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Research article

SEARCHING FOR BACTERIOCIN *PLN* LOCI FROM *LACTOBACILLUS* SPP. ISOLATED FROM FERMENTED FOOD IN BURKINA FASO BY MOLECULAR METHODS

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ABSTRACT: The *pln* locus responsible for plantaricin biosynthesis in *Lactobacillus plantarum* was discover over two decade now. This study identified from fermented food four plantaricin-producing strains of *Lactobacillus* through molecular and bioinformatic methods. Firstly the primers LbF and LbR were used to identify the strains. Secondly, BacF and BacR are primers used in order to identify bacteriocin-producing strains and search for *pln* loci encoding bacteriocin named plantaricin. Each PCR product were sequenced and sequences were aligned through NCBI (identification of strain), NCBI and BAGEL3 (bacteriocin-producing strains identification and mining for *pln* locus). After successive BLASTn, the strains were identified as *Lactobacillus* sp. strain S3; *Lactobacillus* sp. strain S4; *Lactobacillus* sp. strain Y6 and *Lactobacillus plantarum* strain Lf1. Comparison of partial sequences of each product obtained with primers BacF and BacR with available sequences in NCBI and BAGEL3 databases showed that the *pln* gene is a mosaic gene because about 25 genes covering a DNA stretch are found in each locus and they are organized into five operons which are *plnABCD, plnEFI, plnGHSTUVW, plnJKLR* and *plnMNOP*. The *plnK* gene is the most constant gene found. Together these strains produce two different class IIb two-peptide bacteriocins, plantaricins EF and JK, and a pheromone peptide plantaricin A. The strains were also found to harvest for histidine protein kinase (HPK) and response regulator (RR). This study has once again attest the presence of *Lactobacillus* species in fermented foods where they play a key role. These strains can therefore be proposed to local fermented food producers to be used as starter.
Key words: Burkina Faso, *Lactobacillus plantrarum*, Operons, plantaricin, *Soumbala*.

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INTRODUCTION

Lactobacillus spp. are lactic acid bacteria (LAB) that display phylogenetic, phenotypic, and ecological heterogeneity that is reflected in their taxonomic diversity (Claesson et al., 2008). Lactobacilli have been studied extensively because of their importance for the production of fermented foods and beverages (Cho et al., 2011; Raftis et al., 2011). Members of the genus *Lactobacillus* are gram-positive, non-spore forming, mostly non-motile and generally rod-shaped even coccobacilli can be observed. Their cells are often organized in chains. The optimal growth temperature is mostly between 30 and 40°C (Taale et al., 2013), although the overall growth temperature can be ranged between 2 and 53°C; the pH for growth between 3 and 8 (Pot et al., 2014).The large majority of LAB as *Lactobacillus* belongs to the order *Lactobacillales* within the group of low percentage G+C Gram-positive bacteria of the *Firmicutes* phylum (Vandamme et al., 2014). Since the 1990s, identification and description of new species has often been based on a polyphasic approach (Vandamme et al., 1996), involving both genotypic and phenotypic characterization. Genotypically, 16S RNA sequencing was the method of choice, mainly because of the availability of large reference sequences sets. The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been far the most common housekeeping genetic marker used for a number of reasons : first its presence in almost all bacteria (often existing as a multigene family, or operons); secondly the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and finally the 16S rRNA gene (1,500 bp) is large enough for informatics purposes (Patel, 2001; Janda and Abbott, 2007). According to Ludwig and Klenk (2001) and Patel (2001) ribosomal RNA sequences provide a phylogenetic framework that serves as a backbone for modern microbial taxonomy. For that purpose, public databases comprising published and unpublished sequences have been constructed (Olsen et al., 1991; Yarza et al., 2008; Cole et al., 2009). The use of conserved macromolecules such as 16S or 23S rRNA for bacterial classification should reflect as much as possible the natural relationships between bacteria (Woese, 1987; Vandamme et al., 2014). However, classification serves very practical purposes, which is, the recognition of organisms that were encountered previously and the categorization of new ones into a logical and tractable system (Vandamme et al., 2014).

Lactobacillus are used in preservative processes where they, like many other LAB, can contribute with the production of antimicrobial substances (e.g., organic acids and bacteriocins) (Diep et al., 2009). Bacteriocins are ribosomally-synthesized peptides or proteins produced by a wide range of bacteria (Zouhir et al., 2010). Several works show that the *Lactobacillus plantarum* can produce bacteriocin called plantaricin, belonging to bacteriocin of class II. The *pln locus* responsible for plantaricin biosynthesis in *Lactobacillus plantarum* was first unraveled in *Lactobacillus plantarum* C11 and since then it was found in large amount of *Lactobacillus plantarum* strains (Diep et al., 2009). The *pln locus* is a mosaic gene because may contain several genes and operons. For further reading (Ben Omar et al., 2008; Diep et al., 2009). In Burkina Faso, few data on *Lactobacillus* species and its bioactives molecules such bacteriocins isolated from fermented food are available.

In this study, we identified four *Lactobacillus* strains to species level and habor for *pln locus* gene and its operons using bioinformatics tools and molecular methods.

MATERIAL AND METHODS

Bacteria strains

The four *Lactobacillus* spp. strains used in this study were isolated from *Soumbala* (strains S3 and S4), local fermented milk (strain Lf1) and local yogurt (strain Y6) and characterized as previously described by Taale et al. (2013).

DNA extraction

The presumptively identified colony of *Lactobacillus* spp. as described by Taale et al. (2013) were grown overnight on nutritive broth. The cultured broth was centrifuged at 5,000g for 10min . The pellets were then used for bacteria DNA extraction by using QiAamp DNA Mini Kit (QiAgen, France) following the manufacturer's instructions.

DNA amplification

The primers used to amplify 16S rRNA gene of all *Lactobacillus* species and 16S rDNA gene encoding Bacteriocins in *Lactobacillus* genus are all listed in Table 1. All primers were synthesized by Eurofins mwg operon (Germany, www.eurofinsgenomics.eu).

The PCR was run in 50µl reaction mixture containing 6µl of $MgCl_2$25mM, 10 µl of 5X Colorless GoTaq Flexi Buffer (Promega, USA), 1µl of dNTPs Mix10mM (Promega, USA), 3µl LbF and 3µl LbR (when amplifying 16S rRNA gene); 3µl BacF and 3µl BacR (when searching for bacteriocin-producing strains)(table 1), 0.3µl of *Taq* DNA polymerase (Promega, USA), 10µl of the DNA template and nuclease-free water making up the total volume. The concentration of used primer was 20µM.

The thermal cycling (3Prime Thermocycler, United Kingdom) profile was as follows : initial denaturation at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 45s, annealing at 60°C (LbF and LbR)/56°C (BacF and BacR) for 45s, and polymerization at 72°C for 1 min. An extra final polymerization step at 72°C for 5 min was performed to ensure that all PCR fragments were complete and A-tailed. The PCR products obtained were observed an UV plate after they were run on 1.5% (w/v) agarose gel (Eurogenetec) in 1X TEB (Tris-Phosphate-EDTA buffer) at 100 V, 50 mA for 1 h.

PCR products sequencing

The PCR products were used for nucleotide sequencing using LbF and BacF primers. PCR products were purified using Wizard® SV Gel and PCR Clean-Up System (Promega, USA) by dissolving the gel slice and processing PCR products purification (binding of DNA, washing and elution). The purified fragment was used for sequencing. Sequencing of PCR products was determined according to the procedure of GENOSCREEN (Lille, France, www.genoscreen.fr/). The sequences obtained were trimmed to eliminate the ambiguous bases and bases with poor quality.

Identification

The partial sequences were compared with all *Lactobacillus* sp., 16S rRNA gene sequences available in GenBank using online BLASTn software (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple alignments were performed using BLASTn. For identification if the identity is higher than 95%, the isolate could be identify at species level and at genus level if the identity is less than 95%.

Mining for different genes of *pln* locus

The search of bioactive compounds genes like *pln locus* of plantaricin was performed using NCBI and BAGEL3 (http://bagel.molgenrug.nl/index.php/bagel3) using sequences obtained when primers BacF and BacR were used. Those primers were used to identify lactic acid bacteria bacteriocin-producing strains by amplifying their 16S rDNA gene.

RESULTS AND DISCUSSION

Isolates identification

The electrophoretic profile of PCR products showed bands size of 200-300 pairs of bases. Strains S3, S4, Y6 and Lf1 belonged to the genus *Lactobacillus* (Taale et al., 2013).

The partial sequences of the 16S rRNA gene for each *Lactobacillus* isolate was aligned with sequences of *Lactobacillus* 16S rRNA gene available in NCBI database through the link http://blast.ncbi.nlm.nih.gov/Blast.cgi. The different strains and identity average obtained are presented in Table 2.The length of partial sequences of the 16S rRNA gene of strain S3, S4, Y6 and Lf1 is respectively 538 bp, 202 bp, 215 bp and 365 bp.

According to Baxevanis (2011), biological databases play a central role in bioinformatics. They offer scientists the opportunity to access a wide variety of biologically relevant data, including the genomic sequences of an increasingly broad range of organisms. Then, the comparison of partial nucleotide sequences to the16S rDNA gene available in biological database NCBI by alignment gave identification rate which varies from 89% to 98%. NCBI published sequences of *Lactobacillus* species as shown in Table 2 revealed an alignment of 89% sequence similarities of isolate Y6 to 16S rRNA, partial sequence of *Lactobacillus brevis strain* IMAU11348 (YM43-1) and *Lactobacillus brevis* strain NPS-QW-145. However, isolates S3 and S4 showed 95% homology respectively to *Lactobacillus acidophilus* strain FSI4, complete genome and *Lactobacillus plantarum* strain CCB-6 16S ribosomal RNA gene, partial sequence. The isolate Lf1 have 98% identity to *Lactobacillus plantarum* strain J11 16S ribosomal RNA gene, partial sequence (Table 2). These results were obtained by using alignment parameters suggested by Ladunga (2009). From these results the isolates S3, S4, Y6 and Lf1 could be identified respectively as *Lactobacillus* sp. strain S3, *Lactobacillus* sp. strain S4, *Lactobacillus* sp. strain Y6 and *Lactobacillus plantarum* strain Lf1. The figure 1 shows the phylogenetic relationship between strain Lf1 and other species of *Lactobacillus plantarum* available on NCBI database.

Food condiments obtained by alkaline uncontrolled fermentation as *Soumbala* are recognized to be a niche of the genus *Bacillus*(Odunfa and Oyewole, 1986; Ouoba et al., 2004; Azokpota et al., 2006; Ouoba et al., 2007; Ouoba et al., 2008; Parkouda et al., 2009; Uaboi-Egbenni et al., 2009; Savadogo et al., 2011) but also the lactic acid bacteria play a key role in these condiments as previously reported by Olasupo et al. (1997); Uaboi-Egbenni et al. (2009); Ouoba et al. (2010); Ajayi (2014) and Ukwuru and Ibeneme (2014). This study shows that *Soumbala* produce in Burkina Faso may contain *Lactobacillus* spp. strains, and other studies carried out in products based on African locust seeds in other African countries has already shown that *Soumbala* called by various names (Azokpota et al., 2006; Savadogo et al., 2011), may contain species of *Lactobacillus* (Ouoba et al., 2010; Ajayi, 2014). The alignment results have established a phylogenetic relationship between different *Lactobacillus* species tested (Figure 2).

Identification of bacteriocin-producing strains

BacF and BacR are primers used in order to see if *Lactobacillus* sp. strain S3, *Lactobacillus* sp. strain S4, *Lactobacillus* sp. strain Y6 and *Lactobacillus plantarum* strain Lf1 are bacteriocin-producing strains. The PCR products size were greater than 1000 bp (Figure 3). These results are in agreement with those obtained by Diop et al. (2008). Therefore *Lactobacillus* sp. strain S3, *Lactobacillus* sp. strain S4, *Lactobacillus* sp. strain Y6 and *Lactobacillus plantarum* strain Lf1 are bacteriocin-producing strains.

Mining for plantaricin *pln* loci

The PCR products obtain with primers BacF and BacR were partially sequenced. Then those partial sequences were aligned with different *pln* gene sequences available in two databases: NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Bagel3 (http://bagel.molgenrug.nl/index.php/bagel3)(van Heel et al., 2013) to determine if interest strains contain bacteriocin named plantaricin *pln* gene locus synthesized by *Lactobacillus plantarum* strains (Table 3).

Table 1. Primers used for PCR and sequencing

Targeted gene	Primers	Technique	Sequence	Sense	Reference
16S rRNA	LbF	PCR and sequencing	5'- GGAATCTTCCACAATGGACG – 3'	Forward	(Bakar et al., 2010)
	LbR		5'- CGCTTTACGCCCAATAAATCCGG – 3'	Reverse	
16S rDNA of bacteriocin-producing strains	BacF	PCR and sequencing	5'- AAGAGTTTGATCCTGGCTCAG – 3'	Forward	(Diop et al., 2008)
	BacR		5'- CTACGGCTACCTTGTTACGA – 3'	Reverse	

Table 2. BLASTn of 16S rRNA genes, partial sequences of the isolates

Strain	1st BLASTn		2nd BLASTn		Identification
	Description	**Identity**	**Description**	**Identity**	
Strain S3	*Lactobacillus acidophilus* strain FSI4, complete genome	95%	*Lactobacillus acidophilus* strain FSI4, complete genome	95%	*Lactobacillus* sp. strain S3
	Lactobacillus acidophilus La-14, complete genome	95%	*Lactobacillus acidophilus* La-14, complete genome	95%	
Strain S4	*Lactobacillus plantarum* strain SSK03 16S ribosomal RNA gene, partial sequence	93%	*Lactobacillus plantarum* strain CCB-6 16S ribosomal RNA gene, partial sequence	95%	*Lactobacillus* sp. strain S4
	Lactobacillus murinus strain FAM-01 16S ribosomal RNA gene, partial sequence	93%	*Lactobacillus plantarum* strain 1A 16S ribosomal RNA gene, partial sequence	95%	
Strain Y6	*Lactobacillus brevis* strain IMAU11348 (YM43-1) 16S ribosomal RNA gene, partial sequence	89%	*Lactobacillus brevis strain* IMAU11348 (YM43-1) 16S ribosomal RNA gene, partial sequence	89%	*Lactobacillus* sp. strain Y6
	Lactobacillus plantarum strain KM MSU 519 16S ribosomal RNA gene, partial sequence	89%	*Lactobacillus brevis* strain NPS-QW-145 16S ribosomal RNA gene, partial sequence	89%	
Strain Lf1	*Lactobacillus plantarum* strain SSK03 16S ribosomal RNA gene, partial sequence	98%	*Lactobacillus plantarum* strain J11 16S ribosomal RNA gene, partial sequence	98%	*Lactobacillus plantarum* strain Lf1
	Lactobacillus murinus strain FAM-01 16S ribosomal RNA gene, partial sequence	97%	*Lactobacillus plantarum* strain CSCWL 6-8 16S ribosomal RNA gene, partial sequence	97%	

Table 3: Alignment score for the 16S rDNA partial sequence of the four bacteriocins producing strains using BLASTn and BAGEL3 databases

Plantaricin locus	*Lactobacillus* sp. strain S3		*Lactobacillus* sp. strain S4		*Lactobacillus* sp. strain Y6		*Lactobacillus plantarum* strain Lf1	
	NCBI[a]	BAGEL3[b]	NCBI[a]	BAGEL3[b]	NCBI[a]	BAGEL3[b]	NCBI[a]	BAGEL3[b]
plnA	100%	2.10^{-10}	80%	2.10^{-10}		2.10^{-10}	80%	2.10^{-10}
plnB		0.0		0.0		0.0		0.0
plnC	100%		100%		87%		100%	
plnD	87%	0.0		0.0	100%	0.0	100%	0.0
plnE	85%	7.10^{-37}	91%	7.10^{-37}	91%	7.10^{-37}	91%	7.10^{-37}
plnF	100%	6.10^{-35}		6.10^{-35}		6.10^{-35}		6.10^{-35}
plnG	92%	0.0	100%	0.0	100%	0.0	93%	0.0
plnH	100%	0.0	100%	0.0	100%	0.0	94	0.0
plnI	100%	0.0		0.0		0.0	100%	0.0
plnJ	100%	6.10^{-37}	86%	6.10^{-37}		6.10^{-37}	100%	6.10^{-37}
plnK[d]	90%	1.10^{-37}	100%	1.10^{-37}	100%	1.10^{-37}	100%	1.10^{-37}
plnL	92%	3.10^{-159}	100%	3.10^{-159}	92%	3.10^{-159}	100%	3.10^{-159}
plnM	92%		100%		100%		100%	
plnN		3.10^{-35}	100%	3.10^{-35}		3.10^{-35}	92%	3.10^{-35}
plnO	100%	3.10^{-13}		3.10^{-13}		3.10^{-13}	3.10^{-13}	3.10^{-13}
plnP		0,0094		0,0094		0,0094		0,0094
plnQ	93%		100%		100%		100%	
plnR			100%		100%		100%	
plnS	100%		100%		100%		92%	
plnT		9.10^{-167}	100%	9.10^{-167}	92%	9.10^{-167}	92%	9.10^{-167}
plnU		2.10^{-105}	100%	2.10^{-105}		2.10^{-105}	88%	2.10^{-105}
plnV		6.10^{-163}		6.10^{-163}		6.10^{-163}	87%	6.10^{-163}
plnW	92%	4.10^{-161}		4.10^{-161}		4.10^{-161}	93%	4.10^{-161}
plnX		4.10^{-52}	87%	4.10^{-52}		4.10^{-52}		4.10^{-52}
plnY		2.10^{-68}		2.10^{-68}		2.10^{-68}		2.10^{-68}
***pln* gene**	100%						100%	
ABC Tr	100%				100%		100%	

ABC Tr= ABC Transporter [a]Identity [b] Evalue [d] Putative bacteriocin

Table 4: Proteins encoded by *pln* loci of the four *Lactobacillus* strains

Operon	Number of genes	Bacteriocin
plnABCD	4	Plantaricin A or IP Histidine Protein kinase (HKP) Protein RR
plnEFI	3	Plantaricin E Plantaricin F Plantaricin J Plantaricin K Cognate immunity proteins
plnJKLR	4	
plnGHSTUVW	7	ABC-transporter and an accessory protein
plnMNOP	4	Four putative proteins

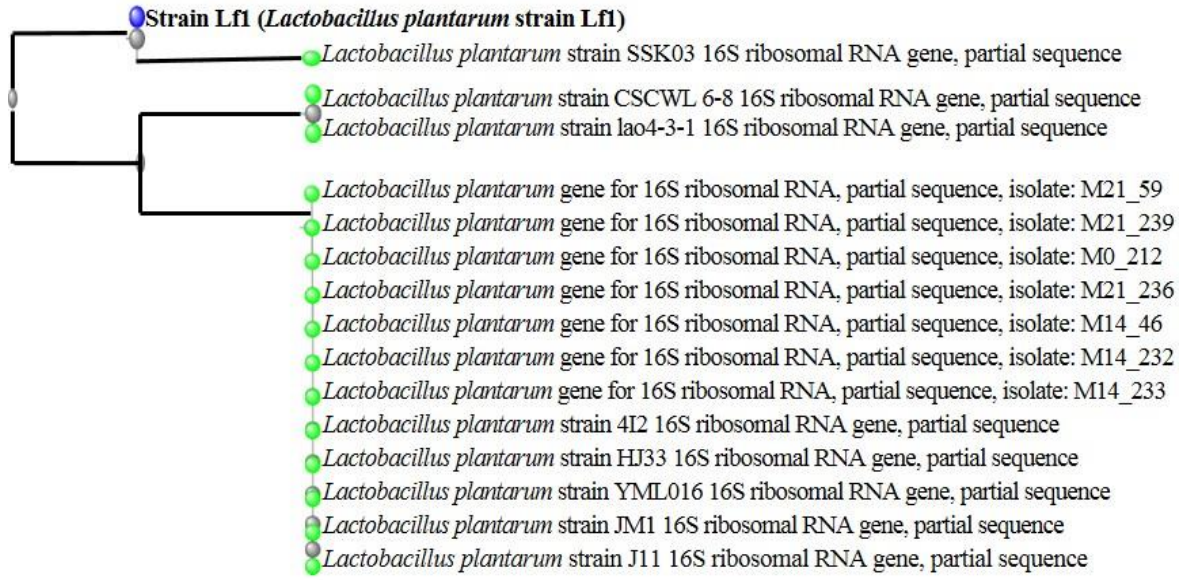

Figure 1: Phylogenetic relationship of Lf1 with related species based on partial 16S rRNA gene sequence analysis. The phylogenetic tree was constructed using the neighbor-joining method. Reference sequences were obtained from the NCBI nucleotide sequence database.

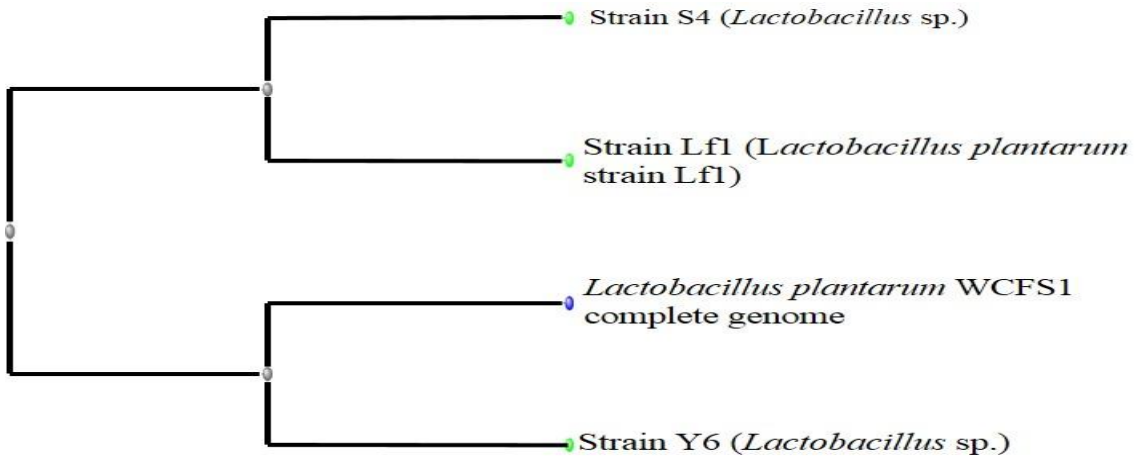

Figure 2: Phylogenetic relationship of three *Lactobacillus* species with *Lactobacillus plantarum* WCFS1 on partial 16S rDNA gene sequence analysis. The phylogenetic tree was constructed using the neighbor-joining method. Reference sequences were obtained from the NCBI nucleotide sequence database.

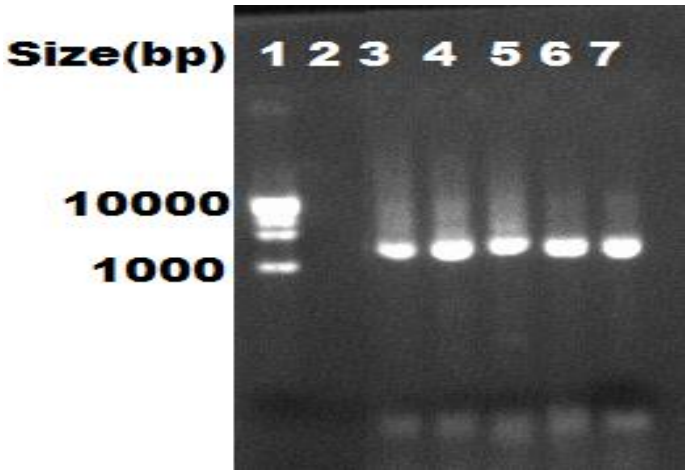

Figure 3: Identification of bacteriocin-producing strains using primers pairs BacF and BacR. Lane 1: 1kb-DNA molecular mass marker, Lane 2: negative control; Lane 3: *Lactobacillus* sp. strain S3, Lane 4: *Lactobacillus* sp. strain S4, Lane 5: *Lactobacillus* sp. strain Y6, Lane 6: *Lactobacillus plantarum* strain Lf1, Lane 7: *Lactobacillus* sp. as positive control.

The alignment identified the potential existence of different loci encoding two bacteriocins components (plantaricin belonging to class IIb bacteriocin) in *Lactobacillus plantarum*. Several screening surveys on the presence of *pln* genes in isolates of *Lactobacillus* sp. and *Lb. plantarum* indicate that determinants of the *pln* locus are relatively present amount them. ABC transporter locus responsible for the synthesized bacteriocin could be detected among *Lactobacillus* sp. strain S3, *Lactobacillus* sp. strain Y6 and *Lactobacillus plantarum* strain Lf1. The locus *pln A, E, F, K, L, T, U, V, W, X* and *Y* could be found in all four strains with constant e-value (Table 3). All strains could produce the pheromone peptide plantaricin A. This mosaic *loci pln* encoding the various plantaricins bacteriocins was shown by several previous studies in different species of *Lactobacillus plantarum* (Ben Omar et al., 2008; Knoll et al., 2008; Diep et al., 2009).

Based on the results, the genetic organization of bacteriocins can be divided into five (5) different inducible operons: *plnABCD* operon; *plnEFI* operon; *pln GHSTUVW* operon, *pln JKLR* operon and *pln MNOP* operon.

plnABCD operon (80-100% identity) codes for the induction peptide plantaricin A, the HPK (gene *plnB*) and Response Regulator Protein RRs (genes *pln*C and *pln*D). Plantaricin A is matured from *pln*A. According to Diep et al. (2009), *plnABCD* is a regulatory operon, coding for a quorum-sensing network necessary to express all genes in the *pln* locus. The presence of operon *plnABCD* amount the four *Lactobacillus* strains suppose that they could be able to produce bacteriocin named plantaricin.

The two two-peptide bacteriocins, plantaricin EF and JK and their cognate immunity proteins are code by *plnEFI* and *plnJKLR*. Plantaricins EF and plantaricin JK are antimicrobial peptides belonging to class IIb bacteriocins, whose activity is, by definition, dependent on the complementary action of two different peptides (Diep *et al*., 2009). As previously reported by Taale et al. (2013), neutral cell-free supernatant of *Lactobacillus* sp. strain S3, *Lactobacillus* sp. strain S4, *Lactobacillus* sp. strain Y6 and *Lactobacillus plantarum* strain Lf1 display large inhibitory spectra because they were active against *Micrococcus luteus*. In summary, their antimicrobial activity may results by permeabilizing M. luteus inner membrane, causing influx and efflux of various molecules across the transmembrane barrier, eventually leading to cell death as shown by inhibitory zone.

Gene's *plnGH* code for an ABC-transporter and an accessory protein, respectively, that together form an ABC-transport system dedicated to secretion of peptides employing a double-glycine leader.

Operon *plnMNOP* (92-100% identity) codes for four putative proteins, of which *plnN* appears to contain an N-terminal double-glycine leader consensus.

The different proteins encoded by these operons are summary in table 4.

These various operons may contain several Opening Reading Frame (ORFs) (Diep et al., 1996).The operon *plnGHSTUV* contains two ORFs (*plnG* and *plnH*) apparently encoding an ABC transporter and its accessory protein, respectively, known to be involved in processing and export of peptides with precursor double-glycine-type leaders (Diep et al., 2009). *Lactobacillus* sp. strain S3 contains less *pln* genes because has 24 genes.

Together these strains produce two different class IIb two-peptide bacteriocins, plantaricins EF and JK, and a pheromone peptide plantaricin A with antimicrobial activity (Taale et al., 2013).

CONCLUSION

This study showed once again the presence of *Lactobacillus* species in local fermented foods. After sequence alignments, the four strains could be identified as *Lactobacillus* sp. and *Lactobacillus plantarum*. The four strains were found to possess the *locus pln* gene encoding plantaricin. This study confirms that the *pln* gene is a mosaic gene because at least four operons were found in producing strains and are probably involved in bioactives compounds production. *Soumbala*, a condiment food may have *Lactobacillus* species playing important function.

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