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ANTIMICROBIAL ACTIVITY OF PROBIOTIC STRAIN *Lactobacillus plantarum* ISOLATED FROM "SHA'A" AND ASSESSMENT OF ITS VIABILITY IN LOCAL HONEY

Zambou Ngoufack François[*], *Moumbe Fossi Georges Perin, Kaktcham Pierre Marie*

Address(es): *Zambou Ngoufack François*
Laboratory of Biochemistry, Food Science and Nutrition (LABPMAN) - Department of Biochemistry - Faculty of Science - University of Dschang. P.O. Box 67 Dschang, Cameroon.

*Corresponding author: fzambou@yahoo.fr

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ABSTRACT

The acid and bile tolerant strain of *Lactobacillus plantarum* 2S isolated from sha'a, a maize-based traditionally fermented beverage from Cameroon, was screened for bacteriocin properties and its viability in local pasteurized honey. Bacteriocin activity were checked after their treatment with different enzymes, organic solvents, sodium chloride (NaCl) and detergents as well as their heat stability and effect of pH was studied. This strain produced an antimicrobial substance sensitive to proteolytic enzymes (trypsin and pepsin), thus confirming its proteinaceous nature. pH changes and heat treatment up to 121°C had no effect on the activity of the bacteriocin produced. This bacteriocins inhibited the growth of various indicator organisms with Lactobacillus *plantarum* 5S having the widest inhibition spectrum. Besides, it showed broad antibacterial activity spectrum against Gram-positive and Gram-negative pathogens including several that are classified as Especially Dangerous Infections by World Health Organization. The mode of action against *Lactobacillus plantarum* 5S and *Salmonella enterica* subsp. *enterica* serovare Typhi ATCC 6539 is bacteriostatic. After 28 days of storage at 4 °C or 25°C, the viable cell numbers of all bacterial strain in both samples were not changed. *Lactobacillus plantarum* 2S exhibited best viability in honey stored at 4 °C for 28 days and did not altered the physico-chemical characteristics of honey.

Keywords: *Lactobacillus plantarum*, probiotic, bacteriocin, honey, viability

INTRODUCTION

Worldwide, food-borne illnesses caused by pathogenic microorganisms are among the largest cause of mortality, killing several millions of persons each year (**WHO, 2006**). In the most developing countries, frequent ones (diarrhea, cholera and typhoid fever) are transmitted by contaminated water and foodstuffs (**Kuete, 2010**). Antibiotics are still widely used for treatments, and the result is an alarming increase and spread of pathogenic bacteria that are resistant to multiple antibiotics, a growing threat in medicine nowadays. Therefore, there is a pressing need to find or develop novel natural antimicrobial agents.

Lactic acid bacteria (LAB) have long been used as supplements to restore intestinal balance disorders by altering the gut flora as well as by their immunomodulatory effects and production of antibacterial substances (**Kaur *et al.*, 2002**) The antimicrobial effects of probiotics are due to their ability to secrete antimicrobial substances such as organic acids, hydrogen peroxide and bacteriocins. The latest are antimicrobial peptides which provide protection against a wide variety of microorganisms in both vertebrates and invertebrates. They could be best alternatives to currently available antibiotics.

For their nutritious and therapeutic benefits, commercial probiotic microorganisms are widely used in dairy products, infant formula or capsules (**Lusby *et al.*, 2005**), but rarely in local food products. Honey is rich in flavonoids and other phenolic compounds, which act as natural antioxidants and are becoming increasingly popular because of their potential role in human health. In developing countries, honey is widely used as traditional and nonconventional medical treatment (**Kalui *et al.*, 2010; Osho and Bello, 2010**). Honey is used pure or in combination with other medicinal substances to treat many diseases. Given the wide use and consumption of honey, it could be a good matrix to convey probiotics into the gastrointestinal tract (GIT) of Humans and animals. Aside, the incorporation of bacteriocin-producing LAB strains in honey could enhance its natural antimicrobial properties or provide this an additional property.

This work reports the characterization of bacteriocin produced by a probiotic strain *L. plantarum* 2S isolated from "*Sha'a*", a maize-based traditionally fermented beverage from cameroon as well as the evaluation of the viability of this strain in the honey.

MATERIAL AND METHODS

Bacterial strains, media and growth conditions

The strain *Lactobacillus plantarum 2S* used in this study was obtained from our laboratory collection. In our previous studies, this strain was isolated from "Sha'a", a Cameroonian maize-based traditionally fermented beverage, and identified as *Lactobacillus plantarum* based on physiological and biochemical characteristics (using API 50 CHL kits, BioMérieux, Marcy l'Etoile France) and rep-PCR genomic fingerprinting; It showed bacteriocinogenic activity using triple agar layer test and proved to be free from virulence factors like hemolytic and gelatinase activities as well as antibiotics resistance (**Kaktcham *et al.*, 2012a**). *Lactobacillus plantarum* 2S strain was maintained as frozen stocks at –20 °C in MRS broth (Biolife®, Milano, Italy) supplemented with glycerol (30% v/v) and propagated twice (1% inoculum) in MRS broth for 14 – 16 h at 37 °C before experimental use. Agar and soft agar media were prepared by the addition respectively of 1.5% and 0.75% (w/v) granulated agar (Merck, Darmstadt, Germany) to broth media. For bacteriocin production, a modified medium (mMRS), was used. The mMRS broth was prepared by dissolving all the ingredients (from Oxoid, Basingstoke, UK; Merck, Darmstadt, Germany; and Sigma, St Louis, USA) of the MRS broth, except ammonium citrate which was replaced by ammonium sulfate. After adding all medium components, the mixture was autoclaved.

The other bacteria strains used as indicator organisms were revived from frozen stocks (at -20 °C on slants) and grown using the appropriate media and temperature as indicated in **Table 3**. *Lactobacillus plantarum* 5S is a bacteriocin's highly sensitive LAB strain resulting from our previous studies.

Assessment of the ability of the strain to resist conditions of the gastrointestinal tract (GIT)

Acid Tolerance

Strain *L. plantarum* 2S was preliminary selected amongst many other lactobacilli using a rapid test according to the method described by **Pelinescu *et al.*, (2009).**

For real assessment of the acid tolerance, strains were cultured on MRS agar medium for 24 h at 37 °C. Colonies were collected and suspended in 0.2 mol.l^{-1} citrate buffer pH 3 (used as representative gastric pH value), and the turbidity of cells suspensions adjusted to 4 Mc Farland standard (12 × 10^8 CFU/ml). This prepared cells suspension was incubated for 0 and 6 h at 37 °C, followed by centrifugation (3500g, 15 min), washing twice using 0.5 mol.l^{-1} phosphate buffer pH 7 and re-suspension in physiological saline. The viable cells were enumerated by pour plate technique. Appropriate serial dilutions in 0.1% peptone water were done and plates were incubated anaerobically in GasPak anaerobic jar (Genbox anaer; BioMérieux, France) at 37 °C for 48 h (**Verdenelli *et al.*, 2009)**. Results were expressed as the percent (log CFU) of resistant cell.

Bile salt tolerance

Bile salts tolerance was also evaluated as described by **Verdenelli *et al.*, (2009).** *L. plantarum* 2S was cultured on MRS agar slant for 24 h at 37 °C. Colonies were collected and suspended in 0.5 M phosphate buffer, pH 7 supplemented with 0.4% (w/v) bile salts (50 % sodium taurocholate and 50 % sodium glycocholate; Sigma, St Louis, USA) and the turbidity of cells suspensions adjusted to 8 Mc Farland standard (24 × 10^8 CFU/ml). The resulting cells suspension was treated as previously described.

Detection and partial characterization of the bacteriocin produced by L. plantarum 2S

Bacterial growth and bacteriocin activity assay

A 14-hour-old culture of *L. plantarum* strain 2S was inoculated (2% v/v) into mMRS broth and incubated at 30 °C without agitation. Changes in cell density ($O.D_{600nm}$) were recorded every 2 h up to 14 h; the pH of the culture and bacteriocin activity were also measured at the same time interval. Bacteriocin activity in liquid medium was detected using Agar Well Diffusion Assay (AWDA) according to **Schillinger and Lücke (1989)**; the culture was centrifuged at 4000 rpm for 30 min at 4 °C and the supernatant was collected, adjusted to pH 6.5 with 6 mol.l^{-1} NaOH, heated at 80 °C for 10 min, and filter-sterilized through a 0.22 µm pore-size membrane filter (Millipore Corporation, Bedford, MA, USA) using an appropriate syringe to prepare Neutralized Cell-Free Supernatant (NCFS). Soft MRS agar seeded with the indicator strain *L. plantarum* 5S (approximately 10^7 CFU/ml) was overlaid onto pre-poured MRS agar plates; six millimeter diameter wells were punched in the plates and filled with 50 µl of supernatant. After incubation of the plates anaerobically at 30 °C for 24 h, diameters of zones of inhibition were measured using a caliper. Experiments were performed in triplicate.

Sensitivity of the bacteriocin to enzymes, temperature and pH

In this set of experiment, neutralized cell-free supernatant was treated with the following enzymes at 1mg/ml final concentrations: trypsin (Fluka Biochemika, Buchs, Switzerland) in 0.05 M Tris-HCl buffer, pH 8.0; pepsin (Sigma-Aldrich, Steinheim, Germany) in 0.05 mol.l^{-1} citrate buffer, pH 2.0; α-Amylase (Sigma-Aldrich, Steinheim, Germany) in 0.05 M phosphate buffer, pH 7.0; lipase (Sigma-Aldrich, Steinheim, Germany) in 0.05 mol.l^{-1} phosphate buffer, pH 7.0; and lysozyme (Fluka Biochemika, Buchs, Switzerland) in 0.05 mol.l^{-1} phosphate buffer, pH 7.0. Incubation was held at 37 °C for 2 h and enzymes reactions were terminated by boiling for 5 min. To determine the effect of pH, the neutralized cell-free supernatant was adjusted to pH 2.0 - 12.0 (at increments of two pH unit) using 6 mol.l^{-1} HCl or NaOH. After incubation at 37 °C for 2 h, the treated samples were neutralized before tested for residual activity. The effect of temperature on the bacteriocin was assayed by heating the neutralized cell-free supernatant at 37 °C and 100 °C for 30, 60, 90, 120 min, and at 121 °C for 15 min. Cell-free supernatants treated with buffers only and sterile MRS broth treated with enzymes served as controls; after each treatment, the residual activity was determined by AWDA using *L. plantarum* 5S as indicator strain. All experiments were performed in triplicate.

Spectrum of inhibitory activity

The antibacterial activity of the neutralized cell-free supernatant was tested against Gram-positive and Gram-negative bacteria. The indicator strains (0.5 Mac Farland suspensions) were inoculated in the appropriate soft agar media (~10^7 CFU/ml) and the antibacterial activities were determined by AWDA as previously described. All experiments were conducted in triplicate.

Mode of bacteriocin action

Twenty milliliter of neutralized cell-free supernatant was added to 100 ml of a 3 h-growing cultures of *Salmonella enterica* subsp. *enterica* serovare Typhi ATCC 6539 and *L. plantarum* 5S respectively in Mueller-Hinton broth and MRS broth at 37 °C. Changes in cell density were recorded at 600 nm hourly for 8 h, and the number of viable cells (CFU) was determined by plating the samples on Salmonella-Shigella agar and MRS agar (1.5% w/v) followed by incubation at 37 °C for 48 h. Growing cultures in which 20 ml of sterilized distilled water was added served as controls.

Assessment of the stability of the bacteriocin-producing *L. plantarum* 2S strain in honey

Preparation of honey samples and inoculation

The honey sample used in this study was a wild polyfloral type, procured from a local beekeeper of the sudano-guinean zone of West-Cameroon. It was obtained by draining the honey after manually uncapping the comb frames, and pasteurized at 63 °C for 30 min.
In another experiment, *L. plantarum* culture were propagated twice in MRS broth and incubated at 37 °C for 16 h. Seventy milliliter of broth media were inoculated with 10% overnight, activated culture of strain *L. plantarum 2S*. The growth of the culture in broth media was monitored using spectrophotometer (Pharmacia LKB NOVASPEC II) for 6 h. At the early stationary growth phase, bacterial cells were harvested from the media by centrifugation of the culture (4000 g, 10 min, 4 °C). The pellet was then washed twice with 0.01 mol.l^{-1} potassium phosphate buffer pH 7.0. The resultant pellet was suspended in 100ml sterile potassium phosphate buffer 0.01mol.l^{-1} and stored at –20°C until used. The viable cell counts of this suspension was determine by enumeration on MRS plate agar and incubation at 37 °C for 48 h in anaerobic. 120 µl of this concentrate (about 2.8 × 10^7 CFU/ml) was introduced into 100 ml of pasteurized honey and thoroughly homogenized. Non-inoculated samples were used as control. Inoculated and non inoculated pasteurized honey samples were stored at 25 °C (room temperature) and 4 °C respectively for 28 days.

Assessment of L. plantarum 2S viability in pasteurized honey

From the inoculated pasteurized honey samples, an aliquot of 10 ml was taken at 1-week interval for viable count. Ten-fold serial dilutions were done in 0.1% peptone water while homogenizing by vortexing, followed by viable cell count by pour plate technique using MRS agar. Plates were incubated anaerobically at 37°C for 48h.

Evaluation of physico-chemical characteristics of honey samples during storage

Physico-chemical characteristics frequently used as best indicators for quality and stability of honey such as pH, moisture, free acidity were determined **(Codex Alimentarius, 1981)**. Moisture was determined using IUPAC method (**IUPAC, 1979**). The pH was measured by a pH-meter (Searchtech) on 10% (v/v) honey sample in CO_2 free distilled water (**Mbogning *et al.*, 2011**). The density of samples was determined by the method described by **AFNOR (1981)**. For the determination of free acidity, the harmonized method of the International Honey Commission was used (**Bogdanov *et al.*, 1997**). In this respect, 10 g of each sample was dissolved in 75 ml of CO_2 free water in a 250 ml beaker, stirred to homogenize and the initial pH of the solution recorded. Afterwards, the solution was titrated with 0.1 mol.l^{-1} NaOH to pH 8.30. The free acidity, express as milliequivalents or millimoles acid.kg^{-1} honey represented the volume (in ml) of 0.1 mol.l^{-1} NaOH used to bring the initial pH of the solution to 8.30, multiplied by the factor 10. All these experiments were performed at 1-week interval.

Statistical analysis

The data obtained were subjected to Analysis of Variance (ANOVA) and multiple comparisons were done by Student-Newman-Keuls test, using the software GraphPad InStat (GraphPad Software Inc, V3).

RESULTS AND DISCUSSION

Tolerance to acidic pH and bile salts

Acid and bile salts tolerance are prerequisite for probiotics to resist the stressful conditions of the stomach and the upper intestine during their journey to the gastro intestinal tract. *L. plantarum* 2S demonstrated a high tolerance to acidic pH after 6 h of exposure in citric acid at 37 °C, by showing a survival percentage of 80.29. It also showed high tolerance to high bile salts concentration (0.4%) after 6 h of incubation at 37 °C, since a survival percentage of 86.32 was recorded.
Probiotics are health-promoting microorganisms. The criteria used to select potential probiotics are related to acid and bile tolerance, production of antimicrobial substances, cholesterol metabolism, production of useful enzymes and safety for food and clinical use (**Ouwehand *et al.*, 1999**). *In vitro* survival of bacterial strains in low pH is a more accurate indication of the ability of strains to survive passage through the stomach. The organisms taken orally have to face stresses from the host which begin in the stomach, with pH between 1.5 and 3.0 (**Corzo *et al.*, 1999**). Resistance to bile salts is generally considered as an

essential property for probiotic strains to survive the conditions in the small intestine. Results suggest that strain *L. plantarum* 2S could successfully transit the human stomach and may be capable of reaching the intestinal environment and functioning effectively there. In fact, many other studies demonstrated the capacity of *L. plantarum* strains to tolerate gastric acid and intestinal bile salts conditions (**Kalui *et al.*, 2009; Sirilun *et al.*, 2010; Sieladie *et al.*, 2011**). Bile resistance of lactobacilli may be due to their ability to de-conjugate bile salts in the duodenum (**De Smet *et al.*, 1995**).

Kinetics of bacterial growth and bacteriocin production

Bacteriocin activity of *L. plantarum* 2S was detected at early logarithmic growth phase (4 h) in mMRS broth at 30 °C (Figure 1). Maximum bacteriocin production (14 mm) was reached after 8 h of incubation (end of logarithmic phase) and the antibacterial activity slowly decreased afterwards. During cultivation (16 h), the pH of the medium decreased from 6.85 to 4.14 and the cell density increased from 0.13 to 2.11 ($O.D_{600nm}$). In this study, the detection of bacteriocin activity at early logarithmic growth phase may suggest that the peptide is secreted as a primary metabolite.

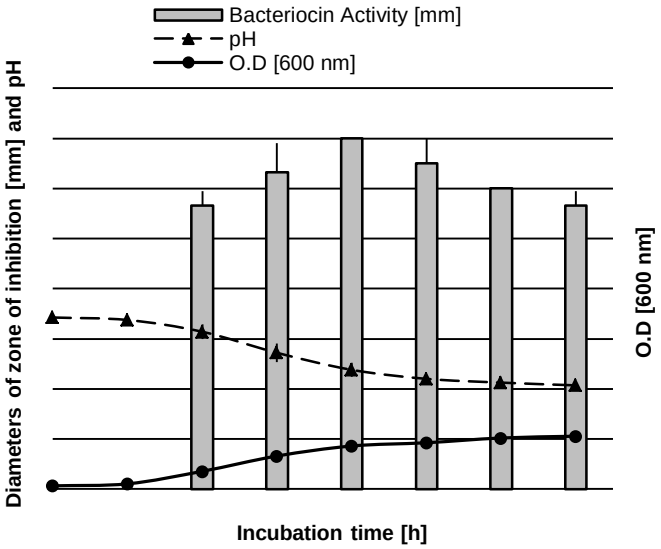

Figure 1 Growth of *L. plantarum* 2S, change in pH and bacteriocin production in mMRS broth at 30 °C for 16 h without agitation. Legend: (-●-) = growth; (-▲-) = change in pH; the bars indicate diameters of zone of inhibition

Sensitivity of the bacteriocin to enzymes, temperature and pH

The effects of proteolytic and non proteolytic enzymes, temperature and pH on the bacteriocins activity were determined (Tab. 1). Complete inactivation of the antimicrobial activity was observed when the neutralized cell-free supernatant was treated by trypsin and pepsin, showing that the antimicrobial substance was of a proteinaceous nature. Treatments with α-Amylase, lipase and lysozyme did not affect the antimicrobial activity, suggesting that the bacteriocin activity does not depend on a carbohydrate or lipid moiety, whatever they are present or not. This bacteriocin is found to be strongly heat stable, as 83.32% of the activity was still recorded after 15 min at 121 °C as compared to the activity at 37 °C. The antimicrobial activity was slightly influenced by neutral pH (8 – 12), as it significantly decreased after treatment at these pH values, comparatively to the activity obtained at acidic to neutral pH (2 – 6).

Treatment of the bacteriocins produced by strain *L. plantarum* 2S with proteolytic enzymes (trypsin and pepsin) resulted in complete loss of activity, confirming its proteinaceous nature containing cleavage-sites suitable for the above proteases. Given that the activity does not depend on a carbohydrate or lipid moiety, this bacteriocin might belong to Class I or Class II of the classification of bacteriocins. The stability of the bacteriocins at 37 °C suggest that it could fully retain its activity once produced in the GIT; its heat stability (up to 121 °C for 15 min) which is advantageous for transport and use as drug, could arise from the complex patterns of disulphide intramolecular bonds that stabilize secondary structures by reducing the number of possible unfolded structures (**Deraz *et al.*, 2009**); but more investigation is required to confirm.

Table 1 Effect of enzymes, pH and heat treatment on the antibacterial activity of the bacteriocin produced by *L. plantarum* 2S

Factors	**Diameter (**mm**) of zone of inhibition**
Enzymes	
Without enzymes	13.00 ± 0.00
Trypsin and pepsin (1 mg/ml)	–
α- Amylase (1 mg/ml)	12.66 ± 0.57
Lipase (1 mg/ml)	12.66 ± 0.57
Lysozyme (1 mg/ml)	13.00 ± 0.00
pH	
2 to 6	13.33 ± 0.57^{ab}
8-10	12.33 ± 0.57^{bc}
12	12.00 ± 0.00^{c}
Heat treatment	
37 °C for 30, 60, 90 and 120 min	14.00 ± 0.00^{d}
100 °C for 30 and 60 min	13.66 ± 0.57^{d}
100 °C for 90 and 120 min	12.33 ± 0.57^{e}
121 °C for 15 min	11.66 ± 0.57^{e}

[a, b, c]: With respect to the effect of pH, values of the activity with the same symbol are not significantly different (P>0.05).
[d, e]: With respect to the effect of temperature, values of the activity with the same symbol are not significantly different (P>0.05).
Diameters of zone of inhibition are mean of triplicate, including the diameter of the wells (6 mm). ''–'': no inhibition zone was observed.

Spectrum of inhibitory activity

The antibacterial activity of the bacteriocin produced by *L. plantarum* 2S was evident not only on Gram-positive bacteria, but also on Gram-negative bacteria. This bacteriocin inhibited several food spoilage bacteria and foodborne pathogens, including *Staphylococcus aureus*, *Bacillus cereus*, *Streptococcus mutans*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Salmonella enterica* subsp. *enterica* serovare Typhi. However, no activity was detected against many other lactobacilli as well as an *Enterococcus faecium* strains tested (**Tab 2**). The bacteriocin had a broad spectrum activity against a range of Gram-positive and Gram-negative pathogens, including several classified as Especially Dangerous Infections (EDI) by the World Health Organization (WHO). Interestingly, the bacteriocin was not active against several related bacteria, including *L. plantarum*, *L. rhamnosus* and *Ec. faecium*, suggesting that *L. plantarum* 2S could share the same niche with other useful LAB. Several other studies reported similar features observed with other bacteriocins produced by *L. plantarum* strains (**Todorov *et al.*, 2007, 2008; Gong *et al.*, 2010; Kaktcham *et al.*, 2012b**], but they were for application as food biopreservatives and not to treat bacterial infections. Nevertheless, the studies of **Charernjiratragul *et al.*, (2010)** and **Mkrtchyan *et al.*, (2010)** reported cases of *L. plantarum* strain that could be used against infectious pathogens such as *vibrio parahaemolyticus* and *Salmonella enterica* serovar Typhimurium ATCC 14028 NA respectively.

Table 2 Antibacterial activity spectrum of the bacteriocin produced by *L. plantarum* 2S

Indicator strains	Sources	Growth conditions	Diameters (mm)* of zone of inhibition
Lactic acid bacteria			
L. plantarum 5S	Our collection	MRS[d], 30 °C	14.00 ± 0.00
L. plantarum 9S	Our collection	MRS, 30 °C	–
L. plantarum 11S	Our collection	MRS, 30 °C	–
L. plantarum 29V	Our collection	MRS, 30 °C	–
L. rhamnosus 18S	Our collection	MRS, 30 °C	–
L. rhamnosus 1K	Our collection	MRS, 30 °C	–
E. faecium DSM 13596	DSM[a]	MRS, 30 °C	–
Other Gram-positive bacteria			
L. innocua ATCC 33099	ATCC[b]	TB[e],PCA, 37 °C	13.66 ± 0.57
S. aureus ATCC 25923	ATCC	TB,PCA[f], 37 °C	12.66 ± 0.57
B. cereus ATCC 11778	ATCC	TB, PCA, 37 °C	13.00 ± 0.00
S. mutans DSM 20523	DSM	MH[g], 37 °C	12.66 ± 1.15
Gram-negative bacteria			
E. coli ATCC 11775	ATCC	MH, 37 °C	13.66 ± 0.57
P. aeruginosa ATCC 27853	ATCC	TB,PCA,37 °C	14.33 ± 0.57
K. pneumoniae (Clinical isolate)	CPY[c]	TB,PCA,37 °C	14.00 ± 1.00
S. flexneri (Clinical isolate)	CPY	N[h], 37 °C	–
S. enterica subsp. *enterica* serovar Typhi ATCC 6539	ATCC	MH, 37 °C	13.66 ± 0.57
P. mirabilis (Clinical isolate)	CPY	TB,PCA,37 °C	–

***:** Inhibition zone Diameters are means of three independent experiments**.** Wells (6 mm in diameter) were filled with 50 µl of Neutralized Cell-free supernatant (NCFS). "–": no inhibition**.**

[a]**DSM:** Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Brau. [b]**ATCC:** American Type Culture Collection. [c]**CPY:** Centre Pasteur Yaoundé. [d]**MRS**: de Man, Rogosa and Sharpe broth and agar. [e]**TB:** Tryptone broth. [f]**PCA:** Plate Count Agar. [g]**MH:** Mueller Hinton Broth and Agar. [h]**N:** Nutrient broth and agar.

Mode of action of the bacteriocin produced by *L. plantarum* 2S

The addition of the neutralized cell-free supernatant containing bacteriocin to cells of *S. enterica* subsp. *enterica* serovare Typhi ATCC 6539 in their early logarithmic growth phase (3 h-old) resulted in a significant decrease ($P<0.05$) in the number of viable cells and cell density 1 h after the treatment; Afterwards, the viable cell number remained constant whereas the cell density slightly but not significantly increase over the following 4 h (Figure 2).

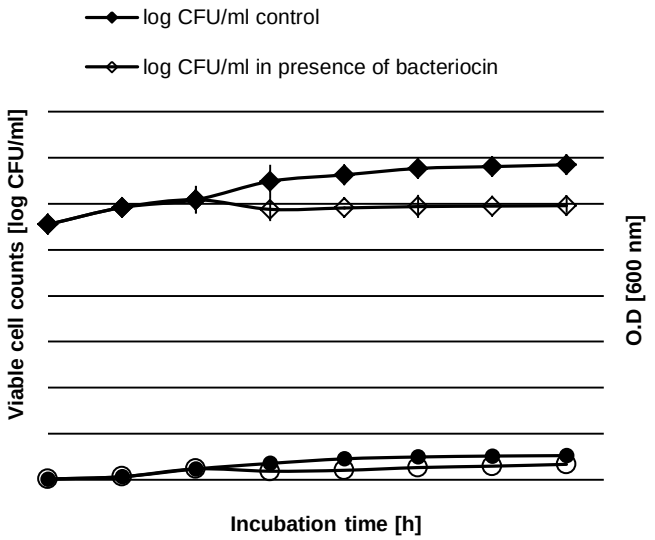

Figure 2 The effect of the bacteriocin produced by *L. plantarum* 2S on the growth of *S. enterica* subsp. *enterica* serovare Typhi ATCC 6539 over a period of 8 h. Optical density recorded in the absence (●) and in the presence (○) of bacteriocin respectively. Viable cell counts recorded in the absence (♦) and in the presence (◊) of bacteriocin respectively. The values are means of three independent experiments and the error bars are the standard deviation (SD)

A similar situation was observed when the neutralized cell-free supernatant containing bacteriocin was added to an early logarithmic growth phase culture of *L. plantarum* 5S (Figure 3). It is worth noting that the addition of sterile distilled water (control) instead of bacteriocin resulted in continuous increase of the number of viable cells and cell density of the two indicators strains. The bacteriocin produced by *L. plantarum* 2S has a bacteriostatic mode of action against *L. plantarum* 5S and *S. enterica* subsp. *enterica* serovare Typhi ATCC 6539. Although the majority of the bacteriocins produced by LAB showed a lethal effect (with or without cell lysis), a bacteriostatic effect has also been described for plantaricins (**Atrih *et al.*, 2001; Hernàndez *et al.*, 2005; Kaktcham *et al.*, 2011; Xie *et al.*, 2011**). It is however worth noting that the determination of the bactericidal or bacteriostatic effect *in vitro* is strongly influenced by several aspects of the assay such as the number of arbitrary units, the buffer and the culture medium, the purity of the antimicrobial agent, the indicator strain and the cell density used (**Hernàndez *et al.*, 2005).**

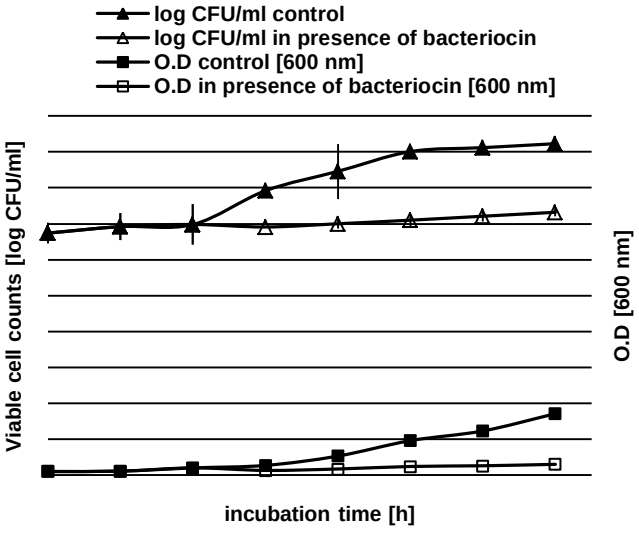

Figure 3 The effect of the bacteriocin produced by *L. plantarum* 2S on the growth of *L. plantarum* 5S over a period of 8 h. Optical density recorded in the absence (■) and in the presence (□) of bacteriocin respectively. Viable cell counts recorded in the absence (▲) and in the presence (Δ) of bacteriocin respectively. The values are means of three independent experiments and the error bars are the standard deviation (SD)

Viability of *L. plantarum* 2S in pasteurized local honey

Figure 4 showed the viable cell counts of *L. plantarum* 2S in pasteurized local honey stored at 4 °C and 25 °C for 28 days. Viable cell counts of *L. plantarum* 2S did not show significant changes ($P>0.05$) after storage at 4 °C for 28 days, as a slight but not significant decrease (from 2.78×10^7 CFU/ml to 5.62×10^6 CFU/ml) was observed (**Figure 4**). The estimated survival percentage at this storage temperature was 90.66%. When the inoculated honey was stored at 25 °C, a significant decrease ($P<0.05$) of the number of viable cell was recorded, going from 2.78×10^7 CFU/ml to 6.30×10^4 CFU/ml after 28 days of storage. At this storage temperature, the estimated survival percentage was 64.47%.

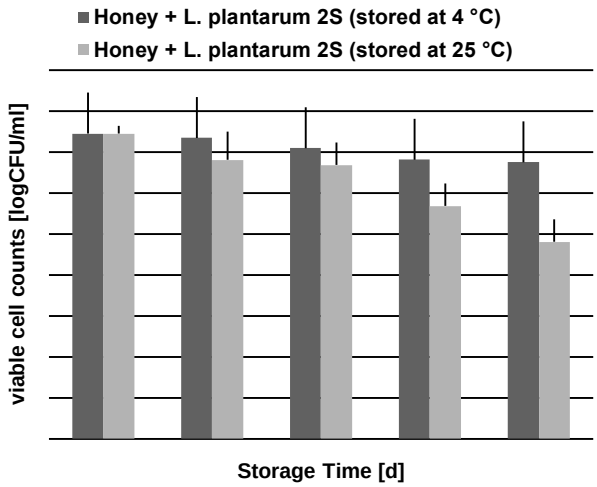

Figure 4 comparison of the number of viable cells counts in honey inoculated with *L. plantarum* 2S during storage at 4 °C and at room temperature (25°C) for 28 days.
Values are means with standard deviation of three independent experiments.

*: The number of viable cell counts of *L plantarum* 2S in honey stored at 25°C for 28 days is significantly different from the initial value ($p<0.05$)

Refrigeration at 4 °C is commonly used to keep cells alive. *L. plantarum* 2S exhibited best viability in honey stored at 4 °C. This survival of strain *L. plantarum* 2S could be attributed to the presence of fructooligosaccharides (FOS) in honey; However, the viable cell number could not increase due to the lower moisture content of honey samples as well as their lower proteins content, as the growth of lactobacilli requires some complex media containing more proteins and growth factors (**De Man *et al.*, 1960**).

Changes in physico-chemical characteristics of inoculated honey during storage

Free acidity, pH, moisture content and density are amongst the physico-chemical characteristic commonly used as main indicators of quality and stability of honey. After 28 days of storage at 4 °C and 25 °C, no significant change was observed in the physico-chemical parameters of the honey samples (**Tab 4**). The moisture, pH, free acidity and density ranged from 17.25 to 17.90 %, 4.22 to 4.28, 101.0 to 102.50 $mEq.kg^{-1}$, and 1.35 to 1.39 $g.ml^{-1}$ respectively, for the honey samples stored at 25 °C . For the samples stored at 4 °C, the values of these parameters ranged from 17.35 to 18.10%, 4.22 to 4.25, 101.25 to 102.50, and 1.34 to 1.39 g/ml respectively (**Tab 3**).

Table 3 Physico-chemical characteristics of honey samples during storage at 4 °C and room temperature (25 °C)

Storage time (days)	Samples (storage temperature)	Moisture* (%)	pH*	Free acidity* (mEq/kg)	Density* (g/ml)
0	Honey	17.35 ± 0.35	4.22 ± 0.07	102.00 ± 1.41	1.38 ± 0.05
7	Honey (25 °C)	17.37 ± 0.17	4.28 ± 0.00	101.00 ± 0.00	1.39 ± 0.04
	Honey + *L. plantarum* (25 °C)	17.87 ± 0.31	4.28 ± 0.00	102.00 ± 1.41	1.39 ± 0.01
	Honey (4 °C)	17.67 ± 0.45	4.23 ± 0.00	102.50 ± 0.70	1.36 ± 0.04
	Honey + *L. plantarum* (4 °C)	17.90 ± 0.14	4.22 ± 0.00	102.00 ± 0.00	1.36 ± 0.09
14	Honey (25 °C)	17.25 ± 0.39	4.28 ± 0.02	102.50 ± 0.70	1.35 ± 0.04
	Honey + *L. plantarum* (25 °C)	17.65 ± 0.91	4.27 ± 0.00	102.25 ± 0.35	1.38 ± 0.03
	Honey (4 °C)	17.80 ± 0.54	4.22 ± 0.01	102.5 ± 0.70	1.37 ± 0.00
	Honey + *L. plantarum* (4 °C)	18.10 ± 0.42	4.23 ± 0.00	101.5 ± 0.70	1.34 ± 0.10
21	Honey (25 °C)	17.90 ± 0.56	4.27 ± 0.01	102.0 ± 0.00	1.36 ± 0.05
	Honey + *L. plantarum* (25 °C)	17.90 ± 0.56	4.27 ± 0.00	102.0 ± 0.00	1.32 ± 0.01
	Honey (4 °C)	18.00 ± 0.10	4.25 ± 0.00	101.25 ± 0.35	1.35 ± 0.04
	Honey + *L. plantarum* (4 °C)	18.10 ± 0.51	4.23 ± 0.01	101.25 ± 1.06	1.35 ± 0.02
28	Honey (25 °C)	17.67 ± 0.45	4.25 ± 0.00	101.50 ± 0.70	1.39 ± 0.02
	Honey + *L. plantarum* (25 °C)	17.75 ± 0.35	4.24 ± 0.00	101.50 ± 0.70	1.36 ± 0.02
	Honey (4 °C)	18.10 ± 0.42	4.22 ± 0.00	102.00 ± 1.41	1.36 ± 0.01
	Honey + *L. plantarum* (4 °C)	18.00 ± 0.10	4.24 ± 0.01	102.00 ± 2.82	1.39 ± 0.02

*: no significance difference ($P>0.05$) was observed between the values for each parameter. Values in this table are means of three independent experiments.

The inoculation of honey samples with *L. plantarum* 2S did not altered their physico-chemical quality over the period and temperature of storage. The pH range was in accordance with the values recommended by the international honey commission **(Bogdanov *et al.*, 1997)** as well as the results of **Mbogning *et al.* (2011)** which shows that the pH of different samples of honey in Western highlands and Adamawa regions of Cameroon is between 4.1 and 5.0.The values of the moisture content were less than the maximum 20% recommended by Codex Alimentarius [12], while the values of the density were in agreement with the standard value defined by IHC. The results of these parameters were also similar to those reported by **Mbogning *et al.* (2011).**

CONCLUSION

In this study we demonstrated the ability of *L. plantarum* 2S to resist stressful conditions of the stomach and small intestine. The properties of its bacteriocin (broad antibacterial activity spectrum, heat stability, resistance to acidic and alkaline pH) indicate that it can be an alternative organism to use as probiotic in the treatment of infectious diseases. Pasteurized locally produced honey has been found to be a good matrix to convey this probiotic strain into the GIT of Humans or animals, provided that it is being stored at 4 °C. These results are promising and more studies are necessary to investigate the survival of other probiotic strains in local honey and the *in vivo* antimicrobial activity of the inoculated honey samples.

REFERENCES

AFNOR. 1981. Recueil des normes Françaises des corps gras, graines oléagineuses, produits dérivés. 438p. ISBN-10: 2123331015

ATRIH, A., REKHIF, N., MOIR, A., J., G., LEBRIHI, A., LEFEBVRE, G. 2001. Mode of action, purification and amino acid sequence of plantaricin C19, an anti-*Listeria* bacteriocin produced by *Lactobacillus plantarum* C19. *International Journal of Food Microbiology*, 68, 93-109.

Bogdanov, S., Martin , P., Lüllmann, C. , Borneck, R., Ch. Flamini, Ch., Morlot, M., Heretier, J., Vorwohl, G. Russmann, H., Persano-Oddo, L., Sabatini, A.G., Marcazzan, G.L., Marioleas, P., Tsigouri, K. Kerkvliet, J., Ortiz, A., Ivanov, T. 1997. Harmonised methods of the European honey commission, Apidologie (extra issue), 1-59.

CHARERNJIRATRAGUL, W., BHOOPONG, P., KANTACHOTE, D., JOMDUANG, S., KONG-NGOEN, R., NAIR,G., B., VUDDHAKUL, V. 2010. Inhibitory activity of lactic acid bacteria isolated from Thai fermented food against pandemic strains of *vibrio parahaemolyticus*. *Journal of Food Safety*, 30, 67–82.

CODEX ALIMENTARIUS. 1981. Codex Standard for honey, Adopted in 1981, Revisions 1987 and 2001.

CORZO, G., GILLILAND, S., E. 1999. Measurement of bile salt hydrolase activity from *Lactobacillus acidophilus* based on disappearance of conjugated bile salts. *Journal of Dairy Science*, 82, 466-4671.

DERAZ, S., F., KARLSSON, E., N., HEDSTRÖM, M., ANDERSSON, M., M., MARTIASSON, B. 2009. Purification and characterization of acidocin D20079, a bacteriocin produced by *L. acidophilus* DSM 20079. *Journal of Biotechnology*, 117, 343–354.

DE MAN, J., C., ROGOSA, M., SHARPE, M., E. 1960. Medium of lactobacilli. *Journal of Applied Bacteriology*, 23, 130-135.

DE SMET, I., VAN HOORDE, L., VANDE WOESTYNE, M., CHRISTIAENS H., VERSTRAETE, W. 1995. Significance of bile salt hydrolytic activities of lactobacilli. *Journal of Applied Bacteriology*, 1995, 292-301.

GONG, H., S., MENG, X., C., WANG, H. 2010. Plantaricin MG active against Gram-negative produced by *Lactobacillus plantarum* KLDS1.0391 isolated from Jiaoke, a traditional fermented cream from China. *Food control*, 21, 89-96.

HERNÀNDEZ, D., CARDELL, E., ZARATE, V. 2005. Antimicrobial activity of lactic acid bacteria isolated from Tenerife cheese: initial characterization of plantaricin TF711, a bacteriocin-like substance produced by *Lactobacillus plantarum* TF711. *Journal of Applied Microbiology*, 99, 77-84.

IUPAC. 1979. Standard methods for the Analysis of oils, fats and derivatives 151p. ISBN-0-632-03337-1

KALUI, C., M., MATHARA, J., M., KUTIMA, P., M. 2010. Probiotic potential of spontaneously fermented cereal based foods – A review. *African Journal of Biotechnology*, 9, 2490-2498.

KALUI, C., M., MATHARA, J., M., KUTIMA, P., M., KIIYUKIA, C., WONGO L. E. 2009. Functional characteristics of *Lactobacillus plantarum* and *Lactobacillus rhamnosus* from ikii, a Kenyan traditional fermented maize porridge. *African Journal of Biotechnology*, 8, 4363-4373.

KAKTCHAM, P., M., ZAMBOU, N., F., FONTEH, A., F., SIELADIE, D., V., TCHOUANGUEP M., F. 2011. Characterization of bacteriocin produced by *Lactobacillus rhamnosus* 1K isolated from traditionally fermented milk in the western highlands region of Cameroon. *New York Science Journal*, 4, 121-128.

KAKTCHAM, P., M., ZAMBOU, N., F., ATIYA, A., FOZIA, A., SYED, A., A., SIELADIE, D., V., TCHOUANGUEP, M., F. 2012b. Characterization of a Bacteriocin Produced by *Lactobacillus plantarum* Lp6SH Isolated from "Sha'a", a Maize-Based Traditionally Fermented Beverage from Cameroon. *International Journal of Biology*, 4, 149-158.

KAKTCHAM, P., M., ZAMBOU, N., F., TCHOUANGUEP, M., F., EL-SODA, M., CHOUDHARY M., I. 2012a**.** Antimicrobial and Safety Properties of lactobacilli Isolated from two Cameroonian Traditional Fermented Foods. *Scientia Pharmaceutica*, 80, 189-203.

KAUR, I., P., CHOPRA, K., SAINI, A. 2002. Probiotics: Potential pharmaceutical applications. *European* Journal Pharmacology Science, 15, 1–9.

KUETE, V. 2010. Potential of Cameroonian Plants and Derived Products against Microbial Infections: A Review. *Planta Medica*, 76, 1479-1491.

LUSBY, P., E., COOMBES, A. L., WILKINSON, J., M**.** 2005. Bactericidal activity of different honeys against pathogenic bacteria. *Archives of Medical Research*, 36, 464-467.

MBOGNING, E., TCHOUMBOUE, J., DAMESSE, F., SOBZE, M., S., CANINI, A. 2011. Caractéristiques physico-chimiques des miels de la zone Soudano-guinéenne de l'Ouest et de l'Adamaoua Cameroun. *Tropicultura*, 29, 2011, 168-175.

MKRTCHYAN, H., GIBBONS, S., HEIDELBERGER, S., ZLOH, M., LIMAKI, H., K. 2010. Purification, characterization and identification of acidocin LCHV, an antimicrobial peptide produced by *Lactobacillus acidophilus* n.v. Er 317/402 strain Narine. *International Journal of Antimicrobial Agents*, 35, 255-260.

OSHO, A., BELLO O. O. 2010. Antimicrobial effect of honey produced by *Apis mellifera* on some common human pathogens. *Asian Journal of Experimental Biological Sciences*, 1, 875-880.

OUWEHAND, A., C., KIRJAVAINEN, P., V., SHORTT, C., SALMINEN, S. 1999. Probiotics: Mechanisms and established effects. *International Dairy Journal*, 9, 43-52.

PELINESCU, D., R., SASARMAN, E., CHIFIRIUC, M., C., STOICA, I., NOHITA, A., M., AVRAM I., SERBANCEA, F., DIMOV, T., V. 2009. Isolation and identification of some *Lactobacillus* and *Enterococcus* strains by a polyphasic taxonomical approach. *Romanian Biotechnological Letters*, 14, 4225-4233.

SCHILLINGER, U., LUCKE, F. 1989. Antibacterial activity of *Lactobacillus sakei* isolated from meat. *Applied and Environmental Microbiology*, 55, 1901-1906.

SIELADIE, D., V., ZAMBOU, N., F., KAKTCHAM, P., M., CRESCI, A., FONTEH A., F. 2011. Probiotic Properties Of Lactobacilli Strains Isolated from Raw Cow Milk In The Western Highlands of Cameroon. *Innovative Romanian Food Biotechnology*, 9, 12-28.

SIRILUN, S., CHAIYASUT, C., KANTACHOTE, D., LUXANANIL, P. 2010. Characterization of non human origin probiotic *Lactobacillus plantarum* with cholesterol-lowering property. *African Journal of Microbiology Research*, 4, 994-1000.

TODOROV, S., D. 2008. Bacteriocin production by *Lactobacillus Plantarum* AMA-K isolated from Amasi, a Zimbabwean fermented milk product and study of the adsorption of bacteriocin AMA-K *to Listeria sp*. *Brazilian Journal of Microbiology*, 39, 178-187.

TODOROV, S., D., NYATI, H., MEINCKEN, M., DICKS, L., M., T. 2007. Partial characterization of bacteriocin AMA-K, produced by *Lactobacillus plantarum* AMA-K isolated from naturally fermented milk from Zimbabwe. *Food Control*, 18, 656-664.

VERDENELLI, M., C., GHELFI, F., STEFANIA S., ORPIANESI, C., CECCHINI, C., CRESCI, A. 2009. Probiotic properties of *Lactobacillus rhamnosus* and *Lactobacillus paracasei* isolated from human faeces. *European Journal of Nutrition*, 5, 25-30.

XIE, Y., AN, H., HAO, Y., QIN Q., HUANG, Y., LUO, Y., ZHANG, L. 2011. Characterization of an anti-*Listeria* bacteriocin produced by *Lactobacillus plantarum* LB-B1 isolated from koumiss, a traditionally fermented dairy product from China. *Food Control*, 22, 1027-1031.

WHO. 2011. Work together for health: report on health situation in the world. [cit. 20 September 2011]. «http://www.who.int/whr/2006/fr/index.html