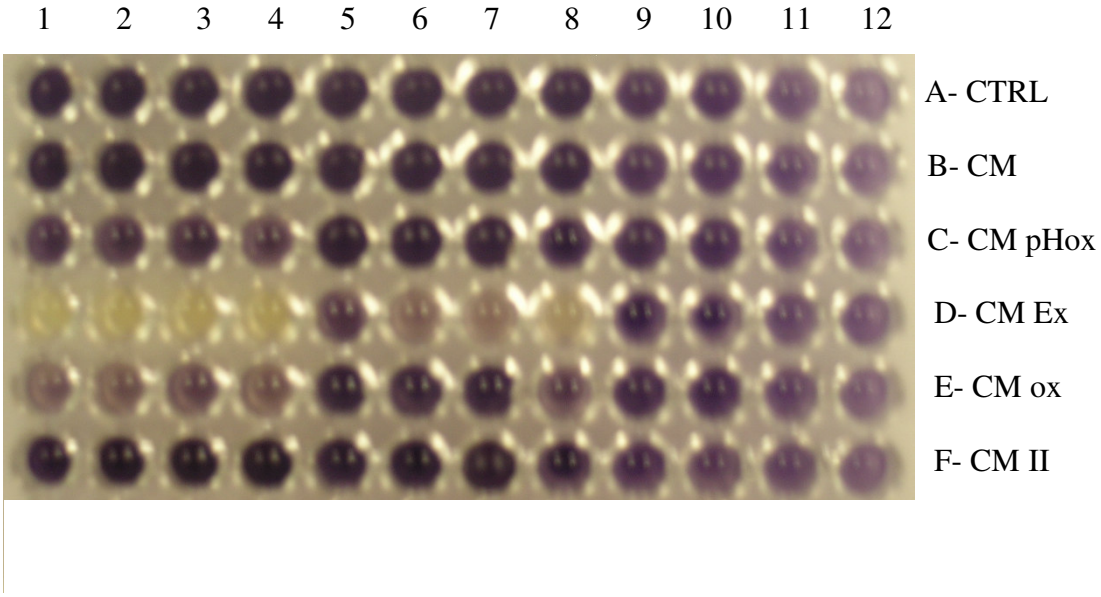


Figure 32- Bioassay with *Chromobacterium violaceum* for the identification of quorum signal antagonists in cell free CMs from *Enterococcus faecalis* exposed to different stresses. Columns 1-4 = 50% of CMs concentrations; columns 5-8 = 50% of SMs concentration; column 9-10 = 10% of CMs concentrations; columns 11-12 = 10% of SMs concentration. Row A = control; row B = CM from unstressed culture (named CM); row C = CM from pH-oxidative stressed culture (named CM-pHOx); row D = CM from hexanal stressed culture (named CM-Ex); row E = CM from oxidative stressed culture (named CM-Ox); row F = CMs from cultures exposed to their own CM (named CM-II).

	50% (v/v) of CM (1-4)	**50% (v/v) of SM (5-8)**	**10% (v/v) of CM (9-10)**	**10% (v/v) of SM (11-12)**
CTRL (A)	2.4 ± 0.3	2.4 ± 0.2	1.8 ± 0.3	1.8 ±0.2
CM (B)	2.2 ± 0.4	2.3 ± 0.2	1.8 ± 0.2	1.8 ± 0.2
CM-pHOx (C)	1.7 ± 0.4	2.3 ± 0.3	1.9 ± 0.3	1.8 ± 0.2
CM-Ex (D)	0.2 ± 0.4	1.3 ± 0.5	1.8 ± 0.2	1.8 ± 0.3
CM-Ox (E)	1.4 ± 0.3	2.1 ± 0.4	1.9 ± 0.3	1.8 ± 0.3
CM-II (F)	2.6 ± 0.2	2.4 ± 0.3	1.8 ± 0.2	1.8 ± 0.2

Table 6- Inhibition effects on violacein production by C. violaceum by different free cell CM from *Enterococcus faecalis* exposed to different stresses on spectrophotometric absorbance (590 nm).

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