African Journal of Microbiology Research

Volume 9 Number 39, 30 September, 2015
ISSN 1996-0808

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Vol. 9(39), pp. 2088-2094, 30 September, 2015
DOI: 10.5897/AJMR2015.7656
Article Number: 61588F455888
ISSN 1996-0808
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African Journal of Microbiology Research

Full Length Research Paper

In vitro antibacterial activity of *Baillonella toxisperma* (Pierre) extracts against *Staphylococcus aureus*, *Salmonella typhi*, *Proteus mirabilis* and *Bacillus cereus* F3748

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Received 7 July, 2015; Accepted 31 August, 2015

This study evaluated the *in vitro* antibacterial activity of the ethyl acetate, acetone, methanol and hydro-ethanol mixture (2: 8) extracts of the leaves and stem-barks of *Baillonella toxisperma* (Pierre), harvested in the East and center regions of Cameroon, on *Staphylococcus aureus*, *Salmonella typhi*, *Proteus mirabilis* and *Bacillus cereus* F3748. These bacteria are usually responsible for diarrheal diseases and in severe cases can lead to the dead of patients. The susceptibility of the bacteria to the extracts was evaluated by the well diffusion method and the inhibition parameters of the bacterial growth were determined by the micro-dilution assay according to the directives of document M27-A9 (2012) of the Clinical and Laboratory Standards Institutes (CLSI). The Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal concentrations (MBC) obtained were between 1.56 and 25.00 mg/ml. Stem-barks ethyl acetate extract from the East region, was most active on *S. aureus*, *S. typhi* and *P. mirabilis* with a MBC of 6.25 mg/ml. The leaves methanolic extracts from the center region was the most active with a MBC of 6.25 mg/ml on *S. aureus*. The ratio MBC/MIC shows that the majority of the extracts were bacteriostatic on the strains tested. The phytochimical screening revealed that the plant contained bioactive substances such as phenols, tannins, flavonoids, steroids, alkaloids, saponins, triterpenes and cardiac glycosides, reported by several authors for their antibacterial activity. The results obtained validate the traditional use of this plant in the treatment of affections of bacterial origin.

Key words: Cameroon, *Baillonella toxisperma* (Pierre), bioactive substances, antibacterial activity.

INTRODUCTION

Bacterial infections constitute a serious public health problem in the world. Among the causative agents, *Staphylococcus aureus*, *Salmonella typhi*, *Proteus mirabilis* and *Bacillus cereus* are cited in most clinical cases. *S. aureus* is one of the principal causes of food toxi-infections which are characterized by a severe appearance of diarrhoea. It is the almost-universal cause of furuncles, carbuncles, and skin abscesses and worldwide is the most commonly identified agent responsible for skin and soft tissue infections (McCaig et al., 2006). *S. typhi* is the causative agent of typhoid fever and diarrhoea. *B. cereus* is responsible for food poisoning and diarrheal syndromes

(Logan and Rodrigez-Diaz, 2006). *P. mirabilis* on its part is responsible for urinary tract, cutaneous, respiratory tract infections, septicemia and bacteremia (Ronald, 2003). These bacterial infections can in extreme cases, lead to the dead of the patient. Moreover, these bacteria have over time developed resistance to certain usual antibiotics. It is the case of *B. cereus* which has become resistant to penicillin, ampicillin, cephalosporines and trimethoprimes (Murray et al., 2007). There is an imperative need for the research and renewal of active ingredients which have become ineffective due to the emergence of the phenomenon of microbial multi-resistance to common antibiotics.

Medicinal plants via their secondary metabolites constitute a potential source of antimicrobial (Li et al., 2007). Many scientific studies have been undertaken in order to study the botanical and therapeutic aspects of the latter and to integrate their medicinal properties in a modern health system by exploiting their active ingredients (Biyiti et al., 2004). Many bioactive compounds isolated from plants such as flavonoids, phenolic alkaloids, saponins, tannins, coumarins, phenolic acids and terpenes, were been used for a long time as active ingredients in the development of anti-infectious drugs (Ghost et al., 2007).

Baillonella toxisperma (Pierre) is a plant of the Cameroonian pharmacopeia, traditional hailed for its medicinal virtues. Commonly called Moabi, this plant is used in treating more than 50 diseases among which are microbial infections (Laird, 2000). *B. toxisperma* (Pierre) develops in hot tropical forests and under wet climates (Louppe, 2005; Angerand, 2006). In Cameroon, it is found abundantly in the East and South regions. This plant is used traditionally to treat infections of microbial origin such as mycoses, rheumatism, hemorroides, diarrheal diseases, sexually transmissible diseases (Dibong et al., 2011; Ngueguim et al., 2009). In this respect, it constitutes a potential source of anti-infectious compounds. With the aim of valorizing this plant, we proposed in this study to evaluate the *in vitro* antibacterial activity of its extracts against *S. aureus*, *S. typhi*, *P. mirabilis* and *B. cereus* F3748. In order to determine the influence of the harvest site on the antimicrobial activity, we used botanical materials from
two regions: the East and Center regions of Cameroon.

MATERIALS AND METHODS

Preparation of the leaves and stem-barks extracts of *Baillonella toxisperma* (Pierre)

Extracts of the botanical material were extracted according to the protocol described by Prakash and Gupta (2005). The leaves and stem-barks were cut out into scraps then, dried at ambient temperature, free from moisture and light. The dried plant materials were finely crushed using an electric blender. The powder obtained was macerated in four solvents: ethyl acetate, acetone, methanol and ethanol-water (8: 2). 100 g of powdered stem-barks and leaves were macerated in 500 ml of each solvent for 48 h. The marcs obtained were filtered through Whatman N° 1 filter paper and the filtrates collected in conical flasks. This process was repeated thrice for complete exhaustion of the plant material and the filtrates obtained were concentrated in a rotarvapor. The dry extracts were preserved at +4°C in a refrigerator. The extraction yields expressed in percentage (%) were determined by the formula below:

Yield (%) = (Mass of macerated powder / Mass of the extract) × 100.

Phytochemical screening

Determination of the phytochemical composition of the various extracts was carried out according to standard methods described by Harbone (1998) and Sofowora (1993).

Preparation of the bacterial inoculum

For each tested micro-organism, overnight cultures of bacterial colonies seeded on Mueller Hinton Agar (Fortress Diagnostics Limited U.K) and incubated at 37°C were suspended in 5 ml saline water in test tubes. This suspension was read thereafter with a spectrophotometer at 625 nm. When the optical density was between 0.08 and 0.13, the bacterial load was 10^8 CFU/mL (0.5 McFarland). After a 100th dilution, the bacterial load was 10^6 CFU/ml (Hernandez et al., 2000).

Preliminary sensitivity test of the strains to the extracts

The preliminary tests of sensitivity of the bacterial strains to the various extracts were carried out as recommended by CLSI (2005). 100 µl of each bacterial inoculum was inoculated on Mueller Hinton agar (Fortress Diagnostics Limited U.K). The Petri dishes were then allowed to dry at ambient temperature under a fumes cupboard for 15 min. 6 mm wells were bored in the agar and the bottom of each well plugged with a drop of Mueller Hinton agar to limit the diffusion of the extracts from below. Fixed volumes of 50 µl of the stock solutions of the extracts (50 mg/ml) and gentamicin (1 mg/ml) were then introduced into each well. After a pre diffusion time of 15 min of the antibacterial substances to be tested at ambient temperature, the Petri dishes were incubated at 37°C for 24 h. The inhibition diameters round each well was measured using a sliding caliper. Each test was carried out in triplicate and the inhibition diameters expressed mean ± standard deviation.

Determination of the inhibition parameters: MIC and MBC

The inhibition parameters of bacterial growth were evaluated according to the M27-A9 guideline described by CLSI (2012). This involved preparing double dilutions of tested substances in 100 µL of glucose supplemented nutrient broth (GNB) medium (Acumedia Manufacturers) into the wells of a microtiter. The range of final

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Table 1. Extraction yields and physico-chemical characteristics of leaves and stem-barks extracts of *Baillonella toxisperma* (Pierre).

Sites of harvest	Extraction Solvents	Parts of the plant	Extraction yield (%)	Physical properties of the extracts	
				Color	**State**
Dimako (East Region -Cameroon) 21/01/15	Hexane	Stem-barks	2,42	Yellow	Fatty
		Leaves	2,59	Dark-green	Pasty
	Ethyl-acetate	Stem-barks	1,53	Brown	Powdery
		Leaves	6,24	Dark-green	Pasty
	Methanol	Stem-barks	14,02	Thick-red	Pasty
		Leaves	8,88	Dark-green	Pasty
	Ethanol-water (8/2)	Stem-barks	18,34	Thick-red	Powdery
		Leaves	13,95	Brown	Pasty
	Acetone	Stem-barks	8,32	Red	Powdery
		Leaves	7,92	Dark-green	Pasty
Mbalmayo (Center Region-Cameroon) Le 09/12/14	Hexane	Stem-barks	2,75	Yellow	Fatty
		Leaves	3,16	Dark-green	Pasty
	Ethyl-acetate	Stem-barks	1,08	Yellowish	Powdery
		Leaves	2,40	Dark-green	Pasty
	Methanol	Stem-barks	4,72	Brown	Pasty
		Leaves	6,80	Light-green	Pasty
	Ethanol-water(8/2)	Stem-barks	6,85	Thick-red	Powdery
		Leaves	10,7	Greenish	Powdery
	Acetone	Stem-barks	4,10	Red	Powdery
		Leaves	4,57	Dark-green	Pasty

concentrations tested were 25 to 0,097 mg/ml for each plant extract and 0,250 to 0,00097 mg/ml for gentamicin (Brunhild Pharmaceutical Private Limited). Each serial dilution was performed in triplicate. The bacterial inoculum was prepared at 10^6 CFU/mL using McFarland. Volumes of 100 µL of this inoculum were distributed to all the wells of the microtiter. A line of the plate without plant extract served as a control for the growth of the organism (negative control) and another (without plant extract and without inoculum) served as sterility testing medium (positive control). The microtitre plates were thereafter sealed with aluminum foil and incubated at 37°C for 24 h. After incubation, 40 µl of 2,3,5-triphenyl tetrazolium chloride (Sigma-Aldrich) (0,2 mg/mL) were introduced into each well (Burdock et al., 2011) . The MIC was defined as the smallest concentration of the extract for which there was no change in the initial yellowish color of the medium to red. The MBC were determined by subculture. 50 µL of the contents of wells greater than or equal to the MIC was introduced into 150 µL of fresh GNB. The microtitre plates were incubated for 48h at 37°C, thereafter revealed as earlier done. The smallest concentration for which no color change was observed was regarded as the minimum bactericidal concentration.

RESULTS

Extraction yield

The extraction yield of the leaves and stem-barks of *B. toxisperma* (Pierre) are shown in Table 1. It is observed that the extraction yields are comprised between 1.08 % (stem-barks ethyl acetate) and 10.07% (leaves hydro-ethanolic extract) for the plant material harvested in the Center and between 1.53 % (stem-barks ethyl acetate) and 18.34% (hydro-ethanolic stem-barks) for the plant material harvested in the East region.

Phytochemical screening

The phytochemical screening revealed the presence of several groups of secondary metabolites such as tannins, the flavonoids, steroids, saponins, terpenoids and phenols in both extracts of *Baillonella toxisperma* (Pierre) harvested from the East and Center regions of Cameroon. Table 2 summarizes the results obtained from the screening depending on the extracts considered.

Susceptibility test

The results obtained from the susceptibility test (Table 3) show that the bacterial strains were sensitive to the leaves and stem-barks extracts of *B. toxisperma* (Pierre). For the plant material harvested in the East, the inhibition diameters of the leaves crude extracts was between 8.66 ± 0.57 mm (hydro-ethanolic extract on *B cereus*) and 11.33 ± 0.57 mm (methanolic extract on *B cereus*), and for the botanical material harvested in the Center, the inhibition diameters ranged from 9.00 ± 1.00 mm (acetone extract) to 11.66 ± 0.57 mm (methanolic extract on *S. typhi*). The inhibition diameters of the stem-barks crude extracts was comprised between 9.66 ± 0.57 mm (hydro-ethanolic extract on *S. aureus*) and 19.66 ± 0.57 mm

Table 2 . Phytochemical screening.

Phytochemical groups	Plant extract															
	East-region								Center-region							
	E1	F1	E2	F2	E3	F3	E4	F4	E'1	F'1	E'2	F'2	E'3	F'3	E'4	F'4
Phenols	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Tanins	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Steroïds	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+
Triterpenes	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
Saponines	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+
Cardiacglycosids	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
Alcaloids	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

E1 and F1, Acetone and ethyl acetate stem-barks and leaves from the East; E2 and F2, Methanol stem-barks and leaves from the East; E3 and F3, hydro-ethanolic stem-barks and leaves from the East ; E4 and F4, acetone stem-barks and leaves extracts from the East; E'1 and F'1, ethyl acetate stem-barks and leaves from the Center; E'2 and F'2, Methanol stem-barks and leaves extract from the Center; E'3 and F'3, Hydro-ethanolic extracts from the Center; E'4 and F'4, Acetone stem-barks and leaves from the Center; +, Presence of compound; -, absence of compound.

(acetone extract on *S. typhi*) for the plant material harvested in the East and between 7.33± 0.57 mm (ethyl acetate and acetone extracts on *S. aureus* and *P. mirabilis*) and 17.00 ± 0.00 mm (acetone extract on *S. typhi*) for the plant material harvested in the Center.

Determination of the inhibition parameters

The results obtained for the inhibition parameters (Table 4) show that the MIC of the plant material from the East was comprised between 1.56 and 25 mg/ml and that of the Center, between 3.12 and 25 mg/ml. The MBC was between 6.25 and 25 mg/ml. According to Fauchère and Avril (2002) when the MBC of an antibiotic on a given strain is close to the MIC (1 ≤ MBC/MIC ≤ 2), the antibiotic is described as being bactericidal. On the other hand, when these values are relatively distant, (4 ≤ MBC/MIC ≤ 16), the antibiotic is known to be bacteriostatic. Lastly if the MBC/MIC >16, it is described tolerant.

DISCUSSION

The extraction yields of the leaves and barks show that the extraction yields (Table 1) were between 1.08% (ethyl acetone stem-barks extracts) and 10.07 % (Hydro-ethanolic leaves extracts) for the plant material from the Center region and between 1.53 % (ethyl acetate stem-barks extracts) and 18.34% (Stem-barks ethanol-water extract) for the plant material collected in the East region. For the same solvent and plant organ (leaves or bark), variations in the extraction yields could be due to edaphic and climatic factors. Globally, methanol and hydro-ethanol extracts gave the best extraction yields. This could be explained by the fact that the secondary metabolites extracted are more soluble in alcohols (Bruneton, 1999).

Results obtained from the phytochemical screening (Table 2) of the extracts of *B. toxisperma* (Pierre) show that this plant is endowed with secondary metabolites such as phenols, saponins, tannins, flavonoids, triterpenes, steroids and cardiac glycosids. These bioactives substances have been reported by several authors for their antibacterial activity. These bioactive compounds have long been used in modern medicine for drug developement (Dawang and Datup, 2012). Several molecules isolated from plants such as pinocembrine, ponciretine, sophora flavanone G and naringine significantly showed antimicrobial activities in both Gram positive and Gram negative bacteria (Tim and Andrew, 2005). For the same solvent and the same plant organ (leaves or bark), variations in the phytochemical composition were observed. This could be due to ecological parameters, which generally differ from one area to another depending on geographic distant. These differences can strongly influence the biology and the physiology of the plants, in particular their composition in secondary metabolites (Etchiké *et al.*, 2011).

The results obtained from the susceptibility test (Table 3) show that at a concentration of 50 mg / ml, the inhibition diameters of the bacterial growth were between 6.66 ± 0.57 and 19.66 ± 0.57 mm. For a given strain, these inhibition diameters were however lower than those of gentamicin (13.00 ± 0.00 to 28.66 ± 0.57 mm). The distinct sensitivity of the strains with regards to the extracts could be due to the intrinsic features specific to each micro-organism (permeability of the cell wall, presence of an external membrane) and with the phytochemical profile of the extracts (Takeo et al., 2004; Achraf et al., 2012). The Gram positive bacteria (*S. aureus* and *B. cereus*) were more sensitive to the toxic effect of the extracts than their Gram negative (*S. typhi*

Table 3. Susceptibility test.

Bacterial strains	Inhibition diameters (mm)																Gen
	East-region								Center-region								
	E1	F1	E2	F2	E3	F3	E4	F4	E'1	F'1	E'2	F'2	E'3	F'3	E'4	F'4	
Bacillus cereus	15.33± 0.57	/	16.66 ± 0.57	11.33 ± 0.57	16.00± 0.00	8.66± 0.57	16.00± 0.00	10.33± 0.57	13.33± 0.57	/	14.00± 1.00	10.00± 1.00	14.00± 0.00	10.33± 0.57	13.33± 0.57	9.33± 0.57	23.33± 0.57
Staphylococcus aureus	12.00 ± 0.57	/	10.33 ± 0.57	/	9.66 ± 0.57	/	10.00± 1.00	/	10.33± 0.57	/	6.66 ± 0.57	/	/	/	7.33 ± 0.57	10.33± 0.57	13.00± 0.57
Salmonella typhi	18.66 ± 0.57	/	18.00 ± 1.00	11.00 ± 0.00	19.00 ± 1.00	/	19.66± 0.57	11.00± 0.00	11.00± 0.00	/	13.66± 1.15	10.33± 0.57	13.66± 0.57	11.66± 0.57	17.00± 0.00	9.00 ± 1.00	28.66± 0.57
Proteus mirabilis	15.00 ± 1.00	/	10.33 ± 0.57	/	14.00 ± 1.00	/	14.33± 0.57	/	7.33 ± 1.15	/	10.66± 0.57	/	11.33± 1.15	/	10.66± 0.57	/	15.00± 1.00

E1 and F1, Ethyl acetate stem-barks and leaves from the East; E2 and F2, Methanol stem-barks and leaves extracts from the East; E3 and F3, Hydro-ethanolic stem-barks and leaves from the East; E4 and F4, Acetone stem-barks and leaves extracts from the East; E'1 and F'1, Ethyl acetate stem-barks and leaves extracts from the Center; E'2 and F'2, Methanol stem-barks and leaves extracts from the Center; E'3 and F'3, Hydro-ethanolic stem-barks and leaves extracts from the Center; E'4 and F'4, Acetone stem-barks and leaves extracts from the Center; Hu ,Oil; Gen, Gentamicin.

and P. mirabilis) counterparts. This could be due to the significant differences in the outer layer of Gram positive and Gram negative bacteria. Gram negative bacteria possess an external membrane and a periplasmic space which is absent in Gram positive bacteria (Duffy and Power, 2001). The surface of Gram-negative bacteria is largely composed of the glycolipid lipopolysaccharide (LPS), serving as one of the initial barriers against extracellular stresses. Specifically, LPS is a major constituent of the outer leaflet of the outer membrane phospholipid bilayer, which envelops the peptidoglycan containing periplasm and the inner membrane (Band and Weiss, 2015).

With regards to the inhibition parameters (Table 3), the MIC ranges from 1.56 to 25.00 mg/ml, and the MBC between 6.25 to 25.00 mg/ml. The ratio MBC/MIC was determined and according to the classification made by Fauchère and Avril (2002), the acetone extracts and the hydro-ethanolic extracts of the leaves and stem-barks harvested in the Center and East were bactericidal on *S. aureus* (1 ≤ CMB/CMI ≤ 2). The antibacterial activity of these extracts can be ascribed to the presence of phenols, terpenoids, tannins and flavonoids whose mechanisms of actions on bacteria are: the destruction of the membrane of the micro-organism through a lipophilic action (Cowan, 1999), bacterial and viral protein precipitation as well as heavy metals (Kansole, 2009), complexing property to soluble extracellular proteins and to the bacterial cell wall (Cowan, 1999) and the inactivation of microbial adhesion, enzymes and extracellular proteins respectively (Ghestem et al., 2001). From the classification of Fauchère and Avril (2002), the extracts presented a bacteriostatic action on most of the strains (4 ≤ MBC/MIC ≤ 16) except acetone and hydro-ethanolic extracts which were bactericidal.

Based on the *in vitro* antibacterial activity obtained with the extracts of *B. toxisperma* (Pierre), this plant englobes a set of criteria which could justify the renewed interest for the exploitation of this natural resource in the development of antibacterial drugs order to mitigate the narrow activity spectrum which the usual molecules pose.

Conclusion

The results obtained in this study bring scientific justification as to the use of *B. toxisperma* (Pierre) in traditional medicine for the treatment of microbial infections, in particular those of bacterial origin. Depending on the harvesting site of the plant (East and Center regions of Cameroon), more or less significant variations in the antibacterial activity was observed. These variations were attributed to edaphic and climatic factors which influenced the qualitative and quantitative chemical composition of the secondary metabolites in the plant at their site of growth. Phytochemical

Table 4. Inhibition parameters: MIC, MBC, MBC/MIC.

Bacterial strains	Inhibition parameters (mg/ml)	Plant extracts																
		East-region								Center-region								
		E1	F1	E2	F2	E3	F3	E4	F4	E'1	F'1	E'2	F'2	E'3	F'3	E'4	F'4	Gen
Bacillus cereus	MIC	1.56	12.5	1.56	6.25	3.12	12.5	1.56	25	12.5	25	3.12	3.12	6.25	12.5	3.12	6.25	0.125
	MBC	12.5	ND	12.5	ND	25	12.5	12.5	ND	25	25	12.5	12.5	25	25	12.5	ND	0.25
	MBC/MIC	8	ND	8	ND	8	1	8	ND	2	1	4	4	4	2	4	ND	2
Staphylococcus aureus	MIC	1.56	6.25	3.12	6.25	3.12	12.5	3.12	12.5	12.5	12.5	3.12	6.25	3.12	6.25	6.25	12.5	0.125
	MBC	6.25	6.25	12.5	ND	6.25	25	6.25	25	25	ND	12.5	6.25	ND	12.5	12.5	25	0.125
	MBC/MIC	4	1	4	ND	2	2	2	2	2	ND	4	1	ND	2	2	2	1
Salmonella typhi	MIC	1.56	6.25	1.56	12.5	1.56	12.5	1.56	6.25	6.25	25	3.12	6.25	3.12	3.12	3.12	6.25	0.0625
	MBC	6.25	ND	12.5	25	6.25	ND	6.25	ND	25	ND	12.5	ND	ND	25	12.5	ND	0.0625
	MBC/MIC	4	ND	8	2	4	ND	4	ND	4	ND	4	ND	ND	8	4	ND	1
Proteus mirabilis	MIC	1.56	6.25	1.56	6.25	1.56	25	3.12	12.5	12.5	25	3.12	12.5	3.12	6.25	3.12	12.5	0.0312
	MBC	6.25	25	12.5	25	6.25	ND	12.5	ND	ND	ND	12.5	ND	ND	12.5	12.5	25	0.25
	MBC/MIC	4	4	8	4	4	ND	4	ND	ND	ND	4	ND	ND	4	4	2	8

E1 and F1, Ethyl acetate stem-barks and leaves from the East; E2 and F2, Methanol stem-barks and leaves extracts from the East; E3 and F3, Hydro-ethanolic stem-barks and leaves from the East; E4 and F4, Acetone stem-barks and leaves extracts from the East; E'1 and F'1, Ethyl acetate stem-barks and leaves extracts from the Center; E'2 and F'2, Methanol stem-barks and leaves extracts from the Center; E'3 and F'3, Hydro-ethanolic stem-barks and leaves extracts from the Center; E'4 and F'4 ,Acetone stem-barks and leaves extracts from the Center; Hu ,Oil; Gen, Gentamicin; ND, not determined.

screening of the extracts of the plant material from the East region and that from the Center region showed that no matter the place of harvest, the two samples were rich in terpenoids, tannins, flavonoids, phenols, saponins, steroids and cardiac glycosides. These bioactive molecules can isolated from this plant and used in the development of pharmaceutical specialties capable of ensuring the treatment of many infectious diseases.

Conflict of interests

The author(s) did not declare any conflict of interest.

ACKNOWLEDGEMENTS

The authors thank the University of Yaoundé I for providing the framework, "Centre Pasteur" of Cameroon for having provided us with the bacteria strains used in this work and the National Herbarium of Yaoundé-Cameroon for botanical identification of *B. toxisperma* (Pierre).

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Vol. 9(39), pp. 2095-2102, 30 September, 2015
DOI: 10.5897/AJMR2015.7575
Article Number: DF9DD5B55891
ISSN 1996-0808
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http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Growth and anti-listerial activity of a nisin Z producer in a pork lean meat broth fermentation system

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Received 9 May, 2015; Accepted 6 July, 2015

Listeria monocytogenes is widely distributed in nature and has been isolated from numerous sources such as meat and fermented meat products. This pathogenic microorganism can resist conditions of low pH, low water activity (Aw), high salt (NaCl) concentrations and the presence of sodium nitrite, being able to survive the commercial sausage manufacturing process. The aim of this work was to evaluate the antilisterial activity of a lactic acid bacterium (_Lactococcus lactis_ subsp. _lactis_ PD 6.9) isolated from Italian-style salami in conditions that simulate salami fermentation. _L. lactis_ PD 6.9 produces nisin Z and grows well in pork lean meat broth, a feature that would be useful to compete with food-borne pathogens. The peak of nisin Z production by _L. lactis_ PD 6.9 in pork lean meat broth occurred after 14 h of fermentation, but the inhibitory activity decreased if the producer organism was maintained in stationary phase. When _L. lactis_ PD 6.9 (10^7CFU ml^{-1}) and _Listeria monocytogenes_ LMA 20 (10^6CFU ml^{-1}) were co-inoculated in pork lean meat broth, growth of _L. lactis_ PD 6.9 was unaffected. The decrease in viable cell number of _Listeria_ coincided with an increase in bacteriocin activity produced by _L. lactis_ PD 6.9 in pork lean meat broth. Co-culture experiments indicated that _L. lactis_ PD 6.9 was able to control the growth of _L. monocytogenes_ even if the _Listeria_ population was 1000-fold greater than the _L. lactis_ population. These results demonstrate the potential application of _L. lactis_ PD 6.9 in controlling the growth of _L. monocytogenes_ during salami fermentation and its usefulness as a starter culture for fermented sausages.

Keywords: *Listeria monocytogenes*, bacteriocins, lactic acid bacteria, co-cultivation, salami.

INTRODUCTION

Fermented sausages are produced by fermentation of minced meat mixed with fat, salt, curing agents (nitrate/nitrite), sugars and spices (Caplice and Fitzgerald, 1999). In order to speed the process and ensure the quality and uniformity of the final product, lactic acid bacteria (LAB) are commonly used as starter

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cultures to decrease the pH (Marchesini et al.,1992; Liu et al., 2010). These LAB produce organic acids that enhance the aroma and extend the shelf life of the fermented product. However, the hurdles faced by microorganisms during sausage fermentation may not prevent the survival of pathogenic bacteria, including *Clostridium, Escherichia coli* O157:H7 and *Listeria* (Bonnet and Montville, 2005; Mor-Mur and Yuste, 2010; Martin et al., 2011; Hospital et al., 2012).

Listeria monocytogenes is a gram-positive food-borne pathogen widely distributed in nature and has been isolated from numerous sources such as meat and fermented meat products, including fermented sausages (Martin et al., 2011; Martins and Germano, 2011; Meloni et al., 2014). *L. monocytogenes* can resist conditions of low pH, low water activity (Aw), high salt (NaCl) concentrations and the presence of sodium nitrite, and is able to survive the commercial sausage manufacturing process (Bonnet and Montville, 2005; Degenhardt and Sant'Anna, 2007). The ingestion of food products contaminated with *L. monocytogenes* is particularly dangerous to young, old, pregnant and immune compromised individuals (Tauxe, 2002; Thévenot et al., 2005; Barlik et al., 2014) and even small number of *Listeria* appear capable to causing disease.

Bacteriocin-producing starter cultures appears to be an effective strategy to control *Listeria* in fermented sausages (Gormley et al., 2010; Freitas de Macedo et al., 2013; Rubio et al., 2014). *Lactococcus lactis* subsp. *lactis* PD 6.9, a lactic acid bacterium isolated from Italian salami (Maciel et al., 2003), was previously shown to inhibit the growth of *L. monocytogenes* in liquid culture and the antimicrobial activity was due to bacteriocin production (Carvalho et. al., 2006). Further work indicated that *L. lactis* PD 6.9 harbor the gene encoding nisin Z on the chromosome and purification of the peptide from the cell free supernatant followed by mass spectrometry analysis confirmed the molecular mass to be 3329.57 Da (Saraiva et al., 2014). Although *L. lactis* PD 6.9 showed great potential to inhibit foodborne pathogens, the production and activity of this bacteriocin had not yet been demonstrated in a liquid model system that simulates the conditions found during salami fermentation. Therefore, we hypothesized that *L. lactis* PD 6.9 could be useful as a starter culture for fermented sausages.

The aim of the present work was to examine the ability of *L. lactis* PD 6.9 to grow and produce bacteriocin in conditions that simulate the fermentation of minced meat and thus to verify its possible antagonism against *L. monocytogenes* in such medium.

MATERIALS AND METHODS

Microorganisms and growth conditions

L. lactis subsp. *lactis* PD 6.9, producer of nisin Z, was previously isolated from Italian salami processed by natural fermentation (Maciel et al., 2003), and kept stored at –80°C in D-MRS (Modified deMan, Rogosa e Sharpe media) (Hammes et al., 1992) supplemented with 20% glycerol. Before use, the culture was activated three times in D-MRS at 30°C.

L. monocytogenes LMA 20, isolated from chicken carcass, was obtained from the culture collection of the Food Microbiology Laboratory at the Universidade Federal de Viçosa, Viçosa, Minas Gerais State, Brazil (Carvalho et al., 2006). It was routinely transferred in tryptic soy broth supplemented with 0.6% yeast extract (TSBYE) and incubated at 37°C. Stock cultures were grown in the same medium and stored at –80°C.

Growth in a liquid model system that simulates the conditions found during minced meat fermentation

Portions of pork fresh lean meat (250 g) were obtained from a local abattoir, aseptically minced, placed into a blender (Walita Beta, São Paulo, Brazil) and mixed with approximately 200 ml of a 0.85% NaCl solution. The volume was adjusted to 500 mL and the mixture was centrifuged twice at 10 000 x *g* for 10 min at 5°C. The supernatant was collected and supplemented with 1.0% glucose, 3.0% NaCl, 120 ppm $NaNO_2$, 200 ppm $NaNO_3$. The pork lean meat broth was filtered through nitrocellulose membranes of 0.45 μm pore size (Schleicher and Schuell BioScience, Keene, USA) and stored at 4°C until use.

Cultures of *L. lactis* PD 6.9, were subcultured in pork lean meat broth (10^7 CFU ml^{-1}) and incubated at 25°C under aerobic conditions. Samples (100 µl) were taken at different time intervals up to 32 h, spread onto D-MRS agar plates and incubated at 30°C. Colony forming units per milliliter (CFU ml^{-1}) at each time interval were determined after 24 h of incubation. *L. monocytogenes* LMA 20 was inoculated into pork lean meat broth (10^5 to 10^6 CFU ml^{-1}), and incubated at 25°C under aerobic conditions. Growth was monitored by enumeration of the viable cell number for up to 32 h on tryptone soy agar supplemented with yeast extract (TSAYE) and incubated at 37°C. The experiments were performed at least in duplicate and the results represent the average from two independent observations. The specific growth rate (µ) of cultures was estimated from the rate of increase in cell number (X) based on the equation $dX/dt = \mu X$, where µ is an absolute rate constant with the units of h^{-1} and t is the growth time in exponential phase.

Antimicrobial activity in pork lean meat broth

L. lactis PD 6.9 was grown in pork lean meat broth to stationary phase. Inhibitory activity and pH were monitored over time. Aliquots (1 ml) of the cultures were centrifuged at 10 000 x *g* for 5 min, the supernatants were collected, and the pH was measured using an Accumet® model 15 pHmeter (Fisher Scientific, Pittsburg, USA). The supernatants were adjusted to pH 6.5 with NaOH 5 M, filtered in nitrocellulose membranes of 0.22 μmpore size (Schleicher and Schuell BioScience, Keene, USA) and tested for bacteriocin activity against *L. monocytogenes* LMA 20 by the agar well diffusion assay (Tagg et al., 1976).

Co-culture experiments in pork lean meat broth were carried out in batch cultures inoculated with 10^4 to 10^7 CFU $ml^{-1}$of *L. monocytogenes* LMA 20 and 10^1 to 10^7 CFU ml^{-1} of *L. lactis* PD 6.9, as indicated in the Figure legends. Samples of the co-cultures were taken at different time intervals and serially diluted (10-fold dilutions) into sterile saline solution (0.85% NaCl). Aliquots (20 µl) were plated onto D-MRS containing $CaCO_3$ (5 g l^{-1})and bromocresol purple (0.04 g l^{-1}) for viable counts of *L. lactis* PD 6.9. *L monocytogenes* was enumerated in TSAYE media supplement with 1.5% lithium chloride after 48 h of incubation. Culture pH was determined as described above. Co-culture experiments were also performed using a no-bacteriocinogenic strain, *Lactococcus lactis*

ATCC 19435. *L. monocytogenes* (10^7 CFU ml^{-1}) and *L. lactis* (10^7 CFU ml^{-1}) were co-inoculated in pork lean meat broth and the viable cell number was monitored up to 48 h as described above. The experiments were performed at least in duplicate and the results represent the mean from two independent observations. Enumeration of *L. monocytogenes* was determined from triplicate plate counts of each dilution.

Experimental design and statistics

The experiments were performed in two biological replicates. To evaluate the growth of *Listeria monocytogenes* and *Lactococcus lactis* PD 6.9 in pork lean meat broth, two samples for each time point were harvested for enumeration. To evaluate the antimicrobial activity pork lean meat broth, triplicate plate counts were prepared for each dilution and for each biological replicate. The error bars in figures indicate the standard deviation of the mean.

RESULTS AND DISCUSSION

Growth of *L. lactis* PD 6.9 and *L. monocytogenes* in pork lean meat broth

In this study, we tested the antagonistic properties of a nisin Z-producing strain of *L. lactis* that was previously isolated from naturally fermented Italian salami. When *L. lactis* PD 6.9 was inoculated (approximately 10^7 CFU ml^{-1}) in pork lean meat broth, the growth rate was 0.59 h^{-1} and the culture reached stationary phase after approximately 8 h of incubation (Figure 1a). The pH of the media decreased rapidly after 6 h of incubation and was as low as 4.2 at the end of the experiment. Bacteriocin activity was generally detected when the cell population was greater than 10^8 CFU ml^{-1}, and the maximum inhibitory activity occurred after 14 h of incubation (Figure 1b).

Bacteriocin purification and DNA sequencing demonstrated that the peptide produced by *L. lactis* PD 6.9 is a natural nisin A variant, nisin Z, as indicated by the substitution of a histidine by an asparagine residue at position 27 of the bacteriocin sequence (Saraiva et al., 2014). Nisin A and nisin Z appear to have similar biological activity, but often differ in their physicochemical properties, such as the diffusion is solid matrices.

In this study, the activity of nisin Z in the cell-free supernatants decreased after 14 h of fermentation, and inhibition zones could not be detected if cultures were maintained in stationary phase for more than 16 h. However, it should be noted that the same observation was done when the bacterium was cultivated alone in D-MRS media (data not shown). These results indicate that bacteriocin stability was not significantly affected by the formulation ingredients of the pork lean meat broth. The decrease in inhibitory activity could be explained by degradation or by adsorption to media constituents, but further experiments will be needed to clarify this point. Previous studies demonstrated that proteinase and peptidase activities associated with producer cells could be responsible for bacteriocin inactivation (Houlihan et al., 2004). Studies also showed that bacteriocins are less effective in food systems in which proteases are not inactivated (Vignolo et al., 1996; Castellano et al., 2004).

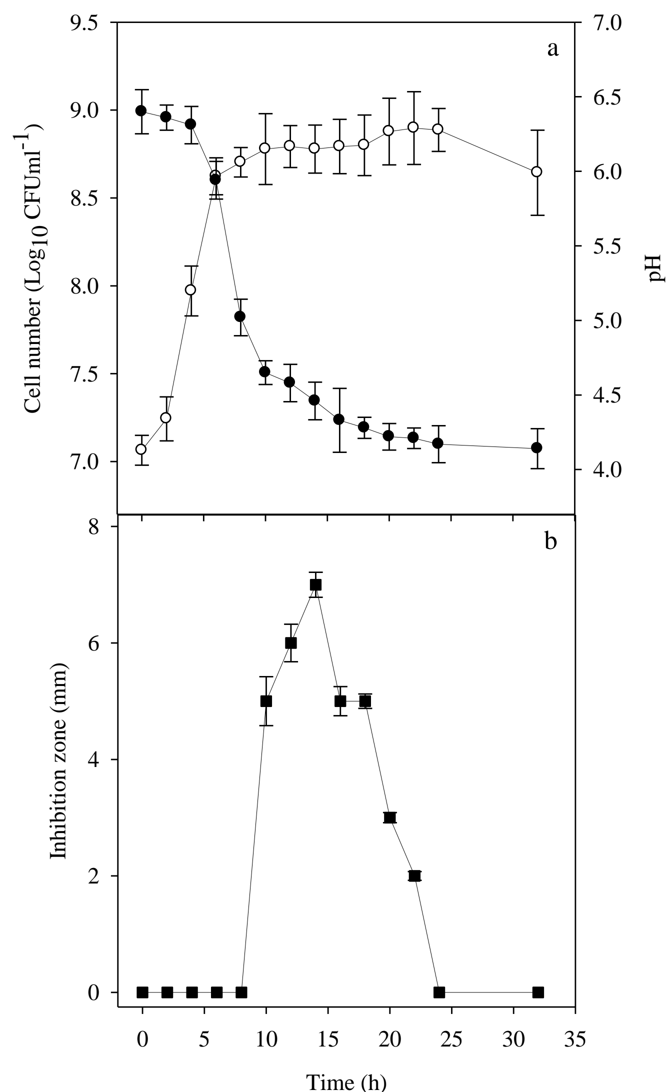

Figure 1. Growth (open circles) and medium pH (closed circles) of *L. lactis* PD 6.9 cultivated in pork lean meat broth at 25°C (a). Nisin Z activity (closed squares) presented as zones of inhibition caused by neutralized supernatants from *L.l actis* PD 6.9 against *L. monocytogenes* LMA 20 is shown in (b). The inoculum of *L. monocytogenes* LMA 20 used for the well diffusion assay was 10^6 CFU ml^{-1}.

Another mechanism for bacteriocin inactivation has also been reported by Rose et al. (1999). The authors used MALDI-TOF/MS to demonstrate that nisin added to fresh meat and meat juices was inactivated due to a reaction with glutathione (GSH), an abundant thiol compound found in animal tissues. GSH appears to react with multiples sites on a nisin molecule in a reaction

catalyzed by glutathione S-transferase (Rose et al., 2002). In our experiments, we used filtered-sterilized pork lean meat broth and the activity of nisin Z produced by *L. lactis* PD 6.9 was less than observed when the bacterium was grown in D-MRS media. These results suggest that a similar mechanism could affect the nisin Z activity of *L. lactis* PD 6.9 in raw meat. Additional experiments will be needed to test this hypothesis.

The fact that antagonistic activity reached its peak when the bacteriocin producer reached stationary phase suggest that *L. lactis* PD 6.9 could have an impact on the composition of the bacterial community that established during the early stages of the salami fermentation. Considering that salami fermentation and maturation can take as long as 30 days, *L. lactis* PD 6.9 could become a dominant culture during the salami manufacturing process. This hypothesis is supported by previous studies in which *L. lactis* PD 6.9 was isolated from salami after 6 days of fermentation and maintained its viability higher than 10^8CFU ml^{-1} even after 22 days of processing (Maciel et al., 2003).

The prevalence and the starter culture potential of *L. lactis* strain during salami fermentation was also showed by Cenci-Goga et al. (2008) and Frece et al. (2014). The authors demonstrated that *L. lactis* helped improving the sensory properties of the fermented product and enhanced the inhibition of pathogens such as *Listeria* spp and *Staphylococcus aureus* (Cenci-Goga et al. 2008; Frece et al., 2014).

If *L. monocytogenes* LMA 20 was inoculated (10^7 CFU ml^{-1}) into pork lean meat broth, growth occurred at a rate of 0.22 h^{-1} and stationary phase was reached after 15 h of cultivation (Figure 2). Processed meat products can provide an excellent environment for the growth of pathogenic organisms such as *L. monocytogenes* (Hereu et al., 2014; Heo et al., 2014). *L. monocytogenes*is a psychrotrophic and ubiquitous bacterium in meat products that is probably transferred from the environment to the food during processing (Mor-Mur and Yuste, 2010; Hereu et al., 2014). An early study performed by Martin et al. (2011) in small-scale factories producing traditional fermented sausage indicated that *L. monocytogenes* could be detected in equipments (11.8% of the samples), raw materials (28.9%), and even in the final products (15.8%). Because *Listeria* has shown resistance to various environmental stresses (heat, acidic pH, low water activity, low storage temperatures, etc.) commonly used in processed foods (Farber et al., 1993; Vogel et al., 2010) food industries have sought methods to control *Listeria* and ensure the safety of food products.

The addition of chemical additives such as nitrite to the meat can inhibit the growth of several pathogens and ensure the safety of the processed product. However, in this study, *L. monocytogenes* LMA 20 was able to grow in pork lean meat broth, even in the presence of NaCl and nitrite concentrations recommended for the fermented sausage manufacture process (Figure 2). This result could be explained in part by the higher water activity in the pork lean meat broth compared to the meat matrix of the fermented sausages, but growth of *Listeria* in model sausages added with salts and chemicals has also been observed (Benkerroum et al., 2003; Albano et al., 2007; Sansawat et al., 2013). Based on higher concentrations of salt and nitrite interfere with desirable characteristics of the processed product and nitrite represent risks to the consumer's health (by affecting the ability of hemoglobin to carry oxygen or producing carcinogenic nitrosamines), alternative methods are needed to prevent the growth of *Listeria* (Ananou et al., 2005; Ammor and Mayo, 2007; Freitas de Macedo et al., 2013).

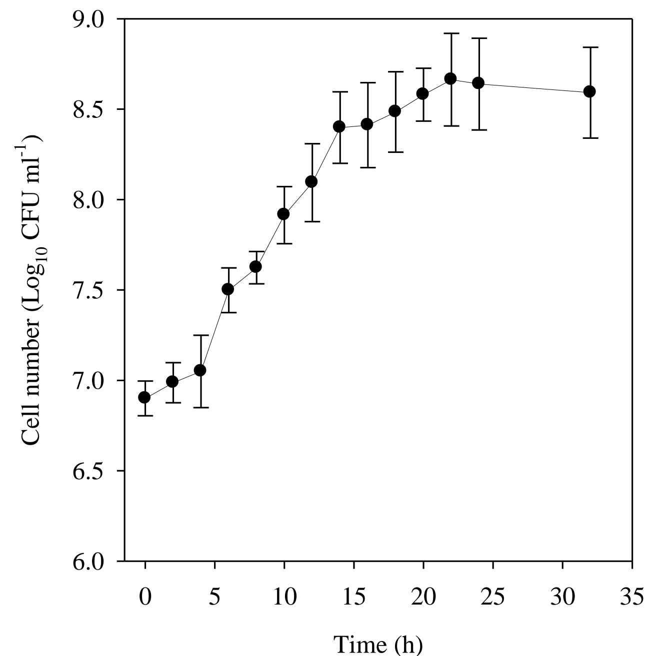

Figure 2. Growth of *L. monocytogenes* LMA 20 in pork lean meat broth at 25°C (open circles). A population of approximately 10^7 CFUml^{-1} of *L. monocytogenes* LMA 20 was used as the initial inoculum. Cell viability was monitored on TSAYE media after 48 h of incubation at 37ºC. Error bars show the standard deviation of the mean.

One such approach could be the use of bacteriocinogenic starter cultures to control potential food-borne pathogens. Bacteriocin are ribosomally-produced, extracellularly released antimicrobial peptides (post-translationally modified or not), which can have a relatively narrow spectrum of antibacterial activity (Deegan et al., 2006; Snyder et al., 2014). Bacteriocins are produced by many gram-positive and gram-negative bacteria and have been shown to inhibit the growth of several food-borne pathogens (Deegan et al., 2006; Snyder et al., 2014). Some bacteriocin-producing LAB have been isolated from fermented meat products and their ability to inhibit the growth of spoilage and pathoge-

nic microorganisms has been demonstrated (Albano et al., 2007; Liu et al., 2010; Snyder et al., 2014).

The potential application of bacteriocins in foods can be limited by properties such as spectrum of inhibition, heat stability and solubility. In general, the following should be considered when selecting bacteriocin-producing strains for food applications: 1) the producing strains should preferably be generally recognized as safe; 2) the peptide should be heat stable and have a broad spectrum of activity against pathogens such as *L. monocytogenes* and *Clostridium botulinum*; 3) the bacteriocinogenic strain and the antimicrobial peptide should pose no associated health risks and 4) the bacteriocinogenic strains should contribute to improve safety, quality and flavour of the food products (Rodríguez et al., 2002; Snyder et al., 2014). Considering that bacteriocin-producing bacteria are frequently isolated from several food sources, it appears that many of these bacteriocinogenic strains have been safely consumed for decades. Therefore, one could argue that the reintroduction of such cultures in a food system might have negligible negative impact on the safety of the consumers (Cleveland et al., 2001).

The main concern regarding a decreased efficiency of bacteriocins as biopreservatives is related with the emergence of nisin resistant strains, particularly in *L. monocytogenes* (Begley et al., 2010; Kaur et al., 2013; 2014). Kaur et al. (2013; 2014) demonstrated that nisin-resistant *L. monocytogenes* were selected after being exposed to high bacteriocin concentrations and nisin resistant strains did not become resistant to other preservation factors, such as low pH, sodium chloride, potassium sorbate or sodium nitrite. These authors also noted that nisin-resistant *L. monocytogenes* strains were generally more sensitive to food preservatives. Therefore, bacteriocins could be used as an additional hurdle to improve food safety without being undermined by resistance (Kaur et al., 2013). However, more studies are needed to determine the distribution of bacteriocin-resistant phenotypes among microorganisms that cause food spoilage and among food borne pathogens.

Co-culture of *L. lactis* PD 6.9 and *L. monocytogenes* in pork lean meat broth

When *L. lactis* PD 6.9 (10^7CFU ml^{-1}) and *L. monocytogenes* LMA 20 (10^6CFU ml^{-1}) were co-inoculated (10:1 ratio) into pork lean meat broth, growth of *L. lactis* PD 6.9 was unaffected and the specific growth rate was similar to that observed when the culture grew alone in pork lean meat broth (Figure 3). However, no increase in *L. monocytogenes* LMA 20 cell number was observed even after 12 h of incubation. Moreover, the decrease in *L. monocytogenes* LMA 20 cell number coincided with the production of nisin Z in the cell-free supernatants of *L. lactis* PD 6.9 cultured in pork lean meat broth (Figure 1b), and viability was approximately

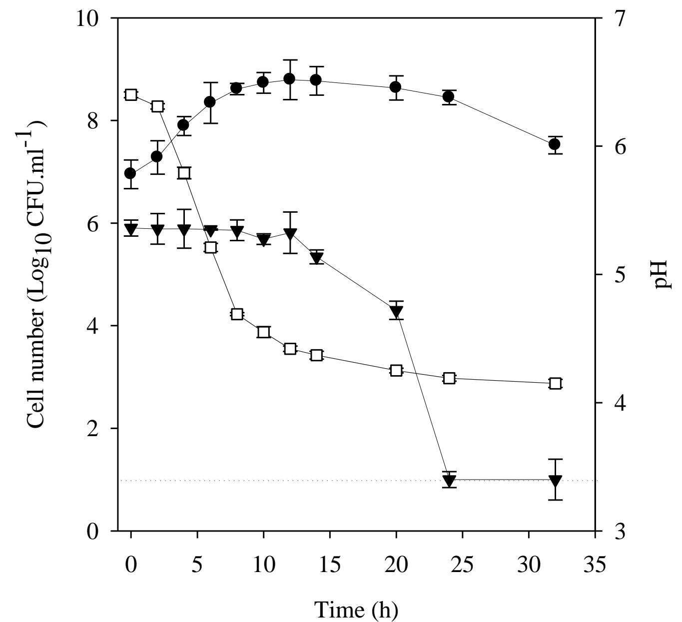

Figure 3. Co-culture of *L. lactis* PD 6.9 (closed circles) and *L. monocytogenes* LMA 20 (closed triangles) in pork lean meat broth at 25°C. Initial inoculums were 10^7CFU ml^{-1} and 10^6 CFUml^{-1} for *L. lactis* PD 6.9 and *L. monocytogenes* LMA 20, respectively. Medium pH (open squares) is also shown. The dotted line represents the detection level of the viable cell counts.

five log units lower after 25 h of co-cultivation (Figure 3).

Preliminary plating experiments showed that *L. lactis* PD 6.9 and *L. monocytogenes* could be unambiguously distinguished and enumerated if spread onto selective media (Figure 4). Co-culture experiments indicated that growth and bacteriocin production by *L. lactis* PD 6.9 was not affected in the presence of a target organism and the inhibitory activity could be inversely correlated with *L. monocytogenes* viability (Figures 1 and 3). *L. monocytogenes* cultivated in pork lean meat broth alone approached stationary phase after approximately 15 h of incubation. However, no growth was observed during the same period if *L. lactis* PD 6.9 was also added to the medium. These results suggest that *L. lactis* PD 6.9 could outgrow *L. monocytogenes* in conditions that simulate the salami fermentation process.

Because *L. monocytogenes* is tolerant to the hurdles of the salami manufacturing process, such as low pH and high osmolarity, the decrease in viable cell number could be explained by the sensitivity of the target organism to nisin Z. This idea was further supported by co-culture experiments using *L. lactis* ATCC 19435, a non-bacteriocinogenic *L. lactis* strain. When *L. lactis* ATCC 19435 (10^7CFU ml^{-1}) was co-inoculated into pork lean meat broth containing *L. monocytogenes* (10^7CFU ml^{-1}), the media pH decreased, but the viable cell number of *L. monocytogenes* did not change even after 48 h of

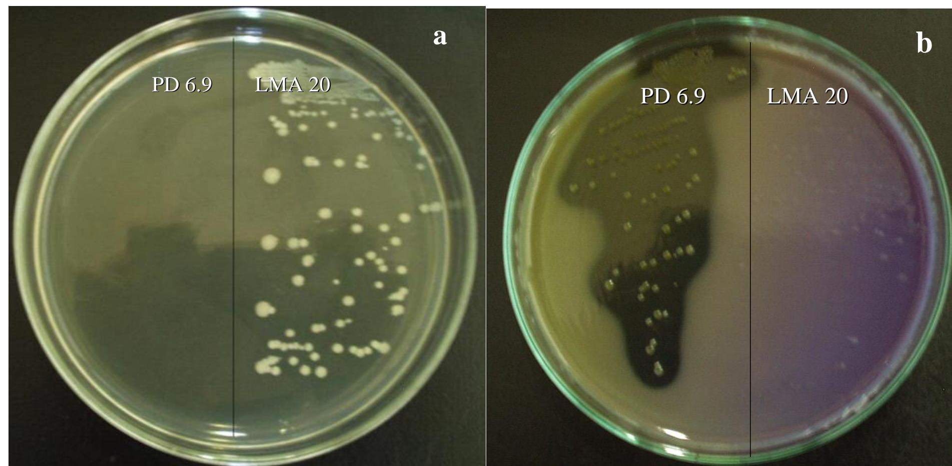

Figure 4. Selective media used for the enumeration of *L. lactis* PD 6.9 and *L. monocytogenes* LMA 20 in co-culture experiments. **a.** The growth of *L. monocytogenes* LMA 20 in TSAYE + 1.5% lithium chloride at 37°C. **b**. The growth of *L. lactis* PD 6.9 in D-MRS supplemented with $CaCO_3$ (5 g l^{-1}) and bromocresol purple (0.04 g l^{-1}) at 30°C. Both sides of the plates were spread with either *L. lactis* PD 6.9or *L. monocytogenes* LMA 20 before the plates were incubated.

incubation (data not shown). Because the cell number of *L. monocytogenes* LMA 20 did not change even when the pH was as low as 4.4 or in the presence of a non-bacteriocinogenic *L. lactis* strain, the bacterial antagonism appeared to be related with the production of nisin Z by *L. lactis* PD 6.9.

L. lactis PD 6.9 showed inhibitory activity against 10^7CFU ml^{-1} of *L. monocytogenes* LMA 20 even at low cell densities (Figure 5). When the cell number of *L. lactis* PD 6.9 inoculated in a co-culture varied from 10^1 to 10^7CFU ml^{-1}(1 million fold ratio variation between *L. monocytogenes* and *L. lactis* PD 6.9), the viability of *Listeria* was reduced 10 fold with a 10^3CFU ml^{-1}inoculum of *L. lactis* PD 6.9 (Figure 5). If the inoculum size increased to values equal to or greater than 10^5CFU ml^{-1}, *Listeria* counts were bellow detection level in the co-culture after 48 hours of incubation (Figure 5). When we tested a co-culture inoculated with a *Listeria* population more plausible to be found in contaminated foods (10^4CFU ml^{-1}), even 10^1CFU ml^{-1} of *L. lactis* PD 6.9 were able to reduce *L. monocytogenes* LMA 20 cell counts bellow the detection level (1 Log_{10} CFU ml^{-1}) in pork lean meat broth (not shown results).

L. monocytogenes is often found in raw meat at populations lower than 10^3 cfu/g (Buchanan et al., 1987; Thevenot et al., 2006), but our results indicate that even counts as high as 10^7CFU $ml^{-1}$of *L. monocytogenes* could be reduced bellow detection level after 48 hof incubation if *L. lactis* PD 6.9 was inoculated into pork lean meat broth at cell numbers greater than 10^4CFU ml^{-1}. Some food industries use starter cultures to ensure the safety and quality of fermented sausages and these cultures are commonly inoculated at 10^6 CFU g^{-1}. In conditions that approached the level of contamination normally found in

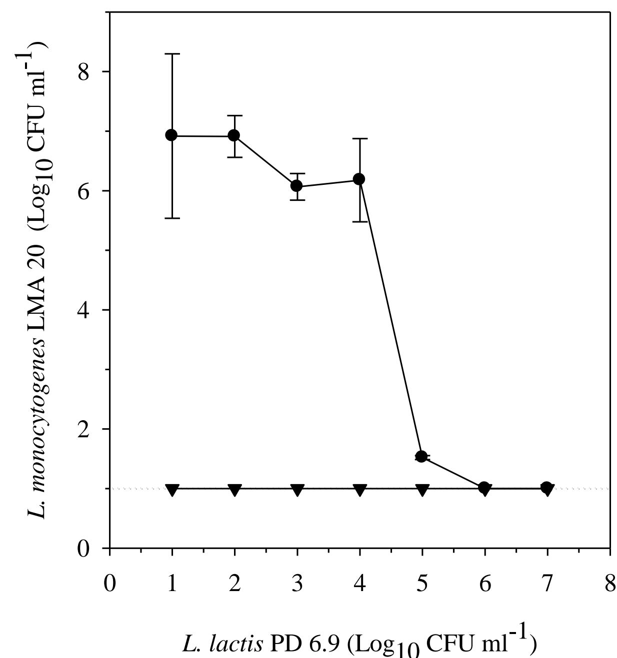

Figure 5. Co-culture of *L. monocytogenes* LMA 20 and *L. lactis* PD 6.9 in pork lean meat broth at various cell densities. Initial inoculum of *L. monocytogenes* LMA 20 was either 10^7 (closed circles) or 10^4 CFU ml^{-1} (closed triangles). The initial inoculum of *L. lactis* PD 6.9 varied from 10^1 to 10^7 CFU ml^{-1}. Viable cell numbers represent counts after 48 h of incubation at 37°C. The dotted line represents the detection level of the viable cell counts. Error bars show the standard deviation.

foods, *L. lactis* PD 6.9 could prevent the growth of *L.*

monocytogenes even if co-inoculated with only 10^1CFU ml^{-1}(1000:1 ratio between *L. monocytogenes* and *L. lactis* PD 6.9). Although the effect of *L. lactis* PD 6.9 against other important food-borne pathogens has not yet been assessed, this bacterium could be a potentially useful starter culture in salami fermentation. Our preliminary results indicate that at least some *Staphylococcus aureus* strains are also inhibited by *L. lactis* PD 6.9. Further studies will address if *L. lactis* PD 6.9 also inhibits starter cultures that are used for salami fermentation or interfere with the organoleptic characteristics of product.

Although our results were obtained in a model system (liquid model) that differs in composition from traditional technological processes for sausage production, the main conditions prevailing during salami fermentation that interfere with bacterial growth were maintained. It is well know that many bacteriocins behave differently in liquid and solid matrices, but the antimicrobial activity of nisin has been demonstrated in real sausages (Hampikyan and Ugur, 2007).

These results indicate that *L. monocytogenes* is inhibited in conditions that prevail during salami fermentation when co-cultured with *L. lactis* PD 6.9. The antagonistic and competitive properties of *L. lactis* PD 6.9 are relevant for its application as a starter culture for fermented sausages, even though the bacteriocin activity might be reduced by components of the salami system. Additionally, because *L .lactis* has been generally recognized as safe and the *L. lactis* PD 6.9 was obtained from a food source (Italian salami), its reintroduction in fermented salami should not impose toxicological problems for consumption of the final product. Furthermore, the use of bacteriocin-producing starter cultures is especially attractive to replace chemical additives or add new hurdles that are effective inhibiting the growth of spoilage and pathogenic bacteria during food processing.

Conflict of interests

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Brazil. R.A. de Paula received a doctoral fellowship from the Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG).

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