



Microbial Characterization of Chocolates of Three Popular Commercial Brands: A Novel and Complete Profiling of Notorious Bacterial and Fungal Contaminants

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Abstract

Our study questioned the assumption that cocoa and low water activity limit contamination in Indian chocolate bars (C1, C2, C3). Each chocolate bar was tested as intact refrigerated (C1 Intact ref, C2 Intact ref, C3 Intact ref), half-eaten refrigerated (C1Href, C2Href, C3Href), or half-eaten at room temperature (C1HRT, C2HRT, C3HRT). After five days, isolation and characterization of notorious food-borne bacterial and fungal pathogens were carried out on them. Tests showed the presence of colonies from C3HRT on Eosin Methylene Blue (EMB) agar plates, typical of *Enterobacter* species, a common food contaminant. Preliminary identification of species of *Staphylococcus* was made from greyish black to completely black colonies with prominent halos on Baird Parker Agar (BPA) plates; *Pseudomonas* was made from green colonies on Hektoen Enteric Agar (HEA) plates; and *Citrobacter* from bright-orange (salmon) colonies on HEA plates. Growth in glucose-free nutrient broth (GFNB) tubes, followed by staining to reveal endospores, indicated the presence of *Bacillus* species. Potentially harmful *Enterobacter*, *Pseudomonas*, *Alcaligenes*, *Bacillus*, and *Staphylococcus* (like *S. aureus* and *S. epidermidis*) were finally confirmed by characteristic biochemical tests. Besides, highly menacing food-borne fungal pathogens, including species of *Rhizoctonia*, *Aspergillus* and *Alternaria*, were also isolated on Yeast Extract Peptone Dextrose Agar (YPDA) plates and presumptively identified microscopically and confirmed using ITS based molecular method sequencing. Antibiotic susceptibility tests (ASTs) of the confirmed bacterial pathogens recorded the highest (80%) resistance against amoxicillin (AMX). Thus, our key findings of high microbial activity and the presence of some of the deadliest food-borne pathogens, including some drug-resistant ones, challenge the long-nurtured notion stated earlier and establish the fact that the overall microbiological quality of the chocolates depends much on their mode of storage.

Keywords: Antibiotic Susceptibility Test; Chocolates; Food-Borne Bacterial And Fungal Pathogens; MPN Index; Total Heterotrophic Count

Introduction

Chocolates, a universally cherished confectionery, are typically composed of roasted and ground cocoa beans, taking various forms from liquid to solid to paste [1]. For a long time, the confectionery industry has largely held the belief that chocolates, especially

those with high cocoa content and inherently low water activity (a_w), are naturally protected against microbial contamination. Consequently, white chocolates, with their lower cocoa content, have been presumed to be most susceptible to microbial spoilage, succeeded by milk chocolate and finally, dark chocolates. However,

this long-standing assumption is now being re-evaluated. This article challenges the notion of chocolates' inherent microbiological safety and investigates how different storage conditions can impact their microbial profile, ultimately aiming to prevent adverse consequences for consumers.

In this pioneering research, chocolate bars from three prominent multinational brands, categorized by their cocoa content (dark, milk, and white chocolate), procured from different stores across Kolkata, India (22.5744° N, 88.3629° E), were carefully selected for analysis. The choice of well-known brands adds a layer of relevance, as these products are widely consumed, making any findings directly impactful on public health. The distinct compositions of each chocolate type were meticulously noted: dark chocolate, primarily cocoa solids, sugar, cocoa butter, emulsifiers, and artificial flavors (cocoa and vanilla); milk chocolate, comprising sugar, cocoa butter, milk solids, cocoa solids, and emulsifiers; and white chocolate, made of skimmed milk powder, sugar, and cocoa butter. A critical aspect of this study's design was the understanding that none of these chocolate formulations contain inherent microbicidal ingredients. Instead, many of their components, such as sugar, milk solids, and cocoa butter, can serve as readily available nutrient sources for a range of bacterial and fungal pathogens. This fundamental insight emphasizes the potential vulnerability of chocolates to microbial growth, even in seemingly inhospitable environments.

The sample preparation methodology was designed to simulate realistic consumer handling and storage scenarios while allowing for controlled experimental conditions. For each chocolate type, one bar was refrigerated, another was refrigerated after being partially broken, and the third was kept at room temperature after being partially broken. All samples were maintained for a uniform duration of four days. This innovative setup was crucial for several reasons - (a) By exposing the chocolates to different temperature conditions, the experiment aimed to comprehensively assess their baseline sanitary quality concerning pathogenic microbe load, and (b) the varied storage conditions (refrigerated versus room temperature, intact versus broken) allowed for a direct investigation into how different procedures influence microbial contamination and subsequent proliferation. The act of breaking a chocolate bar introduces potential points of entry for environmental microbes, mimicking common consumer behavior and testing the robustness of the product's microbial defenses beyond its intact packaging.

While the idea of chocolates being microbiologically safe has been prevalent, previous research on other low-water activity foods has shown that they are not entirely immune to microbial contamination. Studies on products like dried fruits, spices, and nuts have demonstrated the survival and even growth of certain desiccation-tolerant microorganisms, including specific bacterial spores and molds, under seemingly adverse conditions. Furthermore, cross-contamination during processing, packaging, or handling has been identified as a significant route for microbial introduction in various food matrices. Although direct comprehensive studies specifically challenging the microbiological safety of a wide range of chocolate types under varying storage conditions have been limited, these analogous findings from other food systems laid the groundwork for questioning the absolute safety of chocolates. They highlighted the importance of considering not only the intrinsic properties of the food but also extrinsic factors such as environmental exposure and post-production handling. Moreover, acknowledging that prior microbiological studies on chocolate have predominantly focused on temperate regions, this research specifically sought to explore the microbial profile of chocolates in tropical climates. This geographical focus is critical, as tropical conditions, characterized by elevated temperatures and high humidity, present a distinct environment that can significantly influence microbial growth and persistence in food, thereby potentially altering the microbiological landscape of chocolate compared to those observed in cooler, drier environments.

This novel research is a very first of its kind for several compelling reasons. Unlike previous generalized assumptions or fragmented observations, this study provides a systematic and comparative analysis of the microbiological profile of milk, dark and white chocolates across diverse as well as practical storage conditions. This integrated approach of examining bacterial and fungal pathogens, coupled with detailed identification and characterization, fills a critical knowledge gap in food safety.

The methodology employed is scientifically robust. It not only determined the total heterotrophic count (THC) of bacteria and fungi (in terms of colony-forming units (CFU)/mL), but also ensured the isolation, identification, and characterization of pathogens. The specific focus on common food-borne pathogens such as coliforms and species of *Staphylococcus* (for example, *S. aureus* and *S. epidermidis*), *Salmonella*, *Shigella*, and *Bacillus* is highly

relevant given their potential for causing severe human illness. The use of specialized pre-enrichment and selective media, along with characteristic biochemical tests for bacterial confirmation, ensures the accuracy and reliability of pathogen detection. Beyond bacteria, the isolation and identification of extremely threatening food-borne fungal pathogens, including species of *Alternaria*, *Rhizoctonia* and *Aspergillus* using Yeast Extract Peptone Dextrose Agar (YPDA) and Peptone Dextrose Agar (PDA) plates and subsequent ITS-based molecular method sequencing, further elevates the comprehensiveness of this study. Fungal contamination, often overlooked in chocolate safety assessments, can lead to not only spoilage but also the production of harmful mycotoxins.

Ultimately, in the current era of pathogens which are exhibiting an escalation of multi drug resistance, the inclusion of antibiogram analysis to ascertain the antibiotic resistance/sensitivity of the pathogenic bacterial isolates is of paramount importance.

This research's findings very importantly significantly contribute to the existing body of knowledge in food system research related to cocoa. It directly challenges long-held assumptions about the microbiological safety of chocolates, prompting a re-evaluation of food safety protocols in the confectionery industry. The insights gained into the impact of different storage procedures on microbial contamination will be invaluable for developing more precise and effective recommendations for consumers and industry alike. By identifying specific pathogens and their susceptibility profiles, this study will enable more accurate risk assessments for chocolate consumption, leading to better preventative measures. Ultimately, by ensuring the microbiological integrity of chocolates, this research aims to protect consumers from unforeseen consequences associated with contaminated products, thereby enhancing overall public health.

Materials and Methods

Sample collection

27 chocolate bars, 9 each from 3 different popular brands of India, namely white chocolate (C1), milk chocolate (C2), and dark chocolate (C3), with increasing amounts of cocoa, were purchased from different local confectionary shops in Kolkata, and transported carefully to the laboratory for analysis within 2 hours of collection [2].

Sample preparation

From each of these 3 categories (C1-C3), 9 sub-samples were refrigerated as intact chocolate bars labelled as C1 Intact ref, C2 Intact ref, and C3 Intact ref (3 of each), 9 sub-samples were refrigerated as half-broken chocolate bars labelled as C1Href, C2Href and C3Href (3 of each), whereas the remaining 9 sub-samples were kept as half-broken chocolate bars at room temperature (controlled at 25°C) labelled as C1HRT, C2HRT and C3HRT (3 of each). The refrigerators were sanitized completely using 99.9% ethanol (EMSURE® 100983) and acetone (10 mg/ml) (HIMEDIA MB179), followed by simultaneous isolation and characterization of bacteria and fungi following established protocols. After 96 hours, isolation and characterization of notorious food-borne bacterial and fungal pathogens were carried out from these 27 sub-samples. For this, each sub-sample was pulverized, and 5 gm of this was then individually mixed well with 50 ml of sterile saline [0.9% NaCl (HIMEDIA MB023)] to prepare a stock solution of concentration 0.1 gm/ml, put in a rotary shaker at room temperature for 2 hours for maximum possible homogenization, and then refrigerated (at 4°C) overnight for the settling down of larger particles and the separation of a comparatively clearer layer at the top, with which further experiments were performed [2].

Total heterotrophic count

Total bacterial count

100 µl clear aliquot of the saline suspension prepared earlier was spread onto solidified Nutrient Agar (NA) (HIMEDIA M001) plates, and incubated overnight at 37°C, following which the total heterotrophic count (THC) of bacteria in terms of CFU/ml was estimated [2].

Total fungal count

100 µl clear aliquot of the saline suspension prepared earlier was spread onto solidified Yeast Extract Peptone Dextrose agar (YPDA) (HIMEDIA M757) plates containing L-asparagine (2% w/v) (HIMEDIA TC057), amoxicillin (60 µg/ml) (SIGMA - ALDRICH A8523), and tetracycline (20 µg/ml) (SIGMA - ALDRICH T8032), and incubated for 72 hours at 30°C, following which the THC of fungi in terms of CFU/ml was estimated [2].

Isolation of bacterial pathogens

Isolation of coliforms

- **Presumptive Test and MPN Index Determination:** To detect coliform bacteria by the standard Presumptive test, for each sub-sample, 1 tube containing 10 ml sub-sample, 10 ml 2X Lactose Broth (LB) (HIMEDIA M1003S) and Durham's Tube, and 2 tubes, one containing 1 ml sub-sample, 9 ml 1X LB and Durham's Tube, and another containing 0.1 ml sub-sample, 9.9 ml 1X LB and Durham's Tube, were incubated in a rotary shaker overnight at 37°C in order to check for turbidity, acid, and gas production, from which the MPN count/100 ml (MPN Index) was ascertained [3].
- **Confirmed Test:** From the 2X 10 ml sub-samples that tested positive, a loopful of the culture was streaked onto solidified selective and differential Eosin Methylene Blue (EMB) (HIMEDIA MAP022) agar plates and incubated overnight at 37°C, following which the cultural characteristics (colony color and morphology) were checked for [3].
- **Completed Test:** A few representative colonies were picked up from the EMB agar plates, and each inoculated into a tube containing 5 ml 1X LB and Durham's tube, and incubated overnight at 37°C, following which turbidity, and, acid and gas production were noted down [3].

Isolation of *Staphylococcus* species

To 5 ml of the Tryptone Soya Broth (TSB) (HIMEDIA LQ009A) supplemented with 6.5% sodium chloride (HIMEDIA MB023), 100 µl clear aliquot of the saline suspension of each sub-sample was added, and incubated in a rotary shaker overnight at 37°C. Next day, one loopful of all cultures was streaked onto highly selective Baird Parker Agar (BPA) (HIMEDIA M1736) plates, and incubated overnight at 37°C, following which the colony color and morphology was checked for [4,5].

Isolation of *Salmonella/Shigella/other Enterobacteria*

To 5 ml of the pre-enrichment Selenite-Cystine Broth (HIMEDIA M025), 100 µl clear aliquot of the saline suspension prepared earlier was added, and incubated in a rotary shaker overnight at 37°C. Next day, one loopful of all cultures was streaked onto selective Hektoen Enteric Agar (HEA) (HIMEDIA M467) plates, and incubated overnight at 37°C, following which the colony color and morphology were checked for [4,5].

Isolation of *Bacillus* species

100 µl clear aliquot of the saline suspension prepared earlier was added to 5 ml of Glucose Free Nutrient Broth (GFNB) (NB prepared without dextrose), and was incubated in a rotary shaker for 15 days at 37°C for the isolation of species of *Bacillus* [3]. Gram staining was performed according to the standard protocol to observe the Gram character and morphology of the isolated cells microscopically.

Isolation of fungal pathogens

To isolate fungal pathogens, selective colonies grown on YPDA plates were sub-cultured onto Potato Dextrose Agar (PDA) (HIMEDIA MH096) slants, supplemented with tetracycline (20 µg/mL) (SIGMA - ALDRICH T8032), amoxicillin trihydrate: potassium clavulanate (4:1) (2 µg/mL) (SIGMA - ALDRICH SMB00607), and calcium pantothenate (20 µg/ml) (HIMEDIA CMS178), and incubated for 72 hours at 30°C.

Characterization and identification of bacterial pathogens

Microscopy

Gram staining (reagents procured from HIMEDIA) was performed according to the standard protocol, and Gram character and morphology of the cells of the pathogenic bacterial isolates confirmed microscopically [3]. The presence of endospores in the GFNB isolates was confirmed microscopically by Dorner's Nigrosin (HIMEDIA S025) Method of endospore staining [3].

Biochemical tests

The bacterial pathogenic isolates were characterized and identified by a range of standard biochemical tests, including starch hydrolysis (SH), urea hydrolysis (UH) and gelatin hydrolysis (GH), nitrate reduction (NR), catalase production (CP), coagulase production (CoP), oxidase production (OP), carbon source (glucose, sucrose and lactose) utilization [growth in Triple Sugar Iron (TSI) agar] and H₂S production (in TSI agar), and Indole-Methyl Red-Voges Proskauer-Citrate (IMViC) tests [3-11]. All the media were prepared according to the composition prescribed by HIMEDIA.

Characterization and identification of fungal pathogens

Microscopy

Lactophenol Cotton Blue (LPCB) (HIMEDIA S016) staining of the cultures grown on PDA slants was performed to microscopically confirm the morphological details of the pathogenic fungal isolates.

Identification using ITS region based molecular method

To identify the fungal isolates and explore their evolutionary relationships, a molecular approach combining sequence comparison and phylogenetic analysis was employed. The process began with the amplification of the Internal Transcribed Spacer (ITS) region of ribosomal DNA (rDNA), a widely accepted marker for fungal species identification due to its variability between species and conserved flanking regions, which makes it ideal for primer design. Using Polymerase Chain Reaction (PCR), the ITS region was amplified from the extracted fungal DNA with the help of ITS1 and ITS4 primers, the standard choices in fungal taxonomy studies. Following successful amplification, sequencing of both forward and reverse strands was performed using the BDT v3.1 Cycle Sequencing Kit, based on dideoxy chain termination, and run on an ABI 3730xl Genetic Analyzer. The raw reads were then processed and assembled into a consensus sequence using alignment software. The resulting consensus sequences were compared against the NCBI GenBank database using the BLAST algorithm. The ten most similar sequences, based on highest identity scores, were selected for further phylogenetic analyses to ensure a representative set of closely related fungal taxa. These sequences, along with the one obtained from the fungal isolate, were aligned using Clustal W. A distance matrix was then calculated to assess evolutionary divergence between sequences. Phylogenetic trees were built using MEGA 10, applying the Maximum Likelihood method, a robust statistical approach for reconstructing evolutionary history. The Tamura-Nei model was used to account for variations in base frequencies and substitution rates. Initial tree topologies for the analysis were generated using the Neighbor-Joining and BioNJ algorithms, which helped streamline the search for the most likely tree. Final evolutionary interpretations and visualizations were refined in MEGA11, ensuring the use of the most updated computational tools and algorithms [12].

Determination of Antibiotic Resistance in Bacteria by Antibiotic Susceptibility Test (AST) and antibiogram analyses

To determine the antibiotic resistance profiles of the pathogenic bacterial isolates, antibiotic susceptibility testing (AST) was rigorously performed using the standard Kirby-Bauer Disc Diffusion method [13]. Five antibiotics were selected, including both newer generation and conventional ones, to provide a comprehensive resistance profile. The commercially available

antibiotic-impregnated discs used were Ciprofloxacin (CIP, 5 µg/disc) (HIMEDIA SD060), Amoxicillin (AMX, 30 µg/disc) (HIMEDIA MD002), Tetracycline (TE, 30 µg/disc) (HIMEDIA MD056), Streptomycin (S, 25 µg/disc) (HIMEDIA MD048), and Vancomycin (VA, 30 µg/disc) (HIMEDIA MD060). For each pathogenic isolate, a fresh bacterial culture was prepared by inoculating into NB, and incubating it overnight in a rotary shaker at 37°C to ensure optimal growth. The turbidity of each culture was then attuned to match an optical density (O.D.) equivalent to a 0.5 McFarland standard at 625 nm. This standardization is critical, as it ensures a consistent bacterial inoculum concentration of approximately 10^8 colony-forming units per milliliter (CFU/ml) for all ASTs, a crucial factor for reproducible and accurate results. 100 µl of each bacterial culture was then aseptically spread onto the surface of sterile, solidified Mueller-Hinton Agar (MHA) (HIMEDIA M173) plates [14]. The antibiotic discs were then carefully placed on the surface of the seeded plates, the plates allowed to rest at room temperature for approximately 30 minutes for the antibiotics to diffuse into the agar before the initiation of bacterial growth, and then, the plates were inverted and incubated at 37°C for 24 hours. Post-incubation, the diameter of the zones of inhibition (ZOI) of growth around each antibiotic disc were measured in millimeters (mm) using a ruler, and the results interpreted based on Clinical and Laboratory Standards Institute (CLSI) guidelines to determine whether the bacterial isolates were susceptible or resistant to each antibiotic tested [15-19].

Statistical analysis

All results were reported as mean \pm Standard Error of the Mean (SEM), reflecting the precision of the average values. Each assay was performed in triplicate, and the final mean values were calculated from the combined data of these independent replicates to improve accuracy and reproducibility. Wherever applicable, statistical analysis was conducted using Student's t-test to identify significant differences between experimental groups, with a p-value < 0.05 considered statistically significant.

Results

Total heterotrophic count

The observations revealed a significant level of bacterial and fungal contamination in majority of the chocolate samples under study.

Total bacterial count

The total heterotrophic count (THC) of bacteria was found to be a staggering 2960 CFU/ml for the C2HRT sample, followed by 2440 CFU/ml for the C3HREF sample (Figure 1). The colony types varied from white, raised (major) to yellow (minor) surface and sub-surface ones across the different samples.

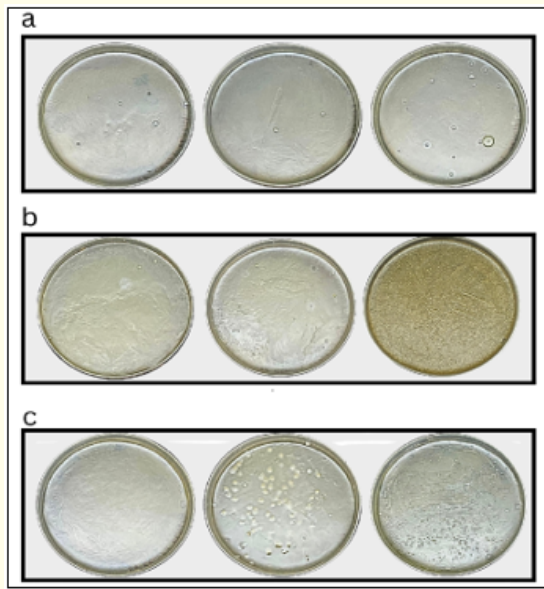


Figure 1: Total heterotrophic count (THC) of bacteria of C1, C2 and C3 sample plates: a (left to right): C1 Intact Ref with 6 white, raised colonies, C1Href with 3 white, raised colonies and C1HRT with 17 white, raised colonies; b (left to right): C2 Intact Ref with 8 white, raised colonies, C2Href with 1 yellow and rest 21 white, raised colonies and C2HRT with 296 white, raised colonies; c (left to right): C3 Intact Ref with 5 white, raised colonies, C3Href with 244 white, raised colonies and C3HRT with 72 yellow and white, raised and semi-raised colonies.

Total fungal count

The THC of fungi for C1HRT was found to be the highest with 200 CFU/ml (Figure 2). In the other fungal plates, isolated single colonies could not be demarcated, apart from plates C2HRT and C3Href, where the THC was 90 CFU/ml and 20 CFU/ml, respectively.

A bar diagram comparative analysis of the THCs of the bacterial and fungal contaminants from across the 3 chocolate samples

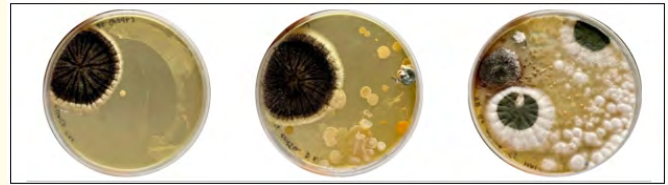


Figure 2: (Left to right): THC of fungi of C3Href showed a luxuriant black fungal colony with distinct white periphery; C2HRT showed a luxuriant black fungal colony with distinct white periphery, and a greyish-black colony; C1HRT showed 2 green colonies with wide white periphery, a greyish-black colony, and numerous white colonies.

showed a greater spoilage by bacteria (Figure 3). This observation challenges the prevailing notion that the presence of cocoa and low *a_w* in chocolates limit their bacterial spoilage, as evident in the contamination in even the dark chocolate sample (C3).

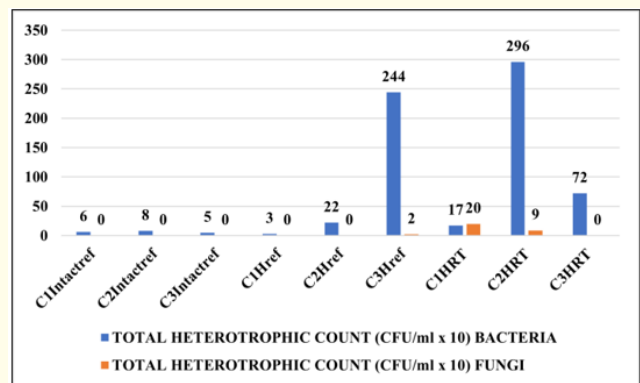


Figure 3: A bar diagram comparative analysis of the THCs of the bacterial and fungal contaminants from across the 3 chocolate samples.

Isolation of bacterial pathogens

Coliforms

Presumptive Test and MPN Index determination

The results obtained indicated a maximum of 6 MPN Index/100 ml (MPN Index) (Table 1, Figure 4) [2, 3].

Sl. No.	Sample	9.9 ml 1X LB (0.1 ml sample)	9 ml 1X LB (1 ml sample)	10 ml 2X LB (10 ml sample)	MPN Index/100ml
1.	C1 Intact Ref	0	0	0	<2
2.	C1Href	0	0	0	<2
3.	C1HRT	1	1	1	6
4.	C2 Intact Ref	0	0	0	<2
5.	C2Href	0	0	0	<2
6.	C2HRT	1	1	1	6
7.	C3 Intact Ref	0	0	0	<2
8.	C3Href	0	0	0	<2
9.	C3HRT	1	1	1	6

Table 1: MPN Index of samples evaluated with Presumptive Test.

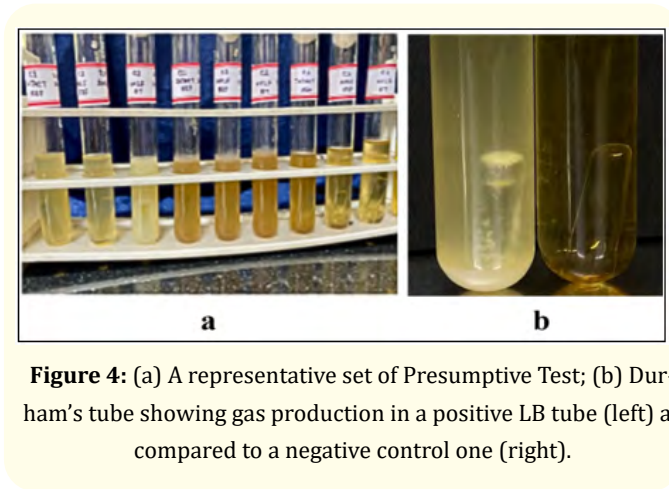


Figure 4: (a) A representative set of Presumptive Test; (b) Durham's tube showing gas production in a positive LB tube (left) as compared to a negative control one (right).

Confirmed test

Confirmed tests showed 42 medium and large blue-black colonies with a greenish-metallic sheen typical of *Pseudomonas* species from C1HRT, and 26 medium and small brown colonies typical of *Enterobacter* species from the C3HRT sample on the EMB agar plates (Figure 5) [3]. However, no fecal coliform contamination was reported from any of the chocolate samples, as evident from the results.

Completed test

Dense turbidity, copious acid production and moderate-to-huge gas production in the Durham's tubes was noted in the LB tubes

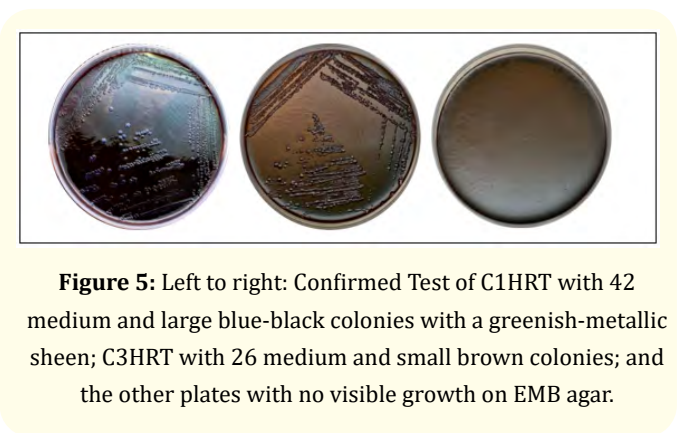


Figure 5: Left to right: Confirmed Test of C1HRT with 42 medium and large blue-black colonies with a greenish-metallic sheen; C3HRT with 26 medium and small brown colonies; and the other plates with no visible growth on EMB agar.

inoculated with representative colonies from the EMB plates, indicative of a huge bacterial contamination (Table 2; Figure 6) [3].

Sl. No.	Sample	pH (Acidity)	Gas Production in Durham's Tube (%)
1.	C1HRT	5.5 (acidic)	40, moderate gas bubble
2.	C1Href	5.0 (acidic)	70, large gas bubble
3.	C2 Intact Ref	5.0 (acidic)	80, large gas bubble
4.	C2HRT	5.5 (acidic)	35, moderate gas bubble
5.	C2HRT(2X)	5.0 (acidic)	85, very large gas bubble
6.	C3HRT	5.0 (acidic)	45, moderate gas bubble

Table 2: Acidity and Gas Production in Completed Test.

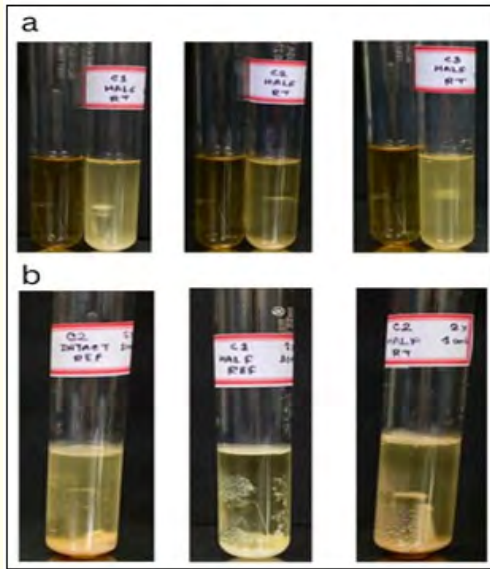


Figure 6: Turbidity along with acid and gas production in Completed Test: a (Left to right): Turbidity with a decent amount of gas production in tubes of C1HRT, C2HRT and C3HRT samples; b (Left to right): Dense turbidity with huge gas production in the tubes of C2 Intact Ref, C1Href and C2HRT samples.

Isolation of *Staphylococcus* species

In line with the understanding that *Staphylococcus* is one of the leading pathogens that can hugely proliferate in dairy products, the presence of colonies of color ranging from greyish-black to black, some of which were surrounded by distinct halos on the BPA plates, indicated the presence of *Staphylococcus* species (Table 3; Figure 7, 8) [2, 3]. C1 Intact ref showed the highest contamination with 1480 CFU/ml, followed by C1HRT with 1360 CFU/ml.

Isolation of *Salmonella*/*Shigella*/other *Enterobacteria*

C1HRT showed a 360 CFU/ml on HEA plate, including both salmon colonies with black center (major) and light-green colonies with black center (minor). C3HRT showed a 440 CFU/ml on HEA plate, including both light-green colonies with black center (major) and salmon colonies with black center (minor) (Figure 9). The salmon colonies with black center are typical of *Enterobacter* species, while light-green colonies with black center are typical of *Citrobater* species [2,3].

Plate Sl. No.	Sample	Colony Count	Plate Sl. No	Sample	Colony Count
A	C1Href	52	e	C2HRT	56
B	C1HRT	136	f	C2 Intact Ref	03
C	C2Href	22	g	C3Href	14
D	C1 Intact Ref	148	h	C3HRT	42

Table 3: Colony Count of *Staphylococcus* species on BPA Plates.

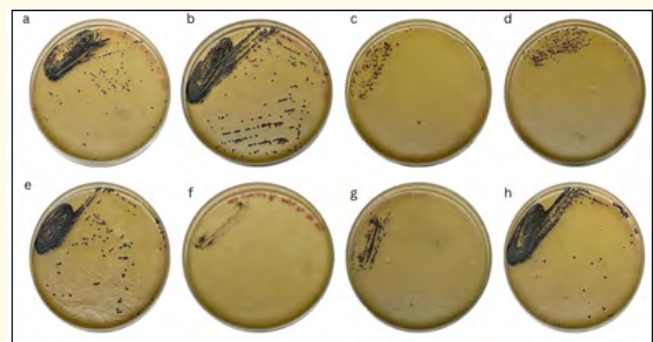


Figure 7: (a-h): Colonies of *Staphylococcus* species growing on BPA plates.

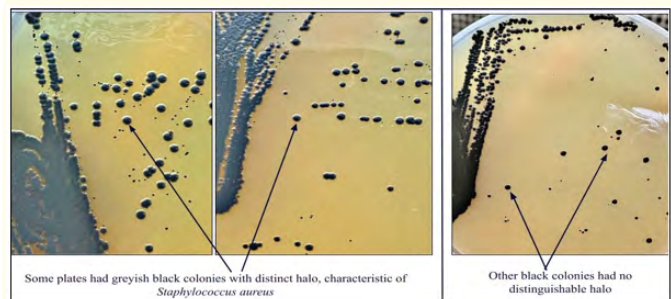


Figure 8: A closer view of greyish-black and black colonies of *Staphylococcus* species observed on BPA plates. Distinct halo, a characteristic feature of *Staphylococcus aureus*, was observed around the greyish-black colonies (left and middle), but absent from the black colonies (right).

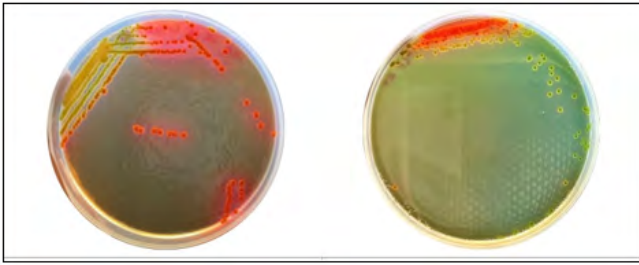


Figure 9: C1HRT showing 36 salmon and light-green colonies, both with black center (left); C3HRT with 44 light- green and salmon colonies, both with black center (right).

However, no contamination by species of *Salmonella* and *Shigella* was reported from any of the chocolate samples.

Isolation of *Bacillus* species

Gram staining of the bacterial culture from the 15-day old GFNB tubes demonstrated Gram positive, medium-sized rods in chain arrangement, typical of *Bacillus* species (Figure 10) [3].

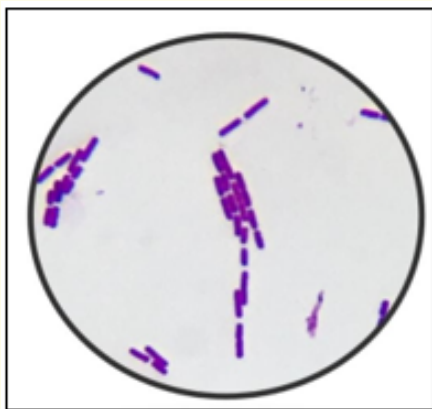


Figure 10: Gram staining observation (400X) of *Bacillus* sp.

Characterization and identification of pathogens

Characterization and identification of bacterial pathogens

Microscopic observation by gram staining

The conventional Gram staining technique to reveal the Gram character and cell morphology of the pathogenic bacterial isolates confirmed the cells from the BPA plates as Gram positive, medium-

sized cocci in clusters and bunch of grape-like arrangements (Figure 11a), from both the EMB agar plates and the HEA plates as Gram negative short rods in single arrangement (Figure 11b-c), and those from the GFNB tubes as Gram positive medium-sized rods in chain arrangement, with visible, unstained, refractile endospores (Figure 11d) [3].

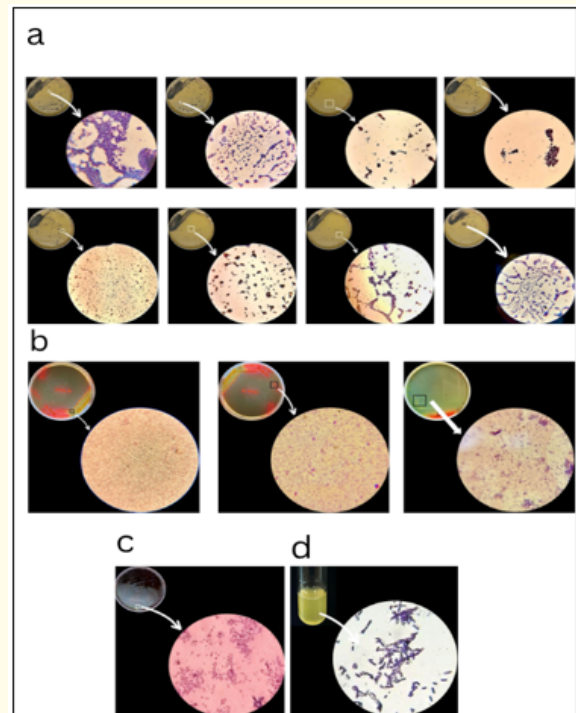


Figure 11: Microscopic observation (400X) of Gram staining from BPA, EMB and HEA plates, and GFNB tube. (a) BPA plates: (top left to right): C1HRT (1) shows Gram positive, medium cocci in clusters; C1HRT (2) shows Gram positive, medium cocci in bunch of grape-like arrangement; C2Href shows Gram-positive, medium cocci in clusters; C2HRT (1) shows Gram positive, medium cocci in bunch of grape-like arrangement and (bottom left to right): C2HRT (2) shows Gram positive, medium cocci; C3HRT (1) shows Gram positive, medium cocci in clusters; C3HRT (2) shows Gram positive, medium cocci in bunch of grape-like arrangement; C3HRT (3) shows Gram positive cocci in bunch of grape-like arrangement; (b) HEA plates: (left to right): All three of C1HRT (1), C1HRT (2) and C3HRT shows Gram positive, short rods in singular arrangement; (c) EMB agar plate: C1HRT shows Gram negative short rods in single arrangement; (d) GFNB broth: C3HRT shows Gram positive medium-sized rods in chain arrangement, with visible refractile endospores.

Endospore staining

The conventional Dorner’s Nigrosin Method of Endospore staining identified endospore-forming *Bacillus* species as the contaminant of the chocolate samples. The endospores appeared red [stained by Carbol Fuchsin (HIMEDIA S006)] within the colorless, medium-sized, rod - shaped vegetative cells against the dark background of Dorner’s Nigrosin (Figure 12).

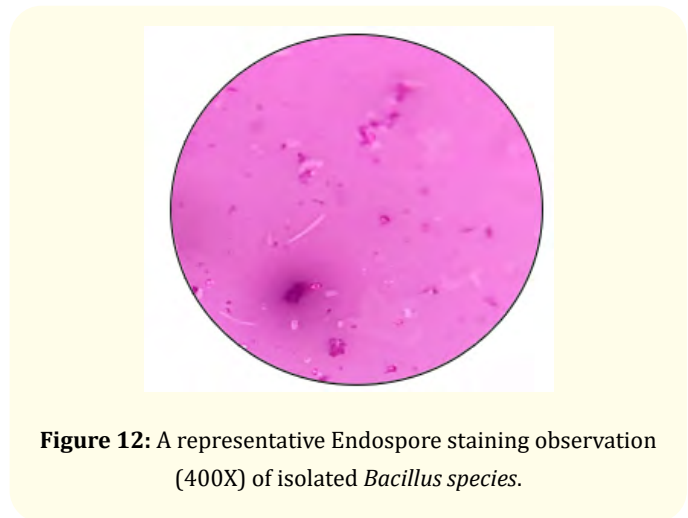


Figure 12: A representative Endospore staining observation (400X) of isolated *Bacillus* species.

Biochemical tests

The pathogenic bacterial isolates were characterized and identified by the set of standard biochemical tests (Table 4, Figure 13).

Characterization and identification of fungal pathogens

Microscopy

From the morphological observations of the respective specimens of three, typical chosen colonies from the PDA plates

Sl. No.	Isolate	IT	MR	VP	CUT	CP	OP	NR	UH	SH	CoP	GH	TSI	Bacterial Pathogen confirmed
1	C1HRTE	-	-	+	+	++	-	+	-	-	NA	-	R/R	<i>Enterobacter</i> sp.
2	C1HRTH (1)	-	-	-	+	+++	+	-	-	+	NA	+	R/Y	<i>Pseudomonas</i> sp.
3	C1HRTH (2)	-	-	-	+	++++	+	-	-	-	NA	-	R/R	<i>Citrobacter</i> sp.
4	C1HRFB (1)	N	N	N	N	+++	-	+	+	-	+	-	R/Y	<i>Staphylococcus aureus</i>
5	C1HRFB (2)	N	N	N	N	+++	-	+	+	-	-	-	R/Y	<i>S. epidermidis</i>
6	C3HRTG	N	N	N	+	+++	-	+	-	-	NA	+	P/Y	<i>Bacillus</i> sp.

Table 4: Summary of Biochemical tests and their results.

C1HRTE = C1HRT from EMB plate; C1HRTH (1) = C1HRT light green colony with black center from HEA plate; C1HRTH (2) = C1HRT salmon colony with black center from HEA plate; C1HRFB (1) = C1HRef greyish black colony with a clear halo from BPA Plate; C1HRFB (2) = C1Href dark grey colony without halo from BPA plate; C3HRTG = C3HRT growth from GFNA slant; IT = Indole Test; MR = Methyl red Test; VP = Voges Proskauer Test; CUT = Citrate Utilization Test; CP = Catalase Production test; OP = Oxidase Production test; NR = Nitrate Reduction test; UP = Urease Production test; SH = Starch Hydrolysis test; CoP = Coagulase Production test; GH = Gelatin Hydrolysis; TSI = Triple Sugar Iron Test; + = Weakly Positive; +++ = Strongly Positive; - = Negative; NA = Test Not Applicable; P = Pink; Y = Yellow; R = Red.

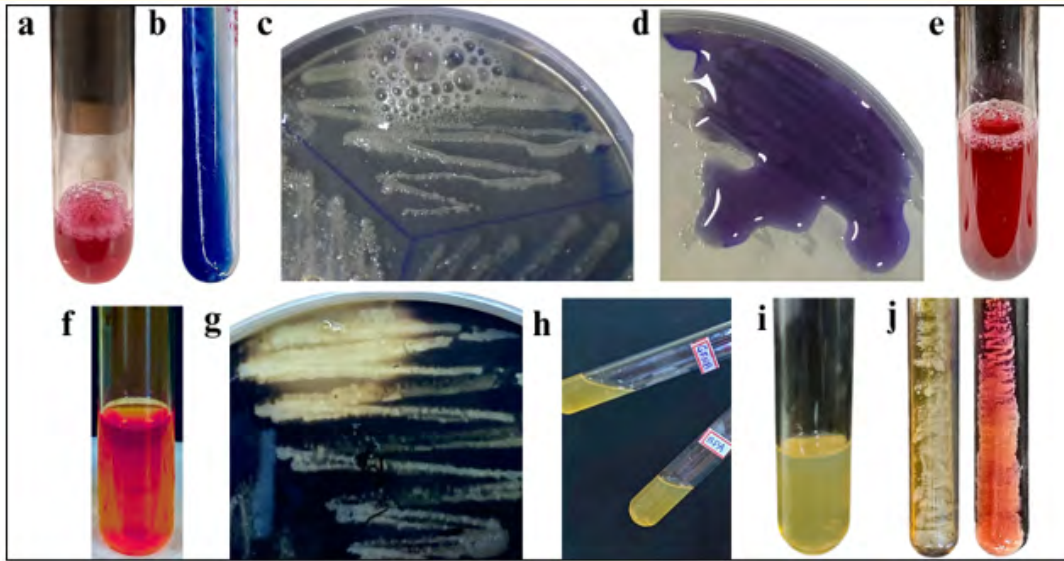


Figure 13: A Representative Set of Positive Biochemical Test Results as a Confirmation of different Pathogenic Bacterial Isolates.

(a) Positive result for Voges Proskauer Test [reddish-pink coloration in MR-VP broth (HIMEDIA LQ082) culture upon sequential addition of, and shaking for 15 minutes Barritt's Reagents A (HIMEDIA R029) & B (HIMEDIA R030)]; (b) Positive result for Citrate Utilization Test [bacterial growth and change in color of Simmon's Citrate Agar (HIMEDIA M411) from green to deep prussian blue upon overnight incubation at 37°C]; (c) Positive result for Catalase Test [evolution of copious oxygen bubbles from a NA culture upon addition of drops of 3% H₂O₂ (EMSURE® 88597)]; (d) Positive result for Oxidase Test [appearance of bluish color on a NA culture within 30 seconds of application of colorless N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride (HIMEDIA GRM445)]; (e) Positive result for Nitrate Reduction Test [red coloration in nitrate broth (HIMEDIA M439) culture immediately upon sequential addition of sulfanilic acid (HIMEDIA GRM428) and α-naphthylamine (HIMEDIA R009) solutions]; (f) Positive result for Urease Test [pinkish-red coloration in the urea broth (HIMEDIA M111) culture upon overnight incubation at 37°C]; (g) Positive result for Starch Hydrolysis [clear halo zone surrounding bacterial growth on a starch-agar (HIMEDIA M107S) plate, observed after addition of Gram's iodine (HIMEDIA S013) solution]; (h) Positive result for Gelatin Hydrolysis [rapid hydrolysis of gelatin at the top of the nutrient-gelatin (HIMEDIA M060) medium by gelatinase of the GFNB isolate observed after 48 hours incubation at 37°C and 30 minutes chilling at 4°C, in comparison to a BPA isolate unable to hydrolyze gelatin]; (i) Positive result for Coagulase Test [gelling in the suitably - diluted EDTA - treated lyophilized rabbit plasma (HIMEDIA FD248A), forming a clot, observed after overnight incubation with NB culture of a BPA isolate at 37°C]; (j) Positive result for Triple Sugar Iron [red slant and yellow butt of TSI agar (HIMEDIA M021I) clearly demonstrates that the isolated bacteria fermented only glucose, but not sucrose and lactose. Glucose fermentation produced acids in the butt; however, the remainder of the aerobic slant reverts to alkaline due to the limited amount of acid and the use of other proteins in the medium. The red color in the slant is due to an alkaline pH, while a yellow color indicates an acidic pH from fermentation].

by LPCB staining, the presence of three fungal species were presumptively identified (Table 5).

Identification using ITS region based molecular method

Following nucleotide homology of ITS region and phylogenetic analyses, the fungal isolate obtained from the C2HRT (2) plate was definitively identified as *Rhizoctonia solani*, from C2HRT

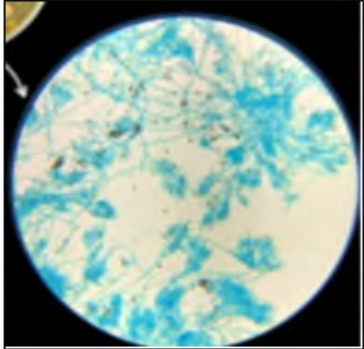
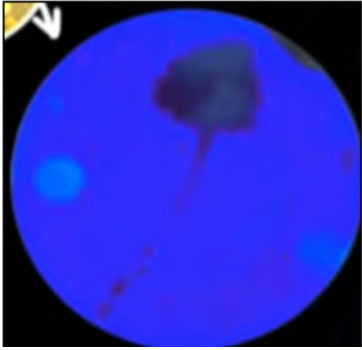
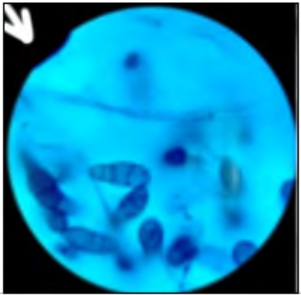
Sl. No.	Isolate	Colony Morphology (on PDA Plate)	Microscopic Observation (400X) after LPCB Staining	Fungal Pathogen	Microscopic Observation (400X)
1	C2HRT (2)	Rapid growth of greyish-black, dense, hardened masses of mycelium, strongly suggestive of darkly pigmented sclerotia.	The hyphae displayed characteristic branching patterns: branches typically originated at a 45-90 degrees angle from the main hypha. Furthermore, a prominent septum was consistently observed near the point where the branch originated, often accompanied by a slight constriction at the very base of the branch. The septa were present at irregular intervals. The hyphae appeared to be surrounded by a thin layer of extracellular matrix.	<i>Rhizoctonia sp.</i> [20,21]	
2	C2HRT (1) and C3Href	Luxuriant black fungal colony with distinct white periphery. The texture was cottony (velvety).	A tubular, septate, hyaline, and branched mycelium, a straight hyphal branch called a conidiophore that enlarges at the apex giving rise to a spherical or elliptical structure with a layer of radiating phialide cells called a vesicle, and clustered chains of exogenous reproductive structures developed from sterigmata called conidia were observed.	<i>Aspergillus sp.</i> [22]	
3	C1HRT (2)	Greyish-black, floccose fungal colony. The texture of the colony was cottony (velvety), with a characteristic "zonate" pattern of growth.	A branched acropetal conidiophore and multi-celled, obclavate to obpyriform conidiospores (conidia), characterized by short conical beaks, was observed. The conidia were large and multicellular, and tapered at the apex, comprising of multiple transverse septa.	<i>Alternaria sp.</i> [23]	

Table 5: Microscopic Confirmation of Different Fungal Pathogens.

(1) and C3Href as *Aspergillus tubingensis*, and from C1HRT (2) plate as *Alternaria alternata* (Figure 14). These conclusive identifications based on very high (99.43%) sequence similarity to the reference strain *Rhizoctonia solani* strain AG-1-1A (Accession ID MK290374.1), complete (100%) sequence similarity to the reference strain *Aspergillus tubingensis* strain USMG11 (Accession ID KF434094.1), and complete (100%) sequence similarity to the reference strain *Alternaria alternata* isolate xiaonei 9-2 (Accession ID MK773579.1), respectively.

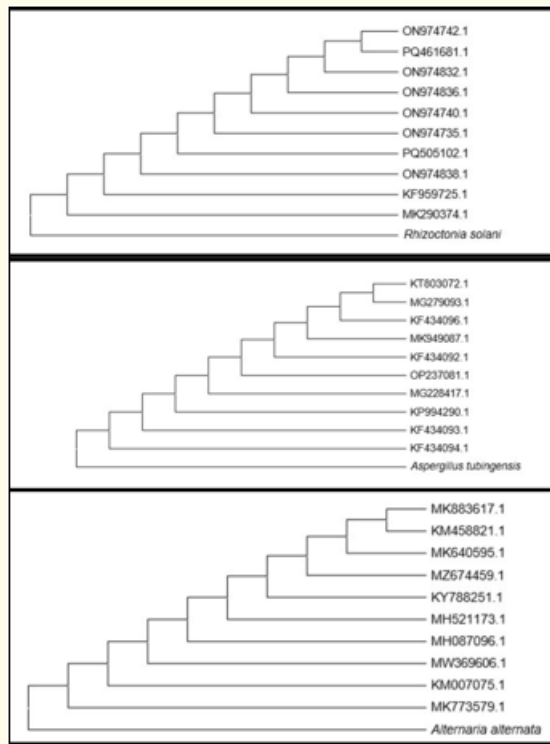


Figure 14: (Top to bottom): ITS region - based study results of the three fungal specimens confirmed the presence of *Rhizoctonia solani*, *Aspergillus tubingensis* and *Alternaria alternata* to be present in the contaminated chocolate samples.

Determination of Antibiotic Resistance in Bacteria by Antibiotic Susceptibility Test (AST) and antibiogram analyses

The results of the AST (Figure 15) and subsequent antibiogram analyses of the typical pathogenic bacterial isolates revealed wide variations in their drug resistance patterns. Very alarmingly, 66.67% of the isolates (*Pseudomonas* sp., *Citrobacter* sp., *S. aureus*,

and *Bacillus* sp.) exhibited complete resistance to Amoxicillin (AMX), while 16.67% of the isolates (*Pseudomonas* sp.) exhibited complete resistance to Vancomycin (VA).

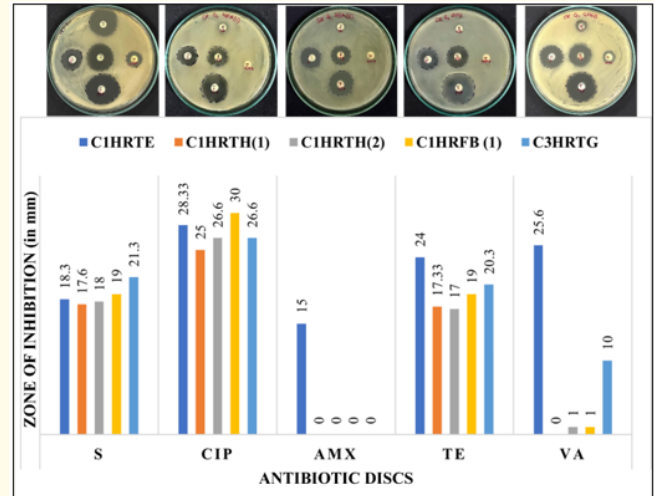


Figure 15: Results of AST and antibiogram analysis: Zones of Growth Inhibition of 5 different pathogenic bacterial isolates observed in MHA plates, and their corresponding bar diagram, indicating zone of inhibition (in mm).

Discussion

The prevailing belief that the low a_w and presence of cocoa in chocolate inherently restrict microbial proliferation and spoilage is significantly challenged by the findings of this study. The observed high levels of bacterial and fungal contamination across chocolate bars of three prominent multinational brands, categorized by their cocoa content as dark, milk, and white chocolates, in particular, the staggering THC of bacteria (2960 CFU/ml), emphasize a critical revisit of the present quality control measures operative in the chocolate-producing industries. The high bacterial THC, notably in C2HRT (2960 CFU/ml) and C3HREF (2440 CFU/ml), directly contradicts the notion that chocolate is a microbiologically-stable food product. This is particularly striking, given the high contamination observed in even the dark chocolate sample (C3), which typically contains a higher cocoa content. The diverse colony morphologies observed, ranging from white - raised to yellow surface and sub-surface ones, suggest a heterogeneous bacterial contaminating population.

The detection of specific bacterial genera offers deeper insight into the nature of contamination. Although the MPN index revealed low levels of coliform contamination (not exceeding 6 MPN/100 ml across all samples), their presence— particularly non-fecal coliforms confirmed through Gram staining and completed tests— points to possible lapses in hygiene maintenance during processing or handling stages. Even in low numbers, coliforms serve as markers of poorly-maintained sanitary conditions. Of particular interest was the isolation of 26 brown colonies from the C3HRT sample on EMB agar plates, resembling *Enterobacter* species. As an opportunistic pathogen, the detection of *Enterobacter* implies a potential breach in hygiene controls. This identification is further supported by the appearance of salmon-colored colonies with black centers on HEA, also consistent with *Enterobacter* morphology. Another significant finding is the recovery of 42 blue-black colonies with a green metallic sheen from the C1HRT sample on EMB agar plates, typical of *Pseudomonas* species. *Pseudomonas* is a notorious human pathogen, and a spoilage organism capable of secreting extracellular enzymes that compromise food quality. In addition, the observation of greyish-black to black colonies on BPA plates, some surrounded by clear zones, strongly indicates the presence of *Staphylococcus* species. The highest colony counts were recorded in C1 Intact Ref (1480 CFU/ml) and C1HRT (1360 CFU/ml) samples, raising concerns due to the ability of *Staphylococcus aureus* to produce heat-stable enterotoxins. Given its preference for dairy-rich environments, its presence in white chocolate is particularly troubling from a public health standpoint. Gram staining of 15-day-old cultures from Glucose Fermentation Nutrient Broth (GFNB) indicated the presence of endospore-forming *Bacillus* species. Known for their environmental ubiquity and heat-resistant endospores, *Bacillus* can survive various processing conditions, potentially explaining their persistence in chocolate samples. Further, light-green colonies with black centers on HEA plates were identified as *Citrobacter* species, predominantly present in C3HRT (440 CFU/ml) and to a lesser extent in C1HRT. Although less virulent than pathogens like *Salmonella* or *Shigella*, *Citrobacter* still reflects possible hygiene shortcomings.

Microscopic examination provided additional confirmation needed to confirm the potential pathogen isolates. Gram negative short rods in single arrangement (from HEA plates) indicated *Enterobacter*, *Pseudomonas*, and *Citrobacter*; Gram positive medium-sized cocci in clusters and bunch of grape-like

arrangements (from BPA plates) indicated *Staphylococcus*; and Gram positive medium-sized rods arranged in chains (from GFNB tubes) indicated *Bacillus* species. A range of biochemical tests, including starch, urea, and gelatin hydrolysis; nitrate reduction; catalase and oxidase production; carbohydrate utilization and hydrogen sulfide production in TSI agar; and the IMViC tests, further confirmed the identities of these isolates.

The significant fungal counts, with C1HRT showing the highest at 200 CFU/ml, highlight that fungal spoilage is also a considerable concern in chocolates. The presumptive identification of *Rhizoctonia*, *Alternaria*, and *Aspergillus* based on observations of colony morphological from PDA plates, and LPCB staining, subsequently confirmed by ITS region nucleotide homology and phylogenetic analyses, represents the comprehensive methodology employed in this study. The definitive identification of *Rhizoctonia solani* (99.43% sequence similarity to reference strain MK290374.1) from the C2HRT (2) plate, *Aspergillus tubingensis* (100% sequence similarity to reference strain KF434094.1) from C2HRT (1) and C3Href plates, and *Alternaria alternata* (100% sequence similarity to reference strain MK773579.1) from the C1HRT (2) plate, is particularly interesting.

The confirmed isolation of these bacterial and fungal pathogens from chocolates carries significant public health implications, as their consumption can lead to a range of illnesses, from mild gastrointestinal distress to severe, life-threatening conditions. While *Staphylococcus epidermidis* is commonly a skin commensal, certain strains can be opportunistic pathogens [24]. *S. aureus* is a major foodborne pathogen. Ingesting food contaminated with *S. aureus* can lead to staphylococcal food poisoning, characterized by rapid onset of severe nausea, vomiting, abdominal cramps, and diarrhea [25,26]. This is primarily due to the production of heat-stable enterotoxins by the bacteria. *Enterobacter* species are opportunistic pathogens capable of causing various infections, particularly in immunocompromised individuals and children [27,28]. Their presence in chocolates can be a concern for vulnerable populations. In food, presence of *Pseudomonas* can indicate spoilage, but if consumed by immunocompromised individuals, it can lead to various infections including gastroenteritis, skin infections, and more severe systemic infections [29]. Primarily, *Bacillus* species is one of the major foodborne concerns. It produces two types of toxins, a heat-stable emetic toxin causing vomiting and

a heat-labile toxin causing diarrhea and abdominal pain [30,31]. Ingestion of any of the spores or preformed toxins in food can lead to *Bacillus* food poisoning [32]. Among the confirmed pathogenic fungal isolates, *Rhizoctonia* species and *Alternaria* species are common environmental molds, but their presence might indicate poor hygiene or environmental contamination during processing [33-36]. They can be allergenic as well. Several *Aspergillus* species are significant human pathogens, and are notorious for producing aflatoxins, potent carcinogens that can cause liver damage and cancer upon chronic consumption [37,38]. *Aspergillus* species can cause Aspergillosis, a severe respiratory infection, especially in immunocompromised individuals, though typically from inhalation of spores rather than ingestion [39]. The presence of *Aspergillus* in chocolate is a major food safety concern due to potential mycotoxin production. *Alternaria* species are common saprophytic fungi and plant pathogens, some of which can produce toxins. Their presence in food indicates potential spoilage and hygiene discrepancies and, similar to *Aspergillus*, raises questions about mycotoxin contamination risk.

Perhaps one of the most alarming findings of this study is the pervasive antimicrobial resistance pattern observed among the pathogenic bacterial isolates. The high percentage of isolates exhibiting resistance to commonly used antibiotics presents a significant public health concern. The finding that 66.67% of the isolates (*Pseudomonas* sp., *Citrobacter* sp., *S. aureus*, and *Bacillus* sp.) exhibited complete resistance against AMX is alarming. AMX is a broad-spectrum antibiotic widely used in treating bacterial infections. Such widespread resistance suggests a serious challenge in effective treatment should these pathogens cause human illness. Furthermore, 16.67% of the isolates (*Pseudomonas* sp.) exhibited complete resistance against VA. Vancomycin is considered an antibiotic of last resort for many severe Gram-positive bacterial infections, particularly those resistant to other drugs. The emergence of VA-resistant *Pseudomonas* species, which are typically Gram-negative, is highly concerning and indicates a potential for novel resistance mechanisms or horizontal gene transfer (HGT) in the food environment.

The isolation of a pathogenic strain of a *Pseudomonas* species that is a multidrug-resistant (MDR) one, exhibiting resistance against both AMX and VA, is a critical highlight of this study. MDR-pathogens pose a severe threat to public health, as treatment options become severely limited, increasing the risk of prolonged

illness, hospitalization, and mortality. This antibiogram analysis not only represents the problem but also provides important information for potential therapeutic strategies. The identification of antibiotics with pharmacophores similar to ciprofloxacin, tetracycline, streptomycin, and vancomycin (for DR *Pseudomonas* sp., *Citrobacter* sp., *S. aureus*, and *Bacillus* sp.), and ciprofloxacin, streptomycin, and tetracycline (for MDR *Pseudomonas* sp.) is invaluable, as the isolated bacteria turned out resistant to those. This information can guide clinicians in choosing appropriate empiric or targeted therapy in cases of chocolate-borne infections.

The study definitively challenges the long-held assumption of inherent microbial safety in chocolates due to their low water activity and cocoa content. It demonstrates that chocolates, including dark chocolate, can harbor a diverse and significant microbial load. The incidence of notorious foodborne pathogens such as antibiotic-resistant *Pseudomonas* species, *Staphylococcus* species (especially *S. aureus*), and *Enterobacter* species, along with mycotoxin-producing *Aspergillus* species and *Alternaria* species, points to a clear food safety risk. The widespread contamination suggests potential deficiencies in hygienic practices throughout the chocolate production chain, from raw material sourcing to processing, packaging, and storage. Contaminants could originate from raw ingredients such as milk solids, sugar, processing equipment, personnel, or the environment. As food products can serve as vehicles for the dissemination of antibiotic-resistant bacteria, contributing to the global crisis of antimicrobial resistance, the isolation of a MDR *Pseudomonas* strain raises grave concern about public health and hygiene.

Conclusion

This pioneering study in Kolkata, India, one of the first comprehensive assessment of both fungal and bacterial contamination in popular commercial chocolate brands, shattering the common belief that chocolate's intrinsic properties prevent microbial spoilage. Contrary to expectation, the research revealed high loads of bacterial and fungal food-borne pathogens across samples. This compelling evidence highlights chocolates' susceptibility to contamination and, critically, emphasizes the need for appropriate storage practices and an urgent re-evaluation of food safety standards in the confectionery industry to protect consumers, especially children. Furthermore, the discovery that the isolated pathogens exhibit complete resistance to tested

antibiotics is a deeply concerning finding, strongly suggesting that contaminated chocolates could be a vector for drug-resistant infections, necessitating enhanced surveillance against antimicrobial resistance in food commodities.

Ethical Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors have no competing interests to disclose.

Authors' Contributions

- SR – Conceptualization, review and editing, preparation of final draft of manuscript, supervision
- DB – Data curation and analysis, review and editing, proofreading, preparation of final draft of manuscript HG – Data curation and analysis, review and editing, proofreading, preparation of final draft of manuscript LC – Overall supervision
- SS – Conceptualization, data curation and analysis, optimization of methodology, review and editing, proofreading, preparation of final draft of manuscript

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