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Structural and functional changes in *Alicyclobacillus acidoterrestris* after the application of several sub-lethal physical or chemical treatments

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A c a d e m i c T r i e n n i u m

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STATEMENT OF ORIGINALITY

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution, and contains no material previously published or written by another person, except where due reference has been made in the text.

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ABSTRACT

Scientific background: *Alicyclobacillus* spp. includes spore-forming and thermo-acidophilic microorganisms, usually recovered from soil, acidic drinks, orchards and equipment from juice producers. The genus includes different species and sub-species, but *A. acidoterrestris* is generally regarded as the most important spoiler for acidic drinks and juices. (i) The investigation of microbial community evolution was performed, since soil species could acquire specific phenotypic traits in relation to available nutrients. (ii) A variety of physical and chemical approaches to control *A. acidoterrestris* were proposed, and the study of sub-lethal injury related to extend of damage was examined. (iii) *In vitro* stress treatments were tested *in vivo*.

Open questions: (i) Few data are available on the genotyping and phenotyping traits of *A. acidoterrestris*; (ii) Few data are available on the effect of innovative approaches to control *A. acidoterrestris*; and no data are available on the sub-lethal injury in *A. acidoterrestris* after invasive treatments; (iii) Few data are available on the control of *A. acidoterrestris* in complex conditions.

Aims: (i) Selection and characterization of wild strains of *A. acidoterrestris* as a contribution to species characterization, (ii) studying the structural and functional changes that may affect alicyclobacilli and *Bacillus* spp. (used as reference) after the application of several sub-lethal physical or chemical treatments, and (iii) validation in a food to assess if an injury could occur also in a complex system.

Planning of the research: In the first part, 25 wild strains of *A. acidoterrestris* from soil (23 strains) and spoiled pear juice (CB-1 and CB-2) were isolated, identified, genotyped and phenotyped. In the second part, 4 strains of *A. acidoterrestris* (the type strain, 2 wild strains isolated from soil and a wild strain isolated from spoiled pear juice) and 2 species of *Bacillus* spp. were studied under *in vitro* conditions after the application of chemical, physical and combined treatments to assess their susceptibility and the presence of sub-lethal injury. The characterization of damage was also performed. Finally, in the last step the presence of sub-lethal injury *in vivo* was investigated.

Materials and Methods: (i) Genotypic traits were performed through examining of 16S rRNA and RAPD PCR (Random-Amplified-Polymophic-DNA). Phenotypical traits were assessed on the opportune laboratory media. (ii) Injury characterization was evaluated by Leakage of UV-absorbing substances and BSA protein assay. (iii) For *in vivo* assay, clarified apple juice was used.

Results: (i) Data of soil-borne strains pinpointed that they could be divided into three blocks, represented by soil strains and by strains moving from soil to other niches. In this context, phenotyping and genotyping did not group the strains in the same way and many strains phylogenetically different showed the same phenotypic trend, thus suggesting that *A. acidoterrestris* could exist as a genomovar. In addition, the strain CB-1 was distant from other alicyclobacilli, although it possessed the same traits than the other isolate from juice (CB-2); therefore, it is probably a fast-clock organism or the beginning on an alternative pathway in alicyclobacilli evolution. (ii) Alternative approaches applied to control 4 strains of *A. acidoterrestris* determined a strain-dependent effect probably related to the isolation source; however, the presence of sub-lethal injury related to released nucleic acids, proteins and DPA by spores pointed out the damage on coat and cortex with loss of barrier properties. (iii) The study performed in apple juice confirmed the results obtained in lab media.

Significance and Impact of PhD research: From the earliest times, *A. acidotererstris* was regarded as an important target in the quality control of acidic beverages. Since soil borne species often contaminate fruit juices and do not need strict extreme requirements of acidity and high temperature for survival, it is a great concern to investigate whether and how soil species could evolve from their ecological niches in microbial community to new environments as fruit juices. The present PhD thesis contributed to species characterization through selection and characterization of wild strains of *A. acidoterrestris*, and provided essential knowledge to validate the genotyping and phenotyping evolution of some strains of *A. acidoterrestris.* Moreover, this PhD project was the first attempt to investigate the changes that may affect alicyclobacilli after sub-lethal stress application; the release of proteins, nucleic acids and DPA was strictly strain dependent. Finally, the novelty of this PhD thesis was the study of sub-lethal injury on *A. acidoterrestris* spores *in vivo*.

Future trends: A future perspective could be a focus on a reassessing of strains nomenclature, considering the evolution of some strains of *Alicyclobacillus* genus, and the different behavior of the type strain compared to the other targets tested.

Key words: bacteria isolation, spore, *Alicyclobacillus acidoterrestris*, inhibition, antimicrobials, HPH, US, heating, sub-lethal injury, DPA, proteins, nucleic acid, apple juice.

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PUBLICATIONS ARISING FROM THIS PhD PROJECT

- Bevilacqua A., **Ciuffreda E.,** Sinigaglia M., Corbo, M.R. (2014). Effects of lysozyme on *Alicyclobacillus acidoterrestris*. *International Journal of Food Science and Technology* 49, 224-229.
- Bevilacqua A., **Ciuffreda E.,** Sinigaglia M., Corbo, M.R. (2015). Spore inactivation and DPA release in *Alicyclobacillus acidoterrestris* under different stress conditions. *Food Microbiology*, 46, 299-306.
- Bevilacqua A., Mischitelli M., Pietropaolo V., **Ciuffreda E.,** Sinigaglia M., Corbo M.R. Genotypic and phenotypic heterogeneity in *Alicyclobacillus acidoterrestris*: a contribution to species characterization. *Plos One*, 10(10), e0141228.
- **Ciuffreda E.,** Bevilacqua A., Sinigaglia M., and Corbo, M.R. *Alicyclobacillus* spp.: new insights on ecology and keeping food quality through new approaches. *Microorganisms*, 3(4), 625-640.

Chapter 1. INTRODUCTION

1.1. Spoilage by *Alicyclobacillus* bacteria in juice and beverage products

1.1.1. The general traits of Alicyclobacillus spp.

The genus *Alicyclobacillus* belongs to the family of *Alicyclobacillaceae* (Da Costa *et al*., 2010), and consists of a group of thermo-acidophilic, strictly aerobic, heterotrophic, and spore-forming bacteria (Wisotzkey *et al*., 1992; Walls *et al.,* 1998).

First, alicyclobacilli were placed in the genus *Bacillus*, as they share with bacilli the characteristic of endospore formation. However, phylogenetic analysis based on sequence comparisons of the 16S rRNA showed that the species of the genus *Alicyclobacillus* belonged to a distinct line of descent within the low G + C (guanine + cytosine) Gram-positive lineage of *Bacteria* also including the closely related facultatively autotrophic species of *Sulfobacillus* spp. (Wisotzkey *et al*., 1992; Tourova *et al.,* 1994; Durand, 1996). Therefore, in 1992 they were allocated to a new genus called *Alicyclobacillus* due to the presence of ω-cyclohexyl or ω-cycloheptyl fatty acids as the major natural membrane lipid component (Oshima *et al.,* 1975; Hippchen *et al.*, 1981). These ω-alicyclic fatty acids may be associated with the heat and acid resistance of *Alicyclobacillus* spp. (Chang *et al.,* 2004), as they are responsible for the ability to survive typical pasteurization regimes applied during juice manufacturing, since *Alicyclobacillus* spp. may be present on fruit surfaces contaminated by soil during production and harvesting (Eiroa *et a.,* 1999). In single-strength juice, these microorganisms find a favourable environment for germination, growth, and spoilage (Chang *et al.,* 2004).

The genus originally consisted of three species, *A. acidocaldarius*, *A. acidoterrestris* and *A. cycloheptanicus*. Nowdays, it consists of 22 species isolated from various habits (Table 1.1).

Table 1.1: Phenotypic characteristics of *Alicyclobacillus* spp.

Species	T Range (°C)	Optimum T (°C)	pH Range	Optimum pH	ω-cyclohexane/ ω-cicloheptane fatty acids	References
A. acidiphilus	20-55	50	2.5-5.5	3.00	ω-cyclohexane	Matsubara *et al.,* 2002
A. acidocaldarius	35-70	55-60	2.5-6.0	4.05	ω-cyclohexane	Wisotzkey *et al.,* 1992
A. acidoterrestris	20-55	40-50	2.0-6.0	3.5-4.5	ω-cyclohexane	Wisotzkey *et al.,* 1992
A. aeris	25-35	30	2.0-6.0	3.05	none	Guo *et al*., 2009
A. cellulosilyticus	40-67.5	55	3.5-6.5	4.08	ω-cyclohexane	Kusube *et al.,* 2014
A. contaminans	35-60	50-55	3.0-6.0	4.0-4.5	none	Goto *et al.,* 2007a
A. cycloheptanicus	30-55	50	3.0-5.5	4.00	ω-cycloheptane	Wisotzkey *et al.,* 1992
A. dauci	20-50	40	3.0-6.0	4.00	ω-cyclohexane	Nakano *et al.,* 2014
A. disulfidooxidans	14702	35	0.5-6.0	1.5-2.5	ω-cyclohexane	Karavaiko *et al.,* 2005
A. fastidiosus	20-55	40-45	2.0-5.5	4.0-4.5	ω-cyclohexane	Goto *et al.,* 2007a
A. ferrooxydans	17-40	28	2.0-6.0	3.00	none	Jiang *et al.,* 2008
A. herbarius	35-65	55-60	3.5-6.0	4.5-5.0	ω-cycloheptane	Goto et al., 2002a
A. hesperidum	35-60	50-53	2.5-5.5	3.5-4.0	ω-cyclohexane	Albuquerque *et al,* 2000
A. kakegawensis	40-60	50-55	3.0-6.5	4.0-4.5	ω-cycloheptane	Goto *et al.,* 2007a
A. macrosporangiidus	35-60	50-55	3.0-6.5	4.0-4.5	none	Goto *et al.,* 2007a
A. pomorum	30-60	45-50	2.5-6.5	4.5-5.0	none	Goto *et al.,* 2003
A. sacchari	30-55	45-50	2.0-6.0	4.0-4.5	ω-cyclohexane	Goto *et al.*, 2007a
A. sendaiensis	40-65	55	2.5-6.5	5.05	ω-cyclohexane	Tsuruoka *et al.,* 2003
A. shizuokaensis	35-60	45-50	3.0-6.5	4.0-4.5	ω-cycloheptane	Goto *et al.,* 2007a
A. tengchongensis	30-50	45	2.0-6.0	3.02	ω-cycloheptane	Kim *et al.,* 2015
A. tolerans	20-55	37-42	1.5-5.0	2.5-2.7	ω-cyclohexane	Karavaiko *et al.,* 2005
A. vulcanalis	35-65	55	2.0-6.0	4.00	ω-cyclohexane	Simbahan *et al.,* 2004

Twelve species of *Alicyclobacillus*, namely *A. acidocaldarius*, *A. acidoterrestris* (Wisotzkey *et al*., 1992), *A. hesperidum* (Albuquerque *et al.,* 2000), *A. acidiphilus* (Matsubara *et al.,* 2002), *A. sendaiensis* (Tsuruoka *et al.,* 2003), *A. disulfidooxidans, A. tolerans* (Karavaiko *et al.,* 2005), *A. fastidiosus*, *A. sacchari* (Goto *et al.,* 2007a), *A. vulcanis* (Simbahan *et al.,* 2004), *A. cellulosilyticus* (Kusube *et al.,* 2014), and *A. dauci* (Nakano *et al.,* 2015), contain ω-cyclohexane fatty acids, whereas *A. cycloheptanicus* (Deinhard *et al*., 1987), *A. kakegawensis*, *A. shizoukensis* (Goto *et al.,* 2007a), *A. herbarius* (Goto *et al.,* 2002a), and *A. tengchongensis* (Tsuruoka *et al.,* 2003) contain ω-cycloheptane fatty acids.

Recent studies (Glaeser *et al.,* 2013) pinpointed the lack of these fatty acids in *A. aeris* (Tsuruoka *et al.,* 2003), *A. ferrooxydans* (Jiang *et al.,* 2008), *A. pomorum* (Goto *et al.,* 2002a), *A. macrosporangiidus* and *A. contaminans* (Nakano *et al.,* 2015). These species also possess a lot of phenotypic characteristics different from the classical traits of *Alicyclobacillus* spp., including growth temperature, assimilation of various carbon sources, production of acids from a range of compounds, and the ability to grow chemoautotrophically using ferrous iron, elemental sulphur and tetrathionate as electron donors (Goto *et al.,* 2003-2007a; Guo *et al*., 2009). However, genotypic analysis showed that they were phylogenetically related to members of the genus *Alicyclobacillus* (Goto *et al.,* 2003-2007a; Jiang *et al.,* 2008; Guo *et al*., 2009).

Generally, interest in *Alicyclobacillus* spp. focused on the study of the role of ω-cyclic fatty acids and hopanoids on membrane function (Poralla *et al*., 1980; Kannenberg *et al.,* 1984). These ω-alicyclic fatty acids could be associated with the strong heat and acid resistance of *Alicyclobacillus* spp. (Chang *et al.,* 2004). Kanneberg *et al.* (1984) demonstrated that lipids, which contain ω-cyclohexane fatty acid, packed densely, resulting in low diffusion at high temperatures. Wisotzkey *et al.* (1992) proposed that this property provided an advantage at high temperatures or low pH. Lipids containing fatty acids with a cyclohexane ring could stabilize the membrane structure and maintain the barrier functions of prokaryotic membranes at high temperatures (Kannenberg *et al.,* 1984). These fatty acids might contribute to the heat resistance of *Alicyclobacillus* by forming a protective coating with strong hydrophobic bonds. These hydrophobic bonds might stabilize and reduce membrane permeability in extreme acidic and-high temperature environments (Poralla *et al*., 1980; Kannenberg *et al.,* 1984; Wisotzkey *et al*., 1992).

However, some authors reported that the presence of ω-cyclohexyl fatty acids is not essential in protecting alicyclobacilli from high temperatures and low pH, because there are other microorganisms, such as *Curtobacterium pusillum* (Suzuki *et al.,* 1981) and *Propionibacterium cyclohexanicum* (Kusano *et al.,* 1997), that also possess ω-alicyclic acids, and are neither thermophilic nor acidophilic (*Propionibacterium cyclohexanicum* is an acidotolerant bacterium).

The possible way to justify the adaptation to extreme environments of alicyclobacilli might be the presence of hopanoids in their cells (Poralla *et al*., 1980; Hippchen *et al.*, 1981; Chang *et al.,* 2004). The hopane glycolipids are structurally similar to cholesterol and have a condensing effect on the cell membrane due to a decrease of the acyl chain lipids' mobility. At low pH the condensing action hinders the passive diffusion of protons through the membrane, facilitating the establishment of an approximately neutral cytoplasmic pH (Poralla *et al*., 1980). The low membrane viscosity induced by branched-chain fatty acids is thus counterbalanced by the presence of a higher concentration of hopanoids, leading to a more stable membrane.

1.1.2. Characteristic of Alicyclobacillus spp.

The genome study of *Alicyclobacillus* spp. was fundamental for its phylogenetic position. In GenBank there are the complete genome sequences of each species. 16S ribosomal RNA (rRNA) sequencing is a common amplicon sequencing method used to identify and compare bacteria present within a given sample. 16S rRNA gene sequencing is a well-established method for studying phylogeny and taxonomy of samples from complex microbiomes or environments that are difficult or impossible to study. Wisotzkey *et al.* (1992) proposed that 16S rRNA gene sequences must be at least 92% similar to belong to the genus *Alicyclobacillus.* Within closely related species, especially belonging to the *A. acidocaldarius* group, the similarity is over 98%.

Table 1.2 reports the similarity level of 16S rRNA and G + C content of DNA in *Alicyclobacillus* spp.

G + C content in DNA is 48.6% to 63.0%; it is *ca*. 62% for *A. acidocaldarius*, and 55% for the other species of *Alicyclobacillus* (Goto *et al.,* 2007a; Walker *et al.,* 2008). The content of G+C in *A. acidoterrestris* varies between 51.5% and 53.3% depending on the strain, with the type strain, *A. acidoterrestris* DSM 3922^{T}, having a G + C amount of 51.5% (Tsuruoka *et al.,* 2003; Walker *et al.,* 2008; Bevilacqua *et al.,* 2008b).

Table 1.2: Genotypic characteristics of *Alicyclobacillus* spp.

Species	DNA G+C content (%)	Homology with 16S rRNA of some other species of the genus	References
A. acidiphilus	54.1	*A. acidoterrestris* (96.6%)	Matsubara *et al.,* 2002
A. acidocaldarius	61.89	*A. acidoterrestris* (98.8%)	Mavromatis *et al.,* 2010
A. acidoterrestris	51.5	*A. acidocaldarius* (98.8%)	Wisotzkey *et al.,* 1992
A. aeris	51.2	*A. ferrooxydans* (94.2%)	Guo *et al.,* 2009
A. cellulosilyticus	60.8	*A. macrosporangiidus* (91.9%)	Kusube *et al.,* 2014
A. contaminans	61.1-61.6	*Alicyclobacillus* (92.3-94.6%)	Goto *et al.,* 2007a
A. cycloheptanicus	57.2	*Alicyclobacillus* (92.7-93.2%)	Wisotzkey *et al.,* 1992
A. dauci	49.6	*A. acidoterrestris* (97.4%) and *A. fastidiosus* (97.3%)	Nakano *et al.,* 2015
A. disulfidooxidans	53	*A. tolerans* (92.6%)	Karavaiko *et al.,* 2005
A. fastidiosus	53.9	*Alicyclobacillus* (92.3-94.6%)	Goto *et al.,* 2007a
A. ferrooxydans	48.6	*A. pomorum* (94.8%)	Jiang *et al.,* 2008
A. herbarius	56.2	*Alicyclobacillus* (91.3-92.6%) and *Sulfobacillus thermosulfidooxidans* (84.7%)	Goto *et al.,* 2002a
A. hesperidum	60.3	*Alicyclobacillus* (97.7-98%)	Albuquerque *et al.,* 2000
A. kakegawensis	61.3-61.7	*Alicyclobacillus* (92.3-94,6%)	Goto *et al.,* 2007a
A. macrosporangiidus	62.5	*Alicyclobacillus* (92.3-94.6%)	Goto *et al.,* 2007a
A. pomorum	53.1	*Alicyclobacillus* (92.5-95.5%)	Goto *et al.,* 2003
A. sacchari	56.6	*Alicyclobacillus* (92.3-94,6%)	Goto *et al.,* 2007a
A. sendaiensis	62.3	*A. vulcanis* (96.9%)	Simbahan *et al.,* 2004
A. shizuokaensis	60.5	*Alicyclobacillus* (92.3-94.6%)	Goto *et al.,* 2007a
A. tengchongensis	53.7	*Alicyclobacillus* (90.3-92.8%)	Kim *et al.,* 2014
A. tolerans	48.7	*Alicyclobacillus* (92.1-94.6%) and *S. thermosulfidooxidans* (87.7%)	Karavaiko *et al.,* 2005
A. vulcanalis	62	*A. acidocaldarius* (97.8%)	Simbahan *et al.,* 2004

Guaiacol production is a common trait of the genus, although the amount of this compound is greatly variable (Bevilacqua *et al.,* 2008b). Regarding sugar metabolism, there is acid but not gas production. Different species could be divided into different phenotypic groups, depending on sugar metabolism, although there is considerable variation within species (Chang *et al.,* 2004; Goto *et al.,* 2007a). As in *Bacillus*, the major respiratory lipoquinone of *Alicyclobacillus* is menaquinone-7 (MK-7) (Goto *et al.,* 2007a). The branched respiratory chain of thermophilic bacilli is quite complex; MK-7 plays a fundamental role, as it is reduced by several dehydrogenases (malate, succinate, NADH).

NADH dehydrogenase is of type II and does not translocate H^+. Energy conservation occurs upon menaquinol oxidation by b_6c_1 complex and Cyt *caa3*. However, at high temperatures the concentration of dissolved oxygen quickly decreases, thus the Cyt pattern of cells suddenly changes (Gennis *et al.,* 2005).

Concerning the other metabolic traits, starch and gelatin hydrolysis, catalase and oxidase tests are generally species- and strain-dependent, as well resistance to 5% NaCl, nitrate reduction, catalase and oxidase activities (Shemesh *et al.,* 2014).

Alicyclobacillus spp. were described as strictly aerobic microorganisms; however, some authors reported alicyclobacilli growth with 0.1% oxygen in the headspace (Goto *et al.,* 2007a). Alicyclobacilli generally grow as planktonic and free cells, but they could also produce a biofilm under favorable conditions (Dos Anjos *et al.,* 2013; Shemesh *et al.,* 2014).

Alicyclobacillus spp. are the type organisms to study and characterize thermostable and non-conventional enzymes (endoglucanase, esterases, α-galactosidase, arabinose isomerase, amylase and many others) (Peng *et al.,* 2013; Boyce *et al.,* 2015). These enzymes represent unique compounds due to their resistance to extreme conditions, as well as to their peculiarities in terms of structure (Bevilacqua *et al*., 2011a), e.g.:

1. Lower number of charged residues. The α-amylases extracted from *Alicyclobacillus* spp. contain *ca*. 30% fewer charged residues than their closest relatives.
2. Acidic and basic residues. More basic residues are exposed on the surface, whereas the acidic groups are buried on the interior.
3. Salt bridges. Pechkova *et al.* (2007) reported that an increase number of salt bridges results in greater compactness of the structure and thereby contributes to thermostability.

4. Cavities. Proteins from alicyclobacilli are more closely packed than the analogue molecules in mesophiles.
5. Proline. Thermostable proteins by alicyclobacilli show a higher content of proline and this amino acid is more common at the second position of the β-turns.

There is also a last detail on a possible benefit and positive role of *Alicyclobacillus* spp. These microorganisms are generally labeled as spoilers or super-spoilers for acidic drinks; however, Yuan *et al.* (2014) heat-inactivated alicyclobacilli cells and used them as adsorbing tools to remove/reduce patulin in apple juice. The removal rate was *ca*. 80% after 24 h.

1.1.3. Ecology of the genus Alicyclobacillus, with a special focus on the species A. acidoterrestris

Spoilage of commercially available pasteurised fruit juice by *Bacillus acidoterrestris* was first reported in Germany in 1982 (Cerny *et al.,* 1984). Several other cases of spoilage by similar bacteria occurred in Japan, Europe and the U.S.A. in 1990 (Splittstoesser *et al.,* 1994; Jensen *et al.,* 2003). Though spoilage by *Alicyclobacillus* spp. was previously regarded as sporadic, a 1998 survey by the National Food Processors Association (NEPA) in the USA reported that 35% of the fruit juice manufactures experienced spoilage caused by acidophilic spore-formers suspected to be *A. acidoterrestris* (Walls *et al.,* 1998; Chang *et al.,* 2004). As a matter of fact, *A. acidoterrestris* caused spoilage of isotonic water and lemonade (Yamazaki *et al.,* 1996), carbonated fruit juice drinks (Pettipher *et al.,* 1997), canned diced tomatoes (Walls *et al.,* 1998) and fruit pulps, Australian shelf-stable iced tea containing berry juice (Duong *et al.,* 2000), apple, pear, orange, peach, mango and white grape juices (Chang *et al.,* 2004). *Alicyclobacillus* strains were also isolated from orchard soil and a fruit concentrate production factory in South Africa; many strains were identified as *A. acidoterrestris*, but *A. acidocaldarius* was also recovered (Groenewald *et al.,* 2009).

Fruit contaminated by soil during harvest or the use of unwashed or poorly washed raw fruits during processing are the most common sources of *A. acidoterrestris* (Chang *et al.,* 2004). Spores are also introduced into the manufacturing facilities by soil associated with employees. Water can also be a source of *A. acidoterrestris* spores; McIntyre *et al.* (1995) isolated the same strain of *Alicyclobacillus* from spoiled juice and from water used by a processing facility. Recently, apple and pear flavourings have been reported as significant sources of *A. acidoterrestris* (Oteiza *et al.,* 2014).

The fruit juice industry now acknowledges *A. acidoterrestris* as a major quality control target for thermal treatment efficacy (Yamazaki *et al.,* 1996; Pettipher *et al.,* 1997; Silva *et al*., 2004; Bevilacqua *et al.,* 2008b; Walker *et al.,* 2008). It represents the greatest threat of spoilage in acidic foods because spores are able to germinate and grow at low pH (Splittstoesser *et al.,* 1994). Spoilage by *Alicyclobacillus* is difficult to detect because *A. acidoterrestris* does not produce gas during growth. The spoiled juice appears normal with little or no change in pH. Occasionally, turbidity and/or white sediment may be formed at the bottom of the container. Members of *Alicyclobacillus* genus cause some clarified fruit juices to have a light sediment, cloudiness or haze (Walls *et al.,* 1998).

However, the most common characteristic of *Alicyclobacillus* contamination is a "smoky", "medicinal", "antiseptic" off-odour associated with guaiacol (2-methoxyphenol) (Pettipher *et al.,* 1997; Jensen *et al.,* 2003), and other halophenols, including 2,6-dibromophenol and 2,6-dichlorophenol. Guaiacol is a product of microbial metabolism in fruit juices and dairy foods. It is formed directly from vanillic acid by nonoxidative decarboxylation (Chang *et al.,* 2004). Many soil bacilli can decarboxylate vanillic acid to guaiacol. Vanillic acid is naturally derived from the plant polymer lignin and can be also introduced to the beverage as an ingredient. *Alicyclobacillus* spp. can also convert vanillic acid to vanillyl alcohol, catechol and methoxyhydroquinone (Chang *et al.,* 2004). Tyrosine is another possible precursor for guaiacol formation. Apple juice contains approximately 4.1 mg tyrosine ml^{-1} juice and orange juice contains 3-13.5 mg tyrosine ml^{-1} (Jensen, 1999). Guaiacol production depends on the viable count of alicyclobacilli, strain, storage temperature, oxygen concentration in beverage, use of heat shock which encourages germination of the spores, and, finally, concentration of precursors to guaiacol, such as vanillin and tyrosine in the fruit juice (Pettipher *et al.,* 1997; Chang *et al.,* 2004; Bahçeci *et al.,* 2005; Goto *et al.,* 2008).

Fortunately, there is no evidence that *A. acidoterrestris* poses a human health risk. Neither the organism nor its metabolites have been associated with any form of illness and *A. acidoterrestris* is considered a non-pathogen (Borlinghaus *et al.,* 1997). However, in 2007 an endospore-forming organism was isolated from a blood sample from a 51-year-old woman on blood agar at 37 °C (Glaeser *et al.,* 2013). There is no evidence that this strain was the causal agent of an infection. Based on 16S rRNA gene sequence similarity comparisons, the strain was grouped into the genus *Alicyclobacillus*, most closely related to the type strain of *Alicyclobacillus pohliae* (94.7%), and was named *A. consociatus*. However, a reclassification was proposed for *A. pohliae* and *A. consociatus*

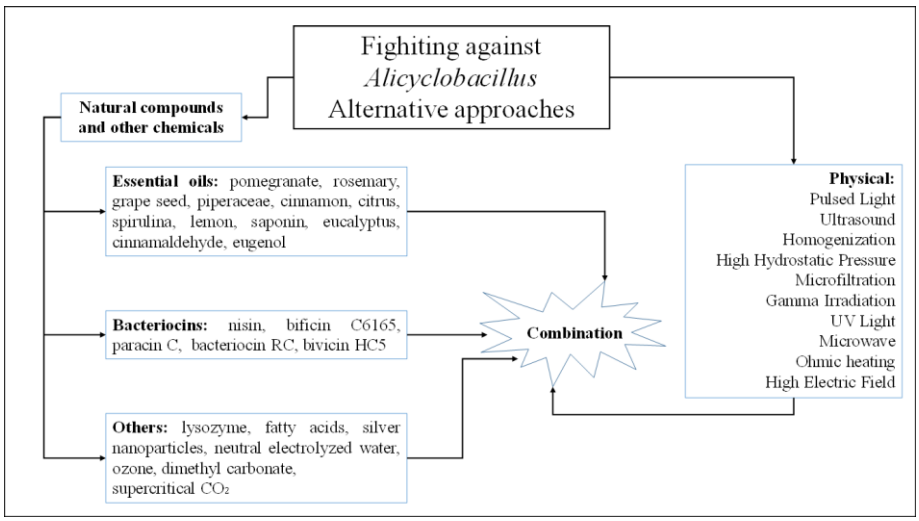
as *Effusibacillus pohliae* and *E. consociatus*, respectively, according to phylogenetic and phenotypic analysis showing that the monophyly of the genus *Alicyclobacillus* had been lost (Watanabe *et al.,* 2014).

1.1.4. Alternative approaches to mitigate Alicyclobacillus species associated with food spoilage

Pasteurization treatments on fruit juice are generally used to control bacterial contamination and increase shelf-life. The U.S. Food and Drug Administration requires all fruit juice sold in the United States to be either pasteurized or subjected to an equivalent process to achieve a mandated 5-log pathogen reduction in the juice (U.S. Food and Drug Administration, 2012). Typically fruit juices are pasteurized through a flash treatment, which uses high temperature/short time to preserve the organoleptic and nutritional properties of the juice (Lado *et al.,* 2002; Rivas *et al.,* 2006); for example, fruit juice is heated to around 88-96 °C for 30 s to 2 min and then rapidly cooled (Choi *et al.*, 2015). Unfortunately, *A. acidoterrestris* spores are able to survive thermal pasteurization and hot-fill hold processes (Splittstoesser *et al.,* 1994; Eiroa *et al.,* 1999; Orr *et al.,* 2002), but pasteurization stimulates the germination of the spores.

An interesting update and overview of the most important alternative approaches to control and/or reduce the contamination by *Alicyclobacillus* spp. is reported in the paper by Tianli *et al.* (2014). Figure 1.1 proposes a graphical abstract of the most used approaches.

Figure 1.1: Alternative approaches to control *Alicylobacillus* spp. (overview from 2005 to 2015)



The use of high-pressure homogenization (HPH) as a germicidal tool is one such promising technology. Its efficacy against cells and spores of *A. acidoterrestris* was reported by many authors (Bevilacqua *et al.,* 2007; Chen *et al.,* 2013); Bevilacqua *et al.* (2007) found that the antimicrobial effect was strain dependent and the spores were less sensible to HPH. The bactericidal activity of HPH could be due to the disruption of the cell wall and outer membrane (Vannini *et al.,* 2004); pressures could induce a structural rearrangement of proteins, an increased exposure of their hydrophobic regions and the disruption of their supramolecular structure under pressure allowing the components to move freely and become independent of the original structure (Vannini *et al.,* 2004).

Ultrasound or ultrasonic waves (US) is another non-thermal process; it is able to disaggregate bacterial clusters and inactivate bacteria through the mechanical, physical, and chemical effects of acoustic cavitation (Joyce *et al.,* 2003). Morales-de la Peña *et al.* (2011) tested the effectiveness of treatment time and power of US on the inactivation rate of *A. acidoterrestris*. The inactivation mechanism of US might lie in intracellular cavitation, localized heating, cell membranes being thinner, and free radical production (Piyasena *et al.,* 2003).

Another interesting approach might be also the use of essential oils (EOs) against alicyclobacilli.

Cinnamaldeyde (100-500 ppm) was able to prevent spore germination of *A. acidoterrestris* for at least 13 days (Bevilacqua *et al.,* 2008a); otherwise, eugenol acted as a strengthening element and, combined with cinnamaldeyde, reduced its amount in the system. Specifically, the experiments were performed in a commercial apple juice, thus highlighting that spore germination could be inhibited through the use of 80 ppm of eugenol and 40 ppm of cinnamaldehyde or alternatively through the combination of 40 ppm of eugenol with 20 ppm of cinnamaldehyde (Bevilacqua *et al.,* 2010). Although many authors have previously reported the suitability of citrus extracts as natural preservatives for the inhibition of a wide range of microorganisms (Fisher *et al.,* 2008; Settanni *et al.,* 2012), to the best of our knowledge there are few data on the spores of *A. acidoterrestris*. The results of *in vitro* assay (Bevilacqua *et al.,* 2013) confirmed that the bioactivity of citrus extracts was related to their concentrations with an effect called the "dose dependence effect" (DDE). Specifically, citrus and lemon extract showed MIC values (minimal inhibitory concentration) from 160 to 500 ppm against *A. acidoterrestris* spores.

The use of essential oils could be considered a new approach, as the stabilization of juices could be achieved through some natural molecules extracted from plants and fruits. Therefore, the consumer would not consider these antimicrobials as chemicals, but rather as natural ingredients of juices that might be added to commercial juices to improve the flavour of the products.

1.2. Sub-lethal injury of microorganisms

Many types of physical or chemical treatments are used to eliminate or inhibit the growth of microorganisms in foods. Food processing often involves the use of acidulants, additives, refrigeration, heating or freezing, drying, irradiation, high hydrostatic pressure or fermentation to control microbial proliferation and pathogens (Rahman, 1999).

After these treatments, microorganisms may be killed, survive (non-injured) or sub-lethally injured (Wu *et al.,* 2001). Many cellular modifications may be observed in sub-injured cells, like the susceptibility to selective agents and antimicrobials, leakage of intracellular material and modified metabolic activities. Injury is characterized by the capability of the microorganism to repair themselves and return to a normal physiological state with initiation of growth and cell division under favorable conditions. The restoration of lost capabilities in injured cells has been termed “resuscitation” because the cells are revived from apparent death (Hurst, 1984).

Sub-lethal damage in spores must be considered because of the complexity of the spore entity and its intrinsic high resistance to stress. Extent of sub-lethal damage and mechanisms of injury and repair are quite variable rely upon the conditions of stress and resuscitation. Knowledge of sub-lethal injury is indispensable in evaluating laboratory data, in developing or modifying food processes, and in preserving culture activity (Busta, 1976).

1.2.1. Effect and changes of microbial cells

Following injury, cells undergo a series of reactions to the injury that constitute the basis of all disease. Reactions of cells to injury can be classified in various ways and defined morphologically and, to some extent, physiologically and biochemically (Trump and Berezesky, 1995).

Many structural and functional components of organisms are affected, such as cell wall, cytoplasmic membrane or inner membrane, ribosomes, DNA, RNA, tricarboxylic- acid-cycle enzymes as well as many other enzymes (Ray, 1993; Wu, 2008).

The cell membrane appears to be the component most commonly affected (Jay *et al.,* 2005). Most injured cells have damaged permeability barriers (surface structures and the cytoplasmic membrane) that render them susceptible to many selective agents or antimicrobials. For example, microbial inactivation by pulsed electric fields (PEF) is believed to be caused by the effects of PEF on the cell membranes. Sublethally injured cells would become leaky during PEF but reseal to some extent after treatment (Weaver and Chizmadzhev, 1996; Garcìa *et al.,* 2003). Injured cells often lose some cellular material such as Mg^{2+}, K^{+}, amino acids, 260 nm absorbing material (nucleic acids), and 280 nm absorbing material (protein) through leakage into their surroundings (Hurst, 1977; Palumbo, 1989). For instance, frozen cells of *Escherichia coli* release amino acids, small molecular weight ribonucleic acids, and peptides. Heat-injured *Staphylococcus aureus* cells release potassium, amino acids, and proteins. Loss of intracellular compounds indicates damage to the cell membrane, which impairs growth and replication of a cell (Busta, 1976). Additionally, some injured cells encounter changed macromolecules within cells, and damage to the functional components that are related to their metabolic activities, thus causing metabolic injury (Ray, 1979; Jay *et al.,* 2005). Iandolo and Ordal (1966) and Allwood and Russell (1968) reported that ribosomal ribonucleic acid was degraded in heated cells of *S. aureus* and *Salmonella* Typhimurium. Heat-injured *S. aureus* have decreased catabolic capabilities and reduced activities of selected enzymes of glucose metabolism (Bluhm and Ordal, 1969).

Lipopolysaccharide molecules on the outer membrane of Gram negative bacteria are damaged by freezing due to destabilization of ionic bonds (Ray, 1986). Gomez and Sinskey (1973) reported that DNA breaks were observed in the heat injury of *Salmonella*. Fung and Vanden Bosch (1975) also showed that injury due to freeze-drying of *S. aureus* S-6 cells caused breakdown of RNA replication. Acid injury has been observed to be different from heat or freeze injuries (Przybylski and Witter, 1979); leakage of cellular constituents following injury was not found after acidification. There were no detectable amounts of 260 or 280 nm absorbing materials leaked during the course of acid injury, but damage of ribonucleic acid was observed (Przybylski and Witter, 1979). Zayaitz and Ledford (1985) reported that coagulase and thermostable nuclease activities were

reduced in injured *S. aureus*. Although acid injury did not affect cell membranes, RNA synthesis was affected (Jay *et al.,* 2005).

1.2.2. Sub-lethal injury detection

Sublethally-injured bacteria exhibit different characteristics than not injured bacteria, including an increased susceptibility to organic acids, dyes, and selective media in general, an increased lag time, a compromised ability to divide, and an overall decrease in metabolic activity (Oliver, 2000-2005). However, sub-lethally injured bacteria are clearly differentiated from dead or dying cells by their gene expression activity and by their level of ATP. Furthermore, although they are non culturable, sub-lethally injured bacteria still sustain their pathogenicity. Thus, the detection of sub-lethally injured bacteria is critical to quality control procedures performed in the food processing industry (Kolling and Matthews, 2001).

Typical quality control measures involve inoculating selective media with food samples in order to detect a specific pathogen of interest, but sub-lethally injured bacteria do not readily grow in the selective media and thereby evade detection (Bosilevac *et al.,* 2004). Therein, the failure of the bacteria to grow in the selective media yields a false-negative result and underestimates the total number of sublethally-injured bacteria (Wu, 2008). Therefore, a more sensitive method of detection is required.

An excellent method available for detecting and enumerating sub-lethally injured bacteria is the application of a selective culturing procedures (Ordal *et al.,* 1976; Hurst, 1977; McKillip, 2001), since sub-lethally injured bacteria become sensitive to many selective compounds due to damage in their membrane and modifications of their permeability, and lose their ability to grow on selective media (Besse *et al.,* 2000; Besse, 2002). As such sub-lethal cells are defined as cells that are able to multiply and form colonies on non-selective media but not on selective media (Jasson *et al.*, 2007).

Chapter 2. AIMS AND PLANNING OF RESEARCH

Alicyclobacillus genus was introduced in the bacterial taxonomy by Wisotzkey *et al.* (1992), when they re-classified *Bacillus acidoterrestris*, *B. acidocaldarius* and *B. cycloheptanicus* as *A. acidoterrestris, A. acidocaldarius* and *A. cycloheptanicus*, respectively, due to the presence in the membrane of ω-alicyclic fatty acids.

Resistance to pasteurization temperatures, low pH and the ability to produce off flavors, led researchers to acknowledge *A. acidotererstris* as an important target in the quality control of acidic beverages (Baumgart *et al.,* 1997; Bevilacqua *et al.,* 2011b).

A. acidoterrestris is composed of bacilli usually isolated from soil (primary source), plants, spoiled juices, tea and equipments (secondary sources) (Bevilacqua *et al.,* 2011a). Since soil borne species often contaminate fruit juices and do not need strict extreme requirements for survival, it is a great concern to investigate whether and how soil species could evolve from their ecological niches in microbial community to new environments as fruit juices (Bevilacqua *et al.,* 2015b). Several questions can be posed: soil species could acquire specific phenotypic traits in relation to available nutrients? Phenotypic diversity reflects genotypic variations? Among soil species could be present a "quasi-species" that could represent an intermediate between different evolutionary states?

Thus, the first phase of my PhD thesis was focused on the isolation of 25 wild strains of *A. acidoterrestris* from soil (23 strains) and spoiled pear juice (2 strains). Hereafter, genotyping and phenotyping characterization of strains was carried out to appreciate differences between environment and juice bacteria.

The main goal of the second part of PhD thesis was to evaluate the possibility of using some natural compound (eugenol, cinnamaldehyde, lemon extract and citrus extract), physical stress (HPH, US and heating), and combined treatments (HPH, US or heating with citrus extract) to inhibit four strains of *A. acidoterrestris* (DSM 3922^{T}, CB-1, C8, and C24) and comparing the effectiveness of stress against 2 species of *Bacillus* genus (*B. coagulans* and *B. clausii*) closely related to the genus *Alicyclobacillus*. Moreover, the purpose of this study was to investigate the occurrence of sub-lethal injury after each stress application, and the relationship between some combinations that determined a significant sub-lethal injury and the kind of damage occurring on spores.

In the last part of this PhD, selected treatments (US or lemon extract addition) were applied against *A. acidoterrestris in vivo* (apple juice) to investigate if a sub-lethal injury could also occur in a complex system.

Chapter 3. MATERIALS AND METHODS

3.1. Reference Cultures

This study focused on 27 strains of *Alicyclobacillus acidoterrestris*:

- DSM 3922^T and DSM 2498, from Deutsche Sammlung von Mikroorganismem und Zellkulturen's collection, Braunschweig, Germany;
- 23 strains, belonging to a private collection of the Department of the Science of Agriculture, Food and Environment (Foggia University), randomly selected from soil of Foggia (orchard, cornfield, ploughed field, garden suburb) and named with capital letter C;
- 2 strains isolated from a spoilage incident in beverage in pear juice, belonging to a private collection of the Department of the Science of Agriculture, Food and Environment (Foggia University), and named CB-1 and CB-2. A partial sequence (1406bp) corresponding to the 16S ribosomal RNA gene of CB-1 strain was submitted to GenBank database and the accession number assigned was KP144333.

and on 2 species of *Bacillus* genus:

- *B. clausii* DSMZ 8716 and *B. coagulans* DSM 1 from Deutsche Sammlung von Mikroorganismem und Zellkulturen's collection.

3.2. Optimal culture conditions

The strains of *A. acidoterrestris* were stored at 4 °C on Malt Extract Agar (MEA) (Oxoid, Milan, Italy), acidified to pH 4.5 through a sterile solution of citric acid (1:1,w/w). Working cultures were prepared by inoculation with 5-6 log cfu ml^{-1} in 5 ml of fresh acidified Malt Extract broth, and incubated at 45±1 °C for 48 h.

B. clausii was preserved at 4 °C in Alkaline Nutrient Agar (Nutrient Agar-Oxoid, Milan, Italy; supplemented with 10% of sterile 1 M Na-sesquicarbonate solution) with 0.5% of NaCl. 1 M Na-sesquicarbonate solution was obtained dissolving 4.2 g of $NaHCO_3$ (Sigma-Aldrich) and 5.3 g of Na_2CO_3 anhydrous (Sigma-Aldrich, Milan) in 100 ml of distilled water. Working culture was prepared by inoculation with 5-6 log cfu ml^{-1} in 5

ml of fresh Alkaline Nutrient broth with 0.5% of NaCl, and incubated for 2 days at 30 °C.

A reference stock of *B. coagulans* was kept at 4 °C in Nutrient Agar; each working culture was prepared by inoculation with 5-6 log cfu ml^{-1} in 5 ml of fresh Nutrient broth and incubated for 2 days at 40 °C.

3.3. Spore suspension

A spore suspension of each strain of *A. acidoterrestris* was produced on acidified MEA, incubated at 45±1 °C for 7 days until approximately 70-80% of cells sporulated. Spores were removed by a mild agitation of plates using a glass spreader after adding 5 ml of distilled water; spore suspension was centrifuged at 1.000 x g for 10 min, after which the supernatant was discarded and the pellet resuspended. Spores were cleaned by washing the pellets with sterile distilled water, followed by centrifugation; then, spore suspension was heated at 80 °C for 10 min to eliminate vegetative cells and stored at 4 °C. Spore number was assessed through the spread plate count on acidified MEA, incubated at 45±1 °C for 2 days and reported as log cfu ml^{-1}.

Spores of *B. clausii* were produced on Alkaline Nutrient Agar with 0.5% of NaCl, supplemented with 10.0 mg l^{-1} of Manganese Sulfate Monohydrate (Sigma-Aldrich), incubated at 30 °C for 11 days until approximately 80-90% of cells sporulated. Spores were removed and heat-treated at 80 °C for 10 min, as reported for *A. acidoterrestris*. Spore number was assessed through the spread plate count on Alkaline Nutrient Agar with 0.5% of NaCl, incubated at 30 °C for 2 days and reported as log cfu ml^{-1}.

B. coagulans spores were prepared from vegetative cells obtained after grown in Trypticase Soy broth (Oxoid, Milan, Italy) with 0.6% Yeast Extract (Oxoid, Milan, Italy), 500 mg l^{-1} of Manganese Sulfate Monohydrate and 3 mg l^{-1} of Dextrose (Sigma-Aldrich) at 37 °C for 2 days. Vegetative cells were then plated and grown on Nutrient Agar at 50 °C for 7 d. When sporulation reached 90%, the spore crop was harvested by adding 5 ml of sterile distilled water, detaching with sterile glass beads, collecting with a pipette and re-suspending in sterile distilled water. The suspension was centrifuged five times at 14.000 × g at 4 °C for 10 min. Heat treatment at 80 °C for 10 min was applied to destroy any remaining vegetative cells and stored at 4 °C. Spore number was assessed through the spread plate count on Nutrient Agar, incubated at 40 °C for 2 days and reported as log cfu ml^{-1}.

3.4. *A. acidoterrestis* wild strain isolation, growth profile and genome sequencing

3.4.1. Isolation of Gram positive spore forming bacteria

10 g of soil were diluted with 90 ml of sterile saline solution (0.9% NaCl) and homogenized for 1 min. Thereafter, this suspension was heat-treated at 80 °C for 10 min, serially diluted in saline solution and plated on acidified MEA at 45±1 °C for 3-5 days. Spoiled pear juice was serially diluted in saline solution, heat treated and analyzed as reported above. From each plates, Gram-positive and spore-forming strains were selected. The isolated strains were stored on acidified MEA slants at 4 °C.

3.4.2. Phenotyping

The phenotyping of the wild strains of *A. acidoterrestris* was based upon the following test:

- Gram staining.
- Catalyse and oxidase test.
- Oxido-fermentative metabolism in Hugh-Leifson broth (Biolife, Milan) acidified to pH 5.5.
- Growth in anaerobiosis in Malt Extract broth, incubated for 7 days, and covered with paraffin oil;
- Hydrolysis of starch on PCA (Plate Count Agar, Oxoid), added with starch (10 g l^{-1}) (C. Erba, Milan), and acidified to pH 5.5.
- Voges-Proskauer reaction, as reported by Tiecco (2001).
- Reduction of nitrate in Nitrate broth (Bacteriological Peptone, 8.6 g l^{-1}; NaCl, 6.4 g l^{-1}-J.T. Baker; KNO_3, 1.5 g l^{-1}-J.T.Baker) acidified to pH 4.5.
- Decarboxylation of lysine on Lysine Iron Agar (Oxoid), acidified to pH 4.5.
- Deamination of arginine in Abd-El Malek broth(Tryptone, 5.0 g l^{-1}-Oxoid; Yeast Extract, 2.5 g l^{-1}-Oxoid; Glucose, 0.5 g l^{-1}-J.T.Baker; K_2HPO_4, 2.0 g l^{-1}-J.T.Baker; Arginine, 3.0 g l^{-1}-Sigma Aldrich).
- Production of indole from tryptophane.
- Hydrolysis of gelatine in Nutrient Gelatine (Oxoid), acidified to pH 5.5.
- Hydrolysis of esculin in acidified Malt Extract broth, supplemented with Esculin (2.0 g l^{-1}) (Sigma-Aldrich) and Fe-ammonium citrate (1 g l^{-1}) (J.T.Baker).
- Urea hydrolysis in Urea broth (Oxoid), acidified to pH 4.5.
- Production of H_2S on SIM medium (Oxoid).

- Growth in acidified Malt Extract broth, supplemented with 0.02% Na-azide (Sigma Aldrich).

All the media, when required, were acidified through a solution of citric acid (1:1, J.T.Baker, Milan, Italy) and incubated at 45±1 °C.

3.4.3. Effect of pH, NaCl, temperature

Growth profile of alicyclobacilli was assessed as follows:

- acidified Malt Extract broth, supplemented with NaCl (2, 5, 7, 8%), incubated at 45±1 °C;
- Malt Extract broth buffered to pH 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, and 7.0 (±0.05) and incubated at 45±1°C;
- acidified Malt Extract broth incubated at 25, 30, 35, 40, 45, 50, 55, 60, 65, and 70 °C (±1 °C).

The samples were inoculated to 6 log cfu ml^{-1} with either cells or spores and incubated for 7 days: growth was assessed every day by measuring absorbance at 420 nm through a spectrophotometer UV-VIS DU 640 Beckman (Fullerton, CA).

Results from phenotyping were converted into quail-quantitative codes (0, no growth or no enzymatic activity; 1, growth or positive enzymatic activity) and used as input data to run a cluster analysis through the add-in component of Excel XLSTAT (Addinsoft, Paris, France).

3.4.4. Sample preparation and DNA extraction

Bacteria were taken at 24 h and 48 h and suspended in 1 ml of distilled water until 1 Mac Farland (about 6 log cfu) was reached.

DNA extraction was carried out using Norgen's Bacterial Genomic DNA Isolation Kit (Norgen Biotek Corp.3430 Thorold Ontario, Canada) according to the manufacturer's instructions. Purification is based on spin column chromatography as the separation matrix. Columns bind DNA under high salt concentrations and release the bound DNA under low salt and slightly alkali conditions.

Extracted DNA was measured at 260 nm and at 280 nm to verify the presence of cellular proteinaceous components. Maximum yields of clean DNA was recovered from 24 h samples, hence, these specimens were used for further analyses.

3.4.5. Strain identification

In order to provide genus and species identification for isolates, biochemical profiles, 16S ribosomal RNA gene (16S rDNA) amplification and sequencing and Random-Amplified-Polymorphic-DNA (RAPD) were performed.

Biochemical profiles. The biochemical profile of the strains was assessed on pure cultures through the miniaturized system API 50 CH, using the suspension medium 50 CHB (Biomeriux, Marcy L'Etoile, France). The API test was incubated at 45±1 °C for 48 h.

16S ribosomal RNA typing. 16S rDNA was obtained by means of a specific Polymerase Chain Reaction (PCR) using two universal primers (Fw: 5'-AGAGTTTGATCCTGGCTCA-3', positions 8 to 26; Rw: 5'-CGGCTACCTTGTTACGGAC-3', positions 1511 to 1493, in the *Escherichia coli* numbering system) (Wisotzkey *et al.*, 1992). Reaction mixture was prepared in a total volume of 25 µl and it was composed of 200 ng of template, 0.1 pmol μl^{-1} of each primer, 200 µM of each deoxynucleoside triphosphate, 2 U μl^{-1} of Taq DNA polymerase (ThermoScientific DreamTaq Green DNA Polymerase) 2.5 µl of 10X PCR buffer supplied with the enzyme and finally 2 mM of $MgCl_2$.

Amplification was carried out in GeneAmp PCR System 9600 (Perkin-Elmer Cetus, Emeryville, CA) and consisted of an initial denaturation at 94 °C for 2 min followed by 35 cycles divided in denaturation step for 20 s at 95 °C, primer annealing for 40 s at 55 °C and elongation for 2 min at 72 °C. A final elongation step was added at 72 °C for 5 min.

All assays included positive (*A. acidoterrestris* DSM 3922^T and *A. acidoterrestris* DSM 2498) and negative (all the PCR components except the template) controls to exclude false-positive and false-negative results.

Amplicons were evaluated with 1.0% agarose gel electrophoresis in TBE buffer using the DNA molecular mass marker O'GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific Inc. MA USA). Proper size products (1500bp) were purified using an appropriate commercial kit (QIAquick PCR purification kit) and sequenced by a service facility (BioFab research s.r.l., Rome, Italy) through Sanger dideoxy sequencing.

Basic Local Alignment Search Tool analyzed acquired sequences at NCBI website whereas alignments were performed with ClustalW2 at the EMBL-EBI website using default parameters.

Evolutionary analysis, based on Neighbor-Joining method, was conducted in MEGA software version 6.0. Adopted reference strains were *A. acidoterrestris* DSM 3922^T, *A. acidoterrestris* DSM 2498 and *A. acidoterrestris* 49025 whose sequence was taken from GenBank, (accession number: AB042057).

B. subtilis IAM 12118^T was used as an outgroup. Its 16S ribosomal RNA sequence was obtained at GenBank, (accession number: AB042061.1).

Random-Amplified-Polymorphic-DNA. Random Amplified Polymorphic DNA (RAPD) was done using primer Ba-l0 (5'-AACGCGCAAC-3) (Yamazaki *et al*., 1997). DNA amplification was carried out in a total volume of 10 µl containing 0.32 pmol μl^{-1} of primer, 200 µM of each deoxynucleoside triphosphate, 2 U μl^{-1} of Taq DNA polymerase (ThermoScientific DreamTaq Green DNA Polymerase), 1 µl of 10X PCR buffer supplied with the enzyme and finally 3 mM of $MgCl_2$. Template was tested at the final concentration of 30 ng μl^{-1}, 20 ng μl^{-1} and 15 ng μl^{-1}. Each sample was tested in triplicate in order to obtain repeatable results and to choose the better DNA concentration suitable with amplification. All assays included positive (*A. acidoterrestris* DSM 3922^T and *A. acidoterrestris* DSM 2498) and negative (all the PCR components except the template) controls to exclude false-positive and false-negative results.

The thermal cycling was done in a GeneAmp PCR System 9600 (Perkin-Elmer Cetus, Emeryville, CA) according to the following program: an initial denaturation at 94 °C for 2 min followed by 50 cycles of 94 °C for 4 s, 45 °C for 8 s, 72 °C for 40 s and a final extension at 72 °C for 3 min. Electrophoresis for PCR products was made on 2.0% agarose gel in TBE buffer with the DNA molecular mass marker O'GeneRuler 100 bp DNA Ladder (Thermo Fisher Scientific Inc. MA USA). DNA profile of each specimen was visualized under UV light (254 nm), captured using photography apparatus (Kodak DC 290) and analysed with KODAK 1D LE 3.6 software.

3.5. Chemical and physical intervention treatments and sub-lethal injury detection

3.5.1. Targets of the experiments

This phase focused on 4 specific strain of *A. acidoterrestris* (DSM 3922^T, CB-1, C8, and C24) and on 2 species of *Bacillus* genus (*B. coagulans* and *B. clausii*).

For each microorganism the targets tested were:

- fresh spores: produced and used within 2 weeks;
- old spores: produced and stored at 4 °C for 4 months before the experiments;

- activated spores: spores heated at 70 °C for 10 min before the analysis;
- cells.

3.5.2. Determination of restrictive (selective) medium

Aliquots of 5 ml of non-selective medium were inoculated with 5-6 log cfu ml^{-1} of each microbial targets and incubated for 48 h at the optimal temperature. The selective medium for each microorganism was determined after plate counting on respective optimal culture medium (non-selective medium) supplemented with different amounts of NaCl (0.3%; 0.5%; 0.8%; 1%; 2%; 3%; 4% and 5%). The experiments were performed twice; regarding the controls, the viable count was assessed on the optimal laboratory medium.

3.5.3. Chemical and physical treatments

Chemical treatments. Eugenol (MP Biomedicals, Aurora, OH), cinnamaldehyde (ICN Biomedicals, Aurora, OH), lemon extract (Spencer Food Industrial, Amsterdam, The Netherlands) and citrus extract (Biocitro ®, Quinabra, Probena, Spagna) were used as the active compounds in this study. Stock solutions (25.000-50.000 ppm) (1 ppm = 1 mg l^{-1}) were freshly prepared before each use in ethanol-water (1:1, v/v) for eugenol, cinnamaldehyde and lemon extract, and in distilled water for citrus extract, and sterilized by filtering through membranes (0.2 μm, Millipore, Milan, Italy).

Physical treatments. High homogenization pressure was performed for 1, 2 or 3 times at 150 MPa through a high-pressure homogenizer PANDA 2K (Niro Soavi s.p.a., Parma, Italy). The circuits of the equipment were cleaned with sterile distilled water (70 °C) and cooled with water (20 °C) to obtain an exit temperature of the samples of 45-50 °C. After the homogenization, samples were collected into 100 ml sterile tubes and immediately cooled to 20 °C in a water bath.

Ultrasound treatment was carried out processing 30 ml of sample for 6 or 8 min (pulse set to 4 s) through a VC Vibra Cell Ultrasound (US) equipment, model VC 130 (Sonics and Materials Inc., Newtown, CT, USA); the power was, also, set to 40 or 60%. Before each treatment, the ultrasonic probe was washed with sterile distilled water and immediately after processing, sample was cooled in ice.

Heat treatment was performed in a water bath previously set to 70, 80, 90 and 95 °C. Immediately after processing, sample was cooled in ice.

3.5.4. Antimicrobial assay

All the targets of *A. acidoterrestris* were submitted to:

- **Chemical treatments:** aliquots of sterile saline solution (9% NaCl) were inoculated with 5-6 log cfu ml^{-1}; each strain was evaluated separately. Then, the samples were added with eugenol (500 ppm), cinnamaldehyde (250 ppm), lemon extract (250 ppm) or citrus extract (250 ppm).
- **Physical treatments:** aliquots of sterile saline solution, inoculated with 5-6 log cfu ml^{-1}, were treated with high homogenization pressure (150 MPa for 1 time; 150 MPa for 2 times, with a rest time of 90 min and 150 MPa for 3 times), or ultrasound (power/time/pulse: 40% 6 min 4 s; 60% 6 min 4 s; 40% 8 min 4 s), or heat stress (70 °C for 10, 20 and 30 min; 80 °C for 10 and 20 min; 90 °C for 10 min; 95 °C for 5 min).
- **Combined treatments:** the strains were treated with HPH or US or heat treatment combined with citrus extract (250 ppm).

Regarding *B. clausii* and *B. coagulans*, the stress treatments were:

- **Chemical treatments:** aliquots of sterile saline solution were inoculated with 5-6 log cfu ml^{-1}; each strain was evaluated separately. Then, the samples were added with lemon extract (250 ppm) or citrus extract (250 ppm).
- **Physical treatments:** aliquots of sterile physiological solution, inoculated with 5-6 log cfu ml^{-1}, were treated with ultrasound (40% 6 min 4 s) or heat stress (temperature of 95 °C for 5 min).
- **Combined treatments:** the strains were treated separately with US or heat treatment combined with citrus extract (250 ppm). The extract was added either before or after the treatment.

Aliquots of sterile saline solution inoculated with the microbial tests but not treated were used as controls. The viable count was assessed both on the optimal laboratory medium (non-selective medium) and on the restrictive one (selective medium with salt), after treatment application (T0), 1 day (T1) and 2 days (T2) at 45±1 °C for *Alicyclobacillus* spp., at 30 °C for *B. clausii* and at 40 °C for *B. coagulans*.

The results from these experiments were modeled as Inhibition Index (%):

$$I.I. = \frac{(A_c - A_s)}{A_c} \cdot 100$$

Where A_c is the level of alicyclobacilli in the control and A_s in the samples added with antimicrobial and/or treated with HPH, US, or thermal processing. The index was evaluated by using the data from the optimal media.

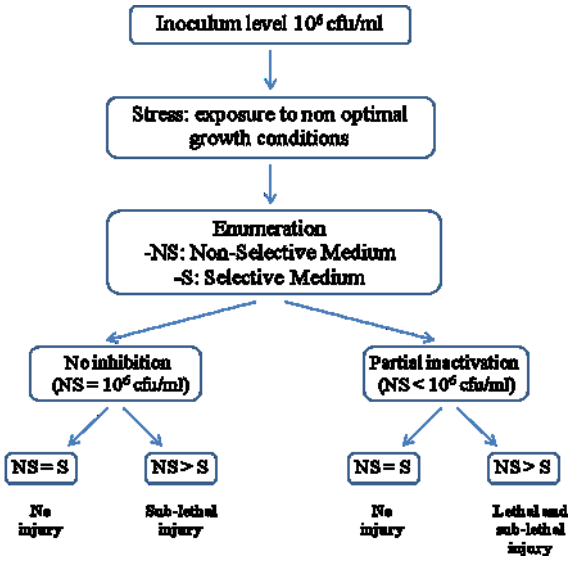
3.5.5. Sub-lethal injury detection

After the application of different stress, damage detection was performed and reported as "Percentage of Sub-lethal Injury", evaluated as follows:

$$\%\ \text{Sub} - \text{lethal Injury} = \frac{\text{counts on Non Selective Media} - \text{counts on Selective Media}}{\text{counts on Non Selective Media}} \times 100$$

Figure 3.1. provides an overview of the performed experimental setup and outlines possible effects of a given stress on the microorganisms.

Figure 3.1: A overview of different kinds of injury



3.5.6. Injury characterization

Sub-lethal injury on fresh and old spores of *Alicyclobacillus* spp. and *B. coagulans* was studied after the application of selected treatments: lemon extract (250 ppm), US (40% 6 min 4 s), heating (95 °C for 5 min), just after stress application (T0) and after 2 days (T2) of incubation at optimal culture conditions, through:

Leakage of UV-absorbing substances. To quantify the intracellular material released from the spores, untreated and treated samples (5-6 log cfu ml^{-1}) were centrifuged at 6.000 x g for 10 min. The UV absorbance of the supernatant was measured at 260 nm and at 280 nm with a spectrophotometer (spectrophotometer UV-VIS DU 640 Beckman (Fullerton, CA) (Virto *et al.,* 2005).

BSA protein assay. To quantify the amount of released proteins from the spores, untreated and treated samples (5-6 log cfu ml^{-1}) were centrifuged at 6.000 x g for 10 min. 0.1 ml of supernatant was mixed with 2.0 ml of BCA Working Reagent (BCA Protein Assay Reagent Kit, Sigma-Aldrich), and the mixture was incubated at 60 °C for 15 min. The absorbance at 562 nm was then measured with a spectrophotometer (spectrophotometer UV-VIS DU 640 Beckman (Fullerton, CA). The BCA protein assay was carried out using bovine serum albumin (BCA Protein Assay Reagent Kit, Sigma Aldrich) as the calibration standard (Wu *et al.,* 2015).

Colorimetric assay for Dipicolinic Acid in bacterial spores. 5 ml of spore suspension (5-6 log cfu ml^{-1}) were thermally-treated for 15 min at 121 °C. Then, the suspension was cooled, acidified with 0.1 ml of 1.0 N acetic acid, and left at room temperature for 1 h. Upon centrifugation at 1.500 g x 10 min, 4 ml of supernatant were put into a clean test tube. 1 ml of freshly prepared reagent, consisting of 1% of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_20$ (Sigma-Aldrich) and 1% of ascorbic acid in 0.5 M sodium acetate buffer solution for optode membranes pH 5.5 (Sigma-Aldrich), was added to the supernatant. The absorbance at 440 nm was then measured with a spectrophotometer (spectrophotometer UV-VIS DU 640 Beckman (Fullerton, CA) within 2 h. The blank was a similarly treated suspension in which 1 ml of the color reagent was substituted by water. The colorimetric assay was carried out using dipicolinic acid (Sigma-Aldrich) as the calibration standard (Chaves-Lòpez *et al.,* 2009).

3.5.7. Experiments in apple juice

The growth/survival of *A. acidoterrestris* (old spores of CB-1 strain) was assessed in a commercial apple juice (clarified juice) purchased from a local market (pH 3.7; soluble solids, 11 °B; carbohydrate content, 10.5%).

The samples were inoculated with 5-6 log cfu ml^{-1} and added to lemon extract (250 ppm) or submitted to US (40% 6 min 4 s). Aliquots of juice, inoculated with spores but not submitted to stress treatments, were used as controls.

Aliciclobacilli number was assessed just after the application of treatments and throughout the storage (11 days) at 25 °C, both on the optimal laboratory medium (acidified MEA) and on the restrictive one (acidified MEA with salt).

The microbiological analysis were performed before and after the treatments of the samples at 80 °C for 10 min, in order to evaluate the total population and the spore number, respectively.

3.6. Statistical analysis

All experiments were performed at least on two independent batches; each analysis was repeated. The significant differences between selective and non-selective media were determined with a T-Student test ($p < 0.05$) using the software Statistic for Windows (Tulsa, OK). Moreover, all the data were submitted to one-way and factorial analysis of variance (one-way and factorial ANOVA) and to Turkey's test ($p < 0.05$). Concerning the factorial ANOVA, the kind of strain, the treatment and the sampling time were used as independent factors (predictors).

A multi-factorial ANOVA offers two kinds of outputs:

1. a table of standardized effects showing Fisher-test and the significance of individual and interactive effects of predictors;
2. hypothesis decomposition graphs, which show the correlation of each predictors or interactive effect vs the output (cell count, spore number etc.).

Chapter 4. RESULTS AND DISCUSSION

4.1. Growth profile and genome sequencing of isolated *A. acidoterrestis* wild strains

In order to understand whether *A. acidoterrestris* could adapt in different ecological niches, 23 isolates of thermo-acidophilic, spore-forming bacteria taken from ground were characterized by using cultural (morphological, physiological and biochemical) and molecular (16S rDNA and RAPD) methods. In addition, 2 strains isolated from a spoilage incident in pear juice were typed to appreciate genomic differences between soil and juice bacteria.

4.1.1. Phenotyping

Phenotyping was based upon the assessment of growth at different temperatures, NaCl concentrations, and pH values, as well as on the evaluation of some biochemical traits (amino acid utilization, production of some secondary metabolites etc. . .). The results from these assays (growth/no growth; positive/negative) were converted into qualitative binary codes (0 and 1) and used as input values to run a Cluster Analysis. Fig 1 shows the results of this multivariate approach. Strains could be grouped into 3 different clusters (A, B and C), containing respectively 9, 6, and 10 isolates. Some strains showed identical phenotypic patterns (C8 vs C12; CB-1 vs CB-2; C4 vs C5; C6 vs C23) or quite similar (C2 vs c19; C13 vs C24; C10 vs C16; C20 vs C25); the strains from spoiled pear juice (CB-1 and CB-2) were in the cluster B, with some isolates from soil (C3, C8, C12, C18).
Tables 1 shows pH and temperature ranges for growth; many strains were able to grow at pH 2.5, with some exceptions to this generalized statement (strains CB-1, and CB-2 from juices and C1, C3, C8, and C12 from soil). The maximal pH was from 5.5 to 6.5. Concerning temperature, the minimal value was quite variable from 25 °C (many isolates from soil) to 35 °C (CB-1, CB-2, C18, and C21); a strong variability was also found for the maximal temperature (from 55 °C for CB-1 and CB-2 to 70 °C for the strains C13, C19, C24, and C25).
These ranges of pH and temperature for growth have been found and described for many species of *Alicyclobacillus*, isolated from soil and acidic beverages (*A. pomorum, A. herbarius, A. contaminans, A. sacchari, A. acidoterrestris, A. sendaiensis*) (Wisotzkey *et*

al., 1992; Goto *et al.,* 2002a; Goto *et al.,* 2007a; Bevilacqua *et al.,* 2012). The description of the genus *Alicyclobacillus* was greatly modified from the 1st report by Wisotzkey *et al.* (1992), due to the recovery of many species and subspecies from different environments and sources.

Nowadays, the genus includes acidophilic species and thermophiles, with growth range of 35-60 °C and pH 3.0-6.0, but some strains can grow at room temperature (20-30 °C) and at pH around neutrality (6.5-7.0) (Bevilacqua *et al.,* 2012).

Some interesting results were recovered from the assays on the qualitative utilization of lysine, arginine and indole production from tryptophan. Two strains used all the amino acids (C3 and C11) and many others used 2 out of 3 compounds (arginine and tryptophan for 5 strains (C2, C6, C14, C15 and C24) and lysine/arginine for 3 strains (C4, C5 and C13)); finally, 13 isolates used either arginine (C16, C17, C18, C19, C22, C23, CB-1 and CB-2) or tryptophan (C1, C8, C12, C20 and C21), but not lysine alone. Indole production was used as a taxonomic test for the characterization and classification of many *Alicyclobacillus* spp. and this trait is variable within the genus, thus confirming the results of this research (Goto *et al.*, 2002a; Guo *et al.,* 2009). This is the 1st report on the deamination of lysine and decarboxylation of arginine by alicyclobacilli, thus further investigations are required to elucidate the biochemical mechanisms underlying these pathways.

Alicyclobacilli growth was found in Malt Extract broth with 5% salt, except for the isolates C6, C15, C23, CB-1 and CB-2; moreover, some strains could also grow in presence of 8% NaCl (C1, C13, C14, C17, C21, C22 and C24). Concerning the other metabolic traits, only one strain could hydrolyze starch and gelatin whilst catalase and oxidase tests were strain-dependent. Resistance to 5% NaCl is a variable trait inside the genus *Alicyclobacillus*, as well as the ability to hydrolyze starch, and gelatin, nitrate reduction, catalase and oxidase activities (Goto *et al.,* 2007a), thus the results of this research confirm these literature evidences, although they also suggested a higher resistance to salt of our strains. Finally, all the strains could grow in Malt Extract broth covered with paraffin oil; the ability to grow in micro-aerophilic environments was first reported by Goto *et al.* (2007b), although *A. acidoterrestris* was described as a strict aerobic species.

4.1.2. Genotyping

16S RNA typing revealed that the isolates belonged to *Alicyclobacillus* genus. The cut-off point for the identification of the genus is a 95% homology (Clarridge 2004); however due to the peculiarity of *Alicyclobacillus* spp., novel species and some strains of the classical species show a lower homology (for example 92.3% *A. contaminans* and *A. fastidiosus*; 91.3% *A. herbarius*; 90.3% *A. tengchnogensis*; 92.1% for *A. tolerans*) (Goto *et al.,* 2002b; Karavaiko *et al.,* 2005; Goto *et al.,* 2007a; Kim *et al.,* 2014). Thus, the strain C2 could be attributed to *Alicyclobacillus*, whilst the results for the strain C4 should be confirmed by further investigations, due to the low homology (89%) and the difficulty to obtain pure DNA. At species level, many isolates (C3, C5, C6, C8, C11, C12, C13, C14, C15, C16, C17, C18, C19, C20, C21, C22, C23, C24, C25, CB-1 and CB-2) possessed high homology values with the type strains *A. acidoterrestris* DSM 3922^T and *A. acidoterrestris* 49025 (Table 2). The strains C1, C2, C10 showed a 93–95% homology; this value is lower than the international recognized cut-off point for species identification. However, the alignment was performed on both 16S ribosomal and NCBI nucleotide databases and the identity score was >200. For the isolates C2 and C5 the lower homology could be due to the length of the sequences (675 for C2 and 698 for C5). A sequence of 600-700 bp could be referred as unbelievable for the identification; however, DNA extraction from alicyclobacilli could be a great challenge. In fact, Groenwald *et al.* (2009) identified these microorganisms with sequences of 800 bp or lower and highlighted the importance of achieving pure and repeatable sequence in the region of genus identification, with a focus on species diversity. For these reasons and for the results reported in the following section, the strains C1, C2 and C5 were attributed to *A. acidoterrestris* species.

The acquired sequences were aligned to evidence nucleotide differences between isolates and reference strains. Sequences analyses revealed the presence of Single Nucleotide Polymorphisms (SNP) that weigh on the phylogenetic tree. In the Figure 2 the optimal tree with the sum of branch length = 0.79567517 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences *per* site. The analysis involved 29 nucleotide sequences.

All the positions containing gaps and missing data were eliminated. There were a total of 573 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

Examining of chronometric molecules, such as 16S rRNA, indicates the tendency of organisms to change on a global level and points out on the highly variable regions of 16S rRNA that exhibit high degrees of sequence divergence in even closely related organisms (Janda *et al.,* 2007). Results showed that isolates were divided into three blocks, the first block included 12 strains which closest relative is *A. acidoterrestris* 49025, the second block incorporated 7 isolates put between reference strains DSM 3922^T and DSM 2498 and finally the third block was represented by 4 strains flanked by reference strain DSM 2498 and strain CB-1. C4 strain was not included together with the others since its taxonomic proximity with *B. subtilis* IAM 12118^T used as an outgroup.

Analyzing these blocks in nucleotide database at NCBI website, a curious distribution was observed: the first block, composed of 12 isolates, is divided in 7 isolates (C22-C11 with the exception of C19) that share a great sequence homology with various strains identified by Zhang et colleagues and overall named CZB-12 (2013) and 4 isolates (C8-CB-2) that aligned with strain XC-6 (accession number: KJ158157) and strain FB40 (accession number: KF880723) (Table 2). Strain CZB-12 was isolated from the whole production line of kiwi products in China whereas strains XC-6 and FB-40 were identified in maize juice and in the debris from the factory floor respectively. The DNA sequences of the second block, composed of 7 isolates (C21-C1), matched with strains XC-6 (accession number: KJ158157) and FB40 (accession number: KF880723) (Table 3) Finally the third block was formed by 4 isolates (C6-C15) that share a good percentage of identity with strains CBZ-12 (Zhang *et al.,* 2013) and strain UFRRJAT1 (Kawase *et al.,* 2013) found in orange juice (Table 2). This distribution is intriguing since it is possible to speculate that soil isolates were clustered in effectively soil species and isolates that are moving from soil to other possible growing source characterized by parameters, like pH, that could strong influence bacterial survive. It is obvious that this assumption is a mere speculation; nevertheless, it is known that evolving organisms typically share relatively low levels of DNA-DNA homology with other members of the same group and that organisms show the phenotype that may be relevant to some environmental or physiological stress (Wisotzkey *et al.,* 1992). As reported above, the strains showed variable phenotypic traits, thus these results point out that bacterial species could exist as a genomovar (DNA group within the same species, i.e. strains showing a 70% DNA homology, with different phylogenetic traits but phenotypically

indistinguishable) that cover different phenospecies dependent from environmental and physiological stimuli received. The concept of genomovar has been extensively used of many species usually found on soil (*Pseudomonas stutzeri*, *Pseuodmonas cichorii*, *Bacillus licheniformis*, *Burkholderia cepacia*) (Mulet *et al.,* 2008; Khan *et al.,* 2013) and could be related to genome plasticity, ability to survive and grow in different ecological niche and show only useful phenotypic traits.

Regarding CB-1, its collocation in the phylogenetic tree is relatively distant from *Alicyclobacillus* type strains, nevertheless its biochemical profile is indistinguishable from strain CB-2.

Genotypically, type strains for many species may not accurately reflect the entire genomic composition of the species; moreover, it is possible to hypothesize that CB-1 is a fast-clock organism that typical has a tendency to change on a global level in chronometric molecules, such as 16S rRNA. An interesting trait of population biology is the so-called "molecular clock"; Zuckerkandl and Pauling (1965) first described this idea. They plotted the numbers of amino acid differences between the cytochrome c molecules of a number of organisms against the evolution time; thus, they suggested that molecules could turn over in a fairly constant or "clock-like" manner. There are several basic points for this theory; the most important one is that the sequence of a given gene evolves at a constant rate as long as its biological function remains unchanged (Novichov *et al.,* 2004). Several violations to this generalized statement have been reported, mainly due to horizontal gene transfer in prokaryotes (Novichov *et al.,* 2004). Nevertheless, the concept of molecular clock is a useful tool to predict expected and unexpected mutations and pinpoint some possible alternative pathways in the evolutionary history. The phylogenetic clustering of the strain CB-1 suggests the beginning of an alternative pathway in alicyclobacilli evolution, mainly for juice strains.

A genotypic indication of a fast-clock organism is an elevated rate of change in more conserved regions compared with the rate of change in the more variable regions of the same molecule (Wisotzkey *et al.,* 1992). On the other hand, the phenotypic traits are identical to those of CB-2 strain that, like CB-1, was isolated from a spoilage incident in pear juice. It confirms that ecological niche is determinant for genomic activation independently from evolutionary state.

In the last decade, RAPD PCR technique has been one of the most commonly used molecular techniques to develop DNA markers. RAPD PCR has found a wide range of

applications in gene mapping, population genetics, molecular evolutionary genetics, plant and animal breeding (Mbwana *et al.,* 2006).

In this study, Yamazaki protocol was employed to generate species-specific banding patterns in order to group strains in clusters (1997). RAPD tests were repeated different times and only the best profile acquired for every studied strain was loaded on the ultimate gel. The results evidenced a great variability in banding patterns and, although it was not possible to obtain genotypically well-distinguished groups, it was feasible to appreciate genetic similarity between strains C19 and C20, strains C21 and C22 and finally between strains C24 and C25 (Figure 3). None of strains recovered in the soil presents correspondence with the adopted reference strains.

Regarding CB-2 and CB-1, only CB-1 shared great genetic match with *A. acidoterrestris* DSM 2498, the considerable concern to fruit juice producers (Deinhard et al., 1987; Wisotzkey *et al.,* 1992). This result suggests that the nature of the growth medium could influence genetic characteristics; moreover, it enables to hypothesize that soil *Alicyclobacillus* could spread into different juice production lines. For instance, CB-1 was first isolated in spoiled pear juice, nevertheless, its surviving and thermal resistance was also assessed in tomato juice causing its spoilage (Bevilacqua *et al.,* 2011a).

Used RAPD primer anneal on DNA regions corresponding to molecular chaperones (DnaJ and DnaK), pivotal enzyme (gyraseB) and both subunits of bacterial ribosomes (23S,16S); therefore, RAPD PCR could trace the pathway of strains from soil to juices evidencing that soil-borne strains could evolve to survive under acidity or high temperature conditions.

4.2. Determination of restrictive (selective) medium

The existence of metabolically injured microorganisms in foods and their recovery during culturing procedures is a great threat to assess the spoiling phenomena. An injured microorganism can be defined as a cell that survives a stress but loses some of its distinctive qualities (Busta, 1976). First Hartsell (1951) defined injured cells as those capable of forming colonies on non-selective media, but hardly on selective media. What is a selective (restrictive) medium? It is a medium containing an hurdle (many times salt or sucrose); this hurdle does not affect cell number, but exerts a kind of stress on the colonies, which appear smaller and stunted.

Metrick *et al.* (1989) and Patterson *et al.* (1995) monitored sub-lethal injury by comparing growth on non selective medium (for non injured and injured

microorganisms) and on selective one added with sodium chloride (for not injured microorganisms), thus in a preliminary step the amount of salt to be added to the optimal medium to obtain a restrictive medium was assessed. Generally a stress results in a reduction of cell number. The reduction can pinpoint a sub-lethal injury, i.e. a non lethal stress which can be highlighted under stress conditions and not when cells are cultured in an optimal medium.

Table 3 shows the optimization/design of the restrictive medium for the different strains of *A. acidoterrestris*, *B. clausii* and *B. coagulans*, as well as the dimension of the colonies on the different medium (Table 3b).

4.3. Chemical and physical treatments

After the design of the restrictive medium, the study focused on a screening procedure to point out some chemical and/or physical treatments able to inhibit, as well as exert a sub-lethal injury on alicyclobacilli.

Four different targets were used: spores used immediately after recovery, spores stored for some months (referred as old spores), outgrowing spores (referred as activated spores) and cells. The experiments were performed on CB-1, C8 and C24 strains, due to the fact that we were able to completely analyze 16S (see Table 2); the results were compared to the type strain DSM 3922 and thereafter to some bacilli, closely related to the genus *Alicyclobacillus* (*Bacilus coagulans* and *B. clausii*).

For each kind of treatment, the data were analyzed through a two-way ANOVA to point out the effect of the kind of treatment (addition of antimicrobial, homogenization, sonication, heating), contact time for the antimicrobials or pressure/power level for the physical treatment; then, we plotted the results as Inhibition Index to quantitatively report the effect of each treatment. This output shows the effective reduction/kill of *A. acidoterrestris*.

Finally, the sub-lethal injury of the surviving population was assessed when the count on the restrictive medium was significantly lower than on the optimal one (t-test, $P<0.05$).

4.3.1. Chemical treatments

The use of EOs was suggested as a suitable method to control the growth of *A. acidoterrestris* in acidic foods (Takahashi *et al.,* 2004). The composition of EOs, however, is quite variable, depending on the mode of extraction, the plants and climate. Therefore, Bevilacqua *et al.* (2008b) proposed the use of active compounds of EOs rather

than the oils; they used, in fact, eugenol and cinnamaldehyde against spores of *A. acidoterrestris* and modeled the data as Inhibition Index (Chaibi *et al.,* 1997). These compounds were compared to citrus extract, as Fisher and Philipps (2008) defined the oils from citrus a promising alternative for the future.

Table 4a shows the statistical effects of eugenol and contact time on alicyclobacilli. The effect of the antimicrobial was strain dependent and also relied on the kind of target (new, old, activated spores or cells); moreover, many times the contact time played a significant role. The spores of the type strain were always affected by eugenol, whereas for the other strains the effect relied upon the kind of the targets. Eugenol played a significant role on both old and activated spores of CB-1 strain; on the other hand, a single target was affected for C8 and C24 strains, respectively the activated and old spores. Figure 4 reports the quantitative effect of eugenol; apart from the statistical weight, a significant inhibition was recovered for the fresh spores of DSM 3922 (inhibition of 17.58%) and activated spores of CB-1 (45.70%).

Concerning sub-lethal injury, it was found only on C8 and C24 strains (respectively on old spores and fresh/activated spores) (Table 4b).

The effect of cynnamaldehyde on alicyclobacilli was slight, as suggested by the results of two-way ANOVA (Table 5a) and Inhibition Indices (Figure 5). These results were not in line with the findings of Bevilacqua *et al.* (2008a); they asserted, in fact, that cinnamaldehyde seemed to exert the strongest antibacterial action against *A. acidoterrestris* spores than other EOs, supporting Burt (2004) hypothesis. They claimed that antimicrobial activity of the natural compounds seems to be related with the phenolic rings, but the type of alkyl group has been found to influence the antibacterial effectiveness. It is also known that cinnamaldehyde inhibits the growth of *Escherichia coli* O157:H7 and *Salmonella* Typhimurium, but it did not disintegrate the outer membrane or deplete the intracellular ATP pool (Helander *et al.,* 1998). However, interesting values of sub-lethal injury due to this chemical were found on the cells of the strains DSM 3922 and CB-1 (Table 5b), highlighting that the susceptibility to cinnamaldehyde was strain dependent and probably related to the isolation source of strains.

Many authors reported in the past the suitability of lemon extract as a natural preservative for the inhibition of a wide range of microorganisms (Fisher and Phillips, 2008; Settanni *et al.,* 2012). The results confirmed these findings; lemon extract exerted a significant effect on all the targets both as single term as well as in interaction with the

contact time (Table 6a). Many times it completely inhibited alicyclobacilli and caused an Inhibition Index of 100% (e.g. on the cells of the strains DSM 3922 and C24 and on the fresh spores of C8 and C24). As expected, old spores were more resistant with an inhibition ranging from 16-20% (strains CB-1 and C24) to 61.51% (strain C8) (Figure 6). These results are in agreement with Maldonado *et al.* (2013).

Lemon extract always determined a significant sub-lethal injury on surviving fresh spores (from 13.75% -strain C8- to 31.71% -type strain); moreover, half of the surviving old spores of the strain C8 were damaged (Table 6b).

The last antimicrobial studied was citrus extract, as in the past we found some promising results in term of juice stabilization (Bevilacqua *et al.,* 2008b). It is a commercial preparation, extracted from *Citrus paradisi* (grapefruit), *Citrus sinensis* (sweet orange) and *Citrus reticulata* (tangerine) and contains glycerol as inert carrier. Differently from lemon extract, it is colorless and odorless and water-soluble.

Citrus extract was always significant on all the targets and many times it played an interactive role with contact time (Table 7a). The value of Inhibition Index was variable and strongly depending on the kind of strain, according to Bevilacqua *et al.,* (2013). They reported, in fact, that the bioactivity of citrus extract against *A. acidoterrestris* was strain dependent. This difference might be explained by a difference in their hydrophobic behaviour; the more hydrophobic an organism behaves, the more sensitive it will be to hydrophobic agents. The maximum values (100%) of inhibition were found on the cells of DSM strain and on the fresh spores of the strain C8. Generally, old and fresh spores were moderately susceptible, with an Inhibition Index ranging from 18.67% (strain C8) to 40% (DSM 3922) (Figure 7). A significant injury (8-10%) on surviving population was, also, found only for some targets (cells and activated spores of CB-1 and old spores of C24 strain).

The antimicrobial activity of EOs active compounds could be due to the inhibition of one or several processes involved in spore-to-cell transition (germination, outgrowth and cell multiplication). Al Kahayat and Blank (1985) reported that *Bacillus subtilis* spores, stored in clove infusion, germinated but it did not outgrow even after sub-culturing in Nutrient Agar.

4.3.2. Physical treatments

The optimization of thermal processing has been considered for a long time as the only way to control *A. acidoterrestris* in acidic drinks and inhibits spore germination (Vieira *et al.,* 2002). A topic of great interest in food microbiology is the use of non-thermal methodologies for food preservation, i.e. some approaches able to prolong food shelf-life and inactivate food-borne pathogens and/or spoiling microorganisms without any significant increase in the temperature, in order to maintain the sensorial quality at acceptable levels (Corbo *et al.,* 2009).

Three kinds of treatments were analyzed in this study: homogenization (HPH), ultrasound (US) and heating (used as reference).

Concerning homogenization at 1 pass, the statistical analysis pinpointed a significant effect on cells (Table 8a), and after 2 days of storage cells experienced an Inhibition Index ranging from 10-12% (strains DSM 3922 and CB-1) to 20% (strains C8 and C24) (Figure 8). Some targets were sub-lethally injured (for example the cells and the fresh spores of the strain DSM 3922, or the cells of the strain C24) (Table 8b). Antimicrobial activity of HPH could be due to the disruption of the cell wall and outer membrane (Vannini *et al.,* 2004); pressures could induce a structural rearrangement of proteins, and increased the exposure of their hydrophobic regions and the disruption of their supramolecular structure under a pressure allowing the components to move freely and become independent of the original structure (Vannini *et al.,* 2004).

Alicyclobacilli were also treated two times at 150 MPa, with a rest time between the two treatments of 90 min. Although the statistical analysis revealed a significant effect of both treatment and storage time (Table 9a), the Inhibition Indices were generally low except for cells (from 18% for the strain DSM 3922 to 40% of the strain C24) (Figure 9); in fact, spores appeared very resistant, as reported also by Bevilacqua *et al.* (2007). They found a reduction of 0.2-0.5 log units on spores submitted to different kinds of HPH, and claimed that the effect of homogenization was strain dependent and the isolation source seemed to play a significant role. However, a significant sub-lethal injury was found on the cells of the strain DSM 3922 (6.84%) and C8 (17.34%) (table 9b).

The last kind of homogenization relied upon the use of a 3-pass treatment at 150 MPa. The standardized effect of two way ANOVA are in Table 10a. Cells were always affected by this approach with an Inhibition Index ranging from 9.57% (strain C8) to 23% (strain CB-1); spores were generally more resistant with some exceptions for fresh spores and activated spores. Variable values of sub-lethal injury were also recovered; the

most interesting result was the effect on the spores of the strain CB-1, which experienced a 18.56% injury (Table 10b).

Alicyclobacilli were also processed through ultrasound, by using different combinations of power and time; pulse was set to 4 s. Ultrasound is able to disaggregate bacterial clusters and inactivate bacteria through mechanical, physical, and chemical effects of acoustic cavitation (Joyce *et al.,* 2003). Cavitation is due to growth and subsequent collapse of microscopic bubbles when ultrasonic waves travel through a liquid. Cavitation can affect a biological system by virtue of the localized temperature rise and mechanical stress (Scherba *et al.,* 1991). Moreover, the dissociation of water molecules into H- and OH- free radicals, as a consequence of the very high temperature and pressures produced by cavitation, may induce adverse chemical changes such as DNA or protein denaturation (Riesz and Kondo 1992). However, the ultimate reason for the lethality of ultrasound on microorganisms is still unknown (Raso *et al.,* 1998).

For this approach the kind of treatment and the strain were used as categorical predictor for the analysis, because the experiments were performed immediately after the treatment. Table 11 shows the output of two way ANOVA. The kind of treatment was found to be significant, along with the effect of the strain and the interactive term strain vs treatment. However, the graph on the Inhibition Index (Figure 11) pinpointed that the quantitative effect of US was strong only on the cells of the strains C8 and C24. Spores were never affected; the highest treatment time (8 min) affected the activated spores.

Surprisingly, old spores of the strains CB-1, C8 and C24 were sub-lethally damaged (the injury was from 5.78% to 7.87%) (data not shown).

Different thermal treatments were also used in this work, since they are still the most common way to control alicyclobacilli in juice (Bevilacqua *et al.,* 2013). Depending on the strain and isolation source, thermal resistance of spores is greatly variable; for example, there are some strains that posses a strong intrinsic resistance to temperature: Goto *et al.* (2007b) reported for *A. acidoterrestris* strain AB-1 a $D_{89°C}$ of 10.9-13.7 min, whereas $D_{95°C}$ was determined to be 2.1-3.2 min. The effect on both cells and spores was always slight.

However, some combinations (e.g. 70 °C/ 20 min; 80 °C/20 min; 95 °C/5 min) determined a sub-lethal injury on fresh and old spores of the type strain (5-14.8%) (data not shown).

4.3.3. Combined treatments

The last step on alicyclobacilli relied upon the combination of citrus extract (250 ppm) with homogenization (150 MPa-2 passes with a rest time of 90 min), ultrasound (40%/4 min/4 s) and heating (95 °C/5 min). The use of antimicrobials and physical treatments as combined hurdles is a common way used for the prolongation of microbial acceptability of food (Bevilacqua *et al.,* 2013). Leistner (1995) introduced the concept of 'multi-target preservation' as an ambitious goal for a gentle but more effective means for prolonging shelf-life. Different hurdles, in fact, could act synergistically (Leistner, 2000) if they simultaneously affect different targets (cell membrane, DNA, enzyme system), disturbing microbial homeostasis. Microorganisms are unable to overcome the combination of several hurdles like mild heat treatments, acidic pH values and the addition of antimicrobials from natural origin. These ideas, as reported by Valero and Frances (2006), could result in a stable and safe product without any loss of sensorial quality.

The results of this step were analyzed through two-way ANOVA, as reported elsewhere; then the values were plotted as Inhibition Index. The statistical analyses generally revealed that the combination could exert a strong effect on alicyclobacilli, although many times the effect was strain-dependent.

As reported in the literature (Vannini *et al.,* 2004), the combination antimicrobials/homogenization acts in two different ways: reducing the initial contamination (homogenization) and controlling the growth of the spoiling microflora throughout the storage (antimicrobials). In some cases (Bevilacqua *et al.,* 2009; Kumar *et al.*, 2009), an interaction HPH/active compounds could occur, thus enhancing their effects. The results of this work are in agreement with the these authors; in fact, for the strain DSM 3922, the interaction citrus/homogenization was significant after 5 days. The interaction antimicrobial/homogenization relied upon the time of the addition of the extract. When citrus was added before homogenization, immediately after the treatment spores were reduced by 40% and were below the detection limit after 2 and 5 days (Table 12).

The combination of citrus with US reduced the cells of the strains DSM 3922 and CB-1 below the detection limit and inhibited by ca. 50% the other two strains; fresh spores were less affected (12-23% the strains DMS 3922 and C8; 40% the strains CB1 and C24) (Figure 12). Old and activated spores were slightly affected. These results are in line with the first findings on the bactericidal effect of US by Berger and Marr (1960). They assessed that the use of ultrasound could be suggested for disinfection and food

preservation but its limited lethal effect on bacterial spores could be a limit for a practical application in a sterilization procedure. The combination of ultrasound with chemical treatments (Ahmed and Russell 1975; Lillard 1993) increased the lethal effect of ultrasound on vegetative cells, but the inactivation of bacterial spores was still low.

The effect of the combination of citrus extract with heat was also less significant; an inhibition of 20-30% of the spores of the strains CB-1 and C8 was found (data not shown).

Periago *et al.* (2006) studied the effects of antimicrobials combined with a heat-shock at 90° C for 25 min, against spores of *B. megaterium*; they suggested that survivors to heat treatment, although able to grow under optimal conditions, were somehow injured and these damages hampered their repair mechanisms in the presence of antimicrobials. In line with the findings of Periago *et al.* (2006), we found that both the combination citrus/US and citrus/heat exerted a sub-lethal injury, as reported in Table 13.

4.3.4. Comparison with B. clausii and B. coagulans

This step of the research focused on two species of the genus *Bacillus* (*B. clausii* and *B. coagulans*) in order to highlight a common stress response for spore-formers and the peculiarity of *Alicyclobacillus* spp.

In particular, *B. coagulans* was chosen for the genotypic and phenotypic similarity with *Alicyclobacillus* genus, as it is able to survive in acid environments and cause spoilage in juice. Tianli *at al.* (2014) suggest that aside from pH and temperature range for growth, *Alicyclobacillus* is similar to *B. coagulans* especially for the DNA G+C content (approximately 62 mol%, compared to 45-50 mol% of *Bacillus*). On the other hand, *B. clausii* was selected for the alkalophilic character (Denizci *et al.,* 2004).

As proposed previously for *A. acidoterrestris*, this part of study focused on a screening practices to select some chemical and/or physical treatments able to exert a sub-lethal injury on *Bacillus* spp.

Also for this step, four different targets were used: fresh, old, and activated spores and cells; for each kind of treatment, the data were plotted as Inhibition Index and analyzed through a two-way ANOVA, with a final focus on the sub-lethal injury of the surviving population.

The qualitative and quantitative effects of lemon extract (250 ppm) on the survival of *B.clausii* and *B. coagulans* are in Table 14a (two-way ANOVA) and Figure 13 (Inhibition Index), respectively. Lemon extract exerted a significant effect on cells

(inhibition of ca. 68% for *B.clausii* and ca. 60% for *B. coagulans*), but not towards spores; a significant sub-lethal injury (ca. 33%) was recovered for *B. coagulans* (Table 14b).

It is not known how EOs can inhibit and/or inactivate spores of *B.clausii* and/or *B. coagulans*. Hayley and Palombo (2009) studied the effects of some EOs towards the spores of *Bacillus subtilis*; they used SEM analysis (Scanning Electron Microscopy) and found that spores treated with EOs appeared withered and deflated with ridges and this effect was more pronounced after a longer exposure time, thus they postulated that the visible damage of spores caused a loss of intracellular material. A different mode of action was proposed in the past by other authors; Cox *et al.* (2001) suggested that tea tree oil components were able to disrupt their structure by increasing fluidity. Cortezzo *et al.,* (2004) studied the mode of action of some compounds towards spores and found that few chemicals (like o-chlorocresol) interrupted the action of, or response to, nutrient receptors involved in the cascade of changes that lead a spore to commit to germination.

The combination of citrus extract (250 ppm) with either US (power/time/pulse: 40% 6 min 4 s) and heat (95 °C for 5 min) was tested on *Bacillus*, too.

B. clausii cells were completely inhibited by citrus, and the biocidal effect of the extract was also significant on old spores (inhibition of ca. 20%); US alone was slighlty effective against old spores (Figure 14a). Citrus extract alone inhibited *B. coagulans* cells by ca. 40% and US alone was not effective. The addition of citrus immeditaly after US increased the Inhibition Index up to 60% (Figure 14b). This result was in agreement with Djas *et al.* (2011); they reported that the additive and synergistic effect of ultrasound with some natural extracts could appear as a suitable approach for juice preservation.

Ultrasound caused, also, a sub-lethal injury on cells and old spores of *B. clausii* (respectively 24.50 and 15.69%). Old spores were also damaged by citrus extract (7.77%). On the other hand, the cells of *B. coagulans* were mainly affected by citrus extract (injury of 45-50%). These results suggest that citrus extract could be a valuable natural antimicrobial compound for the food industry, and the effectiveness of this compound could allow a decrease in the amount of additives used in industrial applications, with effects on both economical and safety aspects of food preservation (Conte *et al.,* 2007).

Heating did not affect strongly bacilli (Figure 15), except for the cells of *B. clausii*, which showed an Inhibition Index of ca. 40%. Moreover, the surviving cells were also sub-lethally damaged (injury of 24.08%) (data not shown).

4.4. Injury characterization

After the screening step, some combinations were chosen to study which kind of damage occurred on spores, i.e. the addition of lemon extract (250 ppm), or the application of US (40% 6 min 4 s) or heating (95 °C for 5 min). The same combinations were studied on *B. coagulans* used as reference.

A stress can be defined as a change in genome, proteome or in environment, producing a decrease in the growth rate or the survival (Vorob'eva, 2004); each stress involves a response mechanism, as microorganisms are dynamic systems able to respond and adapt to environmental changes (Bevilacqua *et al.,* 2015a).

In this study, injury was characterized at different levels, i.e. the release of DPA, nucleic acids (evaluated as Abs at 260 nm) or proteins (absorbance at 280 nm and test of BSA).

Injured cells often lose some cellular material such as amino acids, 260 nm absorbing material (nucleic acids), and 280 nm absorbing material (protein) through leakage into their surroundings (Hurst, 1977; Palumbo, 1989), since the cell membrane appears to be the component most commonly affected (Jay *et al.,* 2005), with damaged permeability barriers. On the other hand, the release of DPA is frequently associated to germination of spores after the activation of the nutrient receptors. It is generally accompanied by activation of spore's cortex lytic enzymes, which are responsible for degradation of the cortex (Paidhungat *et al.,* 2001). However, measurement of DPA could be used as a potential signal indicator of spore injury (Bevilacqua *et al.,* 2015a). Chaves Lopèz *et al.* (2009) defined the DPA released as a sign of spore irreversible damage under the most severe pressurization conditions.

Namely, fresh spores of 3 strains of alicyclobacilli (CB-1, C8, and C24) released variable amounts of DPA (from 2.65 to 12.65 ppm) immediately after the addition of lemon extract (Table 15a). This natural extract also caused the release of protein in the type strain DSM 3922 and on the fresh spores of the strain C8, confirming that there was permeabilization of the coat and cortex when microorganisms were submitted to antimicrobial addition (Virto *et al.,* 2005).

Fresh spores were also injured by the physical treatment and the release of some components was significant for the strains DSM 3922 and C8, although the effect was quite different: the strain DSM 3922 was damaged by heating and released proteins and DNA; the strain C8 released protein, DPA and DNA. Finally, fresh spores of *B. coagulans* released intracellular components (proteins, DPA and DNA) only after the application of US. The cavitations which spores are subjected to, during US treatments,

might cause a mechanical distruption of the coat and cortex, allowing DPA to leak out (Chaves-Lòpez *et al.,* 2009).

The same combinations were also studied on old spores; the results are in Table 15b. Lemon extract only affected the spores of the strain C8, which released ca. 19 ppm of DPA. Old spores of alicyclobacilli were mainly affected by physical treatment (ultrasound towards CB-1, C8 and C24 strains; heating against the type strain). A common trait for these damages was found, as old spores did not release DPA after a physical treatment but mainly proteins or DNA. The release of DPA from the spore core during a thermal treatment is still under discussion and has not been evaluated in full detail (Klijn *et al.,* 1997). Primarily, the high amount of DPA coupled with the low amount of water is responsible for the wet heat resistance. The most likely mechanisms for spore inactivation by heat are a rupture of the spore's inner membrane and the inactivation of core enzymes (Setlow, 2000; Warth, 1980).

Old spores of *B. coagulans* released components after all the treatments although DPA was found only after the application of ultrasound, whereas the main sign of injury after the application of lemon extract or heating was the release of proteins.

4.5. Experiments in apple juice

The last experiment was run in apple juice; the old spores of the strain CB-1 were used as targets. Spores were treated by US or lemon extract. Although the samples were inoculated with spores, some assays in the past revealed that germination could occurr in apple juice. Therefore, both the total population (cells + spores) and spore number (counting after a heat-shock) were evaluated.

The results were analyzed as decrease of total of spore number by using a multi-factoial ANOVA with three categorical predictors: treatment (citrus extract, US, control), storage time (up to 11 days), targets (total population, spores). A multi-factorial ANOVA generally offers two kinds of outputs:

1. a table of standardized effects showing Fisher-test and significance of individual (kind of treatment, target, and storage) and interactive effects of predictors;
2. hypothesis decomposition graphs, which show the correlation of each predictor or interactive effect vs the reduction of alicyclobacilli.

Table 16 shows the standardized effects; concerning the individual weight of predictors, the most significant effect was by the kind of treatment (sample: control, lemon extract

or US), followed by the effect of the kind of target (total population or spores) and finally by the storage time. The interaction sample vs target was the most important interactive term.

The graphs of hypothesis decomposition are a second interesting output of a multi-factorial ANOVA; a graph of decomposition does not show actual values or effective trends. It is a statistic output describing how each independent factor acts on the whole data set. Concerning the effect of the kind of treatment, generally US were more effective than lemon extract as they resulted in a mean reduction of both total population and spores of 0.7 log cfu ml^{-1} (Figure 16a). The ability of ultrasonic vibration to enhance heat transfer and its potential to inactivate microbes in food is also well documented (Sastry *et al.,* 1989; Lima and Sastry 1990; Sala *et al.,* 1995; Piyasena *et al.,* 2003). However, Valero *et al.* (2007) reported that the presence of pulp in orange juice increased the resistance of microorganisms to ultrasound. Yuan *et al.* (2009) asserted that the level of reduction (mortality) of *A. acidoterrestris* in apple juice was higher than that of the aerobic plate counts (APC) in orange juice (Valero *et al.,* 2007), indicating that ultrasonic treatment is more suitable for apple juice than orange juice, as the pulp in orange juice was found to protect the microorganisms against ultrasound.

Storage time also played and interesting quantitative effect, as the population of alicyclobacilli generally decreased thorughout time (Figure 16b). As expected, spores were more resistant than the total population (Figure 16c). Finally, Figure 16d reports the actual trend of both total population and spores.

The antimicrobial effect of US against alicyclobacilli was, also, enhanced by the presence of significant sub-lethal injuries found immediately after treatment application (6.10%) and 2 days of storage (7.48%) (data not shown).

Chapter 5. CONCLUSIONS

5.1. Significance and impact of PhD research

Alicyclobacillus acidoterrestris is the main cause of most spoilage problems in fruit juices and acidic products. Since soil borne species often contaminate fruit, it is a great concern to investigate whether and how soil species could evolve from their ecological niches in microbial community to new environments as fruit juices. In this study, 23 isolates of thermo-acidophilic, spore-forming bacteria from soil were characterized by cultural and molecular methods. In addition, 2 strains isolated from a spoilage incident in pear juice were typed. Strains phenotyping showed that they could be grouped into 3 different clusters, and some isolates showed identical or quite similar patterns. Analyzing pH and temperature ranges for growth, the majority of strains were able to grow at values described for many species of *Alicyclobacillus*. Qualitative utilization of lysine, arginine and indole production from tryptophan revealed, for the first time, deamination of lysine and decarboxylation of arginine. Resistance to 5% NaCl as well as the ability to hydrolyze starch and gelatin, nitrate reduction, catalase and oxidase activities confirmed literature evidences. Examining of 16S rRNA, showed that isolates were divided into three blocks represented by effectively soil species and strains that are moving from soil to other possible growing source characterized by parameters that could strongly influence bacterial survival. RAPD PCR technique evidenced a great variability; in addition, the strain CB-1 was distant from other alicyclobacilli, although it possessed the same traits than the other isolates from juice (CB-2); therefore, it is probably a fast-clock organism or the beginning on an alternative pathway in alicyclobacilli evolution.

Regarding the second part of PhD thesis, the results suggest that it is possible to control *A. acidoterrestris* through alternative approaches; in particular, lemon extract seemed to exert the strongest antibacterial action against *A. acidoterrestris*, determining a significant sub-lethal injury. However, the susceptibility to the antimicrobials was strain-dependent and probably related to the isolation source or to a different composition and/or distribution of fatty acids in the cytoplasmatic membrane.

Physical processing appears as a suitable approach for the inhibition of *A. acidoterrestris*, but an interesting way to improve the control of spoilage in foods could be the

combination of antimicrobial agents with physical treatments. In this study, in fact, the antimicrobial effect was strongly enhanced by the combination of citrus extract with HPH treatment. A similar trend was also observed on *Bacillus* spp., although some treatments (addition of citrus and lemon extract) acted stronger against cells of *B. clausii* and *B. coagulans* than *A. acidoterrestris* ones.

Concerning the other main purpose of this project (sub-lethal injury), I found that the presence of sub-lethal injury was not always related to treatment intensity, and statistical analyses generally revealed that the response was strain-dependent, highlighting different behavior between type and wild strains to stress treatments.

Some effective combinations (lemon extract 250 ppm, or the application of US 40% 6 min 4 s or heating 95 °C for 5 min) were chosen to study which kind of damage occurred on spores, highlighting that it was mainly related to impairment of the coat and cortex. Specifically, some combinations (US or lemon extract addition) determined a release of DPA. Nevertheless, the release of nucleic acids and proteins was found, highlighting that cell membrane could be another target of chemical and physical treatments.

The experiments conducted in apple juice confirmed the suitability of ultrasound treatment as a promising way to control alicyclobacilli and pinpointed that a sub-lethal injury could occur also in a complex system.

TABLES

Table 1: pH (**A**) and temperature (**B**) profiles of *A. acidoterrestris* strains (the error of pH was ± 0.05).

A)

Strains	pH range (min-max)
C2, C4, C5, C6, C11, C19, C21, C22, C23, C24	2.5-6.5
C13, C20, C25	2.5-6.0
C10, C14, C15, C16, C17, C18	2.5-5.5
CB-1, CB-2	3.0-6.0
C1, C3, C8, C12	3.0-5.5

B)

Strains	Growth temperature (°C) (min-max)
C13, C19, C24, C25	25-70
C1, C2, C10, C14, C20	25-65
C3, C4, C5, C6, C8, C11, C12, C15, C16, C22, C23	25-55
C17	30-55
C18, C21	35-60
CB-1, CB-2	35-55

Table 2: Identity percentage between 16S sequences obtained from studied strains and 16S sequences filed at NCBI website.

Sample	Refence Strain (NCBI 16S RIBOSOMAL DATABASE)	Identity	Strain (NCBI NUCLEOTIDE DATABASE)	Identity
C1	DSM 3922	935/982 (95%)	FB40 (KF880723) source:debris from factory floor	935/982 (95%)
C2	DSM 3922	628/675 (93%)	FB40 (KF880723) source: debris from factory floor	628/675 (93%)
C3	DSM 3922	695/698 (99%)	FB40 (KF880723) source: debris from factory floor	695/698 (99%)
C4*	DSM 3922	602/674 (89%)	FB40 (KF880723) source: debris from factory floor	602/674 (89%)
C5	ATCC 49025	680/698 (97%)	UFRRJAT1 (KC783431.1) source:orange juice	686/698 (98%)
C6	DSM 3922	890/898 (99%)	C-ZJB-12-31 (KC354628) source: fruit from the orchard in Bairui kiwi fruit Experimental Base	895/898 (99%)
C8	ATCC 49025	1400/1407 (99%)	XC-6 (KJ158157) source: maize juice	1396/1401 (99%)
C10	DSM 3922	950/995 (95%)	C-ZJB-12-36 (KC354633) source: fruit from the orchard in Bairui kiwi fruit Experimental Base	953/988 (96%)
C11	DSM 3922	771/774 (99%)	C-ZJB-12-65 (KC354683) source: soil from the orchard in Liujiabao village	771/773 (99%)
C12	DSM 3922	927/950 (98%)	C-ZJB-12-44 (KC354669) source: fruit from the orchard in Changdong village	925/945 (98%)
C13	DSM 3922	813/814 (99%)	FB40 (KF880723) source: debris from factory floor	813/814 (99%)
C14	DSM 3922	878/879 (99%)	FB40 (KF880723) source: debris from factory floor	878/879 (99%)
C15	ATCC 49025	708/725 (98%)	UFRRJAT1 (KC783431.1) source: orange juice	714/725 (98%)
C16	ATCC 49025	902/904 (99%)	XC-6 (KJ158157) source: maize juice	902/904 (99%)
C17	DSM 3922	896/914 (98%)	C-ZJB-12-12 (KC193187) source: shop environment (walls)	903/921 (98%)
C18	DSM 3922	945/974 (97%)	C-ZJB-12-10 (KC193186) source: kiwi fruits after washing	950/978 (97%)
C19	ATCC 49025	840/851 (99%)	XC-6 (KJ158157) source: maize juice	845/855 (99%)
C20	DSM 3922	882/884 (99%)	C-ZJB-12-10 (KC193186) source: kiwi fruits after washing	884/885 (99%)
C21	DSM 3922	800/803 (99%)	C-ZJB-12-64 (KC354682) source: soil from the orchard in Liujiabao village	801/803 (99%)
C22	DSM 3922	885/896 (99%)	C-ZJB-12-44 (KC354669) source: fruit from the orchard in Changdong village	886/894 (99%)
C23	DSM 3922	882/889 (99%)	C-ZJB-12-17 (KC193190) source: shop environment (raw material bins)	883/887 (99%)
C24	ATCC 49025	1404/1407 (99%)	XC-6 (KJ158157) source: maize juice	1399/1400 (99%)

C25	ATCC 49025	717/721 (99%)	XC-6 (KJ158157) source: maize juice	717/721 (99%)
CB-1	ATCC 49025	1392/1407 (99%)	CB-1 (KP144333) source: pear juice	/
CB-2	ATCC 49025	878/879 (99%)	XC-6 (KJ158157) source: maize juice	878/879 (99%)

*Identification should be confirmed

**The strain CB-1 was referred as "γ" in Bevilacqua *et al.,* (2011a).

Table 3a: Percentage of salt added to non-selective medium to obtain a selective one for the different microbial targets.

Microbial targets	Percentage of salt
A. acidoterrestris DSMZ 3922	0.5 %
A. acidoterrestris CB-1	1.0 %
A. acidoterrestris C8	1.0 %
A. acidoterrestris C24	0.8 %
B. clausii	3.0 %
B. coagulans	0.3 %

Table 3b: Microorganisms population and colonies diameter on non-selective medium and on selective one. Mean values ± standard deviation.

	non-selective medium		selective medium	
Microbial targets	cell number (log cfu ml^{-1})	colonies diamater	cell number (log cfu ml^{-1})	colonies diamater
A. acidoterrestris DSM 3922	5.81±0.09	5 mm	5.78±0.02	3 mm
A. acidoterrestris CB-1	6.51±0.11	6 mm	6.46±0.05	4 mm
A. acidoterrestris C8	6.32±0.14	6 mm	6.28±0.06	4 mm
A. acidoterrestris C24	7.04±0.19	5 mm	7.11±0.09	3 mm
B. clausii	5.48±0.07	4 mm	5.39±0.12	2 mm
B. coagulans	6.32±0.13	3.5 mm	6.25±0.08	1.5 mm

Table 4a: Statistical effects of eugenol (500 ppm) (**Antimicrobial**), contact time (**Time**) and interaction eugenol/contact time (**Ant*Time**) on the survival of *A. acidoterrestris*. The statistical analysis was performed through the factorial analysis of variance (factorial ANOVA) and Tukey's test ($p < 0.05$), through the software Statistica for Windows (Tulsa, OK). (F_{crit}=2.34).

	cells				fresh spores			
	DSM 3922	CB-1	C8	C24	DSM 3922	CB-1	C8	C24
Antimicrobial	ns	ns	38.10	11.353	12.399	ns	ns	ns
Time	5.214	5.445	55.95	37.311	ns	14.62	66.8	7.30
Ant*Time	6.933	ns	13.79	ns	ns	ns	21.4	ns
MS	0.0605	0.03604	0.0118	0.02794	0.0426	0.0147	0.0025	0.0206
	old spores				activated spores			
	DSM 3922	CB-1	C8	C24	DSM 3922	CB-1	C8	C24
Antimicrobial	294.8	7.16	ns	9.44	12.12	202.43	7.82	ns
Time	147.7	ns	6.696	ns	46.86	76.78	30.62	39.76
Ant*Time	9.5	ns	ns	ns	ns	172.30	ns	ns
MS	0.0030	0.0191	0.0441	0.0079	0.0083	0.0125	0.0220	0.0087

*ns, not significant
**MS, Mean sum of squares

Table 4b: Sub-lethal injury (%) of *A. acidoterrestris* due to eugenol (500 ppm) immediately after application (T0), 1 day (T1) and 2 days (T2) of incubation at 45±1 °C in acidified Malt Extract Agar. Mean values ± standard deviation.

	DSM 3922	CB-1	C8	C24
cells	/	/	/	/
fresh spores	/	/	/	7.18±1.58 (T1)
old spores	/	/	8.54±1.41 (T0)	/
activated spores	/	/	/	28.89±5.56 (T2)

*/, sub-lethal injury not found

Table 5a: Statistical effects of cinnamaldehyde (250 ppm) (**Antimicrobial**), contact time (**Time**) and interaction cinnamaldehyde/contact time (**Ant*Time**) on the survival of *A. acidoterrestris*. The statistical analysis was performed through the factorial analysis of variance (factorial ANOVA) and Tukey's test ($p < 0.05$), through the software Statistica for Windows (Tulsa, OK). (F_{crit}=2.34).

	cells				fresh spores			
	DSM 3922	CB-1	C8	C24	DSM 3922	CB-1	C8	C24
Antimicrobial	ns	6.787	ns	ns	ns	ns	ns	54.7
Time	7.37	7.260	ns	ns	ns	ns	ns	57.9
Ant*Time	13.75	ns	ns	ns	ns	ns	4.93	10.6
MS	0.0049	0.0214	0.0407	0.0175	0.0148	0.1482	0.0142	0.0017
	old spores				activated spores			
	DSM 3922	CB-1	C8	C24	DSM 3922	CB-1	C8	C24
Antimicrobial	22.77	ns	44.94	55.13	26.19	ns	22.71	15.7
Time	12.72	ns	64.85	138.48	9.80	ns	14,86	ns
Ant*Time	19.76	ns	16.21	71.05	ns	ns	ns	ns
MS	0.0123	0.4963	0.0080	0.0032	0.0052	0.2423	0.0265	0.0201

*ns, not significant
**MS, Mean sum of squares

Table 5b: Sub-lethal injury (%) of *A. acidoterrestris* due to cinnamaldehyde (250 ppm) immediately after application (T0), 1 day (T1) and 2 days (T2) of incubation at 45±1 °C in acidified Malt Extract Agar. Mean values ± standard deviation.

	DSM 3922	CB-1	C8	C24
cells	10.14±1.41 (T2)	7.62±0.97(T0)	/	/
fresh spores	/	/	/	/
old spores	/	/	/	3.80±0.01 (T2)
activated spores	4.76±0.14 (T2)	/	/	/

*/, sub-lethal injury not found

Table 6a: Statistical effects of lemon extract (250 ppm) (**Antimicrobial**), contact time (**Time**) and interaction lemon extract/contact time (**Ant*Time**) on the survival of *A. acidoterrestris*. The statistical analysis was performed through the factorial analysis of variance (factorial ANOVA) and Tukey's test ($p < 0.05$), through the software Statistica for Windows (Tulsa, OK). (F_{crit}=2.34).

	cells				fresh spores			
	DSM 3922	CB-1	C8	C24	DSM 3922	CB-1	C8	C24
Antimicrobial	799.95	93.06	110.43	308.159	1462.471	53.20	1049.45	33366.24
Time	270.543	81.542	196.63	497.649	575.32	31.09	394.6	8760.10
Ant*Time	222.502	20.04	51.54	109.25	332.75	22.92	340.9	7384.33
MS	0.0100	0.05841	0.0898	0.05504	0.0053	0.0110	0.0214	0.0013
	old spores				activated spores			
	DSM 3922	CB-1	C8	C24	DSM 3922	CB-1	C8	C24
Antimicrobial	254.6	37.57	590.59	182.67	441.68	479.88	2605.95	1578.85
Time	62.1	36.95	212.268	76.96	36.10	89.52	1042.95	820.18
Ant*Time	53.0	24.72	166.62	91.27	98.98	25.78	654.26	684.57
MS	0.0214	0.0119	0.0219	0.0073	0.0213	0.0061	0.0045	0.0043

*ns, not significant
**MS, Mean sum of squares

Table 6b: Sub-lethal injury (%) *A. acidoterrestris* due to lemon extract (250 ppm) immeditaly after application (T0), 1 day (T1) and 2 days (T2) of incubation at 45±1 °C in acidified Malt Extract Agar. Mean values ± standard deviation.

	DSM 3922	CB-1	C8	C24
cells	/	/	/	/
fresh spores	31.71±0.001 (T0)	17.88±1.69 (T0)	13.75±1.02 (T1)	16.71±3.76 (T0)
old spores	/	/	51.15±9.04 (T2)	/
activated spores	/	13.84±3.70 (T2)	17.01±2.88 (T2)	/

*/, sub-lethal injury not found

Table 7a: Statistical effects of citrus extract (250 ppm) (**Antimicrobial**), contact time (**Time**) and interaction citrus extract/contact time (**Ant*Time**) on the survival of *A. acidoterrestris*. The statistical analysis was performed through the factorial analysis of variance (factorial ANOVA) and Tukey's test ($p < 0.05$), through the software Statistica for Windows (Tulsa, OK). (F_{crit}=2.34).

	cells				fresh spores			
	DSM 3922	CB1	C8	C24	DSM 3922	CB-1	C8	C24
Antimicrobial	69.099	47.260	43.6516	83.455	22.617	53.113	264.088	24.360
Time	63.595	9.831	14.9377	30.120	10.160	18.750	150.861	6.512
Ant*Time	67.748	20.445	6.2913	22.987	ns	6.265	96.484	ns
MS	0.04249	0.0470	0.10948	0.0377	0.0740	0.0828	0.0306	0.0972
	old spores				activated spores			
	DSM 3922	CB1	C8	C24	DSM 3922	CB-1	C8	C24
Antimicrobial	368.36	125.187	522.61	235.11	257.37	214.33	1713.48	317.973
Time	103.34	55.339	127.25	62.75	116.31	123.87	260.74	44.246
Ant*Time	65.47	14.712	119.46	48.58	76.27	32.87	132.74	31.641
MS	0.0134	0.0232	0.0049	0.0066	0.0079	0.0159	0.0102	0.0754

*ns, not significant
**MS, Mean sum of squares

Table 7b: Sub-lethal injury (%) of *A. acidoterrestris* due to citrus extract (250 ppm) immediately after application (T0), 1 day (T1) and 2 days (T2) of incubation at 45±1 °C in acidified Malt Extract Agar. Mean values ± standard deviation.

	DSM 3922	CB-1	C8	C24
cells	/	10.87±0.74 (T2)	/	/
fresh spores	/	/	/	/
old spores	/	/	/	9.20±0.86 (T2)
activated spores	/	8.71±1.87 (T2)	/	/

*/, sub-lethal injury not found

Table 8a: Statistical effects of high homogenization pressure (150 MPa for 1 time) (**Sample**), storage (**Time**) and interaction homogenization/storage (**Sample*Time**) on the survival of *A. acidoterrestris*. The statistical analysis was performed through the factorial analysis of variance (factorial ANOVA) and Tukey's test ($p < 0.05$), through the software Statistica for Windows (Tulsa, OK). (F_{crit}=2.34).

	cells				fresh spores			
	DSM 3922	CB-1	C8	C24	DSM 3922	CB-1	C8	C24
Sample	12.25	ns	34.85	121.94	30.8	ns	34.58	ns
Time	47.96	ns	141.16	105.93	16.8	ns	10.66	ns
Sample*Time	18.41	ns	146.03	103.62	108.0	ns	ns	ns
MS	0.0119	0.0530	0.0072	0.0055	0.0017	0.0220	0.0093	0.0022
	old spores				activated spores			
	DSM 3922	CB-1	C8	C24	DSM 3922	CB-1	C8	C24
Antimicrobial	ns	ns	ns	ns	ns	ns	ns	ns
Time	ns	13.1	109.52	ns	ns	ns	ns	ns
Sample*Time	ns	ns	ns	ns	ns	ns	ns	ns
MS	0.0043	0.0018	0.0068	0.0041	0.0090	0.0193	0.0051	0.0569

*ns, not significant
**MS, Mean sum of squares

Table 8b: Sub-lethal injury (%) of *A. acidoterrestris* submitted to HPH (150 MPa for 1 time) after treatment application (T0) and 2 days (T2) of incubation at 45±1 °C in acidified Malt Extract Agar. Mean values ± standard deviation.

	DSM 3922	CB-1	C8	C24
cells	4.82±0.33 (T0)	/	/	7.26±1.06 (T2)
fresh spores	9.24±0.86 (T2)	/	5.79±0.85 (T2)	/
old spores	/	3.57±0.43 (T2)	/	/
activated spores	/	/	/	3.70±0.84 (T0)

*/, sub-lethal injury not found

Table 9a: Statistical effects of high homogenization pressure (150 MPa for 2 times, with a rest time of 90 min) (**Sample**), storage time (**Time**) and interaction homogenization/storage time (**Sample*Time**) on the survival of *A. acidoterrestris*. The statistical analysis was performed through the factorial analysis of variance (factorial ANOVA) and Tukey's test ($p < 0.05$), through the software Statistica for Windows (Tulsa, OK). (F_{crit}=2.34).

	cells				fresh spores			
	DSM 3922	CB-1	C8	C24	DSM 3922	CB-1	C8	C24
Sample	32.16	25.708	136.53	2026.45	140.0	20.684	278.04	ns
Time	22.49	ns	172.44	65.34	57.4	ns	ns	ns
Sample*Time	32.83	32.379	219.71	747.64	8.6	ns	63.029	ns
MS	0.0136	0.0426	0.0053	0.0018	0.0033	0.0220	0.0036	0.0127
	old spores				activated spores			
	DSM 3922	CB-1	C8	C24	DSM 3922	CB-1	C8	C24
Antimicrobial	35.692	92.171	91.826	8.170	2.372	18.494	141.263	17.585
Time	ns	21.2	549.36	ns	ns	ns	99.441	ns
Sample*Time	ns	12.809	ns	12.044	ns	ns	94.394	ns
MS	0.0043	0.0101	0.0010	0.0008	0.0103	0.0038	0.0063	0.0508

*ns, not significant
**MS, Mean sum of squares

Table 9b: Sub-lethal injury (%) of *A. acidoterrestris* submitted to HPH (150 MPa for 2 times, with a rest time of 90 min) after treatment application (T0) and 2 days (T2) of incubation at 45±1 °C in acidified Malt Extract Agar. Mean values ± standard deviation.

	HPH 2C			
	DSM 3922	CB-1	C8	C24
cells	6.84±0.86 (T0)	/	17.34±5.58 (T0)	/
fresh spores	/	/	/	/
old spores	/	/	/	/
activated spores	/	/	/	/

*/, sub-lethal injury not found

Table 10a: Statistical effects of high homogenization pressure (150 MPa for 3 times) (**Sample**), storage (**Time**) and interaction homogenization/storage (**Sample*Time**) on the survival of *A. acidoterrestris*. The statistical analysis was performed through the factorial analysis of variance (factorial ANOVA) and Tukey's test ($p < 0.05$), through the software Statistica for Windows (Tulsa, OK). (F_{crit}=2.34).

	cells				fresh spores			
	DSM 3922	CB-1	C8	C24	DSM 3922	CB-1	C8	C24
Sample	561.33	439.590	30.73	40.44	22.0	162.103	550.07	10.718
Time	166.29	58.485	77.55	48.69	ns	43.883	1088.75	17.331
Sample*Time	117.01	65.930	39.61	12.97	ns	9.649	340.440	ns
MS	0.0008	0.0040	0.0035	0.0132	0.0106	0.0145	0.0031	0.0144
	old spores				activated spores			
	DSM 3922	CB-1	C8	C24	DSM 3922	CB-1	C8	C24
Antimicrobial	22.926	ns	491.830	ns	34.389	91.638	43.611	ns
Time	ns	ns	11.16	ns	ns	ns	ns	ns
Sample*Time	10.052	ns	94.625	ns	ns	ns	ns	41.008
MS	0.0111	0.0059	0.0016	0.0954	0.0290	0.0141	0.0174	0.0162

*ns, not significant
**MS, Mean sum of squares

Table 10b: Sub-lethal injury (%) of *A. acidoterrestris* submitted to HPH (150 MPa for 3 times) after treatment application (T0) and 2 days (T2) of incubation at 45±1 °C in acidified Malt Extract Agar. Mean values ± standard deviation.

	DSM 3922	CB-1	C8	C24
cells	/	/	5.81±0.01 (T0)	8.60±0.64 (T2)
fresh spores	8.16±0.68 (T2)	/	6.35±0.18 (T0)	/
old spores	1.64±0.16 (T0)	18.56±2.56 (T2)	/	/
activated spores	/	/	/	/

*/, sub-lethal injury not found

Table 11: Statistical effects of ultrasound (power/time/pulse: 40% 6 min 4 s; 60% 6 min 4 s; 40% 8 min 4 s) (**Sample**), *A. acidoterrestris* strain (**Strain**) and interaction treatment/strain (**Sample*Strain**) on the survival of *A. acidoterrestris*. The statistical analysis was performed through the factorial analysis of variance (factorial ANOVA) and Tukey's test ($p < 0.05$), through the software Statistica for Windows (Tulsa, OK). (F_{crit}=2.34).

	cells	fresh spores	old spores	activated spores
Sample	91.89	40.7	8.9	38.5
Strain	13.14	2011.0	1053.0	45.8
Sample*Strain	30.04	11.6	10.9	3.6
MS	0.0394	0.006	0.007	0.010

*MS, Mean sum of squares

Table 12: Inhibition Index (%) of *A. acidoterrestris* (DSM 3922; CB-1; C8 and C24) submitted to HPH (150 MPa for 2 times, with a rest time of 90 min) combined with citrus extract (250 ppm) after treatment application (T0), 2 days (T2) and 5 days (T5) of incubation at 45±1 °C in acidified Malt Extract Agar. Mean values ± standard deviation.

		Inhibition Index %			
		HPH	Citrus Extract	HPH + Citrus Extract	Citrus Extract + HPH
DSM 3922	T0	0.29±0.41	4.75±2.44	7.31±1.00	7.00±0.75
	T2	7.90±1.23	34.43±1.23	31.95±6.42	32.14±0.05
	T5	11.90±4.59	35.81±1.31	70.38±5.14	53.18±1.63
CB-1	T0	0.46±0.66	13.27±2.65	17.22±1.89	41.43±3.05
	T2	0.11±0.001	57.43±5.04	55.93±0.81	100.00±0.00
	T5	15.58±3.50	67.55±1.61	62.55±1.07	100.00±0.00
C8	T0	3.57±0.82	7.66±1.54	10.20±0.53	10.51±0.67
	T2	0.00±0.00	25.74±4.43	25.07±1.10	25.43±0.11
	T5	8.07±2.60	49.92±4.81	49.82±0.03	48.34±1.05
C24	T0	6.01±0.34	5.94±0.24	16.98±3.02	8.6±1.36
	T2	2.41±0.49	63.60±0.02	46.43±2.53	38.31±2.27
	T5	4.36±5.8	63.42±3.51	54.34±1.91	57.03±3.18

Table 13: Sub-lethal injury (%) of *A. acidoterrestris* submitted to ultrasound treatment (power/time/pulse: 40% 6 min 4 s;) combined with citrus extract (250 ppm) (**A**) and to heat stress (95 °C for 5 min) combined with citrus extract (250 ppm) (**B**), after treatment application (T0), 1 day (T1) and 2 days (T2) of incubation at 45±1 °C in acidified MEA. Mean values ± standard deviation.

	DSM 3922	CB-1	C8	C24
cells	/	/	/	30.61±0.99 (T1)
fresh spores	5.52±0.19 (T2)	/	22.42±0.27 (T0)	7.60±0.97 (T0)
old spores	/	/	/	3.61±0.20 (T0)
activated spores	3.40±0.26 (T2)	/	5.97±0.38 (T2)	3.77±1.17 (T1)

*/, sub-lethal injury not found

	DSM 3922	CB-1	C8	C24
citrus extract	/	/	/	/
heating	/	/	/	4.28±0.68 (T2)
citrus extract with heating	9.34±1.11 (T2)	/	13.24±3.41 (T2)	/

*/, sub-lethal injury not found

Table 14a: Statistical effects of lemon extract (250 ppm) (**Antimicrobial**), contact time (**Time**) and interaction lemon extract/contact time (**Ant*Time**) on the survival of *B.clausii* and *B. coagulans*. The statistical analysis was performed through the factorial analysis of variance (factorial ANOVA) and Tukey's test ($p < 0.05$), through the software Statistica for Windows (Tulsa, OK). (F_{crit}=2.34).

	cells		fresh spores	
	B. clausii	*B. coagulans*	*B. clausii*	*B. coagulans*
Antimicrobial	308.741	945.399	ns	ns
Time	14.030	ns	ns	ns
Ant*Time	23.382	ns	25.62	ns
MS	0.0525	0.025300	0.00830	0.031900
	old spores		activated spores	
	B. clausii	*B. coagulans*	*B. clausii*	*B. coagulans*
Antimicrobial	ns	46.93	ns	ns
Time	8.32	45.08	8.801	ns
Ant*Time	ns	38.55	ns	ns
MS	0.02070	0.0062	0.05880	0.0230

*ns, not significant
**MS, Mean sum of squares

Table 14b: Sub-lethal injury (%) of *B.clausii* and *B. coagulans* due to lemon extract (250 ppm) immediately after application (T0), and 2 days (T2) of incubation at 30 °C for *B. clausii* and at 40 °C for *B. coagulans* in Alkaline Nutrient Agar and Nutrient Agar, respectively. Mean values ± standard deviation.

	cells	fresh spores	old spores	activated spores
B. clausii	/	/	/	/
B. coagulans	33.37±3.82 (T2)	/	4.85±0.40 (T0)	/

*/, sub-lethal injury not found

Table 15a: Release of genetic intracellular material, proteins and DPA from fresh spores of *A. acidoterrestris* and *B. coagulans* after lemon extract (250 ppm) addition, or treated by ultrasound (40% 6 min 4 s), or heating at 95 °C for 5 min. Mean values ± standard deviation.

Microorganisms (5-6 log cfu ml^{-1})	Treatment	Time (day)	Sub-Lethal Injury	Damage type (in the bracket there is the protocol used for the experiment)
A. acidoterrestris DSM 3922	lemon extract	0	31.71±0.01 %	Release of 2.7±0.84 ppm of proteins (Wu *et al.,* 2015)
	heating	0	15.58±3.7 %	Release of 7.73±3.21 % of proteins (Virto *et al.,* 2005) Release of 9.96±2.83 % of DNA (Virto *et al.,* 2005)
A. acidoterrestris CB-1	lemon extract	0	17.88±1.69 %	Release of 2.65±0.66 ppm of DPA (Chaves-Lòpez *et al.,* 2009)
A. acidoterrestris C8	lemon extract	0	13.75±1.02 %	Release of 1.02±0.34 ppm of proteins (Wu *et al.,* 2015) Release of 12.65±0.88 ppm of DPA (Chaves-Lòpez *et al.,* 2009)
	ultrasound	0	2.36±0.64 %	Release of 54.58±0.13 % of proteins (Virto *et al.,* 2005) Release of 56.98±0.02 % of DNA (Virto *et al.,* 2005) Release of 14.35±0.63 ppm of proteins (Wu *et al.,* 2015) Release of 7.5±0.66 ppm of DPA (Chaves-Lòpez *et al.,* 2009)
A. acidoterrestris C24	lemon extract	0	16.71±3.76 %	Release of 3.12±0.44 ppm of DPA (Chaves-Lòpez *et al.,* 2009)
B. coagulans	ultrasound	0	10.51±0.90 %	Release of 69.90±0.81 % of proteins (Virto *et al.,* 2005) Release of 68.80±0.81 % of DNA (Virto *et al.,* 2005) Release of 8.75±1.76 ppm of DPA (Chaves-Lòpez *et al.,* 2009)

Table 15b: Release of genetic intracellular material, proteins and DPA from old spores of *A. acidoterrestris* and *B. coagulans* after lemon extract (250 ppm) addition, or treatment with ultrasound (40% 6 min 4 s), or heating at 95 °C for 5 min. Mean values ± standard deviation.

Microorganisms (5-6 log cfu ml^{-1})	Treatment	Time (day)	Sub-Lethal Injury	Damage type (in the bracket there is the protocol used for the experiment)
A. acidoterrestris DSM 3922	heating	0	14.8±1.33 %	Release of 19.58±3.55 % of proteins (Virto *et al.,* 2005) Release of 4.05±0.42 ppm of proteins (Wu *et al.,* 2015)
A. acidoterrestris CB-1	ultrasound	0	7.02±0.01 %	Release of 58.89±0.59 % of proteins (Virto *et al.,* 2005) Release of 59.25±0.71 % of DNA (Virto *et al.,* 2005)
A. acidoterrestris C8	lemon extract	2	51.15±9.04 %	Release of 18.59±1.99 ppm of DPA (Chaves-Lòpez *et al.,* 2009)
	ultrasound	0	7.87±0.71 %	Release of 52.76±0.08 % of proteins (Virto *et al.,* 2005) Release of 48.94±4.42 % of DNA (Virto *et al.,* 2005)
A. acidoterrestris C24	ultrasound	0	5.78±0.85 %	Release of 4.95±0.21 ppm of proteins (Wu *et al.,* 2015)
B. coagulans	lemon extract	0	4.85±0.40 %	Release of 5.4±1.55 ppm of of proteins (Wu *et al.,* 2015)
	ultrasound	0	3.61±0.13 %	Release of 75.95±4.18 % of proteins (Virto *et al.,* 2005) Release of 72.76±4.15 % of DNA (Virto *et al.,* 2005) Release of 12.34±0.66 ppm of DPA (Chaves-Lòpez *et al.,* 2009)
	heating	0	2.40±0.40 %	Release of 57.77±1.87 % of proteins (Virto *et al.,* 2005) Release of 52.67±1.81 % of DNA (Virto *et al.,* 2005)

Table 16: Statistical effects of lemon extract (250 ppm) addition or US treatment (40% 6 min 4 s) (**Sample**), storage (**Time**), total population and spores (**Target**), interaction treatment/storage (**Sample*Time**), interaction treatment/total population and spores (**Sample*Target**), interaction storage/total population and spores (**Time*Target**) and interaction treatment/storage/total population and spores (**Sample*Time*Target**) under *in vivo* conditions. The experiments were carried out in apple juice at 25 °C. The statistical analysis was performed through the factorial analysis of variance (factorial ANOVA) and Tukey's test ($p < 0.05$), through the software Statistica for Windows (Tulsa, OK).

Effect	SS	Degr. of Freedom	MS	F
Intercept	11.92617	1	11.92617	1270.324
Sample	2.92914	2	1.46457	156.000
Time	2.57114	5	0.51423	54.773
Target	0.92516	1	0.92516	98.544
Sample*Time	0.33829	10	0.03383	3.603
Sample*Target	0.27754	2	0.13877	14.781
Time*Target	0.43474	5	0.08695	9.261
Sample*Time*Target	0.52886	10	0.05289	5.633
Error	0.33798	36	0.00939	

*SS, Sum of squares
**MS, Mean sum of squares
*** F, Fisher Test

FIGURES

Figure 1: Clustering of isolated *A.acidoterrestris* strains considering some biochemical traits.

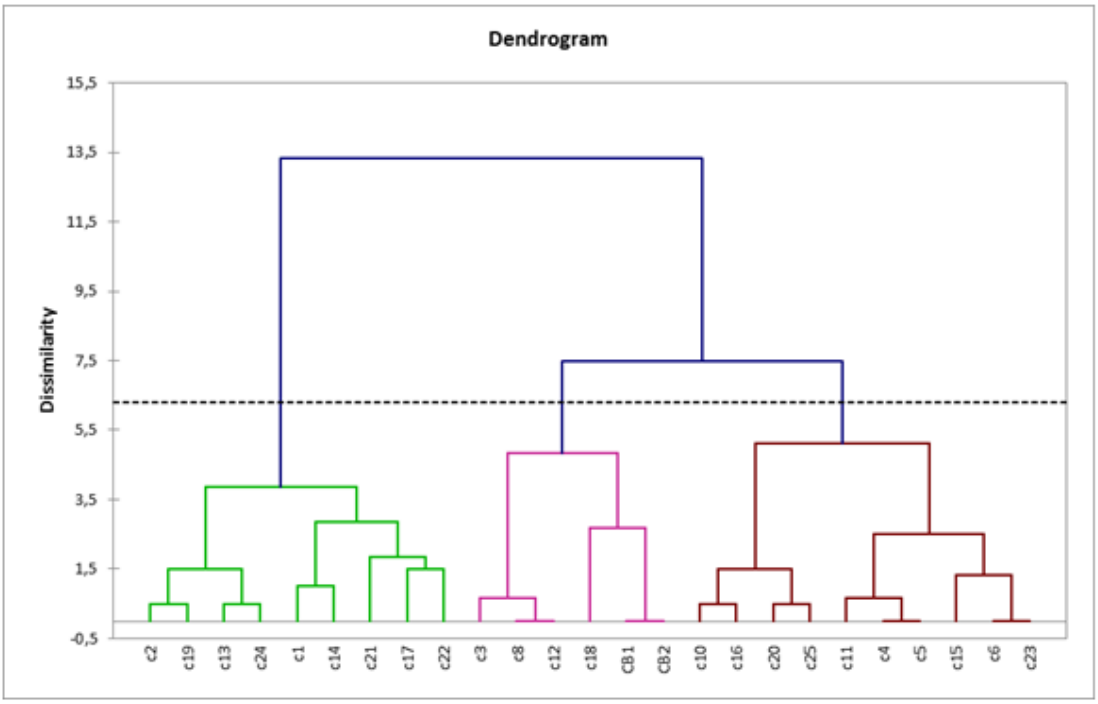


Figure 2: Evolutionary relationships of taxa. (The evolutionary history was inferred using the Neighbor-Joining method.

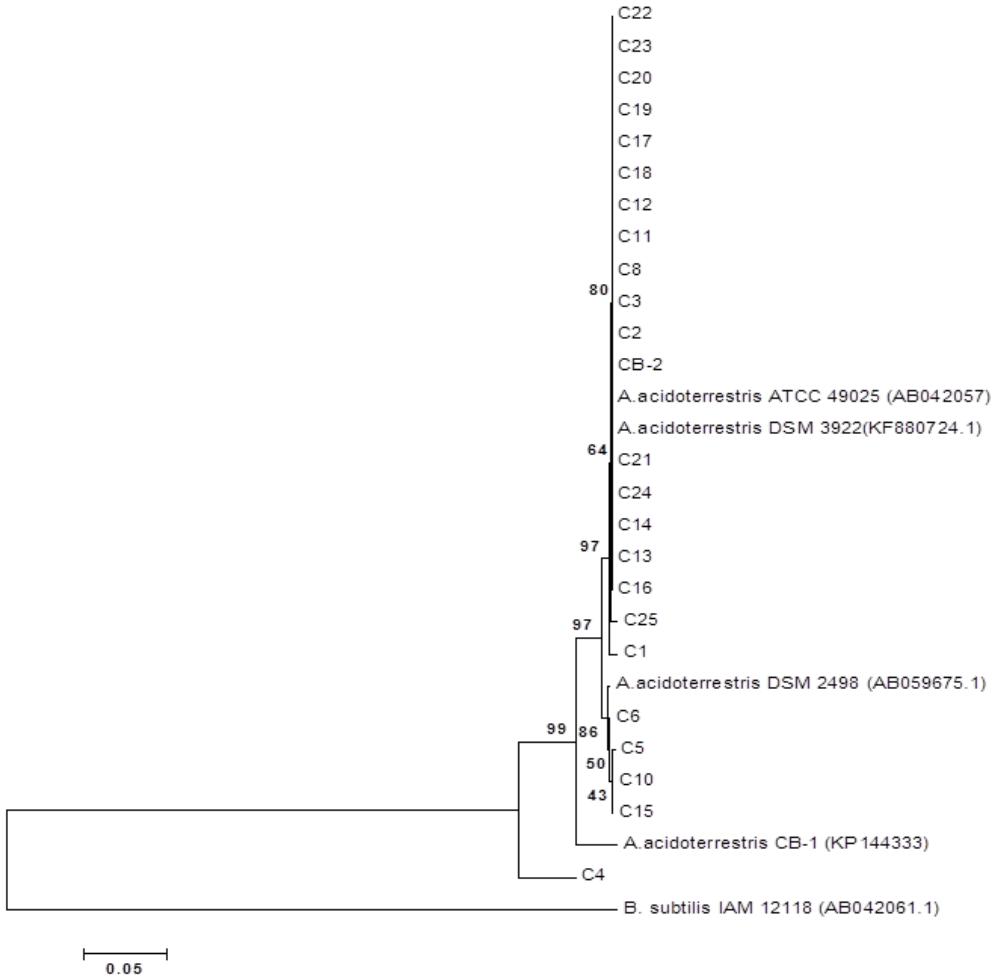


Figure 3: RAPD PCR products obtained amplifying chromosomal DNA of studied *A. acidoterrestris* strains as the template, primer BA-10 was used. Section A: Lanes 1 and 18: *A. acidoterrestris* DSMZ 3922^{T} and DSMZ 2498. Lane 2: C1, lane 3: C2, lane 4: C3, lane 5: C4, lane 6; C5, lane 7: C6, lane 8: C8, lane 9: C10, lane 10: C11, lane 11: C12, lane 12: C13, lane 13: C14, lane 15: C15, lane 16: C17, lane 17: C18. Lanes M: size marker: O'GeneRuler 100 bp DNA Ladder (Thermo Fisher Scientific Inc. MA USA). Section B: Lanes 1 and 11: *A. acidoterrestris* DSMZ 3922^{T} and DSMZ 2498. Lane 2: C19, lane 3: C20, lane 4: C21, lane 5: C22, lane 6; C23, lane 7: C24, lane 8: C25, lane 9: CB-1, lane 10: CB-2. Lanes M: size marker: O'GeneRuler 100 bp DNA Ladder (Thermo Fisher Scientific Inc. MA USA).

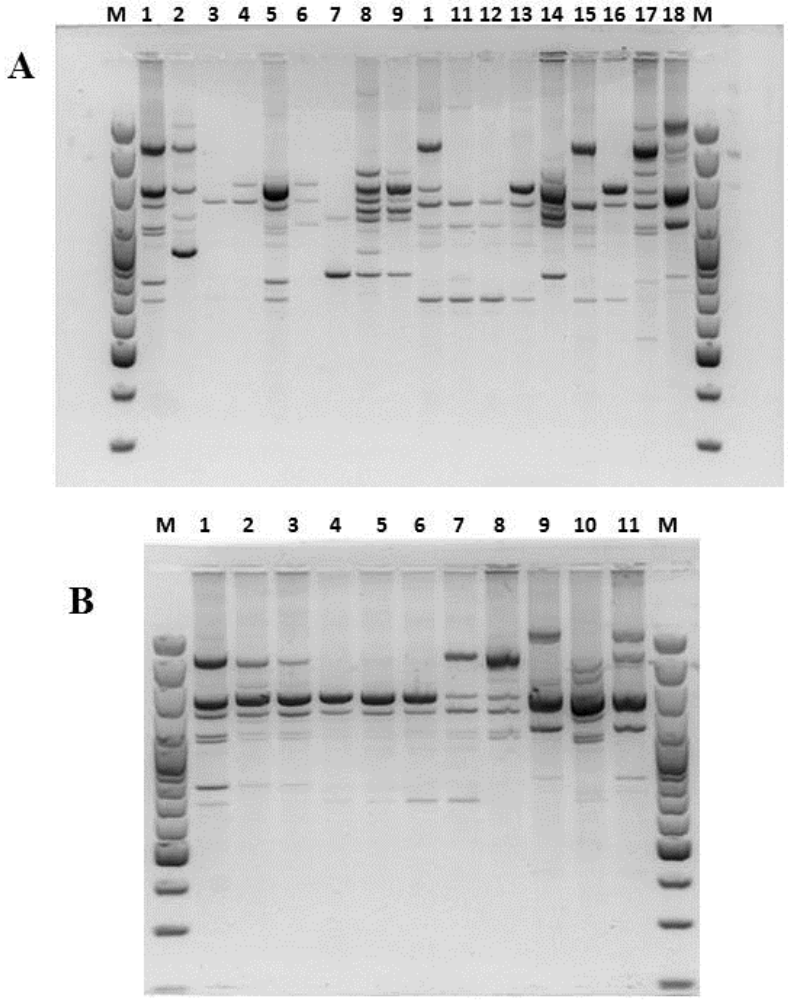


Figure 4: Inhibition Index (%) of *A. acidoterrestris* treated with eugenol (500 ppm) after 2 days of incubation at 45±1 °C in acidified Malt Extract Agar. Mean values ± standard deviation.

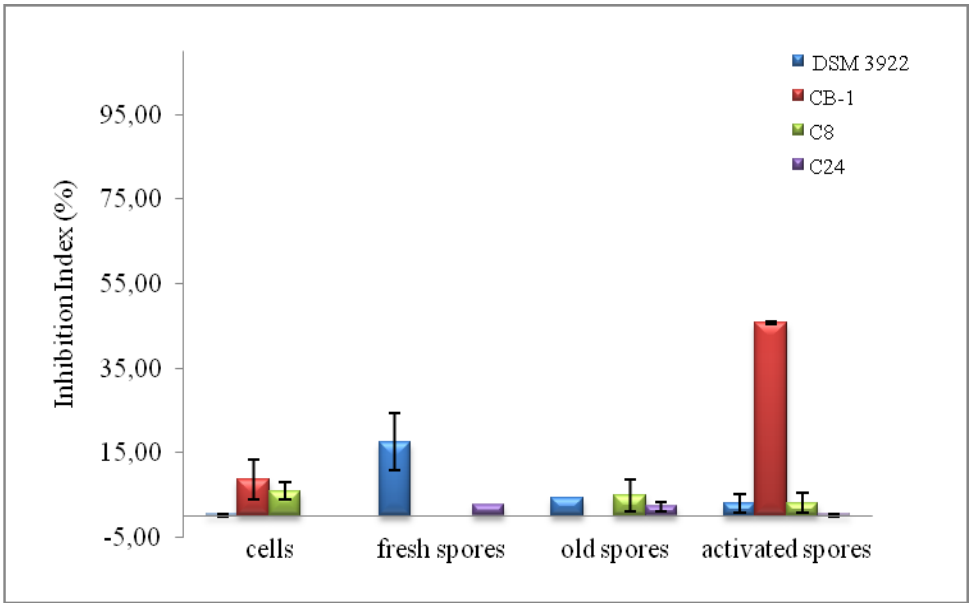


Figure 5: Inhibition Index (%) of *A. acidoterrestris* treated with cinnamaldehyde (250 ppm) after 2 days of incubation at 45±1 °C in acidified Malt Extract Agar. Mean values ± standard deviation.

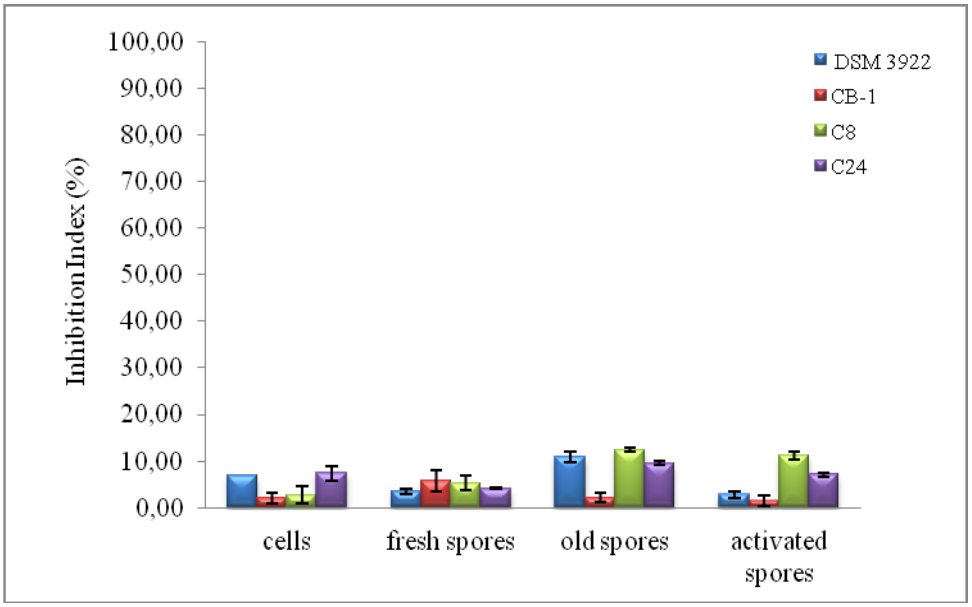


Figure 6: Inhibition Index (%) of *A. acidoterrestris* treated with lemon extract (250 ppm) after 2 days of incubation at 45±1 °C in acidified Malt Extract Agar. Mean values ± standard deviation.

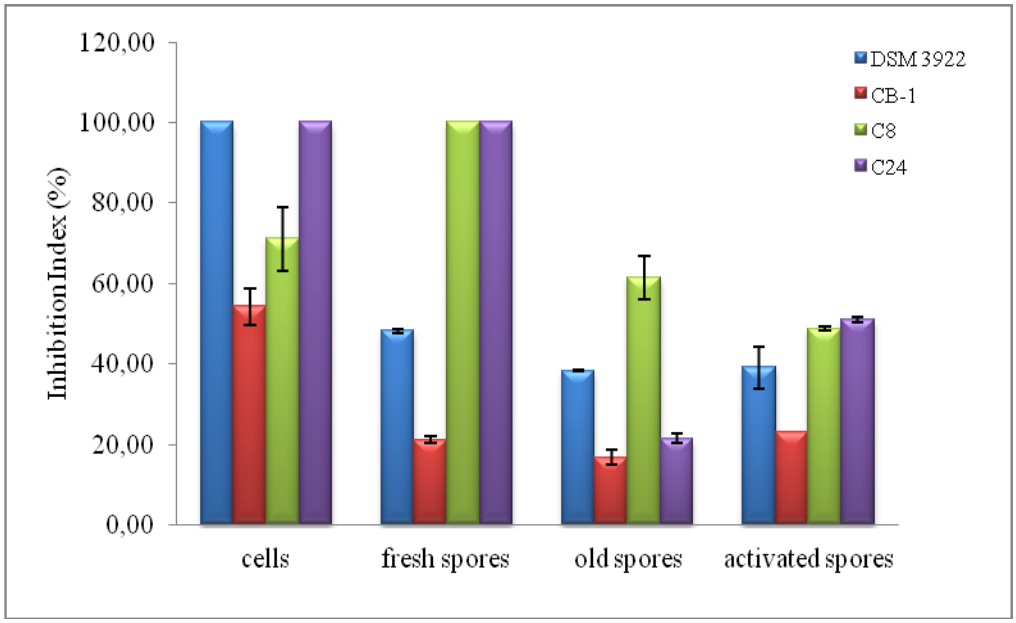


Figure 7: Inhibition Index (%) of *A. acidoterrestris* treated with citrus extract (250 ppm) after 2 days of incubation at 45±1 °C in acidified Malt Extract Agar. Mean values ± standard deviation.

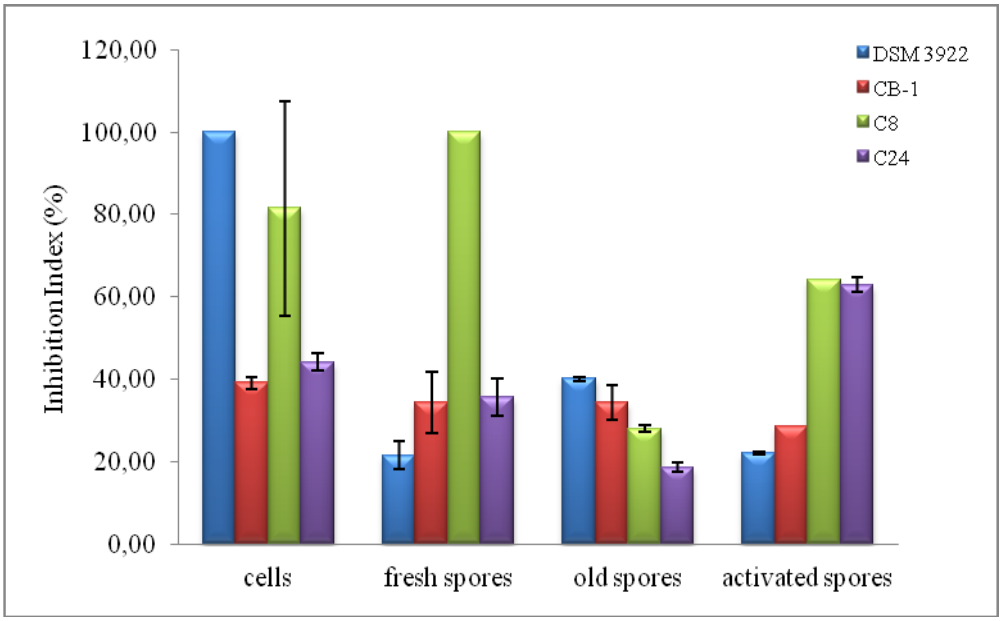


Figure 8: Inhibition Index (%) of *A. acidoterrestris* submitted to HPH (150 MPa for 1 time) after 2 days (T2) of incubation at 45±1 °C in acidified Malt Extract Agar. Mean values ± standard deviation.

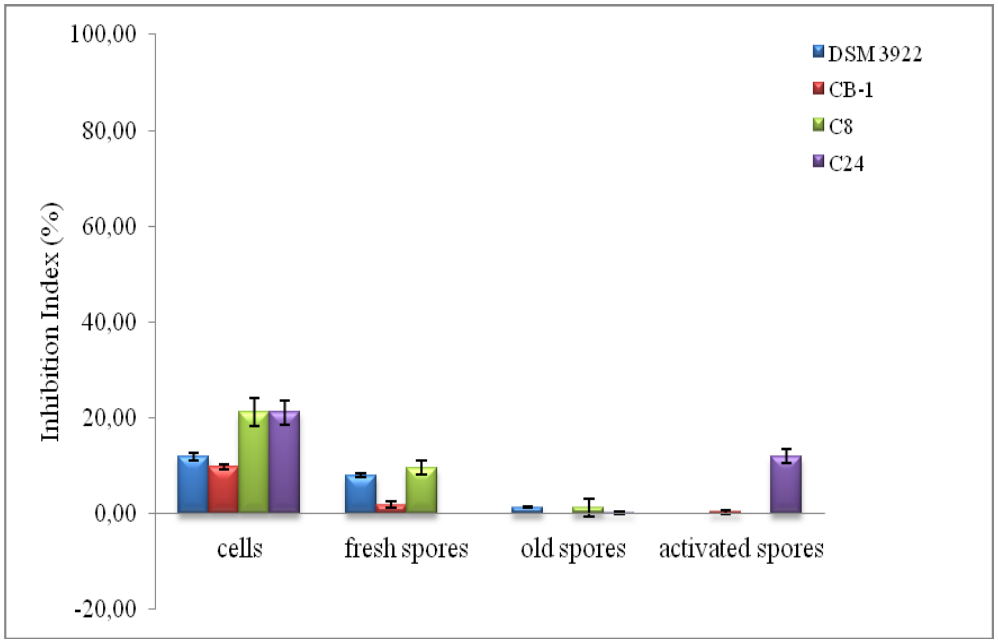


Figure 9: Inhibition Index (%) of *A. acidoterrestris* submitted to HPH (150 MPa for 2 times, with a rest time of 90 min) after 2 days (T2) of incubation at 45±1 °C in acidified Malt Extract Agar. Mean values ± standard deviation.

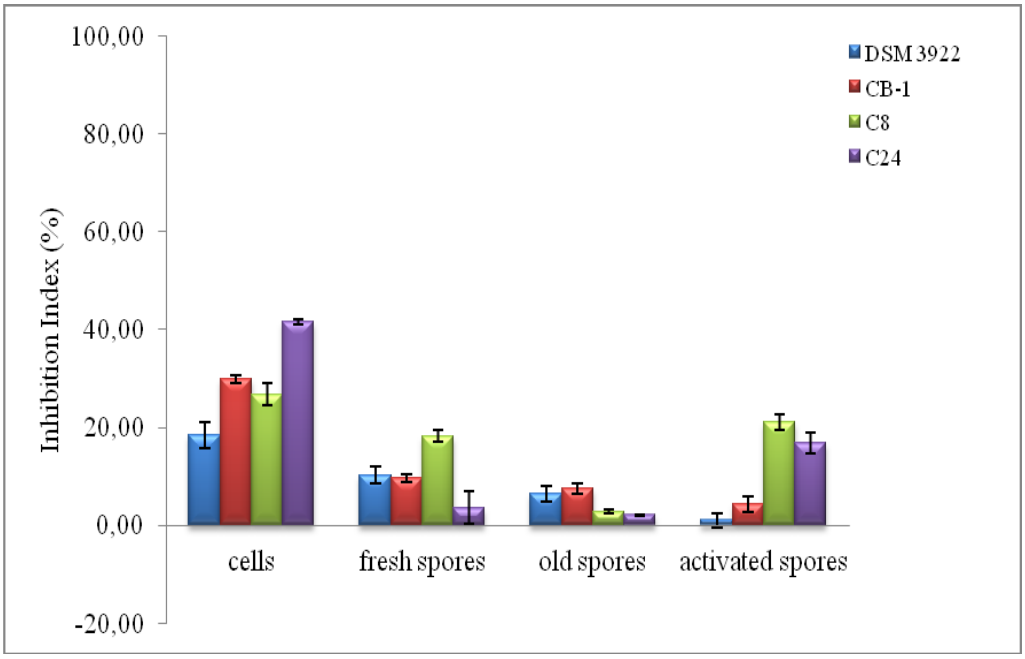


Figure 10: Inhibition Index (%) of *A. acidoterrestris* submitted to HPH (150 MPa for 3 times) after 2 days (T2) of incubation at 45±1 °C in acidified Malt Extract Agar. Mean values ± standard deviation.

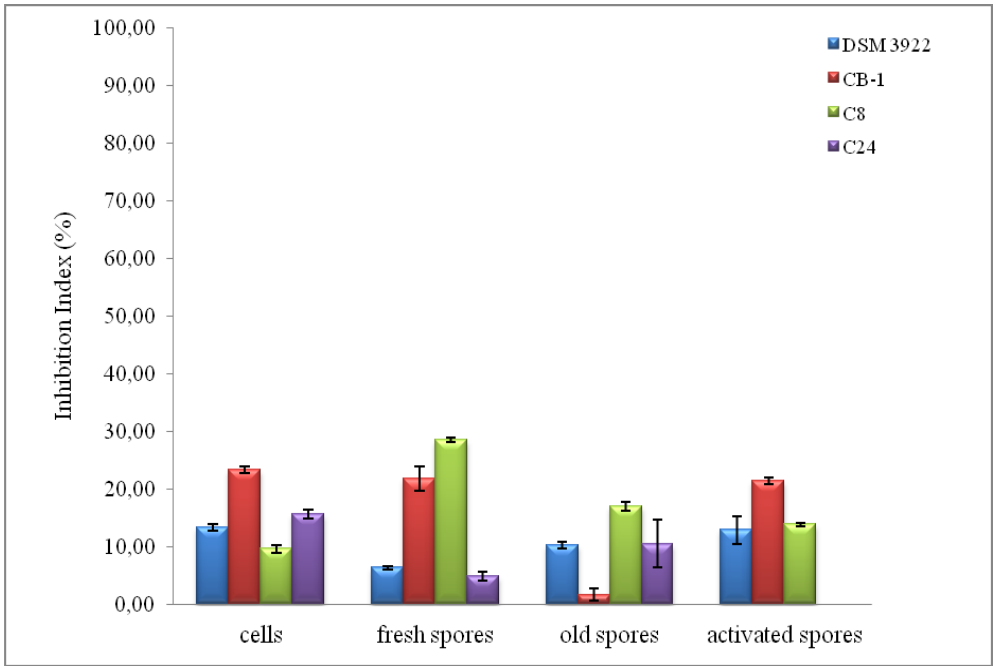


Figure 11: Inhibition Index (%) of *A. acidoterrestris* submitted to ultrasound (power/time/pulse: 40% 6 min 4 s (**A**); 60% 6 min 4 s (**B**); 40% 8 min 4 s (**C**)) after treatment application (T0) at 45±1 °C in acidified Malt Extract Agar. Mean values ± standard deviation.

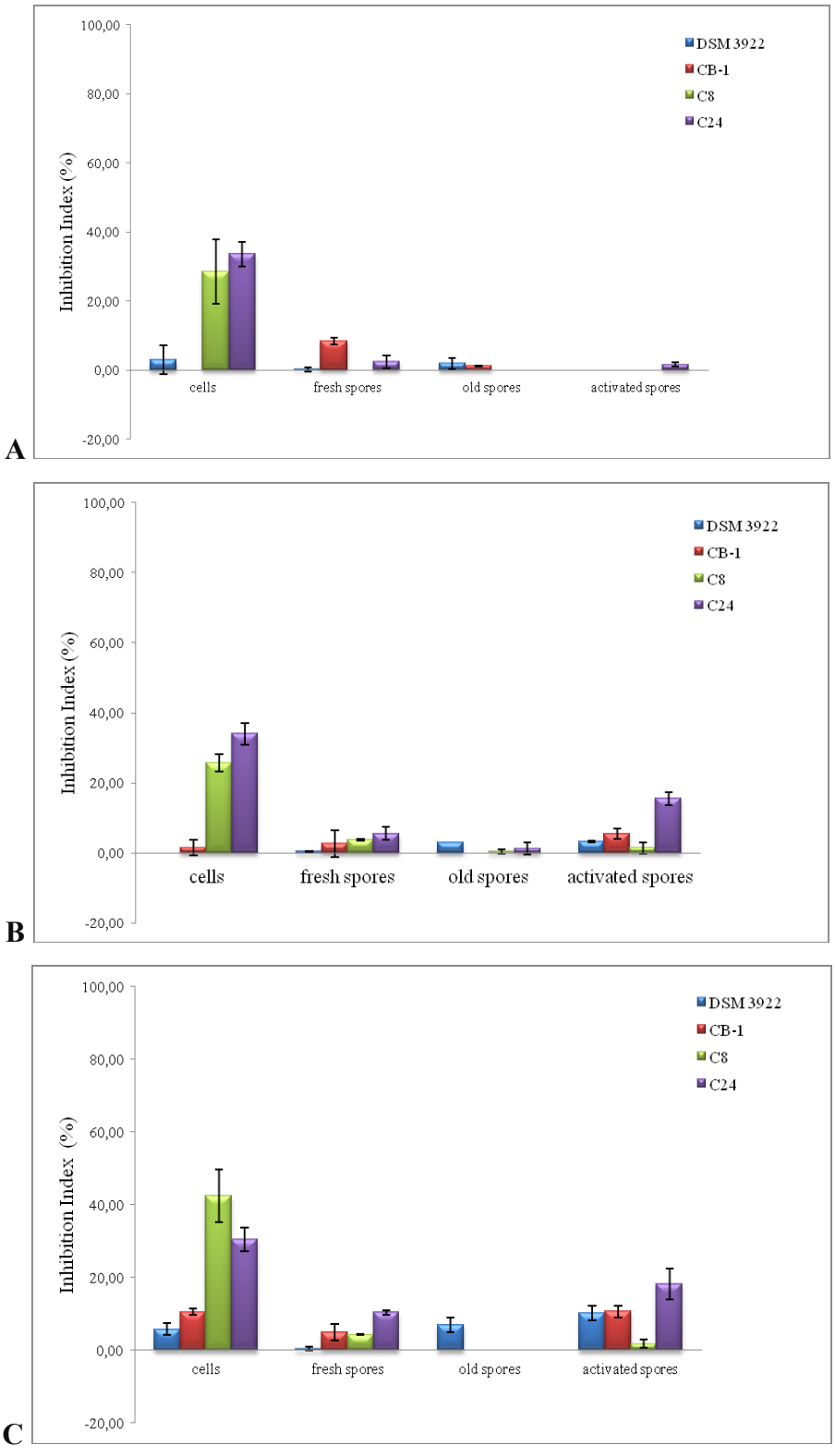


Figure 12: Inhibition Index (%) of *A. acidoterrestris* submitted to ultrasound treatment (power/time/pulse: 40% 6 min 4 s) combined with citrus extract (250 ppm) after 2 days (T2) of incubation at 45±1 °C in acidified Malt Extract Agar. Mean values ± standard deviation.

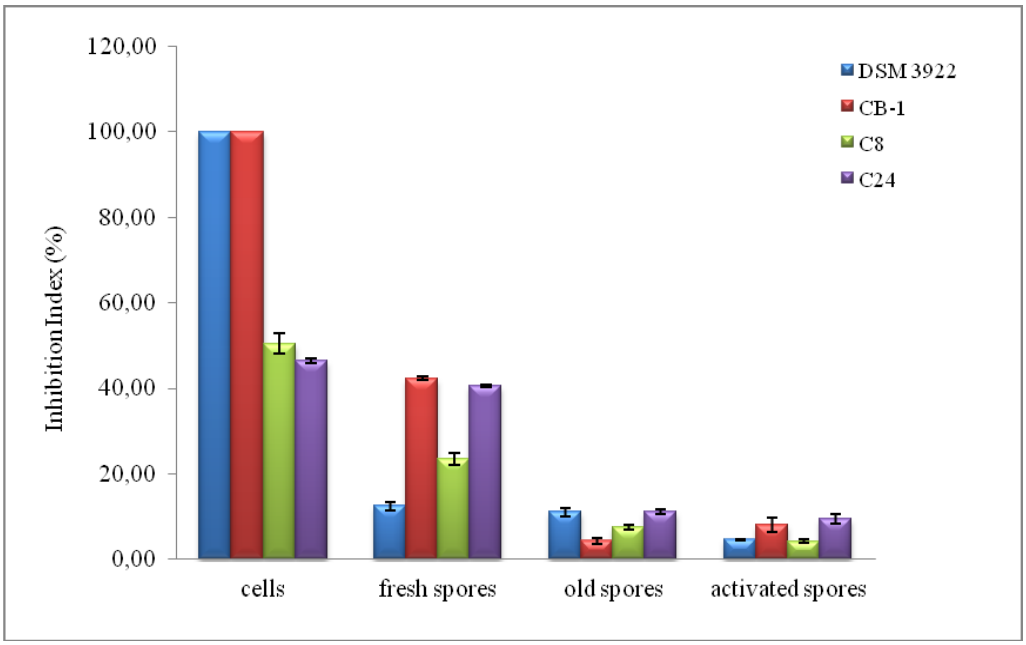


Figure 13: Inhibition Index (%) of *B.clausii* and *B. coagulans* treated with lemon extract (250 ppm) after 2 days of incubation at 30 °C for *B. clausii* and at 40 °C for *B. coagulans* in Alkaline Nutrient Agar and Nutrient Agar, respectively. Mean values ± standard deviation.

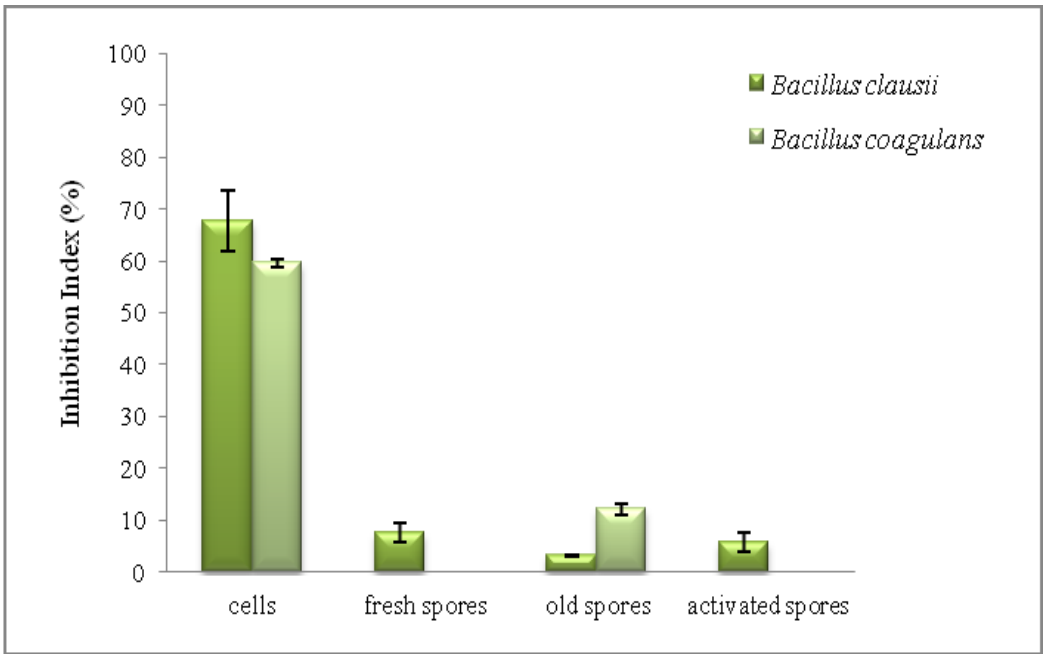
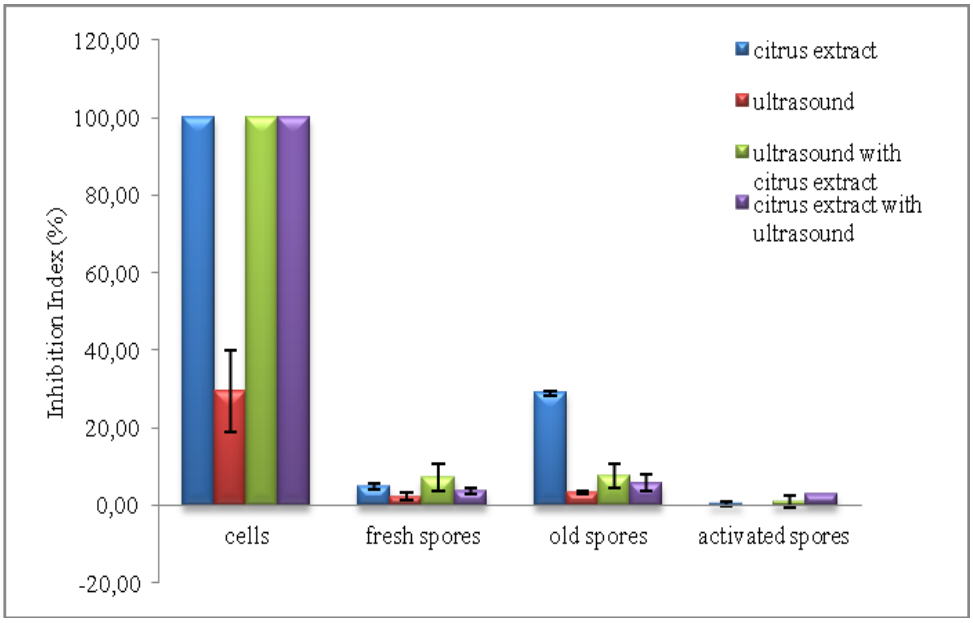


Figure 14: Inhibition Index (%) of *B.clausii* (**A**) and *B. coagulans* (**B**) submitted to ultrasound treatment (power/time/pulse: 40% 6 min 4 s;) combined with citrus extract (250 ppm) after 2 days (T2) of incubation at 30 °C for *B. clausii* and at 40 °C for *B. coagulans* in Alkaline Nutrient Agar and Nutrient Agar, respectively. Mean values ± standard deviation.

A



B

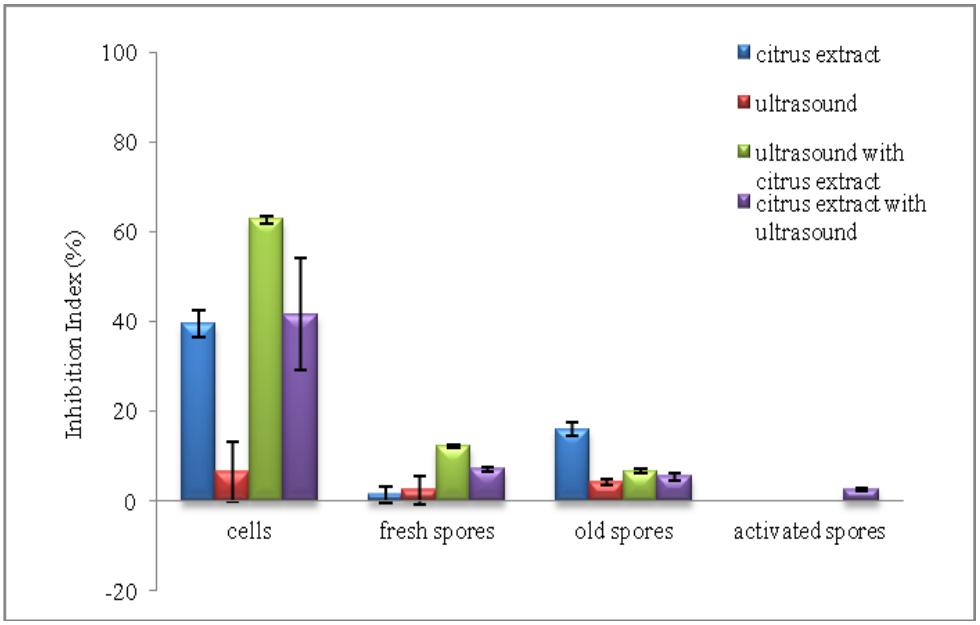
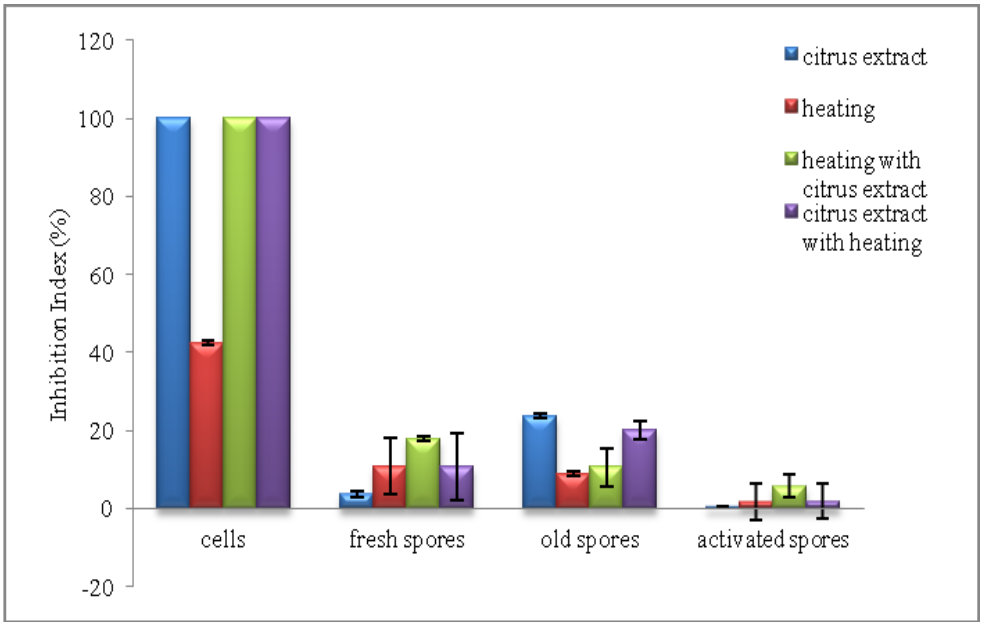


Figure 15: Inhibition Index (%) of *B.clausii* (**A**) and *B. coagulans* (**B**) submitted to heat treatment (95 °C for 5 min) combined with citrus extract (250 ppm) after 2 days (T2) of incubation at 30 °C for *B. clausii* and at 40 °C for *B. coagulans* in Alkaline Nutrient Agar and Nutrient Agar, respectively. Mean values ± standard deviation.

A



B

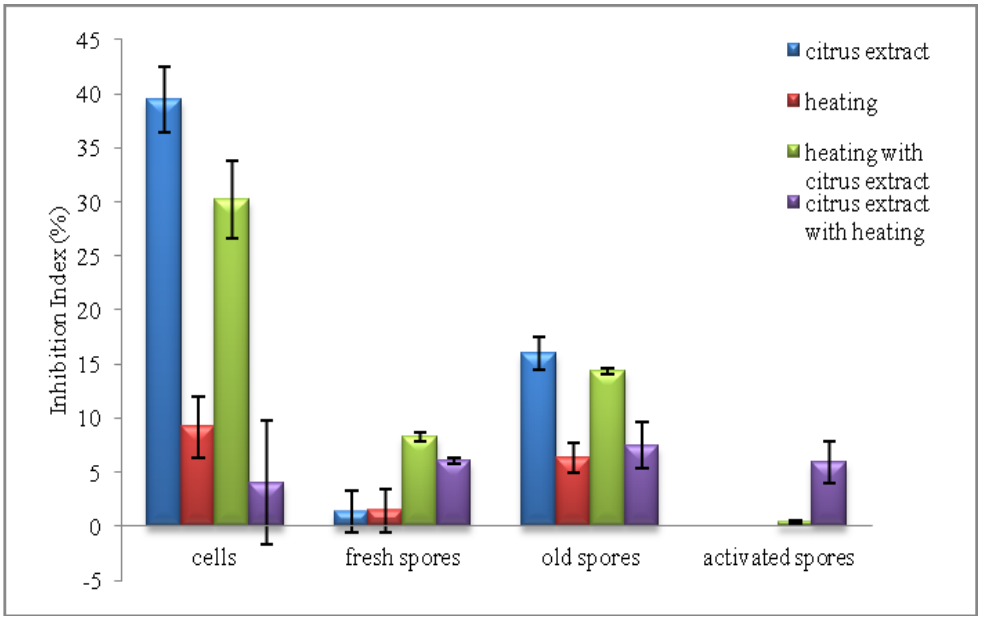
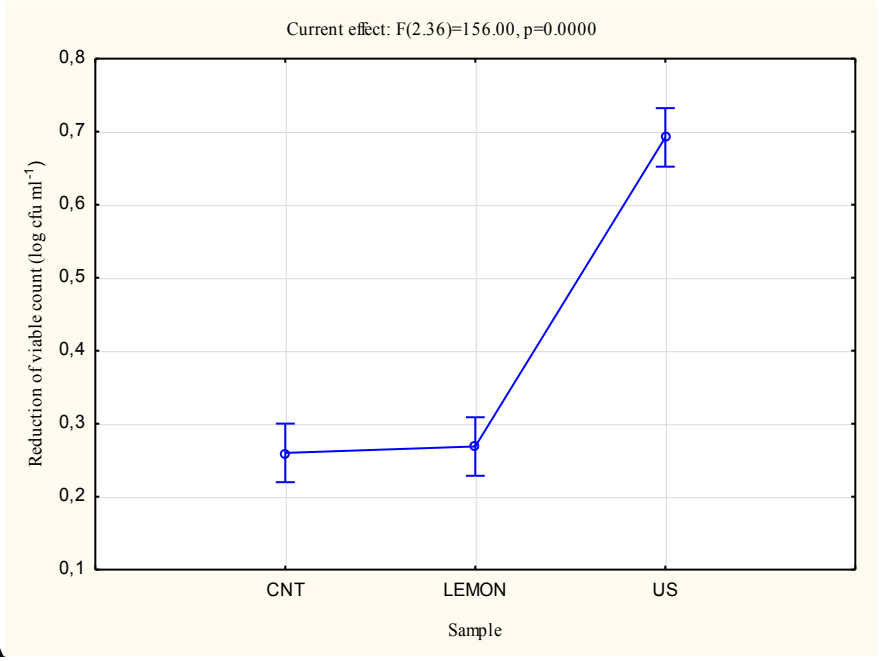
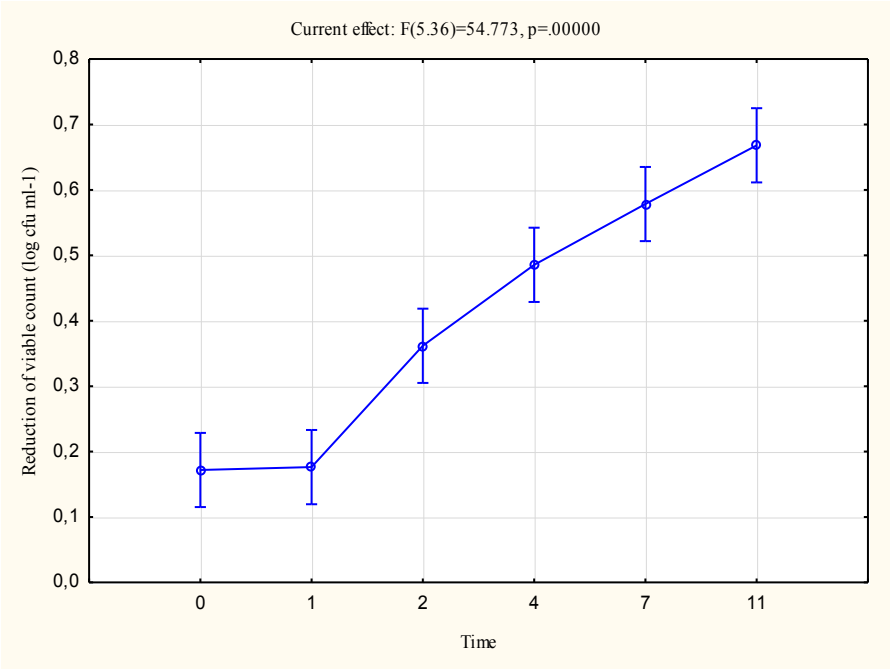


Figure 16: Effective decomposition of factorial ANOVA: effects of lemon extract (250 ppm) addition or US treatment (40% 6 min 4 s) (**Sample** (**A**)), storage (**Time** (**B**)), total population and spores (**Target** (**C**)), interaction treatment/storage/total population and spores (**D**) under *in vivo* conditions. The experiments were carried out in a in a apple juice at 25 °C. The statistical analysis was performed through the factorial analysis of variance (factorial ANOVA) and to Turkey's test ($p < 0.05$), through the software Statistic for Windows (Tulsa, OK).

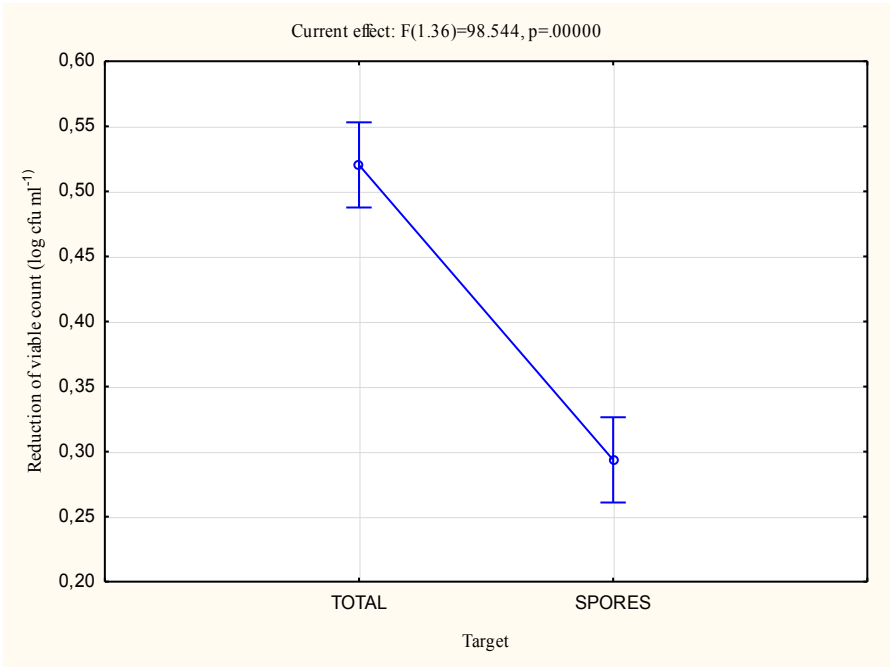


A

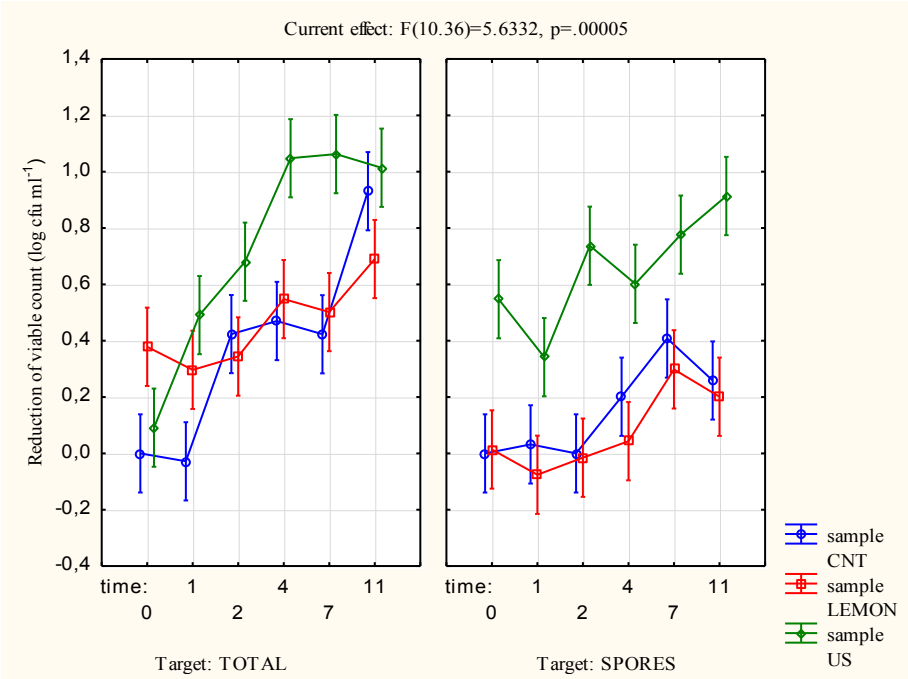
B


Current effect: F(5.36)=54.773, p=.00000
Reduction of viable count (log cfu ml-1)
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
0
1
2
4
7
11
Time

C


Current effect: F(1.36)=98.544, p=.00000
Reduction of viable count (log cfu ml-1)
0,60
0,55
0,50
0,45
0,40
0,35
0,30
0,25
0,20
TOTAL
SPORES
Target

D


Current effect: F(10.36)=5.6332, p=.00005
Reduction of viable count (log cfu ml-1)
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
-0,2
-0,4
time: 0 1 2 4 7 11
Target: TOTAL
Target: SPORES
sample CNT
sample LEMON
sample US

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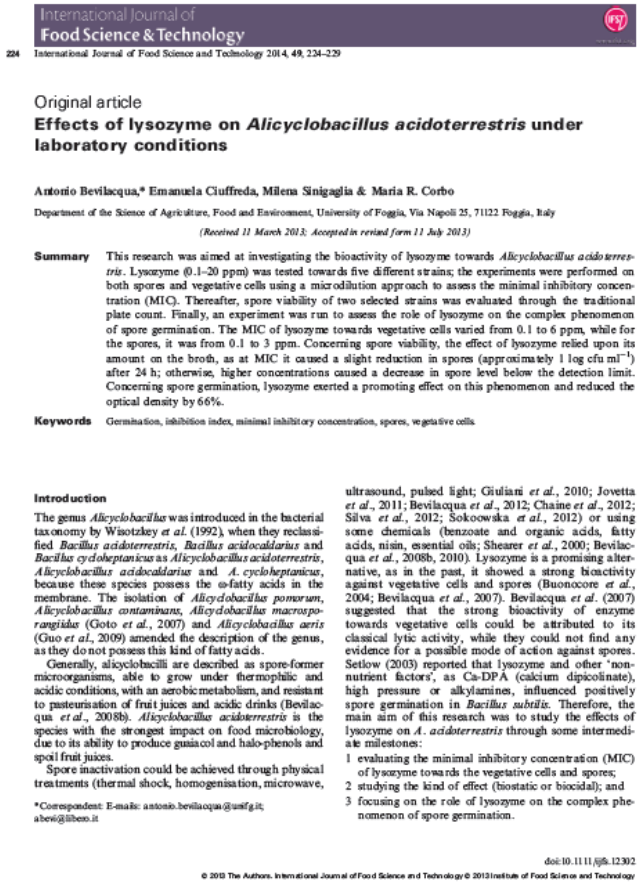
APPENDIX



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Original article

Effects of lysozyme on *Alicyclobacillus acidoterrestris* under laboratory conditions

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Summary This research was aimed at investigating the bioactivity of lysozyme towards *Alicyclobacillus acidoterrestris*. Lysozyme (0.1–20 ppm) was tested towards five different strains; the experiments were performed on both spores and vegetative cells using a microdilution approach to assess the minimal inhibitory concentration (MIC). Thereafter, spore viability of two selected strains was evaluated through the traditional plate count. Finally, an experiment was run to assess the role of lysozyme on the complex phenomenon of spore germination. The MIC of lysozyme towards vegetative cells varied from 0.1 to 6 ppm, while for the spores, it was from 0.1 to 3 ppm. Concerning spore viability, the effect of lysozyme relied upon its amount on the broth, as at MIC it caused a slight reduction in spores (approximately 1 log cfu ml^{-1}) after 24 h; otherwise, higher concentrations caused a decrease in spore level below the detection limit. Concerning spore germination, lysozyme exerted a promoting effect on this phenomenon and reduced the optical density by 66%.

Keywords Germination, inhibition index, minimal inhibitory concentration, spores, vegetative cells.

Introduction

The genus *Alicyclobacillus* was introduced in the bacterial taxonomy by Wisotzkey *et al.* (1992), when they reclassified *Bacillus acidoterrestris*, *Bacillus acidocaldarius* and *Bacillus cycloheptanicus* as *Alicyclobacillus acidoterrestris*, *Alicyclobacillus acidocaldarius* and *A. cycloheptanicus*, because these species possess the ω-fatty acids in the membrane. The isolation of *Alicyclobacillus pomorum*, *Alicyclobacillus contaminans*, *Alicyclobacillus macrosporangiidus* (Goto *et al.*, 2007) and *Alicyclobacillus aeris* (Guo *et al.*, 2009) amended the description of the genus, as they do not possess this kind of fatty acids.

Generally, alicyclobacilli are described as spore-former microorganisms, able to grow under thermophilic and acidic conditions, with an aerobic metabolism, and resistant to pasteurisation of fruit juices and acidic drinks (Bevilacqua *et al.*, 2008b). *Alicyclobacillus acidoterrestris* is the species with the strongest impact on food microbiology, due to its ability to produce guaiacol and halo-phenols and spoil fruit juices.

Spore inactivation could be achieved through physical treatments (thermal shock, homogenisation, microwave, ultrasound, pulsed light; Giuliani *et al.*, 2010; Jovetta *et al.*, 2011; Bevilacqua *et al.*, 2012; Chaine *et al.*, 2012; Silva *et al.*, 2012; Sokoowska *et al.*, 2012) or using some chemicals (benzoate and organic acids, fatty acids, nisin, essential oils; Shearer *et al.*, 2000; Bevilacqua *et al.*, 2008b, 2010). Lysozyme is a promising alternative, as in the past, it showed a strong bioactivity against vegetative cells and spores (Buonocore *et al.*, 2004; Bevilacqua *et al.*, 2007). Bevilacqua *et al.* (2007) suggested that the strong bioactivity of enzyme towards vegetative cells could be attributed to its classical lytic activity, while they could not find any evidence for a possible mode of action against spores. Setlow (2003) reported that lysozyme and other 'nonnutrient factors', as Ca-DPA (calcium dipicolinate), high pressure or alkylamines, influenced positively spore germination in *Bacillus subtilis*. Therefore, the main aim of this research was to study the effects of lysozyme on *A. acidoterrestris* through some intermediate milestones:

1 evaluating the minimal inhibitory concentration (MIC) of lysozyme towards the vegetative cells and spores;
2 studying the kind of effect (biostatic or biocidal); and
3 focusing on the role of lysozyme on the complex phenomenon of spore germination.

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Spore inactivation and DPA release in *Alicyclobacillus acidoterrestris* under different stress conditions

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ABSTRACT

This paper reports on the inactivation of spores of 5 strains of *Alicyclobacillus acidoterrestris* under different stress conditions (acidic and alkaline pH, high temperature, addition of lysozyme, hydrogen peroxide and *p*-coumaric acid). The research was divided into two different steps; first, each stress was studied alone, thus pointing out a partial uncoupling between spore inactivation and DPA release, as H_2O_2 reduced spore level below the detection but it did not cause the release of DPA. A partial correlation was found only for acidic and alkaline pH.

2nd step was focused on the combination of pH, temperature and H_2O_2 through a factorial design; experiments were performed on both fresh and 4 month-old spores and pinpointed a different trend for DPA release as a function of spore age.

1. Introduction

Spoilage of fruit juices and acidic drinks involves lactobacilli (Bevilacqua et al., 2008a), yeasts (*Zygosaccharomyces rouxii*, *Zygosaccharomyces bailii*, *Pichia membranifaciens*, *Saccharomyces* spp. and *Candida* spp.) (Patrignani et al., 2010), heat-resistant species of mycelial fungi (Steyn et al., 2011), anaerobic and aerobic bacilli (*Clostridium butyricum* and *Clostridium pasteurianum*, *Bacillus coagulans* and *Bacillus megaterium*, *Alicyclobacillus* spp.) (Silva and Gibbs, 2004; Brown, 2000), being *Alicyclobacillus acidoterrestris* an emerging spoiling microorganisms for a wide range of high-acid, shelf-stable fruit and vegetables, either hot-filled, pasteurized, canned, ultra heat-treated or carbonated (Steyn et al., 2011).

A. acidoterrestris is a thermophilic, acidophilic and spore-forming bacterium, initially isolated from spoilt apple juice in 1984 (Cerny et al., 1984). It was classified as *Alicyclobacillus*, due to the presence of ω-alicyclic acids and on DNA sequence evidence (Wisotzkey et al., 1992). Spores are able to germinate in acid beverages at pH 3.0–4.5 and heat-resistant with D-values ranging from 16 to 23 min at 90 °C (Splittstoesser et al., 1994). Therefore, they can survive conventional pasteurization and cause spoilage of fruit juice and other acid food products (Chang and Kang, 2004).

Spore resistance towards a wide range of stress (acidic and basic pH, high temperature, radiation and chemical products) was studied for *Bacillus* spp. (Paredes-Sabja et al., 2011) and the resistance and/or spore sensitivity was related to the release of DPA (dipicolinic acid). For example, pressures of 150–400 MPa induce germination in a nutrient-receptor dependent way, through triggering spore's nutrient receptors (Setlow, 2003). The activation of the nutrient receptors causes the release of DPA by opening spore's dipicolinic acid channels. The existence of such DPA-channel proteins is unconfirmed, but it is likely that SpoVAD protein is involved in DPA movement into and out of spores (Li et al., 2012).

Within the core of an endospore, DPA forms a complex with divalent cations (e.g. Ca^{2+}) that excludes water, which contributes to thermal resistance (Setlow, 2006; Gerhardt and Marquis, 1989). As an endospore germinates, DPA is released from its core; DPA is also released when an endospore's structural integrity is compromised by chemicals, heat, or high pressure (Setlow, 2003). The release of DPA is accompanied by activation of spore's cortex lytic enzymes, which are responsible for degradation of the cortex (Paidhungat et al., 2001).

Measurement of DPA has been used to examine the inactivation kinetics of endospores of *Bacillus* spp. following thermal and pressure treatments. The mechanism of spore resistance/susceptibility in *A. acidoterrestris* is not known; thus, the main goal of this research was the evaluation of DPA released by *A. acidoterrestris* as a potential signal indicator of spore injury.

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RESEARCH ARTICLE

Genotypic and Phenotypic Heterogeneity in *Alicyclobacillus acidoterrestris*: A Contribution to Species Characterization

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Abstract

Alicyclobacillus acidoterrestris is the main cause of most spoilage problems in fruit juices and acidic products. Since soil borne species often contaminate fruit juices and do not need strict extreme requirements for survival, it is a great concern to investigate whether and how soil species could evolve from their ecological niches in microbial community to new environments as fruit juices. In this study, 23 isolates of thermo-acidophilic, spore-forming bacteria from soil were characterized by cultural and molecular methods. In addition, 2 strains isolated from a spoilage incident in pear juice were typed. Strains phenotyping showed that they could be grouped into 3 different clusters, and some isolates showed identical or quite similar patterns. Analyzing pH and temperature ranges for growth, the majority of strains were able to grow at values described for many species of *Alicyclobacillus*. Qualitative utilization of lysine, arginine and indole production from tryptophan revealed, for the first time, deamination of lysine and decarboxylation of arginine. Resistance to 5% NaCl as well as the ability to hydrolyze starch and gelatin, nitrate reduction, catalase and oxidase activities confirmed literature evidences. Examining of 16S rRNA, showed that isolates were divided into three blocks represented by effectively soil species and strains that are moving from soil to other possible growing source characterized by parameters that could strongly influence bacterial survival. RAPD PCR technique evidenced a great variability in banding patterns and, although it was not possible to obtain genotypically well-distinguished groups, it was feasible to appreciate genetic similarity between some strains. In conclusion, the investigation of a microbial community entails a combination of metagenomic and classic culture-dependent approaches to expand our knowledge about *Alicyclobacillus* and to look for new subspecies.

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Review

Alicyclobacillus spp.: New Insights on Ecology and Preserving Food Quality through New Approaches

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Abstract: *Alicyclobacillus* spp. includes spore-forming and thermo-acidophilic microorganisms, usually recovered from soil, acidic drinks, orchards and equipment from juice producers. The description of the genus is generally based on the presence of ω-fatty acids in the membrane, although some newly described species do not possess them. The genus includes different species and sub-species, but *A. acidoterrestris* is generally regarded as the most important spoiler for acidic drinks and juices. The main goal of this review is a focus on the ecology of the genus, mainly on the species *A. acidoterrestris*, with a special emphasis on the different phenotypic properties and genetic traits, along with the correlation among them and with the primary source of isolation. Finally, the last section of the review reports on some alternative approaches to heat treatments (natural compounds and other chemical treatments) to control and/or reduce the contamination of food by *Alicyclobacillus*.

Keywords: isolation, source, genotyping, phenotyping

1. Introduction: The General Traits of *Alicyclobacillus* spp.

The genus *Alicyclobacillus* belongs to the family of *Alicyclobacillaceae* [1], and consists of a group of thermo-acidophilic, strictly aerobic, heterotrophic, and spore-forming bacteria [2,3].

"Father of Light and Wisdom,
thank you for giving me
a mind that can know
and a heart that can love.

Help me to keep learning every day of my life
no matter what the subject may be.
Let me be convinced that all knowledge leads to you
and let me know how to find you and love you
in all the things you have made.

Encourage me when the studies are difficult
and when I am tempted to give up
Enlighten me when my brain is slow
and help me to grasp the truth held out to me.

Grant me the grace to put my knowledge to use
in building the kingdom of God on earth
so that I may enter the kingdom of God in heaven."
Amen.

Anonymous