The antimicrobial activity of sodium lactate

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Stellingen

I

Om de antimicrobiële werking van organische zuren of hun zouten bij een bepaalde pH en temperatuur objectief te kunnen vergelijken moeten de concentraties in mM bekend zijn.

II

Als onder bepaalde condities in aanwezigheid van natrium- , kalium-, of calciumlactaat geen microbiële groei optreedt mag niet worden gesteld dat de effectiviteit waarmee deze zouten de groei remmen gelijk is, noch dat deze remming moet worden toegeschreven aan het lactaat ion.
N. Chen and L.A. Shelef. 1992. J. Food Prot. 55: 574–578.

III

Het feit dat er een reactor op de markt is waarmee zowel zeer toxisch afval uit de aluminiumindustrie kan worden verwerkt als vetvrije snacks kunnen worden gemaakt geeft aan hoe ver de integratie van levensmiddelen- en milieutechnologie al is gevorderd.
D. Bolier. Milieumarkt, mei 1996: 40-41.

IV

De drang naar verbetering van de onderwijskwaliteit wordt geïllustreerd door de benaming van onderwijsinstituten, die een stijging van de kennisstandaard suggereert. Wat vóór 1957 nog Middelbare Technische School werd genoemd staat nu te boek als Technische Hogeschool.

V

De in november 1995 door de overheid gevoerde campagne tegen racisme stelde dat er sprake zou zijn van een ernstige huidziekte. In figuurlijke zin zou men beter kunnen spreken van een hartkwaal, terwijl er in essentie sprake is van een geesteszieke.

VI

Gezien de parallellen tussen BSE en AIDS ligt het voor de hand dat er naast een campagne voor "veilig vrijen" ook een campagne voor "veilig eten" wordt opgestart.
W. Daems. EOS 5 (1996): 6–13

VII

De consumptievermindering van Nederlandse honing door import van goedkope maar kwalitatief slechte honing uit andere landen kan worden bestreden door goede voorlichting en een goed imkersbeleid.

VIII

De discussie rond duurzaam consumeren dreigt in een vicieuze cirkel te belanden.De consument wacht op het moment dat het bedrijfsleven met "groene" producten komt, het bedrijfsleven wil eerst duidelijke regelgeving van de overheid en de overheid ziet een centrale rol weggelegd voor de vraag naar "groene" producten door de consument.

IX

Het Arbeidsbureau Veenendaal onderscheidt zich van andere in deze regio. Niet door het feit dat ze academisch geschoolde werkzoekenden niets heeft te bieden, maar door het feit dat ze deze mensen niets wíl bieden.

X

Men zou een promotieplechtigheid ook als volgt kunnen aanvangen: "Geachte promovenda, u heeft niet het recht om te zwijgen en alles wat u zegt kan (of zal) tegen u gebruikt worden".

Pauline C. Houtsma

Stellingen bij het proefschrift "The antimicrobial activity of sodium lactate"

Wageningen, 25 oktober 1996.

Abstract

Houtsma, P.C. (1996) Antimicrobial activity of sodium lactate. Ph.D. thesis, Agricultural University Wageningen (135 pp., English and Dutch summaries)

In this thesis, the action spectrum and mechanism of microbial growth inhibition by sodium lactate were examined, with special emphasis on its use in meat products.

The concentrations (mM) of lactate needed to prevent growth of various spoilage organisms and pathogens in a broth were determined and compared to those of NaCl. The sensitivity towards lactate differed between micro-organisms, as did the effect of pH on the minimum inhibitory concentration (MIC) of lactate. Especially, bacterial strains that were able to grow at low water activity (≤ 0.95) in the presence of NaCl were inhibited by sodium lactate (*Staphylococcus aureus, Listeria monocytogenes, Brochothrix thermosphacta*), indicating that the microbial quality of food products could be increased when part of the NaCl content would be exchanged by sodium lactate. This is also true when the influence of lactate on toxin production, spore germination and heat resistance of spores from *Clostridium botulinum* is considered.

The effects on growth characteristics of pH, temperature and sodium lactate or NaCl concentrations below the MIC, as examined with *Listeria innocua* in a broth, were successfully translated into a relatively simple mathematical model that was subsequently validated in a Bologna-type sausage. Microbial growth in the sausage was much slower than in broth, but this might be related to the growth conditions being less favourable. The parameter value for the maximum specific growth rate in the absence of lactate was re-estimated using the data obtained in sausage and as a result, the model predictions were statistically acceptable.

Listeria innocua and *Lactococcus lactis* which had been grown in the presence of sodium lactate were better capable of regulating their intracellular pH (pH_{in}) than control cells (grown without lactate). This is probably related to an increase in the amount and/or activity of the proton ATPase (F_1F_0–ATPase) in response to a decrease in the pH_{in}. The mechanism of microbial growth inhibition by sodium lactate remains to be further elucidated, since the decrease in pH_{in} was not enough to explain why microbial growth in the presence of lactate is prevented at low pH.

Key words: lactate, MIC, microbial growth inhibition, adaptation, intracellular pH, water activity, spores, predictive modelling.

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1

General introduction

1.1 Lactic acid and sodium lactate

1.1.1 Chemical properties and production process

Sodium lactate or sodium-L-2-hydroxy-propionate ($CH_3CHOHCOONa$, M_r = 112 g/mol) is a very hygroscopic salt with a mildly saline taste and strong humectant properties. It is derived from natural L(+) lactic acid, a weak acid having a dissociation constant of $1.389.10^{-4}$ at 22°C (pK_a=3.857). Lactic acid is produced on an industrial scale through batchwise fermentation by homofermentative lactic acid bacteria using defined substrates such as sucrose or dextrose at temperatures above 50°C (12). During the fermentation process, the pH is kept at a constant value by the addition of lime ($Ca(OH)_2$), which results in the formation of calcium lactate. When the fermentation process has ended, bacteria are killed by heat and calcium lactate is converted into crude lactic acid by the addition of sulphuric acid. The by-product gypsum ($CaSO_4.2H_2O$) is separated by filtration. The yield of lactic acid that is obtained in this way is more than 90% with an L(+) content of about 97% (12). The crude lactic acid solution is then purified and concentrated, resulting in the edible-grade lactic acid solution. This solution can be further purified by distillation of lactic acid esters, resulting in a reagent-grade lactic acid solution (12). Sodium lactate is obtained from lactic acid by the addition of sodium hydroxide. The product is supplied as a colourless (reagent-grade) or slightly yellowish (edible-grade) viscous solution in water with a lactate content of 59-61% (w/w) and a stereochemical purity (L-isomer) of at least 95%. The pH of the reagent-grade solution that was used for the studies described in this thesis varied between 6.5 and 8.5 (10% solution).

1.1.2 Applications in the food industry

Natural L(+) lactic acid, is being used in a large variety of food products such as confectionery, dairy products, meat and meat products, beer, bakery products, mayonnaise and dressings, flavourings and pickles. As a food acidulant, its main functions are flavouring and preservation. Reasons for selecting lactic acid as a food acidulant are its mild acid taste (flavour enhancement), its preserving properties, its natural occurrence in many foodstuffs and its liquid form (3).

The study that is described in this thesis was performed with special interest in the use of sodium lactate in meat products. Therefore, the following paragraphs deal with this subject in more detail.

1.2 The use of lactic acid and sodium lactate in meat products

1.2.1 Legislation

Sodium lactate and lactic acid have been determined safe for the use in foods and are listed as additives generally recognized as save (GRAS) by the Food and Drug Administration (FDA). In 1988, the United States Department of Agriculture (USDA) permitted the use of sodium- and potassium lactate to a level of 2% in those meat products where flavouring agents and flavour enhancers are permitted, and to a level of 4.8% in those products that run an enhanced risk of growth of *Clostridium botulinum* such as vacuum packed hermetically sealed turkey breast. In November 1992, pre-evisceration organic acid rinses were approved by the Food Safety and Inspection Service of the United States (FSIS, USDA) for use in commercial slaughterhouses as a means of enhancing product safety and extending the shelf life of carcasses (5). In the Netherlands, the use of lactic acid for decontamination of carcasses is not permitted. The legislation with respect to the use of sodium lactate in meat products in countries belonging to the European Community is very diverse at the moment. Sodium lactate (E325) and lactic acid (E270) belong to the group of substances for which the *quantum satis* was proposed by the Commission of the European Communities, which means that in the future no maximum levels will be specified, on the condition that they are used according to Good Manufacturing Practice at a level not higher

than is necessary to achieve the intended purpose (*i.e.* pH regulation, flavour enhancement, shelf life extension).

1.2.2 Examples and advantages

Lactic acid is the most widely used organic acid in meat products. Its use concurs with the natural presence of lactic acid in fresh meat muscle tissue (0.2-0.8%) and in commercial meat extracts (up to 12%). It is used for the decontamination of carcasses to reduce the total bacterial count (16,18). It is also applied for the treatment of natural casings and pork rinds (protein swelling and decontamination), in sausages (preservation and pH stabilization) and in preparation of collagen casings.

Sodium lactate is mainly used in vacuum packed meat products that are stored refrigerated. It is a very efficient dehydrating salt for the production of uncured hams. Sodium lactate is further used as a protein dissolving salt in Frankfurter-type sausages. It has a mild salty taste and does not affect the colour of the meat products, and the shelf life is increased. The shelf life extending effect of sodium lactate has been reported for fish and for cured and uncured meat products (1,2,6,8,11,13,14,17). Based on final weight of the meat product as sold, generally 2-3% of the 60% solution is recommended for addition to meat and poultry products. In sausage and ground products, the liquid ingredient may be added during the cuttering process, whereas in whole muscle products the sodium lactate content is added to the brine (3).

1.2.3 Bottlenecks

Many of the meat products in which sodium lactate is used are sliced and vacuum packed. When portioning and packing are performed after cooking or pasteurization, the products are exposed to the risk of recontamination with spoilage or pathogenic flora which may subsequently develop when the products are stored. The microbial safety of meat products is improved by the addition of NaCl and nitrite. Nitrite provides protection against growth of and toxin production by *Clostridium botulinum* in cured meat products (15), and NaCl inhibits growth of micro-organisms in general by decreasing the water activity. Usually, the amount of NaCl added to meat products is 0.1-0.2% (3). The concern that the use of nitrite may result in the formation of carcinogenic nitrosamines when it reacts with secondary amines present in meat has

led to the search for alternatives to overcome the possible health hazards. Moreover, due to undesirable colour developments, nitrite cannot be used (or only in very small amounts) in fish, turkey and chicken.

A vacuum packed cooked meat product without nitrite forms in principle a good environment for the outgrowth of *C. botulinum*, in particular for proteolytic strains (9). The spores of the proteolytic A, B and F strains of this micro-organism are very heat resistant ($D_{100°C}$ = 25 min.) compared with the spores of the non-proteolytic B, E and F strains ($D_{100°C}$ < 0.1 min) (7). The competitive flora thus is eliminated during the heat treatment, while the heat resistant spores survive this process. The anaerobic atmosphere creates a favourable environment for spore germination, growth and toxin production, especially under conditions of temperature abuse, that is, when the products are stored at temperatures above 10°C. Temperature abuse of processed meat products occurs regularly, especially during summer (19). Furthermore, consumers prefer a product with a less salty taste, a fresh appearance and a long shelf life when kept refrigerated, and proteolytic strains can cope with twice as much NaCl as non-proteolytic strains, the inhibitory concentration being 10% and 5%, respectively (7).

With respect to the microbial safety of these products it is therefore very important to look for additives which already occur naturally and which have good preservative properties with the additional advantage of protecting from the danger of toxin production by *C. botulinum*. Sodium lactate is a promising substitute, since it could partially replace NaCl (less salty taste) and/or nitrite, as sodium lactate delays toxin production by *C. botulinum* in cook-in-bag turkey products (10). However, for safe application of sodium lactate in meat products, it is necessary to obtain a better understanding of its antimicrobial activity.

1.3 Outline

The aim of the work described in this thesis is to gain insight into the action spectrum and the mechanism of the antimicrobial effect of lactate. This can be divided into a descriptive, a predictive and an explanatory part.

The descriptive part of the research deals with the action spectrum and the extent of the antimicrobial effect, the effect of lactate addition on

growth parameters (lag time, growth rate, yield) and the influence of growth conditions (pH, temperature, oxygen). It was investigated whether sodium lactate is equally effective in controlling growth of spoilage and pathogenic micro-organisms that may occur in meat products. Information on this subject was obtained by screening in a peptone-yeast extract broth at pH 6.5 and 20°C, according to a method that was described by Eklund (4). Strains used for this study were isolated from spoiled and unspoiled meat products with and without lactate, and consisted almost entirely of lactic acid bacteria and yeasts. Reference strains of both spoilage organisms and pathogens that may occur in meat products were screened as well. In this way, the specific inhibitory effect of lactate was determined for about 200 strains, by comparing the Minimum Inhibitory Concentration (MIC) of sodium lactate to that of NaCl. The results of this study are described in **Chapter 2.**

It is important to know whether there is an influence of temperature on MIC values of sodium lactate and NaCl, as meat products in which sodium lactate is present are stored refrigerated (*e.g.* ham, filet americain) as well as at room temperature (*e.g.* smoked sausage, fermented sausage). Furthermore, the inhibitory effect of weak organic acids on growth of micro-organisms is generally ascribed to the undissociated molecule, and thus it is not only related to the amount of sodium lactate present, but to the pH of the product as well. Therefore, the influences of temperature (4°C-37°C) and pH (5.7-7.0) on the inhibitory effect of sodium lactate were examined with a selection of 33 strains (**Chapter 3**).

Spore-forming micro-organisms form a considerable risk for the safety of meat products, as was explained in the section "bottlenecks" in this chapter. The majority of reported botulism outbreaks that are related to the consumption of meat and poultry products involve *Clostridium botulinum* types A and B and are caused by inadequate heat processing or are related to products that have been grossly temperature abused (20). Therefore, in addition to the screening experiments, information on the effect of sodium lactate on growth and toxin production of proteolytic *C. botulinum* strains under conditions of temperature abuse was collected, and the influence of NaCl and sodium lactate on spore germination and heat resistance of spores was assessed (**Chapter 4**).

Next to information on the type of micro-organisms that are inhibited

by sodium lactate, information on the effect of lactate on growth characteristics (lag time, growth rate and yield) is indispensable for a better understanding of the mechanism of microbial growth inhibition by sodium lactate. This study was performed with *Listeria innocua*, which was routinely used as a model for the pathogenic *Listeria monocytogenes*. About 200 growth curves were obtained in the presence of different concentrations of sodium lactate or NaCl at different temperatures and pH values, and the influences on growth parameters were assessed. A successful attempt was made to develop a relatively simple model that could predict the maximum specific growth rates of *L. innocua* in a broth when incubated under various growth conditions (pH, temperature) in the presence of sodium lactate or NaCl (**Chapter 5**). In **Chapter 6**, it is described how the influences of pH and temperature were included in the model, to make it suitable for predicting growth rates at temperatures and pH values different from those that were used to collect the experimental data. The model was validated in Bologna-type sausages.

Finally, an attempt was made to explain the results obtained in the descriptive and the predictive part, investigating adaptation towards sodium lactate in *L. innocua* and *Lactococcus lactis* in a more detailed study of the antimicrobial effect of sodium lactate (**Chapter 7**). An evaluation of the experimental approach and a general discussion of the work presented in this thesis is given in **Chapter 8**.

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2

Minimum inhibitory concentration (MIC) of sodium lactate for pathogens and spoilage organisms occurring in meat products

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Authors: P.C. Houtsma, J.C. de Wit and F.M. Rombouts.

Abstract

Pathogens and spoilage organisms occurring in meat products were screened in peptone-yeast extract broth, according to a method of Eklund (6), to determine the specific inhibitory effect of lactate on growth of these micro-organisms under optimum growth conditions (pH 6.5, 20°C). In general, the specific inhibitory effect of lactate was more obvious for Gram-positive than for Gram-negative bacteria. It was shown especially, that strains that were able to grow at water activity values of 0.95 and below in the presence of NaCl (*Staphylococcus aureus, Listeria monocytogenes, Brochothrix thermosphacta*) were inhibited by sodium lactate. It appeared that yeasts were able to cope with more than 10% (w/v) sodium lactate. However, sodium lactate had a specific inhibitory effect on growth of these organisms when compared with the effect of NaCl. These results indicate that lactate addition to food products with a pH near neutrality offers good prospects for shelf life prolongation of these products

2.1 Introduction

Sodium lactate is being used in meat products for its shelf life prolonging properties. The products concerned are for the greater part cured meat products such as ham, bacon, corned beef, and turkey salami, all of which are kept at refrigeration temperature. In addition, in the United States the use of lactate in fresh meat, *e.g.* pork sausage, turkey breast and chicken filet, is permitted.

There are several good reasons for the use of sodium lactate in meat products. Addition of sodium lactate has no influence on the pH; it has a mild salty taste in comparison with NaCl, and its natural occurrence in meat makes it a suitable flavouring ingredient (4). It enhances meat flavour, and its humectant properties contribute to the water holding capacity and increase cooking yields (14,15,16). An increase in sodium lactate levels from 0–4% in cooked beef roasts results in a darker red colour with less gray surface, and improves juiciness and tenderness of this meat product (14). Formation of off-flavours is inhibited whereas flavour associated with fresh beef is enhanced. This is possibly due to an indirect inhibition of autoxidation by sodium lactate (12). Levels beyond 2% (w/w) do not further enhance sensory characteristics (14). The most important reason for the use of sodium lactate is its antimicrobial activity, especially when products need handling after heat treatment such as slicing or packaging which can cause recontamination (5). Shelf life of fresh pork sausage is extended by more than two weeks when adding 3% (w/w) sodium lactate (10).

Little work has been published on the antimicrobial effects of sodium lactate. Only few bacteria have been tested. Some of the published results indicate that sodium lactate has a specific inhibitory effect on microbial growth which rises above what would be expected from its water activity (a_w) lowering property (5). The inhibitory effect is dependent on concentration, environmental conditions (temperature, a_w and oxygen availability) and on the micro-organism tested (1,3,5,8,9,13,16). Toxin production by *Clostridium botulinum* is inhibited by sodium lactate (11).

Currently published data are insufficient to conclude whether lactate allows for growth of certain micro-organisms more than that of others and the mechanism of lactate inhibition at a pH near neutrality is not clear. To get more information on microbial growth inhibition by lactate, a wide range of both spoilage organisms and pathogens which may occur in meat products were screened for their sensitivity towards sodium lactate. As a standard for lactate sensitivity we used the minimum inhibitory concentration (MIC), which was defined to be the lowest concentration at which no growth of a certain micro-organism could be observed under the conditions of testing. NaCl was used as a reference because sodium lactate may replace part of this salt in food formulas. Equal molalities of NaCl and sodium lactate bring about an

equivalent decrease in a_w (2).

2.2 Materials and methods

2.2.1 Strains

Strains were isolated from spoiled and unspoiled meat products with and without lactate (Table 2.1). Among these were spreadable raw sausage which had been kept at 7°C for over a month, and vacuum packed sliced ham from a supermarket which was kept at 7°C up until 11 days past the expiry date. The sausages contained 0% (w/w) and 3.3% (w/w) sodium lactate. The lactate content of the ham was not known precisely. The spoilage flora consisted almost entirely of psychrotrophic lactic acid bacteria and yeasts. Lactic acid bacteria were confirmed by catalase tests, Gram staining and microscopic appearance. They were further examined for gas production in culture tubes filled with De Man Rogosa Sharpe agar (MRS, Merck) which was covered with a sterile agar layer (Oxoid, L13). Strains from the three main groups of yeasts that were isolated from spoiled meat products were sent to CBS Yeast Division (Baarn, the Netherlands) for identification. They were identified as *Debaryomyces hansenii*, *Candida zeylanoides* and *Rhodotorula mucilaginosa*, respectively. Reference strains of both spoilage organisms and pathogens from culture collections in the Netherlands, Germany and the United States, which were related to spoilage organisms and pathogens that may occur in meat products, were screened as well (Table 2.1).

Strains were stored in cryotubes (Greiner, Solingen, Germany) at –80°C, in 2-ml portions of Brain Heart Infusion broth (BHI, Difco) or De Man Rogosa Sharpe broth (MRS, Merck) mixed with sterile glycerol (20% (v/v)).

2.2.2 Media

Isolation of strains from meat products was performed with: Plate Count Agar (PCA, Merck) for isolation of aerobic micro-organisms; De Man Rogosa Sharpe agar (MRS, Merck) for isolation of lactic acid bacteria; Schaedler agar (Oxoid) for isolation of anaerobic micro-organisms; Violet Red Bile Glucose agar (VRBA, Difco) with 10 gram glucose added per l, for isolation of *Enterobacteriaceae*; Oxytetracycline Glucose Yeast Extract

Table 2.1: Strains used in MIC studies of sodium lactate (NaL) and NaCl.

Micro-organism	Number of strains	Origin	
		own isolates	reference strains
Gram-positive bacteria			
lactic acid bacteria			
homofermentative rods	42	ham; bacon; s.r.s.[a]	
homofermentative cocci	11	ham	
heterofermentative cocci	17	ham; s.r.s.	
catalase positive rods	6	ham; s.r.s.	
Lactobacillus plantarum	2		WAU[b]
Lactobacillus brevis	1		WAU
Lactobacillus curvatus	3		Vet. Fac.[c]; DSM[d] 20019[e]
Lactobacillus coryniformis subsp. *coryniformis*	1		DSM 20001[e]
Lactobacillus bavaricus	2		Prof. Hammes[f]
Carnobacterium piscicola	1		DSM 20730[e]
Lactococcus lactis subsp. *lactis*	3		NIZO[g]
Lactococcus lactis subsp. *cremoris*	2		NIZO; DSM 20069[e]
Leuconostoc lactis	1		DSM 20202[e]
Enterococccus faecalis	1		DSM 20478[e]
Brochothrix thermosphacta	4		TNO[h]; DSM 20171[e]
Bacillus cereus	3		NIZO
Listeria monocytogenes	6		WAU; DSM 20600[e]
Listeria innocua	2		WAU; DSM 20649[e]
Staphylococcus aureus	5		WAU; DSM 20231[e]
Gram-negative bacteria			
catalase-positive rods	6	ham; s.r.s.; corned beef	
Campylobacter jejuni	1		WAU
Pseudomonas putida	1		NIZO
Pseudomonas fragi	2		NIZO; DSM 3456[e]
Salmonella enteritidis	2		WAU; ATCC[i] 13076[e]
Salmonella heidelberg	1		WAU
Salmonella livingstone	1		WAU
Salmonella typhimurium	1		WAU
Salmonella oranienburg	1		WAU
Yersinia enterocolitica	3		WAU; DSM 4780[e]
Yeasts			
unidentified	4	ham; bacon; chicken breast	
Debaryomyces hansenii	24	ham	CBS[j] T767[e]
Rhodotorula mucilaginosa	4	s.r.s.	
Candida zeylanoides	10	s.r.s.	CBS T619[e]
Candida parapsilosis	1		WAU
Geotrichum candidum	2		WAU
Pichia membranaefaciens	1		WAU
Saccharomyces cerevisiae	6		WAU
Saccharomyces exiguus	2		WAU
Torulaspora delbruckii	1		WAU
Trichosporon beigelii	1		WAU
Zygosaccharomyces bailii	2		WAU

Agar (OGGA, Oxoid) with one vial of oxytetracycline SR73 (Oxoid) added per 500 ml of medium, for isolation of yeasts and moulds; *Pseudomonas* agar (Oxoid) with 1 vial of C-F-C supplement SR103 (Oxoid) added per 500 ml of medium, for isolation of pseudomonads.

MRS agar and modified PCA (containing 13.1 g PCA (Merck), 12.5 g MRS broth (Merck) and 6 g additional agar (Oxoid, L11) per l) were used for purification and cultivation of strains to be used in inoculation studies. When pure cultures had been obtained, they were grown in peptone-yeast extract broth, containing 0.5% (w/v) of each of the following ingredients: glucose (Merck, p.a.), yeast extract (Oxoid) and soytone (Difco), buffered with sodium phosphate (final concentration 0.01 M) and adjusted to pH 6.5 with 4 M HCl prior to autoclaving at 121°C for 15 min. For performance of the screening experiments, concentrated broth was prepared to compensate for the addition of inoculum and solutions of sodium lactate and NaCl.

For determination of MICs, stock solutions of both sodium lactate (60% (w/w) reagent-grade solution, PURAC, Gorinchem, the Netherlands) and NaCl (Merck, extra pure) were diluted in water. After dilution, the solutions were sterilized by filtration using 0.45 µm filters (Schleicher and Schuell, Dassel, Germany). These solutions were mixed with concentrated peptone-yeast extract broth to obtain the desired concentrations of sodium lactate and NaCl (Table 2.2).

2.2.3 Inoculum

Cultures at -80°C were thawed and streaked on to agar plates of modified PCA or MRS to obtain single colonies. The plates were incubated at 20°C for 2.5 days. Material from one single colony was

←

Notes to Table 2.1

a) Spreadable raw sausage.

b) Wageningen, Agricultural University, the Netherlands.

c) Veterinary Faculty, Utrecht University, the Netherlands.

d) Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.

e) Type Strain.

f) Prof. Dr. W.P. Hammes, Hohenheim University, Stuttgart, Germany.

g) Nederlands Instituut Zuivel Onderzoek, Ede, the Netherlands.

h) TNO Nutrition, Zeist, the Netherlands.

i) American Type Culture Collection, Rockville, Maryland, USA.

j) Centraalbureau voor Schimmelcultures, Baarn, the Netherlands.

Table 2.2: Set and measured values for the water activity (a_w) of aqueous solutions after addition of various concentrations of sodium lactate (NaL) or NaCl.

NaL				NaCl			
Set concentration		Set a_w[a]	Meas. a_w[b]	Set concentration		Set a_w[a]	Meas. a_w[b]
mol/l	mol/kg			mol/l	mol/kg		
0.000	0.000	1.000	1.000	0.000	0.000	1.000	1.000
0.089	0.090	0.997	0.997	0.089	0.089	0.997	0.997
0.179	0.181	0.994	0.994	0.179	0.180	0.994	0.995
0.268	0.273	0.991	0.992	0.268	0.269	0.991	0.992
0.357	0.365	0.988	0.989	0.357	0.360	0.988	0.989
0.446	0.459	0.985	–[c]	0.446	0.450	0.985	0.987
0.536	0.555	0.982	–	0.536	0.542	0.982	0.985
0.625	0.651	0.979	–	0.625	0.634	0.979	0.981
0.714	0.749	0.975	0.977	0.714	0.725	0.976	0.978
0.804	0.848	0.972	–	0.803	0.817	0.973	0.975
0.893	0.948	0.969	0.972	0.893	0.910	0.970	0.972
0.982	1.049	0.966	–	0.982	0.983	0.967	–
1.071	1.151	0.962	–	1.071	1.074	0.964	0.967
1.161	1.256	0.959	0.963	1.161	1.167	0.961	–
*[d]	*	*	–	1.250	1.261	0.958	–
1.339	1.467	0.951	0.958	*	*	*	–
*	*	*	–	1.428	1.454	0.951	–
*	*	*	–	1.518	1.553	0.948	–
*	*	*	–	1.607	1.654	0.944	–
*	*	*	–	1.785	1.859	0.936	–

a) Calculated from the set concentration (mol/kg).
b) Calculated from freezing-point measurements.
c) Not determined.
d) Not included.

transferred to a tube with peptone-yeast extract broth. These tubes were incubated at 20°C for 24 h. Then, 1 ml of each culture (approximately 10^8 cfu per ml) was mixed and diluted to approximately 10^5 cfu per ml with sterile physiological salt solution (containing 8.5 g NaCl and 1 g peptone per l). From this diluted culture, 50 µl was dispensed into wells of a tissue culture plate containing 150 µl peptone-yeast extract broth with different amounts of sodium lactate and NaCl to give a final inoculum of approximately 10^4 cfu per ml.

2.2.4 Experimental procedure

Each strain was tested in duplicate or in triplicate at 20°C. The experiments were performed in 96-well tissue culture plates with flat bottom (Greiner), according to a method described by Eklund (6). The

culture plates were covered with special lids (Greiner), to avoid evaporation of water and stored in air at 20°C. For experiments with *Campylobacter jejuni*, the tissue culture plated were incubated in a jar under micro-aerobic conditions at 37°C.

Absorbance was determined at 492 nm in a microtitre plate reader (Easy Reader 400 FW, SLT-instruments, Austria). Sterile peptone-yeast extract broth containing the same amounts of salt as used in the testing served as a reference. Data were stored in a personal computer.

The MIC was taken to be the lowest salt concentration where the absorbance increase did not exceed the threshold value, which was defined as the average increase in absorbance value of the blanks plus three times the standard deviation. Experiments were terminated after 7 days of incubation, since no change in MICs occurred after this period.

2.2.5 Determination of pH and water activity

After concentrated peptone-yeast extract broth had been autoclaved, broth and stock solutions of sodium lactate and NaCl were mixed in culture tubes in proportion to the amounts that were used in wells. Then, the pH was checked with a pH meter (pH 522, IKS, WTW, Weilheim, Germany).

Water activity (a_w) was calculated from the osmotic coefficient of final mixtures of peptone-yeast extract broth and stock solutions of sodium lactate or NaCl. A compensation for the inoculum was made by addition of a physiological salt solution. Salt concentrations were determined by measuring the freezing point by means of a cryoscope (4L2, Advanced Instruments Inc., Needham Heights, Massachusetts). These values were corrected for freezing point reduction caused by nutrients as was determined with peptone-yeast extract broth without sodium lactate or NaCl. From the resulting value, salt concentrations (mol per kg) could be calculated using a molal freezing-point reduction of 1.86°C. While working at pH values of 6.3 or higher, we assumed that both sodium lactate and NaCl had ionized completely. The osmotic coefficient Φ was calculated by means of a model equation developed by Bromley (2). This osmotic coefficient Φ is related to a_w by:

$$a_w = e^{-0.018\Phi\Sigma m_i}$$

where Σm_i is the dissociated molality of the solution (2).

Freezing point measurements of the peptone-yeast extract broth without sodium lactate or NaCl indicated an a_w value of 0.998 for this medium.

2.3 Results and Discussion

The pH of media directly after addition of sodium lactate stock solutions and inoculum, ranged from 6.3 to 6.4 (data not shown). Measurements of freezing points showed good agreement between measured and set a_w values (Table 2.2). Furthermore, equal molar concentrations of sodium lactate and NaCl caused an almost equal reduction of a_w (Table 2.2).

Table 2.3 shows MICs of sodium lactate and NaCl for strains of *Salmonella* and *Listeria monocytogenes*. It can be seen that MICs of sodium lactate varied somewhat more between different species of the genus *Salmonella* than between strains of *Listeria monocytogenes*. It is also seen that *Listeria innocua* was equally resistant as *Listeria monocytogenes*. It appeared that NaCl was less inhibitory than sodium lactate at similar concentrations.

For three separate experiments, Table 2.4 presents ranges of MICs of

Table 2.3: Results from screening experiments with different strains of *Salmonella* and *Listeria*: MICs of sodium lactate (NaL) and NaCl.

Strain	MIC (mM)					
	NaL			NaCl		
	A[a]	B	C	A	B	C
Salm. enteritidis PT1	714	804	804	>893	>893	1080
Salm. heidelberg	893	–[b]	982	>893	–	1080
Salm. livingstone	804	–	893	>893	–	1080
Salm. typhimurium	714	714	714	893	804	982
Salm. oranienburg	893	–	804	>893	–	1080
L. monocytogenes Scott A	893	>893	982	>893	>893	1873
L. monocytogenes A	804	–	893	>893	–	1873
L. monocytogenes B	804	–	893	>893	–	1873
L. monocytogenes C	893	893	982	>893	>893	1767
L. monocytogenes D	893	–	982	>893	–	1873
L. innocua	>893	–	982	>893	–	1767

a) A, B, C: separate experiments.
b) Not tested.

Table 2.4: MICs of sodium lactate (NaL) and NaCl for non-type strains and the *C. piscicola* type strain at 20°C (range for 3 separate experiments).

Micro-organism	n^a	MIC (mM)		a_w limit^b
		NaL	NaCl	
Gram-positive bacteria				
lactic acid bacteria				
homofermentative rods	42	446–714	714–1161	0.976–0.961
homofermentative cocci	11	804–1161	1571–1767	0.947–0.940
heterofermentative cocci	17	446–625	714–1161	0.976–0.961
catalase positive rods	5	446–714	893	0.970
catalase positive rod	1	446	>1873	<0.936
Lactobacillus spp.	8	446–714	625–1080	0.979–0.964
Lactobacillus coryniformis	1	268	625	0.979
Carnobacterium piscicola	1	625	1428	0.951
Lactococcus spp.	4	446–714	804–893	0.973–0.970
Brochothrix thermosphacta	3	625–804	1428–1767	0.952–0.940
Bacillus cereus	3	625–714	714–804	0.976–0.973
Listeria spp.	6	804–982	1767–1873	0.940–0.936
Staphylococcus aureus	4	268–625	≥1873	≤0.936
Gram-negative bacteria				
catalase positive rods	6	893	1375	0.954
Campylobacter jejuni	1	179	179	0.994
Pseudomonas spp.	2	714–982	982–1571	0.967–0.947
Salmonella spp.	5	714–982	804–1080	0.973–0.964
Yersinia enterocolitica	2	714–804	1080–1179	0.964–0.961
Yeasts				
Zygosaccharomyces bailii	2	1339	1873	0.936
Saccharomyces cerevisiae	6	446–804	714–1072	0.976–0.964
Pichia membranaefaciens	1	625	1071	0.964
Geotrichum candidum	2	982	1179	0.961
Trichosporon beigelii	1	>893	>893	<0.970
Torulaspora delbruckii	1	893	>893	<0.970
Rhodotorula mucilaginosa	4	714–893	1767– >1785	0.940– <0.939
Debaryomyces hansenii	23	1161– >1339	1767–1873	0.940–0.936
Candida spp.	10	1339– >1339	1571– >1785	0.947– <0.939

a) Number of strains tested.
b) Water activity limits for growth, obtained from MICs of NaCl.

sodium lactate and NaCl for Gram-positive and Gram-negative bacteria as well as for yeasts. The MICs of NaCl were used for calculation of the minimum a_w permitting growth. The MICs of NaCl for the Gram-

positive bacteria showed a wide variation of about 600–1800 mM. The MICs of sodium lactate (about 300–1200 mM) were always lower than those of NaCl. The differences between MICs of NaCl and sodium lactate reflect the specific inhibitory effect of sodium lactate on growth of the various groups of micro-organisms. Some strains typically tolerated 800 mM NaCl and were sensitive to 400 mM sodium lactate. Others (e.g. homofermentative cocci, *Brochothrix thermosphacta* and *Listeria* spp.) tolerated up to 1700 mM NaCl and were sensitive to 800 mM sodium lactate. *Staphylococcus aureus* appeared to be sensitive to 300–600 mM sodium lactate, while tolerating over 1800 mM NaCl. The *Bacillus cereus* strains were exceptional since they were sensitive to almost equal concentrations (600–800 mM) of NaCl and sodium lactate.

In general, the Gram-negative bacteria tolerated somewhat lower concentrations of NaCl (200–1600 mM) than the Gram-positive strains (600–1900 mM), but they were inhibited by almost the same range of concentrations of sodium lactate as the Gram-positive organisms (Table 2.4). The specific inhibitory effect of sodium lactate was judged from the difference between MICs of sodium lactate and NaCl and the specific inhibitory effect of lactate was therefore more obvious for Gram-positive bacteria than for Gram-negative strains (Table 2.4). *Campylobacter* showed an extremely low tolerance for both sodium lactate and NaCl.

The yeast strains that were isolated from spoiled meat products (*Debaryomyces hansenii, Candida zeylanoides* and *Rhodotorula mucilaginosa*) could cope with high concentrations of NaCl (1600 to >1800 mM) and sodium lactate (700 to >1300 mM) (Table 2.4). These tolerances indicate that such yeasts will be encountered in the spoilage flora of meat products, regardless of whether NaCl or sodium lactate is used for preservation. The other yeasts listed were originally isolated from beer, soft drinks, lemonade and mayonnaise-based salads and with the exception of *Zygosaccharomyces bailii* they were more sensitive to sodium lactate and NaCl.

Since most of the strains used in this study were isolated from meat products, they may have been selected for their tolerance towards NaCl and sodium lactate. Therefore, we also screened a number of type strains (Table 2.5). In general, the MICs of sodium lactate and NaCl were the same as those obtained for the isolated strains, indicating that the micro-

Table 2.5: MICs of sodium lactate (NaL) and NaCl for type strains (duplicate experiments).

Strain	n^a	MIC (mM)		a_w limit^b
		NaL	NaCl	
Lactococcus lactis subsp. *cremoris*	1	446	714	0.976
Leuconostoc lactis	1	≤268	714	0.976
Brochothrix thermosphacta	1	714	1428	0.952
Enterococcus faecalis	1	1071	1785	0.939
Listeria innocua	1	714	>1785	<0.939
Listeria monocytogenes	1	446	>1785	<0.939
Staphylococcus aureus	1	446	>1785	<0.939
Salmonella enteritidis	1	714	1161	0.961
Pseudomonas fragi	1	714	1161	0.961
Yersinia enterocolitica	1	446	1161	0.961
Candida zeylanoides	1	>1339	>1785	<0.939
Debaryomyces hansenii var. *hansenii*	1	1339	>1785	<0.939

a) Number of strains tested.
b) Water activity limits for growth, obtained from MICs of NaCl.

organisms isolated from food products were not particularly adapted towards sodium lactate and NaCl. However, the type strains of *Listeria* appeared to be more sensitive towards lactate than the *Listeria* strains shown in Table 2.4. This was also the case for the *Yersinia* type strain.

The results of this study show that organisms which play an important role in the spoilage of meat products (lactic acid bacteria, *Brochothrix thermosphacta, Pseudomonas* spp.) and bacteria of public health significance (*Yersinia enterocolitica, Staphylococcus aureus, Listeria monocytogenes*) were inhibited more by sodium lactate than by NaCl at a particular a_w. This has also been described for *Staphylococcus aureus* in BHI broth at pH 7.3 (17). Shelef and Yang (16) showed that 4% (w/w) potassium lactate in beef, corresponding to a concentration of 430 mM in the water phase, was more effective in controlling growth of *Listeria monocytogenes* than was 3% (w/w) NaCl, corresponding to a concentration of 662 mM in the water phase.

Experiments were carried out at neutral pH and especially those organisms that are able to grow at low a_w in the presence of NaCl were inhibited by sodium lactate (*Staphylococcus aureus, Listeria monocytogenes, Brochothrix thermosphacta*). The results indicate that sodium lactate addition to food products with a pH near neutrality offers good

prospects for shelf life prolongation although growth of yeasts might be a problem that has to be considered.

Antimicrobial activity is usually ascribed to the undissociated fraction of the organic acid (HL). However, at pH 6.5 less than 1% of the added sodium lactate occurs as lactic acid. Therefore, a possible role of the dissociated molecule (the lactate ion) in the inhibition of microbial growth cannot be excluded (6,7). High intracellular concentrations of the lactate ion could have a non-specific effect on enzymes and when lactate is an intermediate or end-product of metabolism, the anion might even have a more specific effect on enzymes involved in for instance pyruvate-to-lactate conversions. Occurrence and effectiveness of the anion to inhibit growth/metabolism therefore is dependent on the metabolic pathways used and the possibility to shift to for instance pathways that are insensitive to high concentrations of lactate ions. The mechanism of lactate inhibition will be a topic for future research.

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3

Minimum inhibitory concentration (MIC) of sodium lactate and sodium chloride for spoilage organisms and pathogens at different pH values and temperatures

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Abstract

A selection of food spoilage organisms and pathogens were screened to determine the sensitivity towards sodium lactate. The effects of pH (5.7-7.0) and temperature (4°C-37°C) on the minimum inhibitory concentration (MIC) were determined in a peptone-yeast extract medium. NaCl was used as a reference to distinguish between the effect of water activity (a_w) and the specific inhibitory effect of sodium lactate. In general, if MICs of sodium lactate decreased at low temperature, those of NaCl decreased as well. Compared with NaCl, much lower molar concentrations of sodium lactate were needed to prevent growth. For most of the bacteria, MICs of sodium lactate strongly decreased at low pH values. At low pH (5.7), the presence of sodium lactate (≥ 268 mM) often did not allow for growth. However, for *Staphylococcus aureus, Lactococcus lactis* and some lactic acid bacteria isolated from spoiled meat products, the MICs of sodium lactate did hardly change with pH. These organisms were relatively strongly inhibited by sodium lactate at high pH (7.0). Yeasts were less sensitive towards sodium lactate than the bacteria. In most cases, MICs of sodium lactate for *Debaryomyces hansenii, Candida zeylanoides* and *Zygosaccharomyces bailii* exceeded the highest concentration used for screening (1339 mM), independent of the pH of the growth medium. *Rhodotorula mucilaginosa* was more sensitive, the MIC of sodium lactate being about 893 mM. MICs of NaCl appeared to be independent of the pH in the range 5.7 to 7.0 for all micro-organisms that were screened.

3.1 Introduction

Sodium lactate is an important compound in controlling microbial spoilage of meat and meat products. Lactic acid spray treatment of meat carcasses has been shown to result in decontamination of fresh meat (4, 15,21,22). Moreover, in combination with Modified Atmosphere Packaging (MAP), lactic acid treatment prolongs the shelf life and improves the safety of poultry legs at levels sufficiently low to allow for an acceptable organoleptic quality (24). Amongst other factors, the bactericidal efficacy is dependent on acid concentration, temperature of the acid solution, contamination level and type of organism (9). The sprays that are used for decontamination purposes have a low pH (2-3), which may explain the bactericidal effect.

It was only in the 1980s that sodium lactate became popular as a flavouring ingredient in meat products. Sodium- and potassium lactate are generally recognized as safe (GRAS). In 1988, the Food Safety Inspection Service (FSIS) of the United States Department of Agriculture (USDA) approved the use of potassium lactate and sodium lactate (2 g per 100 g) as flavour enhancers and flavouring agents in various meat and poultry products (16). Sodium lactate has a mild salty taste, and partial replacement of NaCl with sodium lactate in food formulas can therefore reduce the salty flavour. Since sodium lactate inhibits toxin formation by *Clostridium botulinum* (11,13), it could also be used to improve the microbiological safety of chicken and turkey products. Furthermore, sodium lactate has been reported to exhibit antioxidant activity (16). In low-sodium foods, potassium lactate is a convenient replacement for KCl, since the latter compound causes a bitter taste.

Addition of up to 3% lactate results in a shelf life extension of cured meat products, fish and uncured meat without negatively affecting sensory characteristics (2,5,14,17,18,20). These products have a relatively high pH (pH 5.5 to 6.5) at which only a small amount of the added lactate is present in the undissociated form. The shelf life extension resulting from lactate is a positive phenomenon, and it is desirable to learn more about the antimicrobial effects of this substance and about the susceptibility of different micro-organisms, in order to benefit from its preservation properties.

Previously, we investigated the sensitivity of spoilage flora and

pathogens that may occur in meat products towards sodium lactate and NaCl in broth at pH 6.5 and 20°C (10). In the present study, the effect of sodium lactate was examined in greater detail, with special emphasis on the effects of pH and temperature on the minimum inhibitory concentration (MIC) of sodium lactate for a selection of important micro-organisms. NaCl was used as a reference to distinguish between the effect of water activity (a_w) and the specific inhibitory effect of sodium lactate. In literature, a large amount of data regarding salt tolerance, acid tolerance and influence of pH and temperature on growth of micro-organisms can be found. However, a systematic study concerning the influences of pH and temperature on MICs of both sodium lactate and NaCl with a variety of micro-organisms is not available.

3.2 Materials and Methods

3.2.1 Strains

Part of the strains that were used for this study were isolated from spoiled meat products (Table 3.1). Reference strains of both spoilage organisms and pathogens that may occur in meat products were screened as well. These strains were obtained from culture collections in the Netherlands, Germany and the United States (Table 3.1). The isolation procedures and the storage conditions for the different strains were described previously (10).

3.2.2 Media

Pure cultures were grown in peptone-yeast extract medium, containing 0.5% (w/v) of the following ingredients: glucose (Merck, p.a.), yeast extract (Oxoid) and soytone (Difco), buffered with sodium phosphate (0.01 M). The pH was adjusted to 6.5 with 4 N HCl before autoclaving.

The screening was performed in peptone-yeast extract medium, supplemented with different amounts of sodium lactate (60% (w/w) reagent-grade solution, PURAC, Gorinchem, the Netherlands) or NaCl. Growth of each strain was examined at four different concentrations of either sodium lactate or NaCl. The concentrations used for screening depended on the sensitivity of the strains, as previously determined at 20°C and pH 6.5 (10). Lactate sensitive strains were incubated in the presence of 268, 446, 714 and 893 mM sodium lactate. For less sensitive

Table 3.1: Strains used for screening of the influences of pH and temperature on MICs of sodium lactate and NaCl.

Strain	Code	Type strain	Origin
Gram-positive bacteria			
Lactic acid bacteria			
homofermentative rod	M18		s.r.s.[a]
heterofermentative coccus	M52		s.r.s.
homofermentative coccus	M75		ham
Lactococcus lactis subsp. *cremoris*		√	DSM[b] 20069
Lactococcus lactis subsp. *cremoris*	Wg2		NIZO[c]
Lactococcus lactis subsp. *lactis*		√	DSM 20481
Lactococcus lactis subsp. *lactis*	SK3		NIZO
Leuconostoc lactis		√	DSM 20202
Enterococcus faecalis		√	DSM 20478
Brochothrix thermosphacta	3538; 3541		TNO[d]
		√	DSM 20171
Carnobacterium piscicola		√	DSM 20730
Listeria innocua			WAU[e]
		√	DSM 20649
Listeria monocytogenes	Scott A		WAU
		√	DSM 20600
Staphylococcus aureus	B		WAU
			ATCC[f] 19095
		√	DSM 20231
Gram-negative bacteria			
Salmonella enteritidis	PT1		WAU
		√	ATCC 13076
Salmonella typhimurium			WAU
Pseudomonas fragi	R579		NIZO
		√	DSM 3456
Yersinia enterocolitica	PR28		WAU
		√	DSM 4780
Yeasts			
Rhodotorula mucilaginosa	G8		s.r.s
Debaryomyces hansenii	GAH		ham
		√	CBS[g] T767
Candida zeylanoides	GE		s.r.s.
		√	CBS T619
Zygosaccharomyces bailii	2877		WAU

strains, the concentrations of sodium lactate added were 714, 893, 1071 and 1339 mM. NaCl sensitive strains were screened in the presence of 446, 714, 893 and 1161 mM NaCl. Less sensitive strains were screened in the presence of 893, 1161, 1428 and 1785 mM NaCl.

After addition of the various amounts of salt, the pH was adjusted with 4 N HCl or 4 N NaOH and water was added until the final volume was reached. pH values used were 6.0, 6.5, 7.0 and 7.5. Each medium was prepared as one batch for the entire experiment to avoid differences in composition and pH as much as possible. The pH values after autoclaving (15 min. 121°C) were checked with a pH meter (pH 522, IKS, WTW, Weilheim, Germany). Those were generally lower than the set values and these measured values were used in the presentation of the results. A_w values had been determined in these media by means of freezing points measurements. This has been described elsewhere (10).

3.2.3 Experimental procedure

The media were dispensed into the wells of tissue culture plates with flat bottom (Greiner, Solingen, Germany) and inoculated to a final level of about 10^4 cfu per ml with 50 µl of a diluted culture in peptone-yeast extract broth that had been incubated for 24 h at 20°C in air. The screening procedure used has been described previously (10). The plates were stored in air at 4, 12, 20, 30 or 37°C. The incubation time varied from 5 days (37°C) to 3 weeks (4°C). At regular time intervals the optical density was determined with a microtitre plate reader (Easy Reader 400 FW, SLT Instruments, Austria). The MIC was defined as the lowest salt concentration where the increase in the optical density was below a certain threshold value (10). Growth of each strain was tested in duplicate in separate tissue culture plates under the different conditions

←
Notes to Table 3.1
a) Spreadable raw sausage.
b) Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.
c) Nederlands Instituut voor Zuivel Onderzoek, Ede, the Netherlands.
d) TNO Nutrition, Zeist, the Netherlands.
e) Wageningen Agricultural University, the Netherlands.
f) American Type Culture Collection, Rockville, Maryland, USA.
g) Centraalbureau voor Schimmelcultures, Baarn, the Netherlands.

for growth.

For most of the micro-organisms listed in Table 3.1, MICs of sodium lactate and NaCl were determined at four pH values. The type strains, however, were screened at pH 6.0 and 6.5 only, as they served as a control for the non-type strains. All strains were screened at the five temperatures mentioned above.

3.3 Results

In most cases MICs were not affected by temperature but if they were, both those of sodium lactate and NaCl decreased at lower temperatures (data not shown). *Debaryomyces hansenii* was exceptional, since it was more sensitive towards sodium lactate at 37°C (MIC 893 mM instead of >1339 mM at lower temperatures), whereas MICs of NaCl did not change.

The MICs of NaCl appeared to be independent of the pH in the range 5.7 to 7.0 (data not shown). The various micro-organisms could be divided into three groups with regard to the influence of pH on MICs of sodium lactate (Table 3.2). Typical results are presented in Fig. 3.1 (T=20°C).

Group 1 comprises both Gram-negative and Gram-positive micro-organisms, namely *Brochothrix thermosphacta, Carnobacterium piscicola, Listeria innocua, Listeria monocytogenes, Salmonella enteritidis, Salmonella typhimurium, Pseudomonas fragi* and *Yersinia enterocolitica*. For all these organisms, the MICs of sodium lactate decreased markedly when the pH of the growth medium was lowered, independent of temperature (Fig. 3.1*A*).

Group 2 includes *Staphylococcus aureus* and the lactic acid bacteria M18, M52, M75, *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* SK3. In contrast to group 1, the pH had minor influence on the MICs of sodium lactate and this group appeared to be relatively sensitive towards sodium lactate at pH 7 (Fig. 3.1*B*). M75 could cope with far higher concentrations of sodium lactate and NaCl than the other lactic acid bacteria (Table 3.2).

Group 3 is composed of yeasts. These micro-organisms were less sensitive towards sodium lactate than the bacteria. In nearly all cases, growth occurred even at the highest lactate concentrations that were applied, independent of the pH of the growth medium (pH 5.7 to 7.0).

Table 3.2: Influence of pH on MICs (mM) of sodium lactate (NaL) for non-type strains and the *C. piscicola* type strain at 20°C.

Strain	Group	NaL				NaCl[a]
		pH= 5.7	pH= 6.3	pH= 6.6	pH= 7.0	
M 18[b]	2	446	446	446	446	1161
M 52[b]	2	446	446	446	446	1161
M 75[b]	2	≤ 714	1339	1339	1339	1785
Lact. lactis subsp. *cremoris*	2	≤ 268	446	446	714	714
Lact. lactis SK3	2	446	446	446	446	893
S. aureus ATCC 19095	2	≤ 268	446	446	446	> 1785
S. aureus B	2	≤ 268	446	446	446	> 1785
B. thermosphacta 3538	1	446	714	714	893	1785
B. thermosphacta 3541	1	446	714	714	> 893	1785
C. piscicola	1	≤ 268	714	893	> 893	1785
L. innocua	1	≤ 714	≤ 714	1339	> 1339	> 1785
L. monocytogenes	1	≤ 714	≤ 714	1339	> 1339	> 1785
Salm. enteritidis PT1	1	≤ 268	714	893	> 893	1161
Salm. typhimurium	1	≤ 268	714	893	893	1161
P. fragi R597	1	≤ 268	714	893	> 893	1161
Y. enterocolitica	1	≤ 268	714	893	> 893	1161
Rhodotorula mucilaginosa	3	> 893	> 893	893	> 893	1785
Debaryomyces hansenii	3	> 1339	> 1339	> 1339	> 1339	> 1785
Candida zeylanoides	3	> 1339	> 1339	> 1339	> 1339	> 1785
Zygosaccharomyces bailii	3	> 1339	> 1339	> 1339	> 1339	1785

a) MIC (mM) of NaCl at 20°C.
b) See Table 3.1 for description.

Rhodotorula mucilaginosa was more sensitive than the other yeasts, the MIC of lactate being about 893 mM (Table 3.2).

MICs of sodium lactate for type strains were similar to those of the corresponding non-type strains with the exception of the *Yersinia* type strain, which was somewhat more sensitive than the non-type strain (data not shown). Experiments with NaCl indicated that the lowest a_w allowing for growth of *Lactococcus lactis, Brochothrix thermosphacta* and *Yersinia enterocolitica* was lower than for the non-type strains (the MICs of NaCl were higher for the non-type strains). The yeast type strains were not able to grow at 37°C, in contrast to the strains that were isolated from spoiled meat products (data not shown).

3.4 Discussion

It is important to know whether there is an influence of temperature on MICs of sodium lactate and NaCl, as meat products in which sodium lactate is present are stored refrigerated (e.g. ham, filet americain) as well as at room temperature (e.g. smoked sausage, fermented sausage). The results of the present study showed that temperature did not have a specific influence on the MICs of sodium lactate (data not shown), except for *Debaryomyces hansenii*.

From literature, it is known that pH has a more pronounced effect on the sensitivity of micro-organisms towards sodium lactate than temperature has, and that inhibition by sodium lactate is not merely due to osmotic or sodium ion effects which has been demonstrated in control experiments with NaCl (7,8,23). The explanation for this phenomenon is that sodium lactate is a weak organic acid, that has a specific inhibitory effect on growth of micro-organisms on top of the effect that is caused by a lowering of the a_w. In general, this specific effect is ascribed to diffusion of the undissociated acid molecule across the plasma membrane, resulting in a lowering of the intracellular pH (pH_{in}) (3,20). When the pH_{in} decreases below a certain threshold value, cellular functions are inhibited (12). The ability of the Gram-positive bacteria to grow at higher concentrations of NaCl compared with Gram-negative strains (Table 2) caused the specific inhibitory effect of sodium lactate to be more obvious for this group (Fig. 3.1*A*). Especially the sensitivity of *Staphylococcus aureus* towards sodium lactate was obvious (Fig. 3.1*B*), as is also reported by Greer and Dilts (9).

For a large number of bacteria that were screened in this study, the antimicrobial effect of sodium lactate increased with decreasing pH values (group 1). It is therefore likely that the undissociated molecule plays an important role in growth inhibition, since the lower the pH, the higher is the concentration of undissociated acid. Even in the absence of weak organic acids, it may be assumed that at lower pH_{out} values, bacteria will be stressed as they have to keep their pH_{in} sufficiently high. It was therefore expected that the MIC of undissociated acid would decrease at low pH_{out}. However, when the MICs of undissociated acid were calculated from the MICs of total lactate (dissociated + undissociated), it appeared that at low pH more undissociated acid was

needed to prevent growth than at high pH (Table 3.3). Similar results are reported for *Lactococcus lactis* subsp. *cremoris* (1).

This phenomenon may at least partly be explained by considering that three antimicrobial factors play a role: pH, undissociated acid and a_w. For the organisms belonging to group 1, the inhibitory effect of sodium lactate at pH 7 seemed to be largely due to a lowering of the a_w, since MICs of sodium lactate and NaCl were almost the same at this pH (Fig. 3.1.*A*). At lower pH values, the difference between MICs of sodium lactate and NaCl increased, indicating that the organic acid character of sodium lactate became more important (Fig. 3.1*A*). Indeed, at low pH the a_w values caused by the MICs of sodium lactate hardly represented any stress factor to the bacteria compared with those at higher pH values (Table 3.3).

There were considerable differences among bacterial species with respect to the ratio of undissociated lactic acid concentrations that caused growth inhibition at pH 5.7 and at pH 7.0. For *Lactobacillus* M18 this ratio was about 20, while for *Salmonella typhimurium* and *Pseudomonas fragi*, it was only 5 (Table 3.3). In general, lactic acid bacteria and some related micro-organisms as well as yeasts were not very sensitive to lactate at low pH values (Table 3.2, Fig. 3.1*B*). The critical concentration of lactate for *Lactobacillus plantarum* at pH 6.0 and 30°C is reported to be 110 g per l (6). This MIC is almost three times higher than that we found for *Lactobacillus* M18, and it approaches the MIC of NaCl (Table 3.2). The strain of Giraud *et al.* (6) was apparently selected for its usefulness as a starter organism for silage production, which would distinguish this organism from a spoilage bacterium as encountered in meat plants.

A possible explanation for the fact that part of the micro-organisms that were screened in this study could cope relatively well with weak organic acids is offered by Russell (19). He shows that some bacteria maintain a more or less constant pH gradient over the cell membrane in stead of keeping the pH_{in} at a high level. Such micro-organisms may be able to avoid the accumulation of high and potentially toxic concentrations of lactate anions at low pH_{out}. In further studies, we will pay more attention to the mechanism of the antimicrobial activity of sodium lactate.

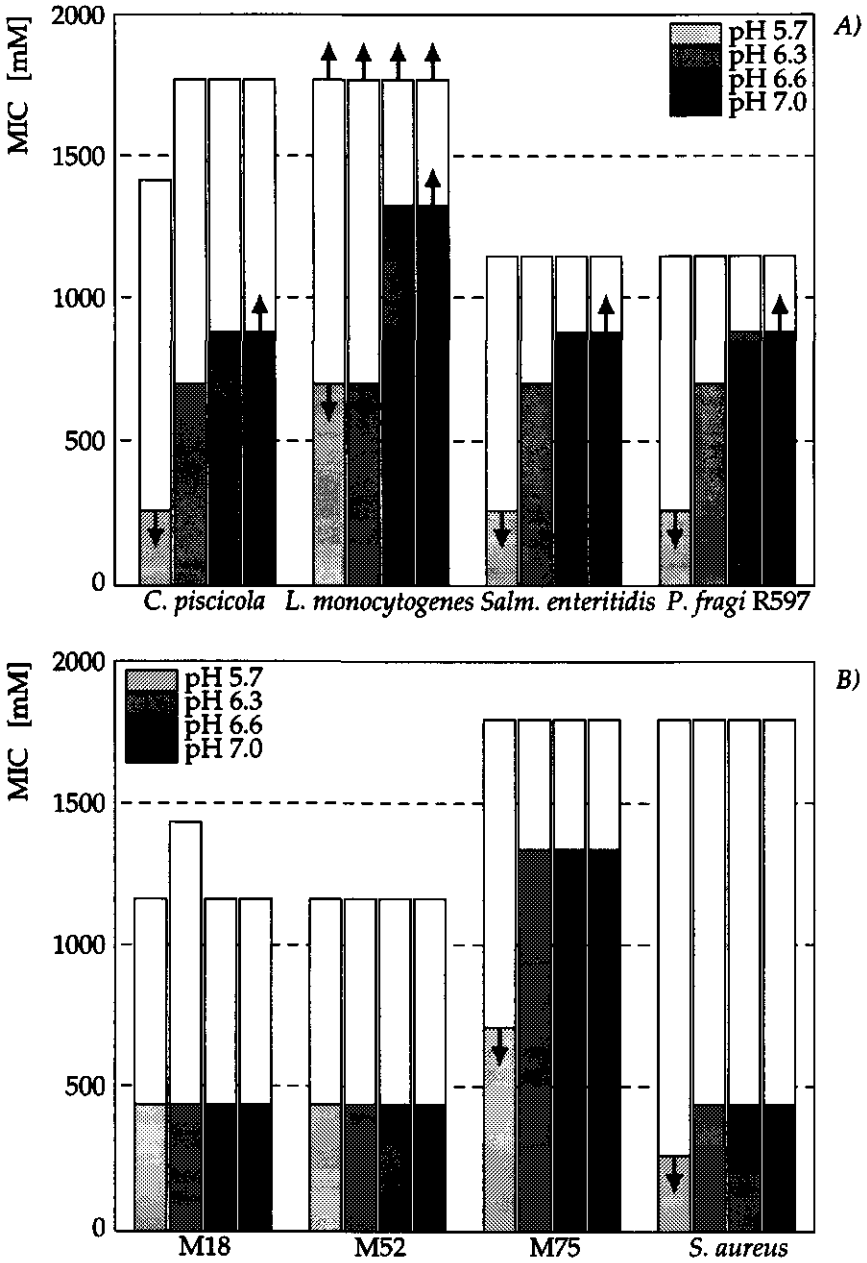

Figure 3.1: MICs (mM) of sodium lactate (grey bars) and NaCl (white bars) at 20°C for strains of group 1 (*A*) and group 2 (*B*) at different pH values. Arrows pointing downwards or upwards indicate that the actual MIC was equal to or beneath the lowest concentration tested or above the highest concentration tested, respectively.

Table 3.3: MICs (mM) of undissociated lactic acid at 20°C.

Strain	Group	pH 5.7	pH 6.3	pH 6.6	pH 7.0
M 18	2	6.35[a] (0.983)[b]	1.61 (0.983)	0.81 (0.983)	0.32 (0.983)
B. thermosphacta 3541	1	6.35 (0.983)	2.58 (0.975)	1.30 (0.975)	0.64 (0.970)
C. piscicola	1	≤ 3.81 (≥ 0.990)	2.58 (0.975)	1.63 (0.970)	> 0.64 (< 0.970)
Salm. typhimurium	1	≤ 3.81 (≥ 0.990)	2.58 (0.975)	1.63 (0.970)	0.64 (0.970)
P. fragi	1	≤ 3.81 (≥ 0.990)	2.58 (0.975)	1.63 (0.970)	> 0.64 (< 0.970)

a) Values calculated from the MIC of total lactate (dissociated + undissociated) by means of the Henderson-Hasselbalch equation.

b) Values between brackets indicate the a_w value at which growth was totally inhibited (related to the total amount of lactate; the contribution of other medium components being negligible).

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4

Effect of sodium lactate on toxin production, spore germination and heat resistance of proteolytic *Clostridium botulinum* strains

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Abstract

The effect of sodium lactate and of sodium lactate combined with NaCl on toxin production by proteolytic strains of *Clostridium botulinum* was determined in peptone-yeast extract medium, pH 6.1. Both inhibitors were also tested for their effect on thermal destruction of spores. Additionally, the effect of sodium lactate on germination of spores was assessed. Toxin production was delayed by sodium lactate concentrations of 1.5 to 2% (w/v) at 15°C and 20°C. In the presence of 3%, 4% and >4% sodium lactate, no toxin was detected within a period of 32-49 days at 15°C, 20°C and 30°C, respectively. The inhibitory effect of NaCl at concentrations resulting in an identical water activity value (a_w) as obtained with sodium lactate was negligible, indicating that the inhibitory effect of sodium lactate on toxin production was not caused by decreasing a_w. No clear synergistic effect of sodium lactate (1.5 or 2.5%) and NaCl (2.1%) on delaying toxin production was observed. It was shown that 4% sodium lactate inhibited germination of the *C. botulinum* spores, which may partially explain the inhibitory effect of sodium lactate on growth and toxin production. In the presence of sodium lactate and NaCl, a tendency of increased heat resistance of *C. botulinum* spores was observed, but the effect was not significant.

4.1 Introduction

In literature, only few data can be found regarding the inhibitory effect of sodium lactate on growth and toxin production by *Clostridium botulinum* strains. Growth of *C. botulinum* is delayed in fish, chicken and turkey

treated with 1.5 to 3.5% sodium lactate (1). Addition of 2 to 3.5% sodium lactate delays toxin production by *Clostridium botulinum* in vacuum-packaged comminuted turkey breast (6). In 1991, Unda *et al.* (10) described the effect of sodium lactate on *C. sporogenes* in microwave-ready beef roasts. Finally, Meng (7) wrote a thesis on the effect of sodium lactate on the probability of growth of and toxin production by non-proteolytic *C. botulinum* strains and on the probability of proteolytic *C. botulinum* spore outgrowth after a heat shock in BHI broth containing sodium lactate or sodium nitrite.

The present study was meant to collect additional information on the effect of sodium lactate on growth and toxin production of proteolytic *C. botulinum* strains under conditions of temperature abuse. In contrast to the experiments of Maas *et al.* (6), the studies were performed in culture media because it is important to have information on the effect of preservative factors in a constant homogeneous environment before the effects are tested under more complicated conditions in a food stuff (2).

Spores of *Clostridium botulinum* that are found in meat products mainly belong to types A and B (7,9). As temperature abuse causes a potential risk for outgrowth of heat resistant proteolytic *C. botulinum* spores in meat products, we focused our experiments on the effect of sodium lactate on spores of this group. In addition, we tested several incubation temperatures (15°C, 20°C and 30°C) as a reference to temperature abuse. The effect of sodium lactate was tested by inoculation of spores to the medium since *C. botulinum* will initially only be present in pasteurized products as spores. Furthermore, we examined the effect of sodium lactate on germination and thermal destruction of spores.

4.2 Materials and Methods

4.2.1 Organisms and production of spores

The following *C. botulinum* strains were used: type A strains 62A, 73A, AV4 and At2K3 and proteolytic type B strains B-Okra, SNF11 and CDI-3. Spores were obtained by growing strains anaerobically at 30°C for two days in fortified egg meat medium (FEM) which contained 10 g yeast extract (Difco), 75 g egg meat medium (Difco 042), 10 g glucose and 10 g $(NH_4)_2SO_4$ per l, and subsequently for another two days after transferring them to fresh FEM medium. Cultures were checked for

purity on brain heart infusion egg yolk agar (BHIEY) which contained 37 g brain heart infusion (Difco), 1 g cysteine HCl (BDH), 20 g agar (So-bi-gel) and 100 ml egg yolk emulsion (Oxoid SR47) per l. Afterwards, 2-3 ml of the FEM cultures were transferred to 100 ml of a sporulation medium which contained 50 g trypticase peptone (BBL 11921) and 10 g $(NH_4)_2SO_4$ per l. After 7 days of anaerobic incubation at 30°C, spores were harvested (13.300 x *g*, 10 min., 20°C) and washed three times in sterile distilled water. The pellets were resuspended in 40 ml of a sterile physiological salt solution. After a heat shock (10 min., 80°C), spore counts were made on BHIEY agar. Then a spore pool of 10^7 spores per ml consisting of equal amounts of spores from each strain was made in a sterile physiological salt solution. The suspension was distributed among sterile plastic tubes (2 ml portions) and stored at -20°C until use.

4.2.2 Peptone-yeast extract medium and incubation procedure

The basal medium used for studies on the effect of sodium lactate and NaCl on spore germination, growth, toxin production and heat resistance of proteolytic *C. botulinum* strains contained 20 g proteose peptone (Difco), 5 g yeast extract (Difco), 5 g glucose and 1 g cysteine HCl (BDH). This medium was supplemented with different amounts of NaCl (Merck, extra pure) and/or different amounts of sodium lactate (60.4% (w/w) reagent-grade solution, PURAC, Gorinchem, the Netherlands). The pH was set at 6.1 with 1 N NaOH or 6 N HCl and 14 ml portions of the media were distributed among polio tubes with screw caps (150 mm, SVM Q0225b). After sterilization (15 min., 121°C) and subsequent cooling in ice water, the media were inoculated with spores from the spore pool to a final level of 10^4 spores per ml. The media were then covered with 2 ml sterile paraffine oil. After a heat shock (10 min., 80°C) and subsequent cooling, the tubes were incubated at 15°C, 20°C or 30°C.

4.2.3 Test for toxin production by *C. botulinum*

At distinct time intervals, for every medium five tubes with the highest visible turbidity were tested for toxicity in mice. If no growth was observed, the five tubes for toxin testing were chosen at random. Samples (1 ml) were first trypsinated by adding 1 ml of a mixture of 0.8 ml 50 mM sodium phosphate buffer pH 6.5 containing 2 g per l gelatin and 2 g per l depomicin, and 0.2 ml 0.2% trypsin in 0.001 N HCl

(Sigma type 111, Sigma Chemical Co., St.Louis, MO, USA). After incubation for 30 min. at 37°C, two mice were injected intraperitoneally with 0.5 ml sample. The mice were observed during 5 days for specific botulism symptoms (difficult breathing, wasp-waist). The type of toxin present was tested by addition of monospecific antiserum (Institut Pasteur, Paris) to the test samples. When all five tubes were found positive for toxin, no further toxin tests were performed.

4.2.4 Effect of sodium lactate on spore germination

The basal peptone-yeast extract medium was supplemented with 0% and 4% (w/v) sodium lactate, respectively. The medium was distributed among tubes and inoculated with spores from the spore pool to a final level of 6.10^3 spores per ml. After a heat shock (10 min., 80°C) and subsequent cooling, the tubes were incubated at 30°C. The number of viable spores was determined at several time intervals. For this, a 2 ml sample was heated (10 min., 80°C) and diluted in a physiological salt solution. Plate counts were made on BHIEY (2 days anaerobic incubation at 37°C).

4.2.5 Effect of sodium lactate and NaCl on heat inactivation of *C. botulinum* spores

Thermal inactivation in the presence of sodium lactate or NaCl was determined for spores of *C. botulinum* 62A, AV4, B-Okra and CDI-3 separately (inoculum level 5×10^4 spores per ml). Thermal resistance was determined at 95°C using the submerged tube procedure (5). The experiments were performed in 50 mM potassium phosphate buffer, pH 6.8, which was supplemented with 0% (w/v) sodium lactate, 4% sodium lactate and 2.1% NaCl, respectively.

Spores of strains 62A, B-Okra and CDI-3, were heated for 5, 20, 40, 60 and 80 minutes and spores of strain AV4 for 5, 30, 60, 90 and 120 minutes, after which the number of cfu per ml was determined on BHIEY agar. $D_{95°C}$ values were calculated from the rate by which the number of viable spores decreased as a function of heating time.

4.3 Results and Discussion

4.3.1 Effect of sodium lactate and NaCl on toxin production

Visible growth was always accompanied by toxin production (data not shown). From Table 4.1, it can therefore be concluded that sodium lactate inhibits growth and toxin production of proteolytic *C. botulinum* strains. At incubation temperatures of 15°C and 20°C, toxin production was delayed in the presence of 1.5 to 2.0% sodium lactate.

Maas *et al.* (6) concluded from their experiments in broth that more than 1.92% sodium lactate was needed to inhibit toxin production by *C. botulinum* at 27°C. This could be due to the higher incubation temperature, since lactate was more effective at low temperatures (Table 4.1). In the presence of 3% and 4% sodium lactate, toxin production could not be detected within 49 and 32 days at 15°C and 20°C, respectively. A concentration of 4% sodium lactate did not prevent toxin production at 30°C. The enhanced antimicrobial activity of lactate with lower storage temperature is also found for non-proteolytic strains of *C. botulinum* (7) and other organisms (3,4,8). The effect of temperature on the antimicrobial activity of sodium lactate is in favour of its use in meat products which are stored at refrigeration temperatures.

In the turkey breast inoculation study of Maas *et al.* (6), 3.5% sodium lactate based on product weight inhibits toxin production for about 8 days at 27°C. The amount of sodium lactate in the water phase under these conditions is about 5%, based on a moisture percentage of 68% (6). In our study, 4% sodium lactate delayed toxin production for more than 32 days at 20°C (Table 4.1). These findings may be explained by interaction between temperature and sodium lactate. However, a discrepancy between results obtained with broth studies and results from meat model systems could also partially account for the observed differences. Compared with our broth studies, an increase in the sodium lactate content had relatively little effect on time to toxin production in turkey breast (6).

NaCl at a concentration of 2.1%, resulting in a a_w of 0.982 (identical to 4% sodium lactate) did not show inhibition of toxin production (Table 4.2). Addition of 2.1% NaCl to media containing sodium lactate resulted in further inhibition of toxin production. However, this was shown to be an additional effect since the time to toxin production with a

Table 4.1: Effect of sodium lactate (NaL) on toxin production of proteolytic strains (type A and B) of *C. botulinum* in peptone-yeast extract medium, pH 6.1, at different temperatures. Inoculation level 10^4 spores per ml.

Time of incubation (d)	15°C				20°C				30°C
	0% NaL	1% NaL	2% NaL	3% NaL	0% NaL	1.5% NaL	2.5% NaL	4% NaL	4% NaL
5	–[a]	–	–	–	5/5	0/5	0/5	0/5	0/5
7	0/5[b]	0/5	0/5	0/5	–	–	–	–	–
11	–	–	–	–		5/5	0/5	0/5	4/5
14	5/5	5/5	0/5	0/5			–	–	5/5
15			–	–			5/5	0/5	
20			–	–				0/5	
21			3/5	0/5				–	
25			–	–				0/5	
28			5/5	0/5				–	
32				–				0/5	
35				0/5				–	
42				0/5				–	
49				0/5				–	

a) Not determined.
b) Number of toxin containing samples/number of tested samples.

combination of the two salts was not longer than the sum of the times to toxin production when the same amounts of the salt were added separately (Tables 4.1 and 4.2). Apparently, sodium lactate has a specific inhibitory effect on both growth and toxin production of *C. botulinum*.

Table 4.2: Effect of percentage (w/v) sodium lactate (NaL) and NaCl on toxin production of proteolytic strains of *C. botulinum* in peptone-yeast extract medium, pH 6.1, at 20°C. Inoculation level 10^4 spores per ml.

Time of incubation (d)	4% NaL 0% NaCl $a_w = 0.982$	0% NaL 2.1% NaCl $a_w = 0.982$	1.5% NaL 2.1% NaCl $a_w = 0.978$	2.5% NaL 2.1% NaCl $a_w = 0.976$
5	0/5[a]	3/5	0/5	0/5
11	0/5	5/5	3/5	0/5
15	0/5		5/5	0/5
20	0/5			5/5
25	0/5			
32	0/5			

a) Number of toxin containing samples/number of tested samples.

However, from the experimental results presented in Tables 4.1 and 4.2, it cannot be concluded whether this is due to an inhibition of spore germination or to a delay of growth of vegetative cells or both. We therefore examined the influence of sodium lactate on spore germination.

4.3.2 Effect of sodium lactate on spore germination

In the presence of 4% sodium lactate, the number of spores had hardly decreased after 168 hours of incubation at 30°C (Table 4.3). At that time no visible growth was observed in the inoculated medium. Without addition of sodium lactate, visible growth was observed after 20 hours of incubation and the spore count decreased rapidly afterwards (Table 4.3). This indicates that sodium lactate inhibits spore germination.

Meng (7) supposed that inhibition of spore germination might be due to competition of lactate with spore germinants such as alanine. From the results in Table 4.3 it is difficult to conclude whether the inhibitory effect of lactate concerns only the time needed to initiate germination, or the germination rate as well. Although the effect of sodium lactate on growth of proteolytic *C. botulinum* strains is related to the inhibition of spore germination, a reduced growth rate of vegetative cells in the presence of sodium lactate may also partially account for the inhibitory effect. The inhibitory effect of sodium lactate on growth of vegetative cells has been

Table 4.3: Effect of sodium lactate (NaL) on germination of spores of proteolytic strains (type A and B) of *C. botulinum* in peptone-yeast extract medium, pH 6.1, at 30°C. Inoculation level 6 x 10^3 spores per ml.

Time of incubation (h)	% NaL (w/v)	
	0	4
0	100[a]	100
7	100	90
20	81 (visible growth)	147
44	43	99
68	–[b]	122
168	–	86
336	–	<1 (visible growth)

a) Percentage of viable spores ((number of viable spores present/number of viable spores after 0 h) x 100).
b) Not determined.

Table 4.4: Effect of sodium lactate (NaL) and NaCl on $D_{95°C}$ values (minutes) of *C. botulinum*. Inoculation level 5 x 10^4 spores per ml.

Type of spore	Heating medium		
	0.05 M phosphate buffer, pH 6.8 a_w = 0.993	Buffer + 2.1% (w/v) NaCl a_w = 0.981	Buffer + 4% (w/v) NaL a_w = 0.981
Strain 62A (type A)	50 (43–58)[a]	79 (51–182)	97 (53–588)
Strain AV4 (type A)	33 (29–39)	44 (34–61)	44 (37–56)
Strain OKRA (type B)	11 (9–13)	18 (13–31)	23 (20–26)
Strain CDI III (type B)	65 (45–120)	91 (61–179)	92 (71–127)

a) Decimal reduction time (95% confidence interval).

observed in the past (3,4,8).

4.3.3 Effect of sodium lactate and NaCl on heat inactivation of *C. botulinum* spores

Sodium lactate and NaCl showed a tendency to protect *C. botulinum* spores against heat inactivation, but the effects were not significant (Table 4.4). Comparable results are obtained for spores of non-proteolytic *C. botulinum* strains (7). Also, the protecting effect of 4% sodium lactate (a_w = 0.981) and 2.1% NaCl (a_w = 0.981) were not different at 95% confidence limit. This may be caused by lowering of the a_w value by addition of salts. However, the presence of sodium lactate inhibits outgrowth of spores after the heat shock (7).

From the foregoing, it is concluded that sodium lactate is a promising tool in inhibition of *C. botulinum* outgrowth and toxin production.

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5

Modelling growth rates of *Listeria innocua* as a function of lactate concentration

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Abstract

The effect of sodium lactate concentration on growth of *Listeria innocua* in a peptone-yeast extract broth at pH 5.5, 6.0, 6.5 and 7.0 at 4, 10, 20 and 30°C was modelled with the modified Gompertz model. NaCl was used as a reference to distinguish between the effect of water activity (a_w) and the specific inhibitory effect of sodium lactate. Minimum inhibitory concentrations (MICs) of NaCl (mM) appeared to be significantly higher than MICs of sodium lactate, indicating that sodium lactate had a specific inhibitory effect on growth of *L. innocua*. The MICs of sodium lactate and NaCl were not much influenced by the temperature. The pH of the growth medium was shown to have influence on the MIC of sodium lactate but not on the MIC of NaCl. Total growth inhibition of *L. innocua* at low pH (5.5) took place at lower sodium lactate concentrations (217 mM) than at neutral pH (1071-1339 mM), indicating that the undissociated lactic acid plays a role in the mechanism of inhibition. However, MICs of undissociated acid increased with decreasing pH from 0.8 mM at pH 7 to 5 mM at pH 5.5. It is therefore likely that besides acidification of the cytoplasm due to diffusion of undissociated acid into the cell, other mechanisms are involved. The maximum specific growth rate (μ) decreased progressively with increasing sodium lactate concentrations down to 0 at the MIC and was strongly influenced by both temperature and pH. In the presence of NaCl, μ was influenced by temperature only. It was shown that a modified Monod equation with 3 parameters was effective for the description of μ of *L. innocua* at sodium lactate and NaCl concentrations over the whole experimental range.

5.1 Introduction

In the meat industry, sodium lactate is gaining interest as a natural flavouring ingredient. Lactate prolongs shelf life of meat products without affecting pH, while improving sensory characteristics (2,3,19,23,24). Research on the influence of sodium lactate on growth of spoilage flora and pathogens occurring in meat products has to be carried out in order to extend knowledge in this field.

Inhibitory action of weak organic acids is generally ascribed to the undissociated molecule and hence is related to the pK_a value of the acid, acetic acid being a more effective inhibitor than lactic, citric or hydrochloric acid (9,17). Eklund (7,8) argues that the undissociated molecule might play a role, but that inhibition due to the dissociated anion is not to be neglected. According to Papadopoulos *et al.* (23), mechanisms which have been proposed for the action of lactic acid and lactate in literature include: lowering of water activity (a_w), acidification of intracellular pH (pH_{in}), inhibition of ATP formation from proton transfer across bacterial cell membranes, feedback inhibition by the lactate anion and/or the ability of the lactate ion to penetrate the microbial cell membrane and inhibition of enzymes involved in the pyruvate-to-lactate conversion.

A micro-organism which has received a lot of attention because of its implication in disease outbreaks involving food products, is *Listeria monocytogenes*. This organism is recognized as a major foodborne pathogen and has also been isolated from meat (products), chicken (products) and fish products (6,10,14,18,29). Growth of *L. monocytogenes* in meat products can be restricted by a combination of salt, sodium nitrite, and pH, especially in fermented sausage (18). However, there is a tendency to decrease nitrite levels, and the pH in most meat products is higher than in fermented sausage. Since *L. monocytogenes* was shown to grow well on a number of refrigerated stored meat products (13), the use of sodium lactate as a precaution against growth of *L. monocytogenes* is worthwhile investigating.

In this survey, growth of *Listeria innocua* was studied. *L. innocua* is a non-pathogenic species of *Listeria* which resembles *L. monocytogenes* in its sensitivity towards sodium lactate (16). Further, the incidence of *L. innocua* on meat is often higher than the incidence of *L. monocytogenes*

(18). These facts justify the use of *L. innocua* as a model organism for *L. monocytogenes*.

The usefulness of predictive modelling in food research has received a lot of attention in recent years. Models can be used to describe the growth of micro-organisms under different environmental conditions, such as temperature, pH and a_w. A model that predicts growth of *L. innocua* as a function of sodium lactate concentration at different temperatures and pH values would be of great benefit in understanding the mechanisms of microbial growth inhibition by sodium lactate. It could also be valuable to predict the shelf life of meat products under various storage conditions and to estimate the inhibition of the microbial flora as a result of lactate addition.

In literature, polynomial models are often used for predictive purposes. However, these models have no theoretical basis and they are not constrained so they can give unexpected predictions, such as negative parameter values (12). Further, they contain many parameters which have no practical significance, and therefore will sooner result in incorrect predictions.

The objective of this research was to develop a relatively simple model that could describe maximum specific growth rates of *L. innocua* in broth, when incubated under various growth conditions (pH, temperature) in the presence or absence of sodium lactate and NaCl. NaCl was used as a reference to distinguish between the effect of a_w and the specific inhibitory effect of sodium lactate.

5.2 Materials and Methods

5.2.1 Determination of minimum inhibitory concentrations (MICs)

Listeria innocua (DSM 20649) was screened for detectable growth in peptone-yeast extract broth with sodium lactate (0-1339 mM) or NaCl (0-1963 mM) at pH 5.5, 6.0, 6.5 and 7.0 at 4, 10, 20 and 30°C to determine MICs of these salts for this organism under the different conditions for growth.

The basal peptone-yeast extract broth contained 5 g per l of each of the following ingredients: glucose (Merck, p.a.), yeast extract (Oxoid) and soytone (Difco) and was buffered with sodium phosphate (final concentration 0.01 M). Various amounts of a stock solution of sodium

lactate (60% (w/w) reagent-grade solution, PURAC, Gorinchem, the Netherlands) or NaCl (Merck, extra pure) were added to the broth to give final concentrations in the range as stated before, with succeeding concentrations increasing no more than 1% (w/v). The pH was set with 4 N HCl or NaOH using a pH meter (pH522, IKS, WTW, Weilheim, Germany). All media were autoclaved at 121°C for 15 min. The method used for screening was described previously (16).

5.2.2 Growth curves

Growth experiments were performed in peptone-yeast extract broth supplemented with 2-8 different concentrations of sodium lactate or NaCl varying from 0 mM up to the MIC, under the same conditions (pH, temperature) as were used for the screening procedure. 250 ml portions of each broth were stored overnight at the intended incubation temperature, prior to inoculation.

The broth then was inoculated to a level of about 10^4 cfu per ml with an overnight culture (30°C) of *Listeria innocua* (DSM 20649) in basal peptone-yeast extract broth (pH 6.5). After that, 10 ml portions were aseptically dispensed among sterile culture tubes. The tubes were immediately transferred to a rotating drum (Model TC 6, New Brunswick Scientific Co., New Brunswick) (20 rev. per min.) at 4, 10, 20 or 30°C, and the time was noted. Directly after dispensing and at regular time intervals, counts were made on modified PCA agar (containing 13.1 g PCA (Merck), 12.5 g MRS (Merck) and 6 g agar (Oxoid, L11) per l) using a spiral plater (Spiral Plater^{TM}, model D, Spiral Systems inc., Cincinnati, USA). For each growth curve, 15-20 samples were taken, using a new tube for each sample. Plates were incubated 2 days at 30°C, and the number of cfu per ml sample (N) was determined.

5.2.3 Modelling

Modelling was carried out in two stages. The first stage involved modelling bacterial growth curves with the modified Gompertz equation, which was fitted to the bacterial counts by nonlinear regression with a Marquardt algorithm, according to Zwietering *et al.* (31). Bacterial counts were expressed as $ln\ (N_t/N_0)$ as a function of time (h). That way, values were obtained for lag time λ (h), maximum specific growth rate μ (h^{-1}), the final level of bacteria A and their 95% confidence intervals.

In the second stage, we focused our attention on modelling the maximum specific growth rate (μ) as a function of sodium lactate or NaCl concentration (mM) for each combination of pH and temperature.

5.3 Results

5.3.1 Minimum inhibitory concentrations (MICs)

MICs of sodium lactate and NaCl for *Listeria innocua* under the conditions of testing are presented in Table 5.1. MICs of NaCl were significantly higher than MICs of sodium lactate, indicating that sodium lactate has a specific inhibitory effect on growth of *L. innocua*. Further, it appeared that the MICs of sodium lactate were strongly dependent upon the pH of the growth medium whereas the MICs of NaCl were not. The influence of temperature on MICs of sodium lactate was less obvious than the influence of pH (Table 5.1). Strikingly, MICs at 20°C were equal to or higher than MICs at 30°C for both sodium lactate and NaCl whereas the optimum temperature for growth of *L. innocua* is 30-37°C (26).

5.3.2 Growth curves

Growth curves were determined with different sodium lactate and NaCl

Table 5.1: Sensitivity of *Listeria innocua* towards sodium lactate (NaL) and NaCl at various combinations of pH and temperature[a]

pH	4°C		10°C		20°C		30°C	
	NaL	NaCl	NaL	NaCl	NaL	NaCl	NaL	NaCl
5.5	0	1071	200	1518	200	1696	200	1518
	97	**1161**	**217**	**1607**	**217**	**1785**	**217**	**1607**
6.0	268	1071	446	1696	500	1874	446	1607
	291	**1161**	**485**	**1785**	**582**	**1963**	**485**	**1696**
6.5	537	1161	803	1696	893	1874	893	1607
	625	**1250**	**872**	**1785**	**969**	**1963**	**969**	**1696**
7.0	803	1161	982	1696	1161	1874	1071	1607
	893	**1250**	**1071**	**1785**	**1250**	**1963**	**1161**	**1696**

a) Numbers in bold typeface are the lowest concentrations (mM) at which no growth occurred (MICs). Values in normal typeface represent the concentrations tested that did just permit growth of the organism.

concentrations at various pH values and temperatures, and growth data were modelled with the modified Gompertz equation. This model gave a good description of the growth of *L. innocua* under the experimental conditions (data not shown).

With N_A being the number of cfu per ml that was reached in the stationary growth phase, experimental data for $ln\ (N_A/N_0)$ varied from 4.3 to 15.0 for sodium lactate (Log N_A varying from 5.8 to 10.3) and from 8.7 to 15.8 for NaCl (Log N_A varying from 7.1 to 10.7). Under most conditions, A (= $ln\ (N_A/N_0)$) was higher than 10. In conclusion, even in the presence of high salt concentrations, values for N_A were so high that a food product should be considered as spoiled, and that leaves this parameter less valuable for modelling.

The maximum specific growth rates (μ) at different sodium lactate or NaCl concentrations, pH and temperatures are presented in Figs. 5.1 to 5.4. There appeared to be a strong interaction between the influence of sodium lactate and pH on μ (Fig. 5.1). This was already expected from the results of MIC determinations. No influence of pH on the inhibitory effect of NaCl was observed (Fig. 5.2). With NaCl concentrations up to

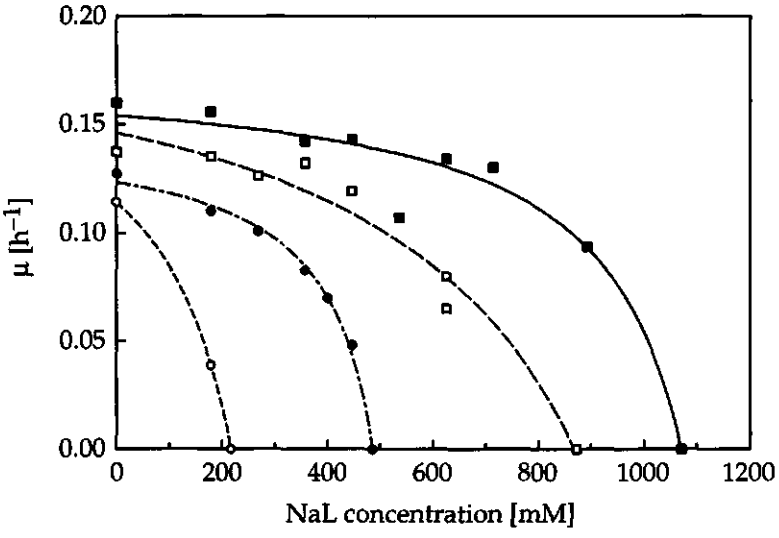

Figure 5.1: Maximum specific growth rate of *Listeria innocua* at 10°C and pH 5.5 (○), pH 6.0 (●), pH 6.5 (□) and pH 7.0 (■) as a function of sodium lactate (NaL) concentration. Both experimental data (symbols) and growth rate as fitted by a modified Monod model (curves) are shown.

800 mM, μ appeared to be lower at pH 5.5 than at pH 6.0, 6.5 or 7.0, but this was mainly due to an effect of pH since this inhibition was also observed in the absence of NaCl (Fig. 5.2).

As can be observed from Fig. 5.3 and Fig. 5.4, temperature had a strong effect on μ. There was no clear interaction of temperature and sodium lactate or NaCl concentration on growth inhibition of *L. innocua* at pH 6.5 as long as temperature was ≥10°C, since MICs of sodium lactate and NaCl were not much influenced in this range. At 4°C, however, for both sodium lactate and NaCl the MIC was significantly lower.

When *L. innocua* was exposed to stressful growth conditions, the estimates that were obtained for the lag time showed large 95% confidence intervals (data not shown). This was also signalled by others (15,25). However, it turned out that $(\mu \times \lambda)$ as a function of μ was constant, although the data had a large variation (data not shown). This was also seen by Zwietering *et al.* (32) and implies that values for λ can be derived from μ on the condition that μ can be estimated with reasonable accuracy. For this reason and because values for μ could be estimated more accurately with the modified Gompertz model, it was decided first to develop a model that describes μ as a function of sodium lactate or NaCl concentration.

5.3.3 Modelling growth rates as a function of sodium lactate or NaCl concentration

The plots of μ versus sodium lactate or NaCl concentration showed a curve that might be described by a Monod type equation. Monod's growth model (22) defines the relation between the growth rate and the concentration of a limiting substrate:

$$\mu = \mu_m \cdot \frac{S}{(K_s + S)} \qquad \text{(eq. 5.1)}$$

where μ is the specific growth rate, μ_m is the maximum specific growth rate (without substrate limitation), S is the concentration of limiting substrate, and K_s is the substrate concentration which supports half-maximum specific growth rate.

In the present study, growth was not dependent on substrate

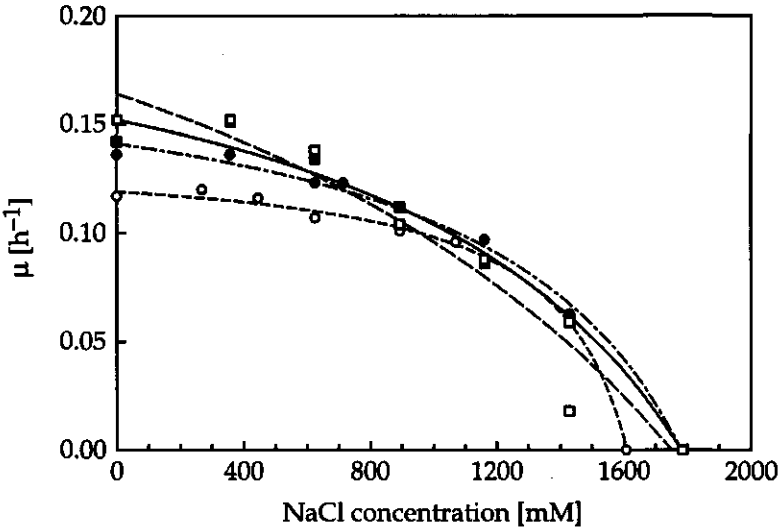

Figure 5.2: Maximum specific growth rate of *Listeria innocua* at 10°C and pH 5.5 (○), pH 6.0 (●), pH 6.5 (□) and pH 7.0 (■) as a function of sodium chloride (NaCl) concentration. Both experimental data (symbols) and growth rate as fitted by a modified Monod model (curves) are shown.

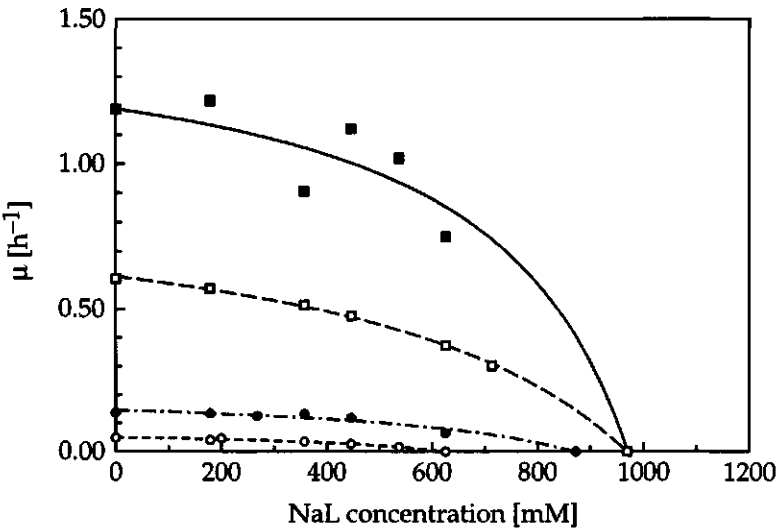

Figure 5.3: Maximum specific growth rate of *Listeria innocua* at pH 6.5 and 4°C (○), 10°C (●), 20°C (□) and 30°C (■) as a function of sodium lactate (NaL) concentration. Both experimental data (symbols) and growth rate as fitted by a modified Monod model (curves) are shown.

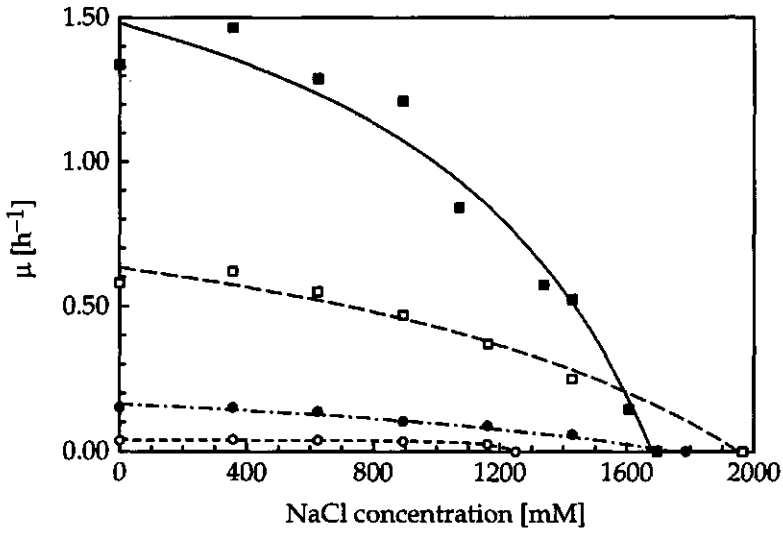

Figure 5.4: Maximum specific growth rate of *Listeria innocua* at pH 6.5 and 4°C (○), 10°C (●), 20°C (□) and 30°C (■) as a function of sodium chloride (NaCl) concentration. Both experimental data (symbols) and growth rate as fitted by a modified Monod model (curves) are shown.

limitation, but influenced by the amount of inhibitor present in the medium. Therefore, μ was related to the salt concentration so that the predicted value was zero at the MIC:

$$\mu = \mu'_m \frac{(p' - p)}{K_s + (p' - p)} \qquad \text{(eq. 5.2)}$$

where p is the salt concentration (mM), μ'_m is the specific growth rate at $p = -\infty$, p' is the MIC (mM), and K_s is a parameter which is used to define the salt concentration that results in half-maximum growth rate at $(p = p' - K_s)$.

Initially, this equation seemed appropriate for describing the specific growth rate as a function of salt concentration. However, since values for μ'_m were calculated as the asymptotic value of μ at $p = -\infty$ a problem was met when μ decreased already at low salt concentrations. In that case, predicted values for both μ'_m and K_s were much higher than the results indicated, because K_s is related to μ'_m and the asymptotic level of μ'_m did not correspond to the value of μ in the absence of salt. In one case the

value for K_s did even exceed the MIC (data not shown).

To avoid this problem, we created a new parameter μ_m which was defined as μ in the absence of salt (p=0):

$$\mu_m = \mu'_m \cdot \frac{p'}{(K_s + p')} \qquad \text{(eq. 5.3)}$$

The new equation for μ_m' was substituted in equation (5.2) to yield:

$$\mu = \frac{(K_s + p')}{p'} \cdot \frac{\mu_m (p' - p)}{K_s + (p' - p)}$$

Finally, this equation was simplified by substitution of $(K_s + p')$ by a new parameter α, resulting in:

$$\mu = \mu_m \cdot \frac{\alpha (p' - p)}{p' (\alpha - p)} \qquad \text{(eq. 5.4)}$$

In which μ_m is the specific growth rate at p=0, p' is the MIC and α is a shape parameter. This equation proved to describe the results satisfactorily, examples of which are given in Figs. 5.1 to 5.4.

5.4 Discussion

Studies concerning the influence of pH on growth of *Listeria monocytogenes* in the presence of NaCl and sodium lactate have been reported in literature, and the results agree with our findings for *L. innocua*. Cole *et al.* (5) conclude that there is no synergistic effect of NaCl and pH on growth of *L. monocytogenes*, as we concluded for *L. innocua* (Table 5.1, Fig. 5.2). The MIC of sodium lactate for *L. monocytogenes* at pH 5.5 (37°C) as found by Young and Foegeding (30) is about 200 mM, whereas for *L. innocua* this value was about 217 mM (Table 5.1). Shelef and Yang (27) report that sodium lactate concentrations higher than 5% (446 mM) delay growth of *L. monocytogenes* in broth at pH 6.5., which was also found for *L. innocua*

(Fig. 5.1). Since experiments were performed in a nutritionally favourable medium without contaminating flora and further inhibitory additives, growth of *L. innocua* in a meat product will probably be inhibited at lower lactate concentrations. Growth rates of *L. monocytogenes* at 37°C and pH 6.0 as reported by Young and Foegeding (30) did not exceed 0.5 (h^{-1}), which is much lower than we found for *L. innocua*. At 30°C and pH 6.0, growth rates of *L. innocua* were 1.2 (h^{-1}) in the absence of sodium lactate, decreasing to 0.6 (h^{-1}) when 357 mM sodium lactate was present. The difference is probably due to the method used for detecting growth. Young and Foegeding (30) use optical density measurements which can only be performed at high cell densities, resulting in apparently reduced growth rates as cells may be at the end of the exponential phase of growth.

Tolerance of *Listeria* to NaCl at different temperatures as described in literature does not always correspond to our findings that NaCl concentrations permitting growth of *L. innocua* were highest at 20°C. Better survival of *L. monocytogenes* in the presence of high salt concentrations at low temperatures (5°C, 10°C) may be due to reduced metabolism and growth at these temperatures (5). Other authors, report that growth inhibition of *L. monocytogenes* by NaCl is minimal at pH 6.6-7.6 and high temperature (30°C, 35°C), the NaCl concentration needed to inhibit growth being higher at 30°C (>10%) than at 20°C (8.8%) and 4°C (21, 28).

Growth of *L. innocua* under the various experimental conditions was modelled with the modified Gompertz equation. The use of the Gompertz model for prediction of bacterial growth in laboratory media has been recommended by various authors (11,12,31). Modelling of bacterial growth curves resulted in values for lag time (λ), max. specific growth rate (μ) and bacterial levels in the stationary growth phase (A). All three parameters are important for modelling the behaviour of micro-organisms in food products, but since the level of stationary growth and the time at which it is reached are largely determined by λ and μ, these latter two are of most interest. In the results section, it was already argued that the best approach is to first develop a model that describes μ as a function of sodium lactate or NaCl concentration.

The modified Monod model meets with the requirements of being a simple model with only a few parameters which have a practical

meaning. It resulted in a good fit of the experimental data, and it also gave a direct relationship between μ and the amount of salt in the growth medium (p). The model has to be extended in order to include temperature and pH as variables.

The effect of pH on growth inhibition by sodium lactate indicates that the undissociated acid molecule plays a role in the mechanism. When MICs of sodium lactate in Fig. 5.1 were interpreted in terms of undissociated acid according to the Henderson-Hasselbalch equation (20), it followed that MICs of lactic acid increased with decreasing pH. At pH 7.0 roughly 0.8 mM lactic acid was needed to prevent growth whereas at pH 5.5 the MIC of lactic acid was about 5 mM. This indicates that the undissociated acid is not the only factor responsible for growth inhibition. Organic acids may inhibit growth by acidifying the cell interior, forcing the cell to use metabolic energy in order to maintain pH homeostasis (1).

It has been proposed that inhibition of *L. monocytogenes* by acids is not caused by a decrease in the pH_{in}, *per se*, but rather by specific effects of undissociated acid molecules on metabolic or other physiological activities (17). In addition, high intracellular concentrations of the lactate anion might influence metabolic pathways, especially when lactate is an intermediate or end-product of metabolism, as is the case for *Listeria*. Since equal molalities of sodium lactate and NaCl bring about an equivalent decrease in the a_w (4), this study proved that lowering of a_w due to lactate addition does not play an important role in the mechanism of microbial growth inhibition by lactate, as is also discussed by Papadopoulos *et al.* (23). Young and Foegeding (30) point at the importance of taking into account factors such as active transport of acids and the ability of the cell to metabolize, excrete or otherwise remove the acids from the cell interior when interpreting the antimicrobial effects of organic acids. Until now, it is not known to what extent this occurs in different micro-organisms. The mechanism of bacterial growth inhibition by lactate will be the topic of our future research.

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6

Model for the combined effects of temperature, pH and sodium lactate on growth rates of *Listeria innocua* in broth and Bologna-type sausages

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Abstract

A modified Monod equation was successfully applied to describe the maximum specific growth rate of *Listeria innocua* in a broth model in the presence of various concentrations of sodium lactate or NaCl. The combined effects of temperature and pH were assessed by translating the parameters of the modified Monod equation (μ_m, α and p') as functions of pH and/or temperature. As a result, the area in which the maximum specific growth rate could be predicted was extended to include as a variable not only the salt concentration, but also pH and temperature. The number of parameters needed to describe the experimental data was thereby reduced from 48 to 4 (NaCl) and from 42 to 5 (sodium lactate). The decline in the goodness of fit that accompanied the reduction in the number of parameters was within statistically acceptable ranges. The resulting model was compared with a polynomial fit, and it was proposed that the former was more suitable for the purpose of this study. The broth model for sodium lactate was evaluated with Bologna-type sausages. Because of the "worst-case" design of the broth model, it was necessary to re-estimate one, or all parameters to obtain a good description of the growth rate of *L. innocua* in the meat product. However, the simplicity of the model and the practical usefulness of its parameters offer considerable prospects for its use in predictive microbiology.

6.1 Introduction

Sodium lactate is used in meat products as a natural ingredient. In the past few years, we have been concerned with the microbial aspects of this application. Besides investigating the boundaries between growth and no growth in a model system (broth) with micro-organisms that are significant for meat products (2), a model was developed that could accurately predict the maximum specific growth rate of a model organism (*Listeria innocua*) under various conditions (pH, temperature, lactate concentration) in the area that allowed for growth of this organism up to the minimum inhibitory concentration (MIC) of sodium lactate (3). In this way, one cannot only discriminate between growth and no growth, but also estimate the time span in which significant microbial growth might occur under certain conditions. The inhibition of microbial growth by sodium lactate was compared with that of NaCl, also a common ingredient in meat products. Sodium lactate could replace part of the NaCl to produce less salty products with a longer shelf life.

The maximum specific growth rate (μ) was modelled as a function of the concentration of sodium lactate or NaCl present in the broth. This resulted in a modified Monod equation with three parameters, each combination of pH and temperature having its own set of parameters. This model accurately described the effect of both NaCl and sodium lactate on the maximum specific growth rate (3). The disadvantage of such a model was that it was not possible to apply it at temperatures and pH values different from those used to collect the experimental data. In the present study, temperature and pH were included in the model to solve this problem. An advantage of doing so is that the number of parameters needed to describe the experimental data is greatly reduced.

Because the model was developed for studying the effect of sodium lactate on microbial growth in meat products, its suitability for predicting growth of the model organism in a Bologna-type sausage was evaluated as well. Although the results for both NaCl and sodium lactate models are presented, the emphasis is on the latter, since our research is focused on the antimicrobial activity of sodium lactate.

6.2 Materials and Methods

6.2.1 Organism

Listeria innocua DSM 20649 (type strain) was used in these experiments.

6.2.2 Data collection

Growth experiments had been performed previously in a peptone-yeast extract broth at 4, 10, 20 and 30°C and pH 5.5, 6.0, 6.5 and 7.0 in the presence of different concentrations of sodium lactate (103 curves) or NaCl (112 curves) (3). This data set (215 growth curves) contained 45 replicate experiments under 20 different conditions. These data were used to determine the variance of the measured growth rate data and to estimate the measurement error.

6.2.3 Analysis of variance

The variance of the maximum specific growth rate (μ) was expressed as a function of the mean value of this variable under a particular condition, and the correlation coefficient was determined by performing a linear regression of the variance data. The Student's t test was used to determine if the correlation was significant (7).

6.2.4 Fitting

The parameters of the modified Monod equation (3) were first plotted as a function of temperature and pH. Depending on the curvature, a constant linear or quadratic behaviour was assumed. A first estimate of the parameters was made by linear regression of the modified Monod parameters as a function of temperature and pH with the help of a commercial spreadsheet program. The equations were incorporated into the modified Monod model. This model was further optimized for describing the original growth rate data, with the help of a nonlinear fitting routine, based on a Marquardt algorithm (5). The resulting model was compared with the fit of a polynomial equation to the experimental data. Finally, the suitability of the model for predicting the growth rate of *L. innocua* in a meat product was demonstrated by using Bologna-type sausage.

6.2.5 Bologna-type sausage experiments

Different amounts of sodium lactate (0, 1, 2, 3 and 4% (w/w) of a 60% reagent-grade sodium lactate syrup (PURAC, Gorinchem, the Netherlands) were added to Bologna-type sausage containing 2% (w/w) NaCl and 120 μg nitrite per g. Three different batches were prepared, one in which the pH (6.2) was not adjusted, one in which the pH was lowered to 5.8 with 2 N HCl and one in which the pH was increased to 6.6 with 2 N KOH. This resulted in fifteen different kinds of sausage. The meat was put into cans (about 1800 g per can), pasteurized in a water bath (160 min. at 78°C) and stored at –20°C until required.

After being thawed at room temperature, 5400 g from each kind of sausage was cut into smaller pieces and inoculated with a suspension of *L. innocua* to a final level of about 10^4 bacteria per g. The bacteria were dispersed throughout the meat by cutting it for 75 s in a sterile cutter. The minced meat was then divided into portions of 40 g, which were put into plastic pouches with an oxygen permeability of 1.5×10^{-11} m Pa^{-1} day^{-1} at 20°C, flattened and vacuum packed. These packages were stored at either 7, 10, 15 or 20°C. Growth of *L. innocua* was monitored by plate counting at intervals. For each sample, a new package was opened. The modified Gompertz equation (5) was used to fit the resulting growth curves, and the calculated growth rates were used for validation of the modified Monod model.

6.2.6 Model comparison

The models were validated statistically with the use of the *F*-ratio test as described by Zwietering *et al.* (7). The measurement error was estimated from replicate experiments, by calculating the sum of squares (RSS_{ME}) of the deviation of the measured values from the mean value of the growth rate (μ) under specific growth conditions. For each model, the residual sum of squares of all μ-data was calculated as:

$$RSS_{model} = \Sigma \left(\mu_{predicted} - \mu_{measured}\right)^2$$

Then, the lack of fit ($RSS_{model} - RSS_{ME}$) was compared with the measurement error. This comparison between the lack of fit and the measurement error can be quantified statistically by the f testing value (7).

6.3 Results and Discussion

6.3.1 Analysis of variance

The t value that was obtained from the regression analysis of the plot of the variance of μ as a function of the mean value of μ under a particular condition (Fig. 6.1A) was 1.70, and the 95% critical t value for 18 degrees of freedom is 2.10. This supports the assumption based on visual inspection of the variance data, that no correlation existed between the variance of μ and the mean value of μ. There is also a theoretical basis for this assumption. The larger the value of μ, the larger the 95% confidence interval of the estimated value supplied by the modified Gompertz model (data not shown). On the other hand, the closer the salt concentration gets to the MIC (μ=0), the larger the variation between the values of replicate measurements. Therefore, high variance values could possibly arise for both high and low μ values. The relatively high variances of the data of four experimental conditions (black dots) shown

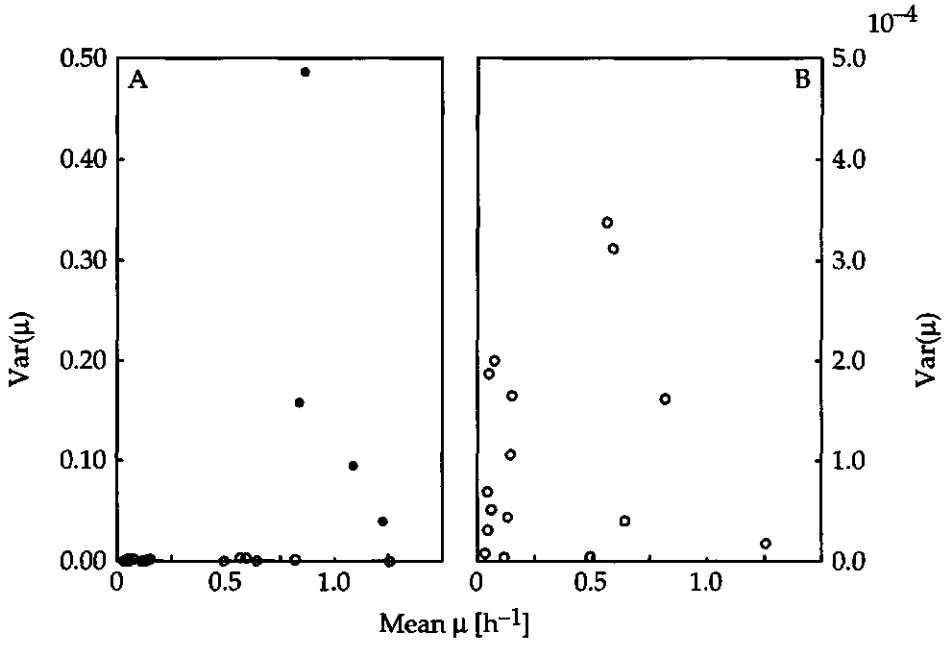

Figure 6.1: Variance of the maximum specific growth rate (Var(μ)), as a function of the mean of μ for all replicate measurements, plotted at two different vertical scales (A) and (B).

in Fig. 6.1*A* correspond to measurements at 536 and 625 mM sodium lactate (pH=6.5, T=30°C), 893 mM NaCl (pH=6, T=30°C) and to measurements in the absence of salt (pH=6.5, T=30°C). If these points were left out (Fig. 6.1*B*), there was still no correlation between the variance of μ and the mean value (t =0.456). For this reason, no transformation was applied to the growth rate data.

6.3.2 Modified Monod equation

The model that was proposed previously (Model 1) (3), describes μ as a function of the concentration of sodium lactate or NaCl present in the growth medium as:

$$\mu = \mu_m \cdot \frac{\alpha(p' - p)}{p'(\alpha - p)} \qquad \text{for } p \leq p' \qquad \text{(Model 1)}$$

where p is the salt concentration (mM), μ_m is the maximum specific growth rate at p=0, p' is the MIC and α is a shape parameter. If $p > p'$ then μ=0.

This equation contains three parameters that have different values for each combination of pH and temperature at which growth experiments were performed. When sodium lactate was present in the growth medium, growth was very poor at pH 5.5 and therefore not enough data were obtained at 4°C and 30°C to determine the parameter values under these conditions. The entire experimental range, therefore, included 48 parameters (4(T) x 4(pH) x 3(parameters)) to describe the NaCl data and 42 ((4 x 4 - 2) x 3) to describe the sodium lactate data. The actual values of the parameters are given in Table 6.1 (sodium lactate) and Table 6.2 (NaCl).

This model was validated statistically (see Table 6.3) with the use of the *F*-ratio test as described by Zwietering *et al.* (7). The measurement error was estimated with the use of replicate experiments as:

$$MS_{ME} = \frac{RSS_{ME}}{DF_{ME}} = \frac{0.123}{45 - 20} = \frac{0.123}{25} = 0.00493$$

This value was compared with the variance as found by Zwietering *et al.* (6). The *F*-test showed that the values were not significantly

different

$F_{25}^{20} = 2.01$:

$$MS_{ME} = \frac{RSS_{ME}}{DF_{ME}} = \frac{0.164}{20} = 0.00820; f = \frac{0.00820}{0.00493} = 1.66$$

The lack of fit of the model was compared with the measurement error. The models for both sodium lactate and NaCl were accepted on the basis of the *F*-test (Table 6.3.).

The disadvantage of Model 1 is that μ cannot be predicted under conditions where either temperature or pH values are different from those used for collecting the data. Furthermore, a large number of parameters are needed. Therefore, the parameters (μ_m, α and p') were

Table 6.1: Values of parameters for the modified Monod model (Model 1) that best described the maximum specific growth rate (μ) of *L. innocua* as a function of sodium lactate concentration in peptone-yeast extract broth

pH	T	Parameter; 95% confidence interval					
	(°C)	μ_m	±	α	±	p'	±
5.5	4	_[a]		_[a]		_[a]	
6.0	4	0.0395	0.0390	313	257	291	20.9
6.5	4	0.0498	0.00547	939	267	624	28.7
7.0	4	0.0499	0.0132	990	252	892	40.0
5.5	10	0.114	∞[b]	367	∞[b]	217	∞[b]
6.0	10	0.123	0.00725	580	34.6	486	6.17
6.5	10	0.145	0.0197	1250	397	869	49.3
7.0	10	0.154	0.0186	1231	172	1067	29.6
5.5	20	0.490	∞[b]	340	∞[b]	217	∞[b]
6.0	20	0.537	0.0556	1082	444	589	33.1
6.5	20	0.614	0.0142	1497	97.9	968	10.4
7.0	20	0.643	0.0464	1875	385	1267	47.3
5.5	30	_[a]		_[a]		_[a]	
6.0	30	1.15	0.0352	731	52.5	485	5.54
6.5	30	1.20	0.108	1283	237	970	48.0
7.0	30	1.26	0.0953	1508	197	1164	27.5

a) Parameters could not be determined because of lack of data.
b) The number of available data (three) was the same as the number of parameters.

Table 6.2: Values of parameters for the modified Monod model (Model 1) that best described the maximum specific growth rate (μ) of *L. innocua* as a function of NaCl concentration in peptone-yeast extract broth

pH	T	Parameter; 95% confidence interval					
	(°C)	μ_m	±	α	±	p'	±
5.5	4	0.0375	0.00512	1193	47.4	1161	7.07
6.0	4	0.0462	0.00622	1210	58.7	1161	10.3
6.5	4	0.0400	0.00319	1317	45.2	1250	8.38
7.0	4	0.0596	0.00147	1365	23.2	1250	3.85
5.5	10	0.119	0.00405	1845	71.2	1607	12.5
6.0	10	0.141	0.00728	2432	263	1782	34.7
6.5	10	0.159	0.0148	3061	984	1784	88.5
7.0	10	0.153	0.0181	2783	952	1781	97.6
5.5	20	0.553	0.111	2289	777	1784	113
6.0	20	0.609	0.0710	3259	1222	1957	116
6.5	20	0.634	0.0900	3589	2091	1951	156
7.0	20	0.661	0.0466	4020	1416	1952	82.8
5.5	30	1.23	0.407	1881	1008	1606	116
6.0	30	1.71	0.539	3299	3638	1687	174
6.5	30	1.48	0.220	2588	979	1682	92.0
7.0	30	1.34	0.235	2459	1022	1690	116

defined as a function of temperature. For sodium lactate, both temperature and pH were included as a variable, since the MIC (p') of this salt was strongly dependent on the pH of the growth medium (3),

Table 6.3: Statistical analysis[a] of the modified Monod model (Model 1) used for predicting the maximum specific growth rate of *L. innocua* in peptone-yeast extract broth in the presence of NaCl and sodium lactate (NaL), respectively

Model	nd	np	DF	RSS	MS	f	F
Model 1 NaCl	112	48	64	0.419	0.00654		
LOF			39	0.296	0.00759	1.54	1.88
Model 1 NaL	103	42	59	0.168	0.00284		
LOF			34	0.045	0.00132	0.268	1.90
Meas. error		20	25	0.123	0.00493		

a) nd = number of μ data; np = number of parameters; LOF = lack of fit; DF = degrees of freedom; RSS = residual sum of squares; MS = mean square; f = $MS_{LOF}/MS_{Meas.\ error}$; F = F table value (95% confidence).

which is also observed from Table 6.1. Table 6.2 shows that the influence of pH on p' was not significant when NaCl was used as the growth-inhibitory substance.

6.3.3 Parameter update - Model 2

Plotting of the derived parameters (μ_m, α and p') of Model 1 as a function of temperature or pH showed either constant, linear or quadratic behaviour that was translated into equations 6.1 to 6.6 (below). Subsequently, parameter values (b_1 to d_8) were determined by linear regression of the μ, α and p' data for sodium lactate (42 parameters) and μ, α and p' data for NaCl (48 parameters) to yield Model 2:

For sodium lactate:

$$\mu_m = b_1^2 \times (T - b_2)^2 \qquad \text{(Ratkowsky } et\ al.\text{, (4))} \qquad \text{(eq. 6.1)}$$

$$\alpha = b_3 \times T^2 + b_4 \times T + b_5 \times pH^2 + b_6 \times pH + b_7 \qquad \text{(eq. 6.2)}$$

$$p' = b_8 \times T^2 + b_9 \times T + b_{10} \times pH^2 + b_{11} \times pH + b_{12} \qquad \text{(eq. 6.3)}$$

And for NaCl:

$$\mu_m = d_1^2 \times (T - d_2)^2 \qquad \text{(Ratkowsky } et\ al.\text{, (4))} \qquad \text{(eq. 6.4)}$$

$$\alpha = d_3 \times T^2 + d_4 \times T + d_5 \qquad \text{(eq. 6.5)}$$

$$p' = d_6 \times T^2 + d_7 \times T + d_8 \qquad \text{(eq. 6.6)}$$

The estimated parameter values of Model 2 are given in Tables 6.4 and 6.5 for sodium lactate and NaCl, respectively.

6.3.4 Parameter update - Model 3

A more accurate parameter update was carried out with the help of the computerized nonlinear fitting procedure, fitting the original μ data (103 data points for sodium lactate and 112 for NaCl) at various values of pH, T and salt concentration, to calculate optimum values for b_1 etc. (Model 3, Tables 6.4 and 6.5). The parameter estimates obtained for

Table 6.4: Parameter estimates of the various models used to predict the maximum specific growth rate of *L. innocua* in peptone-yeast extract broth in the presence of sodium lactate

Parameter	Model 2 np[c] = 12	Model 3 np = 12	Model 3b[a] np = 5	eq.[b] no.	Model 4[a] np = 9	eq.[b] no.
b1	0.0349	0.0342	0.0361	6.1	–26.1	6.7
b2	–1.38	–2.10	–0.927	6.1	0.296	6.7
b3	–2.53	–0.205	0	6.2	–0.0101	6.7
b4	104	–13.6	0	6.2	6.60	6.7
b5	–347	559	0	6.2	–0.00328	6.7
b6	5192	–6670	0	6.2	–0.486	6.7
b7	–18671	21325	1335	6.2	–0.00953	6.7
b8	–1.11	–0.895	0	6.3	0.00133	6.7
b9	47.4	35.0	0	6.3	0.0000157	6.7
b10	–85.5	–35.4	0	6.3	0	6.7
b11	1724	1135	606	6.3		
b12	–7134	–5288	–3066	6.3		

a) Model 2: equations 6.1 to 6.3 fitted to μ, α and *p'*; Model 3: equations 6.1 to 6.3 incorporated in Model 1 and fitted to the growth rate data; Model 3b: Model 3 with exclusion of nonsignificant parameters; Model 4: polynomial equation (eq. 6.7)

b) This number refers to the equation in the text in which the parameter is used.

c) np = number of parameters.

Model 2 were used as initial values. Calculation of the confidence intervals of the parameters showed that this procedure allowed for a further reduction in the number of parameters, without the models being statistically rejected (some parameters were no longer significant, i.e. zero was in the 95% confidence interval). After each parameter elimination the nonlinear fitting procedure was repeated to update the remaining parameter values and to check whether the resulting model was not statistically rejected.

With sodium lactate, α was not necessarily dependent on temperature or pH, but p' obviously was related to the pH of the growth medium so that the initial amount of 12 parameters (equations 6.1 to 6.3) was reduced to 5 (b_1, b_2, b_7, b_{11} and b_{12}) (Model 3b, Table 6.4). With NaCl, α and p' were not necessarily dependent on temperature, so that the initial number of 8 parameters (equations 6.4 to 6.6) could be reduced to 4 (d_1, d_2, d_5 and d_8) (Model 3b, Table 6.5). Model 1 shows that p' and α do not play a role in determining μ when p=0, so that the models for sodium lactate and NaCl were basically the same under this condition (equations 6.1 and 6.4). The values of b_1 and b_2 (Table 6.4) therefore should be equal to the values of d_1 and d_2, respectively (Table 6.5). Indeed, the 95% confidence intervals showed overlap in all cases (data not shown).

6.3.5 Polynomial fit

Also, a polynomial fit (equations 6.7 and 6.8) was applied to the experimental data (Model 4, Tables 6.4 and 6.5). Nonsignificant terms were excluded resulting in the following equations

$$\mu_{NaL} = \exp\{b_1 + b_2 \cdot T + b_3 \cdot p + b_4 \cdot pH + b_5 \cdot T^2 + b_6 \cdot pH^2 + b_7 \cdot pH \cdot T + b_8 \cdot pH \cdot p + b_9 \cdot T \cdot p + b_{10} \cdot p^2\} \quad \text{(eq. 6.7)}$$

and

$$\mu_{NaCl} = \exp\{d_1 + d_2 \cdot T + d_3 \cdot p + d_4 \cdot T^2 + d_5 \cdot p^2\} \quad \text{(eq. 6.8)}$$

MICs could not be incorporated into the polynomial fit, because the regression analysis was performed after a logarithmic transformation of the μ data ($ln\ \mu$), so those conditions under which μ=0 were not taken

Table 6.5: Parameter estimates of the various models used to predict the maximum specific growth rate of *L. innocua* in peptone-yeast extract broth in the presence of NaCl

Parameter	Model 2[a] np[c] = 8	Model 3[a] np = 8	Model 3b[a] np = 4	eq.[b] no.	Model 4[a] np = 5	eq.[b] no.
d_1	0.0385	0.0385	0.0389	6.4	–4.08	6.8
d_2	–0.714	–0.173	–0.0692	6.4	0.240	6.8
d_3	–7.77	–5.69	0	6.5	0.000576	6.8
d_4	313	217	0	6.5	–0.00331	6.8
d_5	155	497	2145	6.5	–0.000000826	6.8
d_6	–2.90	–2.27	0	6.6		
d_7	115	88.3	0	6.6		
d_8	822	934	1629	6.6		

a) Model 2: equations 6.4 to 6.6 fitted to μ, α and p'; Model 3: equations 6.4 to 6.6 incorporated in Model 1 and fitted to the growth rate data; Model 3: Model 3 with exclusion of nonsignificant parameters; Model 4: polynomial equation (eq. 6.8)

b) This number refers to the equation in the text in which the parameter is used.

c) np = number of parameters.

into account. The logarithmic transformation was necessary to obtain sufficient accuracy. When regression was performed on the raw data (no transformation) or when the MICs were taken into account by substitution of μ=0 by μ=10^{-8} or μ=10^{-3}, more parameters were necessary to obtain a sufficiently low *RSS*. The *RSS* was calculated on the basis of the raw μ data.

6.3.6 Comparison of the models

The statistical validation of the various models is shown in Table 6.6 (sodium lactate) and Table 6.7 (NaCl). The various models were compared with the measurement error. The *f*-value was used to discriminate between the number of parameters needed to describe the experimental data for sodium lactate and NaCl.

Figure 6.2 shows that the prediction from Model 3 is less accurate than the prediction from Model 1. However, the decline in the goodness of fit is accompanied by a large reduction in the number of parameters needed (Tables 6.6 and 6.7), whereas the area in which predictions are valid is enlarged from combinations of four temperatures with four pH values to

the whole range of values for these variables that are between the extremities used for data collection. Model 3 had one serious disadvantage compared with Model 1. This is, that the predictions of μ at 4°C (Fig. 6.3*A*) were relatively poor compared with those at 30°C (Fig. 6.3*B*). Since the model was fitted to the data by ordinary (i.e. unweighed) least squares, there is a tendency for the higher growth rates (i.e. those at high temperatures) to be more influential in determining the least-squares line than the data at low temperatures.

It may therefore be questioned whether a polynomial model is more appropriate. Table 6.6 shows that for predicting μ from the sodium lactate data at least nine parameters were needed. The resulting model was significantly less accurate than Model 1, even if ten parameters were included (data not shown). For NaCl, the data were described by a polynomial equation with five parameters in a manner that was statistically acceptable (Table 6.7), so that was comparable to Model 3b. However, the polynomial model had a poor ability to predict μ at salt concentrations close to and at the MIC (Fig. 6.4) which might be because MIC data cannot be included in the polynomial model. Since we were

Table 6.6: Statistical analysis[a] of the different models used for predicting the maximum specific growth rate of *L. innocua* in peptone-yeast extract broth in the presence of sodium lactate (based on 103 growth curves)

Model	np	DF	RSS	MS	f	F
Model 1	42	59	0.168	0.00284		
LOF 1		34	0.0447	0.00131	0.267	F_{25}^{34}=1.90
Model 2	12	89	0.639	0.00718		
LOF 2		64	0.516	0.00806	1.63	F_{25}^{64}=1.82
Model 3	12	91	0.241	0.00265		
LOF 3		66	0.118	0.00179	0.363	F_{25}^{66}=1.81
Model 3b	5	98	0.630	0.00643		
LOF 3b		73	0.507	0.00695	1.41	F_{25}^{73}=1.80
Model 4	9	79	0.458	0.00580		
LOF 4		54	0.335	0.00620	1.26	F_{25}^{54}=1.83
Meas. error	20	25	0.123	0.00493		

a) np = number of parameters; LOF = lack of fit; DF = degrees of freedom; RSS = residual sum of squares; MS = mean square; f= $MS_{LOF}/MS_{Meas.\ error}$; F= F table value (95% confidence).

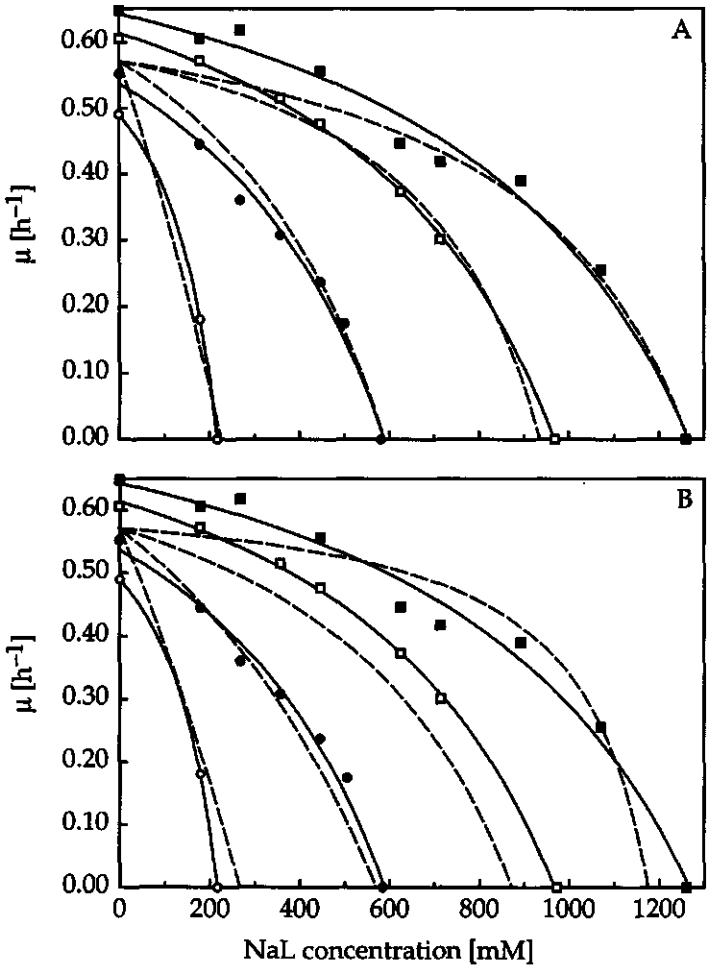

Figure 6.2: Comparison between the fit of Model 1 (solid lines) and Model 3 (dashed lines, Fig. 2A) or Model 3b (dashed lines, Fig. 2B) to the experimental data (symbols) at pH 5.5 (○), pH 6.0 (●), pH 6.5 (□) and pH 7.0 (■), obtained in the presence of different sodium lactate (NaL) concentrations at 20°C.

especially interested in the conditions under which no or only very slow growth was observed and where the growth rate was significantly reduced by sodium lactate, the polynomial equation was less applicable.

Notably, the parameters in the polynomial equations have no practical meaning, whereas p' and μ_m in the modified Monod model have. Both p' and μ_m are easily determined. For this reason, it is more convenient to

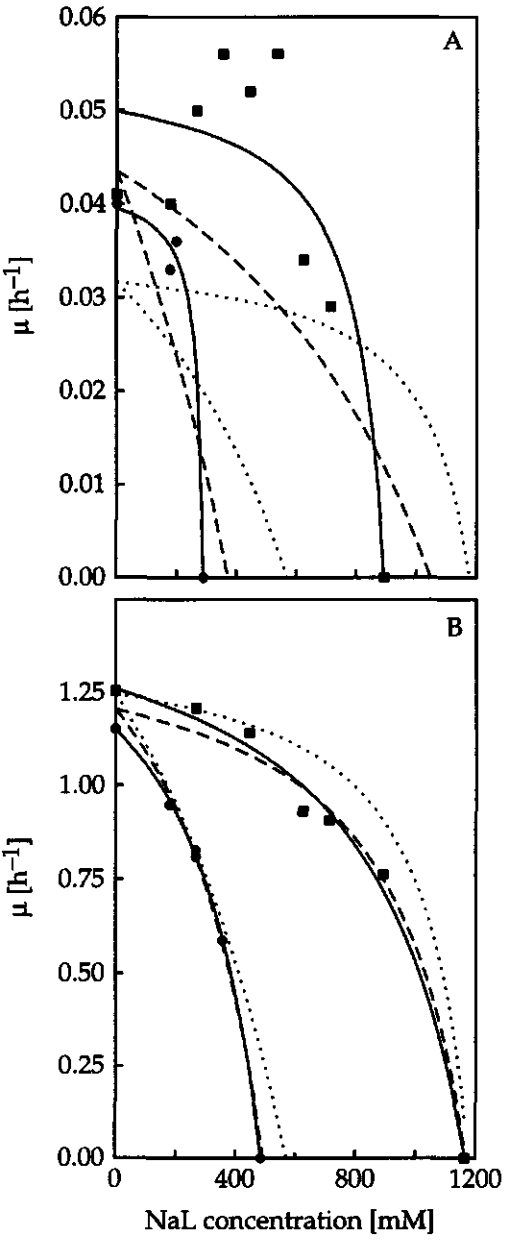

Figure 6.3: Comparison between the fit of Model 1 (solid line), Model 3 (dashed line) and Model 3b (dotted line) to the experimental data (symbols) at 4°C (A) and 30°C (B). The experimental data refer to pH 6.0 (•) and pH 7.0 (■), and were obtained in the presence of different sodium lactate (NaL) concentrations.

Table 6.7: Statistical analysis[a] of the different models used for predicting the maximum specific growth rate of *L. innocua* in peptone-yeast extract broth in the presence of NaCl (based on 112 growth curves)

Model	np	DF	RSS	MS	f	F
Model 1	48	64	0.419	0.00654		
LOF 1		39	0.296	0.00758	1.54	F_{25}^{39}=1.87
Model 2	8	104	0.715	0.00687		
LOF 2		79	0.592	0.00749	1.52	F_{25}^{79}=1.80
Model 3	8	104	0.564	0.00542		
LOF 3		79	0.441	0.00558	1.13	F_{25}^{79}=1.80
Model 3b	4	108	0.625	0.00579		
LOF 3b		83	0.502	0.00605	1.23	F_{25}^{83}=1.79
Model 4	5	91	0.528	0.00580		
LOF 4		66	0.405	0.00613	1.24	F_{25}^{66}=1.81
Meas. error	20	25	0.123	0.00493		

a) np = number of parameters; LOF = lack of fit; DF = degrees of freedom; RSS = residual sum of squares; MS = mean square; f= $MS_{LOF}/MS_{Meas.\ error}$; F= F table value (95% confidence).

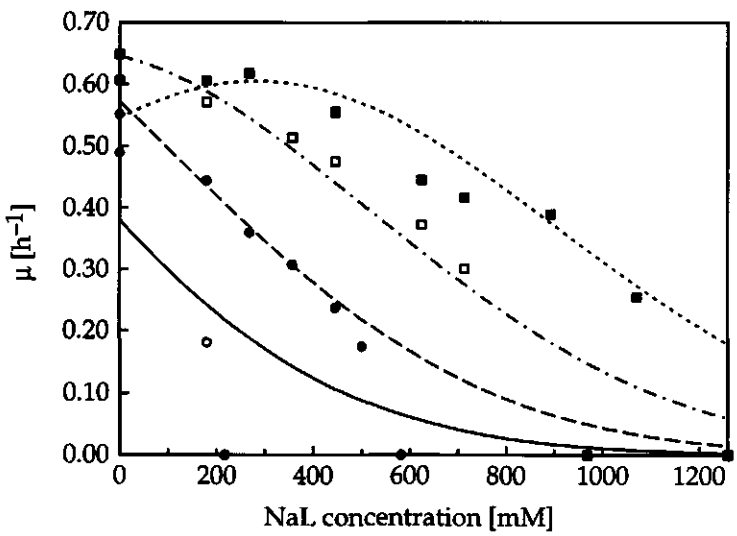

Figure 6.4: Fit of the polynomial model with nine parameters (lines) to the experimental data (symbols) at 20°C at pH 5.5 (solid line, ○), pH 6.0 (dashed line, ●), pH 6.5 (dash-dotted line, □) and pH 7.0 (dotted line, ■).

check the parameters from the modified Monod model; consequently, inaccurate predictions can be easily identified. All these arguments are in favour of the use of the modified Monod model.

In conclusion, the maximum specific growth rate of *L. innocua* was satisfactorily described with a minimum number of parameters by Model 3b which was obtained by translating the relevant parameters in equations 6.1 to 6.3 (sodium lactate) and equations 6.4 to 6.6 (NaCl) into the formula for Model 1. By doing so, the following equation was obtained for sodium lactate:

$$\mu = b_1^2 \cdot (T - b_2)^2 \cdot \frac{b_7 \cdot ((b_{11} \cdot pH + b_{12}) - p)}{(b_{11} \cdot pH + b_{12})(b_7 - p)}$$

The parameters were substituted by the values in Table 6.4 (Model 3b):

$$\mu = 0.0361^2 \cdot (T + 0.927)^2 \cdot \frac{1335 \cdot ((606 \cdot pH - 3066) - p)}{(606 \cdot pH - 3066)(1335 - p)} \quad \text{(eq. 6.9)}$$

The same procedure was followed for NaCl (Model 3b, Table 6.5) and the maximum specific growth rate in the presence of NaCl was described by the following equation:

$$\mu = 0.0389^2 \cdot (T + 0.0692)^2 \cdot \frac{2145 \cdot (1629 - p)}{1629 \cdot (2145 - p)} \quad \text{(eq. 6.10)}$$

6.3.7 Validation of the model for sodium lactate in a meat product

The growth rate of *L. innocua* as predicted by the broth model (eq. 6.9) was much higher than that observed in the Bologna-type sausages that were formulated with different amounts of sodium lactate (Fig. 6.5). The broth model was designed to mimic "worst-case" circumstances and this could explain the observed deviation between the data and the predicted values. The sausage contained not only sodium lactate as a growth-inhibitory substance, but also nitrite (120 µg per g) and NaCl (2% (w/w)). Furthermore, the broth model was related to aerobic conditions whereas growth in the sausage took place anaerobically. *L. innocua* is a facultatively anaerobic micro-organism and in our

experience it preferred aerobic to anaerobic conditions for growth, although the effects of oxygen on growth of the genus *Listeria* in meat (products) as described in the literature are confusing (1).

If no mutual interactions of these factors exist, and if the effects are not correlated with pH and T, the observed deviation can be simply adjusted by re-estimating the value for μ_m (Fig. 6.6*A*). The resulting *RSS*=0.218 gave *MS*=0.00545. Compared with the measurement error of the broth experiments, this resulted in an *f*-value of 1.105 (F(11,25)= 2.20). The measurement error in the experiments with the sausage is expected to be larger than that in the broth experiments. Therefore, it can be concluded that the model described the data in a manner that was statistically acceptable after μ_m was adjusted.

However, careful comparison of the predicted and measured values showed that at high measured values of μ, the predicted value was

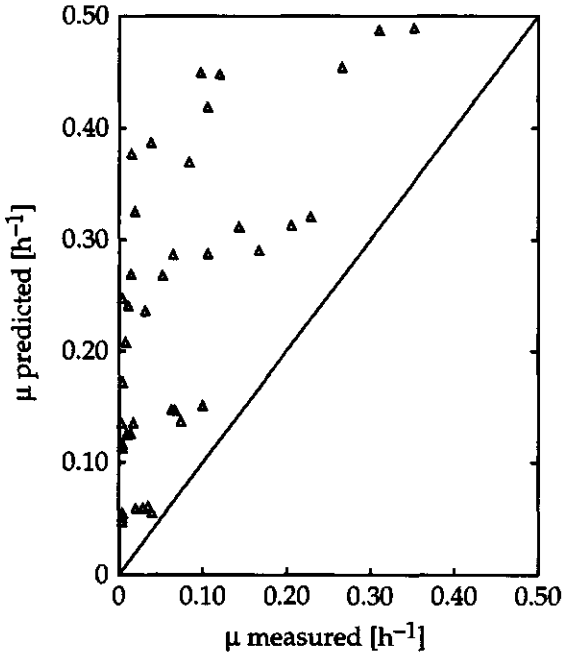

Figure 6.5: Model 3b (eq. 6.9) developed on the basis of experiments with broth is used to predict the maximum specific growth rate of *L. innocua* in a Bologna-type sausage. Predicted values for μ are shown as a function of the measured values (symbols). The line represents the ideal situation, in which the predicted values are exactly the same as the measured values.

clearly too low (Fig. 6.6*A*). Therefore the other parameters of the model were also re-estimated by performing nonlinear regression of the μ data obtained in experiments with the sausage (41 growth curves). The result is shown in Fig. 6.6*B*. Inevitably, the increased accuracy of the model after re-estimating the parameters was accompanied by an increase in the number of cases in which the growth rate was underestimated. This is possibly caused by regular statistical inaccuracy.

More data as well as replicate experiments are necessary to evaluate the usefulness of the model presented in this paper for predicting microbial growth in other meat products. As was shown, the model can be used even when growth conditions differ from those on which the model is based after μ_m is re-estimated. Depending on the degree of agreement between model predictions and measured values and the demands that are made on the accuracy of the model predictions, the modeller has to decide whether it is necessary to collect more experimental data. Overall, the simplicity of the model in combination

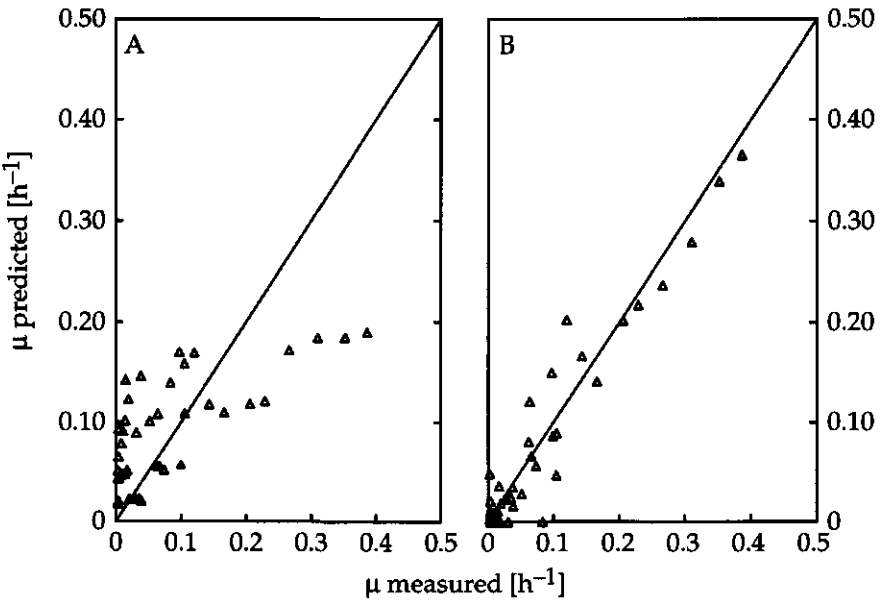

Figure 6.6: Model 3b (eq. 6.9) developed on the basis of experiments in broth is used to predict the maximum specific growth rate of *L. innocua* in a Bologna-type sausage after parameter b_1 has been re-estimated (A): $b_1 = 0.0222$, and after all parameters have been re-estimated (B): $b_1 = 0.0380$, $b_2 = 1.47$, $b_7 = 3000$, $b_{11} = 418$, $b_{12} = -2283$. The line represents the ideal situation, in which the predicted values are exactly the same as the measured values.

with the practical usefulness of its parameters offers considerable prospects for its use in the field of predictive microbiology.

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7

Effect of sodium lactate on acid adaptation of *Listeria innocua* and *Lactococcus lactis*

Authors: P.C. Houtsma, E. Glaasker, B. Poolman, W.N. Konings, F.M. Rombouts and T. Abee.

Abstract

This study deals with lactate sensitivity and acid adaptation of *Listeria innocua* and *Lactococcus lactis* grown in the absence and presence of sodium lactate. Minimum inhibitory concentrations (MIC) of sodium lactate and NaCl were compared. The concentration of sodium lactate needed to prevent growth was much lower than that of NaCl. For *L. innocua*, the MIC of sodium lactate strongly decreased when the pH of the growth medium (pH_{out}) was lowered, but this effect was less obvious for *Lact. lactis*. Intracellular pH (pH_{in}) measurements showed, that when *Lact. lactis* was grown in the presence of sodium lactate, the pH_{in} was higher than that of control cells (grown in the absence of sodium lactate). When *L. innocua* was grown in the presence of sodium lactate this phenomenon was observed only when lactate was present in the assay buffer. The presence of F_1F_0-ATPases was established in both organisms by immunoblotting and acid-adapted cells of *Lact. lactis* and *L. innocua* showed an increased ATPase activity. An electron transfer system has been suggested to be present in *L. innocua*, which could have a role in pH_{in} regulation. Experiments with the inhibitors N,N'-dicyclohexylcarbodiimide (DCCD) and potassium cyanide (KCN) were performed to examine the possible roles in pH_{in} regulation of the F_1F_0-ATPase or the electron transport chain, respectively. In both organisms, addition of 10 mM KCN did allow the pH_{in} to rise after addition of glucose, whereas this was prevented after incubation in the presence of 1 mM DCCD. These results suggest that in both *Lact. lactis* and *L. innocua*, the F_1F_0-ATPase is the major pH_{in} regulator.

7.1 Introduction

Sodium lactate and lactic acid are widely used in food products to

improve flavour characteristics, microbiological quality and chemical stability. Also, these compounds are natural constituents of meats and many fermented foods. The preservative quality of lactic acid primarily due to its pH lowering effect, is well documented. However, its salt sodium lactate also has a significant inhibitory effect on microbial growth. This has led to the widespread application of sodium lactate especially in meat and poultry products. The United States Department of Agriculture (USDA) permits levels of sodium lactate as high as 2% (w/w) (30).

Recent reviews on the inhibitory effects of lactate (35) and organic acids in general (4,10) are available. The bacteriostatic properties of lactate have been confirmed in broth and in meat products, but the underlying mechanism is not yet clear. Sodium lactate decreases the water activity (a_w), a critical factor for microbial growth (15). Lowering of the intracellular pH (pH_{in}) due to either diffusion of the undissociated acid molecule across the cytoplasmic membrane or increased permeability of the plasma membrane to protons in the presence of organic acids is envisaged as a potent inhibitory mechanism that is associated with organic acids in general (10,19). A decrease in pH_{in} presents a potential constraint to various aspects of bacterial growth (metabolism, transport processes, enzyme activities and biosynthesis) (6,29,33).

Inhibition of microbial growth by sodium lactate cannot be ascribed to lowering of the water activity alone (7,15,16,35). It has been concluded that sodium lactate has a specific antimicrobial effect. The nature of this effect was studied in *Listeria innocua* and *Lactococcus lactis* subsp. *lactis*. *L. innocua* is a nonpathogenic member of the genus *Listeria*, and is a common contaminant of meat and meat products (18); the organism is routinely used as a model for the pathogenic *Listeria monocytogenes*. Lactic acid bacteria play a major role in food fermentations, yet they are also frequently a constituent of the spoilage flora in meat products. *Lact. lactis* was chosen as a model, since its physiology is by far best described among the lactic acid bacteria.

In the present study, pH_{in} regulation in the presence and absence of sodium lactate was studied to investigate whether this could explain the differences between the effect of pH_{out} on minimum inhibitory concentrations (MICs) of lactate for various micro-organisms as observed previously (Chapter 3). The presence of a membrane-associated electron

transfer system has been suggested for *Listeria monocytogenes*, on the basis of the inhibition of NADH oxidase activity in lysed protoplasts by respiratory chain inhibitors (32). Therefore, the possible role of this electron transfer system in pH_{in} regulation was investigated. Furthermore, the role of the proton translocating ATPase in regulation of the pH_{in} was assessed by measuring ATPase activities in cytoplasmic membrane vesicles, isolated from cells grown under various acid stress conditions.

7.2 Materials and Methods

7.2.1 Culture conditions

Lactococcus lactis subsp. *lactis* ML3 was grown without shaking at 30°C in a modified MRS broth, pH 6.5, according to Molenaar *et al.* (27). *Listeria innocua* DSM 20649 was grown aerobically at 30°C with shaking (150 rpm) in a peptone-yeast extract broth, pH 6.5 (15). The micro-organisms were cultured with 0.5% (w/v) glucose as a carbon and energy source in the absence and presence of 400 mM sodium lactate (pK_a= 3.86, 0.91 mM undissociated acid at pH 6.5) or 20 mM phenoxy-acetic acid (pK_a= 3.12, 0.01 mM undissociated acid at pH 6.5), and harvested in the exponential phase of growth (*L. innocua*: OD_{660} approximately 0.4, pH 5.8; *Lact. lactis*: OD_{660} approximately 0.9, pH 5.6). Cells were collected by centrifugation at 4°C (8000 x *g*, 10 min.) and washed twice in 50 mM potassium phosphate, pH 7. Pellets were suspended in the same buffer, and the cells were kept on ice until use. For studies of the proton translocating ATPase activity, cells were grown without shaking at 30°C with the pH of the culture medium regulated at 5.0, 6.5 or 7.5 using 5 M NaOH. After harvesting, cytoplasmic membrane vesicles were prepared from these cells as described below.

7.2.2 Minimum inhibitory concentrations

For both organisms, minimum inhibitory concentrations of sodium lactate and NaCl were determined in a soya-peptone broth as described previously (15).

7.2.3 Protein

The amount of protein in membrane vesicles and whole cells was

determined according to the method of Lowry *et al.* (25), using bovine serum albumin (BSA) as a standard. The protein composition of the isolated membranes was analyzed with SDS-PAGE. Gels were stained with Coomassie brilliant blue (5 µg protein per lane) or with silver stain (0.5 µg protein per lane).

7.2.4 Intracellular pH

Cells were harvested in the exponential phase of growth, washed twice and suspended in buffer containing 25 mM 4-morpholine ethane sulfonic acid (MES), 25 mM 4-morpholine propane sulfonic acid (MOPS) and 50 mM KCl, pH 7. The measurements were performed in the same buffer, with the pH set to 5, 5.5, 6, 6.5, 7 or 7.5 with 1 M methylglucamine. The effect of sodium lactate on pH_{in} was assessed at varying external pH values and in buffer containing a total amount of 400 mM lactate (anion + acid), or a fixed amount of 0.9 mM undissociated lactic acid. Experiments were also performed in buffer containing 0.9 mM of undissociated acetic, lactic, formic or phenoxy-acetic acid (pH 5.5). Cell suspensions (0.5 mg protein per ml) were incubated at 30°C for 5 min., after which glucose was added to a final concentration of 11 mM. After another 20 min. of incubation, the pH_{in} was determined from the distribution of ^{14}C-labelled benzoic acid (5 µM; 4 MBq per L) using the silicon oil centrifugation technique (37). Calculations were performed as described by Nannen and Hutkins (28). In control experiments, the proton motive force (pmf) was dissipated by addition of valinomycin plus nigericin to the assay mixture (final concentrations of 1 and 2 µM, respectively). The internal cell volume was determined from the distribution of [^3H]water (37 MBq per L) and [^{14}C]taurine (0.6 µM; 2.58 MBq per L) according to Ten Brink and Konings (37) in 50 mM MES/MOPS (pH 6.5) containing about 5 mg of protein per ml. From the data, internal volumes of 3.2 ± 0.5 µl per mg protein (n=9) and 2.6 ± 0.2 µl per mg protein (n=13) were calculated for *Lact. lactis* subsp. *lactis* and *L. innocua*, respectively.

7.2.5 Preparation of membrane vesicles

Cells were washed with 100 mM potassium-MOPS (pH 7) and suspended in the same buffer. Droplets of this suspension were stored in liquid nitrogen until further use. For preparation of inside-out

membrane vesicles from *Lact. lactis*, the cell suspension was thawed at room temperature and diluted two times with distilled water. $MgSO_4$ was added to a final concentration of 5 mM, together with lysozyme (5 mg per ml) and mutanolysin (250 U). The suspension was gently shaken for 30 min. at 37°C and run through a French pressure cell twice (8000 psi, 0°C). After that, DNase and RNase were added to a final concentration of 100 µg per ml each, and the suspension was incubated for 15 min. at 37°C and subsequently stored on ice. Unbroken cells and cell debris were removed by centrifugation at 4°C (8000 x *g*, 10 min.). Inside-out membrane vesicles were pelleted by high-speed centrifugation of the supernatant at 4°C (48200 x *g*, 40 min.), suspended in 50 mM K-MOPS, pH 7, containing 5 mM $MgSO_4$ to a final concentration of about 7 mg protein per ml, rapidly frozen, and stored in liquid nitrogen.

Membrane vesicles of *L. innocua* were obtained by cell disruption with glass beads (ϕ 184 µm), since no membrane vesicles were obtained with the procedure described above for *Lact. lactis*. The cell suspension in 100 mM K-MOPS, pH 7, was diluted twice in distilled water before adding $MgCl_2$ to a final concentration of 2.5 mM. Glass beads were added (about 1 g per ml) and the cells were treated with high frequency shaking (4000 rpm) for 2 min. in a Braun MSK cell homogenizer, with constant cooling by CO_2 expansion. Membrane vesicles were obtained from this suspension by high-speed centrifugation and suspended in 50 mM K-MOPS, pH 7, containing 5 mM $MgSO_4$ as was described for *Lact. lactis*.

7.2.6 Enzyme assays

ATPase activities of total membrane fractions were obtained by colorimetric determination of inorganic phosphate (P_i) with the malachite green molybdate reagent as described by Driessen *et al.* (9) with some modifications. ATPase activity was assayed in a standard reaction mixture of 50 mM MES/TRIS, containing 10 mM $MgCl_2$ and 0.05% Triton X-100 at pH values ranging from 5.0 to 9.0. Samples of 260 µl, containing about 14 µg and 7 µg protein for *L. innocua* and *Lact. lactis*, respectively, were incubated for 5-10 min. at 30°C in the presence of 5 mM ATP.

To investigate the inhibitory action of N,N'-dicyclohexylcarbodiimide

(DCCD), membrane vesicles were suspended in 20 mM MES/TRIS, pH 8.0, containing 10 mM $MgCl_2$ and 1 mM DCCD, and incubated at 30°C for 30 min. After that, 10 µl of this suspension was added to 250 µl 50 mM MES/MOPS and ATPase activity was determined as described above. All enzyme assays were performed at least in duplicate.

7.2.7 SDS-PAGE and Western blotting

Proteins of cytoplasmic membrane vesicles, were dissolved in sample buffer and incubated for 2 min. at 100°C. Protein (1, 2.5 or 5 µg) was applied to a sodium dodecyl sulfate-polyacrylamide gel (10% (w/v) separating gel) and run with constant current of 80 mA. Proteins were transferred from the gel to a polyvinylidene difluoride (PVDF) membrane (Millipore Corporation, Bedford, Massachusetts, USA) by a semi-dry blotting apparatus (Ancos, Denmark). The PVDF sheets were subsequently incubated with rabbit antibodies raised against subunit ß of the *Escherichia coli* F_1F_0-ATPase, which were a generous gift from Professor K. Altendorf and Dr. G. Deckers-Hebestreit from the University of Osnabrück. Antigen-antibody reactions were detected with alkaline phosphatase coupled to goat anti-rabbit IgG (Biorad), or with the Western-Light™ chemiluminescent detection system (Tropix, U.S.).

7.2.8 Oxygen consumption

The oxygen consumption of *Lact. lactis* subsp. *lactis* ML3 and *L. innocua* DSM 20649 was measured in a biological oxygen monitor (BOM). The experiments were performed at 30°C in the absence and presence of 400 mM sodium lactate in 50 mM potassium phosphate, pH 6.5. Cells were added to a final concentration of about 0.3 mg protein per ml and glucose was added to a final concentration of 15 mM. Oxygen consumption was measured in the absence and presence of 10 mM of the electron transfer system inhibitor potassium cyanide (KCN), and expressed as nmoles of oxygen consumed per min. per mg of protein.

7.2.9 Chemicals

^3H_2O (37 GBq per l; 1 Ci per l), [^{14}C]taurine (4.3 TBq per mol; 115 Ci per mol) and [carboxyl-^{14}C]benzoic acid (0.81 TBq per mol; 22 Ci per mol) were obtained from the Radiochemical Centre, Amersham, Buckinghamshire, Great Britain). The silicon oil was obtained from

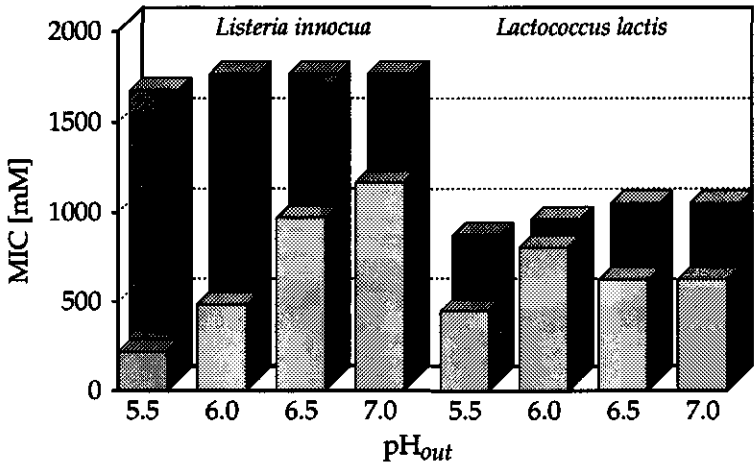

Figure 7.1: Minimum inhibitory concentration (MIC) of sodium lactate (▭) and NaCl (■) for *Listeria innocua* and *Lactococcus lactis* at 30°C at various pH values in a peptone-yeast extract broth.

Wacker Chemicals, Munich, Germany. The sodium lactate was a 60% (w/w) reagent-grade solution, obtained from PURAC, Gorinchem, the Netherlands. All other chemicals were reagent-grade and obtained from commercial sources.

7.3 Results

7.3.1 Minimum inhibitory concentrations

The sensitivities of *L. innocua* and *Lact. lactis* towards sodium lactate were assessed by determination of the MIC values at various pH values. NaCl was used as a control to distinguish between the effect of water activity (a_w) and the specific inhibitory effect of the lactate. The NaCl concentrations needed to prevent growth were much higher for *L. innocua* than for *Lact. lactis* (Fig. 7.1). The a_w values that corresponded to the MICs of NaCl were 0.94 and 0.97 for *Listeria* and *Lactococcus*, respectively, and these values did not change significantly upon lowering the pH of the growth medium (Fig. 7.1). For *L. innocua*, the MIC of lactate decreased markedly when the pH of the growth medium was lowered, whereas for *Lact. lactis*, the MIC for lactate was only slightly affected by pH (Fig. 7.1). Compared with *L. innocua*, *Lact. lactis* could tolerate higher concentrations of lactate at pH 5.5, whereas it was more

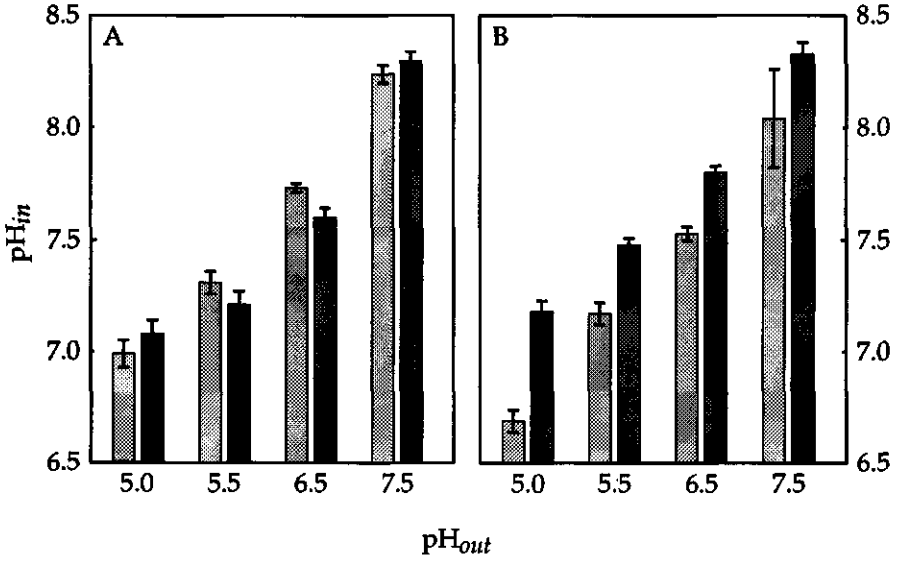

Figure 7.2: pH$_{in}$ as a function of pH$_{out}$ for *Listeria innocua* (A) and *Lactococcus lactis* (B). Cells were grown in the absence (▨) and in the presence (■) of 400 mM sodium lactate, and assayed in buffer without addition of sodium lactate.

sensitive to lactate at pH 7 (Fig. 7.1).

7.3.2 Intracellular pH measurements in *Listeria innocua* and *Lactococcus lactis*

Adaptation of *Lact. lactis* and *L. innocua* to lactate was investigated by analyzing the intracellular pH (pH$_{in}$) of cells grown in the absence or presence of 400 mM lactate. The amount of lactate that was produced by the cells at the time of harvest as a result of metabolism was about 20 mM. In both strains pH$_{in}$ decreased at lower pH$_{out}$ (Fig. 7.2). When cells were grown without lactate (control cells), the decrease of pH$_{in}$ with pH$_{out}$ was more rapid in *Lact. lactis* cells, resulting in lower pH$_{in}$ values at pH$_{out}$ 6.5, 5.5 and 5.0 compared with *L. innocua*. *Lact. lactis* cells that were grown in the presence of sodium lactate had higher pH$_{in}$ values than control cells (Fig. 7.2*B*), a phenomenon that was not observed in *L. innocua* (Fig. 7.2*A*).

When pH$_{in}$ measurements were performed in the presence of lactate, the pH$_{in}$ of control cells of *L. innocua* was higher than of the corresponding *Lact. lactis* cells, irrespective of the lactate concentration in

the buffer (Fig. 7.3). In contrast to the results obtained in assays without lactate, the pH_{in} of *L. innocua* cells grown in the presence of sodium lactate was significantly higher than that of control cells (Fig. 7.3). It thus appeared that both *Lact. lactis* and *L. innocua* are able to adapt to the presence of sodium lactate in the growth medium, resulting in higher pH_{in} values compared with those of unadapted (control) cells. For *L. innocua*, this adaptation was only observed when sodium lactate was present in the assay buffer, whereas for *Lact. lactis*, it was observed both in the presence and in the absence of sodium lactate.

The (undissociated) lactic acid concentration in the buffer also had a strong influence on the effect of pH_{out} on pH_{in}. In the presence of 400 mM sodium lactate at pH_{out} 6.5, the lactic acid concentration in the assay buffer is 0.9 mM. At pH_{out} 6.0 and 5.5, the concentration of lactic acid is higher than 0.9 mM and, accordingly, the pH_{in} of both adapted and unadapted cells of *L. innocua* and *Lact. lactis* was lower in the buffer with 400 mM lactate (Fig. 7.3*A*) than in the buffer with 0.9 mM lactic acid at these pH values (Fig. 7.3*B*). Not only was the absolute pH_{in} value affected, but also the difference between the pH_{in} of adapted and unadapted cells. When no lactate was added to the buffer at pH_{out} 5.0, the pH_{in} of *Lact. lactis* was 6.7 and 7.2 for unadapted and adapted cells, respectively (Fig. 7.2*B*). In the presence of 0.9 mM lactic acid, these values were 6.2 and 6.3, respectively (Fig. 7.3*B*).

The effect of lactate on pH_{in} was compared with the effect of other short-chain organic acids (Table 7.1). Cells grown in the presence of sodium lactate maintained a high pH_{in} when either 1 mM acetic or lactic acid was added to the assay buffer. However, in the presence of 1 mM formic or phenoxy-acetic acid, *L. innocua* as well as *Lact. lactis* cells lost their ability to maintain a high pH_{in} (Table 7.1).

To investigate whether the acid adaptation response was a lactate-specific effect, the experiments were repeated with cells grown in the presence of 20 mM phenoxy-acetic acid (PA). These cells also had higher pH_{in} values compared with cells that were grown in the absence of organic acid. *Lact. lactis* cells maintained a high pH_{in} in the presence and in the absence of lactate, whereas with PA-adapted *L. innocua* cells this adaptation manifested itself in the presence, but not in the absence of lactate (data not shown), a response similar to that observed for lactate-adapted cells (see above, Figs. 7.2 and 7.3).

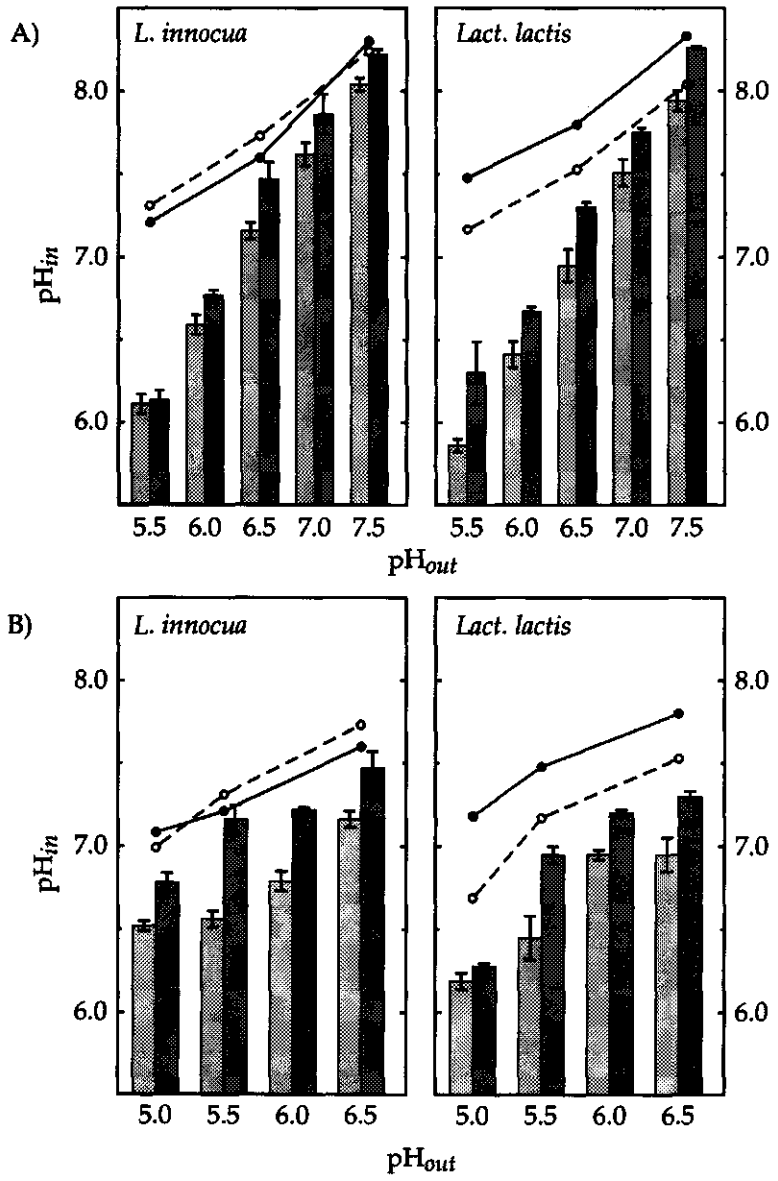

Figure 7.3: pH_{in} as a function of pH_{out} for *Listeria innocua* and *Lactococcus lactis*. Cells were grown in the absence (▨) (control cells) and in the presence (■) of 400 mM sodium lactate, and assayed in buffer containing either 400 mM total lactate (A) or 0.9 mM undissociated lactic acid (B). The amount of undissociated acid present in buffer A is 9.0 mM at pH 5.5, 2.9 mM at pH 6.0, 0.9 mM at pH 6.5, 0.3 mM at pH 7.0 and 0.1 mM at pH 7.5. The solid line represents pH_{in} values of cells grown in the presence of 400 mM sodium lactate assayed in buffer without lactate. The dashed line represents pH_{in} values of control cells assayed in buffer without lactate.

Patchett *et al.* (32) reported a high NADH oxidase activity in aerobically grown *L. monocytogenes* that is possibly associated with the membrane fraction and inhibited by classical respiratory chain inhibitors rotenone and potassium cyanide (KCN). In the present study, the rates of oxygen consumption in cells grown without lactate and without shaking were about 200 and 80 nmol O_2 per min. per mg protein for *L. innocua* and *Lact. lactis*, respectively. When 200 mM lactate was present during the assay, in both organisms the rates of oxygen consumption increased and more acetic acid was produced (data not shown). Subsequently, the influence of KCN and the F_1F_0-ATPase inhibitor N,N'-dicyclohexylcarbodiimide (DCCD) on pH_{in} regulation were examined. In the presence of 10 mM KCN, *Lact. lactis* and *L. innocua* were able to increase their pH_{in} upon addition of glucose, whereas this was prevented in the presence of DCCD (data not shown). This observation indicates that in both organisms the F_1F_0-ATPase is important for pH_{in} regulation.

7.3.3 Proton-translocating Adenosine Triphosphatase activity in *Listeria innocua* and *Lactococcus lactis*

F_1F_0-ATPase was detected by immuno-blotting using rabbit antibodies raised against subunit ß of the *E. coli* F_1F_0-ATPase. A membrane preparation of *E. coli* DW2 served as control. Antigen-antibody reactions were detected in all three strains, and revealed bands with apparent

Table 7.1: Influence of 0.9 mM undissociated organic acid in 50 mM MES/MOPS buffer (pH_{out} = 5.5) on pH_{in} values of *Listeria innocua* and *Lactococcus lactis* cells that were grown either in basal medium or in basal medium supplemented with 400 mM lactate. The pH during growth was not regulated.

			pH_{in}			
			Listeria innocua		*Lactococcus lactis*	
Type of acid	pK_a	total acid (mM)	basal medium	medium + 400 mM lactate	basal medium	medium + 400 mM lactate
No addition	–	0	7.3	7.2	7.2	7.5
Acetic acid	4.75	7	7.0	7.0	6.6	7.1
Lactic acid	3.86	45	6.6	7.1	6.5	7.0
Formic acid	3.75	57	6.4	5.8	6.4	6.4
Phenoxyacetic acid	3.12	240	5.9	5.7	5.9	5.8

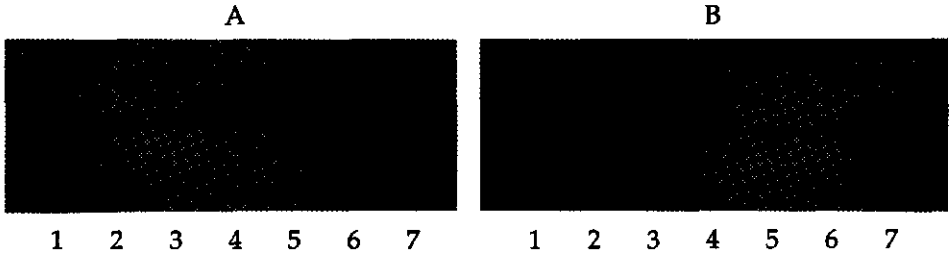

Figure 7.4: The presence of F_1F_0-ATPase in *Listeria innocua* (A) and *Lactococcus lactis* (B). The enzyme was detected in membranes after blotting and incubation with antibodies against the ß subunit of the *Escherichia coli* F_1F_0-ATPase. Cells were grown at different pH values with or without lactate: lane 1+7: reference *E. coli* DW2; lane 2: grown at pH 7.5 without lactate; lane 3: grown at pH 7.5 in the presence of 400 mM sodium lactate; lane 4: grown at pH 6.5 without lactate; lane 5: grown at pH 6.5 in the presence of 400 mM lactate; lane 6: grown at pH 5.0 without lactate.

molecular masses of about 50 kD (Fig. 7.4), which corresponds to the molecular mass reported for the ß subunit of the F_1 part of *E. coli* ATPase (12). No cross reactivity with other proteins was observed. Since *L. innocua* and *Lact. lactis* cells grown at low pH_{out} and in the presence of sodium lactate were able to regulate their pH_{in} better than cells grown at more alkaline pH and without sodium lactate, we anticipated differences in the expression of the F_1F_0-ATPase. However, large differences (more than 2-3 fold) were not seen with the antibody directed against the ß subunit of F_1. Moreover, the shape and the intensity of the bands were not homogeneous, which complicated the comparison (Fig. 7.4).

The pH optima of the *Lact. lactis* and *L. innocua* F_1F_0-ATPase were similar at around 6-7 (Fig. 7.5). At pH 5.0, the ATPase activity of *Lact. lactis* decreased to only 5% of its maximum value, whereas in *L. innocua*, the ATPase activity was relatively high at this pH (65% of the maximum activity), which may give *L. innocua* an advantage over *Lact. lactis* when grown under conditions that result in a low pH_{in} (Fig. 7.5).

The *Lact. lactis* ATPase was inhibited by 1 mM DCCD (80% inhibition, data not shown). ATPase activity in the absence of Triton X-100 was 80% of that observed in the presence of Triton (data not shown), indicating that most of the *Lact. lactis* membrane vesicles had an inside-out orientation. Similar results were obtained with *Lact. lactis* membrane

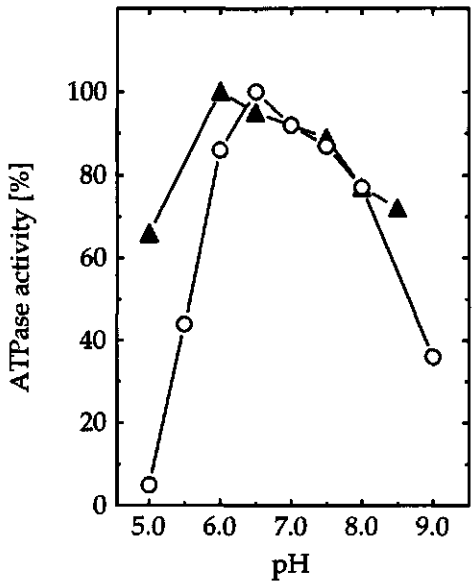

Figure 7.5: The pH dependency of ATPase activity in *Lactococcus lactis* (circles) and in *Listeria innocua* membrane vesicles (triangles). 100% corresponds to the release of 1.3 and 0.3 µmol phosphate per minute per mg protein for *Lact. lactis* and *L. innocua*, respectively. At each pH value, the average of the ATPase activities in membrane vesicles from cells that were grown at pH 5, 6.5 and 7.5 without lactate was expressed as a percentage of the maximum value.

vesicles obtained after glass bead treatment of *Lact. lactis* cells as was described for *L. innocua* in the Materials and Methods section.

ATPase activity in *L. innocua* membrane vesicles in the absence of Triton X-100 was about 40% of that observed in the presence of Triton. Apparently, more than one half of the *L. innocua* membrane vesicles was orientated right-side out. The ATPase was not inhibited by 1 mM DCCD (data not shown), which may be explained by a loose coupling between the F_1 and the F_0 part of the enzyme in this organism.

For both strains it was examined whether or not addition of lactate to the growth medium or lowering of the pH of the growth medium by addition of HCl affected the activity of F_1F_0-ATPase, i.e., whether conditions which caused a lowering of pH_{in} resulted in increased F_1F_0-ATPase activity. Measurements were performed in membrane vesicles obtained from cells grown at different pH values and under different acid stress conditions. ATPase activity in membrane vesicles was indeed

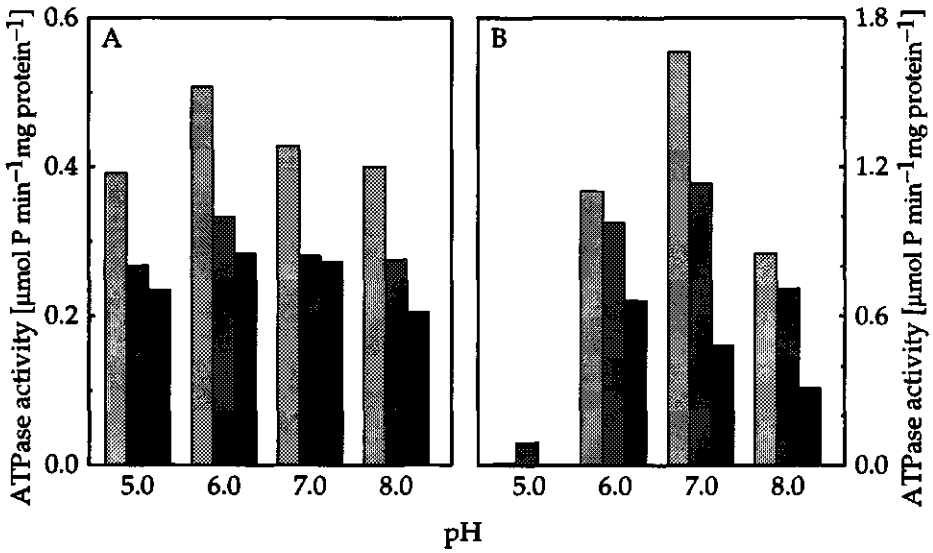

Figure 7.6: ATPase activity in vesicles from *Listeria innocua* (A) and *Lactococcus lactis* (B) of cells that were cultured at different pH values (5.0:▨, 6.5:■, 7.5:■) without addition of lactate to the growth medium. The assay was performed in buffer at the various pH values indicated without addition of sodium lactate to the buffer.

higher in cells grown at low pH (Fig. 7.6). The pH optima of the F_1F_0-ATPase activity after growth at pH 5.0 and pH 7.5 were similar (Fig. 7.6). Addition of 400 mM lactate to the growth medium at pH 6.5 and 7.5 resulted in an increased F_1F_0-ATPase activity in *Lact. lactis* membrane vesicles. With *L. innocua*, this was observed only after addition of lactate at pH 7.5 (data not shown). Addition of 200 mM lactate to the assay buffer did not alter the F_1F_0-ATPase activity, indicating that an increase in the ionic strength and the osmolarity of the buffer had no effect (data not shown). In general, F_1F_0-ATPase activity was higher in *Lact. lactis* than in *L. innocua* as long as the pH of the assay buffer was above 5.0 (Fig. 7.6).

7.4 Discussion

The difference between MICs of NaCl and sodium lactate represents the specific antimicrobial effect of sodium lactate, since equivalent molar concentrations of these substances reduce the water activity to the same extent (15). The correlation between pH and MICs of sodium lactate

suggests that the undissociated molecule plays an important role in the mechanism of lactate inhibition. The dissociated molecule might also play a role in microbial growth inhibition, since the specific antimicrobial effect of lactate was also observed at neutral pH. The antimicrobial effects may arise from high extracellular concentrations of the anion due to its chelating properties (35) or to inhibition of lactate efflux (19).

The pH_{in} values as measured for *Lact. lactis* and *L. innocua* were generally in accordance with data from literature (2,27,33). Both *Lact. lactis* and *L. innocua* were able to adapt to the presence of lactate in the growth medium. Adapted cells had higher pH_{in} values than unadapted cells. Until now, this kind of acid adaptation has been investigated only for *Salmonella typhimurium* (11) and *Enterococcus faecalis* (20). Young and Foegeding (42) reported that growth in the presence of lactate had a negligible effect on pH_{in} of *L. monocytogenes*. The pH_{in} in cells grown at pH 6.0 in the presence of 50 mM lactate (0.3 mM undissociated acid) assayed in a lactate-containing medium did not differ much from that of the control cells grown and assayed in a medium without lactate. These authors thus did not take into account the possibility of adaptation. Assay of the pH_{in} of the control cells in the presence of lactate, would probably have affected the outcome of their experiments, since in our experiments even at pH 7 in the presence of 0.3 mM lactic acid, a difference was observed between the pH_{in} of adapted and unadapted cells (Fig. 7.3*A*).

It is not surprising that the acid adaptation response can be induced also by other acids than lactic acid, as was indeed observed in the present study with phenoxy-acetic acid. Acid adaptation has previously been described for *Salmonella* spp. (23, 24), *Sarcina ventriculi* (14), *E. coli* (13) and *L. monocytogenes* (22) and this was reflected in an increased resistance to inactivation by organic acids and the ability to survive at normally lethal acid pH values. Also, Table 7.1 shows that exposure of *Lact. lactis* to lactate increases its resistance towards acetic acid. Formic acid and phenoxy-acetic acid are more efficient in destroying the pH gradient than the other acids.

To date, considerable research effort has been focussed on the pH_{in} of *Lact. lactis* and of lactic acid bacteria in general and its importance for cellular functioning (6,17,19,20,27,28,33,34). These organisms are able to generate a pH gradient and a membrane potential across the cytoplasmic

membrane by translocating protons outwards via the F_1F_0-ATPase (Fig. 7.7*A*). An alternative mechanism for pmf generation and maintenance in lactococci is the carrier mediated excretion of the metabolic end product lactate in symport with protons (31). The energy of a lactate concentration gradient may be converted into electrochemical gradient of protons according to the energy recycling model (26). It has been described that the efficiency of this process is decreased in the presence of increasing concentrations of extracellular lactate (8,21,38). The conversion of pyruvate to lactate is important for regeneration of NAD^+ needed for glycolysis, and lactate is the main end-product of glucose metabolism under oxygen limited conditions. In the presence of oxygen, however, NAD^+ regeneration can proceed via the cytoplasmic enzyme NADH-oxidase, which is probably widespread among lactic acid bacteria (5). Under these conditions, pyruvate can be converted to acetate as well, which is favourable for the organism since it yields extra

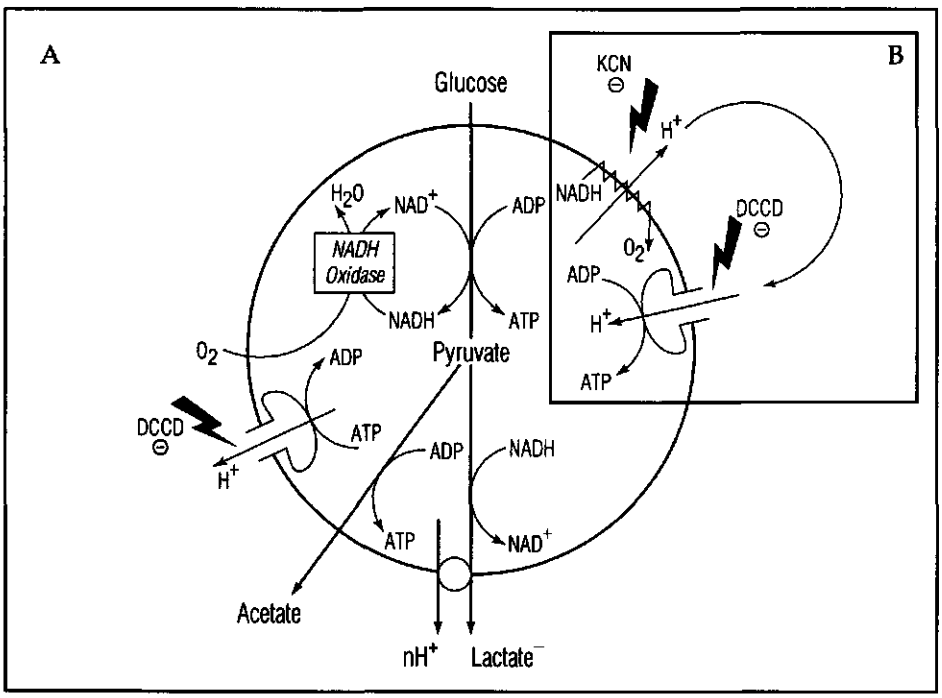

Figure 7.7: Schematic presentation of proton expelling pumps, oxygen consuming reactions and metabolic energy generation as a result of sugar metabolism in *Lactococcus lactis* (A) and *Listeria innocua* (A, B, see text for details).

ATP (Fig. 7.7*A*). In the present study, oxygen consumption concomitant with the production of acetate was observed for *Lact. lactis* under aerobic conditions (data not shown), indicating that NADH-oxidase is present and that the conversions presented in Fig. 7.7*A* can take place. Growth in the presence of 400 mM lactate resulted in increased O_2 consumption rate and an increase in the level of acetate production. Apparently, growth in the presence of lactate induces a metabolic shift towards acetate production concomitant with an increased level of ATP formation (Fig. 7.7*A*). This, in turn, may be used to compensate for increased ATPase activities (see below).

Considerably less is known about the physiology of *L. innocua*. As already stated, the existence of an electron transfer system has been suggested for *Listeria monocytogenes* (32). Under similar conditions, the oxygen consumption rate in *L. innocua* was higher than in *Lact. lactis* (data not shown) which might indicate the presence of an electron transfer system in this organism. During transport of electrons from substrates to oxygen, an electrochemical gradient of protons is generated, which may function as a driving force for the generation of ATP via a proton translocating ATPase (Fig. 7.7*B*). Cyanide is a classical inhibitor of cytochrome oxidases, involved in the final transfer of electrons to oxygen. However, cyanide had a moderate inhibitory effect on oxygen consumption both in *L. innocua* and *Lact. lactis*. It is therefore likely that O_2 consumption in *L. innocua* is also explained by the presence of a cytoplasmic NADH oxidase, similar to that in *Lact. lactis* (Fig. 7.7*A*). Trivett and Meyer (39) reported a specific activity of this enzyme in *L. monocytogenes* of 270 nmoles of oxygen per min. per mg protein which is in accordance with our results. In the presence of DCCD, the pH_{in} in *Lact. lactis* as well as in *L. innocua* cells did not increase upon the addition of glucose, whereas it did in the presence of KCN (data not shown). This argues against an important role of a proton-translocating electron transport chain in proton motive force (pmf) generation in *L. innocua*. The results of Verheul *et al.* (40,41) showed that addition of DCCD to *L. monocytogenes* cells resulted in dissipation of the membrane potential in cells in which the ΔpH was collapsed by nigericin. This observation also suggests that a pmf is merely formed by a proton translocating ATPase in *Listeria* spp. (Fig. 7.7*A*).

Taking all these observations into consideration it is most likely that the

acid adaptation of *Lact. lactis* and *L. innocua* is explained by an increased activity of the F_1F_0-ATPase, induced by growth in the presence of organic acid or at low pH_{out}. Indeed, the ATPase activity in membrane vesicles obtained from cells grown at low pH was higher than from cells grown at pH 7.5. F_1F_0-ATPase activity in *L. innocua* and *Lact. lactis* was stimulated 1.6-1.8 fold and 3.5 fold, respectively (Fig. 7.6). In contrast to *Lact. lactis*, ATPase activity in *L. innocua* membrane vesicles was not inhibited by DCCD. This may be due to a loose coupling between the F_1 and the F_0 part of the enzyme induced during vesicle preparation or storage in liquid nitrogen. Assuming that ATPase activity in membrane vesicles reflects the activity in growing cells, it is concluded that the amount of F_1F_0-ATPase has increased as a result of the adaptation processes. This increase was not directly obvious from the immuno-blots due to the irregular shape and intensity of the bands that hampered a correct comparison (Fig. 7.4).

Kobayashi *et al.* (20) observed an 1.6-fold increase in the ATPase activity of *Enterococcus faecalis* cells grown at pH_{out} 6.0 compared with cells grown at pH_{out} 7.3, which was associated with a 2-fold increase in the amount of F_1F_0-ATPase subunits. Chaia *et al.* (3) reported a 2.5-fold increase in the F_1F_0-ATPase activity from *Lactobacillus helveticus* and somewhat less for *Lactobacillus casei* as a result of growth in the presence of 15 mM propionic acid. The proton translocating ATPase has also been associated with pH homeostasis in other lactic acid bacteria (17). We now supply evidence that the proton translocating ATPase also plays a role in pH_{in} regulation of *Listeria* spp.

The biochemical characteristics (*i.e.* pH-optimum for activity and activity at low pH_{in}) of the F_1F_0-ATPase can also be responsible for differences between acid tolerances of micro-organisms (1,36). However, Fig. 7.6 shows that the pH optimum of the F_1F_0-ATPase of *Lact. lactis* and *L. innocua* did not change under different growth conditions. The ATPase activity of *L. innocua* is less dependent on the pH than that of *Lact. lactis*; the ATPase of *L. innocua* is highly active at pH 5, while the activity of the *Lact. lactis* ATPase is inhibited for 95% (Fig. 7.5).

Cellular functions are inhibited when the cytoplasmic pH decreases below a certain threshold value (19). These threshold values were reported to be about 6.0 (28,34) and 5.5 (42) for *Lact. lactis* and *Listeria monocytogenes*, respectively. Although the present study showed that

pH_{in} decreased in the presence of lactate, the pH_{in} values were well above these limits under all conditions tested (Figs. 7.2 and 7.3). Therefore, the inhibitory effect of lactate on growth at low pH cannot directly be explained by its effect on pH_{in}, and other factors are probably involved as well.

This study has contributed to the understanding of acid adaptation in *Lact. lactis* and *L. innocua*. Increased acid resistance is associated with increased pH_{in} values in these organisms. Accordingly, increased ATPase activities in combination with inhibitor studies revealed that in *L. innocua*, like in *Lact. lactis*, the proton translocating ATPase plays a crucial role in pH_{in} regulation. The amount and activity of the proton translocating ATPase as well as the cell's capacity to shift to other end products from sugar metabolism *i.e.* production of acetate in the presence of O_2 concomitant with production of ATP, probably play a role in the ability to grow in - and adapt to the presence of organic acids.

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8

General discussion

8.1 Introduction

The subject that is being dealt with in this thesis is related to the safety of meat products, and is aimed towards understanding how sodium lactate influences the growth of micro-organisms in meat products with different pH values under various storage conditions (temperature, oxygen). The study was mainly performed in broth as a model for food systems, but part of the results were evaluated in a meat product as well. Recently, the antimicrobial activity of lactate has been reviewed by Shelef (21). Supplementary to this review and other literature data, this thesis aims to present a systematic and integral study which deals with different micro-organisms and different aspects of growth inhibition by sodium lactate. The topic was approached along 4 lines:

1) Screening: For various micro-organisms, the minimum inhibitory concentration (MIC) of sodium lactate was compared with that of sodium chloride, to determine the specific inhibitory effect of sodium lactate and the influence of temperature and pH on this effect.
2) Predictive modelling: Growth studies were performed in the presence of increasing concentrations of sodium lactate or NaCl up to the MIC at various pH values and temperatures. The effects on the maximum specific growth rate of *Listeria innocua* were translated into a mathematical equation that can be used to predict the growth rate under conditions that were not tested.
3) Mechanistic approach: Acid adaptation and the influence of sodium lactate on intracellular pH (pH_{in}) were investigated in *L. innocua* and *Lactococcus lactis* in an attempt to explain the observed differences

between the influence of pH on the MIC of sodium lactate for those organisms.

4) Spores: Next to the effect of lactate on growth of vegetative cells, its influence on spore germination and heat resistance of spores was investigated, with special emphasis on *Clostridium botulinum*. The inhibitory effect of lactate on toxin production by this organism was examined as well.

8.2 MIC values

In Chapters 2 and 3, the minimum inhibitory concentrations (MICs) of sodium lactate, defined as the lowest concentration at which no growth of a certain micro-organism was observed under the conditions of testing, were determined for spoilage organisms and pathogens that may occur in meat products. The influence of temperature and pH on MICs was determined as well. The MIC of sodium lactate was compared with that of NaCl, and the difference was defined as the specific inhibitory effect of sodium lactate. To be sure that the water activity (a_w) in solutions containing equal molarities of sodium lactate and NaCl was the same, a_w measurements were performed which were based on freezing point reductions, measured with a cryoscope 4L2 (Advanced Instruments inc., Needham Heights, Massachusetts), the temperature of which had been set at –7°C, and the data were compared to values obtained with a Novasina a_w meter EEJA-3 at 25°C (Novasina AG, Pfäffikon, Switzerland). Both methods of a_w measurement gave similar results (unpublished data). It turned out that the freezing point measurements were more suitable for solutions with low salt concentrations (up to 0.3 M) which require a longer time to reach equilibrium in the Novasina a_w-meter. This increases the risk of yielding less accurate values. With a further increase of salt concentration the solutions had to be diluted to ensure a rapid and reproducible freezing behaviour. This had the disadvantage of being more labour-intensive, but the dilution did not affect the accuracy of the measurement as the results of the Novasina and freezing point measurements were identical up to salt concentrations of 1.4 M (unpublished data).

Most of the almost 200 strains that were screened were specifically

inhibited by sodium lactate, although differences were observed between different genera as well as between different species of a specific genus (Chapter 2). The medium in which the screening was performed had a pH near neutrality and contained yeast extract as a source of betaine and proline (8).These solutes are important osmoprotectants for Gram-negative bacteria as well as for lactobacilli, *Lact. lactis, Listeria monocytogenes and Staphylococcus aureus* (1,2,3,5,12,13,14,17,18). Yeasts are capable of synthesizing trehalose (9), and dominant species in spoilage of cured meat are very salt tolerant (6). The screening thus was performed under otherwise optimal conditions. It was shown that especially bacterial strains that were able to grow at low a_w (≤ 0.95) in the presence of NaCl were inhibited by sodium lactate (*S. aureus, L. monocytogenes, Brochothrix thermosphacta*). Spoilage bacteria as well as pathogens were inhibited by sodium lactate, and although yeasts were relatively insensitive, the MIC of sodium lactate was always lower than that of NaCl (Chapter 2). This is a positive outcome with respect to the potential reduction in the NaCl content of food products. According to these results, the microbial quality of food products may be increased when part of the NaCl would be replaced by sodium lactate.

If MICs of sodium lactate decreased at low temperatures, those of NaCl decreased as well, indicating that the increased sensitivity of the bacteria at these temperatures is due to a lowering of the a_w. For most bacteria, MICs of sodium lactate strongly decreased at lower pH values (Chapter 3). This might be explained by an increase in the concentration of undissociated molecules, resulting in a decrease of the intracellular pH (pH_{in}) and subsequent effects on metabolism and transport processes. For *S. aureus, Lact. lactis* and some lactic acid bacteria isolated from spoiled meat products, the MICs of sodium lactate changed only slightly with pH (Chapter 3).

In conclusion, considerable differences were observed among MICs of sodium lactate for various micro-organisms, and the influence of pH on those MICs. The question arises how these differences can be explained. It is generally accepted that the potency of weak acids to inhibit growth of micro-organisms is related to the capacity of the undissociated (protonated) molecule to dissipate the pH gradient, thereby reducing the pH_{in}. Besides an effect on pH_{in}, lactic acid has influence on microbial energetics. Organic acids which act as protonophores cause an inward

leak of protons and decrease the pmf. In the presence of oxygen, energy production by respiratory organisms takes place very efficiently by coupling proton extrusion via electron transport to ATP synthesis via the F_1F_0-ATPase. Under anaerobic conditions, energy production by these organisms is much less efficient, even if anaerobic respiration occurs using alternative electron acceptors such as nitrate or fumarate (19). Similarly, *Lactococcus lactis* and *Listeria innocua* may profit from oxygen by their ability to induce a metabolic shift towards acetate production concomitant with an increased production of ATP (Chapter 7). It is therefore expected that more lactic acid will be needed to inhibit growth of these micro-organisms under aerobic than under anaerobic conditions. An alternative way to gain energy which has been described for lactococci is the carrier mediated excretion of the metabolic end product lactate. This process is also inhibited in the presence of increasing concentrations of extracellular lactate which may result in an additional inhibitory effect of lactate on growth of these micro-organisms (7,16,22).

Screening experiments were also performed under semi-anaerobic conditions (unpublished results). After incubation of tissue culture plates in a jar with gaspak system (BBL), it took one and a half day for the media to become completely anaerobic, as was observed from growth media containing the indicator resazurine. As a result, the aerobic indicator organism *Pseudomonas fragi* could grow at low NaCl concentrations but not in the presence of sodium lactate, whereas the growth of the anaerobic indicator organism *Clostridium sporogenes* was not prevented. Yeasts were not able to grow, but this might also be related to the high final H_2 concentration (25%) in the atmosphere (20). In general, MIC values of sodium lactate were more affected by semi-anaerobic conditions than MIC values of NaCl, which is in agreement with the hypothesis mentioned above. Lactic acid bacteria were less sensitive towards the effect of lactate under semi-anaerobic growth conditions than were *Listeria* spp., *B. thermosphacta*, and *Yersinia enterocolitica* (unpublished results). Grau (10,11) reported lactate to be more efficient in inhibiting anaerobic growth of *B. thermosphacta* and *Y. enterocolitica* than in inhibiting aerobic growth of these micro-organisms.

The information that was obtained with screening experiments is very global, but it is a method which is inexpensive and time-saving and

which therefore is appropriate to obtain information on the lactate sensitivity of a large number of different bacterial strains within a relatively short time.

8.3 Predictive modelling

The conclusion that can be drawn from Chapters 2 and 3 is, that although sodium lactate specifically inhibits growth of a large number of food spoilage bacteria and pathogens, the quantities that are needed for totally preventing growth of these bacteria are so high, that in practice we have to consider the possibility of their growth. Therefore, examining the inhibitory effect of sodium lactate at concentrations below the MIC is also necessary. In contrast to screening experiments, growth experiments are very time consuming, but the information that is obtained from growth curves is more specific. Not only is the effect of increasing lactate concentrations on growth assessed, but it can also be observed whether the lag time, the maximum specific growth rate and/or the final yield are influenced by sodium lactate.

The effect of sodium lactate can be compared to that of NaCl. Figures 8.1 and 8.2 show that an increase in the lag time (λ, left y-axis, indicated with circles) as a result of addition of sodium lactate or NaCl is mainly due to a lowering of the a_w, since the lines from both salts run parallel. This is different for the yield (right y-axis, indicated with triangles). Although at concentrations near the MIC, the yield in the presence of sodium lactate was still more than 90% of that in the absence of salt, it can be observed from these figures that sodium lactate decreased the yield more efficiently than sodium chloride, and that at pH 7 (Fig. 8.1), sodium lactate had a smaller effect on the yield than at pH 6 (Fig. 8.2). This indicates that under these conditions the specific inhibitory effect of sodium lactate is confined to the growth rate and to the yield. This information is also valuable for exploring the mechanism of lactate inhibition. It was therefore important not only to determine and describe the inhibitory effect of sodium lactate, but to translate it into a mathematical equation as well.

To come to this equation, the modelling was divided into three stages. The first stage involved modelling of the bacterial growth curves by the

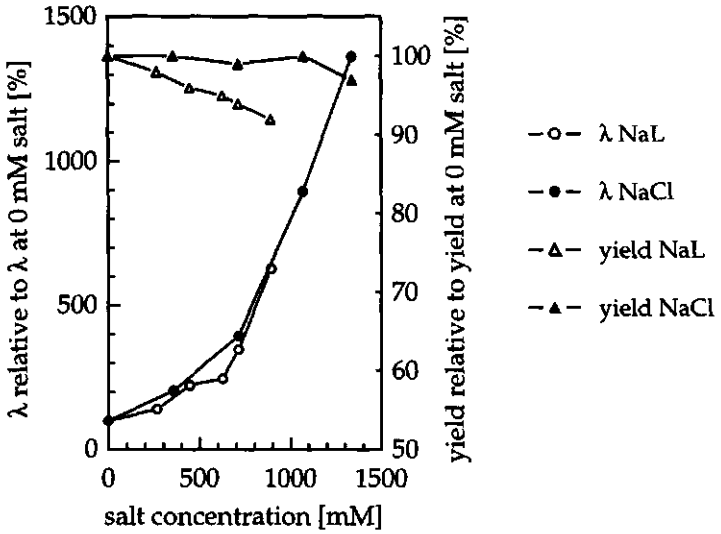

Figure 8.1: Influence of sodium lactate (NaL) or NaCl concentration (mM) on the lag time (left y-axis) and on the number of cfu per ml of *L. innocua* in the stationary growth phase (right y-axis) at pH 7

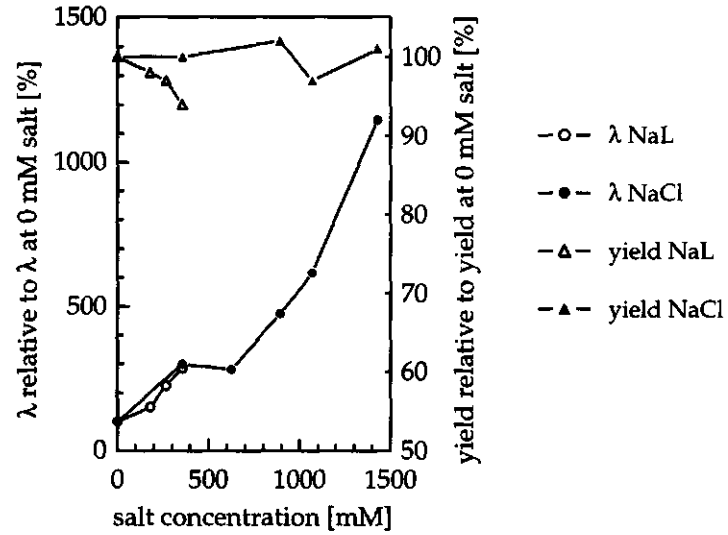

Figure 8.2: Influence of sodium lactate (NaL) or NaCl concentration (mM) on the lag time (left y-axis) and on the number of cfu per ml of *L. innocua* in the stationary growth phase (right y-axis) at pH 6.0

modified Gompertz equation. The second stage was confined to describing the maximum specific growth rate of *L. innocua* as a function of sodium lactate and NaCl concentration. These two stages have been

described in Chapter 5. Initially, the influence of temperature and pH was not incorporated since the goal of this study was to obtain a relatively simple model that could be of help in exploring the mechanism of lactate inhibition. Moreover, in this way it was possible to compare the effect of sodium lactate with that of NaCl apart from the effect of temperature and pH, which was important since Chapter 3 of this thesis showed that the influence of pH on the inhibitory effect of sodium lactate and NaCl was different. The influence of temperature and pH on microbial growth inhibition by sodium lactate and NaCl was assessed in the third stage (Chapter 6). As a result, the number of parameters that were included in the model increased from 3 to 5 for sodium lactate and from 3 to 4 for NaCl, but concomitantly, the number of parameters necessary to describe the whole set of experimental data was substantially reduced from 42 to 5 (sodium lactate) and from 48 to 4 (NaCl). Inevitably, this reduction was accompanied by a decline in the accuracy of the model predictions, but this was a controlled process in which each parameter omission was statistically evaluated by comparing the accuracy with that of the original model.

The growth experiments confirmed the results that were obtained with screening procedures, namely, that much higher concentrations were needed for growth inhibition by NaCl, compared to those of sodium lactate. Again, the pH of the growth medium was shown to exert a strong influence on the MIC of sodium lactate, but not on the MIC of NaCl. At 4°C, the MICs of sodium lactate and NaCl were lower than at the higher temperatures tested. The curves in Fig. 5.1 show that at high pH, the influence of increasing sodium lactate concentrations on the maximum specific growth rate of *L. innocua* is much smaller than at low pH. This again could be linked to the concentration of undissociated acid, increasing more rapidly with total lactate concentration at low pH than at high pH values. The more undissociated molecules are present, the heavier the load on the pH homeostasis mechanisms of the cell will be. This could also be related to the observation that the yield in the presence of sodium lactate is decreased and might be explained by a decrease in the amount of metabolic energy that is available for growth as was discussed before.

Once we had developed a model that could successfully be used to predict the growth of *L. innocua* in broth, the next step was to validate it

in a meat product, since that is the system on which the studies described in this thesis were based. We therefore chose the product composition and packaging method to resemble sausage manufacturing practice although that included the growth conditions for *L. innocua* being less favourable than in broth (absence of O_2; presence of nitrite and NaCl). As a result, the growth rate was lower in the meat product. The growth rate in the presence of sodium lactate was strongly dependent on the pH of the sausage, whereas the effect of pH on growth in the presence of NaCl alone was relatively small, as was observed in broth.

Of course, the question arises of how much use this model is in predicting the growth of other microbial species in other meat products formulated with sodium lactate. This question can only be answered after the microbial growth in the presence of lactate has been investigated with other micro-organisms and other meat products, However, it has been shown that after adjustment of the maximum specific growth rate in the absence of sodium lactate, the model was suitable for predicting the growth of *L. innocua* under conditions that differed largely from those on which the model was based. That is why expectations on the general applicability of the model are high.

8.4 Mechanistic approach

When lactate is frequently used in a meat plant, a microbial flora might develop that becomes more resistant towards the inhibitory effects of this substance. Therefore it was investigated whether acid adaptation occurs and how this is achieved, with *Lact. lactis* and *L. innocua* as target organisms. Initially, the experiments were focused on the intracellular pH (pH_{in}) because this parameter is critical for cellular functioning including transport processes, energy state, metabolism and intracellular enzyme activity. Cook and Russell (4) showed that glycolytic activity in *Lact. lactis* decreased when the pH_{in} declined, and as a result the intracellular ATP level was decreased. From these experiments the authors concluded that the energy available for growth was a key factor in pH sensitivity.

The results in Chapter 3 and 7 of this thesis showed that for most of the organisms tested (including *L. innocua*), pH had a strong influence on the MIC of sodium lactate, suggesting an important role for the

undissociated lactic acid molecule in the mechanism of lactate inhibition. However, *Lact. lactis* belongs to the group of organisms for which pH had not so much effect on the inhibitory activity of sodium lactate (Chapter 3). It was therefore expected that differences would be observed between the effect of lactate on pH_{in} of these two organisms which might be explained by differences in pH homeostasis mechanisms. The adaptation of *L. innocua* towards sodium lactate (*i.e.* increased pH_{in} values) was only observed when sodium lactate was present in the buffer. The signal that triggered the adaptation response was shown not to be specific for the presence of sodium lactate, since the response of the bacteria after they had been grown in the presence of phenoxyacetic acid did not differ from that after growth in the presence of sodium lactate (Chapter 7).

Experiments with whole cells showed that, independent of whether the cells were grown with or without lactate, in both organisms the pH_{in} increased after addition of glucose and that this was prevented in the presence of N,N'-dicyclohexylcarbodiimide (DCCD). The presence of F_1F_0-ATPases has been established in both organisms by immunoblotting. ATPase activity increased as a result of growth at low pH (5.5), compared to that in control cells (pH 7). It is therefore likely that the proton translocating F_1F_0-ATPase plays an important role in pH_{in} regulation in both species (Chapter 7). More attention has to be paid to the question as to why the ATPase activity in membrane vesicles of *L. innocua* was not inhibited by DCCD, and to the possible role of an electron transport chain in pH_{in} regulation in this organism. Next to increased proton extrusion due to the activity of proton pumps, increased acid tolerance may be achieved by decreased membrane permeability to protons or an increased buffering capacity of the cytoplasm. None of these hypotheses explains, however, why acid adaptation in *L. innocua* is only observed in the presence of sodium lactate. Furthermore, complete inhibition of growth at low pH by lactate could not directly be explained by its effect on pH_{in}, because the proposed minimum pH values needed for growth (5.5 (23) and 5.7 (15) for *L. innocua* and *Lact. lactis*, respectively) were not reached. Therefore other factors may be involved in the mechanism of microbial growth inhibition by lactate as well.

8.5 Spore formers

Since many meat products in which sodium lactate is used undergo a heat treatment, it is of significant importance to investigate the influence of sodium lactate on spore germination and subsequent growth of the vegetative cells. In Chapter 4, the results that were obtained with *C. botulinum* were described. In addition, aerobic spore germination and growth in the presence of sodium lactate were investigated with different *Bacillus* strains: *B. cereus* P5; *B. cereus* VC1, *B. cereus* DSM 31 (type strain) and *B. subtilis* BGA (unpublished data). It appeared that the pH of the growth medium had a strong influence on the MIC of sodium lactate: the ratio between MICs of NaCl and sodium lactate for *B. cereus* was about 1.5 at pH 6.5 and pH 7.0, but increased to 4 at pH 6.0. *B. subtilis* was far more tolerant towards NaCl: MICs exceeded 1785 mM under almost all conditions tested, but the MIC of sodium lactate was not different from that of the *B. cereus* strains. At 12°C, MICs of NaCl and sodium lactate were lower than at 20°C and 30°C (unpublished data).

Germination experiments in the presence of various concentrations of NaCl or sodium lactate showed that all spores were able to germinate within 2 to 5 hours at 30°C, pH 6.5. In the presence of equal concentrations of sodium lactate and NaCl, sodium lactate was more inhibitory to spore germination than NaCl (unpublished data).

It thus can be concluded that the specific inhibitory effect of sodium lactate on spore germination and subsequent growth of vegetative cells exists in both aerobic and anaerobic spore formers and that at low temperatures less sodium lactate is needed to inhibit growth, which is favourable for its use in meat products that are stored refrigerated.

8.6 Concluding remarks

The studies described in this thesis have increased our knowledge on the action spectrum and the mechanism of the antimicrobial activity of sodium lactate. It has been shown that the use of sodium lactate in meat products could offer several advantages when compared to the use of NaCl at equal molar concentrations, which include a better inhibition of spore germination and growth of vegetative cells and inhibition of toxin production by *C. botulinum*. Although the specific inhibitory effect was

more pronounced at low pH, it was often observed at pH near neutrality as well.

With respect to the use of lactate in meat products, the consequences of acid adaptation responses in various bacteria need to be studied more closely. It is important to investigate whether the MIC of sodium lactate increases as a result of acid adaptation and whether this effect is reversible or not. Furthermore, in this thesis no attention has been paid to the effect of sodium lactate on osmoregulation. The mechanism of lactate inhibition is probably complex and remains to be further elucidated. The changes that occur in microbial cells as a result of growth in the presence of increasing sodium lactate concentrations may provide important clues to the mechanism of growth inhibition by lactate. Furthermore, experiments with insertion mutants could contribute to a better understanding of the proteins involved in adaptation towards sodium lactate. Once the mechanism is fully understood, it will be easier to fully exploit the potentials of sodium lactate utilization in meat products.

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Summary

In this thesis, several aspects of the antimicrobial activity of sodium lactate were investigated to assess the potentials of lactate for shelf life extension, with special emphasis on its use in meat products. NaCl was used as a reference to distinguish between the effect of water activity (a_w) and the specific inhibitory effect of sodium lactate.

For about 200 strains, including spoilage organisms and pathogens, minimum inhibitory concentrations (MICs) were determined in a peptone-yeast extract broth at pH 6.5 and 20°C (Chapter 2). For almost all strains, the specific inhibitory effect of sodium lactate, defined as the difference between the MIC of sodium lactate and that of NaCl, was demonstrated. Especially strains that were able to grow at a_w limits of 0.95 or below in the presence of NaCl (*Staphylococcus aureus, Listeria monocytogenes, Brochothrix thermosphacta*) were inhibited by sodium lactate.

In Chapter 3, the influences of temperature (4°C-37°C) and pH (5.7-7.0) on MICs of sodium lactate and NaCl were determined with a selection of 20 different strains. If MICs of sodium lactate decreased at low temperature, those of NaCl decreased as well. MICs of NaCl were independent of the pH. For most of the bacteria, MICs of sodium lactate strongly decreased at lower pH values. At pH 5.7, the presence of sodium lactate (≥ 268 mM) often did not allow for growth. However, for *S. aureus*, *Lactococcus lactis* and some lactic acid bacteria isolated from spoiled meat products, the MIC of sodium lactate did not change with pH. These organisms were relatively strongly inhibited by sodium lactate at high pH (7.0). Yeasts were less sensitive towards sodium lactate than the bacteria, but sodium lactate had a specific inhibitory effect on growth of these organisms when compared to the effect of NaCl.

Chapter 4 describes the effect of sodium lactate on toxin production, spore germination and heat resistance of proteolytic *Clostridium botulinum* strains. Toxin production was delayed by sodium lactate concentrations of 1.5 to 2% (w/v) at 15°C and 20°C. In the presence of 3%, 4% and >4% sodium lactate, no toxin was detected within a period of

32–49 days at 15°C, 20°C and 30°C, respectively. The inhibitory effect of NaCl at concentrations resulting in an identical a_w as obtained with sodium lactate was negligible, indicating that the inhibitory effect of sodium lactate on toxin production was not caused by decreasing a_w. No clear synergistic effect of sodium lactate (1.5 or 2.5%) and NaCl (2.1%) on delaying toxin production was observed. It was shown that 4% sodium lactate inhibited germination of the *C. botulinum* spores, which may partially explain the inhibitory effect of sodium lactate on growth and toxin production. In the presence of sodium lactate or NaCl, a tendency of increased heat resistance of *C. botulinum* spores was observed, but the effects were not significant.

The MICs of sodium lactate for spoilage organisms and pathogens were so high, that for practical use, the influence of sodium lactate on growth characteristics at concentrations below the MIC had to be examined as well (Chapter 5). Growth experiments with *Listeria innocua* were performed in a peptone-yeast extract broth at pH 5.5, 6.0, 6.5 and 7.0 at 4, 10, 20 and 30°C, and the resulting data were fitted to a modified Gompertz model. Complete growth inhibition of *L. innocua* at pH 5.5 occurred in the presence of 217 mM sodium lactate, whereas at neutral pH 1071-1339 mM was needed. MIC values for undissociated acid increased with decreasing pH from 0.8 mM at pH 7 to 5 mM at pH 5.5. In the presence of sodium lactate, growth rates decreased progressively with increasing concentrations down to 0 at the MIC value, and were strongly influenced by both temperature and pH. In the presence of NaCl, growth rates were influenced by temperature only. It was shown that a modified Monod equation with 3 parameters was effective for description of growth rates of *L. innocua* at sodium lactate and NaCl concentrations over the whole experimental range (Chapter 5).

Supplementary to this study, pH and temperature were included as variables so that the model can also be used for predictions at temperatures and pH values different from those that were used to collect the experimental data (Chapter 6). The combined effects of temperature and pH were assessed by translating the parameters of the modified Monod equation (μ_m, α and p') into functions of pH and/or temperature. The number of parameters needed to describe the experimental data was thereby reduced from 48 to 4 (NaCl) and from 42 to 5 (sodium lactate). The decline in the goodness of fit that accompanied

the reduction in the amount of parameters was within statistically acceptable ranges. The resulting model was compared to a polynomial fit, and it was proposed that the former was more suitable for the purpose of this study. The broth model for sodium lactate was evaluated in Bologna-type sausages (Chapter 6). Due to the "worst-case" design of the broth model, it was necessary to re-estimate at least one parameter to obtain a good description of the growth rate of *L. innocua* in the meat product. However, the simplicity of the model and the practical usefulness of its parameters offer considerable prospects for its use in predictive microbiology.

A more detailed study of the antimicrobial effect of sodium lactate dealt with lactate sensitivity and acid adaptation in *L. innocua* and *Lact. lactis* grown in the absence and presence of sodium lactate (Chapter 7). Intracellular pH (pH_{in}) measurements showed that when *L. innocua* or *Lact. lactis* cells were grown in the presence of sodium lactate, their pH_{in} increased compared to that in control cells. This was observed both in the absence (*Lact. lactis*) and in the presence (*Lact. lactis* and *L. innocua*) of sodium lactate in the assay buffer. In both organisms, the presence of F_1F_0-ATPase was demonstrated by immuno-blotting. An electron transfer system has been suggested to be present in *Listeria*, which could have a role in pH_{in} regulation. Experiments employing the inhibitors N,N'-dicyclohexylcarbodiimide (DCCD) and potassium cyanide (KCN) were performed to examine the possible roles of the F_1F_0-ATPase or the electron transport chain, respectively, in pH_{in} regulation. In both organisms, addition of 10 mM KCN did allow the pH_{in} to rise after addition of glucose, whereas this was prevented after incubation in the presence of 1mM DCCD. These results suggest that in both *Lact. lactis*, and *L. innocua*, the F_1F_0-ATPase is the major pH_{in} regulator.

In conclusion, this thesis has increased our knowledge on the action spectrum and the mechanism of the antimicrobial activity of sodium lactate. It has been shown that the use of sodium lactate in meat products could offer several advantages when compared to the use of NaCl at equal molar concentrations.

Samenvatting

In dit proefschrift werden verschillende aspecten van de antimicrobiële werking van natriumlactaat belicht om na te gaan welke mogelijkheden het gebruik van deze stof biedt voor verlenging van de houdbaarheid van levensmiddelen. De nadruk lag daarbij op de toepassing ervan in vleesproducten. Natriumlactaat verlaagt de wateractiviteit (a_w), maar heeft daarnaast ook nog een specifieke remming op de groei van micro-organismen. Om onderscheid te kunnen maken tussen deze effecten werd de groeiremmende werking van natriumlactaat vergeleken met die van NaCl.

Voor in totaal ongeveer 200 gist- en bacteriestammen (waaronder zowel bederfveroorzakende als ziekteverwekkende organismen) werd bepaald welke concentraties NaCl of natriumlactaat minimaal nodig zijn om de groei van deze micro-organismen volledig te remmen (de zogenaamde MIC-waarde). Het onderzoek werd uitgevoerd bij 20°C in bouillon met een pH van 6,5 (Hoofdstuk 2). De MIC-waarde van NaCl (uitgedrukt in mM) was in bijna alle gevallen hoger dan die van natriumlactaat. De remming door natriumlactaat kan dus niet enkel aan een verlaging van de a_w worden toegeschreven. Vooral die organismen die in aanwezigheid van NaCl bij een lage a_w kunnen groeien werden in sterke mate geremd door natriumlactaat (*Staphylococcus aureus, Listeria monocytogenes, Brochothrix thermosphacta*).

In Hoofdstuk 3 werd met een selectie van 20 stammen de invloed van temperatuur (4°C–37°C) en pH (5,7–7,0) op MIC-waarden van natriumlactaat en NaCl bepaald. Indien bij lage temperatuur een verlaging van de MIC-waarde optrad, werd dit voor zowel natriumlactaat als NaCl waargenomen. De MIC-waarden van NaCl werden niet door de pH beïnvloed terwijl in de meeste gevallen de MIC-waarden van natriumlactaat bij lage pH beduidend lager waren dan bij hoge pH. Bijna geen enkele van de geteste stammen vertoonde groei bij pH 5,7 in aanwezigheid van 268 mM natriumlactaat of meer. Er bleken echter ook stammen te zijn waarvan de MIC-waarde van natriumlactaat nauwelijks door de pH werd beïnvloed (*Staphylococcus aureus, Lactococcus*

lactis en enkele niet nader geïdentificeerde melkzuurbacteriën die uit bedorven vleeswaren waren geïsoleerd). Deze organismen werden bij pH 7,0 relatief sterk door natriumlactaat geremd. Gisten waren minder gevoelig voor natriumlactaat dan bacteriën, maar ook voor deze organismen werd een specifieke remming van de groei door natriumlactaat aangetoond.

In Hoofdstuk 4 werd onder andere het effect van lactaat op toxinevorming door proteolytische *Clostridium botulinum* stammen beschreven. Daarnaast kwam ook het effect op sporenkieming en hitteresistentie van sporen aan de orde. Remming van toxinevorming bij 15°C en 20°C werd bewerkstelligd door concentraties natriumlactaat van respectievelijk 2% en 2,5% (w/v). Om in een tijdsbestek van 50 dagen toxinevorming bij temperaturen van 15°C, 20°C en 30°C te voorkomen waren concentraties natriumlactaat van respectievelijk 3%, 4% en meer dan 4% nodig. Ook bleek natriumlactaat de kieming van sporen van proteolytische *C. botulinum* stammen te remmen, wat gedeeltelijk zou kunnen verklaren waarom in aanwezigheid van natriumlactaat groei van en toxinevorming door deze stammen werden geremd. Wanneer de experimenten werden uitgevoerd in aanwezigheid van concentraties NaCl die een a_w verlaging bewerkstelligden, vergelijkbaar met die in aanwezigheid van remmende concentraties natriumlactaat, dan werd nauwelijks remming waargenomen. Hiermee is opnieuw de specifieke remming door natriumlactaat aangetoond. Er werd geen duidelijk synergie-effect op de groeiremming waargenomen wanneer natriumlactaat (1,5 or 2,5%) werd gecombineerd met NaCl (2,1%). Verder leek in aanwezigheid van NaCl of natriumlactaat de hitteresistentie van de sporen bij 95°C wat toe te nemen, maar het effect was niet significant.

De MIC-waarden van natriumlactaat en NaCl waren hoger dan die in de praktijk in vleeswaren kunnen worden toegepast. Daarom werd ook onderzocht wat de invloed op microbiële groei was van concentraties natriumlactaat en NaCl beneden de MIC-waarde (Hoofdstuk 5). De groei van *Listeria innocua* werd bestudeerd in dezelfde bouillon die werd gebruikt voor de screeningsexperimenten. Het effect van toenemende concentraties lactaat of NaCl werd bepaald bij verschillende pH waarden (5,5, 6,0, 6,5 en 7,0) en temperaturen (4°C, 10°C, 20°C en 30°C). Groeicurven werden gefit met behulp van een gemodificeerde Gompertz vergelijking. Bij een pH waarde van 5,5 werd geen groei van *L. innocua*

waargenomen indien de concentratie natriumlactaat 217 mM of meer bedroeg. Bij pH 7,0 was de MIC-waarde veel hoger: 1071–1339 mM. De MIC-waarden voor het ongedissocieerde melkzuur namen echter af met toenemende pH: van 5 mM bij pH 5,5 tot 0,8 mM bij pH 7. In aanwezigheid van natriumlactaat werd de maximale specifieke groeisnelheid in hoge mate beïnvloed door de pH van het medium, hetgeen niet werd waargenomen in aanwezigheid van NaCl of in afwezigheid van zout. De temperatuur had ook een grote invloed op de maximale specifieke groeisnelheid maar dit was onafhankelijk van het type zout in het medium. Met toenemende zoutconcentraties nam de maximale specifieke groeisnelheid geleidelijk af tot 0 (MIC-waarde). Dit effect kon worden beschreven met behulp van een gemodificeerde Monod vergelijking met 3 parameters (Hoofdstuk 5).

In aanvulling op deze studie werd het effect van pH en/of temperatuur op de maximale specifieke groeisnelheid in aanwezigheid van NaCl of natriumlactaat ook in het model geïmplementeerd (Hoofdstuk 6). Het resulterende model kan gebruikt worden om de specifieke groeisnelheid van *L. innocua* te voorspellen bij elke willekeurige temperatuur en pH-waarde tussen de experimentele uitersten. Om dit te bereiken werd het effect van pH en/of temperatuur op de parameters van het gemodificeerde Monod model (μ_m, α en p') met wiskundige formules beschreven. Het aantal parameters dat nodig is om alle experimentele data te kunnen beschrijven werd daarmee teruggebracht van 48 naar 4 (NaCl) en van 42 naar 5 (natriumlactaat). Hierdoor weken de door het model voorspelde waarden weliswaar iets meer af van de experimentele waarden, maar deze afwijking bleef binnen statistisch aanvaardbare grenzen. De voorspellende waarde van het uiteindelijke model werd vergeleken met die van een polynoom-vergelijking die minder geschikt bleek voor het doel van dit onderzoek.

Aangezien het model was gebaseerd op experimenten die uitgevoerd waren in een modelsysteem (bouillon) werd ook gekeken naar de voorspellende waarde van het model ten aanzien van de groei van *L. innocua* in boterhamworst (Hoofdstuk 6). Omdat de groeicondities in de bouillon veel gunstiger waren dan die in de boterhamworst en het organisme dus veel langzamer groeide in de worst, moest tenminste één parameter van het model opnieuw worden geschat. Doordat het model relatief simpel is en de parameters ervan direct gerelateerd zijn aan de

praktijk, biedt het goede mogelijkheden voor toepassing op het terrein van de voorspellende microbiologie.

Een meer diepgaande studie naar het antimicrobiële effect van natriumlactaat hield zich bezig met de bestudering van de gevoeligheid van *L. innocua* and *Lact. lactis* voor lactaat en het vermogen van deze organismen zich aan te passen aan minder gunstige groeicondities (Hoofdstuk 7). Meting van de intracellulaire pH (pH_{in}) toonde aan dat cellen die in aanwezigheid van lactaat waren gekweekt onder overigens identieke omstandigheden een hogere pH_{in} hadden dan cellen die in afwezigheid van lactaat waren gekweekt, wanneer tijdens de meting lactaat in de buffer aanwezig was. Met *Lact. lactis* werd dit fenomeen zelfs waargenomen indien tijdens de meting geen lactaat aanwezig was. Met behulp van immunoblots werd voor beide organismen de aanwezigheid van F_1F_0-ATPases vastgesteld en in membraanvesicles van cellen die bij lage pH waren gekweekt werd een verhoogde ATPase activiteit waargenomen. Voor *Listeria* is gesuggereerd dat dit organisme beschikt over een elektronentransportketen, en deze zou een rol kunnen spelen in de regulering van de pH_{in}. Er werden daarom experimenten uitgevoerd met hele cellen waarbij het vermogen werd onderzocht om na toevoeging van glucose de pH_{in} te laten stijgen in aanwezigheid van een remmer van de elektronentransportketen (kaliumcyanide) en in aanwezigheid van N,N'-dicyclohexyl-carbodiimide (DCCD), dat specifiek de werking van proton ATPases blokkeert. Voor beide organismen werd waargenomen dat in aanwezigheid van 10 mM KCN de opbouw van de pH_{in} niet werd geremd en in aanwezigheid van DCCD wel. Dit wijst er op dat het proton ATPase in zowel *Lact. lactis* als in *L. innocua* een cruciale rol speelt in de regulering van de pH_{in}. De resultaten konden echter niet verklaren waarom de remmende werking van lactaat bij lage pH vaak zo groot is en daarom verdient het mechanisme van de remming meer aandacht.

Dit proefschrift draagt bij aan een beter begrip van het werkingsspectrum en het mechanisme van de antimicrobiële activiteit van natriumlactaat. De resultaten tonen aan dat het gebruik van een combinatie van NaCl en natriumlactaat in vleeswaren goede perspectieven biedt voor de microbiologische veiligheid en houdbaarheidsverlenging van deze produkten.

Nawoord

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Curriculum vitæ

Pauline Houtsma werd op 26 november 1965 geboren te Zeist. Zij groeide op in het land van Heusden en Altena, in een klein dorpje genaamd Eethen. In juni 1984 behaalde zij het diploma VWO-B aan het Willem van Oranje College te Waalwijk. In datzelfde jaar begon zij de studie Levensmiddelentechnologie aan de toenmalige Landbouwhogeschool in Wageningen. In de doctoraalfase werden de hoofdvakken Levensmiddelenchemie en Levensmiddelenmicrobiologie gevolgd. Beide hoofdvakken werden afgerond met een stageperiode van drie maanden. De stage levensmiddelenchemie werd doorlopen in Zeist bij de toenmalige Hoofdgroep Voeding en Voedingsmiddelen TNO. De stage levensmiddelenmicrobiologie werd doorlopen in Zutphen bij de Inspectie Gezondheidsbescherming (Keuringsdienst van Waren), waar zij nog 3 maanden bleef werken om zich verder te verdiepen in de eigenschappen van *Campylobacter* species. Tussentijds (januari 1990) studeerde zij af aan de Landbouwuniversiteit.

Van april 1990 tot en met juli 1990 werkte zij als toegevoegd onderzoeker aan de vakgroep Levensmiddelentechnologie (sectie Levensmiddelenchemie en -microbiologie in afwachting van een aanstelling als assistent in opleiding (AIO), per 1 augustus 1990. Het onderzoek dat in dit proefschrift wordt beschreven werd gefinancierd door PURAC te Gorinchem en grotendeels uitgevoerd in Wageningen onder leiding van prof. dr. ir. F.M. Rombouts, dr. ir. M.H. Zwietering en mw. ir. J.C. de Wit. Voor een periode van 1 jaar (december 1992 tot en met november 1993) werd zij gedetacheerd bij de Rijksuniversiteit Groningen, waar zij onder leiding van prof. dr. W.N. Konings en dr. B. Poolman het mechanisme van de microbiële groeiremming door lactaat bestudeerde. Dit onderzoek werd in 1994 o.l.v. dr. T. Abee voortgezet in Wageningen.

In 1995 besloot zij dat zij zich meer specifiek op het bedrijfsleven wilde richten. Sinds januari 1996 volgt zij daarom een éénjarige postuniverstitaire opleiding tot milieuconsulent voor het bedrijfsleven aan de Universiteit Twente.