APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 2004, p. 4807–4813 Vol. 70, No. 8
0099-2240/04/$08.00+0 DOI: 10.1128/AEM.70.8.4807–4813.2004

Effects of Different Spices Used in Production of Fermented Sausages on Growth of and Curvacin A Production by *Lactobacillus curvatus* LTH 1174

Jurgen Verluyten, Frédéric Leroy, and Luc de Vuyst*

Research Group of Industrial Microbiology, Fermentation Technology and Downstream Processing, Department of Applied Biological Sciences, Vrije Universiteit Brussel, B-1050 Brussels, Belgium

Received 6 January 2004/Accepted 5 May 2004

Lactobacillus curvatus **LTH 1174, a fermented sausage isolate, produces the listericidal bacteriocin curvacin A. The effect of different spices relevant for the production of fermented sausages was investigated in vitro through laboratory fermentations with a meat simulation medium and an imposed pH profile relevant for Belgian-type fermented sausages. The influence on the growth characteristics and especially on the kinetics of curvacin A production with *L. curvatus* LTH 1174 was evaluated. Pepper, nutmeg, rosemary, mace, and garlic all decreased the maximum specific growth rate, while paprika was the only spice that increased it. The effect on the lag phase was minor except for nutmeg and especially for garlic, which increased it, yet garlic was stimulatory for biomass production. The maximum attainable biomass concentration (X_{max}) was severely decreased by the addition of 0.40% (wt/vol) nutmeg, while 0.35% (wt/vol) garlic or 0.80% (wt/vol) white pepper increased X_{max}. Nutmeg decreased both growth and bacteriocin production considerably. Garlic was the only spice enhancing specific bacteriocin production, resulting in higher bacteriocin activity in the cell-free culture supernatant. Finally, lactic acid production was stimulated by the addition of pepper, and this was not due to the manganese present because an amount of manganese that was not growth limiting was added to the growth medium. Addition of spices to the sausage mixture is clearly a factor that will influence the effectiveness of bacteriocinogenic starter cultures in fermented-sausage manufacturing.**

Different spices and herbs have been added to various food products for centuries, mainly to contribute to the characteristic flavor of the end product. Several spices are used for the production of fermented sausages in various concentrations, depending on the type of sausage. Commonly applied spices include ground pepper, paprika, garlic, mace, pimento, and cardamom (27). They are mainly added as flavorings and coloring agents. However, they are also a source of many other substances, such as sugars, nitrates, and metallic ions (1). A variety of spices added to meat have been found to accelerate lactic acid production by the lactic acid bacterial starter culture (22, 34). The degree of stimulation of lactic acid production was related to the manganese content of the spices (19, 49). Ground pepper, which is usually present in all types of sausages at the 0.2 to 0.3% level (27), has a relative high manganese content (49). In addition, in special types sausages such as chorizo, containing high amounts of paprika, the sugar, nitrate, and manganese content of this particular spice can also contribute to the fermentation process (1, 16). Furthermore, some spices (garlic, nutmeg, mace, paprika, rosemary, and sage) contain powerful antioxidants that can extend the shelf life of dry-fermented sausages (2, 12, 28, 33, 39). Indeed, the oxidation of lipids in foodstuffs results in the development of off flavors, resulting in a product that is unacceptable for human consumption.

* Corresponding author. Mailing address: Research Group of Industrial Microbiology, Fermentation Technology and Downstream Processing, Department of Applied Biological Sciences, Vrije Universiteit Brussel, B-1050 Brussels, Belgium. Phone: 32-2-6293245. Fax: 32-2-6292720. E-mail: ldvuyst@vub.ac.be.

Besides their antioxidant activity, many spices display antimicrobial activities. The antiseptic potential of spices resides in the essential oils (50). One of the most potent spices is garlic, in which several antimicrobial components are present; the principal active substance was identified as allicin (5, 20). Extensive studies have been performed to determine its inhibitory properties, and many food-borne pathogens, both gram-positive and gram-negative bacteria, have been shown to be inhibited by garlic (23, 26, 41). The main biological effect of allicin is its rapid reaction with thiol-containing proteins (39), and since it can freely permeate through phospholipid bilayers, it can interact with intracellular thiol-containing proteins (31). For spices such as nutmeg, it is interesting that the pathogenic *Escherichia coli* O157:H7 strain is more susceptible than nonpathogenic *E. coli* (45). Finally, rosemary has been shown to possess antimicrobial potential towards different pathogenic bacteria (29), including the food-borne pathogen *Listeria monocytogenes* (38). In contrast to the above-mentioned pathogens, lactic acid bacteria are usually quite resistant to the antimicrobial activity of spices (11, 17, 40).

In most cases, the levels of spices used in the production of fermented sausages are insufficient for their antimicrobial activity to interfere with the growth of food-borne pathogens, and hence they are not very effective as preservatives (17, 50). This is in contrast with fresh meat products, where a mixture of spices can be successfully applied to stabilize the sensory appearance and hence extend the shelf life of the food (18). However, bacteriocins or bacteriocinogenic starter cultures can be applied in various food products and may provide a synergistic effect. In broth, such a positive interaction between nisin and garlic extract has been shown towards strains of *L.*

monocytogenes (42). The concentration of nisin necessary for effective control of *L. monocytogenes* and *Bacillus subtilis* could be lowered when it was used in combination with thymol (13). However, few studies are available on the influence of spices on the activity and/or production of bacteriocins. In the case of *Lactobacillus sakei* CTC 494, the addition of black pepper enhanced sakacin K activity but had no effect on bacteriocin production, either in vitro or in the production of fermented sausage (21). On the other hand, enterocin production by *Enterococcus faecium* CTC 492 was strongly reduced when black pepper was added to the fermentation medium, especially in combination with sodium chloride (6).

One of the uncertainties in the application of bacteriocinogenic starter cultures is sufficient production and effectiveness of bacteriocins in situ and the effect that environmental factors as well as various sausage ingredients such as spices have on bacteriocin kinetics. It is impossible to precisely control actual sausage fermentations, and the determination of bacteriocin activity levels in a meat batter is difficult as well. Hence, proper kinetic studies concerning growth and bacteriocin production cannot be performed during sausage fermentations. An alternative strategy is to mimic the water phase of a fermented sausage by making use of a liquid meat simulation medium that takes into account the kind of nutrients used (meat-derived peptones), the amino nitrogen, sodium chloride, and sodium nitrite content and subjecting it to a pH profile relevant to fermented sausage production. Previously, it was shown that temperature and pH conditions that prevail during sausage fermentations are optimal for bacteriocin production by *Lactobacillus curvatus* LTH 1174 (30). On the other hand, this strain has been shown to be extremely sensitive to nitrite, but anaerobic conditions, as are encountered during sausage fermentation, at least partially reduce the negative effect of nitrite (48).

In this study, the effects of several spices commonly applied in fermented sausage manufacturing were investigated on growth of and bacteriocin production by *L. curvatus* LTH 1174 in a liquid meat simulation medium.

MATERIALS AND METHODS

Microorganisms and media. *L. curvatus* LTH 1174, which produces the bacteriocin curvacin A (46), was used throughout this study. *Listeria innocua* LMG 13568, a curvacin A-sensitive indicator strain, was used to determine bacteriocin activity levels (24). Both strains were maintained and propagated as described previously (30).

For examining growth and bacteriocin production, a liquid meat simulation medium (MSM-BE) was used, containing, per liter, 29.0 g of bacteriological peptone (Oxoid, Basingstoke, United Kingdom), 23.0 g of Lab Lemco (Oxoid), 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.038 g of $MnSO_4 \cdot H_2O$, 1 ml of Tween 80, 5 g of lactic acid, 55.0 g of NaCl, and 0.01 g of $NaNO_2$. The medium was sterilized in situ for 20 min at 121°C. Lactic acid was sterilized separately and added aseptically to the fermentor. A stock solution of $NaNO_2$ (10 g liter^{-1}) was sterilized separately by microfiltration (Acrodisc; Pall Gelman Sciences, Ann Arbor, Mich.). The amount of $NaNO_2$ added was representative of residual $NaNO_2$ levels encountered in fermented sausage, since nitrite is rapidly depleted when added to the sausage batter (4, 16, 32, 44). The complex nutrients source of this medium was composed only of meat-derived peptones, i.e., bacteriological peptone and Lab Lemco. Based on calculations of the amino nitrogen content (36), the medium components approached more closely an actual sausage environment (8). In accordance with actual sausage fermentation conditions, 1.5% (wt/vol) glucose was used. Additionally, a pH profile was imposed as shown in Fig. 1. This profile is representative of the pH decline observed during a Belgian-type sausage fermentation.

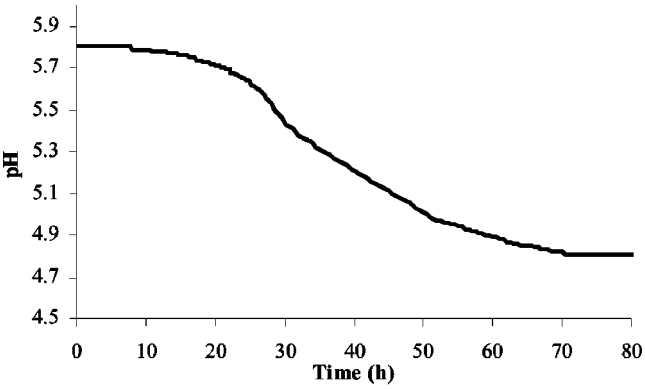

FIG. 1. pH profile imposed during fermentations in liquid meat simulation medium with *L. curvatus* LTH 1174. The pH profile chosen is representative of a Belgian-type sausage fermentation.

Fermentation experiments. To determine the effect of different spices on both growth and bacteriocin production with *L. curvatus* LTH 1174, a series of in vitro fermentations was performed with MSM-BE. All fermentations were performed under anaerobic conditions for better approximation of the actual sausage environment. An anaerobic environment was created by continuously sparging the medium with 1 liter of filtered N_2 gas (N28; Air Liquide, Paris, France) per minute. The fermentation performed in MSM-BE without any spice added will be referred to as the reference fermentation. The concentrations of the spices tested were 1.40 and 0.70% (wt/vol) paprika, 0.80 and 0.40% (wt/vol) pepper, 0.70 and 0.35% (wt/vol) garlic, 0.40 and 0.20% (wt/vol) nutmeg, 0.04% (wt/vol) mace, and 0.04% (wt/vol) rosemary extract. The highest concentration of each spice represents the maximum concentration used in Belgian sausage manufacturing. The concentrations were chosen to ascertain the maximum effects of the spices possible; the other concentrations were chosen as being more representative of practical use.

All spices were obtained locally and used without prior sterilization. The spices were allowed to dissolve in 1 liter of sterile water by stirring overnight at 4°C before addition to the fermentor. The fermentations were performed at a constant temperature of 20°C and with the imposed pH profile mentioned above, which was controlled by automatic addition of 10 N NaOH. The fermentations were carried out in a 15-liter laboratory fermentor (Biostat C; B. Braun Biotech International, Melsungen, Germany) with a working volume of 10 liters. To keep the fermentation liquor homogenous, agitation was performed at 150 rpm. Online control was further performed as described previously (24). The preparation of the inoculum was performed as described previously (30).

Assays. At regular intervals, samples were withdrawn aseptically from the fermentor to determine cell counts (CFU), the optical density at 600 nm (OD_{600}; Uvikon 923; Kontron Instruments, Milan, Italy) was measured, and the level of soluble bacteriocin activity in cell-free culture supernatant, the lactic acid concentration, and the residual glucose concentration were measured. Briefly, the amount of lactic acid produced and the residual glucose concentration were determined by high-performance liquid chromatography (9), and the level of soluble bacteriocin activity in the cell-free culture supernatant was determined by a modified critical dilution method with *L. innocua* LMG 13568 as the indicator organism (9, 24). The twofold critical dilution method displays a variation coefficient of 20% on measurement but has repeatedly been shown to yield good overall reproducibility of the bacteriocin activity curve of *L. curvatus* LTH 1174 (47). The presence of spices did not interfere with the bacteriocin activity assay. The standard deviations for the glucose and lactic acid measurements were 0.04 and 0.02 g liter^{-1}, respectively.

For OD measurements, the samples were homogenized, and the debris from the spices was arbitrarily allowed to settle for 1 min before making the dilution for the OD_{600} measurements. The coefficient of variation for the measurements of OD_{600} was usually lower than 3%. Modeling of bacterial growth was performed with the biomass concentrations obtained from OD_{600} measurements. This was done to allow easier comparison with results obtained previously with this strain (30, 48) and with other data from the literature. The optical density values from a series of previously performed fermentations were calibrated against biomass as cell dry mass (CDM). A change of 1 unit of optical density was shown to be equivalent to an increase of 0.26 g of CDM liter^{-1} ($r^2 = 0.962$).

TABLE 1. Equations used for primary model development[a]

Model	Equation
Cell growth	$dX/dt = [\mu_{max}(1 - X/X_{max})^n - \alpha] X$ when $t > \lambda$
Glucose consumption	$dS/dt = -1/Y_{X/S}dX/dt - m_S X$
Lactic acid production	$dL/dt = -Y_{L/S}dS/dt$
Bacteriocin production	$dB/dt = k_B dX/dt - k_{inact} XB$ when $X > X_B$

[a] *X,* biomass concentration (in grams of CDM per liter); *t,* time (in hours); λ, duration of the lag phase (in hours); μ_{max}, maximum specific growth rate (per hour); X_{max}, maximum attainable biomass concentration (in grams of CDM per liter); *n,* inhibition exponent; α, specific death rate (per hour); *S,* residual glucose concentration (in grams of glucose per liter); $Y_{X/S}$, cell yield coefficient (in grams of CDM per gram of glucose); m_S, maintenance coefficient (in grams of glucose per gram of CDM per hour); *L,* lactic acid production (in grams of lactic acid per liter); $Y_{L/S}$, yield coefficient for the conversion of glucose into lactic acid (in grams of lactic acid per gram of glucose); *B,* bacteriocin activity in the cell-free culture supernatant (in arbitrary units per liter); k_B, specific bacteriocin production (in arbitrary units per gram of CDM); k_{inact}, apparent rate of bacteriocin inactivation (in liters per gram of CDM per hour); X_B, minimum biomass concentration for the onset of bacteriocin production (in grams of CDM per liter).

Primary modeling. Primary modeling of cell growth, glucose consumption, lactic acid production, and bacteriocin production and inactivation was performed to fit the data as well as to estimate the biokinetic parameters representative of growth and curvacin A production. The equations used are listed in Table 1. They are the same as those reported by Messens et al. (30) except that bacteriocin production was made dependent on a value of X_B, the minimum biomass concentration required for the onset of bacteriocin production due to induction (10, 25).

The differential equations were solved numerically in Microsoft Excel 97 (version 8.0a) with the Euler integration technique (7). All parameters needed for the modeling were estimated by minimizing the residual sum of squares between experimental and modeled data. Previously, it was shown that these large-scale and strictly computer-controlled fermentation experiments are highly repeatable, as well as the estimation of the biokinetic parameters for a defined set of fermentation conditions. Based on repetitions, it was estimated for the bacteriocin-producing strains *L. sakei* CTC 494 and *L. curvatus* LTH 1174 that the coefficients of variation of the biokinetic parameters used for modeling are generally lower than 10%, with the exception of k_{inact}, for which variation of up to 25% can be observed (24, 47). Moreover, inherent biological variation within a treatment was small compared to the large differences observed among fermentation experiments under different conditions.

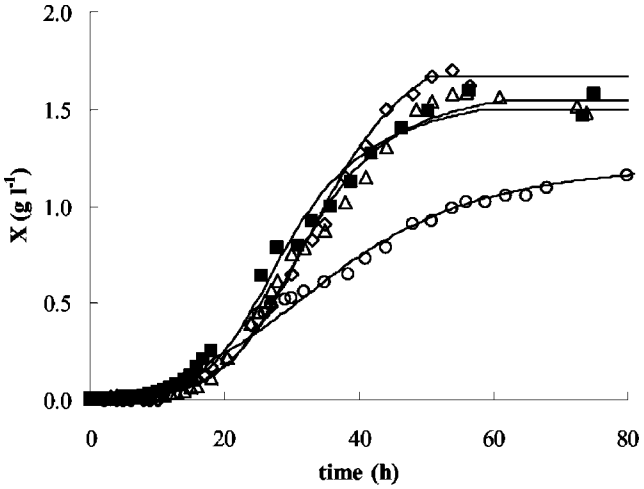

FIG. 2. Modeling of growth (in grams of CDM liter^{-1}) of *L. curvatus* LTH 1174 for different concentrations of various spices as a function of time for the reference fermentation (■), 0.80% (wt/vol) pepper (◇), 0.40% (wt/vol) nutmeg (○), and 0.04% (wt/vol) mace (△). Symbols represent experimental values; lines are drawn according to the model.

RESULTS

Influence of different spices on the growth of *L. curvatus* LTH 1174. All spices at the concentrations tested affected the growth of *L. curvatus* LTH 1174 (Table 2). For both concentrations of paprika tested, both the lag phase (t_{lag}) and the maximum specific growth rate (μ_{max}) increased compared to the reference fermentation to which no spices were added, while the maximum attainable biomass concentration (X_{max}) was comparable. In contrast, both pepper concentrations slowed down growth, resulting in a lower μ_{max}. For 0.40% pepper, X_{max} was the same as for the reference fermentation, while the addition of 0.80% pepper resulted in a higher X_{max} of

TABLE 2. Modeled values of t_{lag}, μ_{max}, X_{max}, X_B, k_B, k_{inact}, B_{max}, $Y_{X/S}$, and m_S of *L. curvatus* LTH 1174 grown in MSM-BE medium at 20°C with an imposed pH profile[a]

Fermentation	t_{lag} (h)	μ_{max} (h^{-1})	X_{max} (g of CDM/liter)	X_B (g of CDM/liter)	k_B (MAU per g of CDM)	k_{inact} (liters per g of CDM per h)	B_{max} (MAU/liter)	$Y_{X/S}$ (g of CDM/g of glucose)	m_S (g of glucose/g of CDM per h)
Reference	4.0	0.23	1.65	0.90	1.50	0.03	0.51	0.19	0.17
Paprika									
1.40%	7.0	0.32	1.50	1.30	0.25	0.15	0.01	0.25	0.23
0.70%	8.0	0.30	1.70	0.95	0.20	0.10	0.05	0.20	0.13
Pepper									
0.80%	3.5	0.19	2.05	NR[b]	0.00	NR	NR	0.21	0.23
0.40%	3.5	0.21	1.65	0.95	0.65	0.04	0.22	0.18	0.15
Nutmeg									
0.40%	10.0	0.13	1.23	NR	0.00	NR	NR	0.50	0.14
0.20%	10.0	0.14	1.70	1.15	0.30	0.30	0.02	0.50	0.30
Rosemary (0.04%)	6.0	0.20	1.60	0.95	0.30	0.03	0.09	0.19	0.21
Mace (0.04%)	5.0	0.21	1.65	0.95	1.00	0.03	0.36	0.19	0.15
Garlic (0.35%)	28.0	0.21	2.00	0.50	1.80	0.04	1.18	0.22	0.15

[a] See Table 1, footnote *a,* for biokinetic parameters. α was zero in all cases except for 0.35% garlic (α = 0.18 h^{-1}) and 0.70% garlic (α = 0.19 h^{-1}).
[b] NR, not relevant.

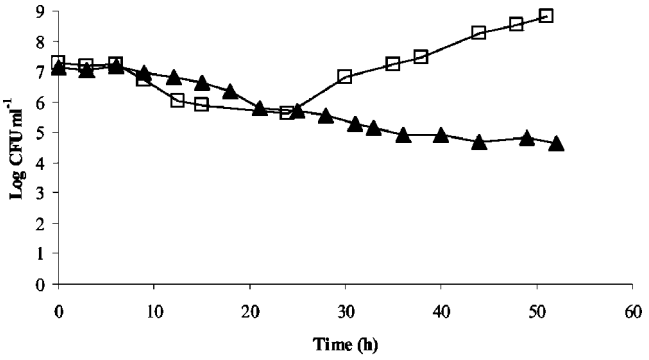

FIG. 3. Inhibitory effect of 0.70% (▲) and 0.35% (□) garlic on growth of *L. curvatus* LTH 1174.

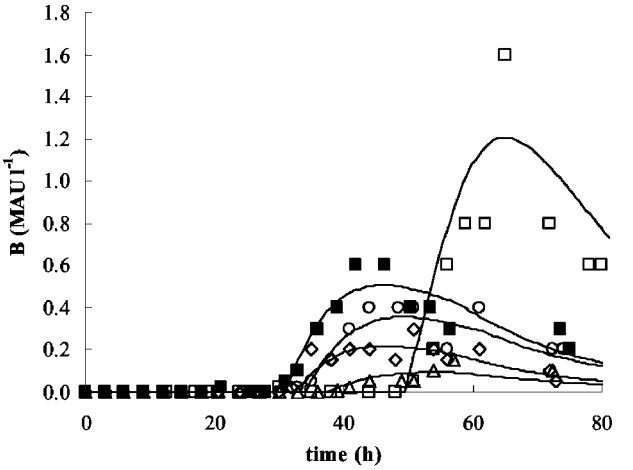

FIG. 4. Modeling of bacteriocin production (in MAU per liter) by *L. curvatus* LTH 1174 for different concentrations of various spices as a function of time for the reference fermentation (■), 0.40% (wt/vol) pepper (◇), 0.04% (wt/vol) mace (○), 0.04% (wt/vol) rosemary (△), and 0.35% (wt/vol) garlic (□). Symbols represent experimental values; lines are drawn according to the model.

2.05 g of CDM liter^{-1} compared to 1.65 g of CDM liter^{-1} for the reference fermentation (Fig. 2).

Nutmeg had the most profound influence on the growth of *L. curvatus* LTH 1174 (Fig. 2). For both concentrations tested, the lag phase was increased to 10 h, compared to 4 h for the reference fermentation. With 0.40 or 0.20% nutmeg, μ_{max} was almost halved, from 0.23 to 0.13 and 0.14 h^{-1}, respectively. Furthermore, 0.40% nutmeg also decreased X_{max} from 1.65 g of CDM liter^{-1} to 1.23 g of CDM liter^{-1}, while for 0.20% nutmeg, X_{max} was comparable to that of the reference fermentation. When 0.04% rosemary was added to the liquid meat simulation medium, t_{lag} was slightly increased to 6 h, while μ_{max} decreased slightly to 0.20 h^{-1} and X_{max} was comparable to that of the reference fermentation. For 0.04% mace, growth was comparable to that of the reference fermentation (Fig. 2). Hence, at the concentrations tested, both rosemary and mace had only minor effects on the growth of *L. curvatus* LTH 1174.

For paprika, pepper, nutmeg, rosemary, and mace, no cell death was observed, and hence α equaled 0 (as determined by cell counts; results not shown). In contrast, when 0.70% garlic was added, a bactericidal action was observed towards *L. curvatus* LTH 1174, which resulted in a decrease of viable cells from 7.1 log CFU ml^{-1} at the time of inoculation to 5.7 log CFU ml^{-1} after 25 h of fermentation. In this case, the specific growth rate was 0, and α equaled 0.19 h^{-1}. Afterwards, the population was not strongly affected and was estimated at 5.6 log CFU ml^{-1} after 52 h (Fig. 3). Adding 0.35% garlic increased t_{lag} considerably, from 4 to 28 h, and during this period garlic again acted as a bacteriocide (α = 0.18 h^{-1}), reducing cell counts from 7.2 log CFU ml^{-1} to 5.6 log CFU ml^{-1}, after which regrowth was observed (Fig. 3). Regrowth was initiated with a μ_{max} of 0.21 h^{-1}, and *L. curvatus* LTH 1174 reached a maximum cell concentration of 2.00 g of CDM liter^{-1} after 80 h of growth, which was higher than for the reference fermentation.

Influence of different spices on curvacin A production by *L. curvatus* LTH 1174. Next to the reference fermentation, the fermentation conditions allowing measurable bacteriocin production were 0.40% pepper, 0.04% mace, 0.04% rosemary, and 0.35% garlic (Fig. 4). All spices tested negatively influenced bacteriocin production by *L. curvatus* LTH 1174 with the exception of 0.35% garlic (Fig. 4). When either 0.80% pepper or 0.40% nutmeg was added, no bacteriocin activity could be detected throughout the fermentation (Table 2). In the presence of 0.35% garlic, bacteriocin production was stimulated, since k_B increased by more then 20% to 1.80 mega arbitrary units (MAU) g of CDM^{-1}, while k_{inact} was comparable. This resulted in an increase of B_{max} by 130% to 1.18 MAU liter^{-1}. This strong increase in B_{max} was a consequence of the fact that more bacteriocin was produced per cell in combination with higher cell numbers reached and an earlier start of bacteriocin production, as shown by a decreased value of X_B (Table 2).

During the first 48 h of fermentation, no bacteriocin activity was observed, and hence the concentration of 0.35% garlic was not inhibitory to the indicator strain, *L. innocua* LMG 13568. The addition of 1.40 or 0.70% paprika caused a severe decrease in k_B and an increase in k_{inact}, which resulted in a B_{max} that was reduced by 97 and 91%, respectively, from the reference fermentation value. Hence, hardly any bacteriocin was produced when paprika was added. For 0.40% pepper X_B and k_{inact} were comparable to those of the reference fermentation, while k_B was more than halved, resulting in a halved B_{max} of 0.22 MAU liter^{-1} (Fig. 4). With 0.20% nutmeg, X_B increased to 1.15 g of CDM liter^{-1}, k_B decreased by 80%, while k_{inact} was the same as for the reference fermentation, resulting in a B_{max} of only 0.02 MAU liter^{-1} (Fig. 4). With the addition of 0.04% rosemary, the values of k_B and k_{inact} were the same as for the fermentation with 0.20% nutmeg. However, X_B was 0.95 g of CDM liter^{-1}. Finally, the addition of 0.04% mace had only a minor effect on bacteriocin production by *L. curvatus* LTH 1174.

Influence of different spices on the sugar metabolism of *L. curvatus* LTH 1174. With regard to the sugar metabolism of *L. curvatus* LTH 1174, it was clear that most spices had little or no influence on the different biokinetic parameters obtained from the primary model (Table 2). The average value for the parameter $Y_{L/S}$ was 0.98 ± 0.04 for all fermentations, and the variations observed were not significant. The addition of 1.40% paprika slightly accelerated lactic acid production in the be-

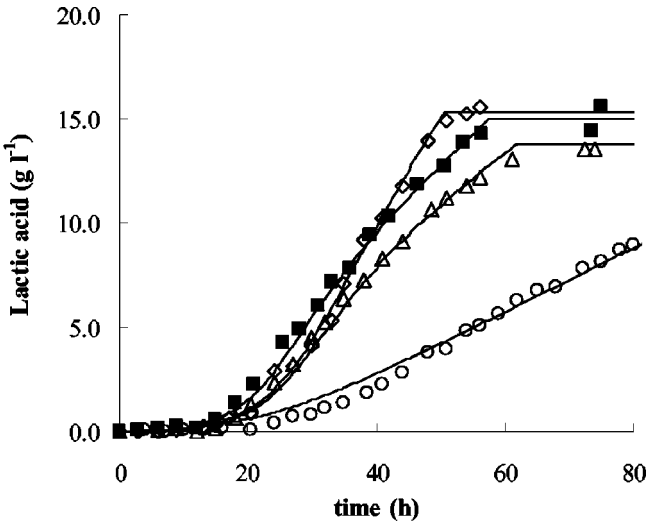

FIG. 5. Influence of different spices on lactic acid production (in grams of lactic acid per liter) by *L. curvatus* LTH 1174 as a function of time for the reference fermentation (■), 0.80% (wt/vol) pepper (◇), 0.40% (wt/vol) nutmeg (○), and 0.04% (wt/vol) mace (△). Symbols represent experimental values; lines are drawn according to the model.

ginning of the fermentation, while the addition of 0.80% pepper showed a clear stimulatory effect on lactic acid production (Fig. 5). On the other hand, 0.04% mace and in particular 0.40% nutmeg caused a severe retardation of lactic acid production (Fig. 5). In the case of nutmeg, lactic acid production started later due to the increased lag phase and was also slower due to the lower growth rate. Additionally, for both concentrations of nutmeg tested, the cell yield coefficient ($Y_{X/S}$) increased from 0.19 g of CDM (g of glucose)$^{-1}$ to 0.50 g of CDM (g of glucose)$^{-1}$. Apparently, the strongly reduced growth rate enabled the strain to produce more cells per unit of substrate. With 0.35% garlic, lactic acid production started later due to the prolonged lag phase, but the rate of lactic acid production was comparable to that of the reference fermentation.

DISCUSSION

Spices and herbs are used for adding desirable sensory properties to food products. Different mixtures of spices are added to many kinds of fermented sausages, all of which have their own specific organoleptic properties. The starter culture that is added to ensure rapid acidification and a safe end product is also influenced by the spices added, and hence it is important to know the effects that different sausage ingredients will have on bacteriocin production.

For all of the spices examined in this study (paprika, pepper, nutmeg, rosemary, mace, and garlic) the maximum specific growth rate (μ_{max}) of the bacteriocin producer *L. curvatus* LTH 1174 decreased, with the exception of paprika. The addition of paprika to the growth medium even stimulated growth, resulting in an increased value for μ_{max}. This might be due to the presence of sugars or microelements (1).

The largest negative effect on growth was ascribed to 0.40% nutmeg, which considerably increased the lag phase (t_{lag}). Previously, a pronounced inhibitory effect was shown, with 500 ppm of oil of nutmeg resulting in delayed bacterial growth for 4 days (49). Also, the food-borne pathogen *L. monocytogenes* is extremely sensitive to oil of nutmeg, with 0.05% showing a bactericidal effect. The concentrations used in food products may be sufficient to result in the stasis of listerial growth, provided that the initial pathogen load is low (43). Additionally, an extract of nutmeg was shown to posses antibacterial activity against *E. coli* O157, while nonpathogenic strains were not reduced (45). Also, the addition of 0.40% nutmeg severely decreased X_{max}. Clearly, nutmeg contains strong inhibitory components active against *L. curvatus* LTH 1174.

With a concentration of 0.35% garlic, an initial 1.5-log reduction in *L. curvatus* LTH 1174 occurred in the first 28 h, after which regrowth was observed. Moreover, *L. curvatus* LTH 1174 was already strongly inhibited by 0.70% garlic. This value is lower than the concentrations inhibitory to lactobacilli reported in the literature, ranging from 1 to 2% (17, 40). The differences might be explained by the natural variations in allicin and allicin content of garlic of different strains (5). Allicin shows a wide range of antibacterial activities against both gram-negative and gram-positive bacteria (5). It seems that garlic oil is consistently more potent than garlic powder when tested in vitro (35). However, when the thiosulfinate content is taken into account, garlic powder is more active than garlic oil against most bacteria (41). Moreover, garlic was shown to differentially inhibit bacteria, lactic acid bacteria being the least sensitive microorganisms (17, 40). Garlic can even stimulate the growth of lactic acid bacteria by providing them with a carbohydrate source for growth (37). *L. curvatus* LTH 1174 may be able to profit from some of these carbohydrates, which may explain the higher final biomass (X_{max}) obtained compared with the reference fermentation. However, it cannot be excluded that other growth-stimulatory components are present.

In addition to garlic, pepper was the only other spice that yielded a higher X_{max} (0.80%). Once more, this may be due to carbohydrates or other stimulatory components present at very low concentrations. However, pepper did not affect the growth rate or lag phase.

All of the spices tested affected bacteriocin production. In all cases except for garlic (0.35%), decreased specific bacteriocin production and hence decreased volumetric curvacin A activity were observed. In the case of garlic, increased biomass production and an earlier start of bacteriocin production, as indicated by the minimum biomass concentration necessary to start bacteriocin production (X_B), was observed. This resulted in more than doubled bacteriocin activity. There are components in garlic that somehow stimulate bacteriocin production. In contrast, in the case of nutmeg and paprika, decreased specific bacteriocin production and a severely increased bacteriocin inactivation rate were observed, in addition to an increased value of X_B. This value indicates interference with the onset of bacteriocin production, indicating that curvacin A is only produced at the end of the fermentation. However, it might be that garlic contains some nutrients that are more water soluble than the other spices. As a result, the observed bacteriocin activity was very low.

Nutmeg has been identified as a spice that has a major negative effect on the amount of bacteriocin produced per cell as well as an unfavorable effect on the start of bacteriocin

production. On the other hand, the addition of 0.04% rosemary did not interfere with the start of bacteriocin production, nor did it affect bacteriocin inactivation. However, this low concentration of rosemary, which is used in sausage production, causes the same severe inhibition of bacteriocin production as 0.20% nutmeg. With respect to pepper, a low concentration (0.40%) halved the observed bacteriocin activity, while a high concentration (0.80%) completely inhibited bacteriocin production. In the case of *Enterococcus faecium* CTC 492, the addition of 0.3% black pepper alone, especially in combination with sodium chloride, strongly inhibited enterocin production (6). In contrast, sakacin K production by *L. sakei* CTC 494 was not affected by the addition of black pepper. Moreover, the inhibitory effect of sakacin K against *L. monocytogenes* was reinforced by the addition of black pepper (21).

Although most spices had a negative effect on bacteriocin production by *L. curvatus* LTH 1174 in vitro and when tested separately, in the sausage environment each spice will have its own effect both on the pathogenic bacteria and on the bacteriocinogenic starter culture. The combination of spices together with bacteriocins that, albeit in a lower amount, can be produced in situ by the bacteriocinogenic starter culture may lead to a synergistic effect, rendering pathogens susceptible to the combined action of bacteriocin and one or more spices. For *L. monocytogenes*, such a synergistic inhibition has been shown between nisin on the one hand and garlic extract (42) or thymol (13) on the other. Moreover, even gram-negative pathogenic bacteria might be affected by the bacteriocins produced (alone or in synergy), since the starter cultures used also produced large amounts of lactic acid, which can permeabilize and disrupt the outer membrane of gram-negative bacteria (3). A synergistic effect has already been shown for curvacin A and sodium chloride, rendering the gram-negative pathogens *Escherichia coli* and *Salmonella enterica* susceptible, while at low pHs these pathogens also show increased sensitivity to the bacteriocins tested (14, 15). Hence, in the actual sausage environment, the bacteriocin that is produced in situ may be aided in its bactericidal action by the lactic acid that is produced and hence the drop in pH, the presence of relative high amounts of salt, and the antimicrobial activity of some of the spices present.

Lactic acid production by *L. curvatus* LTH 1174 was in most cases relatively comparable to that of the reference fermentation. In the case of nutmeg, slower lactic acid production was due to the fact that the spice negatively affected growth. In contrast, lactic acid production was faster in the presence of 1.40% paprika, especially in the presence of 0.80% pepper. Many spices have been reported to stimulate lactic acid production, which is usually attributed to their manganese content (49). However, in this study the liquid meat simulation medium used already contained the same large amount of manganese (0.038 g liter^{-1} or 2.25 $\times$ 10^{-4} M $MnSO_4 \cdot H_2O$) that is present in standard MRS medium. The manganese content is too high to allow the stimulatory effect of spices to be significant (34, 49). Hence, the stimulatory effect of paprika and especially of pepper on lactic acid production is probably due to components other than manganese. Zaika and Kissinger (49) also reported that, with spice extracts, consistently higher acidity values were reached than with a comparable manganese addition, confirming that additional trace minerals or other components present in spices affect acid production.

In this study, it has been shown that growth was not stimulated by most of the spices tested, although spices such as pepper and garlic are a source of trace elements and/or additional carbohydrates that may stimulate lactic acid or biomass production. The spices tested did not seem to be particularly stimulatory for bacteriocin production by *L. curvatus* LTH 1174, with the exception of garlic, a spice with antimicrobial properties that is frequently applied in the production of fermented sausages.

To study the effect of the various spices on the kinetics of *L. curvatus* LTH 1174, a liquid meat simulation medium was used. Although the medium used differs from a real food environment, the information obtained in this study is relevant, as the control of bacteriocin production during food fermentation requires knowledge of the factors affecting growth and bacteriocin production. Other factors that will have to be taken into account are the impact of the solid meat matrix, interactions with other microorganisms present in the sausage environment, substrate gradients and probable diffusion limitations, and bacteriocin activity losses due to adsorption to meat and fat particles and/or degradation by meat proteases. Clearly, in the complex environment of a fermented sausage, the various ingredients, including different spices, will each have its effect on the bacteriocinogenic starter culture. Moreover, the work described here forms a basis for looking at the active ingredients of the various spices or determining the possible interference of these spices with induction factor-regulated curvacin A production in *L. curvatus* LTH 1174.

ACKNOWLEDGMENTS

We acknowledge financial support from the Institute for the Promotion of Innovation by Science and Technology in Flanders, in particular the STWW project Functionality of Novel Starter Cultures in Traditional Fermentation Processes. This work was also supported by the Research Council of the Vrije Universiteit Brussel, the Fund for Scientific Research–Flanders, and different food companies.

The technical assistance of Vincent Schrijvers, Tom Adriany, and Tom De Winter was greatly appreciated. *L. curvatus* LTH 1174 was kindly provided by W. P. Hammes (Institut für Lebensmitteltechnologie, Universität Hohenheim, Stuttgart, Germany).

REFERENCES

1. **Aguirrezábal, M., J. Mateo, C. Domínguez, and J. M. Zumalacárregui.** 1998. Spanish paprika and garlic as sources of compounds of technological interest for the production of dry fermented sausages. Sci. Aliment. **18:**409–414.
2. **Aguirrezábal, M., J. Mateo, C. Domínguez, and J. M. Zumalacárregui.** 2000. The effect of paprika, garlic and salt on rancidity in dry sausages. Meat Sci. **54:**77–81.
3. **Alakomi, H. L., E. Skytta, M. Saarela, T. Mattila-Sandholm, K. Latva-Kala, and I. M. Helander.** 2000. Lactic acid permeabilizes gram-negative bacteria by disrupting the outer membrane. Appl. Environ. Microbiol. **66:**2001–2005.
4. **Alley, G., D. Cours, and D. Demeyer.** 1992. Effect of nitrate, nitrite and ascorbate on colour and colour stability of dry, fermented sausage prepared using 'back slopping'. Meat Sci. **32:**279–287.
5. **Ankri, S., and D. Mirelman.** 1999. Antimicrobial properties of allicin from garlic. Microbes Infect. **2:**125–129.
6. **Aymerich, T., M. G. Artigas, M. Garriga, J. M. Monfort, and M. Hugas.** 2000. Effect of sausage ingredients and additives on the production of enterocins A and B by *Enterococcus faecium* CTC 492. Optimization of *in vitro* production and anti-listerial effect in dry fermented sausages. J. Appl. Microbiol. **88:**686–694.
7. **Burden, R. L., and J. D. Faires.** 1989. Initial-value problems for ordinary differential equations, p. 219–310. *In* R. L. Burden and J. D. Faires (ed.), Numerical analysis, 4th ed. PWS-KENT Publishing Company, Boston, Mass.
8. **Dainty, R., and H. Blom.** 1995. Flavour chemistry of fermented sausages, p. 176–193. *In* G. Campbell-Platt and P. E. Cook (ed.), Fermented meats. Blackie Academic & Professional, London, United Kingdom.

9. **De Vuyst, L., R. Callewaert, and K. Crabbé.** 1996. Primary metabolite kinetics of bacteriocin biosynthesis by *Lactobacillus amylovorus* and evidence for stimulation of bacteriocin production under unfavourable growth conditions. Microbiology **142:**817–827.
10. **Diep, D. B., L. Axelsson, C. Grefsli, and I. F. Nes.** 2000. The synthesis of the bacteriocin sakacin A is a temperature-sensitive process regulated by a pheromone peptide through a three-component regulatory system. Microbiology **146:**2155–2160.
11. **Dorman, H. J. D., and S. G. Deans.** 2000. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. J. Appl. Microbiol. **88:**308–316.
12. **Dorman, H. J. D., A. C. Figueiredo, J. G. Barroso, and S. G. Deans.** 2000. *In vitro* evaluation of antioxidant activity of essential oils and their components. Flavour Fragr. J. **15:**12–16.
13. **Ettayebi, K., J. El Yamani, and B.-D. Rossi-Hassani.** 2000. Synergistic effects of nisin and thymol on antimicrobial activities in *Listeria monocytogenes* and *Bacillus subtilis*. FEMS Microbiol. Lett. **183:**191–195.
14. **Gänzle, M.** 1999. Effect of ecological factors on the inhibitory spectrum and activity of bacteriocins. Int. J. Food Microbiol. **46:**207–217.
15. **Gänzle, M.** 1999. Resistance of *Escherichia coli* and *Salmonella* against nisin and curvacin A. Int. J. Food Microbiol. **48:**37–50.
16. **González-Fandos, M. E., M. Sierra, M.-L. Garcia-Lopez, M. C. Garcia-Fernández, and A. Otero.** 1999. The influence of manufacturing and drying conditions on the survival and toxinogenesis of *Staphylococcus aureus* in two Spanish dry sausages (chorizo and salchichón). Meat Sci. **52:**411–419.
17. **González-Fandos, M. E., M.-L. Sierra, M.-L. Garcia-Lopez, A. Otero, and J. Sanz.** 1996. Effect of the major herbs and spices in Spanish fermented sausages on *Staphylococcus aureus* and lactic acid bacteria. Arch. Lebensmittelhyg. **47:**43–47.
18. **Grohs, B. M., and B. Kunz.** 2000. Use of spice mixtures for the stabilisation of fresh portioned pork. Food Control **11:**433–436.
19. **Hagen, B. F., H. Næs, and A. L. Holck.** 2000. Meat starters have individual requirements for Mn^{2+}. Meat Sci. **55:**161–168.
20. **Harris, J. C., S. L. Cottrell, S. Plummer, and D. Lloyd.** 2001. Antimicrobial properties of *Allium sativum* (garlic). Appl. Microbiol. Biotechnol. **57:**282–286.
21. **Hugas, M., M. Garriga, M. Pascual, M. T. Aymerich, and J. M. Monfort.** 2002. Enhancement of sakacin K activity against *Listeria monocytogenes* in fermented sausages with pepper or manganese as ingredients. Food Microbiol. **19:**519–528.
22. **Kivanç, M., A. Akgül, and A. Doğan.** 1991. Inhibitory and stimulatory effects of cumin, oregano and their essential oils on growth and acid production of *Lactobacillus plantarum* and *Leuconostoc mesenteroides*. Int. J. Food Microbiol. **13:**81–86.
23. **Kumar, M., and J. S. Berwal.** 1998. Sensitivity of food pathogens to garlic (*Allium sativum*). J. Appl. Microbiol. **84:**213–215.
24. **Leroy, F., and L. De Vuyst.** 1999. Temperature and pH conditions that prevail during fermentation of sausages are optimal for production of the antilisterial bacteriocin sakacin K. Appl. Environ. Microbiol. **65:**974–981.
25. **Leroy, F., and L. De Vuyst.** 2001. Growth of the bacteriocin-producing *Lactobacillus sakei* strain CTC 494 in MRS broth is strongly reduced due to nutrient exhaustion: a nutrient depletion model for the growth of lactic acid bacteria. Appl. Environ. Microbiol. **67:**4407–4413.
26. **Leuschner, R. G. K., and J. Zamparini.** 2002. Effects of spices on growth and survival of *Escherichia coli* O157 and *Salmonella enterica* serovar Enteriditis in broth model systems and mayonnaise. Food Control **13:**399–404.
27. **Lücke, F.-K.** 1998. Fermented sausages, p. 441–483. *In* B. J. B. Wood (ed.), Microbiology of fermented foods. Blackie Academic & Professional, London, United Kingdom.
28. **Madsen, H. L., and G. Bertelsen.** 1995. Spices as antioxidants. Trends Food Sci. Technol. **6:**271–277.
29. **Mangena, T., and N. Y. O. Muyima.** 1999. Comparative evaluation of the antimicrobial activities of essential oils of *Artemisia afra*, *Pteronia incana* and *Rosmarinus officinalis* on selected bacteria and yeast strains. Lett. Appl. Microbiol. **28:**291–296.
30. **Messens, W., J. Verluyten, F. Leroy, and L. De Vuyst.** 2003. Modelling growth and bacteriocin production by *Lactobacillus curvatus* LTH 1174 in response to temperature and pH values used for European sausage fermentation processes. Int. J. Food Microbiol. **81:**41–52.
31. **Miron, T., A. Rabinkov, D. Mirelman, M. Wilchek, and L. Weiner.** 2000. The mode of action of allicin: its ready permeability through phospholipid membranes may contribute to its biological activity. Biochim. Biophys. Acta **1463:**20–30.
32. **Miwa, M., A. Okitani, H. Kato, M. Fujimaki, and S. Matsuura.** 1980. Reaction between nitrite and low salt-soluble diffusible fraction of meat. Some compounds influencing nitrite depletion and producing unidentified N compounds. Agric. Biol. Chem. **44:**2179–2183.
33. **Nassu, R. T., L. A. G. Gonçalves, M. A. A. Pereira da Silva, and F. J. Beserra.** 2003. Oxidative stability of fermented goat meat sausage with different levels of natural antioxidant. Meat Sci. **63:**43–49.
34. **Nes, I. F., and R. Skjelkvåle.** 1982. Effect of natural spices and oleoresins on *Lactobacillus plantarum* in the fermentation of dry sausages. J. Food Sci. **47:**1618–1625.
35. **O'Gara, E. A., D. J. Hill, and D. J. Maslin.** 2000. Activities of garlic oil, garlic powder, and their diallyl constituents against *Helicobacter pylori*. Appl. Environ. Microbiol. **66:**2269–2273.
36. **Oxoid Ltd.** 2001. The manual, 8th ed. Oxoid Ltd., Basingstoke, England.
37. **Paludan-Müller, C., H. H. Huss, and L. Gram.** 1999. Characterization of lactic acid bacteria isolated from a Thai low-salt fermented fish product and the role of garlic as a substrate for fermentation. Int. J. Food Microbiol. **46:**219–229.
38. **Pandit, V. A., and L. A. Shelef.** 1994. Sensitivity of *Listeria monocytogenes* to rosemary (*Rosmarinus officinalis* L.). Food Microbiol. **11:**57–63.
39. **Rabinkov, A., T. Miron, L. Konstantinovski, M. Wilchek, D. Mirelman, and L. Weiner.** 1998. The mode of action of allicin: trapping of radicals and interaction with thiol containing proteins. Biochim. Biophys. Acta **1397:**233–244.
40. **Rees, L. P., S. F. Minney, N. T. Plummer, J. H. Slater, and D. A. Skyrme.** 1993. A quantitative assessment of the antimicrobial activity of garlic (*Allium sativum*). World J. Microbiol. Biotechnol. **9:**303–307.
41. **Ross, Z. M., E. A. O'Gara, D. J. Hill, H. V. Sleightholme, and D. J. Maslin.** 2001. Antimicrobial properties of garlic oil against human enteric bacteria: evaluation of methodologies and comparisons with garlic oil sulfides and garlic powder. Appl. Environ. Microbiol. **67:**475–480.
42. **Singh, B., M. B. Falahee, and M. R. Adams.** 2001. Synergistic inhibition of *Listeria monocytogenes* by nisin and garlic extract. Food Microbiol. **18:**133–139.
43. **Smith-Palmer, A., J. Stewart, and L. Fyfe.** 1998. Antimicrobial properties of plant essential oils and essences against five important food-borne pathogens. Lett. Appl. Microbiol. **26:**118–122.
44. **Stahnke, L. H.** 1995. Dried sausages fermented with *Staphylococcus xylosus* at different temperatures and with different ingredients levels. 1. Chemical and bacteriological data. Meat Sci. **41:**179–191.
45. **Takikawa, A., K. Abe, M. Yamamoto, S. Ishimaru, M. Yasui, Y. Okubo, and K. Yokoigawa.** 2002. Antimicrobial activity of nutmeg against *Escherichia coli* O157. J. Biosci. Bioeng. **94:**315–320.
46. **Tichaczek, P. S., J. Nissen-Meyer, I. F. Nes, R. F. Vogel, and W. P. Hammes.** 1992. Characterization of the bacteriocins curvacin A from *Lactobacillus curvatus* LTH 1174 and sakacin P from *L. sake* LTH 673. Syst. Appl. Microbiol. **15:**460–468.
47. **Verluyten, J., W. Messens, and L. De Vuyst.** 2004. Sodium chloride reduces production of curvacin A, a bacteriocin produced by the *Lactobacillus curvatus* strain LTH 1174 originating from fermented sausage. Appl. Environ. Microbiol. **70:**2271–2278.
48. **Verluyten, J., W. Messens, and L. De Vuyst.** 2003. The curing agent sodium nitrite, used in the production of fermented sausages, is less inhibiting to the bacteriocin-producing meat starter culture *Lactobacillus curvatus* LTH 1174 under anaerobic conditions. Appl. Environ. Microbiol. **69:**3833–3839.
49. **Zaika, L. L., and J. C. Kissinger.** 1984. Fermentation enhancement by spices: identification of active component. J. Food Sci. **49:**5–9.
50. **Zaika, L. L.** 1988. Spices and herbs: their antimicrobial activity and its determination. J. Food Safety **9:**97–118.