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RESEARCH ARTICLE

UNVEILING THE CULTURABLE BACILLUS SPP. ISOLATED FROM HONEY IN BURKINA FASO AS A POTENTIAL SOURCE FOR ANTIMICROBIAL PEPTIDES

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Abstract

Backgrounds and objectives: Honey is increasingly recognized as a promising reservoir of beneficial microorganisms with activity against plant pathogens. However it is poorly documented that in the literature. This study contributes to explore the diversity of AMPs genes produced by *Bacillus* spp. isolated from honey in Burkina Faso in order to propose a biological solution against biodiversity loss, water soil pollution and pest resistance caused by chemicals pesticides.
Methods: Methodology was based on isolation and preliminarily characterization of 40 presumptive *Bacillus* spp. followed by their survival under thermal stress, salt tolerance and AMPs related genes identification.
Results: Data from PCR analysis revealed that all isolates belonged to the genus *Bacillus*. In total, 70% of strains carried at least one AMP gene, in which 50% carried one gene. Regarding AMPs-gene associations, 25% carried two genes, 14.3% carried three genes, 7.1% carried four genes, and 3.6% carried five of the six targeted AMP-genes.
Conclusions: These data indicate that honey produced in Burkina Faso could be used to isolate *Bacillus* strains with genetic potential for broad-spectrum antimicrobial activity. This efficacy could be tested in future studies to confirm these findings.
Keywords: Antimicrobial peptides, AMP-genes, *Bacillus* spp., Burkina Faso, Honey microbiota.

INTRODUCTION

Bee-breeding, commonly found in all regions of the globe[1], is the science and practice of raising bees to exploit their products. It contributes to improving the incomes of populations in the Global South[2]. Beyond honey and its multiple virtues, the maintenance of biodiversity and the pollination of flowering plants are undoubtedly the most valuable ecosystem services provided by bees. Bees, therefore, have both ecological and economicstakes[3]. Although bee breeding contributes to fight against poverty and can help create income-generating jobs[4], it is often a secondary activity in Africa. Indeed, honey, which is a viscous sweetener produced by bees from nectar or secretion of floweringplants[5], is a highly valued commodity due to its medicinal and nutritional important values. Its major constituents include 80% of carbohydrates (35% glucose, 40% fructose and 5% sucrose), and water (20%)[6], while its minor components include minerals, proteins, vitamins, organic acids, phenolic compounds, enzymes (hydrolases and oxido-reductases) and otherphytochemicals[7].

The nutritional value of honey dependson the floral monofloral (Manuka, acacia, clover, lavender, and eucalyptus) or multifloral (a mix from different local wild flowers, herbs, and flowering bushes) sources, geographical origin, processing and storage conditions[8]. These factors determine its specific physicochemical composition and bioactive properties that are beneficial to humans[9]. Several studies reveal that honey display several therapeutic traits[10]. Furthermore, honey is an important substance in traditional African medicine[11] in both human and animal medicine.

Although considered as safe with respect to pathogenic microorganisms, it has been reported that yeasts, molds, and aerobic mesophilic bacteria can survive in its matrix[12]. Studies report that *Cladosporium*, *Aspergillus* spp., *Penicillium* spp., and *Bacillus* spp. are the main microbial groups found in honey. These microorganisms can originate from nectar, pollen, and other parts of the plant flower. Their presence in honey has been linked to the water content, which is likely to encourage the growth of existing microorganisms[12]. Recent studies reported the presence of a diversity of *Bacillus* species on flowers foraged by bees and even in the produced honey[13].

Furthermore, several studies have demonstrated that *Bacillus* species have attracted considerable interest due to their ability to produce cyclic lipopeptides and their antifungal properties[14]. Among these interesting molecules are surfactants, fengycins, and iturins. These lipopeptides act as potent biosurfactants known for their antifungal and phytoprotective properties[15].

In this respect this study was performed with the main objective to determine the diversity of antifungal peptides genes of the genus *Bacillus* spp. in honey produced in Hauts Bassins region.

MATERIALS AND METHODS

Sampling

Sampling was carried out in three provinces (Houet, Tuy, and Kenedougou) based in Hauts Bassins Region. These provinces have been reputed to be among the top five honey-producing provinces in Hauts Bassins Region of Burkina Faso. In detail, four samples were collected from Houet province, two samples from Kenedougou province, and four samples from Tuy province. A total of 10 honey samples were collected from producers in sterile jars and transported in a cooler container from sampling sites to the laboratory.

Isolation and purification of presumptive *Bacillus* spp.

Honey samples were treated according to the standard procedure[16]. For each sample, 10 g was taken and diluted into 90 mL of diluent and gently homogenized, then decimal dilutions were obtained. *Bacillus* spp. were isolated and purified according to method applied by Ouoba and co-workers in which 1 mL of each dilution was plated onto Luria Bertani agar medium (Powder, Liofilchemsrl, ITALY) and plates incubated at 37°C for 24 hours[17]. *B. cereus* ATCC 14579 was used in this study as control strain.

Preliminary characterization of isolates

The isolates were described for colony shape, size, color, consistency, and outline, and cultured cells were observed under the microscope (B-292, Optika, Italy) for cellmorphology and ability to Gram stain. Catalase test was performed following the conventional procedure, using a few drops of 3% (v/v) hydrogen peroxide. The sporulation test was carried out according to the method described by Gounina-Allouane and co-workers[18]. If visual growth was observed, the isolate was stained using methylene blue solution at the concentration of 0.5% at pH 12to Schaeffer Fulton method.

Salt tolerance assay

To determine the salt tolerance, the isolates were cultured on LB broth medium (Powder, Liofilchemsrl, Italy) supplemented with NaCl at different final concentrations (2.5, 5.0, and 10%), and the results were read by measuring bacterial growth after incubation at 37°C/ for 24 hours.

Detection and quantification of genes following the RT-PCR reaction

Fresh bacterial cultures were revived in nutrient broth for 18 hours. Then, 200 µL of chelex (Chelex 100, BIORAD, USA) was dispensed into Eppendorf tubes, and 1 µL of the fresh bacterial culture was added.The cultures were incubated at 56°C for 30 - 60 min. The mixture was vortexed for 10 s and returned to incubation at 90°C for 10 min. before being centrifuged at 13,000 rpm for 5 min. Once the extraction was completed, the reaction medium was prepared for the amplification[19].

Primer pairs (Table 1) targeting genes (16S RNA, *ituA*, *hag*, *tasA*, *srf*, *sps*, *mrsH*, *B. cereus*) previously described[13] as beneficial genes for the prevention and control of fungal contamination were used to characterize the 40 selected isolates. Two different amplification programs were performed (a 32-cycles program followed by a 30-cycles program) using a T100 PCR thermo cycler (My Cycler, Bio-Rad Laboratories).

Table 1: Primers sequences used for RT-PCR.

Primers sequences (5'→3')	Gene name	Gene size (pb)
AGAGTTTGATCATGGCTCAG ACGGTTACCTTGTTACGACTT	ARN 16S	1520
ATGAAAATTTACGGAGTATATATG TTATAACAGCTCTTCATACGTT	*itu*A	743
ATGAGAATCAACCACAATATCGC TTAACCTTTAAGCAATTGAAGAAC	*hag*	700
ATGGGTATGAAAAAGAAATTAAG TTAGTTTTTATCCTCACTGTGA	*tas*A	719
ATGAAGATTTACGGAATTTATATG TTATAAAAGCTCTTCGTACGAG	*srf*	743
ATGTCAAAGTTCGATGATTTCACA TTATTTAGAGATTTTGCAGTTACA	*sps*	707
ATGAGTCAAGAAGCTATCATTCG TTAACAAATACATTCAGAAGTTAG	*mrs*H	707
GCGTTTGTGCTGGTGTAAGT	*B. cereus*	621

For amplification, PCR was carried out following a 32-cycles program including one cycle of initial DNA denaturation at 95°C for 10 min followed by a set of 30 cycles program with denaturation for 40s at 95°C, hybridization at 59°C for 40s and elongation for 2 min at 72°C. Then a final elongation (1 cycle) war performed for 6 min at 72°C. The PCR products were run on a 2% agarose gel at 120 volts for 1 hour and visualized on a transilluminator (TM-20 UV, UVP, France). The 100 bp DNA Ladder ready to load (Solis Biodyne, Estonia) was used as a control size.

Data analysis

The results were analyzed using R software version 4.3.2. A multivariate analysis (based on potential differences and associations between the amplified gene fragments) and hierarchical visualization were performed using several packages. The *readxl* package was used to import the raw data from an Excel file, while the *dendextend* package allowed for the manipulation and customization of the dendrograms. Initially, the data were loaded directly from the Excel file containing all the observations. Hierarchical clustering was performed using Ward D2 method based on the size of the amplified fragments associated to the targeted genes.

RESULTS

Isolation and phenotypic characterization of bacterial isolates

A total of 40 isolates belonging to the *Bacillus* genus were obtained. Identification was based on macro-scopic, microscopic, physiological, and molecular characteristics. Macroscopic observation of the bacterial colonies, after 24 hours, revealed two colony types: whitish, creamy colonies with regular outlines and large, sticky, rounded colonies. The isolates exhibited Gram-positive bacillus-shaped cells grouped in chains. All isolates were positive for catalase and able to grow after the heat shock. Results on tolerance to NaCl revealed that all isolates were able to grow in NaCl up to a concentration of 10%.

Amplification of the 16S gene region and genes involved in the synthesis of peptides with antimicrobial peptide

The identification based on the 16S used primer confirmed that all isolates belong to *Bacillus* genus. Isolated strains can be divided into four (04) classes concerning the presence of genes involved in the production of antimicrobial peptides. Cluster 1 includes 28 strains. This group, within which *B. cereus* is strongly represented, is characterized by strains presenting *itu*A, *tas*A, *Srf*, and *hag*genes (from the most extreme to the least extreme). Cluster 2 included strains positive for the presence of *tas*A, *Srf*, and *itu*A genes. Cluster 3 is composed of strains characterized by high*itu*A peptide synthesis values. Cluster 4 is composed of strains characterized by the ability to synthesize antimicrobial peptides, such as *tas*A, *Hag*, *itu*A, and *Srf*.

The search for genes (Table 3) responsible for the synthesis of bioactive microbial secondary metabolites was identified in our strains. However, a negative PCR result for *spa*S was obtained in almost all strains. Twelve out of the 40 strains (BH1.0, BH1.1, BH1.2, BH2.1, HT3.0, HT3.1, HT3.2, BT7.0, OK9.0, OK10.0, KK11.2 and KK12.2) did not show antifungal genes.

DISCUSSION

The genus *Bacillus* spp., a sporulating, Gram-positive bacterium was detected in honey accordingly to previous findings. Moreover, as already reported, the *Bacillus* isolated from honey in Asia has been described as able to secrete various peptides and bacteriocins with antifungal activities[20]. *Bacillus* spp. devote a large portion of their genome to secondary metabolism, as an adaptive response to environmental competition. *Bacillus* spores have been detected in soil and the ocean, where complex microbial communities have been described[21,22]. By producing secondary metabolites that can inhibit closely related species and other microorganisms in the ecological niche, *Bacillus* spp have gained significant survival advantages.

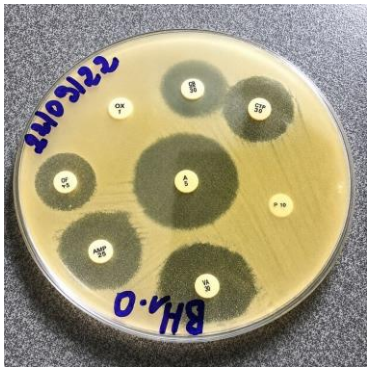

Figure 1: Antibiogram of *B. cereus* against the tested antibiotics.

Table 2: Antibiotics used for *B. cereus* isolates.

Antibiotics	Family	Code	Disc concentration (µg)	Critical diameter (mm)	
				S ≥	R<
Penicillin G	Béta-lactamine	P	10	21	14
Oxacilline		Ox	1	22	17
Ciprofloxacine	Quinolones	CIP	10	21	21
Erythromycine	Macrolides	E	5	21	18
Vancomycine		VA	30	16	16
Gentamicine	Aminosides	CN	10	18	18
Amikacine		AK	30	22	22
Trimethoprime sulfamehoxazole		SXT	25	17	14

Table 3: Distribution of antimicrobial genes among *Bacillus* isolates.

Isolates	*B. cereus*	*Bacillus* sp.	Detected genes					
			ituA	*hag*	*tasA*	*srf*	*spS*	*mrsH*
BH1.0, BH1.1, BH1.2, BH2.1, HT3.0, HT3.1, HT3.2, BT7.0,OK9.0, OK10.0, KK11.2, KK12.2	+	-	-	-	-	-	-	-
BH2.0	+	-	+	+	+	-	-	-
BH2.2	+	-	-	-	-	+	-	-
HT4.0	-	+	+	+	-	+	-	-
HT4.1	-	+	+	+	+	+	-	+
HT4.2	-	+	+	+	+	-	-	-
HT5.0, KK11.3	+	-	-	-	+	+	-	-
HT5.1	-	+	+	+	-	-	-	-
HT5.2	-	+	+	+	+	+	-	-
BT6.0, BT8.2, BT8.3	+	-	-	-	+	-	-	-
BT6.1	+	-	-	+	-	-	-	-
BT6.2	-	+	-	-	-	+	-	-
BT7.1, OK9.1	+	-	+	-	-	-	-	-
BT7.2	-	+	+	-	-	-	-	-
BT8.0	+	-	-	+	-	-	-	+
BT8.1	+	-	-	+	+	-	-	-
OK9.2, KK12.0	-	+	-	-	-	-	-	+
OK9.3, KK11.1	-	+	+	+	-	-	-	-
OK10.1	-	+	-	+	+	+	-	-
OK10.2, KK12.1	+	-	-	-	-	-	-	+
OK10.3	-	+	+	+	+	-	-	+
KK11.0	-	+	-	-	-	-	-	-

In the present study, 6 pairs carrying genes, such as *itu*A (IturinA), *hag* (Flagellin), *tas*A (TasA), *srf* (Surfactin), *spa*S (Subtilin) and *mrs*A (Mersacidin), which are related to the production of antifungal peptides, were investigated. Our results are consistent with those of previous studies[23] who identified *tas*A, *itu*A and *hag* simultaneously in the same strain of *Bacillus* sp isolated from honey and demonstrated that these genes conferred properties to control certain fungal species such as *Fusarium graminearum*, *Rhizoctonia solani*, and *Botrytis cinerea*. The association of multiple AMPs exhibit synergistic effects, particularly among lipopeptides such as surfactin, iturin, and fengycin, which collectively enhance antimicrobial potency beyond the activity of individual molecules. This genomic organization reflects the high plasticity of *Bacillus* genomes, in which horizontal gene transfer and the acquisition of biosynthetic gene clusters contribute to the accumulation of multiple AMP determinants.

Strains possessing multiple AMP genes are therefore strong candidates for biotechnological applications.

According to Zhou and co-workers, the first three antifungal peptides have their own characteristics and antifungal spectrum[23]. The iturin family is a cyclic lipopeptide type containing 7 amino acids, with strong antifungal activity. Iturin's antifungal mechanism places its hydrophobic tail into the plasma membrane of indicator cells and automatically assembles to form an ion channel, thus allowing leakage from the cytoplasm. Additionally, iturin A can release electrolytes and polymer aggregates, increase the electrical conductivity and permeability of the biomass membrane, resulting in surface tension effects on the cell membrane, inhibiting pathogen formation[24].

Table 4: Susceptibility of *B. cereus* strains to tested antibiotics.

Strains	Antibiotics							
	P	OX	CN	VA	SXT	E	AK	CIP
BH1.0, BT8.1	R	R	S	R	R	S	S	S
BH1.1	S	R	S	R	S	R	S	S
BH1.2	R	R	S	S	S	R	S	S
BH2.0, BH2.1	R	R	S	S	S	S	S	S
BH2.2	S	R	S	S	R	S	S	S
HT3.0, OK10.0	R	R	R	S	R	S	R	S
HT3.1	R	R	R	S	S	S	R	S
HT3.2, HT5.0, BT8.2	R	R	S	S	R	S	R	S
BT6.0, OK9.1	R	R	S	S	R	R	R	S
BT6.1, BT8.0	R	R	S	R	R	R	R	S
BT7.0, BT7.1	R	R	S	S	R	R	R	S
BT8.3, OK9.0, KK11.3	R	R	S	S	S	S	R	S
OK10.2, KK12.2	R	R	S	S	R	S	S	S
KK11.2	S	R	S	S	R	S	R	S
KK12.1	S	R	R	S	S	S	S	R

These lipopeptides can modify biofilm formation, motility, and virulence gene expression and one of them (TasA) is involved in sporulation and biofilm formation in *Bacillus*[25].

CONCLUSION

The objectives that prompted the present study can be considered achieved. First, the microbiological analysis of the honey revealed the presence of *Bacillus*. Honey produced in Burkina Faso is a valuable source of interesting bacteria with applications in agronomy and the food industry, where an increasing interest in natural, safe, new, and effective solutions against the harmful effects of global warming has been detected.

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AUTHOR'S CONTRIBUTIONS

Kouhounde SHS: formal analysis, conceptualization, writing original draft. **Kpode UD:** formal analysis, critical review. **Bonane H:** conceptualisation. **Yaovi RC:** critical review, formal analysis. **Adeoti K:** data organization. **Dicko MH:** critical review. Final manuscript was checked and approved by all authors.

DATA AVAILABILITY

The related author can provide the empirical data supporting the study's conclusions upon request.

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CONFLICT OF INTEREST

There are no conflicts of interest in regard to this project.

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