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Optimization of Antilisterial Activity of Cell-Adsorbed Bacteriocin Produced by *Lactobacillus Curvatus* CWBI-B28 By Essential Oils to Control *Listeria Monocytogenes* in Attiéké : Traditional Food Made Côte d'Ivoire

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Abstract

To study the effectiveness of a combination of lyophylized cell-adsorbed bacteriocin (LCAB; a suspension of producer cells on which maximum bacteriocin has been immobilized by pH adjustments) of a *Lactobacillus curvatus* strain with oreganoor savory essential oil to control *Listeria monocytogenes* in Ivorian traditional food, namely « Attiéké » at 4°C, the antimicrobial activity of the LCAB and six different essential oils was tested by the well diffusion assay against *L. monocytogenes, Escherichia coli* and *Salmonella Typhi*. The anti-Listeria activity of the LCAB and oregano or savory essential oils was also investigated in Attiéké matrix. The results of the well diffusion assay showed that LCAB was only inhibitory to *L. monocytogenes* while savory and oregano essential oils had the ability to inhibit the three indicator strains. In Attiéké matrix, Listeria counts have declined from 102 CFU g-1 to below the detectable limit during the first week of storage in samples treated with LCAB or oregano essential oil and in those treated with LCAB combined with oregano or savory essential oil. That said, there is a resumption of growth of *L. monocytogenes* cells after the third week ofstorage in all samples with the exception of those treated with the combination of LCAB and oregano essential oil. The combination of LCAB with savory essential oil resulted in a 2-week delay of the growth rebound compared with samples treated with LCAB alone.

Addition of oregano or savory essential oil exhibited a synergistic effect with LCAB in *Listeria* growth controlling in Attiéké matrix at 4°C.

The combination of LCAB with oregano or savory essential oil may be effectively used in our traditional Attiéké food to enhance it's safety and stability.

Keywords: *Antilisterial Activity; Bacteriocin; Lactobacillus Curvatus; Listeria Monocytogenes*

Introduction

Listeria monocytogenes, a Gram-positive bacteria is a food-borne pathogen that is ubiquitous in soil, vegetation, but also in other sources such as milk and meat [1]. This pathogen bacteria cause invasive disease with high mortality rates in animals and humans. Infection arises when the food chain includes vegetables [2] and fruit [3]. Intermittent outbreaks of human disease associated with the consumption of contaminated dairy, fishery or meat products [4] can result when food-processing plants are contaminated for extended time periods and when such food products were stored for long periods of time prior to consumption. Attiékéis one of the major cassava transformation products developed traditionally by Ivorian women for years. It is a fermented cassava food whose manufacturing process combines fermented cassava called "magnan". This vegetal product (magnan), is the main source of microorganisms involved in

the early stages of Attiéké's manufacturing [5]. It is therefore not excluded that this niche can harbor *Listeria*. Controlling the presence of *L. Monocytogenes* in the Attiéké processing thus appears very important, since environmental contamination or poor hygiene practice are considered to be a source leading to pathogenic contamination in this kind of traditional foods. The potential of bacteriocins of lactic acid bacteria as biological food-grade preservatives is well established [6,7]. However, it is clear that in view of their low-spectrum, their possible inactivation in food systems by proteases or their prospective interactions with food constituents, but also and above all, the loss of antimicrobial activity of bacteriocins against sensitive strains during food storage by spontaneous emergence of resistant mutants, the use of bacteriocin must be combined with other hurdles technology [8]. Looking for new alternatives to overcome these numerous hurdles proves thus necessary to control effectively the pathogen in food products. It should be noted that application of essential oil in food to inhibit spoilage and/or pathogenic bacteria has been scientifically demonstrated since the middle of the last century [9]. The multiple hurdles technology has been also established as an efficient means to improve the safety and keeping quality of foods for some time now [10].

The effect of various combinations of essential oils or their active constituents with other antimicrobial substances has been extensively investigated [11]. A synergetic action between essential oils or their constituents and bacteriocins such as nisin to inhibit spoilage and pathogenic micro-organisms has been demonstrated *in vitro* [12,13]. The present study aimed to investigate the antimicrobial activity of a lyophylizez cell-adsorbed bacteriocin (LCAB) of *Lactobacillus curvatus* CWBI-B28 (i.e. a suspension of producer cells on which maximum bacteriocin has been immobilized by pH adjustment) and selected essential oils among those commonly used in food industry against *Listeria monocytogenes, Escherichia coli* and *Salmonella Typhi* by *in vitro* tests. The potential of LCAB alone or in combination with oregano or savory essential oils to control *L. monocytogenes* was also investigated in Attiéké food during storage at 4°C.

Materials and Methods

Lactobacillus curvatus CWBI-B28wt, a bacteriocin producer strain was previously isolated from meat [14]. *L. monocytogenes*, sensitive to this bacteriocin was used as indicator strain to bacteriocin activity measurement in Attieke's samples. *L. curvatus* CWBI-B28wt were grown on de Man, Rogosa and Sharp medium (MRS broth) (Biokar, Beauvais, France). *Listeria monocytogenes* and *S. Typhi* were grown in trypticase soy broth (TSB; Oxoid) while *E. coli* was grown in lactose broth (Oxoid). All strains were stored at -80°C in the irrespective media with added glycerol (40%). The petri dishes used for bacteria enumerations contain agar media such as MRS agar, PALCAM agar (Oxoid), violet red bile lactose agar (VRBL; Biokar Diagnostics, France) and trypticase soy agar, for *L. curvatus, L. monocytogenes, E. coli* and *S. Typhi* respectively.

Our essential oils were obtained from PranaromTM (Horrues, Belgium) and stored at room temperature, protected from light and air.

Lyophilized cell-adsorbed bacteriocin (LCAB) preparation

The LCAB of *L. curvatus* CWBI-B28 was obtained as described previously [15]. First of all, *L. curvatus* CWBI-B28 was grown at 37°C without aeration, with moderate stirring (80 rev min-1) in MRS broth (20l) in a 100-l fermentor (Biolafite, France). The pH was maintained constant at 6.5 by automatic addition of 2.5 M NaOH. Because at this pH value, the bacteriocin of *L. curvatus* CWBI-B28 was shown to adsorb maximally onto producer cells [14]. After 30h of fermentation, the culture was heat-treated at 70°C for 30 – 35 min causing the inactivation of the cell and protease. Next, Eight liters of supernatant are removed and subjected to centrifugation at 4°C and 4000g for 90 min in a Beckman centrifuge (Avanti J-25I; Beckman, CA, USA).

The resulting concentrate was suspended in 1800 ml supernatant kept at 4°C for 30 - 35 min to enable adsorption of bacteriocins onto producer cells. Then a second centrifugation at 4000g at 4°C for 25 min was carried out to eliminate the supernatant containing any non-adsorbed bacteriocins. The pellet concentrate was then suspended in 150 ml supernatant, allowed to crystallize overnight at -80°C, and lyophilized (Koeltechniek Louw B.V.B.A,Rotselaar, Belgium). The powder obtained was aliquoted,vacuum-packed, and preserved at -20°C.

Antimicrobial activity of LCAB and different essential oils against selected food borne pathogens

Antimicrobial activity of LCAB and essential oils against our three indicator strains *L. monocytogenes, E. coli* and *S. Thyphi* was tested by the well diffusion assay [16]. Wells of approximately 6 mm in diameter were punched in the petri dishes containing M17 agar (Oxoid) previously seeded with0.1 ml of an active culture of each indicator strain and filled with 60 µl of LCAB suspension (0.2g of the active powder in 1 ml of sterile distilled water), or 5 µl of each of the essential oils. The plates were then incubated at 37°C for 16–18 h before measure the diameters of the inhibition zones surrounding the wells.

Minimal inhibitory concentration of essential oils against *Listeria monocytogenes*

The minimal inhibitory concentrations (MIC) of two essential oils (i.e. savory and oregano) that gave the largest diameter of inhibition zones against *L. monocytogenes* by the well diffusion assay (Table 1) were determined in liquid media as described by Wan., *et al* [17]. Savory or oregano essential oil was added to different concentrations ranging from 0 to 5.0 µl ml-1 in a series of test tubes each containing 10 ml of MRS broth.

Tubes were then inoculated with 100 µl of an overnight culture of *L. monocytogenes* and incubated under agitation (120 rev min^{-1}) at 37°C for 48 h to keep the medium homogeneous. A 1-ml sample was withdrawn at 0h and at 48h, to count the numbers of *L. monocytogenes* on PALCAM agar. The MIC was defined as the lowest concentration required for complete inhibition of the test organism after 48 h of incubation in the presence of the essential oil.

Antilisterial activity of LCAB in Attiéké with or without added oregano or savory essential oil

Two independent trials were conducted to study the effectiveness of the LCAB to control *L. monocytogenes* in Attiéké in presence or absence of oregano or savory essential oil. In each trial, a Attiéké block of 250g was divided aseptically into portions of approximately 50 geach to make five different batches (B1 to B5). The batches were placed separately in sterile aluminum foil under a laminar flow hood (Clean Air, VWR, Belgium) for subsequent treatments. Batches B2 to B5 of each trial were artificially contaminated with *L. monocytogenes* with10^2 CFU g^{-1}. The batch B1 (negative control) was not contaminated with *Listeria*. After inoculations, 3 ml of LCAB suspension (1 g of active powder in 5 ml of sterile distilled water) were applied to each of the batches B3and B5. The essential oil (oregano or savory) was added to batches B4 and B5 to a final concentration of 50 µl 100 g^{-1} of Attiéké. For practical reasons (i.e. low volume of the essential oil to be applied), the essential oil was mixed to the required volume of the LCAB suspension (B5) or to an equal volume (3 ml) of sterile distilled water (B4) before application to Attiéké portions. The batch B2 served as a positive control and, hence, was inoculated with *L. monocytogenes* without further treatments. All samples were placed in separate sterile plastic bags, which were then heat-sealed and held at 4°C during the course of the experiments. The trials differed only in the essential oil used in the treatment of batches B4 and B5; while oregano essential oil was used in one of the trials, savory essential oil was used in the other.

Microbiological analyses and bacteriocin activity determinations

Attieké were sampled at regular intervals (1, 2, 3, 4, 5, 6 weeks) of incubation. At each sampling, 15g samples were taken aseptically from the heat-sealed sterile plastic bags, diluted with 150 mL sterile saline solution (0.85% sodium chloride), and pressed manually in another sterile plastic bags to extract as much liquid as possible. From this liquid, serially diluted and a volume of 0.1 ml from each dilution was spread-plated in duplicate onto PALCAM agar (Oxoid) for microbiological analyses.

The CFU of *L. monocytogenes* were determined after incubation at 37°C for 48–72 h. Suspensions obtained from samples treated with LCAB were centrifuged and the supernatant was filter-sterilized to determine the bacteriocin activity in arbitrary units (AU) as previously described [15].

Statistical Analysis

Each trial was repeated twice and each determination was performed in duplicate (analysis of variance α = 0.05% and Student's t-test) of was done with Excel software.

Results

Antibacterial activity of LCAB and different essential oils

Table 1 summarizes the results of the antibacterial activity of the LCAB and selected essential oils against *L. monocytogenes, E. coli* and *S. Typhi.*

Antimicrobial agent	*L. monocytogenes*	*S. Typhi*	*E. coli*
LCAB	25 ± 2	NI	NI
Coriander EO	7 ± 1	14 ± 0	7 ± 0
Savory EO	23 ± 1	23 ± 0	20 ± 1
Clove EO	10 ± 1	13 ± 0	9 ± 0
Oregano EO	23 ± 0	21 ± 0	21 ± 1
Rosemary EO	NI	9 ± 1	10 ± 2
Thyme EO	10 ± 0	11 ± 2	13 ± 1

Table 1 : *Diameter of the zones of inhibition (mm ± SD)* of lyophilized cell adsorbed bacteriocin (LCAB) of Lactobacillus curvatus CW-BI-B28 and selected essential oils (EO) against Listeria monocytogenes, Salmonella Typhi and Escherichia coli strains.*

**Results include the diameter of the well (6 mm).*

- LCAB, Lyophiliezd cell-adsorbed bacteriocin.

- NI : No inhibition.

As can be seen, the LCAB was only active against *L. monocytogenes* while all the tested oils except Rosemary (which is powerless against *Listeria*) are effective at different degrees against our three indicator strains. The antibacterial activity of coriander, clove, rosemary and thyme varied depending on the indicator strain, but their antilisterial activity was weak. Oregano and savory essential oils are the only ones to have an highest inhibitory activity against all bacteria tested and were, therefore, retained for the next experiments. The MIC of each of these two essential oils was determined against *L. monocytogenes* showing complete inhibition of the latter after 48 hours of incubation at concentrations ranging from 0.5 to 5.0 µl ml-1. In contrast, the pathogen grew well in the positive control (i.e. no added essential oil). The level of 0.5 µl ml-1 was, therefore, considered as the MIC for both savory and oregano essential oils.

Antilisterial activity of LCAB in presence or absence of essential oil in Attiéké matrix

The results of the antilisterial activity of the LCAB in presence or absence of oregano or savory essential oil during cold storage of Attiéké matrix are summarized in Table 2.

Time (weeks)	log10 CFU g^{-1} ± SD						AU g^{-1}
	Control	LCAB	OEO	SEO	LCAB+ OEO	LCAB+ SEO	
0	2.70 ± 0.2	2.90 ± 0.1	2.60 ± 0.15	3.0 ± 0.10	2.78 ± 0.0	2.79 ± 0.0	1800 ± 0.0
1	4.10 ± 0.18	0 ± 0	0 ± 0	2.11 ± 0.08	0 ± 0	0 ± 0	750 ± 55.51
2	3.15 ± 0.15	0 ± 0	0 ± 0	1.70 ± 0.75	0 ± 0	0 ± 0	575 ± 60.35
3	2.80 ± 0.10	0 ± 0	0 ± 0	0.78 ± 0.1	0 ± 0	0 ± 0	321 ± 7.06
4	2.10 ± 0.21	1.25 ± 10	0 ± 0	1.75 ± 0.3	0 ± 0	0 ± 0	250 ± 3.60
5	1.90 ± 0.32	1.35 ± 0.25	0.85 ± 0.1	1.81 ± 0.1	0 ± 0	0.90 ± 0.30	170 ± 10.85
6	1.85 ± 0.15	1.50 ± 0.15	1 ± 0.17	2 ± 0.16	0 ± 0	1 ± 0.0	86 ± 26.42

Table 2: *Enumeration of Listeria monocytogenes as function of time in Attiéké treated with lyophilized cell-adsorbed bacteriocin (LCAB) in presence or absence of oregano essential oil (OEO) or savory essential oil (SEO), and bacteriocin production (AU g^{-1}*) in Attiéké samples treated with LCAB*

**AU (arbitrary unit) is defined the reciprocal of the highest dilution giving a definite zone of inhibition by the well diffusion assay (Tagg and McGiven 1971) on a lawn of L. monocytogenes*

Compared to the positive control (i.e. no added LCAB or essential oil), a significant ($P < 0.05$) reduction in *Listeria* cfu counts was observed in the first week of storage in all traited samples. However, the extent of this inhibition and the subsequent protection of the Attiéké product from the growth of *L. monocytogenes* varied depending on the applied treatment. In batches treated with the LCAB in presence or absence of oregano or savory essential oil and in those treated with only oregano essential oil, the counts of *L. monocytogenes* have declined to an undetectable level during the first week of storage. In samples treated with savory essential oil, a reduction of 2 log CFU g^{-1} in *Listeria* counts was observed at the third week of storage but the *Listeria* CFU count did not fall below the detectable level throughout the whole period of the study (Table 2). Moreover in all tested samples, except those treated with the combination of LCAB and oregano oil, an increase in *Listeria* counts was noted after they have reached their lowest level. Such an increase (i.e. rebound phenomenon) was very quickly observed from the fourth week in samples treated with LCAB alone compared with those treated with oregano essential oil alone or with the combination of LCAB and savory essential oil.

Discussion

In vitro antimicrobial activity assays showed that LCAB and six different essential oils inhibited various indicator strains. While the LCAB was only inhibitory to *L. monocytogenes*, the essential oils inhibited the indicator strains to various degrees depending on the essential oil and the bacterial strain (Table 1). No evidence for the difference in sensitivity among gram-positive and gram-negative bacteria to the essential oils could be noted in agreement with earlier reports [13,18,19]. In contrast, other studies suggested that essential oils are generally more active against gram-positive than gram-negative bacteria [20,21]. The high inconsistencies in the chemical composition of an essential oil depending on physiological and ecological conditions of the producing plants [19] may explain the variability in the susceptibility of sensitive strains regardless of their Gram staining. It has been demonstrated that, essential oils containing high levels of carvacrol were reported to be the most inhibitory to microbial growth [22]. This is consistent with the fact that oregano and savory essential oils had the highest antimicrobial activity against all bacteria tested in this study, as carvacrol represents 80% [23] and 57% [24] of these essential oils, respectively. Determinations of the MIC of savory and oregano essential oils showed that low levels of these essential oils effectively inactivate sensitive bacteria. *In vitro* bactericidal effect of essential oils at low concentrations is well documented [25,26]. A lack of knowledge of the amount of essential oil to be applied could have adverse consequences on the taste value of food [12]. Therefore, the search for adequate combinations of essential oils with other hurdles to microbial growth have been the focus of tremendous work in recent years aiming to improve their overall effectiveness in food preservation and, hence, reduce theamount to be added. The study of the in situ effect of LCAB alone or in combinationwith savory or oregano essential oil on the growth of *L. monocytogenes* in Attiéké matrix showed different performances with overall tendency to reduce the counts of the pathogen. However, after an initial decrease in Listeria counts to their lowest levels in all the treated samples, the growth of the pathogen was re-initiated in most of them. The occurrence of this rebound phenomenon upon extended period of storage has been reported previously [5,8]. In the case of the LCAB-treated samples, the steady decrease in the bacteriocin activity during storage may explain the *Listeria* growth recovery (Table 2). Inactivation of bacteriocins in food has been attributed to indigenous or microbial proteases [15]. But this can also be attributed to an adaptation of the pathogen to sublethal injury [27,28], spontaneous development of resistant mutants [28,29], interactions with food constituents [11,18]. In this study, the rebound was not observed in samples treated with the combination of LCAB and oregano essential suggesting a strong synergistic action between these antimicrobial compound. A synergy action was also noted in the samples treated with the combination of LCAB and savory essential oil; however, this combination failed to prevent the growth rebound which was only delayed from the third to the fourth (samples treated with only savory essential oil) or the fifth (samples treated with only LCAB) week of storage.

Conclusion

The combination of a LCAB with oregano or savory essential oil has improved the control of *L. monocytogenes* in our traditional Attiéké food as compared with their utilization separately. However, the synergistic action of LCAB with oregano essential oil provided more efficient and a longer protection of Attiéké food from the growth rebound of *L. monocytogenes*. Therefore, the combination of LCAB of L. curvatus and oregano essential oil may be a useful means to enhance the safety and stability of our Ivoirian traditional Attiéké food.

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