

Cigarette butts, emerging residue on beaches and potential niche of microbiological resources

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Abstract– *Cigarette butts are emerging residues on tourist beaches worldwide, reaching the shores through direct disposal or various transport mechanisms. They persist in marine ecosystems due to their low degradability, becoming potential microbiological niches with the potential to impact health and ecosystems. A pilot study at Bocagrande beach in Cartagena aimed to assess cigarette butts as bacterial habitats. Four samplings were conducted between June and November 2022. Three bacterial isolation stages were employed: 1) Seawater modified LB agar (LB NaCl), 2) morphological and biochemical characterization using Gram staining, catalase, oxidase, and BBL Crystal tests, and 3) 16S rRNA gene sequencing for phylogenetic analysis. Results revealed strains related to *Virgibacillus pantothenicus* and *Virgibacillus dokdonensis*, not previously reported in Cartagena. This Gram-positive bacterium, forming endospores under stress, raises potential concerns for health and the environment. In summary, cigarette butts on Colombian Caribbean beaches serve as bacterial niches, suggesting an assessment of the found bacteria and their potential in bioengineering treatments.*

Keywords– beach sand, bacteria, cigarette butts, emerging pollutants, molecular diagnosis

I. INTRODUCTION

Cigarette butts (CB) are composed of plastic cellulose acetate fibers, and approximately 15,000 strands of fibers are used in the manufacturing process. When discarded in natural environments, regular consumers often believe they degrade easily. However, the degradation times of CB in different natural environments, their level of hazard, and their relationship with microplastics generated from CB are still under study. These microplastics may contain toxic substances derived from the original product [1].

In accordance with the literature, approximately 4.5 trillion cigarette butts (CBs) are annually discarded into the environment [2]. Due to their persistence in the environment and the presence of potentially released polycyclic aromatic hydrocarbons (PAHs), CBs have become a highly relevant.

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CBs discarded in aquatic environments, reducing their

abundance should involve controlling their disposal through mechanisms that include legislative policies and public education, utilizing media and school capacities [3].

Despite the seemingly inhospitable conditions of beach sand environments (temperature, waves, humidity, foot traffic, among others), beach sand serves as a habitat that sustains a myriad of microbes, including viruses, bacteria, fungi, and protozoa [4]. The surface of plastics abandoned in marine environments provides an ideal setting for opportunistic microbial colonizers to form biofilms and may offer a protective niche capable of supporting a diversity of different microorganisms, known as the "Plastisphere." This biotope could act as a significant vector for the persistence and spread of pathogens, fecal indicator organisms (FIOs), and harmful algal bloom species (HABs) in beach and bathing environments [5].

It is crucial to conduct more research on topics related to high-generation waste in coastal marine ecosystems. Cigarette butts are considered highly generated personal waste, with an estimated disposal of around 4.95 trillion butts in various environments, including ecosystems and anthropogenic settings. For instance, studies such as the one conducted on eight beaches in Northeast Brazil indicate that approximately 38.36% of the collected waste consists of cigarette butts [6]. Authors like [7] assert that paper and tobacco remnants decompose more rapidly than the cellulose acetate comprising non-linear decomposition filters. Consequently, the toxic substances found in them can interact with the biota for years.

In the study conducted on the pilot beach, the aim is to identify the potential for bacterial growth in cigarette butt residues, which slowly decompose over time into cellulose acetate fibers that remain in contact with the sand and serve as potential niches for microorganisms. The goal is to identify bacterial species whose effects are related to studies that have determined the harmful effects of microfiber pollution on the environment and human health [8]. Additionally, the study seeks to evaluate, through scientific literature, potential microbial remediation mechanisms for contaminants using treatments that include the isolated bacterial species.

Microbial biomineralization is a process involving the formation of biominerals. Heavy metals with ionic radii

similar to calcium, such as Cu^{2+} , Cd^{2+} , and Zn^{2+} , can be incorporated into the CaCO_3 crystal lattice by substituting calcium ions, thereby preventing the movement of these heavy metals in contaminated soils [9]. Extracellular polymeric substances (EPS) are metabolic products produced by microbial cells. The use of microbial biofilms for heavy metal absorption through EPS secretion has been employed in several soil bioremediation processes with an innovative approach [10].

II. MATERIALS AND METHODS

A. Sampling

Figure 1 shows the sampling area, where 29 composite samples (sand, cigarette butts and fibers) were randomly collected between June and November 2022 at the Bocagrande pilot beach located in Cartagena de Indias, Colombian Caribbean ($75^\circ 33' 42.0'' \text{ W } 10^\circ 28' 56.7'' \text{ NORTH}$).



Figure 1. Location of study beach Cartagena (CO)

The samples were collected on the sand surface in transects of 500 m^2 , in accordance with the usage zones established in the Colombian Sector Technical Standard NTS-TS 001-2, sustainability requirements for beach tourism destinations, which include active, resting, and service zones. The active zone is defined as the area around the waterline, considered as a transition area between the subaerial beach and the water. The resting zone is where most beach users are found, typically with furnishings such as umbrellas, tents, and tables. The service zone encompasses the landward part of the beach and is the area where facilities and services for beach

users, such as showers, dressing rooms, and bars, are located [11].

The collected samples were placed in sterile Ziploc bags, refrigerated at 4 degrees Celsius, and analyzed in the laboratory.

B. Identification of collected material

The methodology used for bacterial identification included three stages, i. Isolation of bacteria: for which broth and LB NaCl agar were used; as reported by Acevedo-Barrios et al. [12], [13], [14], [15]. ii. Phenotypic bacterial identification is mainly based on comparing the characteristics of unknown bacteria with reference cultures, using simple and rapid tests such as Gram staining, growth under different incubation conditions, morphology, enzyme activities like catalase and oxidase, the BBL Crystal test, oxidation-fermentation, spore production, and growth under aerobic conditions. These tests allow for the identification of shape, presence or absence of capsule, endospores, sizes, edges, ends, arrangement, and irregular forms. iii. For the phylogenetic analyses based on our 16S rRNA gene sequence for the genus *Virgibacillus*, we searched 16S rRNA records in the RefSeq database-NCBI. The evolutionary history was deduced through phylogenetic reconstruction of the 16S rDNA gene using the Neighbor-Joining method. The bootstrap test (with 10,000 replicates) determined the percentage of replicate trees where the associated taxa clustered together. Evolutionary distances were calculated using the Kimura 2-parameter method. The final dataset comprised a total of 1477 positions. The evolutionary analyses were carried out using MEGA X [16] [17].

III. RESULTS

Gram staining was performed to identify bacterial growth using LB Agar modified with seawater and fluconazole to inhibit fungal growth, as the goal was to identify bacteria. The samples were incubated at a temperature of 35 degrees Celsius for a growth period of 24 hours. It was found that only 6.9% of bacterial growth corresponded to Gram-negative bacteria, identified in the Sand and Fibers in the resting zone, while 93.1% of the identified samples corresponded to Gram-positive bacteria (in the Figure 2).

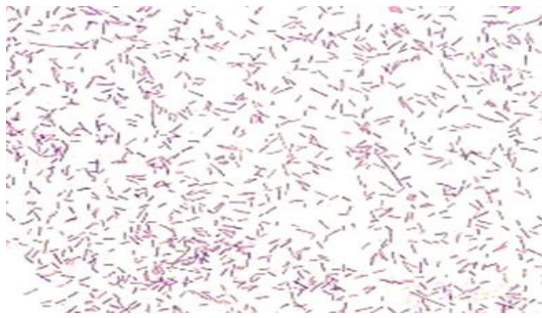


Figure 2. Gram-negative and Gram-positive bacteria identified.

The obtained result with the report of Gram-negative bacteria in the resting area is of interest, where beach users spend much more time sitting or in direct contact with the sand and many of the CB residues found in the sand. This becomes more significant when considering that many Gram-negative bacteria have been reclassified by the World Health Organization (WHO) as critically important for human medicine, as some severe infections caused by Gram-negative bacteria are becoming an increasingly challenging clinical issue [18].

The application of catalase and oxidase tests on samples identified as Gram-negative resulted in a positive catalase outcome and a negative oxidase outcome. Both samples displayed bacillus morphology, suggesting a potential association with the presence of enteric bacteria, which may be linked to the existence of animals like dogs or pigeons. The literature reports an increase in the contamination of beach sand and seawater due to improper disposal of solid waste, discharge of untreated domestic sewage, and animal waste [19].

Samples identified as Gram-positive are morphologically described as long, thin bacilli, arranged in clusters and diplobacilli, non-sporulating. The results of the tests conducted show positive catalase and negative oxidase, which is an indicator of belonging to the same genus.

Molecular tests were conducted on two samples of Gram-positive bacteria (in the Figure 3) isolated from cigarette butts and sand retrieved from the active and service zones (Molecular analyses were conducted through the provision of services at CorpoGen Research and Technology). For these samples, molecular identification was carried out using isolation and purification of DNA, amplification of the 16S ribosomal gene using Polymerase Chain Reaction (PCR) technique, purification of PCR fragments and sequencing using the Sanger method, employing the 337F, 518F, 800R, and 1100R primers of the 16S ribosomal gene. Cleaning and assembling of sequences to obtain the query sequence.



Figure 3. Gram-positive bacteria identified.

Taxonomic analysis of the query sequence using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI), comparing against the reference RNA database "refseq_rna." Taxonomic analysis of the query sequence using the "Classifier" and "SeqMatch" tools, hosted on the Ribosomal Data Project (RDP) website.

The first tool is used to determine the taxonomy of the query sequence, while the second tool is utilized to identify the most similar sequences in the RDP database to the query sequence. Both tools perform the comparison using the RDP's own "16S rRNA training set 18" database.

The results of the molecular identification of the studied bacteria are shown in Table 1 and Figure 4.

Table 1. molecular identification gram-positive bacteria

Identifier Sample		Composite (CB and Sand) Active Zone	Composite (CB and Sand) Service Zone
Bacteria		Gram-positive	Gram-positive
Length of the assembled sequence		1538 pb	1500 pb
Results RefSeq NCBI	Microorganism	<i>V. dokdonensis</i>	<i>V. pantothenicus</i>
		<i>V. pantothenicus</i>	<i>V. dokdonensis</i>
		<i>V. chiguensis</i>	<i>V. chiguensis</i>
	% Identity	99	98
% Coverage	97	98	
Results leBIBI		<i>V. dokdonensis</i>	<i>V. pantothenicus</i>
Conclusion	Genus	<i>Virgibacillus</i>	<i>Virgibacillus</i>
	Species	<i>dokdonensis</i>	* likely that the sample belongs to the species <i>pantothenicus</i>

* The comparison with the RDP 16S sequence database using the SeqMatch tool against cultivated isolates indicates that the assembled query sequence shows a higher homology with sequences of the species *Virgibacillus pantothenicus*.

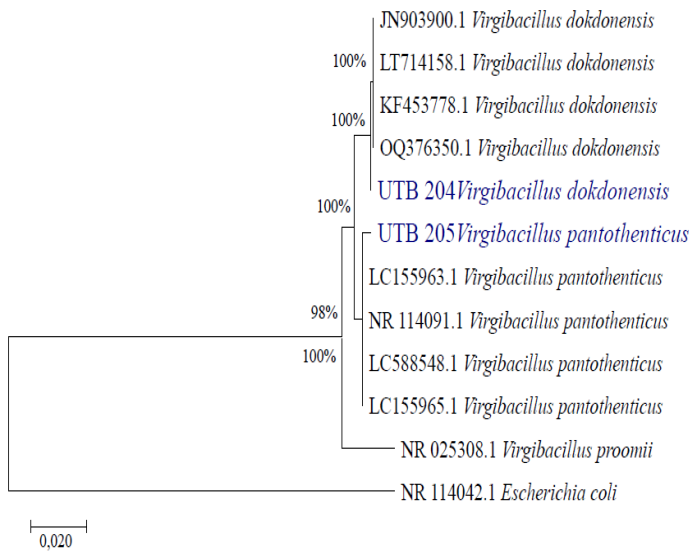


Figure 4. Taxonomic identity of the *Virgibacillus dokdonensis* (UTB 204) and *Virgibacillus pantotheticus* (UTB 205) strains was determined by reconstructing the phylogeny of the 16S rDNA gene using the Neighbor-Joining method.

The identified bacteria belong to the genus *Virgibacillus*, with the species *dokdonensis* identified in the active zone in the composite sample of CB and sand. The second sample, identified in the service zone, is affiliated with the species *pantotheticus* [20] [21]. In cigarette butts and beach sand, this bacterium is reported for the first time in Cartagena, Colombia, to the extent reviewed in the literature.

The significance of reporting the bacterium of the *Virgibacillus* genus is emphasized, which has been documented in halophilic environments but had not been reported as isolated in cigarette butt (CB) waste. It is recommended to further investigate to establish the growth mechanisms at different stages of CB decomposition.

IV. DISCUSSION

The genus *Virgibacillus*, often identified as a bacterium involved in paint deterioration [22], belongs to the Bacillaceae family and Firmicutes phylum, exhibiting aerobic or facultatively anaerobic characteristics and catalase positivity [23]. It has been identified in various habitats, with its species mostly isolated in environments with high salinity. This is consistent with the conditions provided by cigarette butts, primarily composed of cellulose acetate and discarded in the beach sand under study. In environments with humidity and salinity, these cigarette butts constitute a suitable habitat for the growth of *Virgibacillus* bacteria.

According to the bacterial isolations in this study, various microorganisms can be found in marine environments. Gram-

positive bacteria were isolated from fish and marine environments in coastal areas near Australia, with *V. pantotheticus* showing a prevalence of 17% within the identified 16% of bacteria [24]. In Brazil, *V. pantotheticus* bacteria were isolated and characterized from *Haliclona sp.* sponges, and this isolation was identified as having the potential to inhibit the growth of fouling bacteria in natural environments[25]. The above describes the presence of the *Virgibacillus* genus in coastal natural environments. However, there is no reference to the presence of other types of waste, such as plastic, in the sand where these microorganisms may proliferate.

Scientific research has been conducted to characterize halophilic bacteria in saline ecosystems, as demonstrated by a study conducted in arid and semi-arid ecoclimates in northeastern Algeria. In this analysis of 74 bacterial isolates, various genera were identified, including *Virgibacillus*, which exhibited notable antifungal activity against pathogens such as *Botrytis cinerea*, *Fusarium oxysporum*, *F. verticillioides*, and *Phytophthora capsici*. This study on halophilic bacteria in unexplored saline environments provides potential sources of biocatalysts and new bioactive metabolites. Additionally, it presents promising candidates as biocontrol agents and environmentally friendly tools for the bioremediation of heavy metals [26].

It is crucial to highlight the relevance of the isolation carried out in the present study of cigarette butts discarded on the beach sand, as the identification of the bacterium *V. dokdonensis* constitutes a discovery of interest. This becomes significant when considering research on the potential for contaminant biodegradation by certain taxa in the marine environment. This aspect is clearly illustrated in the study conducted in three regions of the Sea of Japan, where 137 strains of mesophilic and hydrocarbon-oxidizing bacteria were isolated, exposed to different levels of anthropogenic pollution. Among the taxonomic affiliations of cultivable bacteria, *V. dokdonensis* was identified, emphasizing its involvement in the biodegradation of petroleum hydrocarbons in those regions [27].

The analysis of the Molecular Characterization and Biocompatibility of the Exopolysaccharide Produced from *V. dokdonensis* isolated from the Kumta Coast Saltern demonstrates how these halophilic bacteria are recognized as innovative sources with diverse biological activities. It has been observed that the exopolysaccharide obtained from *V. dokdonensis* is biocompatible and meets the growing demand for natural polysaccharides in various biotechnological applications, especially in the formulation of biomaterials [28].

Furthermore, research has been conducted related to the formation of calcium carbonate and calcium sulfate with bacteria isolated from seawater and brine from a desalination

plant. Bioprecipitation was observed when the bacteria were cultivated in media containing organic matter. Among the identified colonies with the ability to precipitate carbonate, Gram-positive genera *Bacillus* and *Virgibacillus* were found, demonstrating their capability to precipitate carbonate biominerals. Additionally, it is noteworthy that the ability of this microbiota to precipitate calcium carbonate could be crucial in the bioremediation of CO₂ and calcium in specific environments[29]. Other species of the genus *Virgibacillus* have been investigated in processes for removing Ca²⁺ ions from water at different NaCl concentrations through biomineralization. These studies are important due to the dangers of Ca²⁺ in industrial processes and human health [30].

There are recent studies, such as the one conducted with strains of *Virgibacillus* isolated from the sabkhas of Qatar, where microbial biomineralization has emerged as a promising approach for the bio-removal of heavy metals through immobilization. This process is facilitated by extracellular polymeric substances (EPS) [10]. These studies reaffirm the need to continue research to find ways to harness microorganisms that can be isolated from different sources, such as CB. At the same time, there is a promotion to avoid the disposal of these residues in coastal marine ecosystems, which represent a potential source of contamination.

V. CONCLUSIONS

The microbiological study of composite samples of cigarette butts and sand reported the presence of Gram-negative bacteria at 6.9% located in the beach resting area, and 93.1% of the identified samples correspond to Gram-positive bacteria found in all beach zones.

Molecular identification of Gram-Positive samples resulted in bacteria of the genus *Virgibacillus*, identified for the first time in samples related to cigarette butts in Cartagena, Colombia. Further research is recommended to explore the potential of these isolated bacteria in bioremediation processes, as the literature has described their potential in various biotechnological applications. A challenge for future research is the identification of fungal growth on cigarette butts, associated with the assessment of the risk of dermal contact for beach users.

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