CLÁUDIA VIEIRA PRUDÊNCIO

INFLUÊNCIA DAS CONDIÇÕES DE TEMPERATURA E PH NA SENSIBILIDADE DE *Salmonella* Typhimurium À AÇÃO DE BACTERIOCINAS ASSOCIADAS À EDTA

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola para obtenção do título de *Doctor Scientiae*.

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DEDICO

À minha Família.

“A descoberta consiste em ver o que todos viram e em pensar no que ninguém pensou”.

Szent-Gyorgyi

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Biografia

CLÁUDIA VIEIRA PRUDÊNCIO, filha de José Mauro Prudêncio Vieira e Maria Zulma Vieira, nasceu em Janaúba, Minas Gerais, em 30 de abril de 1984.

Em maio de 2002 iniciou o curso de Nutrição na Universidade Federal de Viçosa, graduando-se, com louvor, em outubro de 2006.

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Em fevereiro de 2010, iniciou o curso de Doutorado em Microbiologia Agrícola no Departamento de Microbiologia na Universidade Federal de Viçosa submetendo se à defesa da tese em fevereiro de 2014.

Sumário

Resumo

PRUDÊNCIO, Cláudia Vieira, D. Sc., Universidade Federal de Viçosa, Fevereiro de 2014**. Influência das condições de temperatura e pH na sensibilidade de *Salmonella* Typhimurium à ação de bacteriocinas associadas à EDTA.** Orientadora: Maria Cristina Dantas Vanetti. Coorientadores: Miguel Prieto Maradona e Hilário Cuquetto Mantovani.

Salmonella é um importante patógeno na área de microbiologia de alimentos e assim como outras bactérias gram-negativas é naturalmente resistente à ação de lantibióticos, como nisina e bovicina HC5. Todavia, a associação com o EDTA é utilizada com sucesso na sensibilização de bactérias gram-negativas à ação destes peptídeos antimicrobianos, por atuar na desestabilização da membrana externa. A ação das bacteriocinas é influenciada por diversos fatores, como temperatura e pH, e embora estas condições ambientais também promovam respostas adaptativas nos micro-organismos, pouco é conhecido sobre o efeito na ação das bacteriocinas, particularmente de bovicina HC5. Neste trabalho, foi avaliada a influência das condições ambientais na sensibilidade de *Salmonella* Typhimurium à ação de nisina ou bovicina HC5 associadas à EDTA. Nisina exibiu atividade bactericida em ampla faixa de temperatura e pH, enquanto bovicina HC5 atuou como agente bacteriostático na maioria das condições utilizadas e exibiu atividade bactericida somente em condições ambientais específicas. Além disso, foi observado que as células pré-adaptadas em condições específicas de temperatura e pH (pH 6,9 e 39,9 °C e pH 7,2 e 27,5 °C) demonstraram maior tolerância ao tratamento com nisina e EDTA, tendo sido detectadas modificações celulares neste grupo de células, relacionadas à membrana celular e à camada de lipopolissacarídeos (LPS), por meio de espectroscopia de infravermelho com transformada de

Fourier (FT-IR). Este comportamento não foi evidenciado com o uso de bovicina HC5, bem como a tolerância cruzada entre estes lantibióticos. Estes resultados indicam que as condições ambientais devem ser consideradas para aplicação de nisina ou bovicina HC5 associadas à EDTA, e que o uso de ambos os lantibióticos pode ser uma alternativa para garantir a segurança contra *S.* Typhimurium.

Abstract

PRUDÊNCIO, Cláudia Vieira, D. Sc., Universidade Federal de Viçosa, February, 2014**. Influence of temperature and pH conditions in the sensibility of *Salmonella* Typhimurium to the action of bacteriocins combined with EDTA.** Advisor: Maria Cristina Dantas Vanetti. Co-advisors: Miguel Prieto Maradona and Hilário Cuquetto Mantovani.

Salmonella is an important pathogen in food microbiology and as well as the other Gram-negative bacteria is naturally resistant to the action of lantibiotics, such as nisin and bovicin HC5. However, the association with the EDTA is successfully employed in the sensitization of Gram-negative bacteria to the action of antimicrobial peptides, for acting in the destabilization of the outer membrane. The action of bacteriocins is influenced by several factors, such as temperature and pH, and although these environmental conditions also promote the adaptive response in microorganism, little is known about the effect on the action of bacteriocin, particularly for bovicin HC5. In this work, we evaluated the influence of environmental conditions of temperature and pH on the sensibility of *Salmonella* Typhimurium to the action of nisin or bovicin associated to EDTA. Nisin to exhibited bactericidal activity in broad range of temperature and pH, while bovicin HC5 acted as bacteriostatic agent in the most conditions utilized and exhibited bactericidal activity in specific environmental conditions. Moreover, was observed that cells pre-adapted in specific conditions of temperature and pH (pH 6.9 e 39.9 °C e pH 7.2 e 27.5 °C) demonstrated greater tolerance to treatment with nisin associated to EDTA. It was detected cellular modifications in this cell group, related to to cell membrane and the lipopolysaccharide layer (LPS), by Fourier transform infrared spectroscopy (FT-IR). This behavior was not evidenced with use of bovicin HC5, as well as the cross-tolerance between these lantibiotics. These results indicate that the environmental conditions should be considered for application of nisin or bovicin HC5 associated to EDTA and the use of both lantibiotics can be an alternative for ensure safety against *Salmonella* Typhimurium.

General Introduction

Salmonella is often cited as a main pathogen cause of foodborne diseases in several countries and one of the most relevant in number of hospitalizations and deaths (Mukhopadhyay and Ramaswamy, 2012; CDC, 2012). This pathogen has been detected in several products of animal and vegetal origin, although it has not been considered one of the most resistant pathogens to the conditions of food processing (Mukhopadhyay and Ramaswamy, 2012). Thus, the security of the foods directs a search for new strategies to control microorganisms, which minimize the alterations in the nutritional and sensory characteristics of the foods, for attend the new demand of consumer, by safe products but less processed (Chalón et al., 2012). Within this context, the use of bacteriocins of acid lactic bacteria stands out as a promising alternative because these are considered as components of the foods and are present naturally in products fermented by lactic acid bacteria (Allende et al., 2007; Settanni and Corsetti, 2008).

The bacteriocins produced by Gram-posite bacteria demonstrate antibacterial activity principally on bacteria of the same group (Balciunas et al., 2013). The use against the Gram-negative cells requires a treatment that destabilizes the outer membrane, the barrier which prevents the passage of these molecules until their site of action (Alakomi et al., 2000; Cao-Hoang et al., 2008). The ethylenediaminetetraacetic acid (EDTA) is one of the most used strategies for achieving this goal and has already demonstrated efficacy in the sensitization of several Gram-negative bacteria like *Salmonella, Enterobacter aerogenes, Shigella flexneri, Citrobacter freudii, Escherichia coli, Pseudomonas aeruginosa* and *Arcobacter butzleri* to bacteriocins such as nisin, gallidermin, piscicolin 126, carnocyclin A, bovicin HC5, and others (Stevens et al., 1991; Boziaris and Adams, 1999; Phillips and Duggan, 2001; Martin-Visscher et al., 2011, Prudêncio et al., 2014).

The activity of bacteriocins can be influenced by several environmental factors such as pH and temperature. These factors also represent signals of microbial adaptations, as observed in the modifications of the cytoplasmic membrane and in the structure of LPS in response to environmental conditions in *Salmonella* (Nikaido, 2003; Álvarez-Ordónez et al., 2008; Kawasaki, 2011; Balciunas et al., 2013). These alterations influenced the permeability of outer membrane, which can alter the resistance to antimicrobial peptides such as bacteriocins (Kawasaki, 2011).

The effects of these factors in the action of nisin and bovicin HC5 associated to EDTA is still scarcely known. In this work, we demonstrated the effect of environmental conditions of temperature and pH on the *Salmonella* Typhimurium sensitivity to nisin or bovicin HC5 associated with EDTA. Moreover, we evaluated the effect of pre-adaptation of cells to nisin and bovicin HC5, and the cross-resistance between both the lantibiotics and also the behavior and molecular changes of the cells pre-adapted to nisin and EDTA.

Chapter 1[*]:

Strategies for the use of bacteriocins in Gram-negative bacteria: relevance in food microbiology

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Strategies for the use of bacteriocins in Gram-negative bacteria: relevance in food microbiology

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Abstract

Bacteriocins are ribosomally synthesized peptides that have bacteriostatic or bactericidal effect on other bacteria. The use of bacteriocins has emerged as an important strategy to increase food security and to minimize the incidence of foodborne diseases, due to its minimal impact on the nutritional and sensory properties of food products. Gram-negative bacteria are naturally resistant to the action of bacteriocins produced by Gram-positive bacteria, which are widely explored in foods. However, these microorganisms can be sensitized by mild treatments, such as the use of chelating agents, heating and freezing. This sensitization is important in food microbiology, because most pathogens that cause foodborne diseases are Gram-negative bacteria. However, the effectiveness of these treatments is influenced by several factors, such as pH, temperature, the composition of the food and target microbiota. In this review, we comment on the main methods used for the sensitization of Gram-negative bacteria, especially *Salmonella*, to improve the action of bacteriocins produced by Gram-positive bacteria.

Key words: bacteriocins, biocontrol, Gram-negative bacteria, outer membrane.

Introduction

Bacteriocins are antimicrobial peptides that are ribosomally synthesized by Gram-negative and Gram-positive bacteria that have bacteriostatic or bactericidal effect on other bacteria (Héchard and Sahl, 2002; Meghrous et al., 1999). These peptides are a heterogeneous group, with differences in their spectrum of activity, biochemical properties, molecular mass, mode of action and others (Abee et al., 1995).

These peptides show great potential for use in foods as a strategy for control of foodborne pathogens and spoilage microorganisms, and they have been recognized as important biological tools for increasing food security and minimizing the incidence of foodborne diseases (Allende et al., 2007; Degan et al., 2006). The use of bacteriocins has minimal impact on the nutritional and sensory properties of foods, thus satisfying consumer demand for products with a lower amount of chemical additives (Galvez et al., 2007; Settani and Corsetti, 2008).

Gram-negative bacteria are naturally resistant to the action of bacteriocins produced by Gram-positive bacteria, because of their outer membrane, which acts as an effective barrier (Cao-Hoang et al., 2008). This barrier is an important physiological tool because of its ability to prevent the entry of macromolecules, such as bacteriocins, possibly due to the size and biochemical characteristics of these peptides, representing an advantage of this bacterial group (Alakomi et al., 2000; Kuwano et al., 2005).

However, the use of chemical compounds, such as chelating agents, or mild treatments, such as heating or freezing, may destabilize the outer membrane and, consequently, reduce the resistance of Gram-negative bacteria to bacteriocin action. This sensitization is relevant in food microbiology, because most pathogens related to foodborne diseases are Gram-negative bacteria (Boziaris and Adams, 1999).

The use of agents or treatments that destabilize the outer membrane enable these peptides to affect Gram-negative bacteria (Belfiore et al., 2007; Martin-Visscher et al., 2011). This strategy has been demonstrated with food additives, such as chelating agents or essential oils; with sanitization treatments using other antimicrobial compounds, such as sodium hypochlorite; and with conservation and food processing treatments, such as freezing, high pressure and pulsed electric fields, on several Gram-negative bacteria, including *Salmonella, Enterobacter aerogenes, Shigella flexneri, Citrobacter freudii, Escherichia coli, Pseudomonas aeruginosa, Shigella sonnei, Arcobacter butzleri, Pseudomonas fluorescens, Yersinia enterocolitica* and *Aeromonas hydrophila* (Tables 1 and 2).

The efficacy of these strategies has already been demonstrated in several tests, both in vitro and in food models. Most such tests have used nisin (Tables 1 and 2), because of its approval for use in foods by the *Food and Drug Administration* (FDA) as a “Generally recognized as Safe” (GRAS) additive (Federal Register, 1988).

Bacteriocins can be subdivided into classes that reflect the similarities and differences of the molecules (Cleveland et al., 2001; Garcia et al., 2010). Class I, also know as lantibiotics, contains small peptides, of 19 to 50 amino acids and a molecular mass of less than 5 kDa, and includes the bacteriocins such as nisin, galidermin and bovicin HC5 (Table 3) (Cleveland et al., 2001). This group is characterized by the presence of unusual amino acids residues, such as lanthionine (Lan) and methyl lanthionine (MeLan), that result from post-translational modifications. These amino acids are formed by the dehydration of serine and threonine, with subsequent condensation with neighboring cysteine residues, producing the characteristic rings of class (Cleveland et al., 2001; Guder et al., 2000; Sahl and Bierbaum, 1998). Lantibiotics are subdivided into subclasses or subtypes according to their chemical structure and activity (Kraaij et al., 1999). Subclass A is composed of amphipathic peptides that are elongated and flexible, with capacity to form pores, such as nisin, galidermin and bovicin HC5. Lantibiotics grouped in subclass B are smaller and globular, such as mesarcidin and cinnamicin, and can inhibit specific enzymes (Kraaij et al., 1999).

Table 1: Use of bacteriocins on Gram-negative bacteria associated to different treatments.

Bacteriocin	Spectrum of Action	Concentration	Associated Treatment	Reference
Nisin	Several sorovars of *Salmonella, E. coli, E. aerogenes, S. flexneri, C. freudii*	50 µg.mL^{-1}	EDTA (20 mM)	Stevens et al. (1991)
	E. coli	200 to 1,000 AU.mL^{-1}	EDTA (10 mM), phosphate (20 mM), citric acid (20 mM) and pyrophosphate (20 mM)	Boziaris and Adams (1999)
	P. fluorescens, S. sonnei, S. Enteritidis	100 AU.mL^{-1}	High pressure processing (155 to 400 MPa, 15 min)	Masschalck et al. (2001)
	Arcobacter butzleri	500 AU.mL^{-1}	EDTA (20 mM) and phosphate trisodium (10 %)	Phillips and Duggan (2001)
	E. coli O157:H7	1,066 AU.mL^{-1}	EDTA (500 and 1000 mM) and sodium lactate (800 mM)	Belfiore et al. (2007)
	E. coli	25 to 1,000 AU.mL^{-1}	Fast freezing	Cao-Hoang et al. (2008)
	E. coli DH5α, *P. aeruginosa, S.* Typhimurium	50 µM	EDTA (20 mM)	Martin-Visscher et al. (2011)

Gallidermin	*E. coli* DH5α, *P. aeruginosa, S.* Typhimurium	50 µM	EDTA (20 mM)	Martin-Visscher et al. (2011)
Bovicin HC5	*S.* Typhimurium	50 and 200 AU.mL^{-1}	EDTA (1.6 mM), citric acid (7 mM), gluconic acid (100 mM) sodium citrate (100 mM), nitrile-tri-acetic acid (6.25 mM) and diethanolamine (50 mM); Tween 80 and Ramnolipid (5X CMC)	Prudencio et al. (2014)
Lactocin 705	*E. coli* O157:H7	2,133 AU.mL^{-1}	EDTA (500 and 1000 mM) and sodium lactate (800 mM)	Belfiore et al. (2007)
Cerein 8A	*S.* Enteritidis,	3,200 AU.mL^{-1}	EDTA (20 to 100 mM), sodium lactate (200 mM)	Lappe et al. (2009)
Carnocyclin A	*E. coli* DH5α, *P. aeruginosa*	25 µM	EDTA (20 mM)	Martin-Visscher et al. (2011)
Carnobacteriocin BM1	*P. aeruginosa*			
Piscicolin 126				

Table 2: Use of bacteriocins on Gram-negative bacteria in foods associated to different treatments.

Bacteriocin	**Spectrum of Action**	**Concentration**	**Associated Treatment**	**Food Matrix**	**Reference**
Nisin	*E. coli*, *S. aureus*	1,280 AU.g^{-1}	High pressure processing (400 MPa, 10 min)	Ham	Garrida et al. (2002)
	Arcobacter butzleri	500 AU.mL^{-1}	Sodium lactate (2 %), sodium citrate (1.5 %)	Chicken	Long and Phillips (2003)
	E. coli, P. fluorescens, L. innocua	500 AU.mL^{-1}	High pressure processing (200 to 500 MPa, 5 min)	Milk	Black et al. (2005)
	S. Typhimurium*, S. aureus*	0.25 to 0.5 µg.mL^{-1}	Essential oil (5 to 30 µL.100mL^{-1})	Barley soup	Moosavy et al. (2008)
	Pseudomonas sp	500 to 1,500 AU.g^{-1}	EDTA (10 mM)	Chicken	Economou et al. (2009)
	S. Stanley, *E. coli* O157:H7, *S.* Newport	300 AU.mL^{-1}	EDTA (20 mM)	Apple juice	Ukuku et al. (2009)
	S. Enteritidis	500 or 1,000 AU.g^{-1}	Oregano essential oil (0.6 or 0.9 %)	Sheep Meat	Govaris et al. (2010)
	Pseudomonas sp.	500 AU.mL^{-1}	EDTA (20 mM), potassium sorbate, sodium benzoate, sodium diacetate (3 %)	Shrimps	Norhana et al. (2012)

Enterocin AS-48	*E. coli* O157:H7	50, 100 or 200 μg.mL^{-1}	EDTA (20 mM), sodium polyphosphate (0.3 or 0.5 %)	Apple juice	Ananou et al. (2005)
	S. enterica sorovar Choleraesuis	30 μg.mL^{-1}	Pulsed electric field (35 kV, 150 Hz)	Apple juice	Viedma et al. (2008)
	S. enterica	25 μg.mL^{-1}	Lactic acid (1.5 %) and polyphosphoric acid (0.1 %), tri-sodium phosphate (1.5 %), sodium hypochlorite (100 ppm)	Soybean sprouts	Molinos et al. (2008)
	E. coli O157:H7, *S. sonnei*, *S. flexneri*, *E. aerogenes*, *Y. enterocolitica* and *A. hydrophila*	25 μg.mL^{-1}	Polyphosphoric acid (different concentrations for each species between 0.2 and 2 %)		

Lantibiotics, particularly those of subclass A, act in sensitive cells by forming pores in the cytoplasmic membrane, which causes an efflux of intracellular metabolites, such as potassium and amino acids, resulting in the depolarization of the membrane and, consequently, cellular death (Helander and Mattila-Sandholm, 2000; Nes et al., 2006). The high efficiency of the bacteriocins in vivo suggests the use of a specific receptor for the anchoring of peptides in the membrane. Nisin, gallidermin and bovicin HC5 appear to use the same receptor molecule, lipid II (Bonelli et al., 2006; Hasper et al., 2004; McAuliffe et al., 2001; Paiva et al., 2011; Wiedemann et al., 2001). Thus, these bacteriocins also interfere with the biosynthesis of peptidoglycan (Güder et al. 2000). Because the lipid II is a molecule that is highly conserved among prokaryotes, if these peptides have access to the cytoplasmic membrane, they will act more efficiently than those peptides that require specific receptors for anchoring to the membrane, such as bacteriocins that use the mannose phosphotransferase system (Martin-Visscher et al., 2011).

Class II bacteriocins are composed of thermostable peptides, containing 20 to 60 amino acids, without post-translational modifications and molecular mass lower than 10 kDa (Table 3) (Deegan et al., 2006; Héchard and Sahl, 2002). Generally, this class is subdivided into subclasses: subclass IIa, is composed of peptides that demonstrate activity against *Listeria* and exhibit a common N-terminal domain (Tyr-Gly-Asn-Gly-Val-X-Cys). Examples of this group include the carnobacteriocin BM1 and the piscicolin 126 (Cintas et al., 2001; Ennahar et al., 2000; Héchard and Sahl, 2002). Subclass IIb is composed of bacteriocins that are formed by two peptides that act synergistically, such as lactocin 705, in which the active molecule is formed by the interaction of two peptides, of 33 amino acids residues each, called 705α and 705β (Castellano et al., 2003). Subclass IIc includes circular peptides that are dependent on the Sec system, such as carnocyclin A and enterocin AS-48 (Cintas et al., 2001; Héchard and Sahl, 2002). Bacteriocins that belong to this class also act by forming pores in the cytoplasmic membrane of target cells (Table 3) (Montalbán-López et al., 2008; Nes and Holo, 2000). Some peptides, such as carnobacteriocin BM1 and piscicolin 126, use the mannose phosphotransferase system as a receptor, which can lead to differences in the sensitivity of target cells, because the amino acid sequence of the receptor may vary among different bacterial species (Martin-Visscher et al., 2011).

Despite some similarities among the bacteriocin molecules of the same class, the sensitivity of target cells varies. These variations are not only due to changes in the bacteriocin molecule, but also to differences in the lipid composition of the target cell membrane (Nissen-Meyer and Nes, 1997).

Bacteriocins associated to food preservatives

The association of bacteriocins with ethylenediaminetetraacetic acid (EDTA) is one of most common strategies in the sensitization of Gram-negative bacteria (Tables 1 and 2). Because the wide use of EDTA as a chelating agent to minimize reactions catalyzed by metals that are related to the spoilage of foods (Branen and Davidson, 2004).

Moreover, it has already been demonstrated that EDTA promotes the release of the LPS layer of *Salmonella* Typhimurium cells, confirmed by the release of the specific lipid material of this structure, at the beginning and at the end of the exponential growth phase (Alakomi et al., 2003). Details of the action mechanism are not yet understood, but it is known that there is at least partial disruption of the lipopolysaccharide layer, possibly due to binding to calcium and magnesium ions, which would establish a cross-link with sugar residues and phosphate radicals, inside the core polysaccharide, reinforcing the structure of the outer membrane (Alakomi et al., 2003; Branen and Davidson, 2004).

Thus, EDTA acts potentiating the bacterial activity of other antimicrobials, in addition to expanding the spectrum of action of bacteriocins, particularly against Gram-negative bacteria, such as *Salmonella, E. aerogenes, S. flexneri, C. freudii, E. coli, P. aeruginosa* and *A. butzleri* (Table 1) (Branen and Davidson, 2004). Generally, low concentrations of EDTA (10 to 20 mM) are sufficient to produce sensitization for bacteriocin activity *(*Tables 1 and 2).

Table 3: General characteristics of bacteriocins used against Gram-negative bacteria.

Classification	Bacteriocin	Producer Strain	Biochemical Characteristics	Mode of Action
Lantibiotic	Nisin	*Lactococcus lactis* subsp lactis	Amphipathic peptide, elongated, positively charged, of 34 amino acid residues and molecular mass of 3.3 kDa. Contains unusual amino acids (lanthionine and methyl-lanthionine), derived from post-translational modifications. Stable to heat and to pH (between 2.0 and 7.0).	Formation of pores in the cytoplasmic membrane and inhibition of cell wall synthesis.
	Gallidermin	*Staphylococcus gallinarum*	Peptide of 22 amino acid residues and molecular mass of 2.2 kDa.	
	Bovicin HC5	*Streptococus bovis HC5*	Peptide of 2.4 kDa, about 22 amino acid residues. Stable to heat and acidic pH.	
-[a]	Cerein 8A	*Bacillus cereus 8A*	Peptide with molecular mass of 26 kDa. Stable at temperatures below 80 °C and pH between 2.0 and 11.0	Possible formation of pores in the cytoplasmic membrane.

Class II	IIc	Carnocyclin A	*Carnobacterium maltaromaticum*	Circular peptide of 60 amino acid residues and molecular mass of 5.9 kDa. Stable to heat and pH between 2.0 and 12.0.	Formation of pores in the cytoplasmic membrane.
	IIa	Carnobacteriocin BM1	*Carnobacterium maltaromaticum*	Peptide of 43 residues of amino acids and molecular mass of 4.5 kDa.	
	IIc	Enterocin AS-48	*Enterococcus faecalis* subsp. *liquefaciens* A 48-32	Circular cationic peptide of 70 residues of amino acids, molecular mass of 7.2 kDa. Presents globular structure, formed by five helices (α_1 a α_5), with pI 10.5. Stable to heat and acidic pH.	
	IIb	Lactocin 705	*Lactobacillus casei* CRL705	Its activity depends of complementation of two peptides, called 705α and 705β, of 33 amino acids residues each.	
	IIa	Piscicolin 126	*Carnobacterium maltaromaticum*	Peptide of 44 residues of amino acids and molecular mass of 4.4 kDa. Stable to heat and acidic pH, but inactivated at neutral and alkaline pH.	

Note: ^{a} Not determined.

However, several factors can alter the sensibility of Gram-negative cells to bacteriocins produced by Gram-positive bacteria. For example, the manner of administration of the chelating agent can make a difference. The simultaneous use of the bacteriocin and the chelating agent is recommended, to increase the efficiency of inhibition (Phillips and Duggan, 2001; Stevens et al., 1991). Furthermore, the inhibition of microbial growth appears to be a time-dependent process, and the method of application can be a critical factor in obtaining the desired effect (Boziaris and Adams, 1999).

The sensitivity to the combination of bacteriocins and EDTA varies between different species of Gram-negative bacteria, and even between different strains of the same species. The concentration of chelating agent necessary for sensitization is also variable, possibly because of the differences in the structure of the LPS layer, which interfere with the permeability (Boziaris and Adams, 1999).

For use in foods, large quantities of the chelating agent may be required, for the removal of exogenous divalent cations that are associated with the food system, and for the effective sensitization of Gram-negative cells (Boziaris and Adams, 1999; Lappe et al., 2009). Moreover, the activity of the chelating agent is influenced by pH: at low pH, a large proportion of the carboxylate group is in its non-ionized form, which is not a particularly effective electron donor, and the EDTA-metal complex is less stable. Therefore, EDTA is preferentially used at neutral pH, when the compound is more active. Otherwise, most bacteriocins, such as nisin and bovicin HC5, are more efficient at low pH value (Ananou et al., 2005; Boziaris and Adams, 1999; Houlihan et al., 2004; Lappe et al., 2009; Norhana et al., 2012).

Other chemical compounds can also be used for the disruption of the outer membrane, such as acids, salts and essential oils (Tables 1 and 2). Lactic, citric and polyphosphoric acids and their salts, as well as tri-sodium phosphate, act as disintegrating agents of the outer membrane, and have demonstrated activity in the sensitization of Gram-negative bacteria, including *E. coli, S. sonnei, S. flexneri, E. aerogenes, Y. enterocolitica, A. hydrophila, Salmonella* and *Arcobacter butzleri* (Tables 1 and 2) (Alakomi et al., 2000; Belfiore et al., 2007; Long and Phillips, 2003; Molinos et al., 2008a; Phillips and Duggan, 2001). These associations are efficient in foods, and different methods of application can be employed, such as in a washing solution for sanitization or addition of bacteriocin with compounds, similar to food additives (Table 2) (Belfiore et al., 2007; Molinos et al., 2008).

Bacteriocins associated with essential oils

Plant essential oils are volatile complex natural substances, characterized by the presence of phenolic compounds with a strong odor that are produced by aromatic plants as secondary metabolites. Essential oils may have bactericidal, fungicidal, virucidal activities and medicinal properties, and have practical applications, such as analgesics, perfumes, anti-inflammatory agents, local anesthetics and food preservatives (Bakkali et al., 2008; Burt, 2004).

Components of essential oils such as thymol and carvacrol, act on the bacterial membrane, resulting in important morphological alterations and the depletion of the intracellular content (Govaris et al., 2010; Moosavy et al., 2008). The mechanism of action of such agents is still poorly understood, but it is known that thymol and carvacrol act to disintegrate the outer membrane, and their activity does not involve the chelation of divalent cations from the outer membrane, because compounds, such as magnesium chloride, do not interfere with their activity, in constrast to the action of EDTA (Helander et al., 1998).

However, the use of essential oils and their derivatives in foods is limited by sensory changes, because high concentrations are needed to exert antimicrobial activity (Govaris et al., 2010; Gutierrez et al., 2008; Nazer et al., 2005). Furthermore, food composition may influence the action of essential oils: high protein concentrations and moderately acidic pH result in an increase in the antimicrobial activity of oregano and thyme essential oils, while concentrations of potato starch or sunflower oil greater than 5% reduce their efficiency (Gutierrez et al., 2008).

Thus, one alternative has been an association with other antimicrobial agents, such as bacteriocins. The effectiveness of this strategy has already been demonstrated with carvacrol and pediocin on *E. coli* O157:H7 (Turgis et al., 2012), and carvacrol and thymol and nisin on *S.* Enteritidis, and others (Table 2) (Govaris et al., 2010). This strategy is viable, because of the consequent reduction of the amount of antimicrobial added to foods, and such a dual application prevents possible undesirable sensory changes due to the presence of large amounts of essential oils (Govaris et al., 2010; Nazer et al., 2005; Turgis et al., 2012). This was demonstrated in *S*. Typhimurium and *S. aureus*, in which the presence of nisin considerably reduced the concentration of essential oil for the inhibition of both bacteria (Mooasavy et al., 2008).

Bacteriocins associated with high-pressure processing (HPP)

High-pressure processing has been evaluated as a food pasteurization technique for inactivating microorganisms at room temperature, and thus minimizing the loss of sensory and nutritional components of the food (Huang et al., 2014; Masschalck et al., 2001). Under normal conditions, the process preserves the original color, flavor and nutritional content because smaller molecules such as pigments, vitamins, volatile compounds and others are less affected by high pressure (Huang et al., 2014).

The mode of action of HPP depends on the level of pressure. Pressures between 30 and 50 MPa can influence gene expression, protein synthesis and reduce the number of ribosomes. A pressure of 100 MPa induces partial protein denaturation, while 200 MPa causes damage to the cytoplasmic membrane and the internal cell structure. An increase of 300 MPa or more induces irreversible damage, such as the denaturation of enzymes and proteins, and the rupture of the membrane (Garrida et al., 2002; Huang et al., 2014). Microorganisms demonstrate differences in their resistance to pressure, depending on the species, strain, physiological state, processing temperature and substrate (Huang et al., 2014; Patterson, 2005). As the different levels of pressure exert distinct effects, it is necessary to evaluate the response of each microorganism in each food system (Huang et al., 2014). In low-acid foods, vegetative cells exhibit great resistance to this process, requiring high pressure for the inactivation of microorganisms, which is not economically feasible, besides the possibility of interference in the sensory characteristics of foods, such as texture and color (Black et al., 2005; Garrida et al., 2002; Masschalck et al., 2001).

Generally, HPP treatment does not completely inactivate microorganisms, allowing the recovery of injured cells, but such recovery is dependent upon the treatment conditions and of the presence of other antimicrobial compounds (Patterson, 2005). Food constituents may protect microbial cells against the increase in hydrostatic pressure or facilitate their recovery after treatment. Thus, to prevent the emergence of resistant cells, this technology has been used in conjunction with other antimicrobial compounds to ensure food safety. One of the alternatives is the use of bacteriocins, because high pressure destabilizes the outer membrane, increasing the activity of bacteriocins in Gram-negative cells. Furthermore, Gram-negative bacteria are more sensitive to HPP, whereas Gram-positive bacteria are more sensitive to bacteriocins. Thus, combined use is complementary (Garrida et al., 2002; Masschalck et al., 2001; Patterson, 2005).

Several works have demonstrated the efficacy of this strategy and report an increase in bactericidal activity against important pathogens, such as *Salmonella* and *E. coli,* in addition to the spoilage microorganisms, that are measured by the total plate count in juice (Tables 1 and 2) (Ponce et al., 1998; Rodriguez et al., 2005; Zhao et al., 2013). Moreover, pressure-resistant strains of *E. coli* have also demonstrated sensitivity to nisin, when treated under high pressure (Masschalck et al., 2000).

However, the process of sensitization can be transient, this is occurs only during the period in which the Gram-negative cells are subjected to high pressure. Therefore, the simultaneous administration of bacteriocin with high pressure is recommended (Black et al., 2005; Masschalck et al., 2001). The sensitivity of different species is variable, and *P. fluorescens* demonstrated more sensitivity than *E. coli,* in milk (Black et al., 2005).

Bacteriocins associated with a Pulsed Electric Field

As well as the use of HPP, the application of a pulsed electric field is considered a non-thermal technology that acts by forming reversible or irreversible pores in the cell membrane. This process improves the sensitization of Gram-negative bacteria to the action of bacteriocins, which work synergistically, increasing the damage to the cytoplasmic membrane (Table 2) (Viedma et al., 2008).

There is little information available about the effect of this strategy in Gram-negative bacteria. However, it is known that the efficiency of the treatment increases with temperature, possibly due to the increase in cell membrane fluidity, which facilitates the process of disorganization promoted by these treatments. However, this parameter can be maintained in mild temperatures. For example: for apple juice, a temperature of 40 °C is adequate, and such a temperature ensures sensory and nutritional qualities and reduces the costs of the process, which is of great interest for the food processing industry (Viedma et al., 2008).

As with other treatments, the method of application is an important parameter, with the simultaneous use of pulsed electric fields with bacteriocins being more efficient. This is primarily due to the high resistance of Gram-negative bacteria to treatments with pulsed electric fields, particularly at acidic pHs, a condition under which bacteriocins demonstrate greater efficiency (Boziaris and Adams, 1999; Houlihan et al., 2004; Viedma et al., 2008).

Bacteriocins associated with physical treatments

Physical treatments may promote perturbations in the outer membrane, either at low or high temperatures, favoring the action of bacteriocins.

A reduction in temperature promotes a change in the structure of the outer membrane. These alterations may permeabilize the cell to bacteriocin, allowing the nisin to act on *S.* Typhimurium and *E. coli* at refrigeration temperatures (Elliason and Tatini, 1999). The chilling process only allows the effective sensitization of Gram-negative bacteria to the action of bacteriocins, when the temperature drops rapidly, because there is not enough time for the reorganization of the outer membrane, altering its permeability. For *E. coli*, it has been demonstrated that the growth phase influences the concentration of bacteriocin required for inhibition. Thus, cells in stationary phase under rapid chilling require higher concentrations of nisin (Cao-Hoang et al., 2008). As observed with other strategies, simultaneous treatment with bacteriocins has a more significant result than the sequential use (Cao-Hoang et al., 2008).

The bactericidal effect varies depending on the strains, bacteriocins and methodologies used, as demonstrated by the results of Kalchayanand et al. (1992), wherein *S.* Typhimurium and *Y. enterocolitica* were more sensitive to nisin, while *A. hydrophila* and *Pseudomonas putida* were more sensitive to pediocin during freezing.

Heat also sensitizes Gram-negative bacteria to the action of bacteriocins. The application of bacteriocin after heat treatment resulted in a synergistic effect, and unlike other treatments, simultaneous use only slightly increases the reduction in viability (Ueckert et al., 1998).

Differences related to strains and bacteriocins were also observed with the use of heat, and pediocin was generally more efficient than nisin (Kalchayanand et al., 1992).

Conclusions

Currently, there is a large number of characterized bacteriocins with potential for use in food. However, the effectiveness of this strategy depends on several factors, such as pH, temperature, food composition and target microbiota. Therefore, it is necessary to establish effective conditions for the use of each bacteriocin in each food matrix.

Although several agents and/or treatments used in combination with bacteriocins inhibit bacterial growth alone, the presence of these peptides provides an additional level of protection by preventing the growth of cells affected by sublethal damage, thus ensuring greater safety for the food product.

The use of bacteriocins in association with chemical compounds or physical treatments allows us to extend the spectrum of action of these peptides on Gram-negative bacteria, in addition to minimizing the emergence of resistant cells. However, for effective application of this technology, more studies are necessary, with different food matrices and in mixed cultures, to understand how bacteria can survive and adapt in a complex environment.

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Chapter 2[*]

Temperature and pH influence the sensitivity of *Salmonella* Typhimurium to nisin combined with EDTA

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[*] Formatting according to International Journal of Food Microbiology author guidelines

Temperature and pH influence the sensitivity of *Salmonella* Typhimurium to nisin combined with EDTA

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Abstract

Lantibiotics, such as nisin, generally act on Gram-positive bacteria, whereas Gram-negative bacteria exhibit natural resistance to the action of these bacteriocins. However, these bacteria can become susceptible to the action of these antimicrobial peptides with the use of chelating agents, such as EDTA. *Salmonella* have great environmental adaptability and can alter their composition of LPS in response to environmental changes, but the effect on nisin sensitivity is unknown. In this work, we examined the effect of variations in temperature and pH on the sensitivity of *Salmonella* Typhimurium to the action of nisin in association with EDTA. The reduction in the number of CFU/mL varied according to the temperature and pH conditions, with a larger reduction in the number of logarithmic cycles with to a temperature increase. These factors can influence the composition of LPS and the susceptibility of *Salmonella* to the action of bacteriocin and thus should be considered for establishing the conditions for nisin use.

Keywords: bacteriocins, *Salmonella* Typhimurium, biocontrol, outer membrane.

1. Introduction

Nisin is a bacteriocin belonging to the lantibiotics group, which is characterized by an elongated shape, amphipathic properties, and a net positive charge (Breukink and Kruijff, 1999; Sahl et al., 1995; Settanni and Corsetti, 2008). Similar to other members of the group, nisin is subjected to post-translational modifications, involving the formation of lanthionine rings, a characteristic of this class (Breukink and Kruijff, 1999; Dawson and Scott, 2012; Settanni and Corsetti, 2008). Moreover, nisin stands out for being approved for use in the food industry, and can replace chemical additives, with minimal changes in sensory characteristics (Dischinger et al., 2014; Nagao, 2009; Settanni and Corsetti, 2008).

Nisin has a dual mechanism of action, that involves the formation of pores in the cytoplasmic membrane, leading to dissipation of the proton motive force, due to the loss of electrolytes and other metabolites, and the inhibition of the cell wall synthesis, by binding to lipid II, which interferes with the biosynthesis of peptidoglycan (Güder et al., 2000; Helander and Mattila-Sandholm, 2000).

Lantibiotics, such as nisin, act primarily on Gram-positive bacteria (Sahl et al., 1995). In contrast, Gram-negative bacteria, such as *Salmonella*, generally exhibit resistance to these bacteriocins (Arques et al., 2011; Cao-Hoang et al., 2008; Dischinger et al., 2014). The outer membrane, and in particular, the lipopolysaccharide (LPS) structure, acts as a barrier, preventing the diffusion of bacteriocin to its site of action (Alakomi et al., 2000). However, this group of bacteria can become susceptible to the action of these antimicrobial peptides, with use of treatments that destabilize the outer membrane, such as heating, freezing, high pressure processes, plant essential oils and chelating agents, such as ethylenediaminetetraacetic acid (EDTA) (Boziaris and Adams, 1999; Govaris et al., 2010; Lee and Kaletunç, 2010; Molinos et al., 2008).

This sensitization can be important due to the high incidence of foodborne diseases caused by Gram-negative bacteria, particularly *Salmonella* (Gálvez et al., 2010). On the other hand, microorganisms demonstrate large adaptability to environmental changes, such as the modifications in LPS composition and structure in response to environmental conditions observed in *Salmonella*

Typhimurium (Kawasaki, 2011; Nikaido, 2003). These modifications regulate features, such as the biological activity of lipid A, and interfere with the permeability of the outer membrane, though their effect on the action of nisin is still unknown (Kawasaki, 2011).

This study was designed to demonstrate the effect of temperature and pH on the sensitivity of *Salmonella* Typhimurium to nisin associated with EDTA.

2. Material and Methods

2.1 Strains and growth conditions

Salmonella enterica serovar Typhimurium ATCC 14028 was grown in Brain and Heart Infusion broth (BHI, Himedia, India), at 37 ± 1 °C for 24 h. *Lactococcus lactis* ATCC 19435 was cultivated in MRS broth (MRS, Himedia, India), at 37 ± 1 ^{o}C, and used to determine the antimicrobial activity of nisin.

2.2 Preparation, activity, and quantification of nisin

Nisin stock solution (Nisaplin, Danisco, Denmark) was prepared by diluting the stock powder in sodium phosphate solution (0.05 M, pH 2.0). *L. lactis* was used to determine the antimicrobial activity by the diffusion method in agar (Tagg, 1976) and quantified by the critical dilution method (Hoover and Harlander, 1993), and the concentration was expressed in arbitrary units (AU/mL).

The concentration of the peptide was determined by HPLC, using a reverse phase column (Acclaim 120, C18, 5 µm, 150 x 4.6 µm (inner diameter)), according to a standard curve with concentrations between 37.5 and 600 µM. The samples were eluted using an isocratic gradient with 35% solution A (0.1% trifluoroacetic acid (TFA) in ultrapure water) and 65% solution B (0.1% TFA in water and 80% acetonitrile), at a flow rate of 1.0 mL/min at 22 °C. Absorbance was monitored at 214 and 280 nm, using a Dionex 3000 Diode Array Detector (DAD) (Paiva et al., 2012).

2.3 Evaluation of the action of nisin with EDTA in S. Typhimurium under different conditions of pH and temperature

Prior to treatments, the cells were centrifuged at 2,500 x *g* for 15 min at 4 ° C (Sorvall RT 6000D, USA), washed, and resuspended in 0.85% saline. The cultures were inoculated with an initial concentration of 10^5 CFU/mL in BHI broth at different pH values (Table 1), with nisin (200 AU/mL, equivalent to 115 μM) and EDTA (1.5 mM) (Reagen, Brazil). These concentrations previously demonstrated an inhibitory effect on *S.* Typhimurium, under optimal growth conditions (Prudencio et al., 2014). Controls included cells grown in BHI broth and in BHI broth with nisin and EDTA, separately, at the same concentrations.

The tubes were incubated at different temperatures (Table 1), according to the experimental design, and growth was assessed at different time intervals for a period of 5 days using the drop plate method, with three replicates, with Plate Count Agar (PCA, Difco, USA). The plates were incubated at 37 ± 1 °C for a period of 8 to 12 h.

2.4 Experimental design

A central composite design with two factors was used to evaluate the effects and interactions of temperature and pH on the inactivation of *Salmonella* Typhimurium by nisin (115 μM) associated with EDTA (1.5 mM). The independent variables were temperature (10 to 45 °C) and pH (5.0 to 7.2). The central point was repeated three times to estimate the variation due to random errors. The remaining tests were performed once, and all experiments were performed at random to minimize possible effects of environmental conditions.

2.5 Statistical analysis

To determine the influence of temperature and pH on the inactivation of *Salmonella* Typhimurium by nisin associated with EDTA, the results were analyzed by multiple linear regressions, by applying the response surface methodology. The data subjected to analysis were based on the average of the results obtained after 0, 2, 4, 6 and 12 h of treatment. The simplest models that best explained the behavior of the data were chosen (Table 2).

3. Results

The growth of *S.* Typhimurium in BHI broth was proportional to the increase in temperature and pH. Temperature appeared to exert more influence than pH, because no growth was detected at low temperature ranges, independent of the pH value (Fig. 1).

The addition of EDTA or nisin separately to the culture medium minimized the growth of the microorganism at a wide range of temperature and pH values, particularly with the addition of the chelator. EDTA and nisin were remarkably influenced by pH. It was noted that the maximum number of cells was attained only with a neutral pH and median or high temperatures (>35 °C) (Figs. 2 and 3). Under conditions of low pH (<5.5) and high temperatures (>40 °C) there was a tendency for a reduction in the number of cells when nisin was added (Fig. 3).

The combination of nisin with EDTA was effective in reducing the growth of *S.* Typhimurium. The reduction varied according to the environmental conditions of pH and temperature, with temperature being the main factor. A larger reduction in the number of viable cells was observed with an increase in temperature (Fig. 4). In the first hours of treatment, there was a greater reduction in the number of log cycles of viable cells under conditions of neutral pH, possibly due to a better performance of EDTA.

The models developed for the behavior of *Salmonella* Typhimurium in BHI with or without addition of nisin or EDTA were the simplest (Table 2). For these cases, it was observed that the growth of the microorganism occured linearly as a function of both factors. There was no significant interaction between temperature and pH, except for the addition of nisin. The addition of EDTA to the medium increased the variability of the data, which prevented a good fit for the observed data (Table 2). The combination of nisin with EDTA produced a second-order response, with a significant effect of interaction between the factors. The results allowed the determination of the best point for inhibitory activity, which was a pH of 6.2 and temperature of 43.6 °C. The concordance of the model was 97.6%, indicating successful data fitting.

Under conditions of low temperature and low pH (condition 1: pH 5.3 and 15.1 °C), a considerable reduction in the number of log cycles was observed, after prolonged exposure to bacteriocin, regardless of the presence of EDTA (Fig. 5). Similar results were obtained at neutral pH (condition 5: pH 6.1 and 10 °C). These results may indicate that under these conditions, environmental factors promote the formation of a layer of LPS that is more sensitive and allows the action of nisin by itself.

Under some conditions, an increase in culture viability was observed after prolonged incubation, suggesting an adaptive effect after prolonged treatment with a combination of bacteriocin with EDTA. These results were observed more frequently under neutral pH conditions and higher or median temperatures (Fig. 6). Under these conditions, the bacteriocin remained stable during the incubation period, thus strengthening the hypothesis of adaptation by the cells (data not shown).

4. Discussion

The combination of bacteriocins with EDTA is one of the most used strategies for the sensitization of Gram-negative bacteria to the action of these peptides (Belfiore et al., 2007; Branen and Davidson, 2004; Martin-Visscher et al., 2011; Prudencio et al., 2014). The chelator acts by promoting the release of the LPS layer, allowing access of the bacteriocin to the cytoplasmic membrane (Alakomi et al., 2003). In general, low concentrations of EDTA (10-20 mM) are sufficient to sensitize Gram-negative microorganisms to the action of bacteriocins (Branen and Davidson, 2004), and minimal concentrations (1.5 mM) were shown to be effective for this purpose in our experiments.

The susceptibility to nisin combined with EDTA can vary among different species of Gram-negative bacteria, or even between different strains of the same species (Boziaris and Adams, 1999). Such differences can be due to variations in the structure of the LPS layer, which could influence the permeability of the outer membrane and alter the susceptibility to bacteriocins (Boziaris and Adams, 1999; Kawasaki, 2011).

The results showed, for the first time, that the susceptibility of *Salmonella* Typhimurium to the combination of nisin with EDTA is influenced by conditions of temperature and pH. Such differences can be related to the activity of the chelator, which is largely influenced by pH. Thus at low pH, a large proportion of the carboxylate group is not ionized and does not function as a good electron donor, while the EDTA-metal complex is less stable. Accordingly, EDTA exhibits higher activity at neutral pH, favoring a greater number of log reductions under these conditions (Fig. 4). Nonetheless, neutral pH does not favor the activity of nisin, which is most effective at a low pH (Ananou et al., 2005; Boziaris and Adams, 1999; Lappe et al., 2009; Norhana et al., 2012).

The variation in growth conditions, such as sub-optimal temperature and pH or lack of nutrients, may cause stress to bacterial cells. These conditions implicate adaptive responses, which can promote survival in limiting or inhibitory environments (Poole, 2012). Changes in temperature can transmit signals through the cell envelope by RpoE (σ^E), and, in *E. coli,* it is known that this mechanism negatively regulates PhoPQ, a two-component system that promotes changes in LPS, which is related to resistance to antimicrobial peptides (Ades, 2004; Coornaert et al., 2010). Such changes may include variations in the region of lipid A, in the fragment of the disaccharide N-glucosamine or in the domain of carbon chains (Bos et al., 2007; Delcour, 2009; Guo et al., 1997; Kawasaki, 2011; Wang and Quinn, 2010). Moreover, the reduction in the negative charge of the molecule, through the removal of phosphate groups from positions 1 and 4', and the addition of amine-containing residues can also be observed (Delcour, 2009; Wang and Quinn, 2010). These modifications influence susceptibility to antimicrobial peptides, such as bacteriocins, which generally have a positive charge and amphipathic characteristics. Thus, their activity depends on hydrophobic and electrostatic interactions with the membrane, and is influenced by any factor that alters these interactions (Ganzle et al., 1999a; Gunn et al., 1998).

Changes in the fatty acids of the cytoplasmic membrane may also be related to differences in susceptibility, because changes in membrane fluidity can modify the process of bacteriocin docking/inserting (Chihib et al., 1999; Crandall and Montville, 1998). In addition, changes in lipid A biosynthesis may alter the fluidity of the outer membrane and influence its permeability. Although this is a well conserved process among Gram-negative bacteria, some changes have been verified in *Francisella novicida* and shown to be regulated by environmental factors, such as temperature (Li et al., 2012; Wang and Quinn, 2010).

Exposure to sublethal growth conditions can cause changes in cellular structures that are aimed at to improving adaptation to environmental conditions (Alvarez-Ordóñez and Prieto, 2010). It has been shown that the exposure of Gram-negative bacteria, such as *E. coli,* to low pH or low temperature conditions favors the production of a cell envelope that is more susceptible to the action of bacteriocins, thereby allowing the action of nisin alone, under conditions of prolonged exposure (Elliasson and Tatini, 1999; Ganzle et al., 1999b). These results agree with our data obtained at low temperature, at which the action of nisin alone under conditions of low pH (6.1 to 5.3) was clearly observed (Fig. 5).

The growth of some strains demonstrates the phenotypic heterogeneity that exists in a microbial population, which is important because it favors the persistence of the population in stressful environments that could inactivate all of the cells, if they were phenotypically homogeneous. This process can be related to environmental conditions that stimulate an adaptive response, and a cross-response mechanism. Thus, the selection of conditions associated with the application of nisin and EDTA must be carefully studied, because environmental stresses that might occur in the food processing chain can impact the activity of these antimicrobials (Alvarez-Ordóñez and Prieto, 2010; Poole, 2012).

6. Conclusions

Environmental conditions of temperature and pH exert a strong influence on the susceptibility of *Salmonella* Typhimurium to the combination of nisin and EDTA. These parameters are largely used in food preservation and should be considered for establishing conditions for nisin use. Different adaptations can be related to these behaviors and further studies are needed to establish the role of each cellular structure.

Acknowledgements

The authors thank to the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for financial support.

Legends to tables

Table 1: Central Composite Design with two factors proposed for evaluating the influence of temperature and pH on the growth of *Salmonella* Typhimurium in BHI broth, with or without the addition of nisin and EDTA.

Table 2: Mathematical models for the log of the number of colony forming units (CFU/mL) of *Salmonella* Typhimurium in BHI broth with or without the addition of nisin and EDTA after 12 h of treatment.

Legends to figures

Fig. 1: Average growth of *Salmonella* Typhimurium in BHI, at different values of temperature and pH, for 12 h. Caption: In log CFU/mL 🟧 2, 🟧 3, 🟨 4, 🟩 5, 🟩 6, 🟦 7, 🟦 8, 🟦 9.

Fig. 2: Average growth of *Salmonella* Typhimurium in BHI, with EDTA (1.5 mM), at different values of temperature and pH, for 12 h. Caption: In log CFU/mL 🟧 2, 🟧 3, 🟨 4, 🟩 5, 🟩 6, 🟦 7, 🟦 8, 🟦 9.

Fig. 3: Average growth of *Salmonella* Typhimurium in BHI, with nisin (115 μM), at different values of temperature and pH, for 12 h. Caption: In log CFU/mL 🟧 2, 🟧 3, 🟨 4, 🟩 5, 🟩 6, 🟦 7, 🟦 8, 🟦 9.

Fig. 4: Average growth of *Salmonella* Typhimurium in BHI, with nisin (115 μM) and EDTA (1.5 mM), at different values of temperature and pH, for 12 h. Caption: In log CFU/mL 🟧 2, 🟧 3, 🟨 4, 🟩 5, 🟩 6, 🟦 7, 🟦 8, 🟦 9.

Fig. 5: Growth of *Salmonella* Typhimurium in BHI at pH 5.3 and 15.1 °C for 5 days. Legend: BHI broth (-•-); EDTA (···○···), Nisin (- -▼- -); Nisin + EDTA (⁻⁻··Δ··⁻⁻). Detection limit of the technique (___).

Fig. 6: Growth of *Salmonella* Typhimurium in BHI, with addition of nisin and EDTA, under different conditions of temperature and pH. Legend: Condition 4: pH 6.9 and 39.9 °C (-●-); Condition 6: pH 6.1 and 45 °C (--○--); Condition 8: pH 7.2 and 27.5 °C (- -▼- -); Detection limit of the technique (___).

Table 1

Test	pH	Temperature (°C)
1	5.3	15.1
2	6.9	15.1
3	5.3	39.9
4	6.9	39.9
5	6.1	10.0
6	6.1	45.0
7	5.0	27.5
8	7.2	27.5
9	6.1	27.5
10	6.1	27.5
11	6.1	27.5

Table 2

Test	Equation	R^2
Salmonella	$UFC=3.65794+0.237893^{a}.pH+0.0634393^{b}.Temp$	0.931
Salmonella + EDTA	$UFC=3.28633+0.39055^{b}.pH+0.0199274^{a}.Temp$	0.642
Salmonella + nisin	$UFC=10.2899-0.827419^{a}.pH-0.351024^{b}.Temp+0.0621156^{b}.Tem.pH$	0.914
Salmonella + nisin + EDTA	$UFC = 47.2489-12.8758^{b}.pH+1.03151^{b}.pH^2-0.192016^{b}.Temp+0.00220294^{b}.Temp^2$	0.976

[a] significant at 5% probability

[b] significant at 1% probability

Figures

Fig. 1

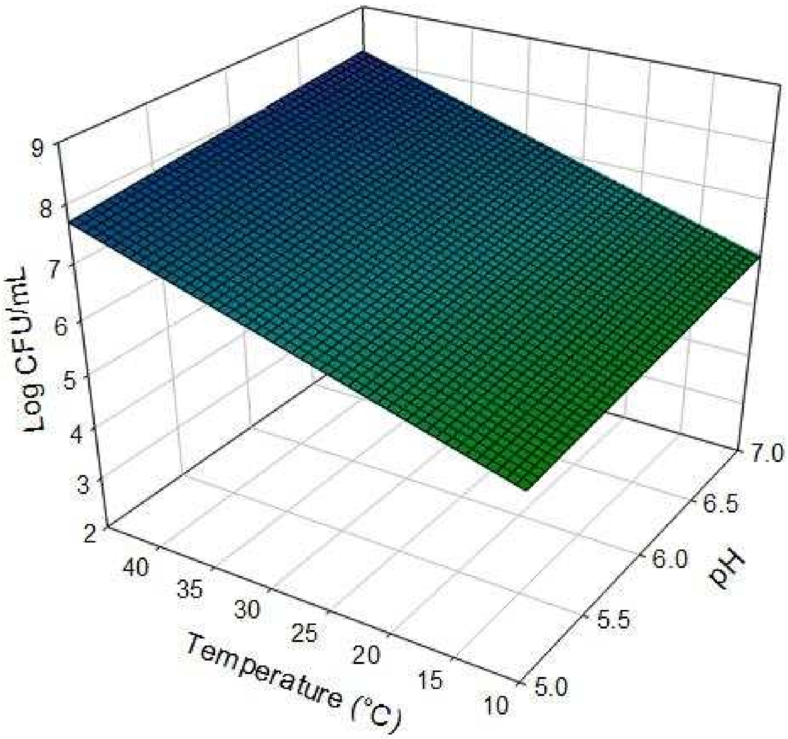
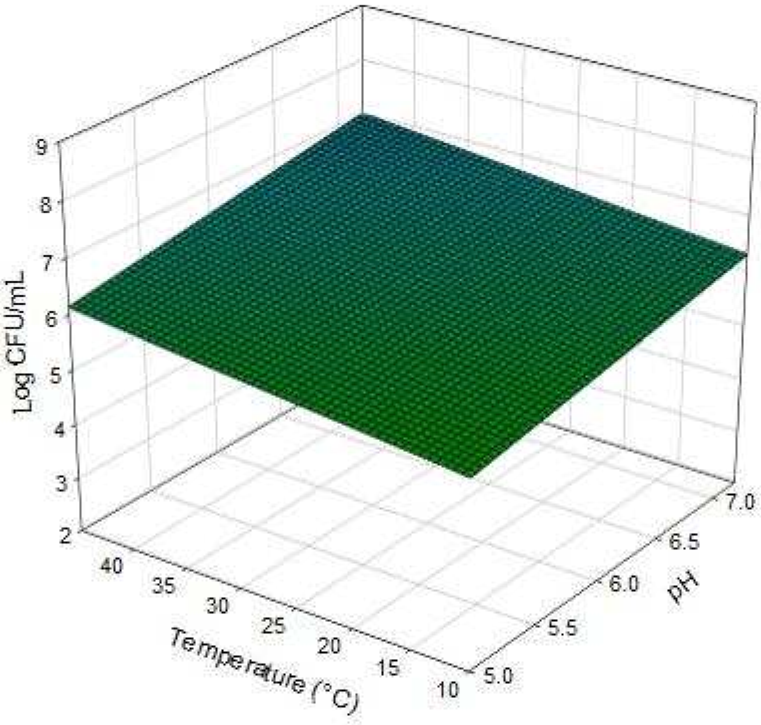
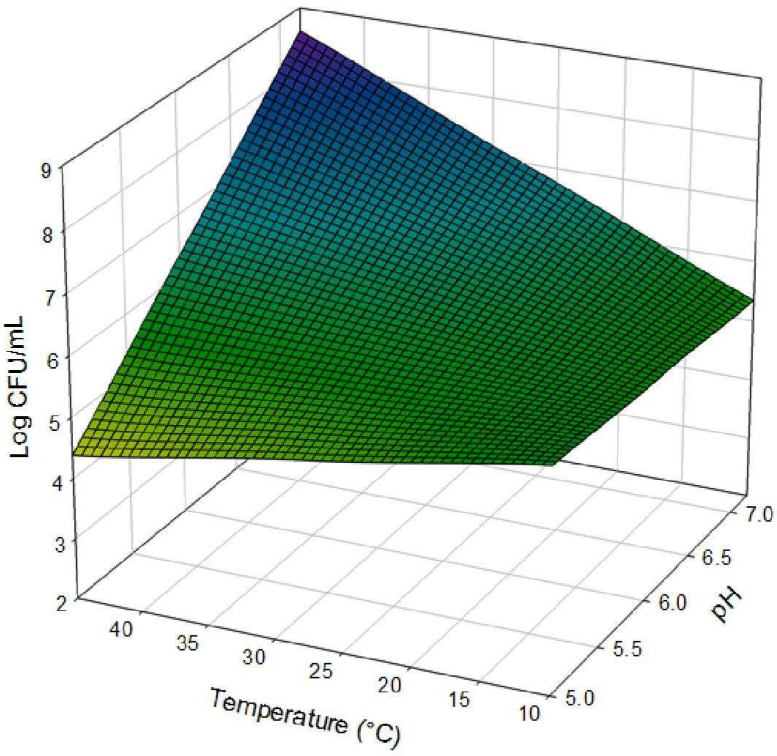


Fig. 2:



Log CFU/mL
9
8
7
6
5
4
3
2
40
35
30
25
20
15
10
Temperature (°C)
5.0
5.5
6.0
6.5
7.0
pH

Fig. 3:



Log CFU/mL
2
3
4
5
6
7
8
9
Temperature (°C)
40
35
30
25
20
15
10
pH
5.0
5.5
6.0
6.5
7.0

Fig. 4:

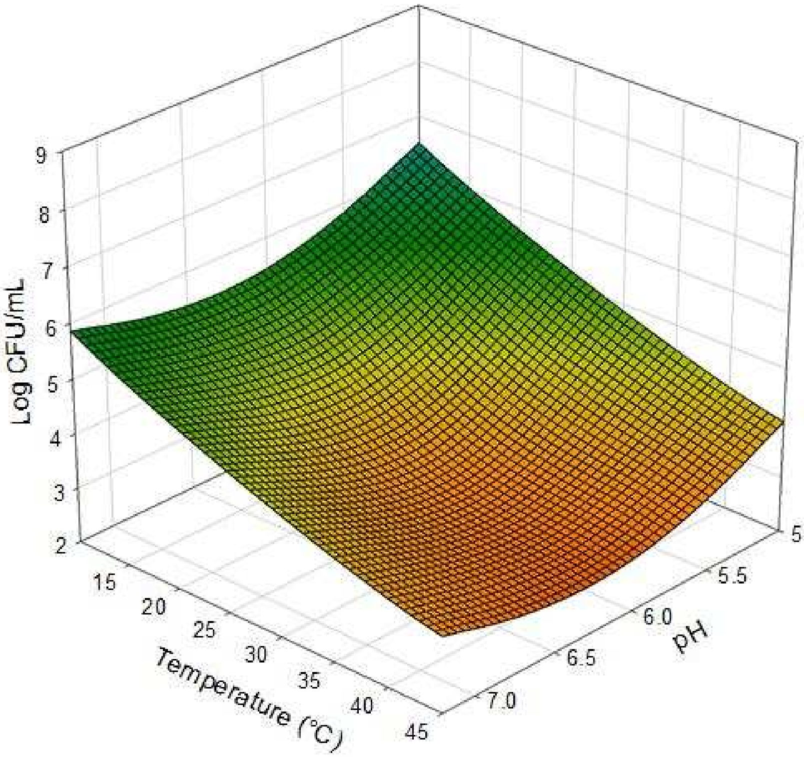
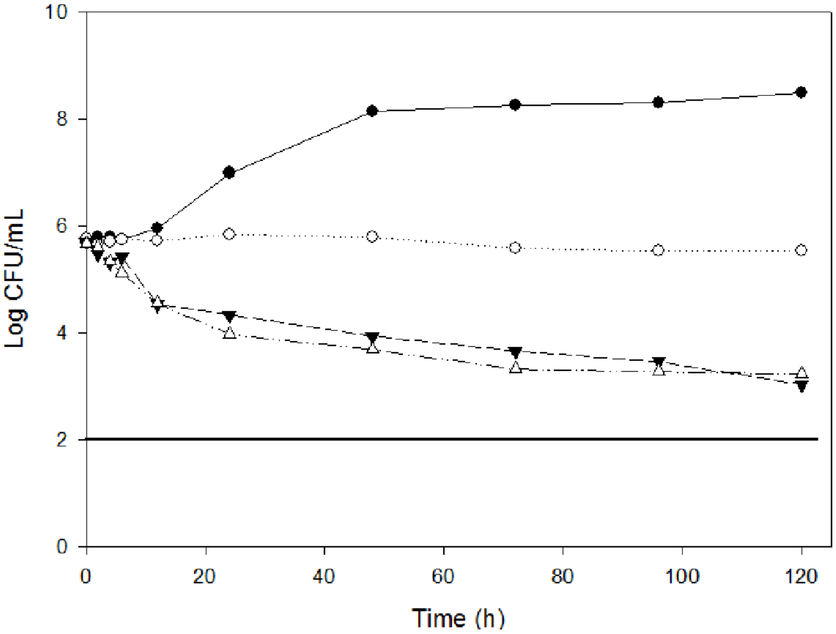
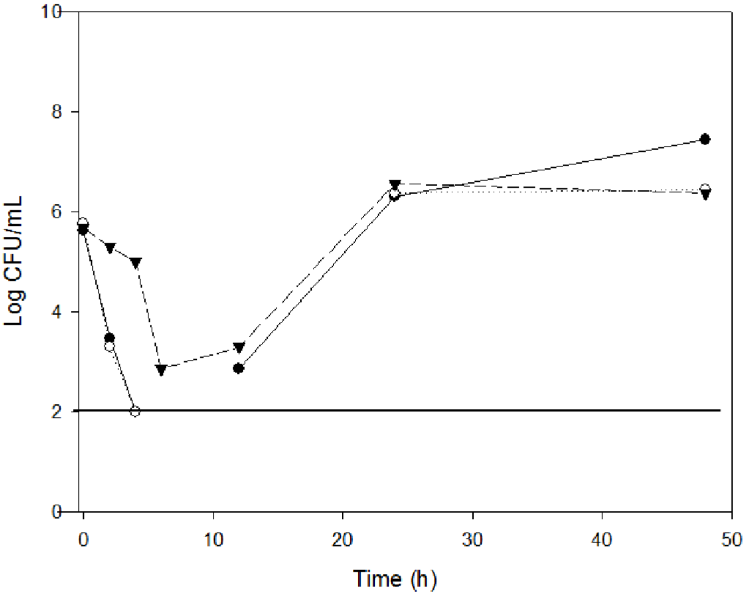

Log CFU/mL
9
8
7
6
5
4
3
2
15
20
25
30
35
40
45
Temperature (°C)
5.0
5.5
6.0
6.5
7.0
pH

Fig. 5:



10
8
6
4
2
0
Log CFU/mL
0
20
40
60
80
100
120
Time (h)

Fig. 6:



10
8
6
4
2
0
Log CFU/mL
0
10
20
30
40
50
Time (h)

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Chapter 3[*]

Tolerance of *Salmonella enterica* serovar Typhimurium to nisin combined with EDTA is related to changes in cellular composition

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[*] Formatting according to International Journal of Food Microbiology author guidelines

Tolerance of *Salmonella enterica* serovar Typhimurium to nisin combined with EDTA is related to changes in cellular composition

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Abstract

Nisin has been widely used in many countries, as a food additive for dairy products and canned vegetables and fruits. Although Gram-negative bacteria have natural resistance to nisin due to the presence of the outer membrane, the use of chelating agents, such as EDTA, allows for their sensitization. However, *Salmonella* can alter the composition and the structure of their lipopolysaccharide layer (LPS) in response to changes in environmental conditions, and thus mitigate their sensitivity to bacteriocins, such as nisin. In this work we demonstrated and characterized tolerance to nisin combined with EDTA in *Salmonella* Typhimurium cultured under specific conditions of temperature and pH by means of assays of growth, viability, leakage of intracellular content and Fourier transform infrared spectroscopy (FT-IR). The modifications related to the tolerance phenotype to nisin combined with EDTA possibly involve changes in the phospholipid acyl chains, in the sugar content of LPS and in the peptidoglycan present in the cell wall. These findings demonstrate the importance of better understanding the behavior the cells treated with nisin and EDTA, especially due to widespread use of this bacteriocin in food preservation.

Key words: nisin tolerance, Fourier Transform Infrared Spectroscopy, outer membrane, bacteriocins, Gram-negative bacteria.

1. Introduction

Bacteriocins are peptides synthesized ribosomally with bacteriostatic or bactericidal effects on other bacteria (Arqués et al., 2011; Héchard and Sahl, 2002). Among the bacteriocins, nisin is the only one approved for commercial use in almost 50 countries. It is used as a food additive for dairy products and canned vegetables and fruits (Dischinger et al., 2014; Nagao, 2009), particularly for the inhibition of *Clostridium* spp.

Nisin is primarily active against Gram-positive bacteria (Sahl et al., 1995). It has a non-enzymatic mechanism of action, characterized by the formation of pores in the cytoplasmic membrane, which promotes the release of electrolytes and metabolites, and so the dissipation of the proton motive force, as well as the inhibition of peptidoglycan synthesis, through the use of lipid II as a docking molecule for pore formation (Breukink and Kruijff, 1999; Guder et al., 2000; Helander and Mattila-Sandholm, 2000).

Like other Gram-negative bacteria, *Salmonella* spp. is naturally resistant to the action of bacteriocins produced by Gram-positive bacteria, such as nisin (Arqués et al., 2011; Cao-Hoang et al., 2008). Its outer membrane acts as a barrier, that prevents the passage of bacteriocin molecules to their target site, acting as a mechanism of innate resistance (Alakomi et al., 2000; Kaur et al., 2011). The use of chelating agents, such as EDTA, increases the susceptibility to nisin by destabilizing the lipopolysaccharide layer (LPS) (Boziaris and Adams, 1999; Martin-Visscher et al., 2011).

However, *Salmonella* can alter the composition and structure of their LPS layer in response to environmental conditions (Kawasaki, 2011; Nikaido, 2003). Known responses include modifications of lipid A, which may occur in the disaccharide N-glucosamine fragment, or in the hydroxymyristic-acid-derived carbon chains; or through reduction of the overall negative charge of the molecule (Bos et al., 2007; Delcour, 2009; Guo et al., 1997; Kawasaki, 2011; Wang and Quinn, 2010). Such modifications regulate important characteristics, including the biological activity of lipid A, and interfere with the permeability of the outer membrane, which can alter the resistance it provides against antimicrobial peptides, such as the bacteriocins (Kawasaki, 2011).

Several authors have reported the ability of *Salmonella* to exhibit adaptive responses to environmental factors, such as pH, salt concentration, temperature and oxygen stress, that allow the cells to survive under more extreme stress conditions by expressing resistant phenotypes (Álvarez-Ordóñez et al., 2008; Greenacre et al., 2003; Greenacre and Brocklehurst, 2006; Papadimitriou et al., 2008; Sampathkumar et al., 2004; Wilde et al., 2000).

Previous research has shown that Fourier transform infrared spectroscopy (FT-IR) is a powerful tool to monitor and detect changes in the cellular composition of *S.* Typhimurium in response to changes in environmental conditions (Álvarez-Ordóñez et al., 2010). FT-IR spectroscopy can also be used to detect compositional changes in cells under environmental stress (Ede et al., 2004; Moen et al., 2009; Papadimitriou et al., 2008). So, the bacterial exposure and subsequent adaptation under certain environmental conditions lead to the modification of certain cell structures, and at the same time, render changes in the IR spectra.

The resistance to nisin is a complex phenomenon and may be related to alterations in fatty acid composition, cell wall changes and the presence of enzymes degrading nisin, among other mechanisms (Kaur et al., 2011). This phenotype is relatively common in Gram-positive bacteria, and has already been detected in *Listeria*, *Lactobacillus* and *Clostridium*, among others (Crandall and Montville, 1998; Kaur et al., 2011). However, this behavior has still not been demonstrated in Gram-negative bacteria. The aim of this work was to demonstrate the tolerance of *S.* Typhimurium to nisin combined with EDTA when cultured under specific conditions of temperature and pH, and to characterize the molecular changes associated with this phenotype, focusing on modifications to membrane structure.

2. Materials and methods

2.1 Bacterial strains and culture conditions

Salmonella enterica serovar Typhimurium ATCC 14028 was grown in Brain and Heart Infusion broth (BHI, Merck, Germany) at 37 ± 1 °C, for 24 h. The cells were centrifuged at 3,220 *g* for 15 min (Eppendorf 5810R, Germany) and washed with 0.85% saline. Stocks of the microbial cultures were maintained in appropriate culture medium containing glycerol (50%), at - 20 °C.

2.2 Preparation and activity of nisin

Nisin A was obtained from commercial products (2.5% wt/wt, Sigma, USA) and extracts were prepared by diluting the stock powder in sodium phosphate solution (0.05 M, pH 2.0). The concentration of the peptide was determined by HPLC as described by Paiva et al. (2012), with reference to a standard curve with concentrations between 37.5 and 600 μM.

2.3 Evaluation of tolerance to nisin associated to EDTA in S. Typhimurium

The possible development of tolerance by *S*. Typhimurium to a combination of nisin with EDTA was observed using a response surface design which modeled the influence of pH (range 5.0 to 7.2) and temperature (range 10 to 45 °C) on growth. In these assays, under specific conditions of pH and temperature, an increase in the number of viable cells after 24 h of treatment was observed (results unpublished). From the behaviour observed in these results, two given combinations of pH and temperature were chosen for this study: condition 1: pH 6.9, temp 39.9 °C, and condition 2: pH 7.2, temp. 27.5 °C.

S. Typhimurium cells were subjected to a pre-adaptation period to promote tolerance to nisin and EDTA. 10^5 CFU/mL of *S.* Typhimurium cells were incubated in BHI broth, containing nisin (115 µM) and EDTA (1.5 mM, Fluka, China), under the conditions previously specified (pH 6.9, temp. 39.9 °C; pH 7.2, temp. 27.5 °C), for 24 h. After this time, cells were collected by centrifugation at 3,220 *g* for 15 min, washed two times, resuspended in 0.85% saline and grown with the same concentrations of nisin and EDTA under the same incubation conditions.

Viability was assessed at different time intervals using the drop plate method on Plate Count Agar (PCA, Difco, USA). Plates were counted after incubation at 37 ± 1 °C for a period of 8 to 12 h. Similar assays were also performed in polystyrene microplates (Honeycomb II plates, Labsystems, Finland). The microplates were incubated at 39.9 or 27.5 ± 1 °C and the growth was evaluated by absorbance at 600 nm (Bioscreen C, Finland), for a period of 24 h. The test was repeated twice, with at least five replicates.

Cells not subjected to any pre-adaptation period were used as controls. Controls were grown in BHI broth for 24 h. The treatment was performed under the same conditions of temperature and pH (conditions 1 and 2), with nisin and EDTA (sensitive cells), only with EDTA (EDTA cells), only with nisin (nisin cells) and without nisin and EDTA (control cells).

The two cell populations of interest (sensitive and tolerant cells) demonstrated considerably different viability after 6 h of treatment. Therefore, samples were collected at this time, and assays intended to evaluate the differences in membrane integrity, chemical composition and ultrastructure were subsequently performed.

2.4 Measurement of cellular leakage by spectrophotometry

Cells were resuspended in citrate-phosphate buffer (0.1 M) with pH 7.2 or 6.9, containing nisin (115 µM) and EDTA (1.5 mM), and incubated at 27.5 or 39.9 ± 1 °C, according to conditions specified. After 6.0, 6.5 and 7.0 h, 2 mL aliquots of cell cultures were collected and centrifuged at 11,700 *g* for 5 min (Eppendorf 5810R, Germany). Concentrations of nucleic acids and proteins in the supernatant were quantitated by measuring the absorbance of the supernatant at 260 nm and

280 nm, respectively (Beckman DU 7400 Spectrophotometer, USA). The test was repeated twice. The analysis was conducted from the UV spectrum by a relation (N_x – N_0), where N_x is a value obtained at given time and N_0 at the beginning of the experiment (Moosavy et al., 2008).

2.5 Acquisition of FT-IR spectra

The different populations to of cells, subjected or not to the pre-adaptation period, were incubated in BHI broth under conditions 1 and 2 for 6 h. Then, the cells were harvested by centrifugation at 8,000 *g* for 5 min, washed three times in phosphate buffer (Calbiochem, USA and Canada), dried and placed in a ZnSe window.

IR spectra were measured with a Fourier transform infrared spectroscope (Perkin-Elmer System 2000 FT-IR, USA) equipped with a DGTS detector. Measurements were recorded over the wavelength range 3,000 to 700 cm^{-1}, with interval of 1 cm^{-1} and spectral resolution of 4 cm^{-1}. The final IR spectrum was obtained by averaging 20 scans. Digitized spectra (represented by a total of 1,300 points which included the critical ranges) were saved for additional transformation. The reproducibility of these data was ensured through the development of a strict experimental protocol regarding medium preparation, incubation time and temperature, cell harvesting conditions, sample preparation and FT-IR measurement. The FT-IR experiments were conducted in triplicate using three different fresh cultures grown and processed on different days, performing a separate measurement for each experimental run.

2.6 Transformation of spectra

Spectra were transformed (normalization, smoothing and second derivative using the Savitzky-Golay algorithm), recorded in ASCII format and imported into an Excel worksheet (Mouwen et al., 2005). Transformation was intended to improve the quality of the spectrum by increasing spectral resolution and removing baseline shifts.

Five spectral ranges or windows were targeted for calculation purposes: the window between 3,000-2,800 cm^{-1}, dominated by the influence of functional groups of membrane fatty acids (w_1); the window between 1,800-1,500 cm^{-1}, with the influence of the amide I and amide II groups belonging to proteins and peptides (w_2); the window between 1,500-1,200 cm^{-1}, mixed region influenced by proteins, fatty acids and other phosphate-carrying compounds (w_3); the window between 1,200-900 cm^{-1}, revealing mostly for polysaccharides present within the cell wall (w_4); and the window between 900-700 cm^{-1}, which is named the true fingerprint because of very specific spectral patterns (w_5).

2.7 Spectral reproducibility and clustering

To study variability between replicates and within windows, samples were processed, in independent experiments, yielding three replicates for each growth condition. A differentiation Index (*DI* (Naumann, 2000)) was calculated for each pair of IR spectra, for the whole IR range (3,000-700 cm^{-1}) and independently for the ranges described (w_1-w_5), according to the equation

$DI_{y1y2} = (1 - r_{y1y2}) * 1000$

Where r_{y1y2} is the Pearson's correlation coefficient, $y1_i$ and $y2_i$ are the individual absorbance values of the two spectra to be compared, *n* is the number of data points in the given range, and $y1$ and $y2$ are the arithmetic mean values of $y1$ and $y2$.

$$r_{y1y2} = \frac{\sum_{i=1}^{n} y1_i\, y2_i - n\,\overline{y1}\,\overline{y2}}{\sqrt{\sum_{i=1}^{n} y1_i^2 - n\,\overline{y1}^2}\ \sqrt{\sum_{i=1}^{n} y2_i^2 - n\,\overline{y2}^2}}$$

The three replicates for each combination are considered and the mean is obtained. *DI* may adopt values between 0 and 2000, with 0 for identical spectra, 1000 for completely noncorrelated spectra and 2000 for completely negatively noncorrelated spectra (Naumann, 2000).

The transformed IR spectral data were subjected to multivariate statistical methods (Hierarchical Cluster Analysis (HCA) and Factor Analysis (FA)). The Pearson's product moment correlation coefficient was used to measure the similarity between spectra, and final clustering was achieved using Ward's algorithm. In FA, a set of correlated variables is transformed to a set of uncorrelated, hidden variables ranked by variability in the descending order, called factors, which are graphically represented. The analyses were carried out with Statistica for Windows (version 7.0, Statsoft Inc., Tulsa, Okla, USA).

2.8 Determination of membrane fluidity using FT-IR

Membrane fluidity was evaluated by monitoring the vibrational modes of the acyl chain $\nu_s CH_2$ symmetric stretching band located approximately at 2,850 cm^{-1}. The exact location of the $\nu_s CH_2$ peak was calculated through second derivative analysis, by obtaining the second derivative of the spectra and determining the location of the peak maxima.

3. Results

3.1 Effect of pre-adaptation on S. Typhimurium in the presence of a combination of nisin and EDTA

The growth data demonstrated that the cultures of *S.* Typhimurium that had been subjected to a pre-adaptation period with nisin and EDTA exhibited distinct behavior. When exposed to both nisin and EDTA, the sensitive cells showed no growth, while tolerant cells exhibited a lag phase lasting approximately 6 h, 3 h more than the control (without nisin and EDTA), and growth similar to the culture in medium containing EDTA only (EDTA cells) (Fig. 1). This behavior was supported by the viability data: tolerant cells showed less viability reduction and faster growth compared to sensitive cells (Fig. 2). This difference tends to decrease with time, possibly due to the adaptation of sensitive cells.

The same pattern between the sensitive and tolerant populations is also observed when measuring the absorbance at 260 and 280 nm of the supernatant from cell centrifugation. Tolerant cells leak less of their intracellular protein and nucleic acid content into the environment than sensitive cells (Fig. 3 A and B).

These results demonstrate a change in behavior of the cell population previously exposed to the combination of nisin with EDTA under specific environmental conditions, suggesting that adaptive changes had occurred in the population after exposure. To check the stability of the bacteriocin, activity assays were performed which showed that the bacteriocin was active in incubation conditions (data not shown), strengthening the hypothesis that the *Salmonella* has undergone adaptive physiological changes. Data for condition 2 (pH 7.2, temp. 27.5 °C) are not shown, but similar results were obtained regarding growth, viability and cellular leakage.

3.2 FT-IR spectroscopy

FT-IR spectroscopy is a physicochemical technique that is able to detect compositional changes occurring in the cell membrane. In our study, FT-IR combined with multivariate statistical analysis was used to detect and interpret changes in the chemical composition of the cell membrane occurring when populations were pre-adapted to nisin and EDTA under specific conditions of temperature and pH.

3.2.1 Reproducibility analysis

Reproducibility analysis for FT-IR measurements was performed as a means of studying variability between spectra replicates and discriminatory capacity of different IR ranges. Reproducibility was measured for the whole working range (3,000 to 2,800 and 1,800 to 700 cm^{-1}) and separately for the five window ranges, using the DI value. The lowest mean results of DI for all the conditions corresponded to windows w_1 (range 0.60 to 6.46) and w_4 (range 1.03 to 19.20). Intermediate DI values were obtained for windows w_2 (range 1.75 to 21.12) and w_3 (range 2.99 to 29.52) while window w_5 (range 2.42 to 113.52) yielded higher values. Based on DI results obtained, low-quality replicates were discarded and the spectral ranges w_1 and w_4 (or subranges) were selected for the subsequent transformation, HCA and FA mathematical analyses.

3.2.2 Transformation and clustering of FT-IR spectra

The IR spectra were further processed with the aim of minimizing methodological variability and, simultaneously, amplifying the chemically based spectral differences. Normalization of the spectra balanced the differences in path strength, smoothing eliminated the instrumental noise, and the conversion of the spectra to their second derivative separated absorption bands, removed baseline shifts, and increased spectral resolution. These transformations more prominently revealed the differences between the spectral features of the five populations of cells.

Major differences in the untransformed FT-IR spectra for all conditions tested were found within the two spectral regions: regions w_1 (Fig. 4A and B) and w_4 (Fig. 5A and B). The spectra obtained from cultures grown under condition 1 showed the most evident differences.

Cells treated with both nisin and EDTA (both the tolerant and sensitive populations) showed important variations in the shape and intensity of several spectral bands, compared to the other populations (Figs. 4 and 5). Visible differences were evident in the untransformed spectra in the range w_1 (Fig. 4A), around the subregion 2,850 cm^{-1}, where the ν_sCH_2 peak is missing in both the sensitive and tolerant populations. This is clearly noticeable in the transformed spectra for the subrange 2,930-2,830 cm^{-1} (Fig. 4B). Vibrational modes of the acyl chain ν_sCH_2 symmetric stretching band located approximately at 2,850 cm^{-1} are used normally to monitor membrane fluidity, by calculating the location of the ν_sCH_2 peak through second derivative analysis and determining the location of the peak maxima. However, the $\nu_s CH_2$ peak was absent for the IR spectra belonging to the sensitive and tolerant populations (Fig. 4B). Differences between the tolerant and the sensitive populations are much less prominent and not perceptible around the $\nu_s CH_2$ peak (Fig. 4B).

In the w_4 range, differences in the untransformed spectra were visually less pronounced, but were nonetheless concentrated around of 1,120-1,190 cm^{-1} region (Fig. 5A). In contrast with window w_1, no major changes such as the loss of absorption peaks were observed, and changes were limited to the reduction of intensity of absorption of IR radiation. When transformation was performed, differences in IR spectrum were obvious in the transformed spectra (Fig. 5B). These differences are attributed to ring vibrations of several chemical groups such as the C–O–C and C–O structures in carbohydrates.

The dendrograms obtained by HCA on the second derivative spectra for the w_1 and w_4 spectral regions showed differences in the populations treated with nisin and EDTA (both the sensitive and tolerant cells), discriminating the groups with a global linkage distance of 1.2 and 0.8, respectively (Fig. 6A and B). Moreover, the region w_4 could additionally differentiate between sensitive and tolerant populations (Fig. 6B). An FA of this region confirmed this differentiation by discriminating the five groups of samples (Fig. 7). Spectral regions w_2, w_3 and w_5 were also capable to completely discriminate all phenotypes tested (including nontreated control cells) except tolerant and sensitive samples (results not shown).

4. Discussion

4.1 Effect of pre-adaptation on S. Typhimurium in the presence of a combination of nisin and EDTA

Tolerance to stress caused by environmental conditions, processing treatments or sublethal concentrations of compounds has been observed in many bacteria (Skandamis et al., 2008; Cebrian et al., 2010; Huang and Wong, 2012, Bradley et al. 2012). This phenomenon has not been demonstrated in the presence of nisin and EDTA and scarce information is available about the molecular basis of this behavior. Generally, the combined action of nisin and EDTA results in a substantial reduction of viable cells presents in the medium (Fig. 2). However, under certain conditions of temperature and pH, when *S*. Typhimurium cells were pre-exposed to these agents, growth occurred and a smaller reduction in viability during the treatment was observed (Figs. 1 and 2). The difference of behavior between these populations (sensitive and tolerant cells) is also revealed when measuring the absorbance at 260 and 280 nm of cell supernatant (Fig. 3A and B), strengthening the idea that the cells adapt.

The susceptibility of Gram-negative bacteria to the combination of nisin with EDTA can vary between different species of bacteria or even between different strains of the same species (Boziaris and Adams, 1999). Such variation can be due to alterations in the structure of LPS layer, which possibly influences the permeability of the outer membrane (Boziaris and Adams, 1999). These changes may involve the structure of lipid A, in the disaccharide N-glucosamine, or in its carbon chains, or through a reduction in the negative charge of the molecule by removal of phosphate groups from positions 1 and 4' or by the addition of amine-containing residues (Bos et al., 2007; Delcour, 2009; Wang and Quinn, 2010). Changes in the hydrophilic region, composed of the core and the polysaccharide O, may also influence the resistance phenotype, because the core region exerts an important role in the barrier function (Delcour, 2009; Loutet et al., 2006).

These modifications may reduce the negative charge of the LPS molecule, or may influence the fluidity of the outer membrane, which would decrease the frequency of interactions with the membrane and reduces its permeability to bacteriocin, favoring resistance (Breukink and Kruijff, 1999; Erridge et al., 2002; Gunn et al., 1998; Kawasaki, 2011)

4.2 FT-IR spectra: interpretation of changes in spectral bands

FT-IR is a physicochemical technique capable of detecting compositional changes in *S.* Typhimurium caused by environmental changes, and is additionally useful for the study of molecular aspects of bacterial stress response (Álvarez-Ordóñez et al., 2010; Álvarez-Ordóñez and Prieto, 2010).

FT-IR spectra of bacterial biomass are usually composed of broad and superimposed absorbance bands deriving from many cellular components, although there is evidence that the elements of the outer membrane have a predominant influence (Mouwen et al., 2011). In our study, the untransformed IR spectra of *S.* Typhimurium cells in control conditions (without nisin and EDTA) were comparable to the spectra reported in other study (Álvarez-Ordóñez and Prieto, 2010), confirming the effectiveness of the technique.

It had previously been demonstrated that the w_1 (3,000 to 2,800 cm^{-1}) and w_4 spectral regions (1,200 to 900 cm^{-1}) were the most informative regions (Álvarez-Ordóñez et al., 2010). The region w_1 (3,000-2,800 cm^{-1}) reflects the dominance of the C-H stretching vibrations of -CH3 and -CH2 functional groups, abundantly found in membrane fatty acids. The region w_4 is dominated by ring vibrations overlapped with stretching vibrations of (C-OH) side groups and the (C-O-C) glycosidic bond vibration. It also displays (a broad band at ~1,100 to 950 cm^{-1}) the symmetric stretching vibration of PO_2^- groups found in nucleic acids and the C-O-C and C-O-P stretching from carbohydrates and polysaccharides in the cell wall.

Several authors have used FT-IR spectroscopy to study changes occurring in cells after exposure to bacteriocins (Bizani et al., 2005; Motta et al., 2008). Generally, they have observed changes in the spectral region w_1, associated with membrane fatty acids (Bizani et al., 2005). These changes are related to the dissipation of proton motive force and leakage of intracellular contents, characteristic of bacteriocin activity. Considerable changes in the assignments for C–H antisymmetric stretching of CH_2 bonds (2,920 cm^{-1}), symmetric C=O bonds (1,400 cm^{-1}), and P=O antisymmetric bonds (1,225-1,220 cm^{-1}) have been demonstrated in *Listeria monocytogenes* and *Bacillus cereus* treated with cerein 8A (Bizani et al., 2005). A similar effect in the membrane was also described to BLS P34, a bacteriocin-like substance produced by a novel *Bacillus* sp. strain P34 (Motta et al., 2008). We also observed similar changes in the w_1 region in our results, such as the loss of the $\nu_s CH_2$ peak around wavenumber 2,850 cm^{-1}, in both the sensitive and tolerant populations (Fig. 4A and B).

Additionally, the sensitive and tolerant populations could be differentiated on the basis of major IR spectral changes revealed in the assignments for C–H antisymmetric stretching of -CH_2 bonds (Fig. 4A and B, wavenumber 2,930 cm^{-1}), as was similarly observed in a previous report (Bizani et al., 2005). The changes in the antisymmetric and symmetric stretching bands in region w_1 suggest a strong modification within the phospholipid acyl chains, resulting in a net fluidizing effect of the apolar part of the bilayer.

Furthermore, we also observed changes in the w_4 region, which were generally related to modifications in the sugar content of the LPS and the peptidoglycan present in the cell envelope, although other molecules may also be involved. These changes have also been reported to result from exposure to bacteriocin-like substances (Bizani et al., 2005; Motta et al., 2008; Zoumpopoulou et al., 2013). In addition, the bacteriocin resistance phenotype in Gram-positive bacteria has been linked to the introduction of distinct changes mainly in the polysaccharide, fatty acid and protein regions, which represent the major cell membrane components in the resistant phenotype (Tessema et al., 2009).

In agreement with IR spectral plots, results from HCA and FA using the second derivative of IR spectra (Figs. 6 A, 6B and 7) allows concluding that IR spectra from the two studied conditions are unambiguously different and that the mathematical distances (as reflected in dendrograms and scatterplots) between these spectra are due to the distinct chemical composition of the populations. This degree of differentiation between populations subjected to conditions 1 and 2 is consistent with previous results from several authors (Bizani et al., 2005; Motta et al., 2008; Zoumpopoulou et al., 2013). In addition, tolerant and susceptible cells could be clearly separated, which possibly indicates that the development of tolerance in populations is due to changes in the membrane which are reflected in the IR spectrum.

5. Conclusions

Here we describe the changes occurring in cellular structures of *Salmonella enterica* serovar Typhimurium cultures that were pre-adapted to nisin and EDTA. There are strong indications that these changes are related to changes in the structure of the cellular membrane, most likely of the LPS layer, however, further studies in the area should be conducted in order to confirm this hypothesis. These findings are important because this is the first study to observe the development of tolerance to nisin combined with EDTA in Gram-negative bacteria. We speculate that this behavior can occur in other bacteria of the same group, but more studies are needed to understand this behavior, to clarify the mechanisms of resistance, and to better understand the use of nisin in combination with EDTA in food preservation.

Acknowledgements

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Figure captions:

Fig. 1:

Growth of *S.* Typhimurium in BHI broth with pH 6.9, with or without the addition of nisin (115 μM) and EDTA (1.5 mM), incubated 39.9 ± 1 ºC, for 24 h. Caption: Cells in BHI broth (-●-), cells in BHI broth containing EDTA (▼), cells in BHI broth containing nisin (-■-), cells in BHI broth containing nisin and EDTA (◆), and cells pre-adapted to nisin and EDTA in BHI broth containing nisin and EDTA (-▲-).

Fig. 2:

Viability of *S.* Typhimurium in BHI broth with pH 6.9, containing nisin (115 μM) and EDTA (1.5 mM), at 39.9 ± 1 ºC for 24 h. Caption: Cells not adapted (sensitive cells) (-●-) and cells pre-adapted to nisin and EDTA (tolerant cells) (-▽-). Error bars indicate standard deviations (*n* = 2)

Fig. 3:

Leakage of material absorving at 260 nm (A) and 280 nm (B), from *S.* Typhimurium cells in citrate-phosphate buffer with pH 6.9, with or without the addition of nisin (115 μM) and EDTA (1.5 mM), at 39.9 ± 1 °C, after 6, 6.5 and 7 h. Caption: Cells in citrate-phosphate buffer (■), cells in citrate-phosphate buffer containing EDTA (▨), cells in citrate-phosphate buffer containing nisin (▩), cells in citrate-phosphate buffer containing nisin and EDTA (▧), and cells pre-adapted to nisin and EDTA in citrate-phosphate buffer containing nisin and EDTA (▤).Error bars indicate standard deviations (*n* = 2)

Fig. 4:

Untransformed FT-IR spectra (A) and transformed FT-IR spectra (B), of the w_1 spectral range (3,000 to 2,800 cm^{-1}) of different populations of *S.* Typhimurium grown BHI broth, with pH 6.9, with or without nisin and EDTA, at 39.9 ± 1 °C for 6 h. Dotted lines indicate major differences in the IR spectra. Caption: Cells grown in BHI broth (—), cells grown in BHI broth containing EDTA (—), cells grown in BHI broth containing nisin (—), cells grown in BHI broth containing nisin and EDTA

(—), and cells pre-adapted to nisin and EDTA in BHI broth containing nisin and EDTA (—).

Fig. 5:

Untransformed FT-IR spectra (A) and transformed FT-IR spectra (B), of the w_4 spectral range (1,200 to 900 cm^{-1}), of different populations of *S.* Typhimurium grown BHI broth, with pH 6.9, with or without nisin and EDTA, at 39.9 ± 1 °C, for 6 h. Dotted lines indicate major differences in the IR spectra. Caption: Cells grown in BHI broth (—), cells grown in BHI broth containing EDTA (—), cells grown in BHI broth containing nisin (—), cells grown in BHI broth containing nisin and EDTA (—), and cells pre-adapted to nisin and EDTA in BHI broth containing nisin and EDTA (—).

Fig. 6:

Dendrogram obtained from IF-TR spectra of *S.* Typhimurium cells grown BHI broth, with pH 6.9, with or without nisin and EDTA, at 39.9 ± 1 °C, for 6 h, with cluster analysis performed with the Pearson product moment correlation coefficient (*r*) and using Ward's algorithm. Three replicates of each experimental condition are represented. The w_1 spectral region (3,000 to 2,800 cm^{-1}) (A), and the w_4 spectral region (1,200 to 900 cm^{-1}) (B). Caption: control cells (cells grown in BHI broth), EDTA cells (cells grown in BHI broth containing EDTA), nisin cells (cells grown in BHI broth containing nisin), sensitive cells (cells grown in BHI broth containing nisin and EDTA) and tolerant cells (cells pre-adapted to nisin and EDTA and grown in BHI broth containing nisin and EDTA).

Fig. 7:

Scatter plot obtained from the Principal Components and Factor Analysis of w_4 (1,200 to 900 cm^{-1}) spectral region, showing the differentiation between the five populations. Three replicates of each experimental condition are represented. Caption: control cells (cells grown in BHI broth) (●), EDTA cells (cells grown in BHI broth containing EDTA) (●), nisin cells (cells grown in BHI broth containing nisin) (●), sensitive cells (cells grown in BHI broth containing nisin and EDTA) (●) and tolerant cells (cells pre-adapted to nisin and EDTA and grown in BHI broth containing nisin and EDTA) (●).

Figures:

Fig. 1:

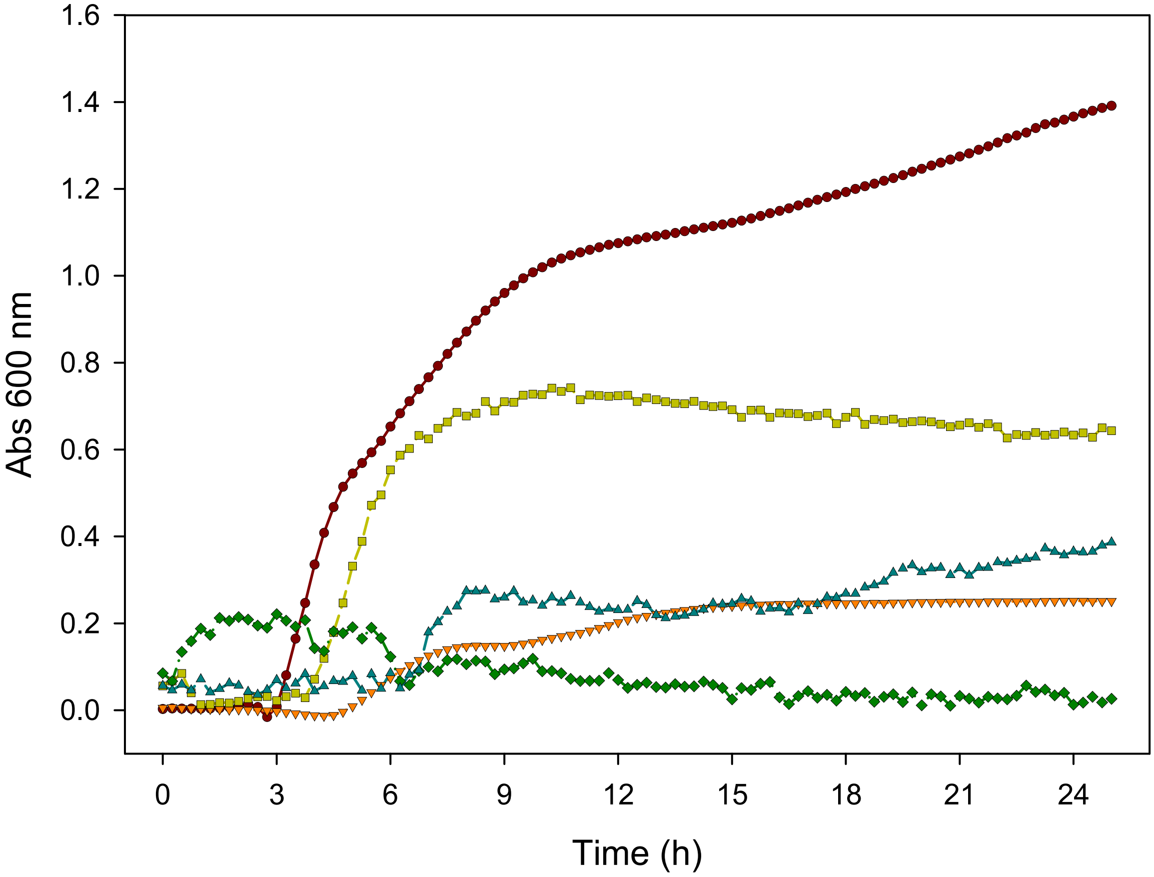


Fig. 2:

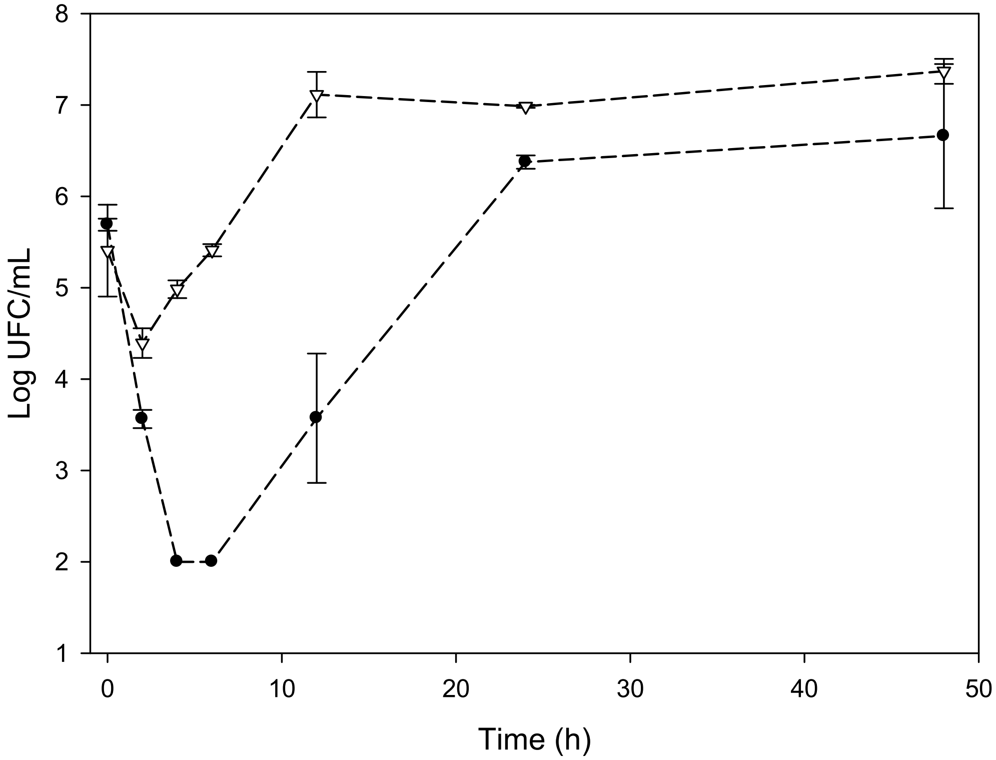
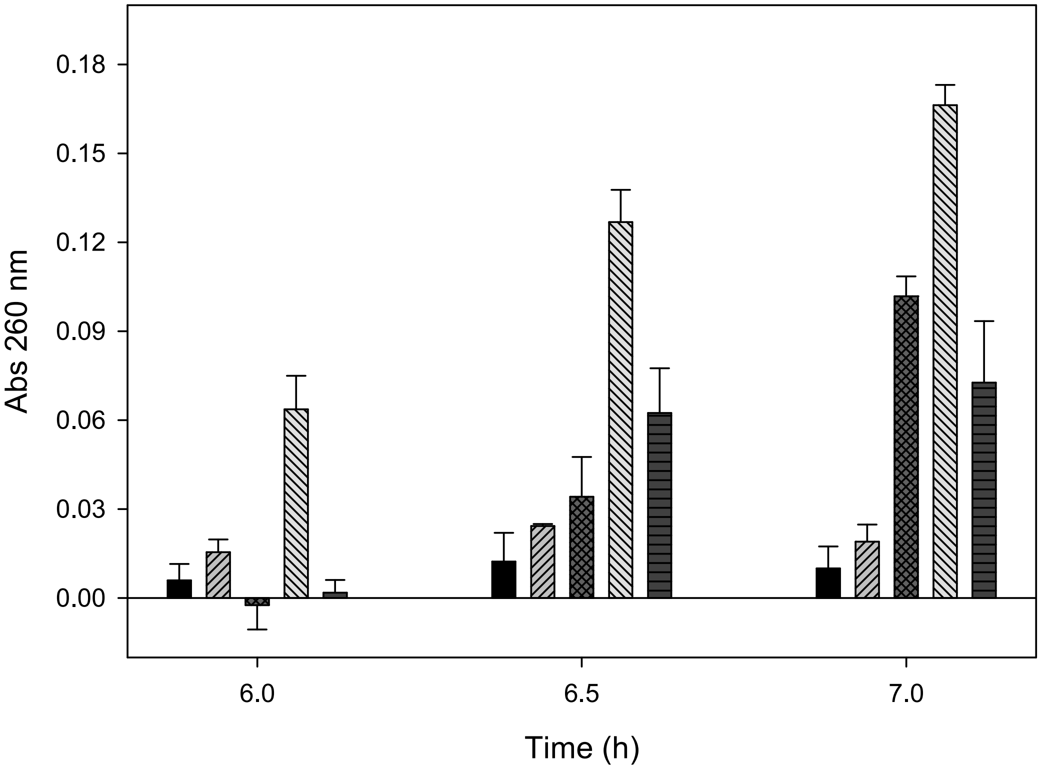


Fig 3a:


Abs 260 nm
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0.15
0.12
0.09
0.06
0.03
0.00
6.0
6.5
7.0
Time (h)

Fig. 3b:

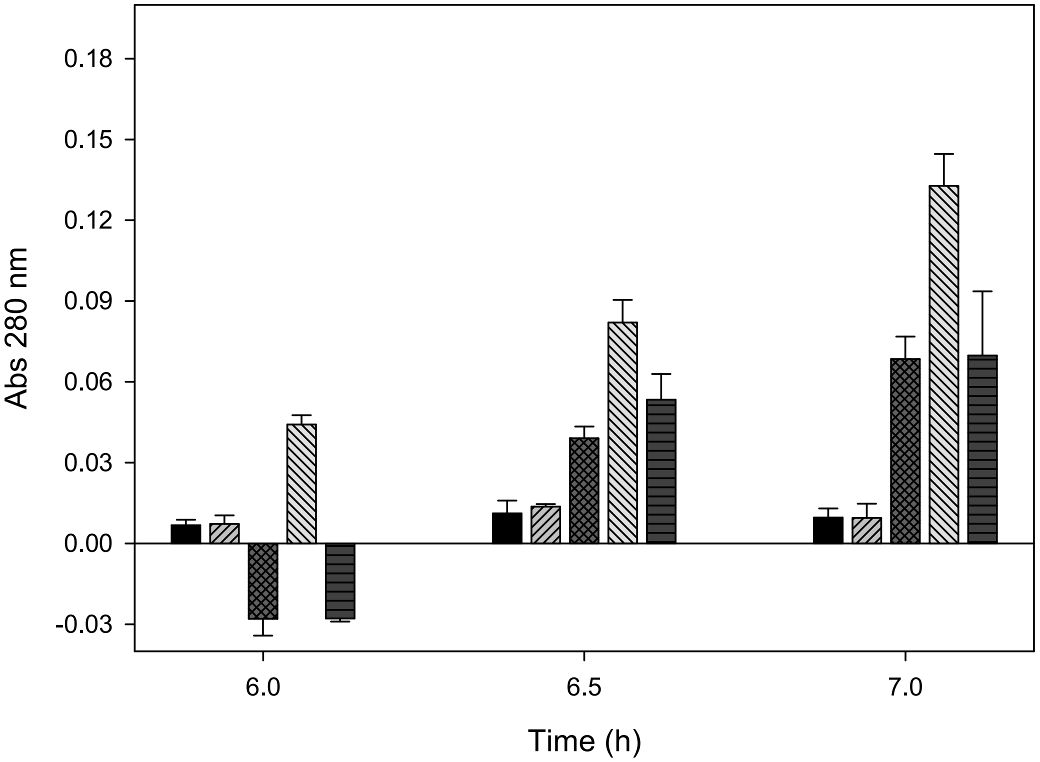
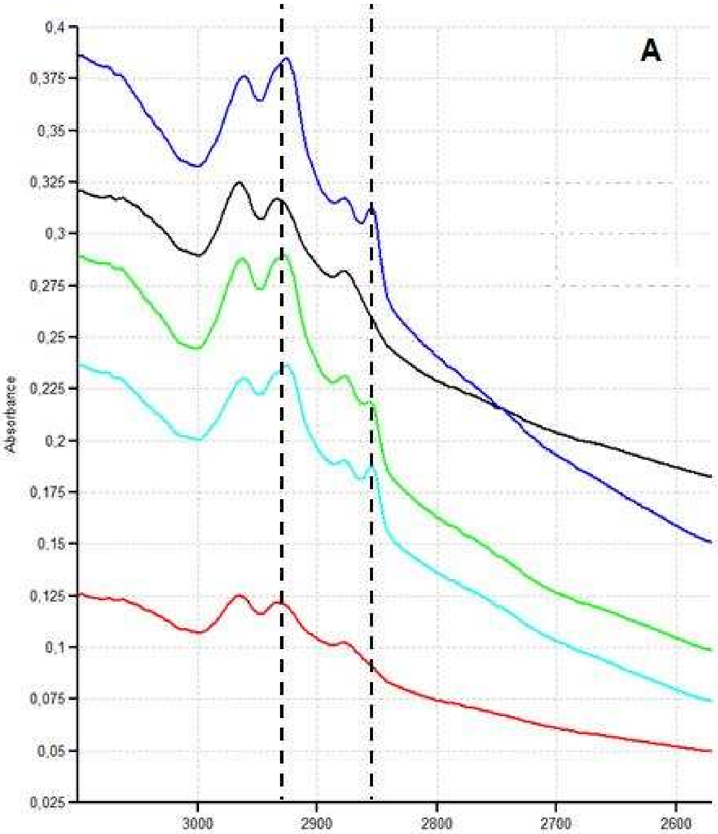
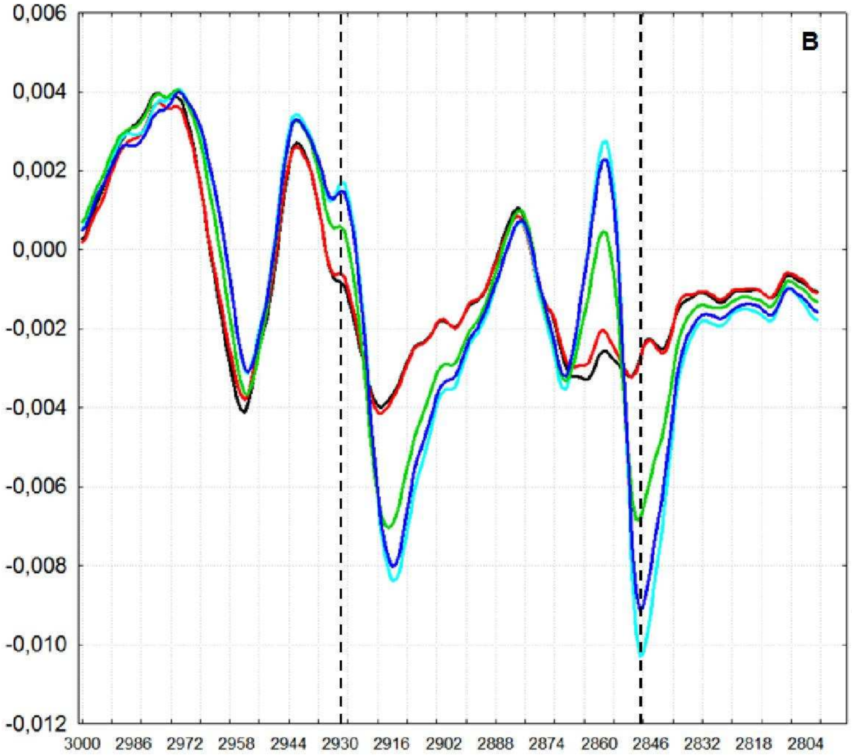

Abs 280 nm
0.18
0.15
0.12
0.09
0.06
0.03
0.00
-0.03
6.0
6.5
7.0
Time (h)

Fig. 4a:



A
Absorbance
0,4
0,375
0,35
0,325
0,3
0,275
0,25
0,225
0,2
0,175
0,15
0,125
0,1
0,075
0,05
0,025
3000
2900
2800
2700
2600

Fig. 4b:



B
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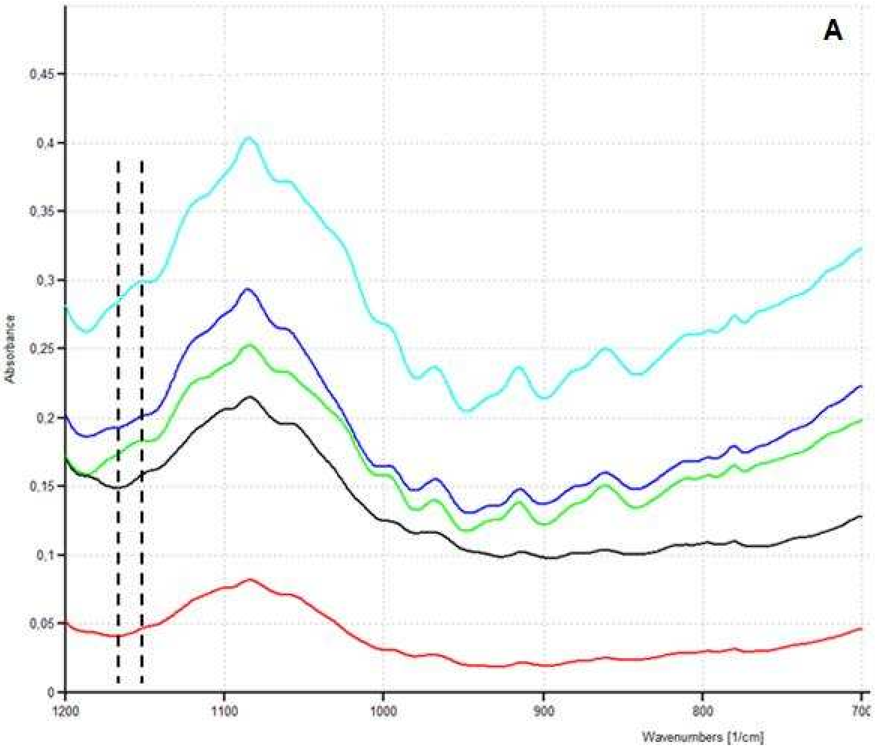
0,004
0,002
0,000
-0,002
-0,004
-0,006
-0,008
-0,010
-0,012
3000
2986
2972
2958
2944
2930
2916
2902
2888
2874
2860
2846
2832
2818
2804

Fig. 5a:


A
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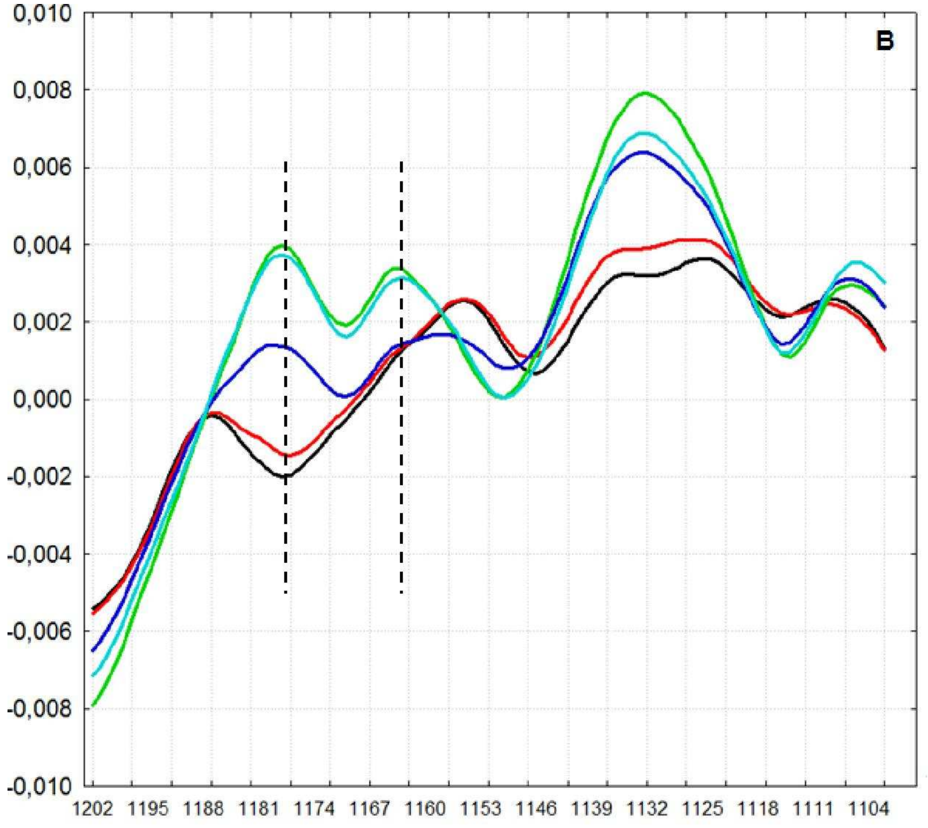
0,4
0,35
0,3
0,25
0,2
0,15
0,1
0,05
0
Absorbance
1200
1100
1000
900
800
70
Wavenumbers [1/cm]

Fig. 5b:


B
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0,006
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1202
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1125
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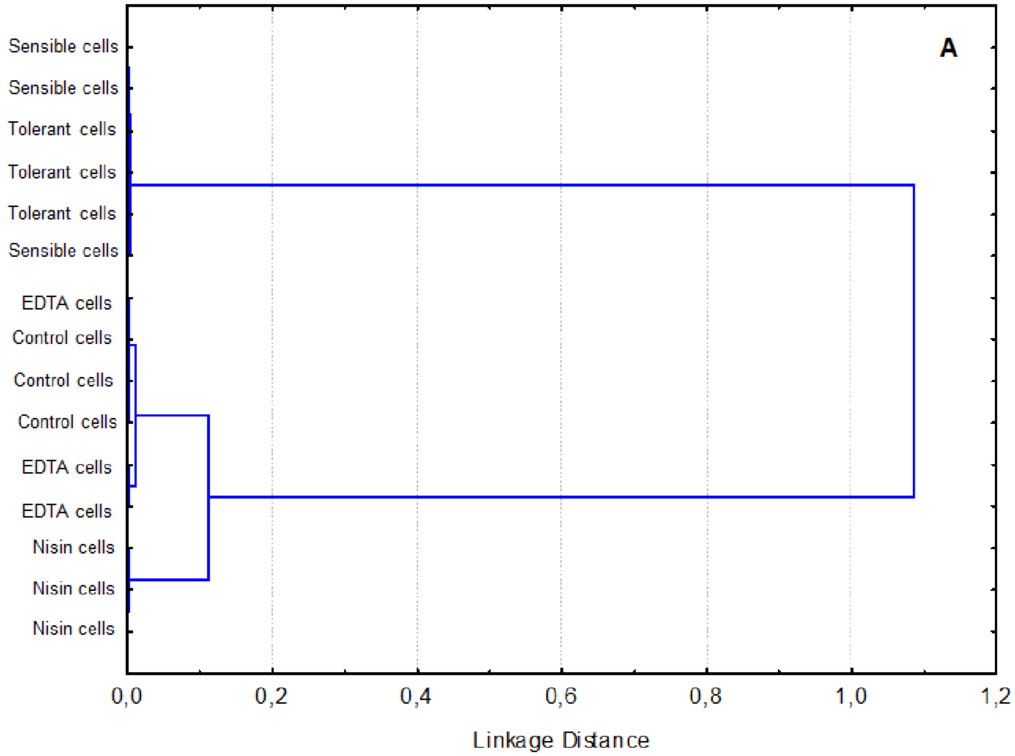
1111
1104

Fig. 6a:



A
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Sensible cells
Tolerant cells
Tolerant cells
Tolerant cells
Sensible cells
EDTA cells
Control cells
Control cells
Control cells
EDTA cells
EDTA cells
Nisin cells
Nisin cells
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0,2
0,4
0,6
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1,0
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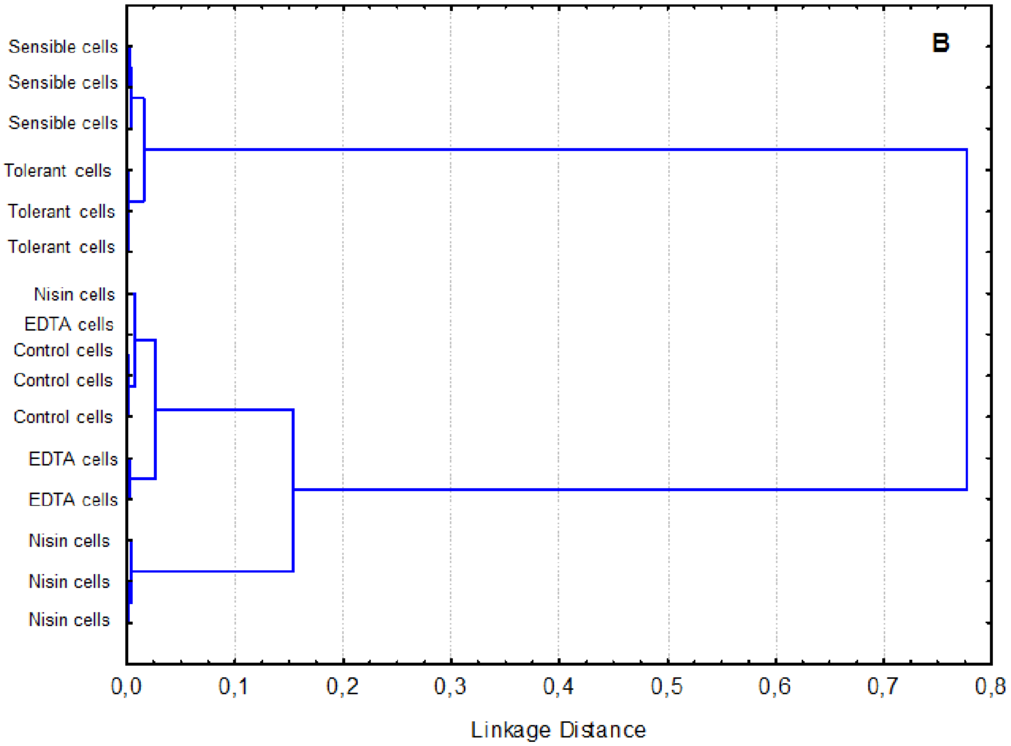
Linkage Distance

Fig. 6b:



B
Sensible cells
Sensible cells
Sensible cells
Tolerant cells
Tolerant cells
Tolerant cells
Nisin cells
EDTA cells
Control cells
Control cells
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EDTA cells
EDTA cells
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Nisin cells
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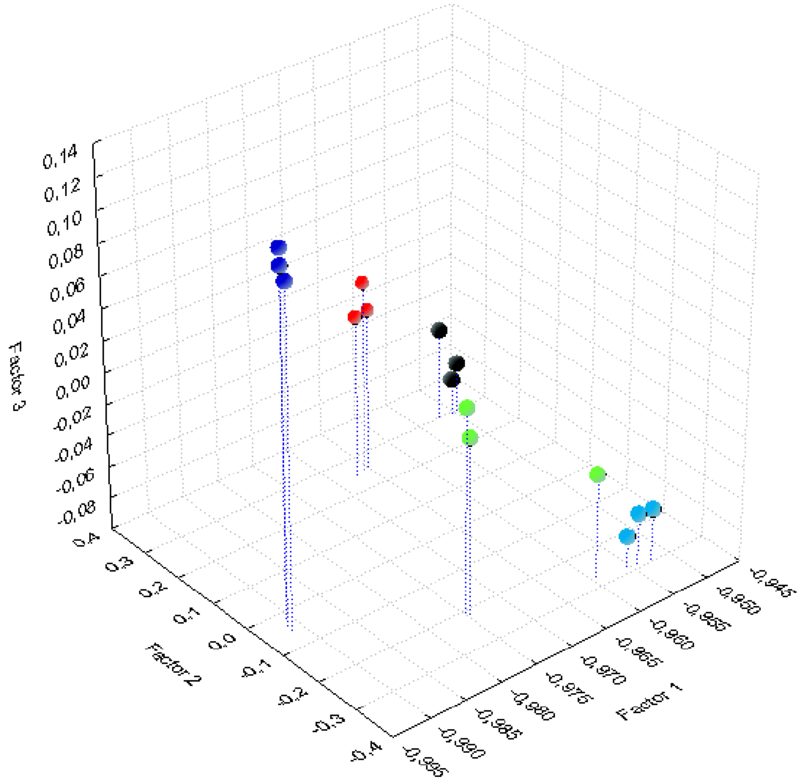
Linkage Distance

Fig. 7: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0,08
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0,00
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-0,04
-0,06
-0,08
Factor 3
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0,3
0,2
0,1
0,0
-0,1
-0,2
-0,3
-0,4
Factor 2
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-0,990
-0,985
-0,980
-0,975
-0,970
-0,965
-0,960
-0,955
-0,950
-0,945
Factor 1

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Chapter 4[*]

Differences in the antibacterial activity of nisin and bovicin HC5 against *Salmonella* Typhimurium under different temperature and pH conditions

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[*] Formatting according to Journal of Applied Microbiology author guidelines

Differences in the antibacterial activity of nisin and bovicin HC5 against *Salmonella* Typhimurium under different temperature and pH conditions

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Abstract

Aims: To compare the action of nisin and bovicin HC5 in combination with EDTA on *Salmonella* Typhimurium, under different environmental conditions.

Methods and Results: *Salmonella* Typhimurium was treated in BHI broth containing EDTA (1.5 mmol l^{-1}) and nisin or bovicin HC5 (200 AU ml^{-1}) under different pH and temperature conditions, and according to a central composite design with two factors (temperature and pH). Cell viability was evaluated on plate count agar for 48 h. The combination of nisin or bovicin HC5 with EDTA was able to inhibit the growth of *Salmonella,* but the temperature and pH conditions promoting inhibition were distinct for each bacteriocin. Nisin was bactericidal over a broad range of temperature and pH, while bovicin HC5 was bacteriostatic in most conditions and bactericidal only in specific conditions (pH >6.0 and temperature >30 °C). *S.* Typhimurium did not show tolerance to bovicin HC5 or cross-tolerance between these lantibiotics.

Conclusions: Nisin and bovicin HC5 both inhibited the growth of *Salmonella*, but the activity of each bacteriocin was differently influenced by environmental conditions.

Significance and Impact of the Study: These results suggest that environmental conditions should be considered to establish optimal conditions for application of these lantibiotics.

Keywords: bacteriocins, lantibiotics, environmental conditions, biocontrol, gram-negative bacteria.

1. Introduction

Salmonella is a heat sensitive bacterium that is not highly resistant to food processing conditions, but it is still the main foodborne pathogen in several countries, and is related to foodborne illnesses vehicled by several products (Mukhopadhyay and Ramaswamy, 2012). Like other Gram-negative bacteria, *Salmonella* is resistant to the action of the bacteriocins classified as lantibiotics. The use of lantibiotics has increased due to their desirable characteristics, including low toxicity and stability against proteases and temperature (Dischinger et al., 2014).

Lantibiotics are bacteriocins of 19 to 38 amino acids that are characterized by the presence of unusual amino acids formed by post-translational modifications (Cleveland et al., 2001; Dawson and Scott, 2012). They act mainly on the cell membrane by forming pores that promote the efflux of metabolites and ions through the membrane and/or by inhibiting cell wall synthesis (Sahl and Bierbaum, 1998; Dischinger et al., 2014). They exhibit antibacterial activity primarily on Gram-positive bacteria and have been presented as viable alternatives for use in several applications, such as in veterinary medicine, and in the pharmaceutical, agricultural and food industries (Dawson and Scott, 2012; Balciunas et al., 2013; Dischinger et al., 2014).

The use of lantibiotics against Gram-negative bacteria depends on the destabilization of the outer membrane by chelating agents, such as ethylenediaminetetraacetic acid (EDTA), by treatment with essential oils, or by physical treatments such as freezing, heating, or high pressure processing (Belfiore et al., 2007; Govaris et al., 2010; Lee and Kaletunç, 2010).

Nisin is the main representative of the lantibiotic class and is the only lantibiotic approved for commercial use, as a food additive for dairy products and canned vegetables and fruits, in several countries (Dischinger et al., 2014; Nagao, 2009). The antibacterial activity of nisin and other lantibiotics against Gram-positive bacteria has been extensively demonstrated (Millette et al., 2007; Solomakos et al., 2008; Ruiz et al., 2010; Pinto et al., 2011; Wijnker et al., 2011; Avila et al., 2014) and less demonstrated against Gram-negative bacteria (Boziaris and Adams, 1999; Govaris et al., 2010; Martin-Visscher et al., 2011). Bovicin HC5 is a bacteriocin produced by *Streptococcus bovis* HC5, a bacterium isolated from the bovine rumen. Its effectiveness against Gram-positive bacteria (Mantovani and Russel, 2003; Carvalho et al., 2007a, Carvalho et al., 2007b; Carvalho et al., 2008) and Gram-negative bacteria (Prudencio et al., 2014) has also been investigated.

The activity of bacteriocins in foods is influenced by several factors, including the chemical composition of the food, and physical conditions, like pH and temperature (Balciunas et al., 2013). Additionally, *Salmonella* can adapt to environmental conditions and alter its outer membrane composition, which can also influence its resistance to antimicrobial peptides, such as bacteriocins (Kawasaki, 2011).

Because the activity of each bacteriocin can be influenced differently by environmental conditions, the aim of this work was to compare the action of nisin and bovicin HC5 in combination with EDTA on *Salmonella* Typhimurium under different temperature and pH conditions.

2. Material and Methods

2.1. Strains and growth conditions

Salmonella enterica serovar Typhimurium ATCC 14028 was grown in brain and heart infusion broth (BHI, Himedia, India), at 37 ± 1 °C for 24 h. *Lactococcus lactis* ATCC 19435 was cultivated in MRS broth (MRS, Himedia, India), at 37 ± 1 °C, and was used to determine the antimicrobial activity of bacteriocins. Stock cultures were maintained at – 20 °C in appropriate culture medium with glycerol (50%).

2.2. Preparation and activity of bacteriocins

Extracts of nisin (Nisaplin, Danisco, Denmark) were prepared by diluting the stock powder in sodium phosphate solution (0.05 mol l^{-1}, pH 2.0). Extracts of bovicin HC5 were prepared as described by Mantovani and Russell (2003).

The antimicrobial activity was determined using the diffusion method in agar (Tagg, 1976), and was quantified by the critical dilution method (Hoover and Harlander, 1993). The concentration was expressed in arbitrary units (AU ml^{-1}).

2.3. Evaluation of the action of bacteriocins in combination with EDTA against S. Typhimurium under different temperature and pH conditions

Cells were centrifuged at 2,500 *g* for 15 min at 4 °C (Sorvall RT 6000D, USA), washed once and resuspended in 0.85 % saline. BHI broth at different pH values were supplemented with EDTA (1.5 mmol l^{-1}) (Reagen, Brazil) and bacteriocins (nisin or bovicin HC5, 200 AU ml^{-1}) and was inoculated with 10^5 CFU ml^{-1} of the test microorganism. These concentrations previously demonstrated to have an inhibitory effect on *S.* Typhimurium under optimal growth conditions (pH 7.2 and 37 °C) (Prudencio et al., 2014).

The tubes were incubated at different temperatures, according to the experimental design and growth was assessed at different time intervals over a period of 48 h using the drop plate method on plate count agar (PCA, Difco, USA). Plates were incubated at 37 ± 1 °C, for a period of 8 to 12 h.

Growth analysis was conducted using the count results by a log reduction factor (Log N_T-Log N_o), where Log N_T is the log of CFU ml^{-1} at a given time and Log N_o is the log of CFU ml^{-1} at the beginning of the experiment.

2.4. Experimental design

A central composite design with two factors was used as an experimental design to evaluate the effects and interactions of temperature and pH on the inactivation of *S.* Typhimurium by nisin or bovicin HC5 in combination with EDTA. The independent variables were temperature (10 to 45 °C) and pH (5.0 to 7.2) (Table 1). The central point was repeated three times to estimate the variation due to random errors. The remaining tests were performed once, and all experiments were performed at random, to minimize the possible effects of environmental conditions.

Table 1: Central composite design with two factors, proposed for assays on *S.* Typhimurium in BHI broth, with or without addition of the bacteriocins (nisin or bovicin HC5, 200 AU ml^{-1}) and EDTA (1.5 mmol l^{-1}).

Test	pH	Temperature (°C)
1	5.3	15.1
2	6.9	15.1
3	5.3	39.9
4	6.9	39.9
5	6.1	10.0
6	6.1	45.0
7	5.0	27.5
8	7.2	27.5
9	6.1	27.5
10	6.1	27.5
11	6.1	27.5

2.5. Evaluation of tolerance to bovicin HC5 and cross-tolerance between nisin and bovicin HC5

Condition 8 (pH 7.2 and temperature 27.5 °C, see table 1) was chosen to evaluate the tolerance of cells to bovicin HC5 and EDTA, and to evaluate the cross-tolerance between the bacteriocins. In this condition, the culture demonstrated tolerance to treatment with nisin and EDTA (unpublished data), and cell growth was observed under treatment with bovicin HC5 and EDTA, after long treatment times.

Pre-adaptation to bovicin HC5 and EDTA was performed with an initial concentration of 10^5 CFU ml^{-1} *S.* Typhimurium cells, in BHI broth at pH 7.2, that contained bovicin HC5 (200 AU ml^{-1}) and EDTA (1.5 mmol l^{-1}), and was incubated at 27.5 ± 1 °C for 48 h. To induce tolerance to nisin and EDTA, the same concentration of cells was pre-adapted to nisin (200 AU ml^{-1}) and EDTA (1.5 mmol l^{-1}) in BHI broth at pH 7.2 by incubation at 27.5 ± 1 °C for 24 h. After this time, cells were collected by centrifugation at 8,000 *g* for 5 min, washed twice and resuspended in 0.85 % saline. The culture was then cultivated with the same concentrations of bovicin HC5 and EDTA under the same incubation conditions.

Cell growth was evaluated at different time intervals over 48 h, by the microdrops method on PCA. Plates were counted after incubation at 37 ± 1 °C for a period of 8 to 12 h. The test was repeated two times and statistical analyses were performed using the Assistat program, version 7.7 beta, 2009 (Silva and Azevedo, 2009). Tukey's test was used to determine the existence of differences between the treatment and the control groups. A significance level of 0.05 was adopted.

Similar assays were also performed in polystyrene microplates, in a final volume of 200 µl. Unadapted cells (grown in BHI broth for 24 h) were used as controls and were treated with bovicin HC5 and EDTA, with EDTA only (1.5 mmol l^{-1}), with bovicin HC5 only (200 AU ml^{-1}) or without bovicin HC5 or EDTA. The plates were incubated at 27.5 ± 0.5 °C for 24 h, and the growth evaluated by absorbance at 600 nm (Thermo Scientific, USA). The test was repeated twice with at least five replicates.

3. Results

3.1. Antimicrobial activity of nisin and bovicin HC5 combined with EDTA on S. Typhimurium

During the first 2 h, the growth of *S.* Typhimurium in BHI broth only occurred under optimal conditions, but the bacterium rapidly extended its range of growth to lower pH and temperature conditions, 5.0 and < 20 °C, respectively, demonstrating the adaptability of the microorganism. The greatest amount of growth after 24 h of incubation, seen as the largest increase in the number of log cycles (3.5 CFU ml^{-1}), was observed in optimal conditions (pH of 7.2 and temperature near to 35 °C), as expected for a mesophilic and neutrophilic microorganism (Fig. 1, A).

The growth of *S.* Typhimurium was partially reduced in the presence of EDTA and this chelating agent affected the final number of cells even at a low concentration (1.5 mmol l^{-1}), as visualized by a smaller increase in the number of log cycles (Fig. 1, B). The behavior of the culture was similar to that observed without the addition of the agent, as an increase in the number of cells was observed in most conditions, although there was less growth at acidic pH values (5.0 to 5.5) (Fig. 1, A and B).

The presence of 200 AU ml^{-1} of nisin or bovicin HC5 did not exert a great influence on the final number of cells, but it was possible to note differences in the action of both lantibiotics (Fig. 2, A and B). During the first 6 h of growth, nisin exerted antimicrobial activity at higher temperatures and lower pH values, although the culture recovered its the growth after 24 h of incubation (Fig. 2, A). This behavior was not observed when bovicin HC5 was added to the medium (Fig. 2, B). Furthermore, after prolonged treatment time under low temperature and low pH conditions, nisin alone promoted a significant reduction in the number of viable cells, while bovicin HC5 did not affect the growth of the culture (Fig. 2, A and B).

The combination of nisin with EDTA was bactericidal and promoted a rapid reduction in the number of viable cells at all studied pH values, at temperatures above 25 °C (Fig. 3, A). After 6 h of treatment, its inhibitory activity extended to lower temperatures, although greater activity occurred under optimal growth conditions (pH near 6.2 and temperature near 35 °C), as clearly visualized by a reduction in growth of 4 log cycles (Fig. 3, A). Increased treatment time also increased the area of the optimal inhibitory activity, although the activity was slightly weaker (reduction of 3 log cycles) (Fig. 3, A). On the other hand, there was a reduction in antimicrobial activity at temperatures above 25 °C and near-neutral pH (> 6.3), possibly due to the enhanced growth of the culture in some conditions (Fig. 3, A).

After 6 h of treatment, the combination of bovicin HC5 with EDTA exerted bacteriostatic action over a large pH and temperature range, while bactericidal activity was only demonstrated under specific conditions namely: pH values above

6.0 and temperatures above 30 °C (Fig. 3, B). Moreover, bovicin HC5 acted more slowly than nisin; with bovicin HC5 considerable reductions in the number of viable cells were only observed after 4 h of treatment (data not shown), while nisin exhibited a similar effect after 2 h. After 24 h, there was an increase in the inhibitory activity of bovicin HC5 at low temperatures, generally at near-neutral pH values (Fig. 3, B).

3.2. Analysis of tolerance to bovicin HC5 and EDTA and cross-tolerance between nisin and bovicin HC5

Increased culture viability was observed after long treatment times with nisin and EDTA (Fig. 3, A), under conditions in which the bacteriocin remained active in the culture supernatants (results not shown). Similar behavior was observed for bovicin HC5 and EDTA, but in a smaller number of experimental conditions, and generally requiring more time (48 h) for similar growth (results not shown). This behavior could suggest an adaptive effect after prolonged treatment with a combination of bacteriocin and EDTA. We tested this possibility with growth assays.

In the absence of bovicin HC5 and EDTA, pre-adapted cells exhibited growth similar to the control, demonstrating the viability of pre-adapted cultures (Fig. 4). However, when pre-adapted cells were treated with bovicin HC5 and EDTA, the cultures exhibited behavior in microplate assays that was similar to non-adapted cells. The decrease in the number of viable cells was also substantial, suggesting that the pre-adapted cultures had not developed tolerance (Fig. 4 and 5).

We also evaluated cross-tolerance between nisin and bovicin HC5 at pH 7.2 and 27.5 °C, conditions under which cells demonstrated tolerance to nisin (unpublished results). The results showed that pre-adaptation to nisin and EDTA did not influence the response of the culture to treatment with bovicin HC5 and EDTA. Although the reduction in the number of viable cells was smaller than in cultures pre-adapted with bovicin HC5 and EDTA, the reduction was higher and significantly different from those presented by non-adapted cells (Fig. 5).

4. Discussion

Our study revealed that both nisin and bovicin HC5 were able to inhibit *S.* Typhimurium in combination with EDTA. However, nisin clearly acted more rapidly, primarily with a bactericidal mode, whereas bovicin HC5 showed bacteriostatic activity and bactericidal activity only under specific conditions. This difference in the mode of action of the lantibiotics has already been demonstrated, and it is related to the bacteriocin and bacterial concentration, the physiological state of cells and the treatment conditions (Delves-Broughton et al., 1996). Generally, bactericidal activity is favored under optimal conditions for growth (Delves-Broughton et al., 1996), as noted in our results for both lantibiotics (Fig 3, A and B). However, it should be considered that optimal growth conditions may also promote the growth of the culture after long incubation times, as observed after 24 h in the presence of nisin (Fig. 3, A).

Nisin is more stable and soluble at low pH (Liu and Hansen, 1990; Garcera et al., 1993; Delves-Broughton et al., 1996) and, like bovicin HC5, is more active at acidic pH (Liu and Hansen, 1990; Houlihan et al., 2004). However, our results demonstrated that the maximal activity of both lantibiotics, but particularly of bovicin HC5, occurred at near-neutral pH. It is possible that this behavior was due to the action of EDTA, which exhibits higher activity at neutral pH, due to the greater stability of EDTA-metal complexes and the higher proportion of ionized carboxylate group that act as good electron donor groups (Boziaris and Adams, 1999). At near-neutral pH, there may be enhanced EDTA binding to calcium and magnesium ions, destabilizing the lipopolysaccharide layer (LPS), and thereby allowing the diffusion of bacteriocins (Boziaris and Adams, 1999).

Microbial adaptations to environmental conditions and the structure of lantibiotics also appear to influence the mechanisms of action. The bacterial response to temperature variations involves changes in the fatty acid composition of the cytoplasmic membrane. At low temperature, there is a substantial increase in the proportion of unsaturated fatty acids, primarily C18:1 in *S.* Typhimurium, to maintain membrane fluidity (Álvarez-Ordónez et al., 2008). However, it has been demonstrated that bovicin HC5 is small to permeabilize lipid bilayers formed by phospholipids with C18:1 fatty acids or longer acyl chains, preventing its bactericidal action under these conditions (Paiva et al., 2011). At low pH, there is a reduction in membrane fluidity, due to a decrease of unsaturated fatty acids and an increase of cyclic fatty acids, but in *S.* Typhimurium these adaptations can be less intense at low temperature and may not significantly change the length of fatty acids (Álvarez-Ordónez et al., 2008; Álvarez-Ordónez et al., 2010). Changes in the cellular membrane can modify the bacteriocin docking and insertion process and thereby alter the susceptibility of the target microorganism (Crandall and Montville, 1998; Chihib et al., 1999).

Another adaptive response that can influence resistance to antimicrobial peptides involves changes in LPS (Ades, 2004; Coornaert et al., 2010). Modifications in LPS structure may alter the permeability of the outer membrane and the negative charge of the molecule, which can affect the electrostatic and hydrophobic interactions that are necessary for bacteriocin activity (Gunn et al., 1998; Ganzle et al., 1999; Delcour, 2009; Wang and Quinn, 2010). Therefore, environmental conditions can promote the formation of a more sensitive LPS structure that allows nisin to act on its own, as demonstrated in our results (Fig. 2, A). These results agree with those obtained by Elliason and Tatini (1999), wherein low temperature (6.5 °C) favored the action of nisin alone against *S*. Typhimurium and *Escherichia coli*. However, this behavior was not demonstrated in the presence of bovicin HC5, most likely because of the effect of low temperature on the length of the fatty acids present in the cellular membrane (Fig. 2, B).

On the other hand, it has already been observed that *Salmonella* cells can display tolerance after exposure to a combination of nisin and EDTA under some treatment conditions, in a process related to modifications in the cytoplasmic membrane and in LPS (unpublished data). Despite being a complex phenomenon arising from modifications in the cytoplasmic membrane or cell wall or from the presence of enzymes that degrade nisin, resistance to nisin is relatively common and has already been detected in various Gram-positive bacteria, including *Listeria*, *Lactobacillus* and *Clostridium*, among others (Crandall and Montville, 1998; Kaur et al., 2011). Such resistance has not yet been demonstrated with bovicin HC5, and cross-tolerance between these lantibiotics was not detected, possibly due to differences in their modes of action (Paiva et al., 2011). Our results suggest that a combination of bovicin HC5 with nisin and EDTA can be an alternative for *Salmonella* control, under specific environmental conditions.

5. Conclusions

In this study, we showed that nisin and bovicin HC5 exhibit differences in their modes of action with respect to environmental conditions. Nisin acted over a greater temperature and pH range than bovicin HC5. Neither tolerance of *S.* Typhimurium to bovicin HC5 nor cross-tolerance between nisin and bovicin HC5 were observed. These findings are important, as few studies have evaluated the influence of environmental conditions on the action of bacteriocins, particularly of bovicin HC5, against *Salmonella*. Moreover, we consider that bacteriocins can have different effects on other Gram-negative bacterial species, as each microorganism may respond to environmental changes in a distinct way. Further studies are needed to understand this behavior and to allow these lantibiotics to be appropriately employed as antimicrobial products in foods and in other applications.

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Legends to figures:

Fig. 1: Variation of log CFU ml^{-1} of *Salmonella* Typhimurium in BHI broth with different pH values and incubated at different temperatures: A) BHI broth; B) BHI broth with added EDTA (1.5 mmol l^{-1}).

Fig. 2: Variation of log CFU ml^{-1} of *Salmonella* Typhimurium in BHI broth with different pH values and incubated at different temperatures: A) BHI broth with added nisin (200 AU ml^{-1}); B) BHI broth with added bovicin HC5 (200 AU ml^{-1}).

Fig. 3: Variation of log CFU ml^{-1} of *Salmonella* Typhimurium in BHI broth with different pH values and incubated at different temperatures: A) BHI broth with added nisin (200 AU ml^{-1}) and EDTA (1.5 mmol l^{-1}); B) BHI broth with added bovicin HC5 (200 AU ml^{-1}) and EDTA (1.5 mmol l^{-1});

Fig. 4: Growth of *Salmonella* Typhimurium in BHI broth at pH 7.2, with or without added bovicin HC5 (200 AU ml^{-1}) and EDTA (1.5 mmol l^{-1}), and incubated at 27.5 ± 1 °C for 24 h. Legend: Cells in BHI broth (●), cells in BHI broth containing EDTA (▼), cells in BHI broth containing bovicin HC5 (■), in BHI broth containing HC5 and EDTA (◆), cells pre-adapted to bovicin HC5 and EDTA in BHI broth containing bovicin and EDTA (▲), cells pre-adapted to bovicin HC5 and EDTA in BHI broth (●), and cells pre-adapted to nisin and EDTA in BHI broth containing bovicin HC5 and EDTA (●).

Fig. 5: Viability of *Salmonella* Typhimurium in BHI broth at pH 7.2 with added bovicin HC5 (200 AU ml^{-1}) and EDTA (1.5 mmol l^{-1}), and incubated at 27.5 ± 1 °C for 48 h. Legend: Cells not pre-adapted (-●-), cells pre-adapted to bovicin HC5 and EDTA (...○…), and cells pre-adapted to nisin and EDTA (-▼-).

Figures:

Fig. 1:

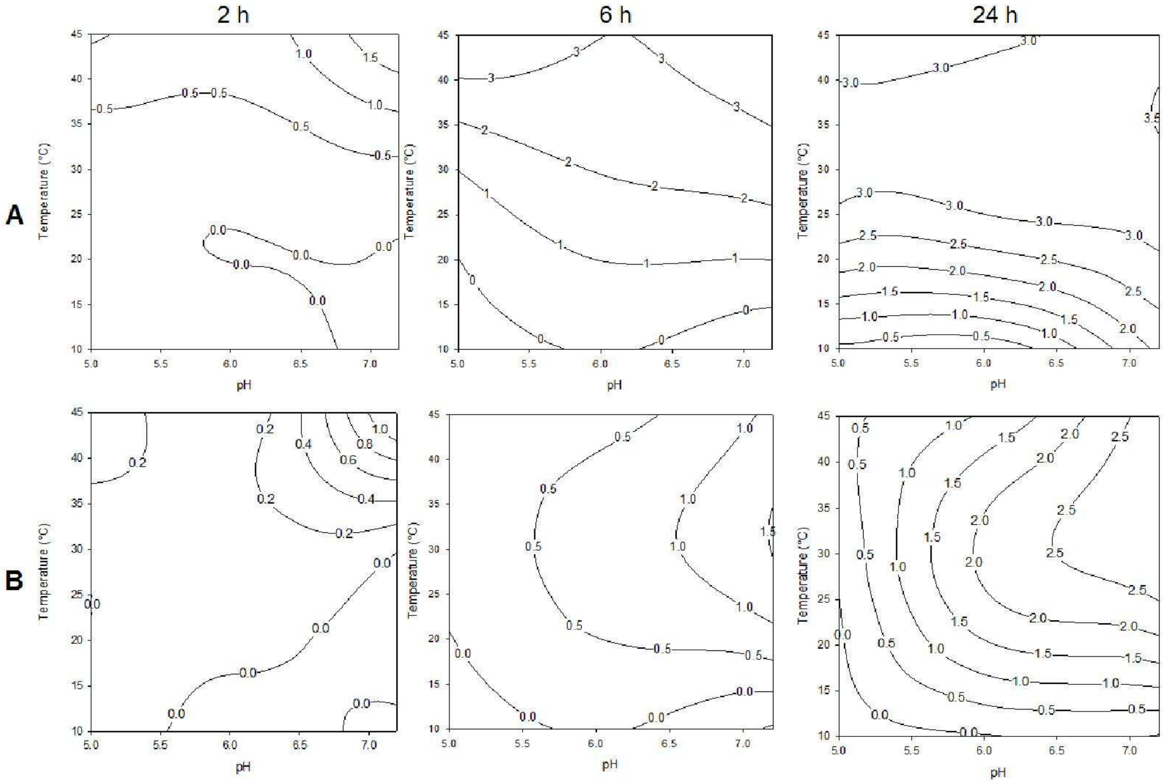
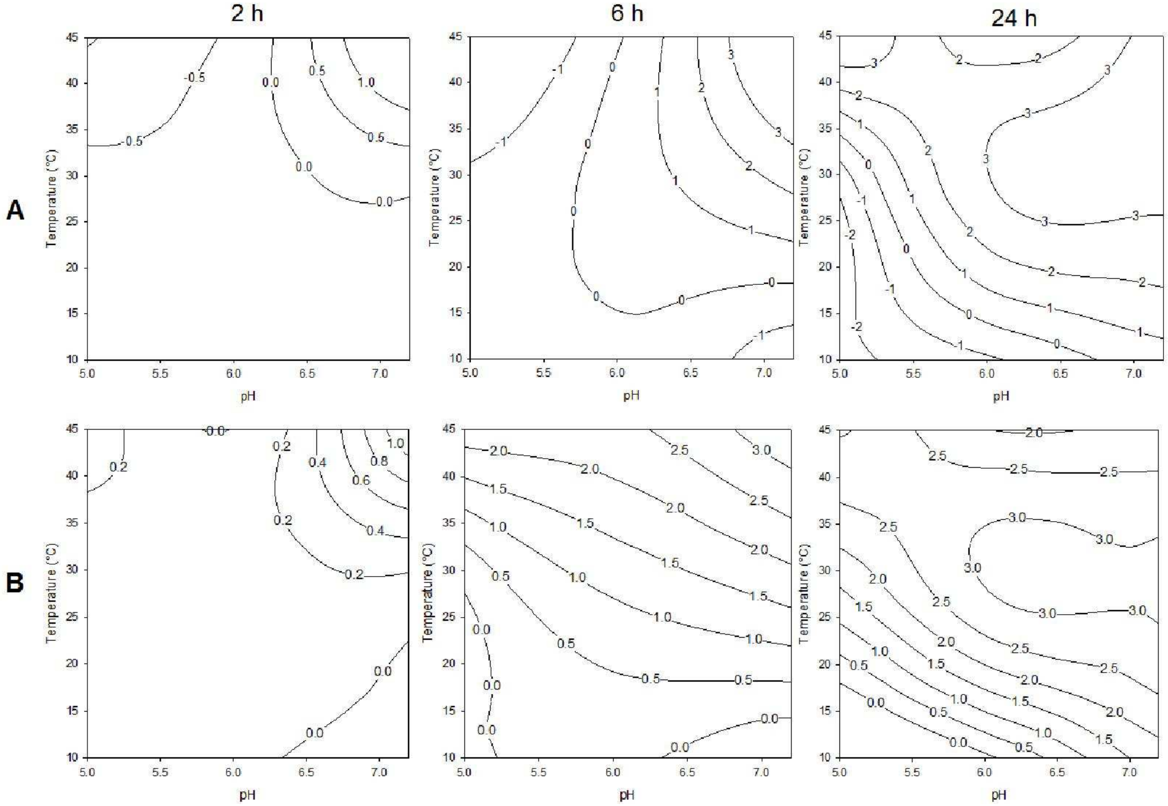


Fig. 2:


2 h
6 h
24 h
A
B
Temperature (°C)
pH
5.0
5.5
6.0
6.5
7.0
10
15
20
25
30
35
40
45
-0.5
0.0
0.5
1.0
-1
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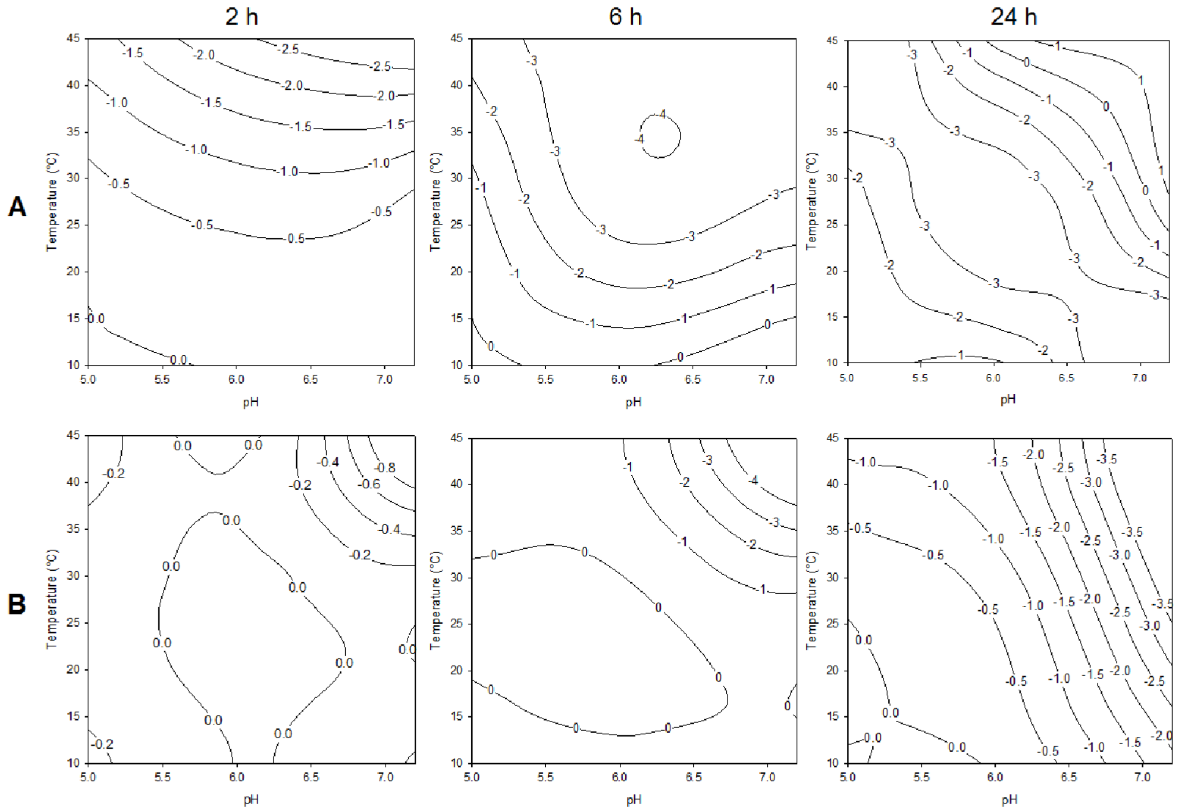
0.2
0.4
0.6
0.8
1.5
2.0
2.5
3.0

Fig. 3:



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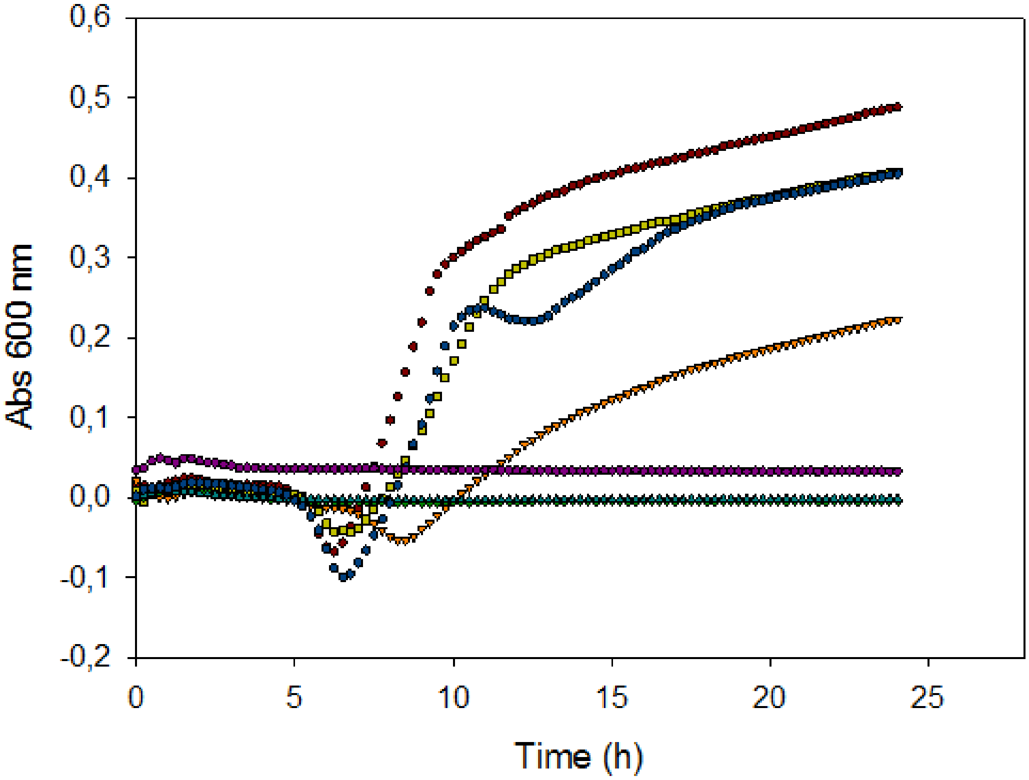
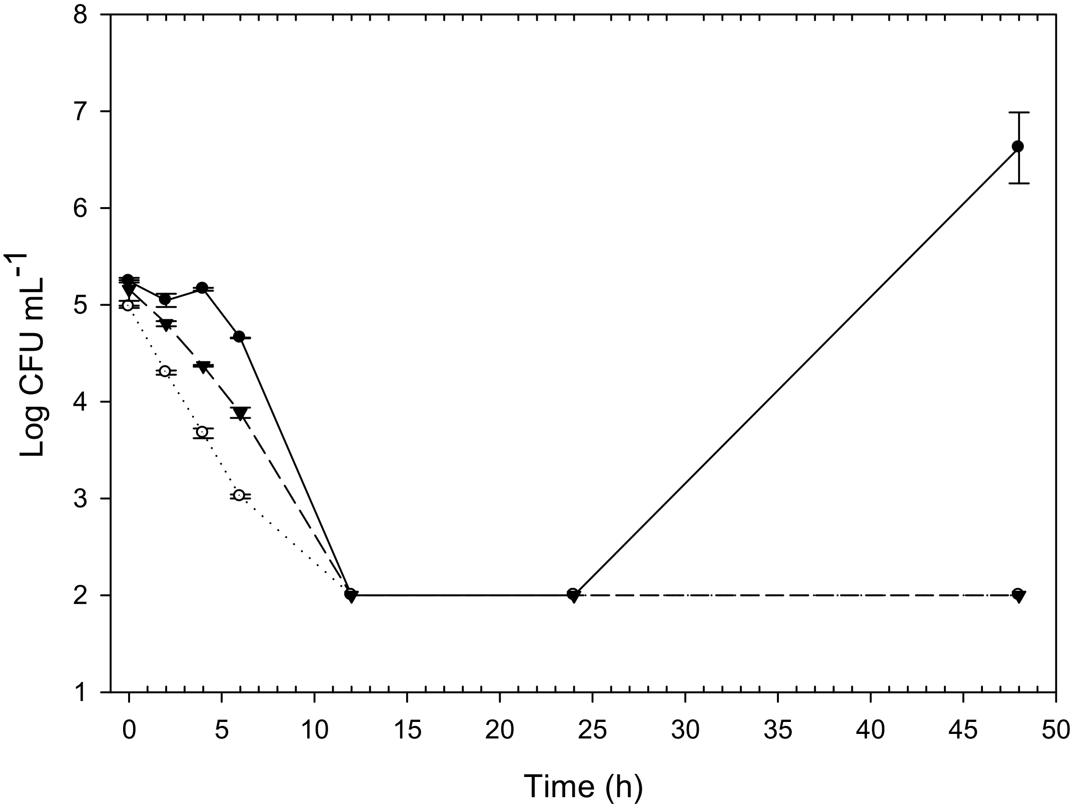


Fig. 5:



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