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Isolation, identification, and functional characterization of gut-derived microbiota from *Acheta domesticus* (House cricket): Exploring probiotic potential and bioactive metabolite profiling for pharmaceutical applications

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Abstract

Background: The house cricket (*Acheta domesticus*) has recently emerged as a promising source of novel probiotic bacteria and bioactive compounds, yet comprehensive systematic reviews of its gut microbiota isolation and characterization are lacking. This review provides a critical synthesis of isolation methodologies, characterization approaches, and functional properties of cricket-derived bacteria with translational potential for probiotic and pharmaceutical applications.

Methods: A systematic literature review was conducted covering studies on the isolation, cultivation, and characterization of gut microbiota from *Acheta domesticus*. Methodological approaches were critically evaluated, including sample collection and preparation, culture-dependent techniques, culture-independent metagenomic analyses, targeted functional group isolation, phenotypic and genotypic characterization, and functional and safety assessments. Data on microbial diversity, functional activities, and probiotic potential were systematically extracted and analyzed.

Results: The cricket gut microbiota is dominated by Proteobacteria (44%), Firmicutes (31.5%), and Bacteroidetes (24.25%) at the phylum level. Notable isolates include *Lacticaseibacillus paracasei* AL01, *Pediococcus pentosaceus* GL05, and cellulolytic *Lactobacillus* sp. FF02041. These strains exhibit diverse functional properties including uric acid clearance (90–100% of aerobic isolates), antifungal activity against *Aspergillus flavus*, exopolysaccharide (EPS) production with antioxidant (DPPH: 15.93% at 10 mg/mL; ABTS: 25.59% at 10 mg/mL) and α-amylase inhibitory (49.61% at 10 mg/mL) activities. Selected isolates meet probiotic criteria including gastric pH tolerance (>80% survival at pH 2.5–3.0 for 2 hours), bile salt tolerance (>70% survival at 0.3% oxgall), and favorable safety profiles (γ-hemolysis, absence of mucinolytic and gelatinase activities).

Conclusion: *Acheta domesticus* harbors functionally diverse bacteria with significant probiotic and pharmaceutical potential. The identification of LAB with anti-*Aspergillus* activity, cellulolytic capabilities, and EPS with dual antioxidant and α-amylase inhibitory properties represents novel discoveries. However, translational gaps remain including limited strain collections, absence of human clinical trial data, and incomplete mechanistic understanding. Standardized methodologies, expanded isolation campaigns, rigorous *in vivo* testing, and progression to human studies are essential to realize the translational potential of cricket-derived microbiota.

Keywords: Gut microbiota, *acheta domesticus*, lactic acid bacteria, probiotics, exopolysaccharides, antifungal activity, cellulolytic bacteria, metagenomics, 16s rrna sequencing, functional characterization, gut-brain axis, bile salt hydrolase, uric acid metabolism, insect microbiome, pharmaceutical microbiology

Introduction

1. Background and Rationale

The common house cricket, *Acheta domesticus*, has gained significant scientific and commercial attention beyond its traditional roles in pet food and ecological studies. In recent years, the European Union and other jurisdictions have authorized *A. domesticus* as a novel food under regulations such as (EU) 2015/2283, prompting increased research into its nutritional and microbiological properties. This regulatory milestone has revealed that the cricket gastrointestinal tract contains a rich and functionally diverse microbial community with potential applications in human and animal health.

Insect gut microbiota performs essential functions including digestion, immune modulation, pathogen resistance, and even behavioural regulation through the gut-brain axis. In crickets specifically, gut bacteria influence acoustic signalling, territorial aggression, mating success, and longevity. These traits are not only ecologically significant but also commercially relevant for insect farming operations. The ectothermic physiology of crickets renders their microbiota particularly responsive to environmental variables such as temperature, diet, and humidity, which in turn affect host fitness [1].

The global search for novel probiotics has expanded from conventional mammalian sources primarily dairy-derived

lactic acid bacteria—to include insects, which have co-evolved with diverse microbial communities under demanding environmental conditions. Concurrently, the antimicrobial resistance crisis has intensified interest in natural sources of bioactive compounds, including bacteriocins, antifungal metabolites, and exopolysaccharides. Insects, with their high microbial density and specialized gut niches, represent an underexplored reservoir of such compounds.

2. The Cricket Gastrointestinal Tract as a Microbial Niche

The alimentary canal of *Acheta domesticus* is anatomically divided into three regions: foregut, midgut, and hindgut. Each region presents distinct physicochemical conditions that support specialized microbial communities. The foregut receives ingested material and has a relatively short transit time, limiting extensive microbial colonization. The midgut, lined with a peritrophic membrane composed of chitin and glycoproteins, provides attachment surfaces for adherent bacteria. The hindgut, characterized by cuticular structures, slower flow rates, and near-anaerobic conditions, harbours the densest and most metabolically diverse bacterial populations [2].

Quantitative culture-based studies have demonstrated that the hindgut contains approximately three times more culturable bacteria than the midgut, reflecting its role as the primary fermentation chamber. In many insect species, the hindgut microbiota contributes to the breakdown of recalcitrant dietary components such as cellulose, hemicellulose, and lignin. Additionally, the hindgut serves as a site for nitrogen conservation through the recycling of uric acid and other nitrogenous wastes.

The oxygen gradient along the cricket gut—from relatively oxygenated foregut to near-anoxic hindgut—supports a mixture of aerobes, facultative anaerobes, and obligate anaerobes. This diversity makes the cricket gut a valuable model for studying microbial adaptation and functional redundancy. For probiotic and pharmaceutical applications, both aerobic and anaerobic isolates must be considered, as different production conditions may Favor one group over the other [3].

Fig 1: *Acheta domesticus* (House Cricket)

3. Composition of the Cricket Gut Microbiota

Metagenomic sequencing and culture-dependent analyses have provided a progressively refined picture of the bacterial communities inhabiting the *Acheta domesticus* gut. At the phylum level, the microbiota is dominated by three lineages: Proteobacteria, Firmicutes, and Bacteroidetes. Meta-analyses of 16S rRNA gene amplicon data indicate that Proteobacteria constitute approximately 44% of the bacterial community, followed by Firmicutes at 31.5% and Bacteroidetes at 24.25%. Minor phyla, including Actinobacteria and Tener cutes, are detected variably depending on rearing conditions. at the genus level, culture-based studies have recovered *Citrobacter*, *Klebsiella*, *Yersinia*, *Bacteroides*, and *Fusobacterium*. More recent targeted isolations have identified multiple lactic acid bacteria strains, including *Lacticaseibacillus paracasei*, *Pediococcus pentosaceus*, and *Lactobacillus* sp. strain FF02041. The discovery of cellulolytic lactic acid bacteria is particularly noteworthy, as it challenges the conventional view that cellulolysis is primarily mediated by non-LAB bacteria or fungi [4].

The taxonomic composition of the cricket gut microbiota is not static. It varies with age, diet, temperature, and rearing density. For example, rearing crickets at 30°C Favors the presence of Actinobacteria, while lower temperatures reduce their abundance. This plasticity has implications for industrial cricket farming and for the reproducibility of probiotic strain isolation protocols.

4. Functional Significance of Cricket Gut Bacteria

The functional attributes of cricket-associated bacteria extend considerably beyond host nutrition. One of the most striking findings is the uric acid-clearing capacity of hindgut bacteria. Studies have shown that 40–85% of hindgut isolates clear uric acid under anaerobic conditions, while 90–100% exhibit this capability under aerobic conditions. This nitrogen-recycling capacity is ecologically significant, enabling crickets to thrive on low-protein diets and contributing to their efficiency as protein producers for human consumption.

From a biotechnology perspective, cricket gut bacteria have demonstrated several promising activities. Lactic acid bacteria isolated from edible crickets exhibit potent antifungal activity against *Aspergillus flavus*, a ubiquitous fungus that produces carcinogenic aflatoxins. These antifungal effects manifest through inhibition of mycelial expansion and suppression of spore germination, indicating the production of diffusible bioactive metabolites. Gas chromatography-mass spectrometry analysis of cell-free supernatants from these LAB strains has identified multiple organic acids, fatty acids, and other compounds with known antifungal properties [5].

Exopolysaccharides produced by cricket-derived LAB represent another class of bioactive molecules with pharmaceutical potential. EPS from *Lactobacillus* sp. FF02041 has demonstrated concentration-dependent antioxidant activity, including DPPH radical scavenging (15.93% at 10 mg/mL), ABTS radical scavenging (25.59% at 10 mg/mL), and ferric reducing antioxidant power (21.74 mg Fe^{2+} equivalents per gram). Furthermore, this EPS exhibits α-amylase inhibitory activity (49.61% inhibition at 10 mg/mL), suggesting potential applications in managing postprandial hyperglycaemia.

5. Research Objectives and Scope

The convergence of taxonomic diversity, functional richness, and bioactive potential of cricket gut bacteria provides a compelling rationale for systematic investigation. This research review addresses four primary domains:

1. **Isolation methodologies**: Critical evaluation of culture-dependent and culture-independent approaches for recovering gut bacteria from *Acheta domesticus*,

including regional sampling (foregut, midgut, hindgut), selective media, and incubation conditions.

2. **Characterization frameworks**: Analysis of phenotypic (morphological, biochemical, physiological), genotypic (16S rRNA sequencing, phylogenetic analysis), and functional methods for assessing cricket-derived bacterial isolates.

3. **Probiotic potential assessment**: Systematic review of *in vitro* and in *vivo* evidence for probiotic properties, including acid and bile tolerance, adhesion capacity, antimicrobial activity, exopolysaccharide production, and safety considerations.
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4. **Pharmaceutical applications**: Examination of bioactive compounds produced by cricket gut bacteria, including antifungal agents, exopolysaccharides with antioxidant and antidiabetic properties, and enzymes (cellulases, uricase) with industrial and therapeutic potential.

This review synthesizes peer-reviewed literature published between 2015 and 2025, with emphasis on studies that include both culture-dependent and culture-independent analyses. Where data are limited, this review identifies gaps and proposes research priorities for future investigation.

Isolation Methodologies

1. Sample Collection and Preparation

The isolation of gut microbiota from *Acheta domesticus* requires rigorous attention to sample collection and processing to ensure representative recovery of microbial diversity. Crickets are typically obtained from commercial insect farms or laboratory colonies in Babu Mahipati Singh Mahavidyalaya Tiloi, Amethi UP, India. Under the supervision of Miss. Roshni Singh Assistant Professor Department of Zoology Babu Mahipati Singh Mahavidyalaya Tiloi, Amethi Reference No-BMSMV/Bio.024/2026/27 with detailed documentation of rearing conditions—including temperature, humidity, photoperiod, diet composition, and population density—to contextualize microbial findings.

Prior to dissection, crickets are usually surface-sterilized to eliminate external contaminants. A common protocol involves sequential immersion in 70% ethanol (30–60 seconds), followed by sterile distilled water (three rinses), and optionally a 0.1% sodium hypochlorite solution (30 seconds) with additional rinses. Sterility of the surface is confirmed by rolling the sterilized specimen across nutrient agar plates and checking for growth after incubation.

Dissection is performed under aseptic conditions, typically using a laminar flow hood. Crickets are immobilized by chilling at 4°C for 10–15 minutes or by brief exposure to carbon dioxide. Using fine for cells and dissecting scissors, a longitudinal incision is made along the ventral surface. The alimentary canal is gently removed and can be sectioned into foregut (from the pharynx to the proventriculus), midgut (gastric caeca to Malpighian tubule attachments), and hindgut (from Malpighian tubules to anus). Each section is placed in pre-weighed sterile microcentrifuge tubes for downstream processing.

Gut contents are expelled by gentle squeezing with sterile forceps or by homogenizing the gut tissue in sterile phosphate-buffered saline (PBS, pH 7.4) or reduced transport medium for anaerobes. Homogenization can be performed manually using a sterile pestle or mechanically using a bead beater with 0.1 mm glass beads (30 seconds at low speed). Serial ten-fold dilutions (10^{-1} through 10^{-8}) are prepared in sterile diluent (PBS, 0.85% saline, or peptone water).

Several factors influence bacterial recovery at this stage. The time between dissection and plating should be minimized to prevent overgrowth of faster-growing organisms or death of oxygen-sensitive anaerobes. For anaerobic isolates, all steps from dissection to plating should be performed under an anaerobic atmosphere (85% N_2, 10% H_2, 5% CO_2). Additionally, the choice of diluent affects recovery: reduced transport medium containing cysteine, resazurin, and other reducing agents improves survival of strict anaerobes compared to PBS or saline.

Mucolytic agents such as N-acetylcysteine (0.1%) can be added to the homogenization buffer to disrupt the peritrophic membrane and release adherent bacteria. This step is particularly important for isolating LAB and other bacteria that colonize the midgut epithelium. Comparative studies have shown that mucolytic treatment increases culturable counts by 0.5–1.5 $\log_{10}$ CFU/gut section all the Procedure done in Microbiology and Chemistry lab in BMS College of Pharmacy Tiloi, Amethi UP Under the supervision of Prof. (Dr.) Subhashish Tripathy

2. Culture-Dependent Approaches

Traditional culture-based methods remain essential for recovering viable bacterial strains for subsequent probiotic and pharmaceutical evaluation. A range of selective, differential, and non-selective media have been employed for cricket gut samples.

For total aerobic and facultative anaerobic bacterial enumeration, nutrient agar (NA) and tryptic soy agar (TSA) provide baseline estimates. For selective isolation of lactic acid bacteria, de Man, Rogosa, and Sharpe (MRS) agar—often supplemented with 0.02% sodium azide and 0.01% cycloheximide to suppress Gram-negative bacteria and fungi, respectively—is standard. For bifidobacteria, modified MRS with 0.05% L-cysteine hydrochloride and 0.05% mupirocin is used. For enterococci, bile esculin azide agar is appropriate.

For Gram-negative facultative anaerobes, MacConkey agar and eosin-methylene blue (EMB) agar are employed. For strict anaerobes such as *Bacteroides* and *Fusobacterium* species, Bacteroides bile esculin (BBE) agar and supplemented Brucella blood agar are used. For cellulolytic bacteria, carboxymethylcellulose (CMC) agar (containing 0.5–1.0% CMC as the sole carbon source) followed by Congo red staining (0.1% solution) to detect clearing zones is effective.

Incubation conditions significantly influence recovery outcomes. Cultures are typically incubated at 25°C, 30°C, or 37°C. Aerobic incubations are performed in standard microbiological incubators. Anaerobic incubations require anaerobic chambers or gas-generating packets (AnaeroGen) in sealed jars with an indicator strip (e.g., methylene blue) to confirm oxygen exclusion.

Incubation times vary by target group: LAB and facultative anaerobes typically require 24–72 hours, while strict anaerobes may require 5–7 days. For slow-growing

organisms, extended incubation up to 14 days may be necessary.

Quantitative culture results from cricket gut samples consistently show regional differences. Total culturable counts (CFU/gut section) are approximately three-fold higher in hindgut compared to midgut preparations. For example, one study reported 7.2×10^6 CFU/midgut and 2.1×10^7 CFU/hindgut on NA, with corresponding values of 4.8×10^5 and 1.5×10^6 CFU on MRS agar. These regional differences reflect hindgut specialization for microbial fermentation and nutrient recovery.

After colony formation, isolates are selected for purification based on distinct colony morphology (size, color, shape, edge, surface texture, opacity). Subculturing onto fresh plates is performed at least twice to obtain pure cultures. Isolates are preserved for long-term storage in 20–40% glycerol at −80°C, in lyophilized form with skim milk cryoprotectant, or in liquid nitrogen [6].

3. Culture-Independent and Metagenomic Approaches

While culture-dependent methods recover viable strains for application development, they capture only a fraction of total microbial diversity—typically 10–50% depending on the ecosystem. Metagenomic approaches, particularly 16S rRNA gene amplicon sequencing, provide complementary insights into the unculturable majority and enable quantitative comparisons of community composition across samples.

The typical metagenomic workflow begins with total DNA extraction from gut contents or tissue. Mechanical lysis (bead beating with 0.1 mm zirconia beads) combined with chemical lysis (SDS, proteinase K, lysozyme) and heat (65–95°C) yields the highest DNA recovery from Gram-positive bacteria. Commercial kits such as the Qiagen D Neasy Power Soil Kit or the MoBio Power Fecal Kit are widely used and validated for insect gut samples.

PCR amplification of the 16S rRNA gene employs universal bacterial primers targeting hypervariable regions. Commonly used primer pairs include 27F/1492R (full-length, ~1,500 bp), 341F/805R (V3-V4, ~464 bp), and 515F/806R (V4, ~291 bp). The V3-V4 and V4 regions offer a balance between taxonomic resolution and amplicon length compatibility with Illumina sequencing platforms.

High-throughput sequencing platforms include Illumina MiSeq (2 × 300 bp paired-end) for cost-effective profiling, Oxford Nanopore Technologies (MinION, GridION) for full-length 16S rRNA or long-read metagenomics, and PacBio Sequel for high-accuracy long reads. Illumina remains the most widely used for microbial community comparisons.

Bioinformatics processing typically includes: (1) quality filtering using Phred scores (e.g., Q20 or Q30 thresholds); (2) trimming of adapters and primers; (3) merging of paired-end reads; (4) chimera detection and removal (UCHIME, VSEARCH); (5) clustering into operational taxonomic units (OTUs) at 97% similarity or denoising into amplicon sequence variants (ASVs) using DADA2 or Deblur; (6) taxonomic assignment against reference databases (SILVA, Greengenes, RDP, or GTDB); and (7) alpha diversity (Shannon, Simpson, Chao1) and beta diversity (Bray-Curtis, Uni Frac) calculations.

Metagenomic studies have confirmed that the cricket gut microbiota is dominated at the phylum level by Proteobacteria, Firmicutes, and Bacteroidetes. Alpha diversity estimates typically range from Shannon values of 2.5–3.5, indicating moderate diversity compared to mammalian guts (Shannon 3.5–5.0). Beta diversity analyses reveal that rearing temperature and diet are the major drivers of microbial community variation.

A significant methodological finding is that culture-dependent and culture-independent approaches produce different rankings of abundant taxa. For example, while metagenomics identifies *Bacteroides* as a dominant genus, culture-based recovery of *Bacteroides* is often low due to its strict anaerobic requirements and fastidious growth conditions. Conversely, some LAB species are readily cultured but represent less than 1% of metagenomic reads. These discrepancies underscore the necessity of combining both approaches [7].

4. Isolation of Specific Functional Groups

Targeted isolation strategies have been developed to recover bacteria with particular functional attributes relevant to probiotic and pharmaceutical applications.

For cellulolytic bacteria, isolation is performed on CMC agar plates incubated at 30°C for 3–5 days. After colony growth, plates are flooded with 0.1% Congo red for 15 minutes, destained with 1 M NaCl for 15 minutes, and examined for clearing zones around colonies. Cellulase activity (ratio of clearing zone diameter to colony diameter) is calculated to semi-quantitatively rank isolates. Positive isolates are further characterized for endoglucanase, exoglucanase, and β-glucosidase activities using colorimetric assays with carboxymethylcellulose, Avicel, and p-nitrophenyl-β-D-glucopyranoside, respectively. Using this approach, *Lactobacillus* sp. FF02041 was identified as a cellulolytic LAB.

For antifungal activity screening, isolates are tested against target fungi such as *Aspergillus flavus*, *Aspergillus niger*, *Fusarium oxysporum*, and *Candida albicans*. Dual culture overlay assays involve spotting bacterial isolates on agar plates, allowing growth for 24–48 hours, then overlaying with soft agar containing fungal spores (10^6 spores/mL). Alternatively, well diffusion assays utilize cell-free culture supernatants (filter-sterilized, 0.22 µm) placed in wells bored into fungal-seeded agar plates. Both methods measure zones of inhibition after 48–72 hours of fungal growth. This approach successfully identified *Lacticaseibacillus paracasei* AL01 and *Pediococcus pentosaceus* GL05 as potent antifungal probiotic candidates.

For uric acid-clearing bacteria, minimal media containing uric acid (0.1–0.5%) as the sole nitrogen source are used. Isolates are streaked on uric acid agar plates and incubated under aerobic and anaerobic conditions. Clear zones around colonies indicate uric acid hydrolysis. The high prevalence of this trait (90–100% of aerobic isolates, 40–85% of anaerobic isolates) suggests that nitrogen recycling is a conserved function in the cricket gut microbial community.

For exopolysaccharide production, isolates are grown on MRS agar containing 2–5% sucrose or glucose, supplemented with bromocresol purple as a pH indicator. EPS-producing colonies appear mucoid, ropy, or glistening. Semi-quantitative assessment involves measuring colony diameter after 48 hours growth and scoring mucoidy on a 1–4 scale. Positive isolates are confirmed by EPS extraction: overnight broth cultures are centrifuged, supernatants

treated with chilled ethanol (1:3 ratio), and the precipitated polysaccharide collected, dialyzed, and lyophilized.

For bile salt hydrolase activity, isolates are streaked on MRS agar containing 0.5% Tauro deoxycholic acid and 0.37 g/L $CaCl_2$. Precipitated bile acid zones around colonies indicate positive activity. Bile salt deconjugation is often considered an undesirable trait in probiotics due to potential negative effects on lipid metabolism, although the clinical significance remains debated [8].

5. Methodological Considerations and Limitations

Several factors influence the comprehensiveness and reproducibility of cricket gut microbiota isolation. Rearing conditions—particularly temperature, diet, and housing density—significantly affect microbial community composition. Crickets reared at 30°C show higher proportions of Actinobacteria compared to those reared at 20°C or 25°C, while high-density rearing increases the abundance of stress-associated bacteria such as *Yersinia* and *Klebsiella*. Researchers must therefore document all rearing conditions and ideally obtain crickets from standardized commercial sources.

The age and developmental stage of crickets also influence microbial community structure. Newly molted adults (days 1–5 post-final molt) have different microbial communities than mature breeding stock (days 15–30). Additionally, females and males show subtle differences in microbial composition, possibly related to differential reproductive investment. For probiotic strain discovery, sampling young adults is recommended as they are actively feeding and have stable gut physiology.

Seasonal variation, even in laboratory-reared colonies, is an underappreciated confounder. Studies that sample over multiple time points should explicitly analyze temporal variation and consider blocking by collection date.

Culture-dependent methods inevitably select for organisms that tolerate laboratory conditions—temperature, oxygen exposure, nutrient media, and handling stress—potentially missing obligate symbionts, fastidious species, and organisms with stringent growth requirements. For example, spirochetes and some Bacteroidetes are consistently detected by metagenomics but rarely cultured from cricket guts using standard media.

Conversely, culture-independent methods provide community composition data but do not yield viable strains for functional characterization or product development. A combined approach—using metagenomics to guide selective isolation strategies—represents the optimal framework. For example, metagenomic detection of abundant genes for specific metabolic pathways (e.g., cellulose degradation, uric acid metabolism) can inform the design of targeted isolation media and growth conditions.

Methodological standardization is currently lacking in the field. Variability in sample preparation (gut content versus gut wall plus contents), homogenization methods, media formulations, incubation conditions, and data analysis pipelines limit cross-study comparisons. The development of standardized protocols—similar to those developed for human gut microbiota research (IHMS, Earth Microbiome Project)—would accelerate progress.

Finally, laboratory domestication effects should be considered. Bacteria isolated from crickets and subsequently passaged on laboratory media may lose traits that were adaptive in the cricket gut but are no longer selected *in vitro*. For traits of interest (EPS production, antifungal activity), isolates should be screened immediately after isolation and re-tested after cryopreservation to assess stability. Long-term subculturing should be avoided, and master cell banks should be prepared from early passage isolates [9].

Characterization of Cricket Gut Isolates

1. Phenotypic Characterization

Initial characterization of bacterial isolates from cricket gut samples employs standard microbiological techniques. Colony morphology—size (pinpoint to >5 mm), shape (circular, irregular, rhizoid), colour (cream, white, yellow, pink), elevation (flat, raised, convex, umbonate), margin (entire, lobate, filamentous), and surface texture (smooth, rough, dry, mucoid)—provides preliminary discrimination among isolates.

Gram staining reveals cell wall architecture (thick peptidoglycan layer staining purple for Gram-positive, thin layer staining pink for Gram-negative) and is the first step in taxonomic assignment. Cricket gut isolates show both Gram-positive (Firmicutes, LAB, Actinobacteria) and Gram-negative (Proteobacteria, Bacteroidetes, Fusobacteria) profiles, with Gram-negative isolates typically comprising 50–70% of the culturable community.

Additional staining methods provides complementary information. Endospore staining (malachite green-safranin) identifies spore-forming isolates, predominantly within the Firmicutes phylum. Capsule staining (India ink negative staining or Hiss method) identifies encapsulated bacteria, which often show enhanced survival and adhesion. Motility testing in semi-solid agar (0.3–0.4% agar) detects flagellated isolates.

Biochemical profiling using commercial kits provides rapid genus-level assignment. The API 50 CHL system (bioMérieux) is specifically designed for LAB and tests carbohydrate fermentation of 49 substrates. The API 20E system is used for Enterobacteriaceae and other Gram-negative rods, testing 20 biochemical reactions (e.g., β-galactosidase, arginine dihydrolase, lysine decarboxylase, citrate utilization, indole production). The API ZYM system evaluates 19 enzymatic activities (e.g., alkaline phosphatase, esterase, leucine arylamidase, acid phosphatase, β-glucuronidase).

Physiological characterization includes determining optimal growth temperature (temperature range: 15–45°C), pH range (pH 2.0–9.0), and oxygen requirements. Oxygen tolerance is assessed by comparing growth under aerobic, microaerophilic (5% O_2, 10% CO_2, 85% N_2), and anaerobic conditions using anaerobic jars or chambers. Cricket gut isolates typically grow at 25–37°C, with many LAB strains tolerating pH 2.5–3.0 for at least 2 hours.

Salt tolerance (NaCl concentrations of 2%, 4%, 6.5%, and 10%) is relevant for probiotic survival in food matrices. Most LAB isolates tolerate 4–6.5% NaCl, while some halotolerant strains grow at 10% NaCl. Bile salt tolerance (0.3%, 0.5%, 1.0%, 2.0% oxgall) is assessed by comparing viable counts after 2–4 hours exposure to counts in control buffer. Isolates that retain ≥70% viability in 0.3% bile and ≥50% viability in 1.0% bile is considered bile-tolerant [10].

2. Genotypic Identification and Phylogenetic Analysis

Molecular identification has become the gold standard for definitive taxonomic assignment of bacterial isolates. The

16S rRNA gene—present in all bacteria, containing nine hypervariable regions (V1–V9) interspersed among conserved regions, and with an extensive reference database—is the most widely used phylogenetic marker.

PCR amplification uses universal bacterial primers: 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) for near-full-length sequence (~1,500 bp). For routine identification, shorter fragments (~500–900 bp) targeting V1–V3 or V3–V4 regions are sufficient for genus and often species identification. PCR conditions typically include initial denaturation at 95°C for 5 minutes; 30–35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 60–90 seconds; and final extension at 72°C for 10 minutes.

Sanger sequencing of PCR products is performed using the same primers or internal primers (e.g., 518F, 800R). Sequencing reaction products are run on automated capillary sequencers (e.g., ABI 3500, 3730xl). Sequence chromatograms are manually inspected for base-calling quality and ambiguous positions.

Phylogenetic analysis begins with sequence assembly and editing using software such as Bio Edit, Gene Studio, or MEGA. Sequences are compared against the NCBI 16S rRNA database using BL ASTn for initial identification. For definitive placement, sequences are aligned with reference sequences from type strains using alignment algorithms (Clustal W, MUSCLE) [11].

Phylogenetic trees are constructed using: (1) neighbor-joining (NJ) with Kimura 2-parameter or Jukes-Cantor distance models; (2) maximum likelihood (ML) with appropriate substitution models (e.g., GTR+G+I) selected by Model Test or j Model Test; and (3) maximum parsimony (MP) for character-based analysis. Bootstrap resampling (1,000 replicates for NJ/MP, 100–500 for ML due to computational constraints) assesses statistical support for phylogenetic groupings.

Species delineation thresholds: ≥98.7% 16S rRNA gene similarity to a recognized type strain generally indicates the same species; 95–98.7% similarity indicates the same genus; <95% similarity may indicate a novel genus. For strain FF02041, 1,282 base pairs of the 16S rRNA gene were sequenced, showing 99.2% similarity to *Lactobacillus crispatus* ATCC 33820, confirming its placement within the genus *Lactobacillus*.

For LAB isolates, additional molecular markers (*pheS*, *tuf*, *rpoA*, *recA*) provide higher resolution than 16S rRNA alone, particularly for distinguishing closely related *Lactobacillus*, *Lacticaseibacillus*, *Levilactobacillus*, and *Pediococcus* species. For strain typing (differentiating isolates of the same species), pulsed-field gel electrophoresis (PFGE), repetitive element palindromic PCR (rep-PCR), or multilocus sequence typing (MLST) can be employed.

3. Functional Characterization

Beyond taxonomic identification, the utility of cricket gut isolates for probiotic and pharmaceutical applications depends on their functional properties. A standardized panel of *in vitro* assays has been established.

Antifungal activity screening: employs dual culture assays: bacterial isolates are streaked as a line across agar plates, incubated for 24–48 hours, then fungal target spores (10^6 spores/mL in soft agar) are overlayed. Alternatively, well diffusion assays use cell-free supernatants (CFS) from overnight bacterial cultures (centrifuged, filter-sterilized). For heat stability testing, CFS is heated at 60°C, 80°C, or 100°C for 15–30 minutes before testing. For pH sensitivity, CFS is adjusted to pH 6.0–7.0 with NaOH to distinguish acid-mediated from non-acid inhibition. For proteinase sensitivity, CFS is treated with trypsin, proteinase K, or pepsin (1 mg/mL, 37°C, 1–2 hours) to identify proteinaceous bacteriocins.

Cellulolytic activity: is quantified by: (1) CMC-Congo red plate assay for qualitative detection; (2) DNS (dinitrosalicylic acid) assay for endoglucanase activity using CMC substrate, measuring reducing sugar release; (3) filter paper assay (FPase) for total cellulase activity; (4) p-nitrophenyl-β-D-glucopyranoside (pNPG) assay for β-glucosidase activity. One unit of enzyme activity is defined as the amount of enzyme releasing 1 µmol of glucose (or p-nitrophenol) per minute under assay conditions.

Exopolysaccharide production: involves standardized extraction: overnight culture (18–24 hours) in MRS broth with 2–5% glucose or sucrose at 30°C; centrifugation (10,000 × g, 20 minutes, 4°C); supernatant filtration (0.22 µm) to remove residual cells; precipitation with chilled ethanol (1:3 v/v, overnight at 4°C); centrifugation to collect EPS pellet; dialysis (1 kDa cut-off) against distilled water for 48–72 hours with multiple changes; lyophilization to constant weight; and yield calculation (mg EPS/L culture). Chemical composition is analyzed by: total carbohydrate (phenol-sulfuric acid method), protein (Bradford or Lowry method), and uronic acid (m-hydroxydiphenyl method). Monosaccharide composition is determined by HPLC or GC-MS after acid hydrolysis [12].

Antioxidant activity: of EPS or bacterial cultures is assessed by: DPPH radical scavenging (517 nm, measuring reduction in absorbance due to stable DPPH radical); ABTS radical scavenging (734 nm, measuring reduction of pre-generated $ABTS^{\bullet+}$ radical cation); ferric reducing antioxidant power (FRAP) (measuring reduction of Fe^{3+}-TPTZ to Fe^{2+}-TPTZ); and hydroxyl radical scavenging (using deoxyribose or benzoate degradation assays). Results are expressed as percent scavenging or as ascorbic acid equivalents.

α-Amylase inhibitory activity: is measured using the starch-iodine or PNPG method. Reaction mixtures containing α-amylase (from porcine pancreas or human saliva), starch substrate, and EPS (or bacterial CFS) are incubated at 37°C for 10–15 minutes. Residual enzyme activity is measured by color development with iodine (absorbance at 580–620 nm) or by p-nitrophenol release (absorbance at 405 nm). Acarbose is used as a positive control. Percent inhibition is calculated as [(A_control − A_sample) / A_control] × 100.

4. Metabolite Profiling

The bioactive compounds responsible for observed functional activities can be identified through metabolomic approaches. Gas chromatography-mass spectrometry (GC-MS) analysis of cell-free supernatants requires derivatization of non-volatile compounds (e.g., organic acids, sugars, amino acids) using methoximation

(methoxyamine hydrochloride in pyridine) followed by trimethylsilylation (N, O-bis(trimethylsilyl)trifluoroacetamide, BSTFA). Separation is achieved on polar or medium-polar capillary columns (e.g., DB-5, HP-5, or VF-5ms) with temperature programming (60°C to 320°C at 10°C/min). Compound identification uses mass spectral libraries (NIST, Wiley) and retention indices compared to authentic standards.

Liquid chromatography-mass spectrometry: (LC-MS) is preferred for thermolabile, non-volatile, or larger compounds. Reversed-phase columns (C18) with water-acetonitrile gradients (with 0.1% formic acid) are commonly used. High-resolution mass spectrometry (Q-TOF, Orbitrap) provides accurate mass determination for molecular formula assignment. Tandem mass spectrometry (MS/MS) generates fragmentation patterns for structural elucidation.

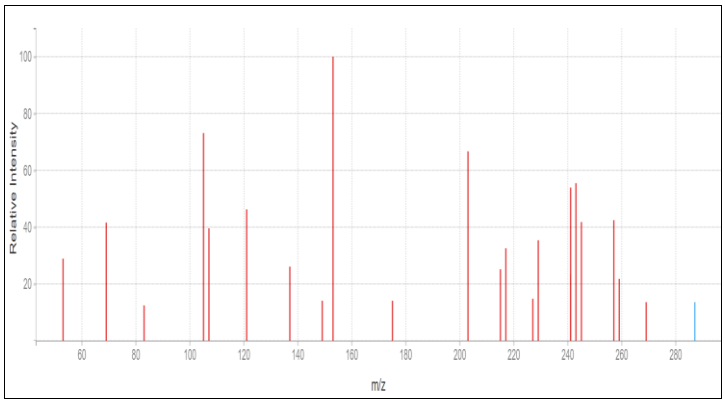

Fig 2: Mass Spectroscopy thermolabile, non-volatile, or larger compounds

For exopolysaccharide structural characterization, Fourier-transform infrared spectroscopy (FTIR) identifies functional groups (e.g., broad O-H stretch at 3400 cm^{-1}, C-H stretch at 2920 cm^{-1}, C=O of carboxyl groups at 1650 cm^{-1}, C-O of glycosidic bonds at 1000-1200 cm^{-1}). Nuclear magnetic resonance (NMR) spectroscopy—^{1}H NMR (400-600 MHz), ^{13}C NMR (75-150 MHz), and 2D techniques (COSY, HSQC, HMBC)—determines linkage positions (α vs. β, 1→3, 1→4, 1→6) and monosaccharide sequence.

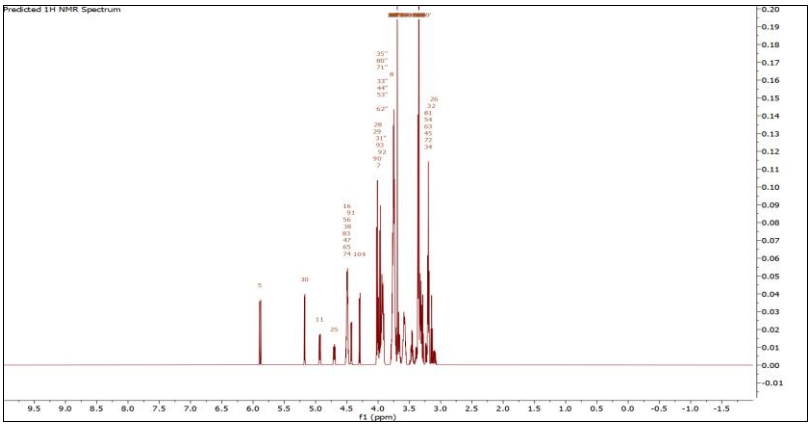

Fig 3: ^{1}H NMR

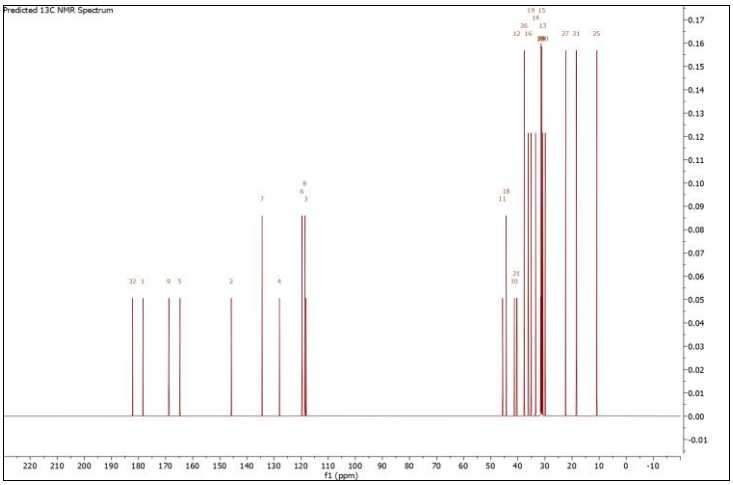

Fig 4: ^{13}C NMR

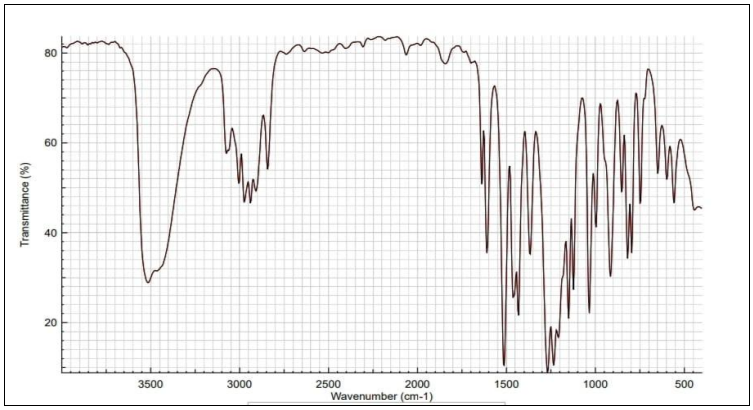

Fig 5: FTIR

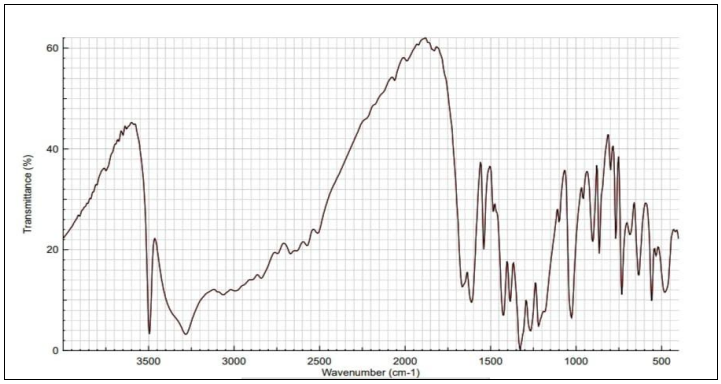

Fig 6: FTIR

Size exclusion chromatography: (SEC) with multi-angle light scattering (MALS) and refractive index (RI) detectors determines molecular weight distribution (number average molecular weight, Mn; weight average molecular weight, Mw; polydispersity, Mw/Mn). Pullulan standards (0.18-800 kDa) calibrate the system.

5. Safety Assessment

For strains intended for probiotic or pharmaceutical use, safety characterization is essential and follows established guidelines (FAO/WHO, EFSA QPS, FDA GRAS).

Hemolytic activity: is assessed on blood agar plates (5% defibrinated sheep or horse blood). After 24-48 hours incubation, colonies are examined for: β-haemolysis (complete lysis, clear zone around colony) — generally disqualifying for live biotherapeutics; α-haemolysis (partial lysis, greenish discoloration) — acceptable but requires careful risk assessment; γ-haemolysis (no activity) — desirable. Cricket-derived LAB typically shows γ-haemolysis.

Antibiotic susceptibility testing: uses disc diffusion (Kirby-Bauer) or broth microdilution to determine minimum inhibitory concentrations (MICs) for antibiotics specified by regulatory authorities (EFSA-recommended panel for LAB: ampicillin, vancomycin, gentamicin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline, chloramphenicol). The absence of acquired resistance genes—confirmed by PCR screening or whole genome sequencing—is essential. Breakpoint values distinguish susceptible from resistant strains.

Bile salt deconjugation: (BSH activity) is assessed by measuring free bile acids by HPLC or by monitoring deoxycholic acid production. Positive strains convert taurocholic or glycocholic acid to cholic plus taurine/glycine.

Mucin degradation: is tested by growing isolates in broth containing 0.5% porcine gastric mucin as the sole carbon source. After 5-7 days, mucin degradation is assessed by residual mucin precipitation with cetyltrimethylammonium bromide (CTAB) or by SDS-PAGE analysis. Mucolytic activity is undesirable as it may compromise intestinal barrier function [13].

Additional safety tests: include: gelatinase activity (gelatin liquefaction test), DNase activity (DNase test agar with methyl green or with HCl flooding), urease activity (Christensen's urea agar), indole production (Kovac's reagent), and production of biogenic amines (tyramine, histamine, putrescine, cadaverine) by HPLC. Absence of these activities supports safety of candidate strains.

Conclusions

1. Summary of Key Findings

The systematic review of literature on the isolation and characterization of gut microbiota from the common house cricket, *Acheta domesticus*, reveals several significant findings with implications for probiotic and pharmaceutical applications.

Taxonomic diversity: The cricket gut microbiota is dominated at the phylum level by Proteobacteria (44%), Firmicutes (31.5%), and Bacteroidetes (24.25%). Cultured isolates from the gut include representatives of *Citrobacter*, *Klebsiella*, *Yersinia*, *Bacteroides*, *Fusobacterium*, and multiple lactic acid bacteria species. The isolation

of *Lacticaseibacillus paracasei* AL01, *Pediococcus pentosaceus* GL05, and *Lactobacillus* sp. FF02041 demonstrates that the cricket gut harbors LAB strains with functional properties comparable to or exceeding those from traditional dairy sources.

Functional richness: Cricket gut bacteria exhibit diverse activities with translational potential. Uric acid clearance is nearly universal among aerobic isolates (90–100%) and common among anaerobes (40–85%), reflecting the cricket's nitrogen conservation strategy. Cellulolytic activity, demonstrated by *Lactobacillus* sp. FF02041, challenges conventional assumptions about the phylogenetic distribution of cellulose degradation. Antifungal activity against *Aspergillus flavus*—including complete inhibition of spore germination and reduction of mycelial growth—is present in multiple LAB isolates. Exopolysaccharide production yields biopolymers with antioxidant and α-amylase inhibitory activities.

Probiotic potential: Selected cricket-derived isolates meet standard *in vitro* probiotic criteria: tolerance to simulated gastric conditions (pH 2.5–3.0 for 2 hours, survival >80%); bile salt tolerance (0.3% oxgall, survival >70%); adhesion-associated properties (auto-aggregation, co-aggregation with pathogens); and antimicrobial activity against Gram-positive and Gram-negative pathogens. Safety assessment reveals absence of hemolytic (γ-hemolysis), mucinolytic, and gelatinase activities, with antibiotic susceptibility profiles lacking transferable resistance genes.

Gut-brain axis evidence: In crickets, antibiotic- and 5-HTP-induced dysbiosis increases agonistic chirping and aggression compared to probiotic-treated groups, demonstrating that gut microbiota composition influences behaviour in the host species. In mice, supplementation with a cricket-containing functional food (MexMix) reduces body weight, liver weight, visceral fat, and metabolic parameters (triglycerides, cholesterol, LDL, insulin, glucose), while improving cognitive performance in novel object recognition testing. These effects are associated with increased abundance of *Lachnospira*, *Blautia*, and *Eubacterium coprostanoligenes*.

Pharmaceutical applications: Cricket-derived LAB metabolites, including exopolysaccharides from *Lactobacillus* sp. FF02041, show concentration-dependent antioxidant activities (DPPH: 15.93% at 10 mg/mL; ABTS: 25.59% at 10 mg/mL; FRAP: 21.74 mg Fe^{2+} equivalents/g). The same EPS exhibits α-amylase inhibitory activity (49.61% at 10 mg/mL, equivalent to 22.74 mg acarbose/g). These dual activities suggest potential applications in managing postprandial hyperglycaemia and oxidative stress. Additionally, cellulolytic and uricolytic activities have industrial and therapeutic relevance [14].

2. Significance and Novelty

This review establishes *Acheta domesticus* as a previously underexplored source of functionally diverse bacteria with probiotic and pharmaceutical potential. The significance of these findings rests on several pillars.

First, the identification of LAB with potent anti-*Aspergillus* activity addresses a critical gap in food safety. Aflatoxin contamination affects 25% of global crop production annually, causing economic losses and contributing to hepatocellular carcinoma. Current control methods rely on chemical fungicides with environmental and regulatory limitations. Cricket-derived LAB, or their metabolites, offer a biological alternative that could be deployed at multiple points in the food supply chain—from pre-harvest field application to post-harvest storage.

Second, the discovery of a cellulolytic *Lactobacillus* expands the known functional repertoire of LAB. Traditional commercial cellulase production depends on fungal systems (*Trichoderma reesei*) or bacterial systems (*Clostridium thermocellum*). LAB offer advantages including GRAS status, simpler growth requirements, and the possibility of in situ production during food fermentation. The same strain simultaneously producing cellulase, EPS, and α-amylase inhibitor suggests potential for consolidated bioprocessing.

Third, EPS with dual antioxidant and α-amylase inhibitory activity is unusual among microbial exopolysaccharides, which typically show either prebiotic effects or immune modulation but rarely direct enzyme inhibition. The potential for a single biopolymer to address two axes of metabolic syndrome (oxidative stress and postprandial hyperglycemia) is commercially attractive.

Fourth, the gut-brain axis findings—both in crickets and in mice—suggest that cricket-derived probiotics might influence neurological and behavioral outcomes. While the mouse study used whole cricket powder rather than isolates, these results justify direct testing of cricket-derived LAB strains in animal models of mood, cognition, and behaviour.

3. Limitations, Gaps, and Future Directions

Despite promising findings, significant limitations must be acknowledged. The number of thoroughly characterized cricket gut isolates remains small (fewer than 20 publicly deposited strains with comprehensive functional data). Most functional data derive from *in vitro* assays, which do not always predict in *vivo* efficacy. Human clinical trial data are entirely absent for cricket-derived probiotics. Mechanistic understanding is incomplete: the specific antifungal metabolites, the structure-activity relationships for EPS bioactivities, and the molecular pathways linking cricket gut bacteria to host behaviour remain undefined.

Eight priority research directions are identified: (1) systematic isolation campaigns targeting multiple cricket populations (geographic, commercial, wild) to expand strain collections; (2) whole-genome sequencing of promising isolates to identify biosynthetic gene clusters and predict safety profiles; (3) rigorous in *vivo* testing in validated animal models (murine metabolic syndrome, *Aspergillus* colonization, cognitive testing) before human trials; (4) mechanistic studies including bioassay-guided fractionation to identify active antifungal compounds and structural elucidation of EPS; (5) formulation and stability studies to enable commercial development; (6) clinical trials progressing from safety (Phase I) to efficacy (Phase II) for lead indications (e.g., postprandial glucose management, aflatoxin exposure reduction); (7) comparative genomics to place cricket-derived strains within the broader context of insect and mammalian probiotics; and (8) regulatory engagement to clarify pathways for cricket-derived probiotics as foods, supplements, or biotherapeutics [15].

4. Final Conclusion

The gut microbiota of *Acheta domesticus* represents a promising frontier in the search for novel probiotics and pharmaceutical agents. The combination of taxonomic novelty, functional diversity (uric acid clearance, cellulolytic, antifungal activity, EPS production), and emerging in *vivo* evidence for metabolic and behavioral effects distinguishes this system from conventional probiotic sources. While significant translational work remains—including expanded isolation campaigns, mechanistic studies, and clinical trials—the foundation established in the current literature supports continued investment in cricket-derived microbiota research. With standardized methodologies, rigorous safety assessment, and progression to human studies, cricket gut bacteria may transition from laboratory curiosities to viable products addressing food safety, metabolic health, and beyond.

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