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THE ANTIMICROBIAL EFFECT OF RED WINE ON *Bacillus Cereus* IN SIMULATED GASTROINTESTINAL CONDITIONS

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under the supervision of

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Resumo

Diversos estudos têm vindo a descrever uma panóplia de efeitos benéficos na saúde humana, potencialmente atribuíveis ao consumo de vinho, incluindo efeito anti-oxidante, anti-carcinogénico, anti-inflamatório, anti-cardiovascular, assim como propriedades antimicrobianas. Este estudo foi conduzido com o objectivo de avaliar a actividade antimicrobiana do vinho sobre *Bacillus cereus*, células vegetativas e esporos. Os resultados apresentados neste trabalho indicam claramente, via testes *in vitro*, que o vinho inactiva com eficácia as células vegetativas das duas estirpes de *B. cereus* utilizadas. O vinho tinto inactivou as células vegetativas em fase estacionária, atingindo-se números de colónias não detectáveis (< 500 CFU mL^{-1}), em menos de 10 s de exposição. Como tal, os ensaios de inactivação subsequentes foram efectuados com vinho diluído com água (diluição de 1:4 e 1:8). O vinho diluído 1:4 causou uma redução de 4.5 ciclos logarítmicos nas contagens de células viáveis**,** em 20 s de ensaio. No entanto, os esporos de *B. cereus* apresentaram uma elevada resistência à exposição directa ao vinho, com reduções nas contagens inferiores a 1.0 ciclo logarítmico, em 3 h. A influência de componentes do vinho (etanol, ácidos orgânicos, baixo pH e compostos fenólicos) também foi contemplada neste estudo, em células vegetativas. A combinação de ácidos orgânicos e etanol resultou numa actuação sinergética, que provocou padrões de inibição de viabilidade celular similares aos do vinho. Os compostos fenólicos testados não causaram inactivação das células (nas concentrações utilizadas). Relativamente aos resultados obtidos em condições gástricas simuladas, em contexto de refeição simulada, podemos concluir que o consumo de vinho ao longo de uma refeição pode diminuir consideravelmente o número de células de *B. cereus* que poderá persistir no tracto gastrointestinal. O queijo fresco pasteurizado conferiu maior protecção às células do *B. cereus*, quando comparado com a matriz arroz com frango. Nesta investigação também foi avaliado o comportamento de esporos de *B. cereus* quando submetidos a condições gastrointestinais na presença e na ausência de vinho. A presença de vinho inibe a multiplicação das células resultantes da germinação de esporos no fluido intestinal sintético, dando origem a contagens totais (células vegetativas e esporos) de *B. cereus* mais baixas do que na ausência de vinho. Esta tese gerou resultados que indicam que o consumo de vinho durante uma refeição conduz à redução do número de células viáveis de *B. cereus* no tracto gastrointestinal, assim como à diminuição do impacto da eventual germinação de esporos que pode ocorrer no intestino, reduzindo, consequentemente, o risco de infecção que o referido patogénico pode causar.

Abstract

Several studies describe the burgeoning health benefits of red wine consumption, including anti-oxidative, anti-carcinogenic, anti-inflammatory, anti-cardiovascular and antimicrobial properties. This study aimed to evaluate the antimicrobial activity of wine against *Bacillus cereus* vegetative cells and spores. The results of this work clearly show, via *in vitro* tests, that wine exerts a strong inactivation effect against vegetative cells of two *B. cereus* strains. The red wine tested inactivated *B. cereus* stationary phase vegetative cells to undetectable numbers (< 500 CFU mL^{-1}) in less than 10 s. Thus, further inactivation assays were carried out with wine diluted with water (1:4 and 1:8). Wine diluted 1:4 caused a reduction of 4.5 log cycles on viable cell counts, in 20 s. Nevertheless, *B. cereus* spores were found to be highly resistant to the wine exposure, with decreases in the counts lower than 1.0 log cycles, after 3 h. The influence of wine components (ethanol, organic acids, low pH and phenolic compounds) was investigated on vegetative cells. Organic acids, when combined with ethanol, acted synergistically and conduced to a similar inhibition pattern as that of wine. The wine phenolic compounds assayed displayed no activity against the vegetative cells at the concentrations studied. Regarding data obtained in simulated gastric conditions, in a simulated meal context, we can conclude that the ingestion of wine during a meal diminishes considerably the number of *B. cereus* cells persisting in the alimentary tract. Pasteurized fresh cheese was found to be more protective to the cells than the chicken-rice matrix**.** We also evaluated the behavior of *B. cereus* spores under gastrointestinal conditions. In a consumption-like scenario, the treatment SGF (synthetic gastric fluid)-SIF (synthetic intestinal fluid) +Food+Wine, when compared to the system SGF-SIF+Food+Water, led to lower total counts of *B. cereus* in the intestine, showing that wine inhibits the multiplication of the cells obtained from the germination of spores.

This work provides evidence that drinking wine with meals leads to a reduction of the number of viable cells of *B. cereus* and reduces the impact of the germination of spores that may occur in the small intestine, thus lowering the risk of infection the aforementioned pathogen may cause.

Acknowledgements

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I would like to deeply thank my supervisory team of Dr. José António Gomes Couto and Dr. Timothy Alun Hogg, for their outstanding knowledge, encouragement, guidance and constructive advice throughout this thesis which have contributed to the success of this study.

I am grateful to my colleagues for all the help and advice. You have been great colleagues and wonderful friends.

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Contents

Abbreviations

ATCC	American Type Culture Collection
a_w	Water activity
B.	*Bacillus*
bp	Base pair(s)
CFU	Colony Forming Units
DNA	Deoxyribonucleic acid
DPA	Dipicolinic acid
EFSA	European Food Safety Authority
EU	European Union
Fig.	Figure
g	gram
GIT	Gastrointestinal tract
h	Hour(s)
M	(10^6), molar
m	Milli (10^{-3}), meter
Mb	Megabase
MHL	Million Hectolitres
min	Minute(s)
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
rpm	Revolution per minute
s	Second(s)
SGF	Synthetic/Simulated Gastric Fluid
SIF	Synthetic/Simulated Intestinal Fluid
TSA	Tryptone Soy Agar
TSB	Tryptone Soy Broth
UK	United Kingdom
USA	United States of America
vol.	Volume
v/v	Volume/volume
w/v	Mass/volume
µ	Micro (10^{-6})

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1. Introduction

1.1. *B. cereus* taxonomy

1.1.1. *Bacillus* genus

The genus *Bacillus* includes members that demonstrate a wide range of diversity from physiology and ecological niche to DNA sequence and gene regulation. The bacteria in this genus belong to the family *Bacillaceae*, phylum Firmicutes. The *Bacillus* genus comprises species distributed ubiquitously in the environment, being commonly isolated from soil, air, water and dust (Harwood, 1989; Drobniewski, 1993). The bacteria found in this genus are aerobic or facultative anaerobic, Gram positive endospore-forming rod shaped organisms (Harwood, 1989; Drobniewski, 1993). The species important for human health are species belonging mostly to two groups, *B. cereus* and *B. subtilis*, both members of *Bacillus* RNA group 1 (Stackebrandt and Swiderski, 2002).
The vegetative cells range from approximately 0.5 by 1.2 to 2.5 by 10 µm in length and most *Bacillus* species grow optimally at temperatures from 25 °C to 37 °C. Several thermophilic and psychrophilic species exists which can grow at temperature up to 75 °C and down to 3 °C, respectively (Drobniewski, 1993). Many bacilli produce extracellular hydrolytic enzymes capable of breaking down polymers such as polysaccharides, proteins or peptides and nucleic acids allowing the bacteria to use the monomers/oligomers as carbons sources and electron donors (Maidgan, 2003). Some species in the *Bacillus* genus are responsible for the production of several antibiotics, such as bacitracin, polymyxin and cirulin. (Maidgan, 2003) Traditionally they are classified as low GC gram positive bacteria, however their GC level range from 32% to 69% (Drobniewski, 1993; Maidgan, 2003).

1.1.2. *B. cereus* group

The *B. cereus* group, a very homogeneous cluster within the *Bacillus* genus, comprises *B. cereus*, *B.anthracis*, *B. thuringiensis*, *B. weihenstephanensis*, *B. mycoides* and *B. pseudomycoides* (Drobniewski, 1993; Lechner *et al.*, 1998; Nakamura, 1998). These six species are genetically closely related, but with regard to pathogenicity they differ; *B. cereus* – food poisoning, systemic infections; *B. thuringiensis* – insect pathogen and *B.anthracis* – the causative agent of anthrax. Recent data have proposed that *B. cereus sensu stricto*, *B. anthracis*, and *B. thuringiensis* should be considered as one species designated *B. cereus sensu lato* (Helgason *et al.*, 2000; Drobniewski, 1993). Nevertheless, other studies indicate sufficient genetic discrimination between *B. anthracis*, *B. cereus* and *B. thuringiensis* (Keim *et al.*, 1997) and the genetic differentiation of *B. cereus* and *B. thuringiensis* (Cherif *et al.*, 2003). Thus, at the moment there is no consensus as to whether these bacteria should be considered separate species or specialized variants of a single species. Despite these arguments, members of the *B. cereus* group retain their status as separate species due to their varying and distinctive pathogenic features (Priest *et al.*, 2004). In general, species of the *B. cereus* group have a

low G+C content of DNA (35%) (Drobniewski, 1993), hydrolyze lecithin and do not ferment mannitol to acid (Maidgan, 2003). Formerly, these species were classified as distinct because of the great relevance of their phenotypical differences, which formed the basis for their classification. These three species differ in virulence, which is encoded by genes located on plasmids recognized as mobile genetic elements (Van der Auwera *et al*., 2007). These include the *cry* gene encoding δ-endotoxins of *B. thuringiensis*, pXO1 plasmid carrying genes for the anthrax toxin complex and the pXO2 encoding the poly-γ-D-glutamic acid capsule of *B. anthracis* as well as the positive regulator of the virulence factor AtxA located on pXO1 (Arnesen *et al*., 2008). The *B. cereus* emetic toxin genes (*ces*) are also present on a large plasmid (Hoton *et al*., 2005; Rasko *et al*., 2007). *B. anthracis* is capable of capsule formation and the production of toxins that lead to carbuncles in animals and in humans, causing the disease known as anthrax (Mock and Fouet, 2001). The species *B. thuringiensis* is an insect pathogen which produces insecticidal δ-endotoxins during sporulation and is commercially used for crop protection (Drobniewski, 1993). The species *B. mycoides* and *B. pseudomycoides* are phenotypically distinguishable from the species *B. cereus sensu stricto* by their rhizoidal colony shape and whole cell fatty acid composition (Nakamura, 1998). *B. weihenstephanensis* is the psychrotolerant species within the *B. cereus* group, characterized by the ability to grow aerobically at 7 ºC or lower in agitated liquid culture but not at 43 ºC, possessing a signature sequences in the major cold shock gene *cspA* and in the 16 rDNA sequence (Lechner *et al*., 1998). Finally, *B. cereus* lacks those characteristics and can cause food contamination. Later, however, despite these phenotypic differences, comparison of their 16S rRNA nucleotide sequences revealed less than 1% divergence between them (Ash *et al*., 1991), but a cutoff of 3% divergence is recommended as a conservative criterion for demarcating species, supporting the suggestion as a single species. Therefore, the sequences of 16S rDNA and the phenotypical traits are some of the main factors that give rise to the phylogenetic discussions about the classification of these species. The main diagnostic features of *B. cereus sensu lato* are their ability to hydrolyze lecithin and an inability to ferment mannitol.

1.1.3. *B. cereus sensu stricto*

B. cereus is a Gram-positive, motile, spore forming, aerobic rod-shaped bacteria, generally 0.9 to 1.2 µm by 2 to 4 µm and the spore is ellipsoidal, central or paracentral, rarely distending the sporangia (Granum, 1994). It is ubiquitous in nature and is the most frequently isolated soil bacterium, also contaminating agricultural products, playing a major role in spoilage of food products (Kramer and Gilbert, 1989). It may also be present in a diversity of foods such as pasta, rice, dairy products, dried foodstuffs, vegetables, fruit, seafood, meat and poultry (Schoeni and Wong, 2005). The organism has also been identified in cocoa bean fermentations (Ardhana and Fleet, 2003) and cocoa powders (Te Giffel *et al*., 1997; Ardhana and Fleet, 2003). Optimum growth occurs between 28 and 37 °C although the temperature range is between ≤ 5 and 55 °C (Forsythe, 2000). Growth may occur over a wide range of pH, from 4.1–9.3 and at salt concentrations of up to 7.5% (Clavel *et al*., 2004). The minimum water activity (a_w) for growth is 0.93 (Forsythe, 2000). Under optimum conditions generation time for *B. cereus* may be as short as 18–27 min, however at temperatures around 5 °C this increases

substantially (Choma *et al*., 2000). It is also possible for *B. cereus* to adhere to the surface of stainless steel, a material commonly used within the food industry. Once attached, cells may form a biofilm, which confers a number of benefits to the cells with regards to survival in unfavourable conditions. The process of biofilm formation has been described by Wijman *et al*. (2007) and involves attachment of the organism to a surface within a matrix of exopolymeric substances.

As has been previously discussed, *B. cereus* is genetically linked to other organisms in the *B. cereus* group. However, the genetic characteristics, virulence factors and growth/survival characteristics of organisms within the species *B. cereus* can vary considerably (Carlin *et al.,* 2006). Nevertheless, emetic strains appear to share a distinct cluster of characteristics. There is also evidence to link emetic *B. cereus* virulence with other toxin producers in the *B. cereus* group (Ehling-Schulz *et al*., 2006). Carlin *et al*. (2006) demonstrated that when compared to diarrhoeal and non-toxigenic strains, emetic strains were unable to grow below 10 °C, but were able to grow at 48 °C. On average the spores from emetic strains were more heat resistant at 90 °C but showed reduced germination potential, especially at lower temperatures. No differences were observed at growth over the range 24–37 °C or at pH 5, 7 or 8. This confirms the special risk involved from emetic strains in foods which are kept warm after cooking, but perhaps not in refrigerated foods. Ehling-Schulz *et al*. (2006) comment on the close relations between emetic strains in contrast to the diversity discovered among diarrhoeal isolates.

The method for the enumeration of *B. cereus* in foods has been standardized by the International Organization for Standardization (ISO, 2004). The method is based on growth on mannitol egg yolk polymyxin (MYP) agar. Other plating media are commonly used for the isolation, detection and enumeration of *B. cereus* from foods, including PEMBA (polymyxinpyruvate-egg yolk-mannitol-bromthymol blue-agar) (Holbrook and Anderson, 1980; Mossel *et al*., 1967). In addition to selective compounds like polymyxin, these media utilize the bacterium's lecithinase production (egg-yolk reaction giving precipitate zones) and lack of mannitol fermentation. A thorough description of these media is found in Kramer and Gilbert (1989). More recently, chromogenic media have been developed for several food pathogens, including *B. cereus* (for instance Cereus–Ident-Agar from heipha Dr Müller GmbH, and chromogenic *B. cereus* Agar from Oxoid Ltd). *B. cereus* ATCC 14579 is the type strain of *B. cereus* (Fig.1), being an environmental isolate and harboring the three enterotoxins, but not the emetic toxin (Ivanova *et al*., 2003).

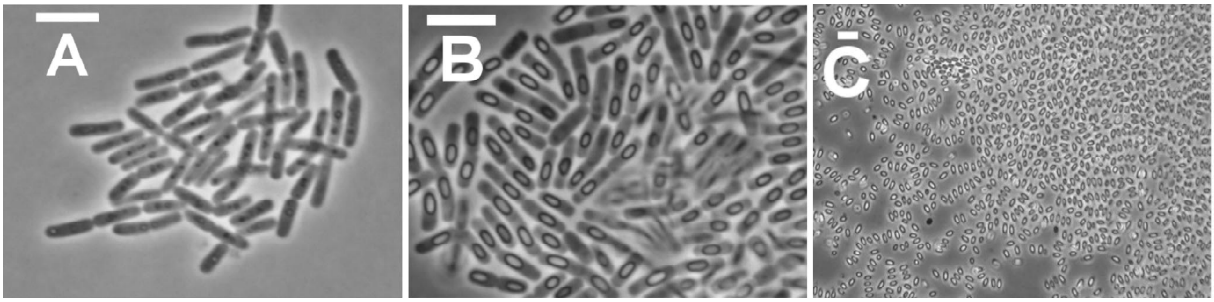

Fig.1. Microscopic images of *B. cereus* cells. Bars, 5 µm .(A) Vegetative cells aggregating in stationary phase; (B) aggregated cells forming spores; (C) spores present in foam. Images adapted from de Vries *et al.* (2004).

1.2. *B. cereus* spores

1.2.1. Bacterial spores general considerations and *B. cereus* spore structure

Bacterial spores are described as the ultimate survival vehicles, and have astonished scientists for over a century. Spore-formation is a very successful survival and dispersal strategy: spores are encountered virtually everywhere, and may persist for long periods as they are difficult to destroy. Considering these characteristics, it is not hard to imagine that spores cause major problems in settings where sterility and hygiene are factors of extreme importance, such as the medicare and food-industry. Consequently, bacterial spores are of prime interest from both fundamental and applied perspectives. Moreover, several spore-formers have pathogenic properties, extending the occurrence of their spores from a hygiene issue to a health issue. In this thesis, such a pathogenic spore-former is introduced in a food context: *B. cereus*.

Bacterial spores are highly specialized, differentiated cell types, designed for the survival of adverse conditions. They are formed inside the bacterial cell and hence called endospores. All *Bacillus* species can form heat stable endospores (Harwood, 1989). The bacterial endospore is a resting, dormant, tough, non reproductive structure and it is the most resistant living structure known (Atrih and Foster, 1999). Endospores formed by *Bacillus* and related aerobic endospore-forming Firmicutes are a strategy to survive during unfavourable conditions. The structures of the mature spore are: 1. The core which is the analog of the vegetative cell protoplast as it contains DNA, ribosomes, tRNA and a high concentration of dipicolinic acid (DPA) and of Ca^{+2} (Setlow, 2006). It contains only 25–50% of water, i.e. the content of free water is extremely low such that the macromolecular movement is greatly restricted (Cowan *et al*., 2003). 2. The germ cell wall is composed of peptidoglycan identical to that of the vegetative cell (Setlow, 2006). 3. The cortex consists of a peptidoglycan which is different from the vegetative cell peptidoglycan (Atrih and Foster, 1999). The cortex is essential for the formation of a dormant spore and for the reduction of its water content (Andersson, 1998).4. The spore coat complex consisting of several layers of different proteins, mostly spore-specific and important for resistance towards chemicals and lytic enzymes (Setlow, 2006). 5. The exosporium which is a loose fitting balloon-like structure (Yan *et al*., 2007). The *B. cereus* exosporium contains more than 20 proteins

(Todd *et al*., 2003), amino and neutral polysaccharides, lipids and ash (Matz *et al*., 1970). Alanine racemase protein is a major component of the exosporium of *B. cereus* spores (Yan *et al*., 2007). It converts reversibly L-alanine to D-alanine (Todd *et al*., 2003). The structures of the spores of the *B. cereus* emetic strain F4810/72 are displayed in Fig. 2. The exosporium of the *Bacillus* spores is surrounded by a hair like external protein layer. It has been a suggested that these proteins are tightly absorbed on spore surface after the cell lyses or are included between the coat and the exosporium (Todd *et al*., 2003). *B. cereus* spores are covered with appendages not present in many other *Bacillus* species (Arnesen *et al.,* 2008). The appendages consist of protein as the main part together with a small amount of carbohydrate and lipid (Arnesen *et al.,* 2008). There was a large difference in the protein profiles of the appendages of different strains of *B. cereus* (Granum, 2007) and a variation in the surface characteristics of *B. cereus* spores between strains (Tauveron *et al*., 2006).

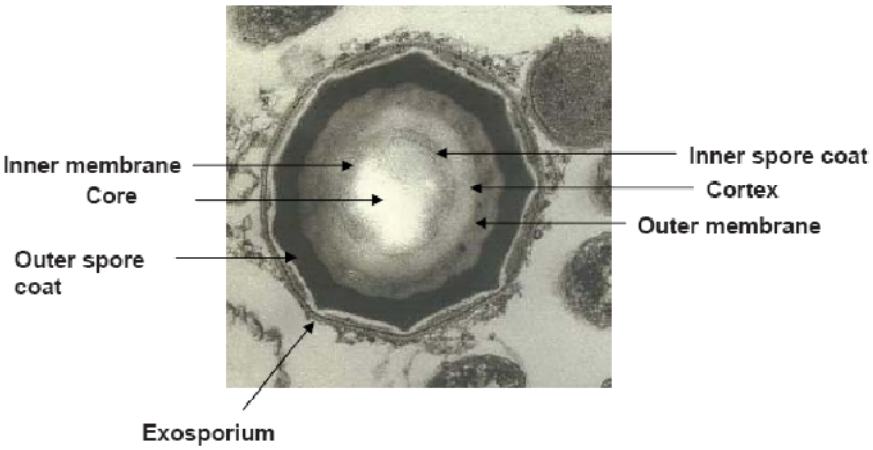

Fig. 2. Transmission electron micrograph of a sporulating culture of the emetic *B. cereus* strain F4810/72. Adapted from Shaheen (2009).

1.2.2. *B. cereus* spore properties

Diverse properties reported for the spores of *B. cereus* make them a problem for the food industry. *B. cereus* spores are highly resistant to adverse conditions such as heat, dehydration, desiccation, starvation, ionizing radiation, mechanical abrasion, hydrolytic enzymes, extreme pH values, antibiotics, disinfectants and cleaning agents (Setlow, 2000)**.** The spores of *B. cereus* are hydrophobic and adhere to the processing equipment and subsequently form biofilm (Andersson *et al*., 1995; Peng *et al*., 2002). The resistance properties reported for the spores of *B. cereus* are also a problem for human health. *B. cereus* spores are highly resistant to acidity in a range of media simulating the conditions in the human stomach after food ingestion. The decrease in the spore counts was less than 1.5 log CFU mL^{-1} after 6 h of incubation at pH 1 and 1.5 (Clavel *et al*., 2004). The conditions prevailing during the sporulation: the temperature (Gonzales *et al*., 1999) and the composition of the sporulation medium (de Vries *et al*., 2004) affect the properties of the formed spores. The heat resistance of the *B. cereus* spores increases with the increase in sporulation temperature (Gonzalez *et al*., 1999). *B. cereus* spores showed higher survival at 90 ºC when the spores were produced at 37 ºC as compared

to 15-20 ºC (Gounina-Allouane *et al*., 2008). Spores of *B. cereus* have extreme metabolic dormancy with respiratory activity of low as 10^{-4} of the maximum rate for vegetative cells metabolizing substrate (Andersson, 1998). Several components are important for the resistance properties of the spores. Dipicolinic acid (pyridine-2,6-dicarboxylic acid) is responsible for the reduction of the spore core water content during sporulation and for the UV photochemistry of the spore DNA. This molecule comprises ~5 to 20% of the dry weight of *Bacillus* spores. It is chelated with divalent cations, mainly Ca^{+2} (Setlow, 2007). The small acid-soluble proteins (SASP) in the spore core play an important role of spore resistance. SASP proteins (α, β) represent 5-10% of the total core protein which is sufficient to saturate and protect the spore DNA (Setlow, 2007) especially against UV radiation. SASP also play a role in the osmoresistance of spores (Tovar-Rojo *et al*., 2003).

1.2.3. Spore formation and germination of *B. cereus* spores

The spore formation, also called sporulation, involves asymmetric cell division with a copy of the genome partitioned into each of the sister cells. The smaller cell develops into the mature endospore and the mother cell contributes to the differentiation process of the endospore and then autolyses releasing the mature spore into the environment (Henriques and Moran, 2007). It takes approximately 6 h for the process of spore formation of *B. cereus* to complete (Henriques and Moran, 2007). Spore germination involves a series of rapid degradative reactions, leading to dismantlement of the unique spore structure and loss of spore dormancy and resistance. The subsequent steps that lead to cell-enlargement and cell-division are termed outgrowth, which is considered a separate process, distinct from germination (Campbell and Leon, 1958). Germination can be enhanced by several treatments, including heat-treatments, time, and certain chemicals (Keynan and Evenchick, 1969). This enhancement is called activation (Foster and Johnstone, 1990), and the underlying mechanisms have not been resolved yet. Germination is a non-log-linear event. Some spores form vegetative cells within 2 h, others only after many hours or even days. For 12 *B. cereus* strains tested, 2 out of the 10 strains did not germinate and the maximum spore germination was obtained after 100 min with no additional germination was observed up to 160-200 min (Broussolle *et al*., 2008). Germination occurs without need for synthesising any new macromolecules and all the needs are present in the mature dormant spores (Moir, 2006). In the process of germination, substances acting as germinants permeate the outer coat and cortex layers of the spores and interact with receptors located in the inner spore membrane (Moir, 2006). Then compounds such as monovalent cations (H^+, Na^+, K^+), divalent cations (Ca^{+2}, Mg^{+2}, Mn^{+2}) and DPA are released from the spore core (Moir, 2006). The germ cell wall becomes the bacterial cell wall when the spore germinates. The release of Ca-DPA triggers the hydrolysis of the spores peptidoglycan cortex by activating the cortex lytic enzymes (Moir, 2006). Hydrolysis of the peptidoglycan is required for germination and outgrowth of the spores (Atrih and Foster, 1999). The spore core rapidly takes up water so that the core water content rises to that in the protoplast of growing cells and the macromolecular motion and enzyme activity in the core are restored. In *B. cereus* L-alanine and the purine ribonucleoside inosine are effective germination-promoting compounds (Gounina-Allouance *et al*., 2008) and D-alanine is an effective inhibitor of L-

alanine-induced germination. The most rapid germination of *B. cereus* spores was observed in a mixture of 0.1 mM L-alanine and 0.1 mM inosine. *B. cereus* spores failed to germinate in minimum salts medium with glucose plus yeast extract in 0.1 mM inosine (Warren and Gould, 1968). Other germinants have also been identified, like L-phenylalanine, L-glutamine, a mixture of L-asparagine, glucose, fructose and K^+. Some mammalian cells like Caco-2 cells were reported to induce germination of enterotoxigenic *B. cereus* spores whereas HEp-2 cells did not trigger germination (Wijnands *et al*., 2007). A number of germination receptors are present in the spores of *B. cereus*. *B. cereus* type strain ATCC 14579 spore may contain seven functional receptors (Hoornstra *et al*., 2006). *ger*P-encoded protein of *B. cereus* is believed to be important in establishing a coat that is permeable to germinants. Variability of response to inosine or to L-alanine was observed between spores of *B. cereus.* Some strains can germinate at low germinant concentration (i.e. 0.05 mmol L^{-1}) (Broussolle *et al*., 2008). Mild preheating activates spores to germinate, in the presence of germination permissive environment. Optimal heating temperature depends on the sporulation temperature. *B. cereus* spores that were formed at 37 ºC require 80-90 ºC heat shock for activation, whereas those formed at room temperature, need only heat shock of 70-75 ºC (Becker *et al*., 2005). Gamma-radiation, reducing agents such as thioglycolate or mercaptoethanol and oxidizing agents also may activate the spores (Andersson, 1998).

1.2.4. Using *B. cereus* as a probiotic

Probiotics are live microbial feed supplements which beneficially affect the host by improving its intestinal microbial balance. The potential benefits that are claimed include improved efficiency of feed, protection against infectious disease (Duc *et al.* 2004; Fuller, 1989), enhancement of the host immune responses or inhibition of tumor growth in animal models (Spinosa *et al*., 2000). Among the bacteria used as probiotics, bacteria belonging to the genus *Bacillus* represent a peculiar situation. Unlike other bacteria, *B. subtilis*, *B. cereus*, *B. clausii* (Spinosa *et al*., 2000) and *B. coagulans* are given orally as spores, not as vegetative form *Bacillus*, even if they are suspected (for example in the case of *B. natto)*. The following three basic mechanisms have been proposed for how orally ingested nonindigenous bacteria can have a probiotic effect in a host: (i) immunomodulation (that is, stimulation of the GALT-gut-associated lymphoid tissue) (e.g., induction of cytokines), (ii) competitive exclusion of gastrointestinal pathogens (e.g., competition for adhesion sites), and (iii) secretion of antimicrobial compounds which suppress the growth of harmful bacteria (Duc *et al.,* 2004). Since the mammalian intestine is an anaerobic environment and *Bacillus* spp. are preferentially aerobic, germination and outgrowth of spores in the intestine seem difficult to attain. Moreover, the capacity to survive the lytic action of bile salts, one of the criteria used to select potentially probiotic strains, is highly uncommon in nonenteric microorganisms (Spinosa *et al.,* 2000). Although recent evidence suggests that *Bacillus* spores do germinate in the gastrointestinal tract, it remains unclear which form, cell, spores, or both, is actually responsible for the competitive exclusion and probiotic effects. *B. cereus* belongs to the few species of *Bacillus* genus that are used as probiotics (Barbosa *et al.,* 2005). Probiotic *B. cereus* are

used as animal feed supplements (Schierack *et al*., 2009), for aquaculture (Ravi *et al*., 2007) and for human (Duc *et al*., 2004).

1.3. *B. cereus* ecological niches and food spoilage

B. cereus has a ubiquitous presence in nature and can be isolated from a wide variety of environmental samples (Granum, 2007; Kramer and Gilbert, 1989). It can be found in many types of soils, sediments, dust and plants (Kramer and Gilbert, 1989; von Stetten *et al*., 1999; Schoeni and Wong, 2005). Spores may be passively spread and thus found also outside natural habitats. It is believed that *B. cereus sensu lato* exists in soil as spores, and germinates and grows when brought in contact with organic matter or an insect or animal host. Interest in the ecology of this bacterium spurred a study showing that *B. cereus* could germinate, grow and sporulate in soil, thus demonstrating a saprophytic life cycle (Vilain *et al*., 2006). Furthermore, a multicellular phenotype with a filamentous mode of growth was observed and suggested to be a means of translocation through soil (Vilain *et al*., 2006). A multicellular, filamentous mode of growth has also been observed in the gut of insects. The intestines of insects were suggested as a habitat for *B. cereus* when sporeforming bacteria, later identified as *B. cereus*, were isolated from guts of different soil-dwelling arthropod species, where the bacteria appear to exist in symbiosis with their invertebrate host. The role of the insect gut microbial communities as a natural niche for part of the *B. cereus* life cycle is further discussed by Jensen *et al*. (2003), and it is also suggested that the existence of different morphological modes used by *B. cereus*, such as the filamentous mode, may be adaptations to different life cycles like the 'normal' cycle of life as a symbiont or the more infrequent pathogenic life cycle with rapid growth. *B. cereus* has been reported to be present in stools of healthy humans at varying levels (Kramer and Gilbert, 1989; Jensen *et al*., 2003). Its ubiquitous low level presence in environments, feed and foods would ensure *B. cereus* a transient presence in the mammalian gut (Kramer and Gilbert, 1989). However, genomic data from the *B. cereus* type strain ATCC 14579 and from *B. anthracis* suggested that their metabolic capacity is more adapted to the use of proteins as a nutrient source than carbohydrates, and furthermore that genes for establishment within a host were conserved (Ivanova *et al*., 2003). Another nuance to the scenario is a recent genomic and phenotypic comparison between *B. cereus* strains ATCC 14579 and ATCC 10987 which revealed that ATCC 14579 actually has the capacity to metabolize a larger number of carbohydrates than what was initially believed based on genomic analysis alone (Mols *et al*., 2007). These data suggest that in addition to a full life cycle in soil, where it is richly present, *B. cereus* is also adapted to a lifestyle in a host, as a pathogen or perhaps as a part of intestinal flora, as well as to growth in foods. The possible adaptation of *B. cereus* to the environment of the animal gut could be the basis of their proposed probiotic effect. Being present in so many environments, it is expected that *B. cereus* should also be found in water; however, there are not many data on the presence of *B. cereus* in water sources, and standard methods for the detection from water are not available. Norwegian surface waters were investigated for presence of *B. cereus* spores, and cytotoxic strains were isolated from several rivers (Østensvik *et*

al., 2004). This suggests the possibility that the water supply may be a means by which *B. cereus* enters the food processing chain.

Growth of unwanted bacteria can cause enormous expenses for food industry, as this may lead to food spoilage. *B. cereus* can be isolated from a wide range of different foods and food ingredients, including rice, dairy products, spices, dried foods and vegetables (Kramer and Gilbert, 1989). Cross-contamination can distribute spores or cells to other foods, such as meat products (Gilbert and Kramer, 1986; Granum, 2007). At harvest, *B. cereus* cells or spores may accompany plant material into food production areas and establish on food-processing equipment. Food spoilage caused by *B. cereus* occurs mainly in dairy industry, thereby for instance shortening the shelf-life of milk. *B. cereus* is present in soil, on cattle feed and in cattle faeces and is thus ubiquitously present in the dairy farm environment. From these sources raw milk can be easily contaminated with *B. cereus*, as its spores germinate more easily in milk than spores from other bacilli (Wilkinson and Davies, 1973). Spores and vegetative *B. cereus* cells present in food products can attach to processing equipment and form biofilms. Biofilms are multicellular complexes embedded in a matrix of exopolysaccharides that grow attached to a surface. Cells embedded in a biofilm are more resistant to cleaning agents and other anti-microbial substances, making them difficult to eradicate from processing equipment (Peng *et al.*, 2002). Biofilms in processing equipment are a continuous source of contamination for food products by detachment of cells and spores from the biofilm. Biofilm formation may also cause economic losses by causing equipment failure (Kumar and Prasad, 2006). Modern large-scale food production technology, with extended use of refrigeration as a means of conservation, has created a cold niche well suited for bacteria that are not very competitive, but that can survive heat treatment and also grow at low temperatures. In addition to dairy products, lightly heat-treated foods with extended refrigerated storage also represent a new and favourable environment for *B. cereus* group species. Considering the ubiquitous presence of *B. cereus*, its resilient spores, and the non-fastidious nature of this microorganism, no type of food with pH > 4.8 (Gilbert and Kramer, 1986) can be excluded as a possible vehicle or as representing a risk of food spoilage or food-borne disease. Failure by consumers to follow basic food preparation rules, i.e. slow or inadequate cooling, storage at ambient temperature or prolonged heat-keeping at < 60 ºC, may allow growth of *B. cereus* and it happens often in cases of food-borne disease. *B. cereus* strains isolated from food spoilage incidents generally do not produce cereulide. In contrast, enterotoxin producing strains are commonly isolated from food. Recently, however two psychrotrophic strains were also shown to produce cereulide (Thorsen *et al.*, 2006). Therefore, advanced knowledge is needed about *B. cereus* diversity, behavior and pathogenic capacity in order to allow for better control of this pathogen in foods and in food production environments.

1.4. *B. cereus* human infections

B. cereus and related species can cause two forms of food poisoning, in addition to several forms of nongastrointestinal disease.

1.4.1. *B. cereus* nongastrointestinal infections

B. cereus can give rise to a number of local and systemic clinical infections. *B. cereus* spores can be found within hospital environments and as such may contaminate dressings, intravenous catheters and linens (Drobniewski, 1993). Local infections from *B. cereus* may develop in post surgical situations, traumatic wounds, burns and also in the eye. Ocular infections may take the form of keratitis, endophthalmitis and panophthalmitis (Gigantelli *et al.*, 1991). It is usually the case that such infections will arise in immunocompromised patients and also in those engaged in intravenous drug use via contamination of the drug or injection equipment. However, the introduction of a foreign body into the eye and contaminated contact lens solutions has also been implicated in infections (Pinna *et al.*, 2001). Systemic disease may take the form of bacteraemia or septicaemia, bacterial endocarditis, respiratory or central nervous system infections. Drobniewski (1993) provides a thorough review of reported cases, which includes incidences of meningitis, pneumonia and endocarditis. It has been speculated that bacterial toxins and enzymes are the key virulence factors in *B. cereus* infections. Beecher *et al.* (1995a) concluded that ocular virulence was multifactoral and that the toxin hemolysin BL was only partly responsible for ocular infections.

1.4.2. *B. cereus* gastrointestinal infections

1.4.2.1. *B. cereus* gastrointestinal infections: Outbreak reports

Two distinct food-borne disease types, emetic and diarrhoeal, are associated with *B. cereus*. Both are generally mild and self-limiting, although more serious and even lethal cases have occurred (Granum, 1994; Lund *et al*., 2000).

B. cereus is an important cause of food-borne disease worldwide (Clavel *et al*., 2007; Granum, 2007), although it is probably highly under-reported in official lists of food-borne disease causes. In the European Union, *Bacillus* species (including non-cereus) were reported to be responsible for 1.4% of food-borne outbreaks in 2005 (Anonymous, 2006). In the years 1992–2006, 45 outbreaks of gastroenteritis attributed to *Bacillus* spp. in England and Wales were reported to the Health Protection Agency Centre for Infections (www.hpa.org.uk/infections/topics_az/bacillus/fp/fpdata.htm). Between 1993 and 1998 in the Netherlands, *B. cereus* accounted for 12% of food-borne disease outbreaks where a causative agent was identified (Schmidt, 2001). Several factors contribute to the number of food-borne *B. cereus* disease being largely under-reported. It is a consequence of the generally short and mild course of disease, which does not motivate the patient to seek medical attention. Furthermore, when diagnosed, the disease is not reportable. In addition, cases and/or outbreaks may not always be attributed to *B. cereus*, because the symptoms of the emetic disease are not easily distinguished from those caused by *S. aureus* intoxication, and the *B. cereus* diarrhoeal disease shows the same symptoms as *C. perfringens* type A food poisoning. The number of cases of *B. cereus* food-borne disease is reportedly increasing in industrialized countries (Gilbert and Kramer, 1986; Kotiranta *et al*., 2000). However, as the surveillance systems for food-borne disease differ

between countries, it is difficult to compare data and obtain true incidence estimates. Examples of cases and outbreaks are well described in several publications (Gilbert and Kramer, 1986; Kramer and Gilbert, 1989; Granum, 2007). Somewhat different distribution between countries is observed for the emetic and diarrhoeal diseases, which could partly be a reflection of the association of the two types of disease with different food vehicles: in Japan and the UK, the emetic disease dominates (Gilbert and Kramer, 1986; Shinagawa *et al*., 1995), while in Northern Europe and North America, the diarrhoeal disease seems more prevalent (Kotiranta *et al*., 2000). At least part of the difference in disease pattern is probably due to different eating habits, but it is difficult to document whether the distribution is truly different and not a result of reporting differences.

1.4.2.2. *B. cereus* gastrointestinal infections: Emetic food-borne disease and detection of cereulide

The emetic food poisoning was identified in the UK in the 1970s and was frequently associated with the consumption of cooked rice dishes (McElroy *et al*., 1999). The emetic form of illness is characterised by nausea, vomiting, abdominal cramping and malaise (Granum, 1997). Symptoms may appear between 0.5 and 6 hours after consumption of the contaminated food (Arnesen *et al*., 2008). The illness is usually self limiting, with recovery occurring within 24 h. The rapid onset of the symptoms is due to the ingestion of a preformed toxin, cereulide. Cereulide is a small, cyclic, heat stable dodecadepsipeptide, identified and named by Agata *et al*. (1995). Ehling-Schulz *et al.* (2005) hypothesised that its chemical structure was (D-*O*-Leu-D-Ala-L-*O*-Val-L-Val)$_3$ and demonstrated that it was in fact produced by a nonribosomal peptide synthetase (NRPS). This hypothesis was based on the characteristics of the toxin and its synthesis in the late exponential and stationary phase of the organism's growth (Häggblom *et al*., 2002). Ehling-Schulz *et al*. (2006) have confirmed the chemical structure as above and further sequenced the cereulide synthetase (*ces*) gene cluster and placed it on a 208 kb megaplasmid (now named pCER270 – Rasko *et al.*, 2007). This has many similarities to the pXO1-like plasmids implicated in *B. anthracis* toxin production (Hoton *et al*., 2005). This similarity provides further evidence of the close relationship between the various members of the *B. cereus* group. The genetic regions flanking the *ces* gene also have a high homology to the virulence plasmids of other *B. cereus*, *anthracis* and *thuringiensis*. There is also evidence to suggest that the ability to produce emetic toxin is restricted to a single evolutionary lineage, owing to very low diversity among emetic isolates (Ehling-Schulz *et al.,* 2005). In contrast, considerable diversity has been found among diarrhoeal isolated and other non-emetic strains. Cereulide acts as a K^+ ionophore through mitochondrial membranes and interferes with oxidative phosphorylation. The effects on mitochondria have been demonstrated on boar spermatozoa and rat liver cells, with a stimulation of swelling and respiration of the mitochondria observed (Mikkola *et al.,* 1999). Mahler *et al*. (1997) report on the death of a boy from liver failure associated with impaired function of liver mitochondria due to food-borne cereulide. A similar case has also been reported more recently by Dierick *et al*. (2005), in which a 7 year old girl died from (among other symptoms) liver failure, 13 h after consuming a pasta dish containing > 10^8 CFU g^{-1} *B. cereus*. Paananen *et al*. (2002) have also demonstrated that cereulide can

inhibit human natural killer (NK) and killer T cells and suggest that it may have immunomodulating properties. For illness to occur the *B. cereus* must be able to produce the toxin within the contaminated food prior to consumption. Kramer and Gilbert (1989) have reviewed earlier studies of the levels of *B. cereus* present in foods causing both forms of illness. A total of 107 cases of emetic poisoning were identified with the number of the organism present in the food ranging from 10^3–10^{10} CFU g^{-1}. Granum (1997) found counts between 200 and 10^9 CFU g^{-1} in incriminated foods and concluded that although foods with a level of > 10^3 CFU g^{-1} could not be considered safe. The real infective dose was above 10^5 CFU g^{-1}. While this level is generally accepted, Häggblom *et al*. (2002) warn that food poisoning risk cannot be evaluated on microbial load alone and that toxin production should also be measured. Many foodstuffs have been implicated in emetic food poisoning cases including beef, poultry, pasta, infant formula, milk and cream (Granum and Lund, 1997; Schoeni and Wong, 2005). By far the most common cause of the disease, however, is cooked rice dishes. Kramer and Gilbert (1989) review a number of studies, which found between 10 and 100% of raw and cooked rice samples to be contaminated with *B. cereus*. Cooked rice provides a model for the formation of cereulide within foods. *B. cereus* spores, present in the rice, are heat activated during the cooking or cooling process and can multiply and produce toxin if the rice is held at room temperature. Finlay *et al*. (2002) found that toxin was produced by emetic *B. cereus* in cooked rice at 15–30 °C. Toxin production was significantly greater between 15 °C than 20 or 30 °C. Re-heating of the rice is usually insufficient to inactivate the toxin. Pasta has also been implemented and Rajkovic *et al*. (2006) found that penne pasta and potato puree provided a better substrate for cereulide production than rice when incubated at 28 °C. At lower temperatures background flora was found to be a determining factor, aeration and agitation were also found to reduce toxin production. Finlay *et al*. (2000), in addition to their work on cereulide production in cooked rice, have shown that toxin production in skimmed milk is significantly greater at 12 and 15 °C than at 30 °C. This was in agreement with their later study (2002) which showed toxin production on solid laboratory media was greatest at 12 °C. However, Häggblom *et al*. (2002) suggest that 21 °C was the optimum temperature for toxin production. Agata *et al*. (2002) also found that toxin production in cooked rice was greatest at the higher temperature of 35 °C. Toxin was also produced at 20 and 30 °C, but not in such quantities. All studies largely agree that the minimum and maximum limits for growth and toxin production are 10 and 37 °C respectively (Finlay *et al*., 2000). Although *B. cereus* is frequently isolated from soil, agricultural products and milk, Altayar and Sutherland (2005) have found that emetic isolates are rare. Of the 271 isolates from soil, animal faeces, raw and processed vegetables 45.8% were found to be *B. cereus*. Of these isolates only 4 were found to produce emetic toxin. This is in contrast to other research which showed that 44% of *B. cereus* isolated from rice paddies in Bangladesh were emetic toxin producing isolates (Ueda and Kuwabara, 1993), suggesting that rice paddies provide a selective environment for emetic isolates. Svensson *et al*. (2006) examined 5668 isolates obtained from dairies and dairy farms. No emetic strains were found in milk at the farm during the cow's outdoor grazing period. Up to 3.8% of milk and environment isolates were found to be emetic while the cow's grazed in indoor stalls. In total, 0.05% of isolates from dairies were found to produce emetic toxin although there was evidence for 1 silo in particular having a significant emetic flora.

Rhesus monkey was used as the experimental animal to demonstrate that the heat stable toxin (cereulide) produced by *B. cereus* isolates from emetic outbreaks was associated with the emetic syndrome (Melling *et al*., 1976). The monkey feeding test and ligated rabbit ileal loop (LRIL) methods were used with success to show that the factors responsible for the vomiting and the diarrhoeal illnesses were distinct. The LRIL and the vascular permeability reaction (VPR) methods were found to be of value for studying the diarrhoeal toxin but not the vomiting factor (Turnbull *et al*., 1979). A quantitative chemical assay was introduced by Häggblom *et al*. (2002) based on liquid chromatography followed by ion trap mass spectrometry (HPLCMS). This method allows measuring the exact contents of the molecule cereulide in the *B. cereus* biomass as well as in food or samples from environmental origins. A rapid sperm bioassay was developed in 2004. It allows detecting the toxicity of *B. cereus* bacterial extract in a short period of time (Andersson *et al*., 2004). Research on the emetic toxin was hampered by the fact that rodents are insensitive to the orally given toxin (Yokoyama *et al*., 1999). Therefore primates were needed for each test until Hughes *et al*. (1988) developed an in vitro assay based on the vacuolisation of the human larynx carcinoma cells (HEp-2 cells). These authors tested samples connected to food poisoning and cultured isolates connected to food poisoning in rice. They noticed that the extracts obtained from some isolates caused vacuoles in the HEp-2 cells. Sakurai *et al*. 1994 observed that the vacuoles formed in the HEp-2 cells were swollen mitochondria. Agata *et al*. (1995) extracted and purified the factor which causes the vacuolation in HEp-2 cells from the culture supernatant of *B. cereus* strain NC7401 connected to a case of emetic syndrome food poisoning and named the toxin cereulide. Shinagawa *et al*. (1995) and Agata *et al*. (1995) found that the factor causing vacuolation of HEp-2 cells also caused vomiting when fed to rhesus monkey (*Macaca mulatta*) and the house musk shrew (*Suncus murinus*) and concluded that this was the emetic toxin. Cereulide was chemically synthesized by Isobe *et al*. (1995) and shown to possess the same emetic and pathogenic activities. Andersson *et al*. (1998) developed a bioassay based on loss of the motility of boar spermatozoa upon 24 h exposure to the toxin. Finlay *et al*. (1999) developed the metabolic staining assay MTT using as an indicator 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a water soluble yellow tetrazolium salt. This salt was converted to an insoluble purple formazan (MTT) in HEp-2 cells but not in cells exposed to the emetic toxin. This allowed detecting the cytotoxicity of cereulide towards the HEp-2 cells.

1.4.2.3. *B. cereus* gastrointestinal infections: Diarrhoeal food-borne disease and detection of cytotoxins

Perhaps the first description of the diarrhoeal form of the illness comes from Hauge (1950, 1955) in 1950s. The latter study covers an outbreak of diarrhoeal illness, involving around 600 patients in 4 Norwegian hospitals. Hauge (1955) found that cornstarch used in the preparation of a vanilla sauce contained 10^4 *B. cereus* spores g^{-1}. Incubation of sterile vanilla sauce with a solution containing approximately 10^4 cells g^{-1} of the *B. cereus* isolate led to the presence of 10^7 cells mL^{-1} in the sauce. After consumption of a portion of the sauce, diarrhoea and abdominal pain lasting 8 h were reported. The mechanism of infection differs from the emetic illness. In the case of diarrhoeal food poisoning

bacterial spores and/or vegetative cells are consumed, which survive the acidic conditions within the stomach and germinate within the small intestine (Wijnands *et al*., 2006). It is here that enterotoxins are produced, causing illness (Drobniewski, 1993). Symptoms can include diarrhoea, abdominal pain and rectal tenesmus, with nausea, vomiting and fever less frequently reported (Hauge, 1955; Granum and Lund, 1997). The illness has an incubation period of between 8 and 16 h and symptoms may last for 12 to 24 h. Many foods have been implicated with the diarrhoeal syndrome including cooked and raw meats, poultry, fish, vegetables, dairy products, desserts and sauces (Schoeni and Wong, 2005). It appears that *B. cereus* is able to produce a number of different enterotoxins capable of causing the diarrhoeal syndrome, although identification of the toxins themselves attracts some debate. The first toxin to be discovered was a three component haemolysin–HBL (Beecher and Macmillan, 1990). In addition to haemolytic activity, this toxin was found to be dermonecrotic, cause fluid accumulation in ligated rabbit illial loops (Beecher *et al*., 1995b) and be cytotoxic to Chinese hamster ovary cells. HBL has been suggested as the primary virulence factor in the diarrhoeal syndrome (Granum and Lund, 1997) although this has been frequently questioned due to the wider presence of NHE genes among *B. cereus* isolates (Moravek *et al*., 2006). A non-haemolytic, three protein enterotoxin has also been identified – NHE (Lund and Granum, 1996). The 3 components of NHE (Nhe A, B and C) show some similarities to those of HBL and each is required in specific concentration (in a ratio of 10:10:1) for cytotoxic activity (Lindbäck, *et al*. 2004). NheB has been found to be the binding component for the enterotoxin complex. Other possible enterotoxins such as Cytotoxins CytK-1 and CytK-2 (Lund *et al*., 2000), Enterotoxin T (Guinebretière *et al*., 2002) and Enterotoxin FM (Moravek *et al.,* 2006) have been suggested as virulence factors for the illness, however, further research is needed. In addition, several other haemolysins and enzymes have been described as potential contributors to the diarrhoeal disease (Arnesen *et al*., 2008). It has been demonstrated that preformed enterotoxins are not able to pass through the human digestive system and that live cells may not survive transit through the stomach (Shinagawa *et al*., 1991). However, Clavel *et al*. (2004) have shown that vegetative cells may pass through the stomach if the pH is raised sufficiently by the presence of foodstuffs. The degree of survival was found to depend on the pH and the type of food consumed. In extreme conditions (pH > 5.0) *B. cereus* may even be able to grow during gastric transit (Clavel *et al*., 2004). Wijnands *et al*. (2009) comment that up to 26% of vegetative cells ingested may survive the gastric passage under normal conditions. The enterotoxins are thought to be produced during the late exponential phase of growth (Kramer and Gilbert, 1989) so it is necessary for spores to germinate within the human small intestine. Recently, Wijnands *et al*. (2007) demonstrated that germination of *B. cereus* was induced by Caco-2 cells, a human cell line which mimics the epithelial cells of the small intestine. This study also demonstrated that the spores were able to adhere to the intestinal cells at a rate of around 1%. Andersson *et al*. (1998) had previously discussed the possibility of spore adhesion to epithelial cells providing an additional virulence mechanism. Four out of ten strains tested produced spores able to adhere to human epithelial cells. One strain used was involved with an outbreak of diarrhoeal food poisoning where symptoms were more severe and longer lasting than would usually be observed. As with attachment to food and processing equipment surfaces, the hydrophobicity of the spores is a contributing factor in the adhesion mechanism. Minnaard *et al*. (2001) have also discussed the effect

of exocellular factors on human intestinal epithelial cells, including the loss of plasma membrane asymmetry of mitochondrial activity.

B. cereus is expected to be present in different foods and raw materials, and thus detection of the bacterium is not always the main issue for food safety purposes. Instead, ability to detect the possibly harmful strains, or their toxic products, is the highly desired goal. As cereulide and the three cytotoxins Hbl, Nhe and CytK are the main known virulence factors in *B. cereus* food-borne disease, focus has been on their detection. Antibodies have been produced for the three-component toxins Nhe and Hbl (Dietrich *et al*., 1999), and two antibody-based detection kits targeting these toxins are commercially available (Buchanan and Schultz, 1994; Day *et al*., 1994). The BCET-RPLA kit (Oxoid Ltd., UK) is a semi-quantitative assay detecting, by reversed antibody agglutination, the L_2 component of Hbl in foods and in cultures of *B. cereus* (Beecher and Wong, 1994a). The sensitivity of the test is reported to be 2 ng mL^{-1} test extract. The TECRA-BDE kit (Tecra International Pty Ltd., Australia) detects the NheA component of the Nhe toxin by an enzyme-linked immunosorbent assay (ELISA) sandwich test (Beecher and Wong, 1994a). The sensitivity reported by the manufacturer is 41 ng mL^{-1} prepared sample, and the kit is intended for use on foods and environmental samples. Neither of the kits will confirm the presence of biologically active toxin, because only one of each of the three-component toxins is detected. For the third and more recently described toxin CytK, there is at present no commercially available detection kit. For nonspecific detection and characterization of *B. cereus* enterotoxins, different laboratory animal and tissue culture assays have been employed. Among the tests involving live animals are the rabbit ileal loop (RIL) test, performed by injection of *B. cereus* cultures or extracts into ligated rabbit intestinal loops followed by observation of fluid accumulation, the guinea pig skin reaction, and the vascular permeability assay (Kramer and Gilbert, 1989). The use of tissue culture assays for detecting *B. cereus* enterotoxins has been shown to correlate well with results from traditional methods, and represent a convenient alternative for screening purposes (Gilbert and Kramer, 1984; Thompson *et al*., 1984; Shinagawa *et al*., 1991). The cell culture lines used include CHO cells (Buchanan and Schultz, 1994), McCoy cells (Fletcher and Logan, 1999), Caco-2 cells (Hardy *et al*., 2001) and Vero cells (Lund and Granum, 1996; Dietrich *et al*., 1999). For specific detection of the genes encoding the *B. cereus* toxins Hbl, Nhe and CytK, several PCR schemes, including multiplex PCR, have been developed (Mäntynen and Lindström, 1998; Yang *et al.*, 2005). Considering the wide distribution of cytotoxin genes among *B. cereus* strains (Mäntynen and Lindström, 1998; Ehling-Schulz *et al*., 2005), the use of PCR techniques to identify diarrhoeal strains is of little use for practical food safety purposes, because detection of a toxin gene does not reveal the level of toxin production and thus cannot predict the potential pathogenicity of a particular *B. cereus* strain.

1.5. Gastrointestinal barrier

The mammalian gastrointestinal tract (GIT) and its accessory organs form an essential organ system with intriguing biology. It is composed of 11 main organs: the mouth, pharpharynx, oesophagus, stomach, intestine, colon, anus, and accessory organs which are the salivary glands, liver, gall

bladder, and pancreas. In consideration of its main function in digestion and absorption of food, the GIT and its accessory glands secrete a huge volume of secretions that aid in this purpose. The salivary glands, stomach, gall bladder, pancreas, and intestines together secrete 7 L of endogenous secretions daily. Together with an average daily oral intake of 2 L of fluids, the mammalian GIT handles 9 L of fluid, of which 8.8 L (98%) is efficiently absorbed and only 0.2 L is excreted in stool. These secretions include digestive enzymes, immune-related proteins, and specialized factors that are necessary for the proper functioning of the GIT in its role in digestion, absorption as well as protection of the individual against pathogens, since the GIT mucosa is the largest vulnerable surface in contact with the external environment (Tan *et. al.,* 2007). The extremely low pH of the stomach (~pH 1-2) is considered a major defense barrier against food-borne infection (Barmpalia-Davis *et al.*, 2008; Tamplin, 2005). This has been recognized for various bacterial pathogens, such as *Vibrio*, *Salmonella*, and *Campylobacter* spp., where reduced gastric acidity via bicarbonate, gastrectomy, or proton pump inhibitor medication increases human susceptibility to infection (Tamplin, 2005). The degree of bacterial susceptibility to low pH varies among bacterial pathogens, and it has been suggested that pH susceptibility is associated with infectious dose (Tamplin, 2005). The survival of any bacterial pathogen in the stomach cannot be modeled with 100% confidence due to the complex nature of diverse food matrices and individual human physiological conditions. Vegetative cells of bacteria, such as *Shigella flexneri*, are very acid resistant and do not need the protection of food matrix for survival of pH-values simulating the gastric conditions (Wijnands *et al*., 2009). However, it is possible to estimate pathogen behavior under controlled conditions that approximate different types of gastric conditions. During the passage of contaminated food through the stomach, *B. cereus* vegetative cells are more acid-sensitive than spores, and this sensitivity depends on the growth pH and on the type of food (Clavel *et al*., 2004). However, it has been shown that *B. cereus* vegetative cells, like many other bacteria, are able to induce an acid tolerance response (ATR) (O'Hara and Glenn 1994; Jobin *et al*., 2002). These mechanisms of resistance to acid may involve (i) *F*0*F*1 ATPase and/or glutamate decarboxylase, which is implicated in intracellular pH (pHi) homeostasis, (ii) metabolic modifcations and (iii) protein synthesis to protect and/or repair macromolecules (Cotter and Hill, 2003). It has been shown that *B. cereus* TZ415 grown in regulated batch culture is more tolerant to acid shock when cells are cultivated at low pH (pHo). Concerning acid stress, Clavel *et al*. (2004) suggested that the probability of viable *B. cereus* cells reaching the small intestine (where HBL enterotoxin is produced) depends (i) on the form of the cells ingested (vegetative cells or spores), (ii) on the food they have contaminated and (iii) on the pH of the stomach (Clavel *et al*., 2004). Moreover, Giannella *et al.* (1972) consider the bactericidal effect of the gastric juice is also dependent of the buffering of gastric content and the rate of gastric emptying. After experiencing this harsh environment, cells that survive gastric passage and reach the small intestine must withstand the presence of bile and high-osmolarity conditions (Barmpalia-Davis *et al.*, 2008). Thus, food intoxication may be due to toxin production by cells resulting in a germination of spores that have resisted acid stress, or vegetative cells which have adapted and survived the acid stress. Therefore, it is particularly important to characterize the precise behavior of *B. cereus* in a low pH environment.

Adaptation and survival at low pH are important factors in the pathogenicity of vegetative cells, and are of great concern in food safety and health.

1.6. Wine

1.6.1. Biochemical composition of wine and actual global wine consumption demand

Wine is one of the world's most popular alcoholic beverages. It has been produced and consumed throughout history for cultural, economical, social, religious, and, more recently, health reasons (Soleas *et al*., 1997).

The term wine describes a diverse commodity class composed of the yeast fermentation products of the must, or juice, pressed from grapes, the fruit of species *Vitis vinifera*. Wine is a fruit product, but fermentation produces a variety of chemical changes in the must, and so wine is not simply grape juice with ethanol added. Fermentation alters the must by altering the conjugation of organic acids and phenolics, by extraction and formation of copigments and the development of an anaerobic and protective redox potential (German and Walzem, 2000). The taste and mouth-feel sensations of wine are due primarily to the few compounds that occur individually at concentrations > 100 mg L^{-1}. These include water, ethanol, organic acids, sugars, and glycerol. Tannins occur in red wine and rarely in significant amounts in white wines unless maturation in oak cooperage took place. Water is the predominant chemical constituent of grapes and wine and is critical in establishing its fundamental characteristics. It is an essential component in many of the chemical reactions involved in grape growth and juice fermentation and in wine aging. Compounds insoluble or only slightly soluble in water rarely play a significant role in wine (Soleas *et al.,* 1997*)*. Dry wines, where the sugars have been completely fermented, contain small quantities of hexose (on the order of 1 g L^{-1}) (Ribéreau-Gayon *et al.*, 2000). This is mainly fructose, because glucose is preferentially fermented by the great majority of yeasts. Residual sugars in dry wines, generally below 1.5 g L^{-1}, consist mostly of pentoses such as arabinose, rhamnose, and xylose. Their levels may increase slightly during maturation in oak cooperage via the breakdown of glucosides in the wood, as well as from their synthesis and release by yeast cells. Besides water**,** ethanol (ethyl alcohol) is the most plentiful compound in wine. Under standard fermentation conditions, ethanol can accumulate to ~14–15%, but generally ethanol concentrations in wine range between 10–13% (Soleas *et al.,* 1997). The primary factors controlling ethanol production are sugars, temperature, and yeast strain. Ethanol is crucial to the stability, aging, and sensory properties of wine. Together with other alcohols, it slowly reacts with organic acids to produce esters and influences their stability (Soleas *et al.,* 1997). Organic acids make major contributions to the composition, stability and organoleptic qualities of wines (Ribéreau-Gayon *et al*., 2000). In wine, organic acids are divided into two categories: volatile and fixed. The first refers to acids that can be readily removed by distillation, whereas the latter refers to the carboxylic acids. The most common volatile acid in wine is ascetic acid. Quantitatively, carboxylic acids such as tartaric, malic, lactic, succinic, oxalic, fumaric, and citric acids control the pH of wine (Soleas *et al.,* 1997). The pH values of wines range from 2.8 to 4.0. It is surprising to find such low, non-physiological values in a

biological, fermentation medium such as wine (Ribéreau-Gayon *et al.,* 2000). Low pH hinders the development of microorganisms, while increasing the antiseptic fraction of sulfur dioxide (Ribéreau-Gayon *et al.,* 2000). The most abundant phenolic compounds in red wine are phenolic acids, flavonoids, and non-flavonoid phenolic compounds (Soleas *et al.,* 1997). The most common phenolic acids in red wine include cinnamic acids (coumaric acid, ferulic acid, caffeic acid, and chlorogenic acid) and benzoic acids (*p*-hydroxybenzoic acid, protocatechuic acid, vanillic acid, and gallic acid). Flavonoids include flavanols (catechin, epicatechin, and their ester forms), flavonols (quercetin), red and blue anthocyanins, and the polymeric tannins. The most abundant non-flavonoid phenolic compound in red wine is resveratrol (Tagliazucchi *et al.,* 2005). White wine contains more phenolic acids (mainly cinnamic acids) but fewer flavonoids and resveratrol with respect to red wine (Tagliazucchi *et al.,* 2005). The concentration of total phenols ranges from 800 to 4000 mg L^{-1} for red wines and from 200 to 1000 mg L^{-1} for white wines (Ronca *et al.,* 2003; Frankel *et al.,* 1995). Phenolic compounds have bactericide, antioxidant and vitamin properties that apparently protect consumers from cardiovascular disease (Ribéreau-Gayon *et al.,* 2000).

The global viticulture situation in 2009 continues to reel from the impact of the world economic downturn. In a global context of diminishing demand, the overall wine consumption, estimated to be 236.6 MHL, continues to decrease in 2009, with a deficit of 6.8 MHL compared with 2008. The European Union has seen a particularly important decrease of wine consumption, between 2008 and 2009, due to lower demand levels in the main consumer countries (France, -0.9 MHL; Italy, -1.7 MHL; Spain, -1.5 MHL) and importer countries, (Germany, -0.5 MHL/2008; the United Kingdom, -0.8 MHL) in Europe. And so in the USA, a first analysis showed a relatively important decrease estimated to be -0.7 MHL, between 2008 and 2009, i.e. -2.5%. Despite the fact that the New-Zealand consumption maintains a high level and is only slightly eroded, and that the Brazilian consumption reached in 2009 its 2007 levels, significant demand reductions have also been recorded in Argentina (-0.4 MHL) and in South Africa (-0.15 MHL). Wine consumption is expected to decline in 2010/11 primarily to the continued general economic crisis, which continues to affect domestic EU wine consumption, and this situation is not expected to change in the short term. Another important limiting factor is represented by the anti-alcohol campaigns conducted in some countries, primarily France and Italy, where advertising of wine is virtually impossible. In addition, health concerns and concerns over drinking and driving have pushed the local authorities to implement more stringent legislations, which further penalize alcohol beverage consumption (OIV, 2010a, 2010b).

1.6.2. Red wine consumption and its health benefits

Following the “60 Minutes” news broadcast on the “French Paradox” in 1991, it became fashionable in the United States to drink wine with meals. The so-called term “French Paradox” was used to describe the relatively low incidence of cardiovascular disease in the French population when compared to American population, despite a relatively high dietary intake of saturated fats by the French. This fact is potentially attributable to the consumption of red wine. After nearly 20 years, several studies have investigated the fascinating, overwhelmingly positive biological and clinical associations of red wine

consumption with cardiovascular disease and mortality (Lippi *et al.,* 2010; Renaud and Lorgeril, 1992). Since then, various studies suggest that light to moderate wine consumption, corresponding to 1–2 drinks daily (with a meal) for men, 1 drink daily for women and the elderly) produces a kaleidoscope of potentially beneficial effects on human health (Lippi *et al.,* 2010; Goldfinger *et al.,* 2003). Both clinical and experimental evidence suggest that red wine offers greater protection to health (German and Walzem, 2000; Goldfinger *et al.,* 2003). The antioxidant and antiradical properties, particularly of red wine, attributed mainly to a high polyphenol content (the concentration of total phenols ranges from 800 to 4000 mg L^{-1} for red wines and from 200 to 1000 mg L^{-1} for white wines (Frankel *et al.,* 1995; Ronca *et al.,* 2003), appear to protect against the risk of coronary heart disease and cancer. Red wine also possesses anti-carcinogenic, anti-inflammatory and antimicrobial properties (Dolara *et al*., 2005; Just and Daeschel, 2003; Moretro and Daeschel, 2004).

1.6.2.1. Cardiovascular disease prevention

Cardiovascular disease (CVD) is the leading cause of death and disability in the United States and is responsible for 53% of deaths in women and 46% of deaths in men (Goldfinger *et al.,* 2003). Coronary heart disease (CHD) affects 12 million people in the United States of which 1.1 million have a myocardial infarction annually and about one third die. Worldwide, CVD is the foremost cause of death, accounting for 57% of deaths among developing nations, and the second most cause of disability (Goldfinger *et al.,* 2003). A cohort study following 6,051 men and 7,234 women, 30-70 years of age for 10-12 years, showed a significant decreased risk for coronary artery disease among wine drinkers. Compared to non-wine drinkers, people who consumed several glasses of wine per day had a 50% reduced risk of death from all causes. The intake of beer or spirits, however, was not associated with risk reduction (Gronbaek *et al.,* 1995). A recent case-control study conducted in Spain also found that a moderate alcohol intake, particularly from red wine, significantly decreased the risk of heart attacks (Fernandez-Jarne *et al*., 2003). The results showed that moderate wine drinkers had a 32% average decreased risk of cardiovascular disease compared to non-drinkers. A J-shaped curve was observed for the relationship between red wine and cardiovascular disease risk, as a very heavy consumption increased the risk in a linear fashion. The antioxidant properties of red wine phenolic compounds were shown to protect *in vitro* and *in vivo* LDL free radical–mediated oxidation (Frankel *et al.,* 1995). There is evidence that oxidatively modified LDL play a crucial role in atherogenesis (Frankel *et al.,* 1995). Thus, wine polyphenols may be important in preventing cardiovascular disease. Alcohol, particularly red wine, has been shown to increase serum concentrations of HDL “good” cholesterol (Serrano-Martinez *et al.,* 2004). A multitude of studies has been performed both in animals and humans to examine the relationship between alcohol, wine and particularly red wine in relation to cholesterol levels, atherosclerosis, as well as other risks, and protective effects associated with heart disease (Serrano-Martinez *et al.,* 2004). The results from a 3-week study involving pre-menopausal women on the contraceptive pill and post-menopausal women, showed that after wine consumption, the overall HDL “good” cholesterol level was increased in post-menopausal women, while the LDL “bad” cholesterol levels were reduced in pre-menopausal women

as compared with those who received only grape juice (Ivanov *et al.,* 2001). Another 2-week long study involving 20 healthy males found that red wine, but not white wine, significantly increased HDL levels by 26% and increased plasma apolipoprotein A-I levels by 12% (Lavy *et al.,* 1994).
Studies, *in vitro*, have found that red wine, but not white wine, has a beneficial effect on vascular smooth muscle cells, thus, having a protective effect by preventing smooth muscle cell proliferation and consequently slowing the development of atherosclerosis (Rosenkranz *et al*., 2002). Moreover, red wine consumption caused significant decreases in fibrinogen, factor VII, plasma C-reactive protein, and oxidized LDL antibody, while causing significant increases in total plasma antioxidant capacity. All of these factors suggest a protective effect of red wine against cardiovascular disease.

1.6.2.2. Cancer prevention

Although excessive alcohol intake has been shown to increase the risk of various cancers, there is some evidence to suggest that frequent moderate red wine intake may have some cancer preventing effects, due largely to the high level of polyphenols. In recent years, experimental studies have shown that polyphenols from red wine, like resveratrol, quercetin, (+)-catechin and gallic acid, were potential cancer chemopreventive agents (Soleas *et al*., 1997; He *et al.,* 2008). Research indicated that red wine reduced the function of proteins in pancreatic cancer cell membranes that are responsible for pumping chemotherapy out of the cell, making the cells chemo-sensitive and also triggered the production of reactive oxygen species (ROS), which are substances circulating in the human body that have been implicated in a number of diseases (when ROS is increased, cells burn out and die). It also caused apoptosis, which is likely the result of increased ROS and depolarized the mitochondrial membranes, which indicates a decrease in the cell's potential to function (Timothe *et al*., 2007). Most of the available data describe effects in rodents in vivo. Wine polyphenols have been reported to delay neurofibroma-like tumours in transgenic mice and to slow down the process of chemical carcinogenesis in the colon of rats (Dolara *et al.,* 2005).

1.6.2.3. Antimicrobial properties

Although it has often been stated that the United States has the safest food supply in the world, there are approximately 76 million illnesses and 325,000 hospitalizations estimated to occur annually (Mead *et al*., 1999). *Escherichia coli* O157:H7 and non-typhoidal *Salmonella* are the potent food-borne pathogens (Mead *et al.,* 1999). *B. cereus* is an important cause of food-borne disease worldwide (Clavel *et al*., 2007; Granum, 2007), although it is probably highly under-reported in official lists of food-borne disease causes. In the European Union, *Bacillus* species (including non-cereus) were reported to be responsible for 1.4% of food-borne outbreaks in 2005 (Anonymous, 2006). In the years 1992–2006, 45 outbreaks of gastroenteritis attributed to *Bacillus* spp. in England and Wales were reported to the Health Protection Agency Centre for Infections (www.hpa.org.uk/infections/topics_az/bacillus/fp/fpdata.htm). Between 1993 and 1998 in the Netherlands, *B. cereus* accounted for 12% of food-borne disease outbreaks where a causative agent

was identified (Schmidt, 2001). One main reason for the capability of the above-mentioned pathogens to cause diseases is through their ability to resist low pH, and a high acid environment (Peterson *et al.,* 1989). Bacteria, when exposed to environmental stress, may respond and adapt to new conditions. However, wine is unique beverage as it also has a high alcohol content in addition to high amounts of organic acids and low pH. All these factors together contribute to wine's antimicrobial property. This has been confirmed by diverse studies where various food-borne pathogens were exposed to wine, and the results indicate the reduction of their viable counts in the presence of wine. Several studies have been conducted that demonstrate the antibacterial property of wine against a notable amount of relevant food-borne pathogenic bacteria (Fernandes *et al.,* 2007; Carneiro *et al.,* 2008; Moretro and Daeschel, 2004; Sugita-Konishi *et al.,* 2001; Weisse *et al.,* 1995). Various *in vitro* studies indicated that the potency of wine as an antibacterial agent was higher than a given ethanol concentration and was, in fact, due to a combination of ethanol and organic acids (tartaric, malic, lactic and acetic) (Just and Daeschel 2003; Weisse *et al.,* 1995). Reports indicate that the consumption of red wine had a protective effect during food-borne outbreaks of *Salmonella enteritidis* (Bellido-Blasco *et al.,* 2002) and hepatitis A (Desenclos *et al.,* 1992). Alcohol consumption also has a protective effect against *Helicobacter pylori* (Brenner *et al.,* 2001), which is a major cause of stomach ulcers. Various *in vitro* studies indicate that viable counts of *Enterobacteriaceae* are more rapidly reduced when treated with wine than other alcoholic beverages (Harding and Maidment, 1996; Weisse *et al.,* 1995). Weisse *et al.* (1995) showed that wine was able to reduce the viable counts of *S. enteritidis*, *Shigella sonnei*, and *E. coli* by 5 to 6 logs after a 20-min exposure. Other studies show a 5 to 6 log reduction in viable counts of *Salmonella* sp. and *E. coli* after exposure to wine for 5 to 30 min and 20 to 60 min, respectively (Harding and Maidment, 1996; Just and Daeschel 2003). Carneiro *et al.* (2008) focused their study on the activity of red wine against the important food-borne pathogen, *Campylobacter jejuni.* Undiluted red wine was found to rapidly inactivate *C. jejuni* and further inactivation data were obtained from experiments performed in red wine diluted with water. Their experiments also indicated that the two components in wine, namely ethanol and certain organic acids, act synergistically. Red wine was found to be anti-*Campylobacter*, which suggested that ingestion of wine during a meal may lower the risk of infection by this pathogen. Moretro and Daeschel (2004), when testing different combinations of ethanol, organic acids and acidity, found that a mixture of 0.15% of malic acid, 0.6% of tartaric acid, 15% of ethanol, and pH 3.0 had the strongest bactericidal effect and suggested that all these compounds acted synergistically and represented the major components for the bactericidal effect of wine. Drinking white wine with raw oysters can help prevent diarrhoea and this was reconfirmed by studies conducted by Desenclos *et al.* (1992). These authors studied the effects of an oyster-borne hepatitis A exposed to beverages with alcohol concentrations of > 10% which had resulted in a reduced rate of illness. Just and Daeschel (2003) evaluated the survival of *E. coli* O157:H7 and *Salmonella typhimurium* in a model stomach system. Bacteria were inactivated in both red and white wine within 60 min, but survived up to 16 days in grape juice. When a model stomach system was designed, wine had little effect on *E. coli* O157:H7 survival, whereas *Salmonella* was undetectable after 120 min. A nonvolatile wine fraction containing acids was more powerful in killing *Salmonella* than a volatile wine fraction containing alcohol, thereby, suggesting that the antibacterial activity of

wine is acid dependent (Just and Daeschel, 2003). Moretro and Daeschel (2004) studied the effects of red and white wines without added sulfite against wild type strains and sigma mutants of foodborne pathogens, like *E. coli* O157:H7, *S. typhimurium*, *Staphylococcus aureus* and *Listeria monocytogenes*. They deduced that the wines had bactericidal activity against all strains, with red wine being more potent. S. *typhimurium* was most sensitive with a 6 log cycles reduction and *S. aureus* was the least sensitive to the wines tested. Mutants having the gene encoding the alternative sigma factor disrupted were more sensitive to wine than their wild-type counterparts. The principal organic acids in wine, namely malic and tartaric acids, in conjunction with ethanol, exerted an immense effect on cell viability in either natural wine or constructed model wine. Weisse *et al.* (1995) reported that red and white wines are as potent as bismuth salicylate against several bacteria that are responsible for traveler's diarrhea. They also demonstrated that diluted alcohol did not induce any significant reduction in colony counts. Sugita-Konishi *et al.* (2001) examined the antibacterial activity of red and white wines against three potential entero-pathogenic bacteria, *S. enteritidis*, *E. coli* O157:H7 and *Vibrio parahaemolyticus*, *in vitro*. They also identified that the evaporated fraction present in wine had antibacterial activity and examined the ability of wine and the fraction to protect against infection and concluded that there is little evidence for the use of wine as a digestive aid *in vivo*. Daglia *et al.* (2007) studied the antimicrobial action of commercial red and white wines against oral streptococci responsible for caries development and against *Streptococcus pyogenes* responsible for pharyngitis. The compounds responsible for the former activities were succinic, malic, lactic, tartaric, citric and acetic acids. Wine polyphenols, however, exerted no effect against oral streptococci or *S. pyogenes*. Fernandes *et al.* (2007) designed a model stomach, containing a food matrix and a synthetic gastric fluid, and studied the bactericidal effect of ingested wine on *Listeria innocua*. The influence of ethanol and organic acids, wine constituents with known antimicrobial properties, was also investigated. Their study showed that ethanol had a higher bactericidal effect than a mixture of the main wine organic acids. When the organic acids were tested separately, malic and lactic acids had the strongest effect. The synergetic effect of ethanol combined with the organic acids provides evidence that the ingestion of wine during a meal might reduce the quantity of *Listeria* persisting further in the alimentary tract. Murray *et al.* (2002) associated alcohol consumption with a significant decrease in *H. pylori* infection. Another research indicated that modest consumption of wine and beer protects against *H. pylori* infection (Daroch *et al.,* 2001). There are some key phenolic phytochemicals in grape that have antimicrobial properties and inhibit the bacteria that cause common types of food poisoning (Weisse *et al.,* 1995). It has already been stated that the antimicrobial agent in wine is a polyphenol, resveratrol, produced during the fermentation process. This is active in the acidic environment and may be linked to inhibition of *H. pylori* (Daroch *et al.,* 2001; Murray *et al.,* 2002). Gañan *et al.* (2009) investigated the antimicrobial power of wine and its components against *Campylobacter jejuni*. The two compounds which had the most significant effect on the viability of *C. jejuni* LP1 were the gallic acid, and the *p*-hydroxybenzoic acid; even at concentrations as low as 1 mg/L they were able to significantly reduce the viability of this microorganism. Red wines have been reported to contain from 5.8 to 2.2 mg L^{-1} of these compounds (Salagoite-Auguste and Bertrand, 1984), thus the antimicrobial power of wine may be attributed to them.

Studies incorporating wine as a food additive in the form of marinades and other similar treatments provide further evidence of its protective role (Friedman *et al.,* 2006). It is known that many naturally occurring compounds found in dietary and medicinal plants, herbs and fruit extracts, possess antimicrobial activities (Kouassi and Shelef, 1998; Larson *et al*., 1996). Lin *et al.* (2005) aimed at determining the potential of phenolic phytochemical-enriched wine and vodka to inhibit *H. pylori* in laboratory media. Their study also indicated that raspberry, cinnamon and peppermint-enriched wines had the highest antimicrobial activity. The results indicated that the synergistic contribution of phenolics and the antioxidant activity might be more important for inhibition than any specific phenolic concentration. This study clearly demonstrates the feasibility of the use of plant extracts as antimicrobial ingredients in alcoholic beverages. Such phenolic profiles also have the added benefit of enhancing host tissue and cellular response through enhanced antioxidant activity (Shetty and Wahlqvist, 2004). Studies incorporating wine as a food additive in the form of marinades and other similar treatments provide further evidence of its protective role (Friedman *et al.,* 2006). They developed wine formulations containing plant essential oils and oil compounds effective against the food-borne pathogenic bacteria *E. coli* O157:H7 and *Salmonella enterica*. The results showed that wines containing essential oils/oil compounds added or extracted from oregano or thyme leaves could be used to reduce pathogens in food and other environment. In a further study, Friedman *et al.* (2007) have evaluated bactericidal activities against *B. cereus*, *E. coli* O157:H7, *Listeria monocytogenes*, and *Salmonella enterica* of several antimicrobial wine recipes, each consisting of red or white wines extracts of oregano leaves with added garlic juice and oregano oil. The wine marinades were highly effective against the four pathogens. Moreover, the red wine polyphelonics isolated by chromatography from red wine exhibited exceptionally high activity at nanogram levels against the two *B. cereus* strains tested.

1.7. Aim of the research

In this thesis, the aim was to evaluate the effect of red wine on *B. cereus* vegetative cells and spores. Firstly, we have characterized the effect of direct exposure of cells and spores to wine. Secondly, the antimicrobial activity of selected wine components was tested separately and in combination (organic acids, ethanol, low pH and phenolic compounds). Thirdly, in food consumption scenarios (wine/food/bacteria), the kinetics of inactivation of *B. cereus* vegetative cells in simulated gastric conditions, with and without the addition of wine, were evaluated. Finally, we aimed to monitor the spore counts of *B. cereus* on simulated gastric and intestinal conditions, with and without a food matrix or wine addition. This is, to our knowledge, the first study that takes into consideration the effect of direct wine exposure on *B. cereus* vegetative cells and spores. Yet, this is the first report where behavior of spores is characterized in direct wine immersion or under gastrointestinal conditions in a simulated meal context. The results obtained are considered in risk assessment circumstances.

2. Materials and methods

2.1. Bacterial strains and growth medium

Two *B. cereus* strains purchased from American Type Culture Collection (ATCC) were used for the experiments (*B. cereus* ATCC 11778 and *B. cereus* ATCC 14579). The cultures were preserved in slants of Tryptone Soy Agar (TSA) medium (Pronadisa, Madrid, Spain) and kept at 4 ºC. Stock cultures of vegetative cells were kept at -80 ºC in Tryptone Soy Broth (TSB) medium (Pronadisa, Madrid, Spain) with glycerol 20% (v/v). For each assay, the inoculum was transferred from TSA slants to TSB medium and incubated at 30 ºC for 16 h to attain the stationary growth phase. Concentrations of vegetative cells and spores were determined by plating serial decimal dilutions on TSA.

2.2. Spore production

Pure *B. cereus* strains were grown on TSA for 24 h at 30 ºC. These cells were then used to inoculate TSA plates supplemented with $MnSO_4$ 40 mg L^{-1} (Merck, Darmstad, Germany) and $CaCl_2$ 100 mg L^{-1} (Merck, Darmstad, Germany) to stimulate sporulation. TSA plates were incubated at 30 ºC for 7 days. Sporulation was checked by microscopic visualization to guarantee the cells had produced spores (Malachite Green was used as the primary stain for the spores). Spores were collected by scraping colonies from the plates and suspended in sterile phosphate buffer at pH 7.0 and washed by centrifugation (10.000 g for 10 min). Washings and centrifugation were performed until the suspension was milky white (about three times). After the final centrifugation procedure, the pellet was suspended in 5 mL distilled water and 5 mL ethanol and the resulting suspension was kept at 4 ºC for 12 h in order to eliminate vegetative cells and washed again three times by centrifugation in distilled water. Lastly, the final suspension (about 10^{10} spores per mL) was distributed in sterile Eppendorf microtubes and kept at – 20 ºC.

2.3. Wine

Red wine, from the Douro demarcated region (Portugal, 2008) with an ethanol concentration of 13% (v/v) was used. The wine was filter sterilized using 0.45 µm cellulose acetate membranes (Orange Scientific of GyroDisc, CA) and was kept at 4 ºC, in 1 L sterile bottles, until use. The pH was determined by potentiometry using a Crison micro pH 2002 (Crison Instuments SA, Barcelona, Spain) pH meter with an electrode calibrated using freshly buffer solutions at pH 4.0 and pH 7.0.

2.4. Wine components

Solutions of wine components were analyzed for their antimicrobial properties. A mixture of organic acids was made, containing the final concentrations of 2 g L^{-1} lactic acid, 5.5 g L^{-1} tartaric acid, 0.5 g L^{-1} of acetic and 0.5 g L^{-1} of citric acid, with pH adjusted to 3.3 with 1 M HCl (Pronalab, Lisbon,

Portugal), in the presence or absence of 13% (v/v) of ethanol. Tartaric acid and lactic acid were obtained from Sigma (St. Louis, USA), while acetic acid and citric acid were obtained from Merck (Darmstad, Germany). A solution of 0.15 M KH_2PO_4 at pH 7.0 was used as control**.** The individual effect of each organic acid was tested too.

The potential antimicrobial activity of five phenolic compounds was also checked. Solutions of 1 mg L^{-1} of resveratrol, 5 mg L^{-1} of ferulic acid, 5 mg L^{-1} of *p*-coumaric acid, 2 mg L^{-1} of kaempferol and 10 mg L^{-1} of quercetin were made, with pH adjusted to 3.3 and 3.25% (v/v) of ethanol. Contents of phenolic compounds and organic acids are comparable to those reported in literature (Soleas *et al*., 1997; Daglia *et al*., 2007; Avar *et al*., 2007; Rodríguez-Delgado *et al*., 2002).

The effect of ethanol alone was also tested at concentrations of 13% (v/v) and 3.25% (v/v), at pH 7.0. Finally, the individual effect of a solution at pH 3.3 was considered. All the above mentioned solutions were filter sterilized using 0.45 µm cellulose acetate membranes (Orange Scientific, Brain L' Alleud, Belgium).

2.5. Behavior of *B. cereus* vegetative cells and spores in wine and wine components

Suspensions of stationary phase vegetative cells and spores of *B. cereus* ATCC 11778 and *B. cereus* ATCC 14579 grown in TSB medium for 16 h at 30 ºC were used in the inactivation experiments. When the wine components were tested only vegetative cells of *B. cereus* were used. 1 mL of each suspension was transferred onto 100 mL Erlenmeyer flasks containing 49 mL of wine, solutions of wine components, or a control solution (0.15 M KH_2PO_4, pH 7.0), all at a final volume of 50 mL. These flasks were immersed in a thermostatted water bath at 37 ºC. At selected times, 1 mL samples (vegetative cells or spores) were collected and serially diluted in 9 mL Ringer's solution (Lab M). 20 µL of each ten-fold dilutions were plated in triplicate onto TSA medium by the drop count technique (described by Miles and Misra,1938) and incubated overnight at 30 ºC for 24 h. Cell concentrations were expressed as colony forming units per milliliter (CFU mL^{-1}). The detection limit was taken as one colony on the lowest dilution plate, i.e. 500 CFU mL^{-1}.

2.6. Synthetic gastric and intestinal fluids preparation

The synthetic gastric fluid (SGF) was produced following an adapted formulation from Beumer *et al*. (1992). SGF consisted of NaCl (2.05 g), KH_2PO_4 (0.6 g), $CaCl_2$ (0.11 g), KCl (0.37 g) and pepsin (0.0133 g). The aforementioned constituents were mixed in 1 L deionised water. The SGF was adjusted to pH 1.5 with 1 M HCl and sterilized through 0.22 µm cellulose acetate membranes.

The synthetic intestinal fluid (SIF) was produced following an adapted formulation from Rotard *et al*. (1995) and consisted of NaCl (6.58 g), KH_2PO_4 (0.66 g), KCl (0.043 g), $MgCl_2$ (0.17 g), $CaCl_2$ (0.060 g), pancreatin (2.25 g) and bile salts (1.5 g), in 1 L of deionised water. The SIF solution was sterilized by filtration through a 0.22 µm filter. The pH of SIF was approximately 7.5 (adjusted by 0.5 M NaOH solution).

Both SGF and SIF were prepared fresh daily to avoid possible changes in their intrinsic bactericidal properties. KH_2PO, $MgCl_2$ and $CaCl_2$ were obtained from Merck (Darmstad, Germany). NaCl, KCl, pepsin and pancreatin were purchased from Sigma (St. Louis, USA), while bile salts were obtained from Difco.

2.7. Behavior of *B. cereus* vegetative cells in simulated gastric medium

The synthetic gastric mixture was transferred onto Erlenmeyer flasks. Vegetative cells of *B. cereus* ATCC 14579 were suspended in 50 mL of SGF and in 43 mL of SGF supplemented with 7 mL of sterile wine, at pH 1.5 for 4 min in a water bath with a shaker at 200 rpm, at 37 ºC. 1 mL samples were taken at selected times and ten-fold dilutions of the samples were carried out. 20 µL aliquots were plated in triplicate onto TSA and plates were incubated overnight at 30 ºC.

Regarding the study of *B. cereus* vegetative cells in the presence of a food matrix, other trials were performed. Then, 7 mL of sterile wine and 21.5 mL of SGF were added to 21.5 g of solid food matrix (sterile chicken-rice baby meal, from Nestlé, and pasteurized fresh cheese, from Santiago, to a final volume of approximately 50 mL). Control solutions consisted of 43 mL of sterile water added to 7 g of food or 21.5 mL of sterile water and 21.5 mL of SGF supplemented with 7 g of food. In proportion to the amount of food used in these experiments, the volume of wine tested corresponds, approximately, to a glass of wine ingested in a regular meal 100-150 mL).

Samples were collected and plated as described above (see section 2.5).

2.8. Behavior of *B. cereus* spores considering simulated gastrointestinal passage

The trials were carried out with spore suspensions of *B. cereus* ATCC 14579. The solutions tested (involving a food matrix) were prepared as described above (section 2.6) for the vegetative cells. Additionally, other experiment, doubling the volume of wine (14 mL of sterile wine, to a final volume of 57 mL) was carried out. Spores were exposed to SGF and SIF for 60 and 240 min, respectively, as desbribed by Wijnands *et al.* (2006)**.** After 60 min of incubation at 37 ºC in the water bath, 1 mL suspensions of the mixture of SGF, wine and food, were transferred to 50 mL of SIF and then incubated at the same temperature, for 240 min.

Total counts, i.e. total number of colony forming units (vegetative cells+spores) and spore counts (after heat-treatment of the ten-fold serial diluted samples at 80 ºC, for 10 min) were determined.

2.9. Data analysis

The survival of *B. cereus* strains was calculated by enumeration on TSA and the counts converted to $\log_{10}$ CFU mL^{-1}. The experiments were performed in triplicate and the results were expressed as the mean value ± standard error. Statistical analysis (ANOVA, significance level: $P < 0.05$ was recorded in an Excel spreadsheet (Microsoft Corporation, Redmond, WA, USA).

3. Results and discussion

3.1. Behavior of *B. cereus* vegetative cells and spores in wine and wine components

The red wine tested was found to be strongly effective against stationary phase vegetative cells of *B. cereus*, whereas spores of both *B. cereus* strains tested showed a marked resistance to the wine exposure. The results presented here show that the wine treatment immediately decreased initial *B. cereus* vegetative cells numbers from 10^7–10^8 CFU mL^{-1} to non detectable levels (detection limit of 500 CFU mL^{-1}) in less than 10 s (data not shown). Therefore, inactivation experiments were carried out in diluted wine, 1:4 and 1:8. The results displayed in Fig. 3 and Fig. 4 show an almost 5.0 log cycles reduction of the cells of ATCC 11778 strain and a 4.5 log reduction of the cells of ATCC 14579 strain in the viability of cultures exposed to diluted wine 1:4, in 20 s. No colonies were detected (< 500 CFU mL^{-1}) in the following sampling times. The dilution 1:8 provoked a 5.0 log cycles reduction on the viable cell counts of ATCC 14579 strain after 40 s, and a similar reduction was also noticed in ATCC 11778 strain, but only after 30 s exposure. The initial populations of vegetative cells in the control solution (KH_2PO_4) remained constant until the end of the trial. Only a small decrease on the spore counts, less than 1.0 log cycle, was observed after 180 min of undiluted wine treatment (Fig. 5).

No significant differences were found between the two strains ($P > 0.05$), both for vegetative cells and spores. The following experiments were performed only with the strain ATCC 14579.

Activity against *B. cereus* has been reported previously for grape juice and wine marinades (Rhodes *et al*., 2006; Friedman *et al.,* 2007) but not, to our knowledge, for wine exposure. Rhodes *et al.* (2006) reported a reduction of 1 log cycle within 10 min of contact with the grape juice, but no further reduction after 60 min. Friedman *et al.* (2007) have evaluated bactericidal activities against *Bacillus cereus*, *E. coli* O157:H7, *Listeria monocytogenes*, and *Salmonella enterica* of several antimicrobial wine recipes, each consisting of red or white wines extracts of oregano leaves with added garlic juice and oregano oil. The wine marinades were highly effective against the four pathogens.

Numerous studies have been conducted that demonstrate the antibacterial property of wine against a notable amount of relevant food-borne pathogenic bacteria (Fernandes *et al.,* 2007; Moretro and Daeschel 2004; Sugita-Konishi *et al.,* 2001; Weisse *et al.,* 1995). Studies incorporating wine as a food additive in the form of marinades and other similar treatments provide further evidence of its protective role (Friedman *et al.,* 2006, 2007). Comparison with published data suggests that *B. cereus*, while considering vegetative cells**,** is more sensitive to wine than other food-borne bacteria. Carneiro *et al.* (2008) showed that cells of *Campylobacter jejuni* CIN55c exposed to diluted wine 1:2 suffered a reduction of almost 6 log cycles in 30 s. Weisse *et al*. (1995) showed that wine reduced the viable number of *Salmonella enteritidis*, *Shigella sonnei* and *E. coli*, 5–6 log cycles, in 20 min of exposure. Other works obtained the same extent of inactivation of *Salmonella* spp. and *E. coli* in 5–30 min and of 20–60 min, respectively (Harding and Maidment, 1996; Just and Daeschel, 2003; Marimon *et al*., 1998; Moretro and Daeschel, 2004). Moretro and Daeschel (2004) investigated the bactericidal effect of the wine on *E. coli* O157:H7, *Listeria monocytogenes*, *S. typhimurium* and *Staphylococcus aureus*

and concluded that *S. typhimurium* was the most sensitive species, with a reduction of 6 log cycles after 10 min of exposure. *S. aureus* was the most resistant. Bacterial inactivation experiments are notoriously sensitive to medium and conditions variations (ex: variability of wine composition) and direct comparison in this case must also contemplate the special cultivation conditions of *B. cereus*. However, the results presented here suggest that *B. cereus* vegetative cells are, amongst the food-borne pathogens studied, at the lower end of the range of resistance to wine. Concerning spores, there is no comparison term, since other studies focusing the activity of wine against other sporulating food-borne bacteria have not yet been documented.

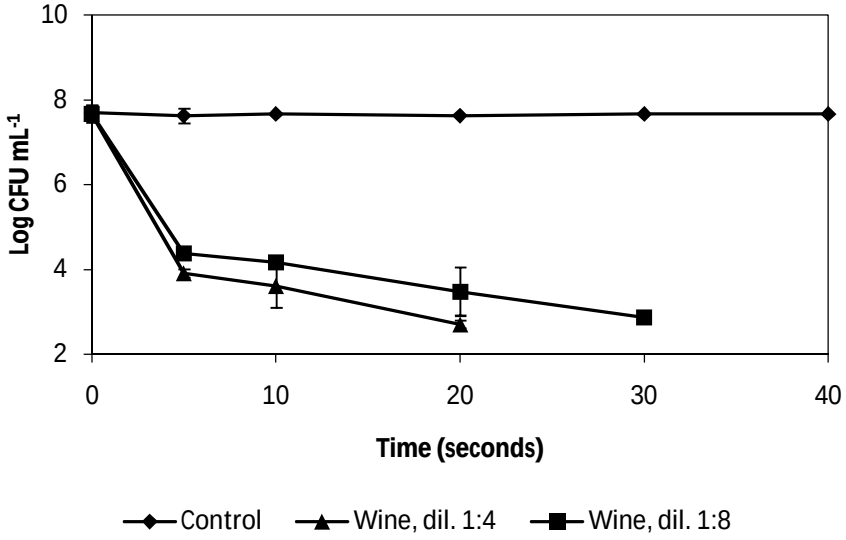

Fig. 3. The effect of wine, dilutions 1:4 and 1:8, on the vegetative cells of *B. cereus* ATCC 11778. Error bars represent the standard deviation of the mean of three replications.

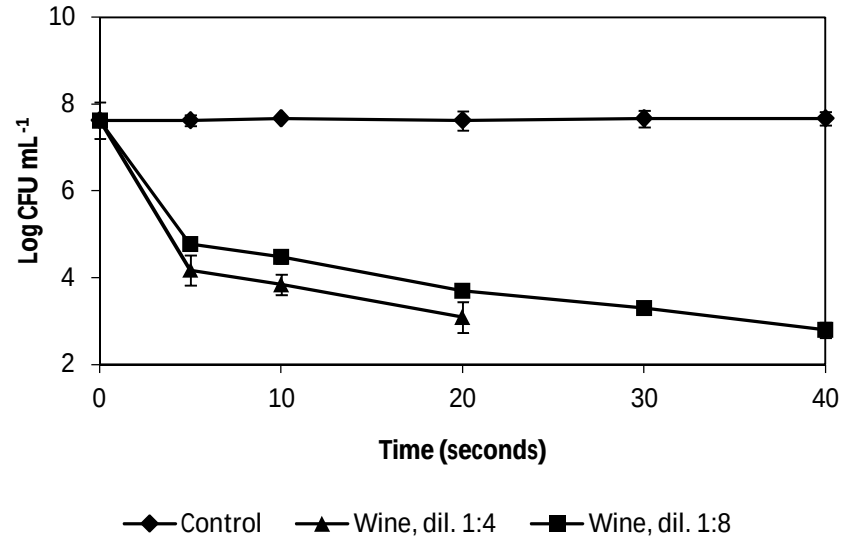

Fig. 4. The effect of wine, dilutions 1:4 and 1:8, on the vegetative cells of *B. cereus* ATCC 14579. Error bars represent the standard deviation of the mean of three replications.

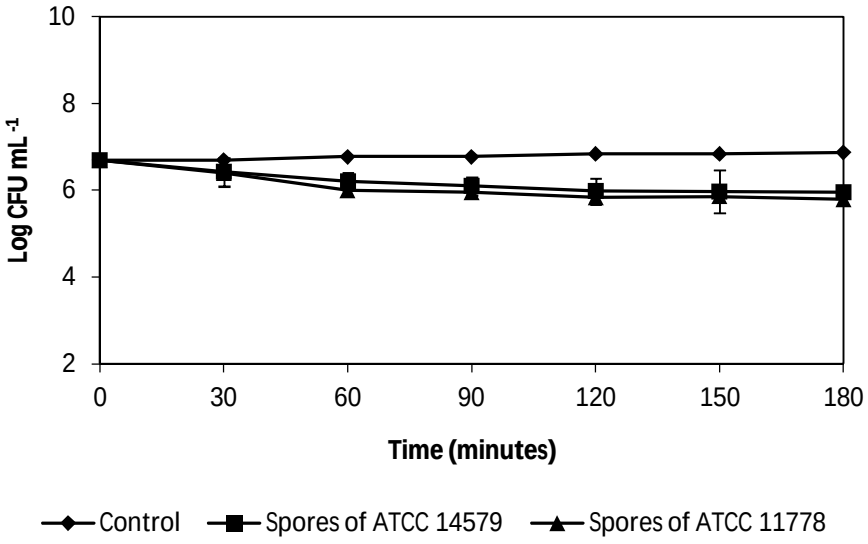

Fig. 5. The effect of wine on the spores of *B. cereus* ATCC 11778 and ATCC 14579. Error bars represent the standard deviation of the mean of three replications.

We then studied the contribution of certain wine components (organic acids, low pH, ethanol and phenolic compounds) to the antibacterial effect of wine on *B. cereus* ATCC 14579 vegetative cells. The main organic acids of red wine (tartaric, acetic, lactic and citric acids (Jackson, 2000) – and mixtures of the acids with and without ethanol, at concentrations commonly found in wines, were tested. Resveratrol, ferulic acid, *p*-coumaric acid, kaempferol and quercetin were the polyphenols tested. These compounds were found to have antibactericidal properties in previous works (Aziz *et al.*, 1998; Vaquero *et al.*, 2007; Gañan *et al.*, 2009). The solutions of organic acids were diluted 1:4 and the pH was adjusted to 3.3 (pH of the wine tested) to make meaningful comparisons with the antimicrobial activity of wine. With regard to phenolic compounds, only the ethanol content was diluted 1:4, and pH was equally adjusted to 3.3.The effect of ethanol was studied at the concentrations of 3.25% (corresponding to the ethanol content of the wine diluted 1:4) and 13% (v/v) (ethanol content of the wine), at pH 7.0 and at pH 3.3.

As can be seen in Fig. 6**,** the mixture of organic acids with ethanol displayed a higher bactericidal effect than the mixture of acids alone, but the divergence was not statistically significant ($P > 0.05$). At the end of the exposure time (40 s), a reduction of about 4.2 log cycles was provoked by the mixture of acids with ethanol (only 0.4 log cycles more than the mixture of organic acids). It can be assumed that the organic acids mixture contributes notably to the antimicrobial effect of wine, but it is important to note that there is a difference between the effect of the mixture of the organic acids (Fig. 6) and the effect of wine itself (Fig. 3 and Fig. 4). The effect of the organic acids was studied separately at the same concentrations used in the mixture. Fig. 7 shows that lactic acid was found to be the most effective in the inhibition of *B. cereus* ATCC 14579 vegetative cells, being responsible for a 3.6 log cycles reduction after 40 s of exposure, whereas citric acid was the least effective, causing only a 1.3 log cycles reduction. Acetic acid was responsible for a 3.1 log cycles reduction in the viable counts, while tartaric acid caused a 2.7 log cycles reduction.

Based on the results of the bactericidal activity exhibited by the mixture of acids with ethanol and the individual compounds of the mixture, it can be said that the antimicrobial effect of the organic acids fraction is mainly due to lactic acid. Various *in vitro* studies indicated that the potency of wine as an antibacterial agent was higher than ethanolic solutions at concentrations equivalent to the wine (Just and Daeschel, 2003; Weisse *et al.,* 1995). Fernandes *et al.* (2007) reported that malic acid and lactic acid were amongst the most effective organic acids in the inactivation of *Listeria inocua* NCTC 11288, causing a 3 log reduction in 120 min. Weisse *et al.* (1995) found the combination of ethanol and low pH to be important when determining the inactivation of *E. coli, Salmonella* sp., and *Shigella sonnei.* Marimon *et al.* (1998) found similar results with ethanol and pH combinations against *H. pylori*. Moretro and Daeschel (2004) found that the combination of organic acids (malic and tartaric) with ethanol (15%) and low pH (≤ 3.0) had significantly stronger antimicrobial activity than the effect of these components individually against various food-borne pathogens, indicating potential synergistic interactions between these components. Fernandes *et al.* (2007) also demonstrated that the combination of the acids with ethanol showed a higher antibacterial effect than the mixture of acids and ethanol in separate. Lower pH values will lead to higher concentration of the undissociated form of organic acids, which are considered to be the antimicrobial active species (Doores, 1983). Ethanol is known to damage the cytoplasmic membrane, causing changes in the permeability of the membrane (Barker and Park, 2001). These changes may lead to enhanced efficacy of organic acids and may partly explain the difference in antimicrobial activity between grape juice and wine (Harding and Maidment, 1996; Barker and Park, 2001; Just and Daeschel, 2003).

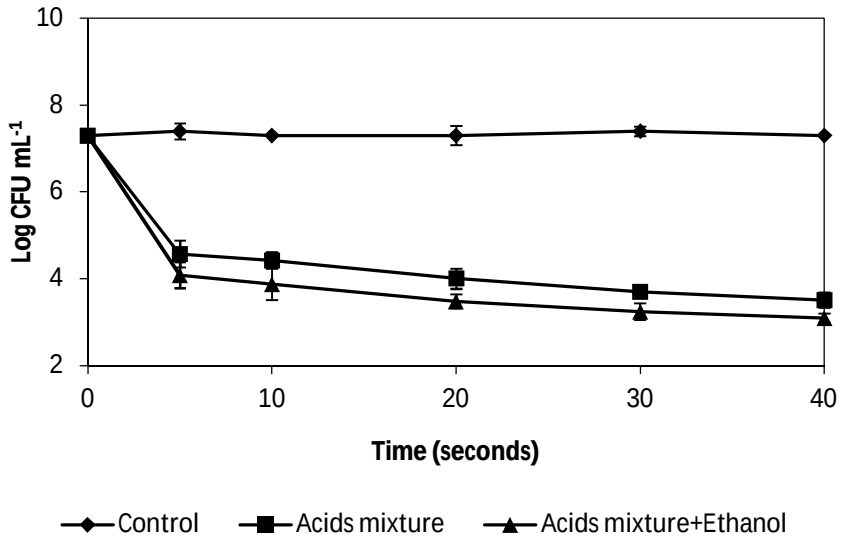

Fig. 6. The effect of wine organic acids mixed (5.5 g L^{-1} tartaric acid, 0.5 g L^{-1} acetic acid, 2 g L^{-1} lactic acid and 0.5 g L^{-1} citric acid) with and without ethanol 13% (v/v) , diluted 1:4, on the vegetative cells of *B. cereus* ATCC 14579. Both solutions of organic acids were adjusted to pH 3.3. Error bars represent the standard deviation of the mean of three replications.

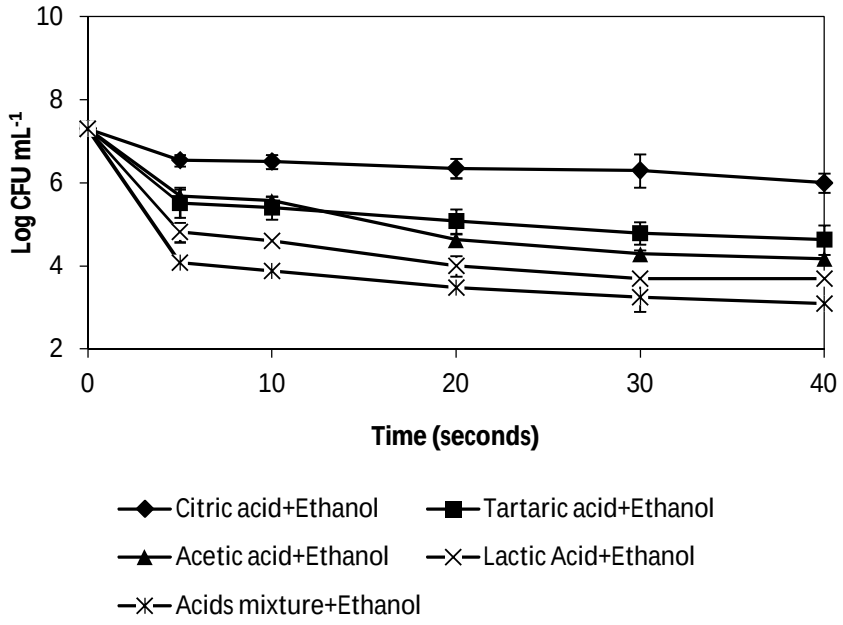

Fig. 7. The effect of wine organic acids mixed and separated (5.5 g L^{-1} tartaric acid, 0.5 g L^{-1} acetic acid, 2 g L^{-1} lactic acid and 0.5 g L^{-1} citric acid) supplemented with ethanol 13% (v/v) , diluted 1:4, on the vegetative cells of *B. cereus* ATCC 14579. All solutions were adjusted to pH 3.3. Error bars represent the standard deviation of the mean of three replications.

Regarding the experiments investigating the effect of ethanol and low pH (3.3), cell inactivation rates after 40 s were negligible (data not shown). Another time scale was employed (exposure time extended to 7 min) to better understand the potential contribution of the aforementioned wine components (see Fig. 8). As can be seen in Fig. 8, ethanol concentrations of 3.25% (v/v) caused a 0.7 log cycles reduction, while a concentration of 13.0% was responsible for 1 log cycles reduction of the initial population of *B. cereus* cells

The low pH (3.3) alone exhibited a higher inactivation than the ethanol, causing a 1.5 log survival reduction. However, the combination of the low pH with ethanol was found to be more effective in the inactivation experiments. At low pH, ethanol at 3.25 and 13.0%, (v/v) caused a 3 and 3.6 log cycles reduction respectively. Contrarily to our work, where ethanol showed scarce activity against *B. cereus* cells, Fernandes *et al*. (2007) demonstrated that the effect of ethanol was found to be higher than any of the organic acids at the concentrations they have used, being responsible for an almost 4 log cycles reduction of the viable cells, after 120 min of exposure. We can assume that *B. cereus* cells are less sensitive to the ethanol content than other bacterial species. Waite and Daeschel (2007) showed that an ethanol concentration of 12.08% (v/v) caused more than 3 log reduction cycles on the viable CFU of *Staphylococcus aureus* (in our study, the reduction was negligible, when tested in the same time exposure of the wine organic acids and wine itself).

Additional components in wine have been documented to possess antibacterial properties. In our study, the wine phenolic compounds assayed displayed no activity, at the concentrations tested, against *B. cereus* vegetative cells.

Soleas *et al.* (1997) quantified the levels of phenolic compounds (*trans* and *cis*-resveratrol, gallic acid, ferulic acid, caffeic acid, *p*-coumaric acid, vanillic acid and gentisic acid) in different red and white wines. Red wines, in general, contain much higher levels of both *cis*- and *trans*-resveratrol, gallic acid, vanillic acid, ferulic acid, and genistic acid. These differences have been used to explain the additional effectiveness often seen with red wines compared to white wines (Moretro and Daeschel, 2004). Papadopoulou *et al.* (2005) investigated the effectiveness of phenolic extracts of red and white wines at inactivating *S. aureus*, *E. coli*, and *Candida albicans*. Vaquero *et al.* (2007) performed a similar study using red wines and investigating effectiveness against 7 different bacterial species. Phenolic fractions from wines showed marked antimicrobial activity, indicating some contribution of the phenolic compounds in inactivation of microorganisms by wine treatment. Several studies have investigated the impact of specific phenolic compounds on inhibiting microbial growth. Mahady and Pendland (2000) and Mahady *et al.* (2003) determined a MIC50 value of 12.5 μg mL^{-1} of resveratrol against *H. pylori* strains using an agar disk diffusion assay. Chan (2002) used a broth dilution assay to determine the MIC of resveratrol against *S. aureus*, *Enterococcus faecalis*, and *Pseudomonas aeruginosa* to be 171 to 342 μg mL^{-1}. Aziz *et al.* (1998) investigated the inhibitory effect of several phenolic compounds against *E. coli*, *Klebsiella pneumoniae*, and *B. cereus* using a suspension test. Caffeic acid and protocatechuic acid were effective at inhibiting the growth of *E. coli* and *K. pneumoniae* at levels of 0.3 mg mL^{-1}. Vanillic acid and *p*-coumaric acid were capable of inhibiting growth of *E. coli, K. pneumoniae*, and *B. cereus* at levels of 0.4 mg mL^{-1}. The reason why the phenolic compounds we studied have not exhibited activity against *B. cereus* vegetative cells, might be related with the concentrations tested. The efficacy of phenolic compounds that has been observed against various bacterial species was attained using concentrations 10 to 1000 times greater than found in commercially available wines.

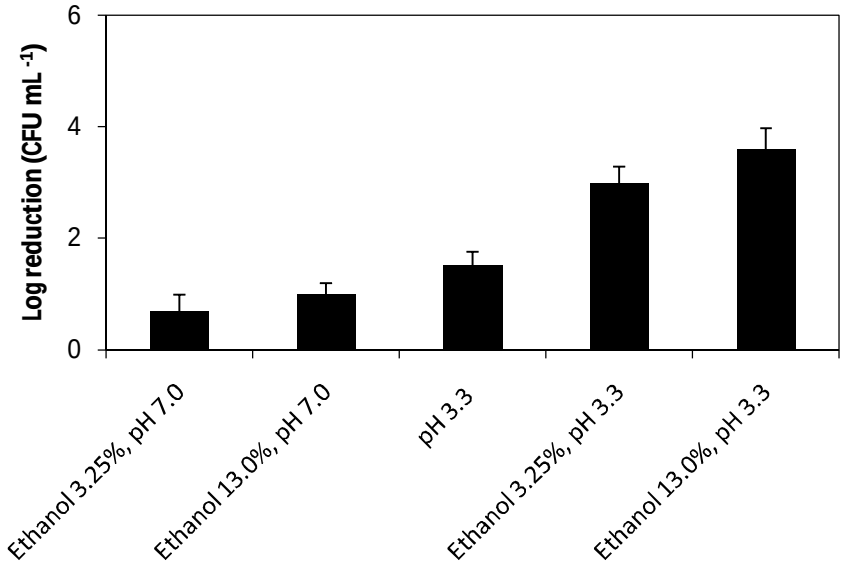

Fig. 8. The effect of ethanol 3.25% and 13% (v/v) at pH 7.0 and pH 3.3, and the effect of low pH (pH 3.3), on the vegetative cells of *B. cereus* ATCC 14579, after 7 min. Error bars represent the standard deviation of the mean of three replications.

3.2. Behavior of *B. cereus* vegetative cells in simulated gastric medium

Taking into account the low pH in the stomach, vegetative cells of *B. cereus* may be expected to be inactivated upon gastric passage. Thus, we opted to investigate the behavior of *B. cereus* cells only under simulated gastric conditions and not intestinal conditions. However, during the consumption of food, the pH in the stomach varies and may even reach values up to pH 5, a condition allowing the survival of *B. cereus* vegetative cells (Kramer and Gilbert, 1989; Dressman *et al*., 1990).

As depicted in Fig. 9, wine combined with synthetic gastric fluid (SGF) was more effective on decreasing the number of cells of *B. cereus* ATCC 14579 than SGF used alone, despite significant differences were not observed ($P > 0.05$). The inhibitory action of wine against *B. cereus* vegetative cells, under a simulated meal consumption context, was studied. Figure 10 depicts the results obtained with a sterile chicken-rice baby meal used as food matrix. In the control assay (Food+Water), the culture densities of *B. cereus* cells remained constant until the end of the 60 min exposure. With regard to the assay Food+SGF+Water, *B. cereus* cells suffered an almost 2.0 log reduction in the number of detected colonies, in 20 min, and almost a 4.0 log reduction occurred at the end of the experiment (60 min). The treatment Food+SGF+Wine exhibited the strongest inactivation effect ($P < 0.05$, for 6 min of exposure), causing a reduction on viable cell counts of 4.5 log cycles, in 6 min. No viable cells were detected (< 500 CFU mL^{-1}) after the 6 min exposure. The influence of another food matrix, pasteurized fresh cheese, was also used in the experiments conducted in simulated gastric conditions (Fig. 11). The assay Food+SGF+Water led to inactivation of the vegetative cells, but to a lesser extent than the inactivation presented previously by the other foodstuff. With the addition of wine to the system (Food+SGF+Wine), a significant decrease ($P < 0.05$) of 3.7 log cycles in the cell survival was noticed after 20 min. After this time on, no viable cells were detected (< 500 CFU mL^{-1}). Doubling the quantity of wine of the system (Food+SGF+Wine2x), a short period of exposure as 3.5 min caused a 4 log reduction with no subsequent detectable colonies in the following exposure times. Taken these results into account, the fresh pasteurized cheese exhibits a higher protective effect on the vegetative cells of *B. cereus* ATCC 14579 than the chiken-rice meal. This result may be explained with some findings of some published data. Clavel *et al.* (2004), when using a gastric electrolyte solution (GM) and 1 volume of J broth (JB) or some food media in their experiments, showed that survival of *B. cereus* cells was higher in GM-milk than in the other GM. This protective effect of a dairy product against inactivation by low pH was previously observed on *Salmonella* in cheese (D'Aoust, 1985) and might be due to lipids, proteins or particles of the food. Conway *et al*. (1987) have hypothesized that microorganisms are trapped in hydrophobic lipid moieties and may consequently survive acid conditions. This effect may be responsible for the strong protective effect on *B. cereus* that they observed in GM-milk. Milk proteins may also explain the gastric tolerance of some strains of lactic acid bacteria (Charteris *et al*., 1998). Waterman and Small (1998) showed the survival on beef particles of acid-sensitive bacteria such as *Salmonella typhimurium*, *Shigella flexneri* and *Escherichia coli* and suggested that ground beef could raise the pH of the acidified medium in the microenvironment of the bacteria. Growth of, and toxin production by *Clostridium botulinum* at pH 4.3 required proteins from skimmed milk or soya (Smelt *et al*., 1982). The protective effects of food

matrices on bacteria often observed in laboratory conditions are also observed in vivo: in rats, a 100 fold higher *Lactococcus lactis* survival rate in the stomach was reported with bacteria ingested in feed (Drouault *et al*., 1999).

The importance of the gastric juice as a bactericidal barrier has been known for almost a century, being one of the first lines of defence (host unspecific immunity) against ingested pathogenic organisms (Tortora *et al*., 2002). It has been already described, in some previous works, that the bactericidal activity of stomach is predominantly pH (chloridric acid) dependent (Just and Daeschel, 2003). But the acidification of the ingested food is not the only physiological mechanism responsible for this activity. For example, salivary nitrite, under the acid conditions of the stomach is converted to nitrous acid and other unidentified nitrogenous metabolites that have considerable antibacterial activity (Xu *et al*., 2001). In comparison to the SGF, recognized as a bactericidal barrier against the ingested pathogens, the presence of wine, in an equivalent amount of a glass of wine in a meal (proportionally to the food in the model stomach), led to a significant additional cell inactivation effect.

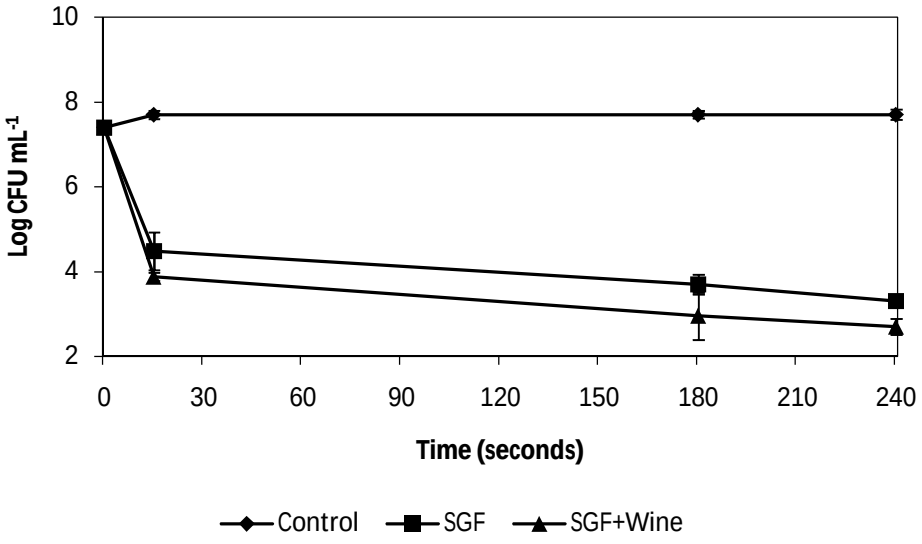

Fig. 9. The effect of synthetic gastric fluid (SGF) and the effect of SGF combined with wine on the vegetative cells of *B. cereus* ATCC 14579. Error bars represent the standard deviation of the mean of three replications.

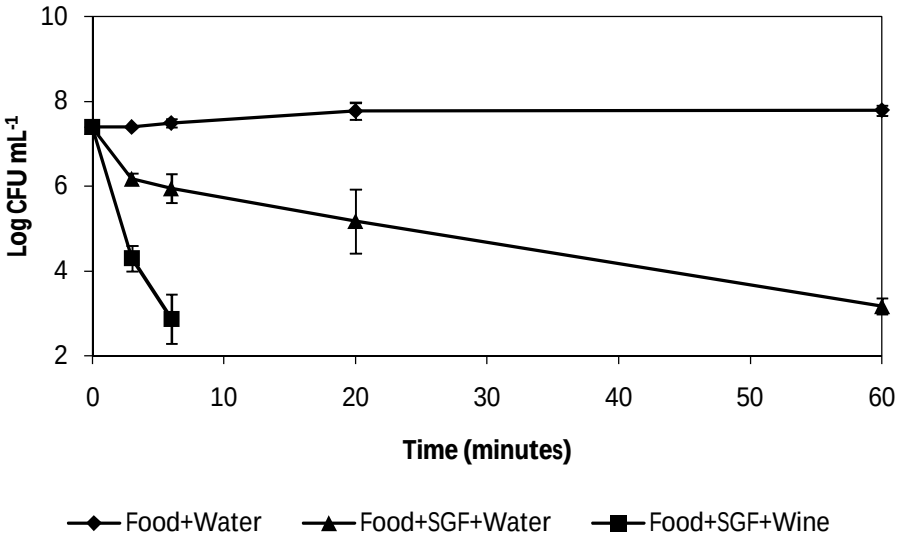

Fig. 10. The effect of different treatments on the vegetative cells of *B. cereus* ATCC 14579, in a model stomach system. Chicken-rice baby meal was the food matrix used. Error bars represent the standard deviation of the mean of three replications.

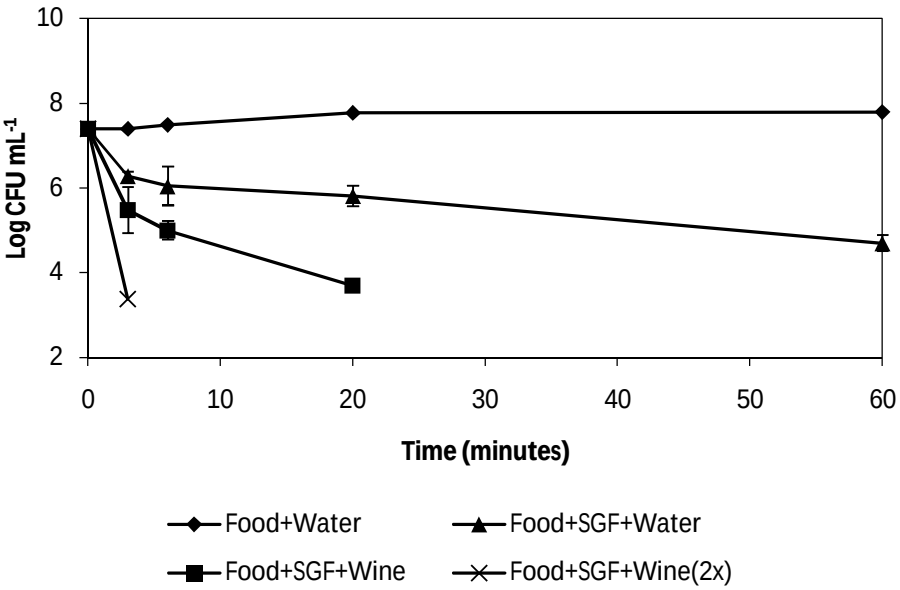

Fig. 11. The effect of different treatments on the vegetative cells of *B. cereus* ATCC 14579, in a model stomach system. Pasteurized fresh cheese was the food matrix used. Error bars represent the standard deviation of the mean of three replications.

3.3. Behavior of *B. cereus* spores during the simulated gastrointestinal passage

The experiments were carried out with spore suspensions that were first incubated in SGF and subsequently transferred to SIF. The spore counts of *B. cereus* ATCC 14579 are depicted in Fig. 12

and Fig. 14 and the total counts are presented in Fig. 13 and Fig. 15. In the SGF-SIF+Food+Water system there was a decrease in spore counts (reduction of 1.6 log cycles at t=240 min), which can be explained by spore germination, and an increase in the total counts (1.9 and 2.1 log cycles in chicken-rice and fresh cheese respectively), due to growth of vegetative cells. Wijnands *et al.* (2006) reported that the low pH of SGF may have a triggering effect on germination, resulting in subsequent growth of vegetative cells in SIF. The changes in spore counts were not always comparable to the changes in total counts, as only a small percentage of germinating spores can result in clear vegetative growth. It is interesting to observe that the treatment SGF-SIF+Food+Wine led to a lower increase in the total counts, showing that wine exerted an inactivation effect on the cells originated from the germination of the spores. Thus, we can say that wine, even in the presence of certain foodstuff, may hinder the growth and/or inactivate vegetative cells of *B. cereus* in the small intestine. Once again, pasteurized fresh cheese appeared to be more protective to the *B. cereus* cells than the chicken-rice food (see a possible explanation in section 3.2)

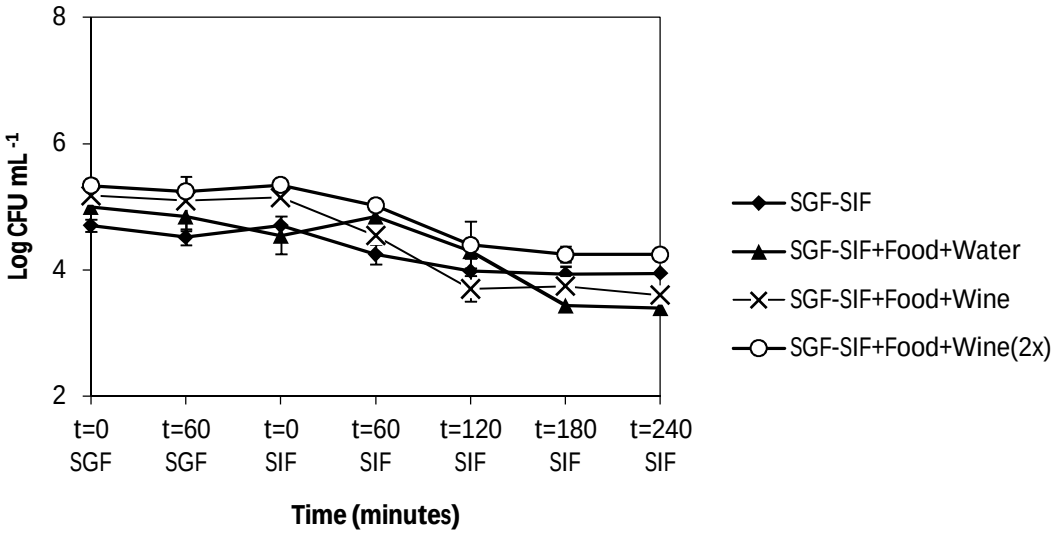

Fig. 12. The effect of different treatments on the spore counts of *B. cereus* ATCC 14579, considering the passage from simulated gastric fluid (SGF) to simulated intestinal fluid (SIF). Chicken-rice baby meal was the food matrix used. Error bars represent the standard deviation of the mean of three replications.

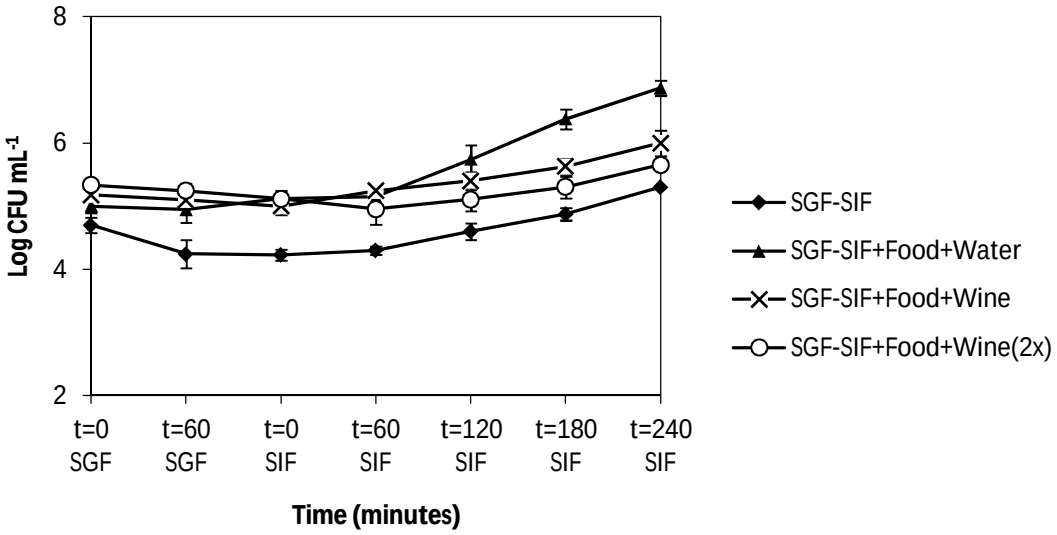

Fig. 13. The effect of different treatments on the total counts of *B. cereus* ATCC 14579, considering the passage from simulated gastric fluid (SGF) to simulated intestinal fluid (SIF). Chicken-rice baby meal was the food matrix used. Error bars represent the standard deviation of the mean of three replications.

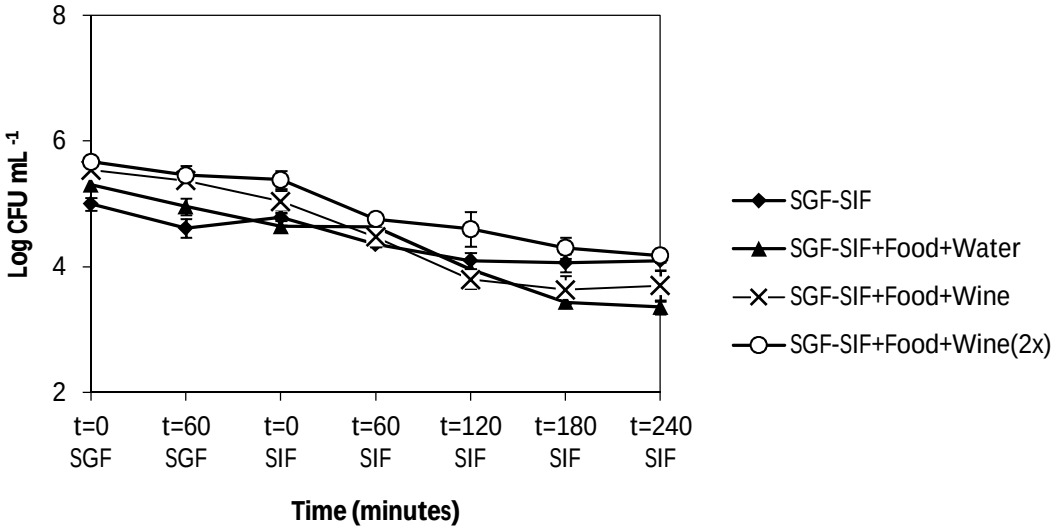

Fig. 14. The effect of different treatments on the spore counts of *B. cereus* ATCC 14579, considering the passage from simulated gastric fluid (SGF) to simulated intestinal fluid (SIF). Pasteurized fresh cheese was the food matrix used. Error bars represent the standard deviation of the mean of three replications.

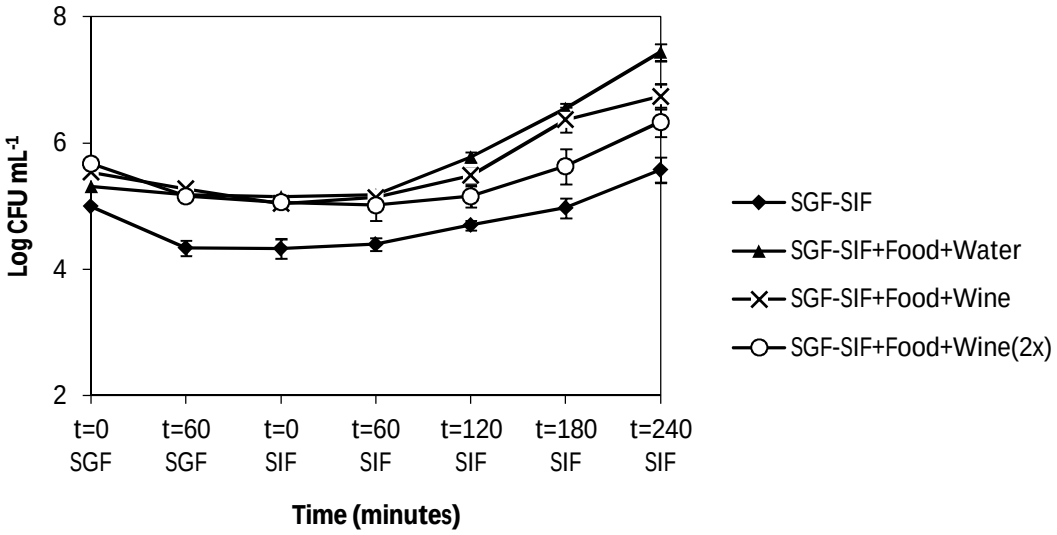

Fig. 15. The effect of different treatments on the total counts of *B. cereus* ATCC 14579, considering the passage from simulated gastric fluid (SGF) to simulated intestinal fluid (SIF). Pasteurized fresh cheese was the food matrix used. Error bars represent the standard deviation of the mean of three replications.

The probability of becoming ill after ingestion of *B. cereus* cells or spores depends on the number of viable *B. cereus* surviving gastric transit and then entering the small intestine. Clavel *et al*. (2004) showed that this number strongly depends on the form of the ingested cells (spores or vegetative cells), on the food they are ingested with and on stomach acidity. Stomach acidity is subject to variations with time (in particular that elapsed after eating) and age, and to variability among individuals. Situations (i.e. pH ≥ 4.5) in which *B. cereus* cells are not or only weakly affected by stomach transit to the small intestine are frequent: ingestion at the end of a copious meal, when stomach pH is at its maximum (Dressman *et al*., 1990), or ingestion by elderly people or people suffering from achlorhydria (the absence of gastric secretion) (Russell *et al*., 1993). In extreme situations with stomach pH ≥ 5.0, *B. cereus* can even grow during gastric transit. In addition, it has previously been reported that pre-exposition of cells to nonlethal acid pH induced an acid tolerance response (ATR) in *B. cereus* (Browne and Dowds, 2002; Jobin *et al*. 2002). Such an inducible ATR is an important component of bacterial survival. Reliable quantitative risk assessment, and establishing minimal infective dose–response or dose–response curves for enterotoxigenic *B. cereus* must thus allow for these multiple factors affecting survival during gastric transit. Wijnands *et al.* (2006) described a static system, mimicking the gastric and intestinal fluids in a state resembling feeding, i.e. including pepsin, bile salts and pancreatic secretion, but without food components present. Thus, possible protection of microorganisms by food or food components, possible induction of germination of spores by food or food components, and possible influences of the small intestinal epithelium are not taken into account. Conversely, in this thesis, the potential protective effect of food on spore and vegetative cell counts was evaluated and our results demonstrated that in fact food induces germination of spores and subsequent growth of vegetative cells.

In our study, we opted to expose the spore suspensions 1 h in SGF, as the spore counts after the initial drop verified after 30 min of exposure to SGF seem not to vary significantly (data not shown).

Some strains used by Wijnands and co-workers (2006) showed a similar behavior. According to this author**,** several explanations could be given for this phenomenon. Firstly, this initial drop in spore count could be due to the production procedure, by combining freezing (storage temperature) and heating the spores at 80 °C for 10 min, resulting part of them to become "activated". This activation caused these spores to die in the heat treatment needed to assess the numbers of spores during the exposure to simulated gastric fluid. A second explanation could be that aggregation of spores as a protection against adverse conditions resulted in an under-estimation of the spore content. When subsequently transferred to SIF, the change of conditions can then result in dissolving the aggregates, and hence better estimates of spore counts. And thirdly, the phenomenon may be explained by the method of investigation of the samples. For the determination of the total count dilutions were made from the sample taken from SGF, but for determination of the spore count in the first tenfold dilution of the original sample was heat treated. Therefore the composition of the heat treated sample was different from the original sample, which may have modified the behavior of *B. cereus* strain tested.

Accordingly to Wijnands *et al*. (2006) we reaffirm that approximately 3 h of intestinal conditions is necessary to note clear increases in CFU counts. Consequently, we can say the time in these experiments is about 4 h, i.e. 1 h exposure to gastric conditions and 3 h to intestinal condition. To evaluate the impact of these findings, several issues have to be taken into consideration. Firstly, *in vitro* production of enterotoxins, the actual perpetrators of diarrhoeal disease, is observed to start when the total count is about 10^7 CFU mL^{-1}. Such counts are normally encountered in the (late) exponential growth phase. Comparatively, for routine production of enterotoxins, a growth period of 5 to 6 h is used (Beecher and Lee Wong, 1994b; Dietrich *et al*., 1999). Within this time-span the total count increases from approximately 10^7 to 10^9 CFU mL^{-1}. Secondly, the time of onset of symptoms lies between 6 and 24 h after consumption of contaminated food (Kramer and Gilbert, 1989). And thirdly, the production of enterotoxin in micro-aerobic conditions like the small intestine is comparable with aerobic conditions but growth is slower (Granum *et al*., 1993). Therefore, relatively fast adapting strains producing high amounts of enterotoxins would therefore be the most important to set off the diarrhoeal syndrome. Since the minimal incubation period for the diarrhoeal syndrome is approximately 6 h, relatively fast adapting strains producing high amounts of enterotoxins are required for onset of the symptoms. Wijnands *et al.* (2006) also conclude that spores of mesophilic strains of *B. cereus* germinate better and grow faster in simulated gastrointestinal conditions than psychrotrophic strains. Therefore, these researchers conclude that mesophilic strains, when ingested in equal amounts as psychrotrophic strains, may be more important for the onset of diarrhoeal symptoms caused by *B. cereus*. Although psychrotrophic strains are most important as food contaminants, mesophilic strains appear most important for the onset of disease. However, other factors are also important with respect to the onset of disease: The level of contamination, the ability to produce enterotoxins, and the level at which enterotoxins are produced. Their results do not exclude psychrotrophic strains as provokers of diarrhoeal disease, they emphasize the ability of mesophilic strains to cope better with certain circumstances in the intestinal tract than psychrotrophic strains. In our work, we used mesophilic strains (*B. cereus* ATCC 11778 and *B. cereus* ATCC 14579). Based on our data, it is reasonable to affirm that drinking wine at a meal may lowering the probability of

becoming ill due to ingestion of *B. cereus*, as wine is capable to significantly reduce the number of vegetative cells in the stomach and reduce the impact of the germination of spores that may occur in the small intestine.

4. Conclusions

This is, to our knowledge, the first study reporting the antimicrobial activity of wine against *B. cereus*. The results of this work clearly show, via *in vitro* tests, that wine exerts a strong inactivation effect against vegetative cells of two *B. cereus* strains. Wine almost instantly inactivated *B. cereus* stationary phase vegetative cells to undetectable numbers. Thus, further inactivation assays were carried out with wine diluted with water (1:4 and 1:8). Nevertheless, *B. cereus* spores were highly resistant to direct wine exposure, being the changes in the counts lower than 1.0 log cycles. The influence of wine components (ethanol, organic acids, low pH and phenolic compounds) was investigated. Ethanol, when used in separate, had a negligible influence on the survival of *B. cereus* cells over the timescale studied. Wine organic acids were found to have a noteworthy inactivation effect. When organic acids were combined with ethanol, a slight synergistic effect was observed leading to a similar inhibition pattern as that of wine. This apparent synergistic effect is in accordance with the reports of other authors, with concern to other food-borne pathogens (Moretro and Daeschel, 2004). In this study, lactic acid supplemented with ethanol, when tested alone, was found to have the strongest activity (amongst the organic acids tested). The phenolic compounds (ferulic acid, *p*-coumaric acid, quercetin, kaempferol and resveratrol) showed no activity against *B. cereus* cells at the concentration tested, even during an extended time-scale. Regarding data obtained in simulated gastric medium, in a meal context, we can conclude that the ingestion of wine during a meal substantially diminishes the number of *B. cereus* cells persisting in the alimentary tract. The level of inactivation depends, however, in the type of food present (protective role). We also evaluated the behavior of *B. cereus* spores under gastrointestinal conditions. In a consumption-like scenario, the treatment SGF-SIF+Food+Wine led to lower total counts when compared to the treatment SGF-SIF+Food+Water, showing that wine partially inhibited the cells growth that occurs under simulated intestinal medium.

B. cereus vegetative cells/spores play a major role in spoilage of food products (Kramer and Gilbert, 1989). It may also be present in a diversity of foods such as pasta, rice, dairy products, dried foodstuffs, vegetables, fruit, seafood, meat and poultry (Schoeni and Wong, 2005), thus detection of the bacterium is not always the main issue for food safety purposes. Instead, ability to detect the possibly harmful strains, or their toxic products, is the highly desired goal. Our work provided some evidence that suggests drinking wine with meals leads to a reduction of the number of viable counts of *B. cereus* cells or spores in the gastrointestinal tract, thus lowering the risk of infection the aforementioned food-borne pathogen may cause. The antimicrobial effect of wine in marinades can be expected to be higher than this, due to the relatively large periods of exposition time (one to several hours), and to the synergistic effect with other substances (spices, vinegar, etc) commonly used in these preparations. Friedman *et al.* (2007) have evaluated bactericidal activities against *B. cereus*, *E. coli* O157:H7, *Listeria monocytogenes*, and *Salmonella enterica* of several antimicrobial wine recipes, each consisting of red or white wines extracts of oregano leaves with added garlic juice and oregano oil. The wine marinades were highly effective against the four pathogens. Moreover, the red wine polyphenolics isolated by chromatography from red wine exhibited exceptionally high activity at nanogram levels against the two *B. cereus* strains tested. These observations showed that wine

marinades and their constituents can also be used to inactivate pathogenic *B. cereus* strains. Consumption of feeds and foods treated with antimicrobial wine formulations may also benefit therapy of infectious diseases of animals and humans. Wine, used as a beverage or as a marinade, may be expected to diminish the incidence of *B. cereus* illnesses.

5. Future work

- It would be important to extend the study of the inactivation effect of wine to a larger number of strains of *B. cereus* to evaluate possible “strain-to-strain” variations on the resistance to wine.
- Further work may include the study of the effect of wine on the production of enterotoxins (haemolysin BL, non haemolytic toxin and cytotoxin K) in the simulated intestinal fluid (SIF), since these toxins are produced in the small intestine and are responsible for the diarrhoeal syndrome, and thus may contribute to the onset of disease.
- It would be interesting to test more phenolic compounds (since there is a wide diversity of these compounds present in wine) and use other red wines and/or white wines, to better understand the role of the phenolic compounds on inhibiting microbial growth.
- Further studies may include the use of different amounts and types of food commodities, as the resistance of *B. cereus* strongly depends on the type of food present in the simulated gastric/intestinal fluids, as seen in this work.
- Another step we may take is the improvement of the gastrointestinal system used in our trials, getting closer to the *in vivo* human digestion conditions. This may be obtained considering peristaltic movements, the specific immune responses or competition with natural microflora, which are present in the human digestion.

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