



Isolation and Molecular Identification of Bacteria Associated with Commercial Tea Brands in Al-Bayda, Libya

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العزل والتعريف الجزيئي للبكتيريا المرتبطة بالعلامات التجارية للشاي في مدينة البيضاء، ليبيا

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Abstract:

Dried tea is a low-moisture plant product in which microbial growth is limited, but viable bacteria may persist through cultivation, harvesting, processing, transport and storage. This study isolated and identified culturable bacteria from five commercial tea products sold in Al-Bayda, Libya: two green teas (Nabet and Al-Jawhara) and three red/black teas (Al-Ghazaleen, Al-Baraka and Zahra red/black tea). One gram of each product was suspended in 9 mL of sterile 0.85% saline, serially diluted and spread on nutrient agar, and plates were incubated at 30°C for 24 h. Seven morphologically distinct colonies (T1–T7) were purified and characterised by colony morphology and Gram staining, and the 16S rRNA gene was amplified by PCR, sequenced by the Sanger method and compared with NCBI records using BLAST. All isolates were rods; T1, T4 and T6 were Gram-positive and T2, T3, T5 and T7 were Gram-negative. The isolates were assigned to *Psychrobacillus psychrodurans* (T1; PV000778), *Pseudomonas palmensis* (T2; PV000779), *P. kilonensis* (T3; PV000780), *Priestia megaterium* (T4; PV000781), *P. fragi* (T5; PV000782), *Bacillus cereus* (T6; PV000783) and *Sphingobacterium anhuense* (T7; PV000784), with accession numbers reported as PV000778–PV000784; their live records and correspondence with the isolates should be verified before submission. The assemblage combines endospore-forming bacteria, pseudomonads and a genus typical of soil and water, which is consistent with several possible routes of contamination along the supply chain. Because selected isolates were examined without viable counts or toxin tests, the study documents the presence of culturable bacteria but not their abundance, prevalence or risk to consumers. The deposited sequences provide a reference for quantitative studies of tea microbiology in Libya.

Keywords: commercial tea, bacterial isolation, 16S rRNA gene, molecular identification, *Bacillus cereus*, *Pseudomonas*, Libya.

الملخص:

هدفت الدراسة إلى عزل وتشخيص البكتيريا المصاحبة لخمس علامات تجارية من الشاي المتداولة في مدينة البيضاء، ليبيا. جُمعت عينات من نوعين من الشاي الأخضر وثلاثة أنواع من الشاي الأحمر/الأسود، وأجري العزل البكتيري باستخدام التخفيفات المتسلسلة والزرع على الوسط الغذائي Nutrient Agar، ثم نُقِيت المستعمرات المختلفة ووصفت مورفولوجيًا، وأجري صبغ غرام لتحديد تفاعل الخلايا. وللتشخيص الجزيئي، ضُخِّم جزء من جين 16S rRNA بتقنية تفاعل البوليميراز المتسلسل (PCR)، ثم سُلِّسِلَت النواتج وُحِلَّت باستخدام BLAST بالرجوع إلى قاعدة بيانات المركز الوطني لمعلومات التقنية الحيوية (NCBI). أسفرت الدراسة عن الحصول على سبع عزلات بكتيرية (T1–T7)، وصُنِّفَت اعتمادًا على نتائج التسلسل إلى *Psychrobacillus psychrodurans* و *Pseudomonas palmensis* و *Pseudomonas kilonensis* و *Pseudomonas fragi* و *Priestia megaterium* و *Bacillus cereus* و *Sphingobacterium anhuiense*. كانت العزلات T1 و T4 و T6 موجبة لصبغة غرام، بينما كانت T2 و T3 و T5 و T7 سالبة، وكانت جميع الخلايا عصوية. أوردت الرسالة أرقام تسجيل GenBank للعزلات من PV000778 إلى PV000784، وينبغي التحقق من إتاحة السجلات ومطابقتها للعزلات قبل التقديم للنشر. وتوثق النتائج وجود تنوع في البكتيريا القابلة للزرع المرتبطة بعينات الشاي المدروسة، مع اقتصار الاستنتاجات على العينات والعزلات التي فُحصت في هذه الدراسة.

الكلمات المفتاحية: الشاي التجاري، العزل البكتيري، جين 16S rRNA؛ التشخيص الجزيئي؛ *Bacillus cereus*؛ *Pseudomonas*؛ البيضاء، ليبيا.

Introduction:

Tea (*Camellia sinensis*) is consumed worldwide and reaches the market after a chain of agricultural and post-harvest operations. The microbial quality of the finished product may reflect the conditions under which leaves were grown, harvested, dried, sorted, blended, packaged, transported and stored. Although the low moisture content of dried tea usually limits multiplication during storage, drying should not be interpreted as proof of sterility. Some microorganisms survive unfavorable conditions, and contamination may be introduced after the main drying step. Microbiological assessment of dry tea is therefore relevant to product quality and to understanding possible routes of contamination, while the significance of any recovered organism depends on its identity, abundance, viability and intended use.

The microbial profile of tea is influenced by product type and processing. Green tea and black tea differ in processing, including the degree of enzymatic oxidation, and these differences may affect microbial survival, but they cannot by themselves predict which bacteria are present in a retail product. Storage humidity, packaging integrity, contact with equipment, handling and environmental exposure may also contribute. A culture-based survey recovers organisms able to grow under the chosen laboratory conditions and does not describe the whole microbial community, because organisms with other nutritional, atmospheric or temperature requirements may not be recovered by a single protocol.

Several studies have examined the microbiological quality of commercial tea. Carraturo *et al.* (2018) compared commercial black and green teas using microbiological analyses, and Dharmarathna *et al.* (2018) examined microbes associated with a commercial black tea blending factory and traced contamination mainly to incoming raw material. A Canadian Food Inspection Agency (CFIA) survey of dried herbs and teas assessed selected bacterial pathogens in retail products and illustrates the difference between occasional pathogen detection and general statements about the safety of a whole product category (CFIA, 2019). Results from one country or product set cannot be directly generalised to another, and data for tea sold in Libya are limited.

Initial bacterial characterisation commonly relies on colony appearance, cell morphology and Gram staining. These observations are useful for describing isolates, but they have limited discriminatory power at species level, because different taxa may share similar colony morphologies and the Gram reaction depends on culture age, smear preparation and decolourisation (Beveridge, 2001). Sequencing of the 16S rRNA gene is widely used for identification, because conserved regions allow amplification and variable regions support taxonomic comparison, although its resolving power differs among bacterial groups and a top BLAST match must be interpreted together with sequence quality, identity, query coverage and the reliability of reference records (Janda and Abbott, 2007; Yarza *et al.*, 2014; Franco-Duarte *et al.*, 2019).

This study describes bacteria cultured from five commercial tea products purchased in Al-Bayda, Libya. Its objectives were to (i) recover and purify morphologically distinct culturable bacteria, (ii) document their colony and cellular characteristics, including Gram reaction, and (iii) identify them by 16S rRNA gene sequencing and BLAST analysis and discuss the diversity found in relation to tea quality and microbiological safety. The study is descriptive and does not support comparisons of microbial load among brands or tea types.

Materials and Methods

1. **Tea products:** Five commercial tea products were obtained from markets in Al-Bayda, Libya: two green teas (Nabet and Al-Jawhara) and three red/black teas (Al-Ghazaleen, Al-Baraka and Zahra red/black tea).
2. **Bacterial isolation and purification:** For each tea sample, 1 g of dried material was suspended in 9 mL of sterile physiological saline (0.85% NaCl). Serial dilutions were prepared, and aliquots were spread onto nutrient agar. Plates were incubated at 30°C for 24 h. Colonies that differed in colour, form, margin or surface texture were selected and subcultured repeatedly until pure, and pure isolates were maintained on agar slants at 4°C (Figure 1).

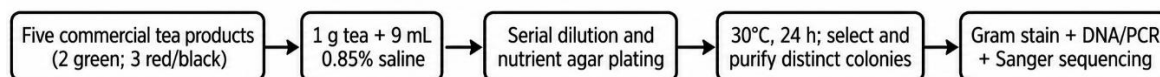


Figure (1): Workflow used for isolation, characterization and molecular identification of bacteria from commercial tea.

3. **Gram staining:** For Gram staining, smears from purified cultures were treated with crystal violet for 60 s, Gram's iodine for 60 s, alcohol or alcohol–acetone decolouriser for about 10–20 s, and safranin for 30–60 s, and were examined by light microscopy with a 100× oil-immersion objective.
4. **DNA extraction:** Genomic DNA was extracted from purified cultures with a commercial bacterial DNA extraction kit (MacroGen, Asia) following the manufacturer's instructions, and DNA quantity and quality were assessed with a NanoDrop spectrophotometer.
5. **PCR amplification of the 16S rRNA gene:** The 16S rRNA gene was amplified with primers P0 and P6 (Table 1) in 25 µL reactions containing 12.5 µL Thermo Scientific PCR Master Mix, 1 µL of each primer (10 µM), 2 µL template DNA and 8.5 µL nuclease-free water. Cycling consisted of initial denaturation at 95°C for 3–5 min; 35 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 1 min; and a final extension at 72°C for 10 min. Products were checked by agarose-gel electrophoresis, and the expected amplicon size was approximately 1,550 bp.

Table (1): Primers used for PCR amplification of the 16S rRNA gene.

Primer	Sequence (5'–3')	Target
P ⁰ (forward)	GAAGAGTTTGATCCTGGCTCAG	Bacterial 16S rRNA gene
P ⁶ (reverse)	CTACGGCTACCTGTTACGA	Bacterial 16S rRNA gene

6. **Sequencing and BLAST analysis:** PCR products were sequenced by the Sanger method (Sanger et al., 1977) at MacroGen (Republic of Korea). The sequences, returned in FASTA format, were compared with NCBI nucleotide records using BLAST (Altschul et al., 1990), and isolates were assigned according to the reported best matches when sequence similarity exceeded 97%.

Results:

1. **Isolates recovered:** Seven isolates (T1–T7) were obtained from the five tea products: two from Nabet, one each from Al-Jawhara, Al-Ghazaleen and Al-Baraka, and two from the Zahra red/black tea (Table 2). Tea type and brand are used to describe the samples and were not used as factors in a statistical comparison. Plate counts were not recorded in a form that allows colony-forming units per gram (CFU/g) to be calculated, so the study reports recovery of selected isolates and not microbial load.

Table (2): Tea products sampled and the isolates recovered from each.

Isolate code	Tea category	Brand
T1	Green tea	Nabet
T2	Green tea	Nabet
T3	Green tea	Al-Jawhara
T4	Red/black tea	Al-Ghazaleen
T5	Red/black tea	Al-Baraka
T6	Red/black tea	Zahra red/black tea
T7	Red/black tea	Zahra red/black tea

Results are summarized per isolate in tables. No inferential statistics were applied, and no comparison of bacterial abundance between brands or between green and red/black tea is made. The

number of isolates from a brand is not interpreted as a measure of prevalence or contamination, because colony selection and sampling effort were not standardized.

2. **Colony characteristics:** Colony characteristics were described on nutrient agar by recording color, shape, margin and texture. Colonies were beige-white, greyish white, creamy white, white to light grey or pale yellow (Figure 2). Most were circular and smooth, whereas T1 was relatively dry, T4 formed large colonies with a smooth to slightly rough surface, and T6 formed rough colonies with an irregular margin. The colony and cell characteristics of each isolate are summarized in Table 3. These traits supported the selection of distinct colonies for purification but are not diagnostic of species.



(a). Colonies on agar



(c). Straked culture



B. Pale colony growth



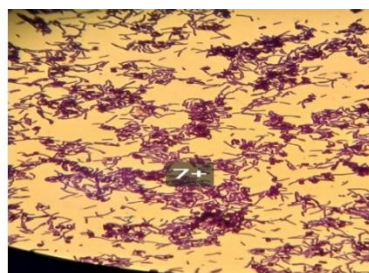
D. Colony morphology (additional plate view)

Figure (2): Colonies of the bacterial isolates on nutrient agar.

Table (3): Colony and cell characteristics of the bacterial isolates on Nutrient Agar.

Isolate	Brand / tea type	Cell shape	Gram reaction	Colony colour	Colony form / texture
T1	Nabet / green	Rod	Positive	Beige-tinged white	Circular; relatively dry
T2	Nabet / green	Rod	Negative	Greyish white	Circular; smooth
T3	Al-Jawhara / green	Rod	Negative	Creamy white	Circular; smooth
T4	Al-Ghazaleen / red/black	Rod	Positive	Creamy white	Large circular; smooth to slightly rough
T5	Al-Baraka / red/black	Rod	Negative	White/light grey	Circular; smooth
T6	Zahra red/black tea / red/black	Rod	Positive	Greyish white	Irregular margin; rough
T7	Zahra red/black tea / red/black	Rod	Negative	Pale yellow	Circular; smooth

3. **Cell morphology and Gram reaction:** All seven isolates were rod-shaped. T1, T4 and T6 were Gram-positive, and T2, T3, T5 and T7 were Gram-negative (Figure 3). This split describes the seven selected isolates only and not the proportion of Gram-positive and Gram-negative bacteria in the products.



(a). Gram-stained cells



(b). Gram-stained cells (second file/image)

Figure (3): Gram-stained cells of the bacterial isolates viewed under oil immersion (100×).

4. **Molecular identification:** BLAST analysis of the partial 16S rRNA gene sequences assigned the isolates to five genera in three families: *Psychrobacillus*, *Priestia* and *Bacillus* (Bacillaceae), *Pseudomonas* (Pseudomonadaceae) and *Sphingobacterium* (Sphingobacteriaceae). Three isolates were pseudomonads. The species assignments and GenBank accession numbers are given in Table 4. The Gram reaction of every isolate agreed with its taxonomic placement: the Bacillaceae isolates were Gram-positive, and the *Pseudomonas* and *Sphingobacterium* isolates were Gram-negative.

Table (4): Molecular identification of the isolates by 16S rRNA gene sequencing and GenBank accession numbers.

Isolate	Brand	Species (BLAST)	GenBank accession
T1	Nabet (green)	<i>Psychrobacillus psychrodurans</i>	PV000778
T2	Nabet (green)	<i>Pseudomonas palmensis</i>	PV000779
T3	Al-Jawhara (green)	<i>Pseudomonas kilonensis</i>	PV000780
T4	Al-Ghazaleen (red/black)	<i>Priestia megaterium</i>	PV000781
T5	Al-Baraka (red/black)	<i>Pseudomonas fragi</i>	PV000782
T6	Zahra red/black tea	<i>Bacillus cereus</i>	PV000783
T7	Zahra red/black tea	<i>Sphingobacterium anhuiense</i>	PV000784

The accession numbers PV000778–PV000784 were reported for the seven partial 16S rRNA sequences (Benson et al., 2013); their accessibility and record-to-isolate correspondence should be confirmed against the live GenBank records before submission. Example sequence traces are shown in Figure 4.

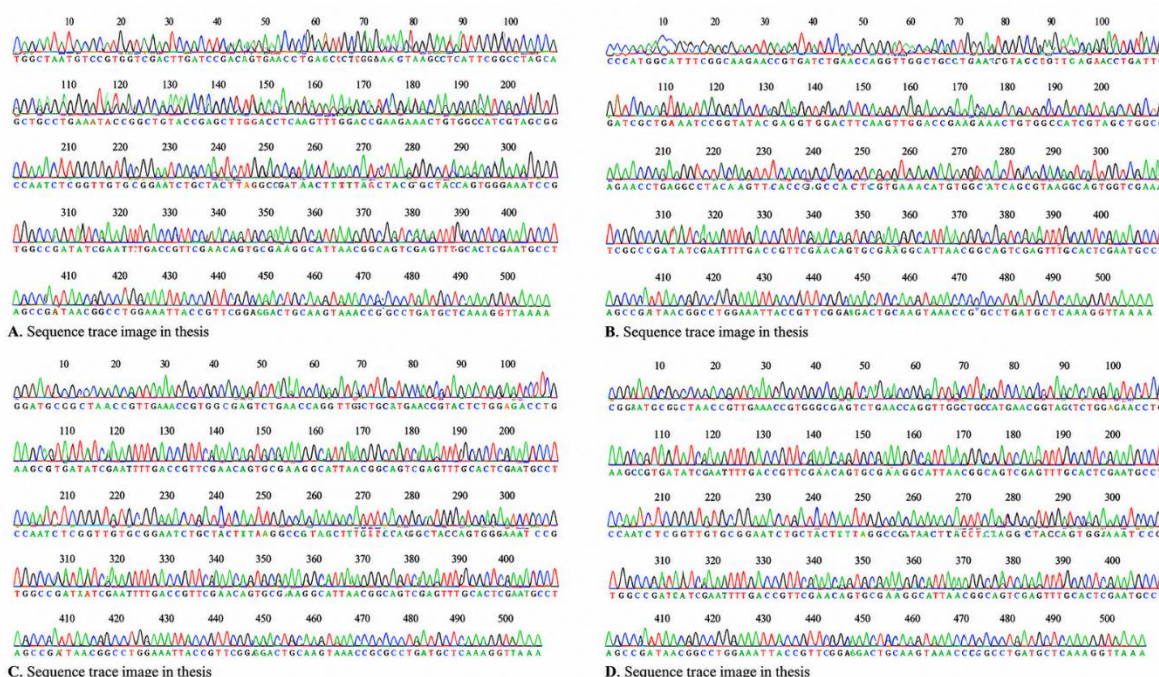


Figure (4): Examples of sequencing chromatogram traces.

Discussion:

1. Diversity of culturable bacteria in dried tea:

Seven culturable isolates representing five genera show that the bacterial community recoverable from the tea products was taxonomically varied, which agrees with earlier reports that commercial dried tea is not sterile (Carraturo et al., 2018; Dharmarathna et al., 2018). A positive culture does not show that bacteria multiplied in the dry product or when contamination occurred. Possible points of entry include cultivation, harvest, drying, processing equipment, packaging, transport, retail handling and storage, and distinguishing among them would require sampling across the production chain.

Reduced water availability generally limits microbial growth, but some cells survive desiccation and grow when transferred to a suitable medium. The procedure used here recovers organisms able to grow on nutrient agar at 30°C within 24 h and may miss slow-growing, nutritionally demanding or anaerobic bacteria. The isolates therefore represent a selected culturable fraction and not the whole tea microbiome.

2. Pseudomonads and environmental bacteria:

Three isolates were pseudomonads (*P. palmensis*, *P. kilonensis* and *P. fragi*). The genus is widespread in plant-associated and moist environments, and some species cause food spoilage; *Pseudomonas* has also been detected in commercial tea in Italy (Carraturo *et al.*, 2018). Because pseudomonads are generally associated with moist habitats, their recovery from dry tea may reflect contamination during processing, blending, packaging or handling, exposure to moisture during storage, or persistence in protected microsites. These are plausible explanations, not conclusions demonstrated by the present experiment: water activity, storage humidity and processing hygiene were not measured. *Sphingobacterium anhuense* (T7) belongs to a genus commonly found in soil and water, which is consistent with possible transfer from soil, water or dust, although these routes were not tested. Taken together, the isolates indicate possible points for hygiene control along the supply chain.

3. Endospore-forming bacteria and *Bacillus cereus*:

Bacillus cereus (T6), *Priestia megaterium* (T4) and *Psychrobacillus psychrodurans* (T1) belong to Bacillaceae, a family that includes endospore-formers whose spores resist drying and heat, which is a plausible explanation for their persistence in a dry product. *Psychrobacillus* was separated from *Bacillus* on taxonomic grounds and includes species tolerant of cool conditions (Krishnamurthi *et al.*, 2011). Its presence may reflect storage or transport conditions, but it may equally reflect its natural habitats, and the data do not distinguish these.

The detection of *B. cereus* deserves attention in food-hygiene monitoring because some strains cause food poisoning. However, 16S rRNA sequencing cannot reliably separate *B. cereus* from closely related members of the *B. cereus* group (Janda and Abbott, 2007) and does not show whether an isolate produces toxin. No toxin assay, virulence-gene test or enumeration was performed, so T6 should be regarded as a member of the *B. cereus* group of unknown toxigenic potential, and its isolation is not evidence of a hazard to consumers. Tea is normally brewed with hot water, which kills vegetative cells, and a risk would arise mainly if brewed tea were kept warm for long periods, which was not investigated.

4. Phenotypic and molecular identification:

Colony morphology and Gram staining separated the isolates but are affected by medium, incubation time and culture age, and similar-looking colonies can belong to different taxa (Beveridge, 2001). Here, T3 (*Pseudomonas*) and T4 (*Priestia*) both formed creamy-white colonies, and only sequencing showed that they belong to different families. Phenotype is best used for screening and description, with species identification resting on sequence data (Franco-Duarte *et al.*, 2019). 16S rRNA gene sequencing resolves some closely related taxa poorly, and confidence depends on sequence length and quality, alignment coverage and the reliability of the reference record (Janda and Abbott, 2007; Yarza *et al.*, 2014). BLAST is a similarity search and not a taxonomic validation (Altschul *et al.*, 1990), so species assignments should be interpreted as preliminary where closely related taxa are difficult to distinguish.

5. Implications for monitoring tea quality:

This study gives an initial descriptive record of bacteria cultured from selected tea brands marketed in Al-Bayda. Surveys of tea at larger scale, such as that of the CFIA (2019), show the sampling effort needed to estimate how often organisms of concern occur. A future study should use independently purchased packages and lots of each brand, define the sampling frame, record dilution and plating details, include negative controls, and count colonies on countable plates so that loads can be expressed as CFU/g. Selective media and toxin-gene screening would be appropriate for *B. cereus*. These results should not be used to rank the brands, to infer that one tea type is more contaminated than another, or to characterise all tea sold in Libya. Two isolates came from Nabet and two from the Zahra red/black tea, but isolate counts are not contamination rates, because sampling effort, package replication and a quantitative denominator were not established for each product. The study is best seen as a small exploratory survey and a foundation for a larger quantitative investigation.

6. Limitations:

Five products and seven selected isolates were examined, and independent packages or lots per brand were not documented. A single medium and one incubation condition were used, so fastidious, anaerobic and psychrophilic bacteria and fungi were not captured. Colony counts and replicate data are lacking, so CFU/g and prevalence cannot be estimated. The sequences are partial 16S rRNA sequences, and additional genes (for example *gyrB* or *rpoB*) would strengthen species assignment of closely related taxa. No virulence or toxin characterisation was done for the isolate assigned to *B. cereus*. Detection of bacteria in dry tea does not show that the brewed beverage is a health risk.

Conclusions:

Seven culturable bacterial isolates from five commercial green and red/black tea products sold in Al-Bayda were assigned by 16S rRNA gene sequencing to *Psychrobacillus psychrodurans*, *Pseudomonas palmensis*, *P. kilonensis*, *P. fragi*, *Priestia megaterium*, *Bacillus cereus* and *Sphingobacterium anhuense* (GenBank PV000778–PV000784). All were rods, three Gram-positive and four Gram-negative. The mix of endospore-formers, pseudomonads and a soil-associated genus is consistent with several possible contamination points along the supply chain. The findings document recoverable bacteria under the conditions tested but do not quantify contamination, establish prevalence or demonstrate a food-safety risk. Hygienic handling and routine microbiological monitoring of tea are warranted, and future studies should use independent product replicates, quantitative enumeration and targeted tests for organisms of food-safety concern.

Practical recommendations:

The findings support routine, risk-based microbiological monitoring of imported and locally marketed tea, with attention to hygienic handling during processing, blending, packaging and retail storage. Tea should be kept in dry conditions in intact, moisture-resistant packaging and protected from dust and contact with contaminated surfaces. Follow-up studies should sample independent packages and production lots, document storage conditions, include appropriate positive and negative controls, and enumerate colonies so that bacterial loads can be expressed as CFU/g. Where *B. cereus*-group isolates are recovered, targeted identification and toxin/virulence testing should be considered before drawing food-safety conclusions. The present study does not establish that any sampled brand is unsafe and cannot be used to rank brands.

Declarations:

Data availability: The thesis reports GenBank accession numbers PV000778–PV000784 for the seven partial 16S rRNA sequences. Before submission, the authors should confirm that each record is publicly accessible and corresponds to the isolate listed in Table 4.

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