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Investigation of the bactericidal effect of Nisin on lactic acid bacteria of wine

by

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Untersuchung der bakteriziden Wirkung von Nisin auf Milchsäurebakterien des Weines

Zusammenfassung: Geringe Konzentrationen des Polypeptids Nisin genügen, um das Wachstum der für Wein wichtigen Milchsäurebakterien zu hemmen. Die Hemmkonzentrationen sind sehr verschieden: *Leuconostoc oenos*, *Pediococcus damnosus* und *Lactobacillus brevis* werden bei 4, 8 und 100 IU Nisin/ml gehemmt. Unterhalb der Hemmkonzentration wird der Carboxylsäurestoffwechsel nicht beeinflußt. Nisin ist für nicht wachsende Milchsäurebakterien bakterizid, aber erst bei wesentlich höheren als den wachstumshemmenden Konzentrationen. *Lactobacillus casei* erwies sich als extrem resistent und wurde erst bei Konzentrationen von > 2000 IU Nisin/ml abgetötet.

Key words: wine, lactic acid bacteria, *Leuconostoc*, *Pediococcus*, *Lactobacillus*, Nisin, bactericide, growth inhibition, carboxylic acid, metabolism.

Introduction

Lactic acid bacteria are important in wine-making in various ways. They carry out the malolactic fermentation that is regarded as essential for certain types of wine, but they may occasionally spoil the wine by producing undesirable metabolites, off-flavours and turbidity. The antibacterial effect of sulphur dioxide is exploited to prevent an unwanted development of lactic acid bacteria in wine. However, this compound is rather toxic. Its use is not only legally restricted but also many attempts have been made to reduce the amount of sulphur dioxide or to replace it — at least partially — by a less harmful substitute. Nisin has been suggested as an antimicrobial agent to suppress the growth of lactic acid bacteria of wine (RADLER 1990 a, b). Earlier, OGDEN (1986) and OGDEN *et al.* (1988) have described the effect and the advantages of a possible use of Nisin in brewing.

Nisin is a polypeptide that is produced by the lactic acid bacterium *Streptococcus lactis* (HURST 1981). Its antimicrobial activity is rather specific and restricted to lactic acid bacteria and other mainly gram-positive organisms. Such proteinaceous compounds that are produced by bacteria are called bacteriocins (TAGG *et al.* 1976). The use of Nisin as a food additive is authorized in several countries. It is mainly employed in the dairy industry, particularly in cheese-making (HURST 1981).

The purpose of this work was to determine the inhibitory concentrations of Nisin on growing cells and the bactericidal effect on non-growing cells of species of lactic acid bacteria that are of importance in wine-making. Furthermore it was investigated whether concentrations of Nisin that do not prevent growth, exert an influence on the metabolism of carboxylic acids by lactic acid bacteria.

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Materials and methods

1. Microorganisms, culture media and conditions

The lactic acid bacteria used in this work (*Lactobacillus casei* B48, *Lactobacillus brevis* B18, *Leuconostoc oenos* B211, and *Pediococcus damnosus* B42) were from the collection of this institute.

The media were tomato juice broth (TJB) : glucose (0.5 %), tryptone (2 %), peptone (0.5 %), yeast extract (0.5 %), Tween 80 (0.1 %), pH 4.0 or as indicated. Tomato juice agar (TJA) was TJB at pH 5.5 solidified with 1.2 % agar. Basal medium was as described previously (RADLER and BRÖHL 1984). Acid medium was the same as basal medium except that the glucose content was increased to 2 % and the various carboxylic acids were added separately at concentrations of 37 mmol/l. Pasteurized grape must (GM) was adjusted to pH 4. Enriched grape must (GM + YE) was GM diluted 1:1 with a solution of 1 % (w/v) yeast extract. All media were sterilized at 121 °C for 15 min, except the grape juice which was steamed only.

Nisin (a gift from Aplin and Barrett Ltd., Beaminster, Dorset, U.K.) was dissolved in 0.02 N hydrochloric acid and sterilized by filtration. Appropriate amounts of this solution were added to the media as required. 1 mg preparation of Nisin = 40 000 IU (international units).

Viable cells (cfu) were determined by plating on TJA. The colonies were counted after 4—7 d.

2. Analytical methods

L-malate, citrate, oxoglutarate and fumarate were monitored by thin layer chromatography on pre-coated 20 cm × 20 cm TLC aluminium plates (cellulose, Merck), in the liquid medium, after removing the cells by centrifugation as described (RADLER and BRÖHL 1984). The solvent for citrate, malate and oxoglutarate was a mixture of dichloromethane : n-propanol : formic acid (75:8:17). The solvent for fumarate analysis consisted of 2-butanol : formic acid : water (75:15:10). Acid spots became visible after spraying the chromatogram with a reagent containing 2 g of glucose, 2 ml of aniline in 20 ml of water, 20 ml of ethanol and 60 ml of n-butanol (SCHWEPPE 1959).

Gluconic acid was determined by the enzymatic method (MÖLLERING and BERGMEYER 1974), following the recommendations of Boehringer-Mannheim. Sugars were determined by the Nelson-Somogyi copper reduction method.

Results and discussion

A previous investigation has shown that very different concentrations of Nisin were required to inhibit the growth of various lactic acid bacteria of wine (RADLER 1990 a). *L. oenos* was found to be most sensitive, whereas higher concentrations of Nisin were tolerated by *P. damnosus* and *L. brevis*. *L. casei* was very resistant. These experiments showed that Nisin prevented the growth of lactic acid bacteria, but did not demonstrate a bactericidal effect.

At first the threshold concentration of Nisin was determined for the growth of the 3 most important species of lactic acid bacteria. In accordance with previous results no growth was observed at 4 IU Nisin/ml with *L. oenos*, 8 IU Nisin/ml with *P. damnosus* and 100 IU Nisin/ml with *L. brevis* in tomato juice broth at pH 4.5 (Table).

Although lactic acid bacteria require carbohydrates for growth, the metabolism of carboxylic acids, which is concomitant to growth, is of great practical importance in wine-making. Therefore it was analysed if concentrations of Nisin that do not inhibit

Determination of the minimum inhibitory concentration of Nisin that prevents growth of lactic acid bacteria

Bestimmung der minimalen Hemmkonzentration von Nisin, die das Wachstum von Milchsäurebakterien aus Wein hemmt

Lactic acid bacterium	Nisin (IU ml^{-1})							
	0	2	4	5	8	10	30	100
L. oenos B211	+	+	–	–	–	–	–	–
P. damnosus B42	+	+	+	+	–	–	–	–
L. brevis B18	+			+		+	+	–

Tomato juice broth, pH 4.5, 25 °C; – = no growth; + = turbidity as in control without Nisin.

growth affect the metabolism of carboxylic acids. Carboxylic acid metabolism that is non-essential for the lactic acid bacteria was studied in the acid medium to which 2 % glucose was added. The concentrations of Nisin were below the inhibitory level. 1 and 2 IU for *L. oenos*, 2 and 5 IU for *P. damnosus* and 10 and 30 IU were used for *L. brevis*. No difference was detected in the metabolism of carboxylic acids in the presence of the bacteriocin. All acids that were metabolized in the absence of Nisin were also metabolized in its presence. At the higher concentration of Nisin, the effect of acids on bacterial growth varied among the strains. In both *L. oenos* and *P. damnosus*, oxoglutarate,

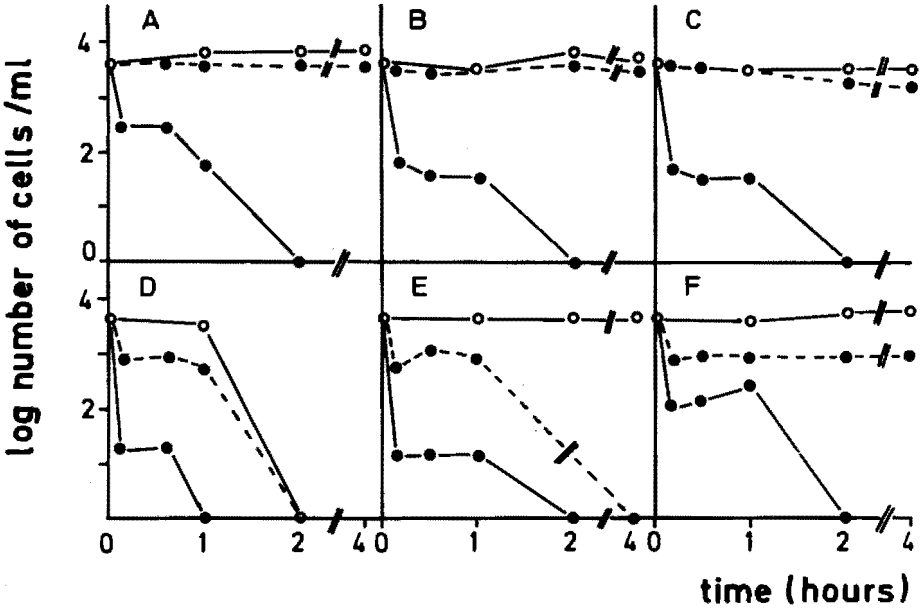

Fig. 1: The action of Nisin on cells of *Pediococcus damnosus* in different media. A) Tomato juice broth. B) Grape must. C) Enriched grape must (GM + YE). D—F) Citrate buffer, pH 4.0, 5.0 and 6.0, respectively. — ○——○ = Controls without Nisin; ●---● = 10 IU Nisin/ml; ●——● = 100 IU Nisin/ml.

Die Wirkung von Nisin auf Zellen von *Pediococcus damnosus* in verschiedenen Medien. A) Tomatensaftlösung. B) Traubenmost. C) Angereicherter Traubenmost. D—F) Citratpuffer, pH 4,0, 5,0 oder 6,0. — Erläuterung der Kurvensymbole s.o.

fumarate and K-gluconate significantly decreased the lag phase in spite of the fact that the acids were not metabolized by *P. damnosus*. On the other hand, malate, which was decarboxylated by both strains, and citrate, that was only cleaved by *L. oenos*, significantly increased the lag phase. Only slight growth was observed with *P. damnosus* when citrate and 5 IU Nisin/ml were added to the medium. The final pH of the culture medium increased significantly when L-malate had been added and the media were inoculated with *P. damnosus* and *L. oenos*. These results might be attributed to a decrease in sugar consumption that was observed when both Nisin and L-malic acid were present in the medium.

One possible application of Nisin could be its use as a sanitizing agent to remove lactic acid bacteria from equipment and from yeast starters if required. For any such use a rather rapid action of the antimicrobial substance is essential. Therefore the time

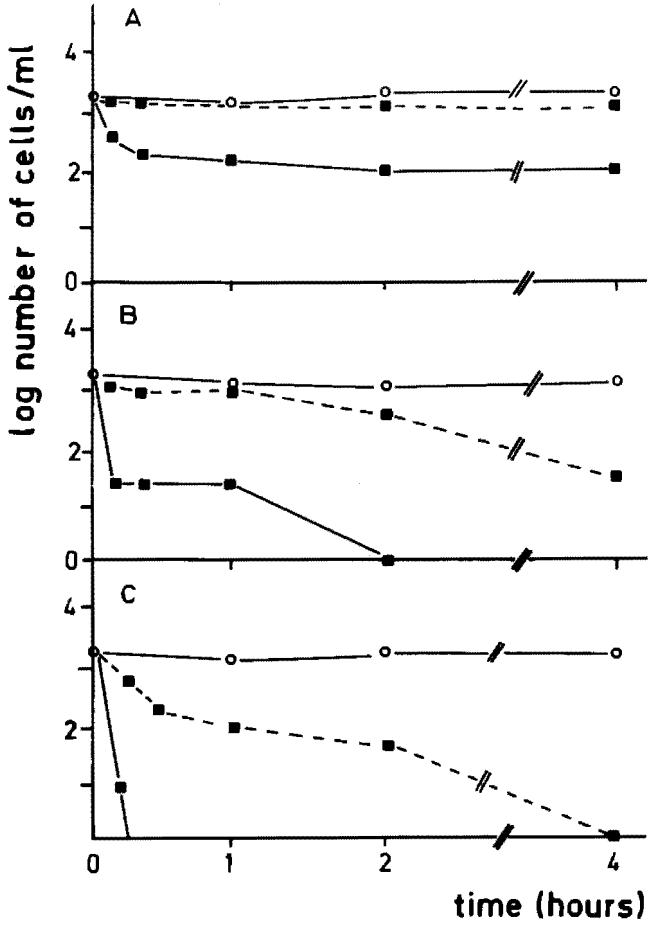

Fig. 2: The action of Nisin on cells of *Lactobacillus casei* in tomato juice broth (A), grape juice + yeast extract (B), and citrate buffer, pH 4—6 (C). — ○——○ = Controls without Nisin, ■ --- ■ = 2000 IU Nisin/ml; ■——■ = 20000 IU Nisin/ml.

Die Wirkung von Nisin auf Zellen von *Lactobacillus casei* in Tomatensaftlösung (A), angereichertem Traubenmost (B) und Citratpuffer (pH 4—6) (C). — Erläuterung der Kurvensymbole s.o.

course of the action of Nisin on lactic acid bacteria was determined, not only in citrate buffer pH 4—6 but also in several media. All assays were carried out with cells in the late stationary phase (grown for 72 h in tomato juice broth at 25 °C) since the effect of Nisin is dependent on the physiological condition of the cells (OGDEN and WAITES 1986). Although experiments were performed with several bacteria (*L. oenos*, *L. brevis*), only the results obtained with *P. damnosus* are presented as example (Fig. 1). In the previous experiment, no growth of this organism occurred at 10 IU Nisin/ml. Under all conditions tested, this concentration of Nisin showed almost no effect when compared with the controls. In citrate buffer at pH 4, all the cells were killed in less than 2 h, but this was independent of the presence of Nisin. At a concentration of 100 IU Nisin/ml the bacteria were killed within 2 h, but in all cases (except at pH 4) viable cells were detected after 1 h.

Completely different results were observed with *L. casei*. At the extraordinarily high concentration of 2000 IU Nisin/ml, no or almost no effect was observed in tomato juice broth or diluted grape juice (Fig. 2). An almost identical figure was obtained in grape juice, therefore these results are not depicted. As with the other organisms, Nisin proved to be more bactericidal in citrate buffer than in growth media. In citrate buffer the bacteria were rapidly killed by 2×10^4 IU Nisin/ml but at 2000 IU Nisin/ml viable cells were detected after 2 h.

Summary

Even low concentrations of the antimicrobial polypeptide Nisin inhibit the growth of the most important lactic acid bacteria of wine. The inhibitory concentrations vary. *Leuconostoc oenos*, *Pediococcus damnosus*, and *Lactobacillus brevis* were inhibited by 4, 8, and 100 IU Nisin/ml, respectively. At concentrations of Nisin below the inhibitory level the carboxylic acid metabolism was not affected. Nisin is bactericidal for non-growing lactic acid bacteria, but only at concentrations above those that inhibit growth. *Lactobacillus casei* is most resistant, it is only killed at extremely high concentrations of Nisin (> 2000 IU/ml).

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Literature cited

HURST, A.; 1981: Nisin. Adv. Appl. Microbiol. **27**, 85—123.

MÖLLERING, H.; BERGMEYER, H. U.; 1974: Gluconat. Bestimmung mit Gluconat-Kinase und 6-Phosphogluconat-Dehydrogenase. In: BERGMEYER, H. U. (Hrsg.): Methoden der enzymatischen Analyse. Bd. **II**, 1288—1292. Verlag Chemie, Weinheim/Berstraße.

OGDEN, K.; 1966: Nisin: a bacteriocin with a potential use in brewing. J. Inst. Brew. **92**, 379—383.

— — ; WAITES, M. J.; 1986: The action of Nisin on beer spoilage lactic acid bacteria. J. Inst. Brew. **92**, 463—467.

— — ; — —; HAMMOND, J. R. M.; 1988: Nisin and brewing. J. Inst. Brew. **94**, 233—238.

RADLER, F.; 1990 a: Possible use of Nisin in winemaking. I. Action of Nisin against lactic acid bacteria and wine yeasts in solid and liquid media. Amer. J. Enol. Viticult. **41**, 1—6.

— — ; 1990 b: Possible use of Nisin in winemaking. II. Experiments to control lactic acid bacteria in the production of wine. Amer. J. Enol. Viticult. **41**, 7—11.

— —; BRÖHL, K.; 1984: The metabolism of several carboxylic acids by lactic acid bacteria. Z. Lebensm.-Untersuch. -Forsch. **179**, 228—231.

SCHWEPPE, H.; 1959: Papierchromatographischer Nachweis von Carbonsäuren. In: LINSKENS, H. F. (Hrsg.): Papierchromatographie in der Botanik, 107. Springer-Verlag, Berlin, Göttingen, Heidelberg.

TAGG, J. R.; DAJANI, A. S.; WANNAMAKER, L. W.; 1976: Bacteriocins of gram-positive bacteria. Bacteriol. Rev. **40**, 722—756.

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