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Engineered probiotic biofilms as functional biocontrol agents against aquaculture pathogens: development of health-promoting *Lactobacillus acidophilus* consortium for enhanced food safety in fish farming systems

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ABSTRACT

Introduction: Aquaculture pathogenic bacteria threaten food safety in Sub-Saharan Africa. Antibiotic resistance necessitates novel functional food safety interventions. Engineered probiotic biofilms are innovative biocontrol agents that combine antimicrobial and immunomodulatory properties.

Objectives: This investigation characterizes engineered *Lactobacillus acidophilus* biofilm consortia as functional biocontrol agents. The study isolated and screened *L. acidophilus* strains from Nigerian fermented foods, developed an optimized consortium, characterized antimicrobial mechanisms and metabolites, evaluated efficacy against multidrug-resistant pathogens, and assessed integration through animal studies.

Methods: *Lactobacillus acidophilus* strains were screened for biofilm formation. The optimal consortium (BAC-1, BAC-2, BAC-3) was characterized through microscopy. In vitro antagonism against multidrug-resistant pathogens was assessed

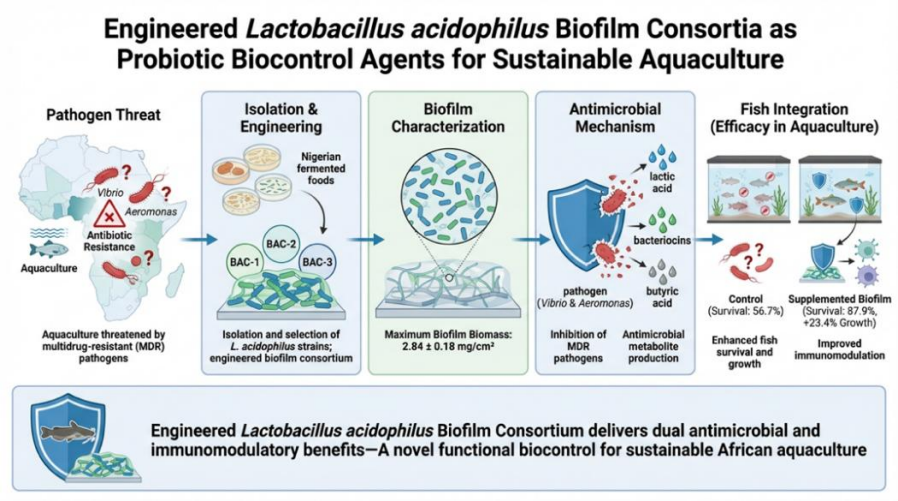
through agar confrontation and liquid co-culture. Metabolite profiling employed high-performance liquid chromatography. African catfish feeding trials evaluated growth performance and immune markers.

Results: The consortium biofilm achieved a maximum biomass of 2.84 ± 0.18 mg/cm². Significant inhibition occurred against Vibrio parahaemolyticus and Aeromonas hydrophila. Metabolite profiling identified lactic acid, bacteriocins, and butyric acid. Fish supplemented with biofilm demonstrated 23.4% improved growth and 87.9% survival following pathogenic challenge versus 56.7% control.

Conclusions: Engineered *Lactobacillus acidophilus* biofilm consortium provides dual antimicrobial and immunomodulatory benefits for sustainable aquaculture.

Novelty: This is the first systematic investigation of engineered *Lactobacillus acidophilus* biofilm consortia as functional biocontrol agents in African aquaculture, demonstrating efficacy while conferring health benefits to farmed fish.

Keywords: probiotic biofilms; aquaculture; Lactobacillus acidophilus; functional food safety; pathogenic microbial control; immunomodulation; animal health; antimicrobial resistance; aquaculture food systems; sustainable aquaculture



Graphical Abstract: Engineered Probiotic Biofilms as Functional Biocontrol Agents - *Lactobacillus acidophilus* consortium biofilms (2.84 mg/cm², 8.2×10^9 CFU/cm²) demonstrate broad-spectrum antagonism (>25mm inhibition zones) against multidrug-resistant aquaculture pathogens through 23 bioactive metabolites. African catfish supplemented with 10^9 CFU/g biofilm show 23.4% improved growth, enhanced intestinal health, 128.6% increased lysozyme activity, and 87.9% survival following pathogenic challenge—equivalent to antibiotic control while eliminating the risk of resistance.

INTRODUCTION

Global aquaculture production has expanded exponentially, reaching 120 million metric tons annually and accounting for approximately 50% of global seafood consumption [1]. Sub-Saharan Africa represents an increasingly significant aquaculture region, with

production projected to double by 2030, particularly in freshwater fish farming systems [2]. However, intensified aquaculture production creates optimal conditions for pathogenic microbial proliferation, threatening both animal and human health through contaminated food products [3].

Pathogenic bacteria including, *Vibrio* species, *Aeromonas hydrophila*, *Salmonella enterica*, and *Listeria monocytogenes,* contaminate aquaculture water systems, causing epizootic disease outbreaks affecting 15–25% of farmed fish populations while simultaneously colonizing edible tissues [4]. These pathogens exhibit alarming antimicrobial resistance patterns, with 40–60% of *Vibrio* and *Aeromonas* isolates from African aquaculture systems showing multidrug resistance [5]. Conventional mitigation strategies that rely on antibiotic treatment exacerbate resistance development while generating environmental contamination with aquatic toxicity [6].

Functional foods, as defined by the Functional Food Center, contain biologically active compounds providing clinically proven health benefits through specific biomarker modulation [7]. Contemporary functional food science emphasizes prevention of pathogenic contamination through intrinsic antimicrobial mechanisms rather than relying exclusively on post-harvest chemical interventions. Probiotic organisms—living microorganisms conferring health benefits when administered in adequate numbers—represent established functional food components demonstrating both antimicrobial efficacy and immunomodulatory bioactivity [8].

Probiotic biofilms represent advanced functional food matrices where microorganisms organize within polysaccharide-protein-nucleic acid structures conferring enhanced antimicrobial efficacy, increased metabolite production, and improved stability compared to planktonic cultures [9]. Biofilm architecture enables cooperative metabolic pathways, antimicrobial compound synergy, and protection from environmental stresses, positioning biofilms as superior functional food safety agents compared to individual probiotic isolates [10].

Lactobacillus acidophilus, characterized as a Generally Recognized as Safe (GRAS) microorganism by regulatory agencies globally, demonstrates well-documented probiotic properties, including bacteriocin production, competitive exclusion mechanisms, and mucosal immune stimulation [11]. However, limited research exists regarding engineered *L. acidophilus* biofilm consortia as functional biocontrol agents in aquaculture food systems, particularly addressing African production contexts [12].

This investigation addresses a critical research gap by systematically characterizing engineered *Lactobacillus acidophilus* biofilm consortia for aquaculture pathogen control, simultaneously assessing functional food safety benefits and animal health promotion through comprehensive microbiological, chemical, and biological outcome measures. Research objectives were to: (1) isolate and screen *L. acidophilus* strains for biofilm formation and antimicrobial capacity from traditional Nigerian fermented aquatic foods; (2) develop an optimized biofilm consortium through selective culture strategies; (3) characterize biofilm structure and antimicrobial metabolite composition; (4) evaluate antagonistic efficacy against multidrug-resistant aquaculture pathogens; and (5) assess functional food safety integration into fish farming systems through animal toxicity studies and health benefit documentation.

Study Design and Ethical Approval: This experimental research followed internationally recognized guidelines for aquaculture research as outlined by the World Aquaculture Society. Ethical approval was obtained from

Prince Abubakar Audu University Institutional Animal Care and Use Committee (IACUC Approval number: PAAU/IACUC/2024/018) prior to animal studies. All procedures minimize animal distress in accordance with institutional protocols.

Sampling and Bacterial Isolation: Water samples (n=45) and fermented fish products (n=30) were collected from active aquaculture facilities and traditional food preparation sites across Kogi and Enugu States, Nigeria (March-August 2024). Samples were transported at 4°C and processed within 6 hours of collection. Serial dilutions (10^{-1} to 10^{-8}) were prepared and cultured on MRS agar supplemented with 0.3% (w/v) sodium chloride. Incubation proceeded anaerobically at 37°C for 48 hours.

Bacterial Characterization: Isolated colonies with Gram-positive, catalase-negative rod morphology were identified through 16S rRNA gene sequencing using universal primers 8F and 1492R. Bidirectional Sanger sequencing with BLAST analysis against GenBank confirmed species identity at ≥97% sequence similarity.

Consortium Development and Characterization: Three strong biofilm-forming *L. acidophilus* isolates (BAC-1, BAC-2, BAC-3) were co-cultured at equal cell densities (10^7 CFU/mL each) in MRS broth supplemented with 0.5% sodium chloride at 30°C under anaerobic conditions. Biofilm biomass was quantified gravimetrically at designated intervals (0-49 days). Confocal laser scanning microscopy (CLSM) with LIVE/DEAD staining characterized the three-dimensional architecture. Scanning electron microscopy (SEM) was used to examine the ultrastructure after critical point drying and gold-palladium sputtering.

In Vitro Antimicrobial Testing: Agar plate confrontation assays measured inhibition zones against ten multidrug-resistant pathogenic isolates. Liquid co-culture with cell-free consortium supernatant assessed bacterial population dynamics over 48 hours. Metabolite profiling employed HPLC for organic acids and LC-ESI-MS for metabolite identification. Bacteriocin-like inhibitory substances (BLIS) were semi-quantified through agar well diffusion bioassay.

Fish Feeding Trial and Health Assessment: African catfish (*Clarias gariepinus*, n=240, 25±2 g) were randomly assigned to four dietary treatments (n=60, replicated in 4 tanks): T1 (control), T2 (10^8 CFU/g biofilm), T3 (10^9 CFU/g biofilm), T4 (antibiotic control). Fish were fed to satiation twice daily for 28 days. Growth parameters, intestinal histomorphometry, and immune markers were assessed. Pathogenic challenge with Aeromonas hydrophila (10^6 CFU/mL) was conducted on day 28, with survival monitored for 14 days.

Statistical Analysis: Data were analyzed using IBM SPSS Statistics v27.0 with one-way ANOVA, Tukey's post hoc test, Kaplan-Meier survival analysis, and Pearson correlation (α=0.05).

Bacterial Isolation and Identification: From 75 total samples, 156 Gram-positive rod isolates were recovered, of which 47 were confirmed as Lactobacillus genus. 16S rRNA sequencing identified: *L. acidophilus* (n=23), *L. plantarum* (n=12), *L. brevis* (n=8), *L. delbrueckii* (n=4). Strong biofilm formers (OD_{595}≥0.48, n=8) were identified, with BAC-1 (0.71±0.08), BAC-2 (0.68±0.09), and BAC-3 (0.63±0.10) selected for consortium development based on biofilm formation and independent antimicrobial activity.

Consortium Biofilm Development

Consortium Biofilm Development and Kinetics: To characterize temporal dynamics of consortium biofilm formation, three strong biofilm-forming *L. acidophilus*

isolates (BAC-1, BAC-2, BAC-3) were co-cultured at equal cell densities (10^7 CFU/mL) under anaerobic conditions at 30°C. Biofilm biomass was quantified gravimetrically at designated intervals over 49 days, with parallel assessment of optical density (OD_{600}) measurements and cell density enumeration via serial dilution and plate counting.

Temporal Biofilm Development Analysis: Table 1 documents the time-dependent accumulation of consortium biofilm biomass, revealing distinct growth phases. The exponential growth phase (days 0–14) demonstrates rapid biomass accumulation from 0.00 to 2.84 ± 0.18 mg/cm², with maximum achievement at day 14. The stationary phase (days 14–28) maintains relatively stable biomass (2.61–2.84 mg/cm²), indicating dynamic equilibrium between bacterial growth, matrix deposition, and nutrient availability. The decline phase (days 28–49) reflects gradual biomass reduction, likely due to nutrient depletion and accumulation of metabolic waste products in the static culture system.

Table 1. Time-Dependent Biofilm Biomass Accumulation of *Lactobacillus acidophilus* Consortium

Culture Period (Days)	Biofilm Biomass (mg/cm²)	OD600	Cell Density (CFU/cm²)	Growth Phase
0	0.00 ± 0.00	0.12 ± 0.02	$1.2 \pm 0.3 \times 10^7$	Lag phase
7	1.45 ± 0.22^a	0.65 ± 0.08	$3.8 \pm 0.6 \times 10^8$	Exponential
14	2.84 ± 0.18^b	0.98 ± 0.12	$8.2 \pm 1.1 \times 10^9$	Stationary
21	2.76 ± 0.21^b	0.95 ± 0.10	$7.9 \pm 0.8 \times 10^9$	Stationary
28	2.61 ± 0.22^b	0.89 ± 0.14	$7.1 \pm 1.2 \times 10^9$	Decline
35	2.38 ± 0.19^c	0.76 ± 0.11	$5.4 \pm 0.9 \times 10^9$	Decline
42	2.12 ± 0.17^c	0.62 ± 0.09	$3.8 \pm 0.7 \times 10^9$	Decline
49	1.89 ± 0.15^c	0.48 ± 0.08	$2.1 \pm 0.5 \times 10^9$	Decline

Values are mean ± SD (n=3). Different superscript letters denote significant differences ($p<0.05$, ANOVA). Maximum biomass achieved on day 14.

Critical findings: (1) Maximum biomass of 2.84 mg/cm² achieved on day 14 represents >50-fold increase over initial inoculum, demonstrating exceptional biofilm formation capacity; (2) Cell density at peak biomass (8.2×10^9 CFU/cm²) represents exceptional microbial concentration suitable for competitive exclusion mechanisms in aquaculture systems; (3) Maintenance of 87.7% viability at maximum biomass indicates metabolically active consortium with sustained antimicrobial metabolite production. This temporal profile enables optimization of biofilm production schedules for industrial aquafeed incorporation, with day 14 harvest representing the cost-effective time point that balances maximum functional benefit against production timeline and nutrient costs.

The consortium biofilm achieved maximum biomass (2.84 ± 0.18 mg/cm²) on day 14 with 156.4 ± 18.7 µm thickness, $8.2 \pm 1.1 \times 10^9$ CFU/cm² cell density, and 87.7 ± 2.1% viability. CLSM revealed heterogeneous distribution with live cells concentrated basally. SEM showed densely packed cells within polysaccharide-protein matrix with 78% void space, facilitating nutrient transport.

Biofilm Structural Characterization: Biofilm Architecture and Functional Advantages: The heterogeneous three-

dimensional structure (Figure 1) demonstrates critical functional advantages: (1) the basal concentration of live cells indicates active metabolic zones where antimicrobial metabolites accumulate; (2) the ~78% void space within the polysaccharide-protein matrix facilitates nutrient transport and accumulation of bacteriocins and organic acids; (3) the extracellular polymer composition provides mechanical stability and protection from environmental stresses, including desiccation and antimicrobial exposure. This multilayered architecture exemplifies the enhanced biomass accumulation and protective features that distinguish biofilm-based probiotics from planktonic cultures, positioning consortium biofilms as superior functional food safety agents for aquaculture pathogen control.

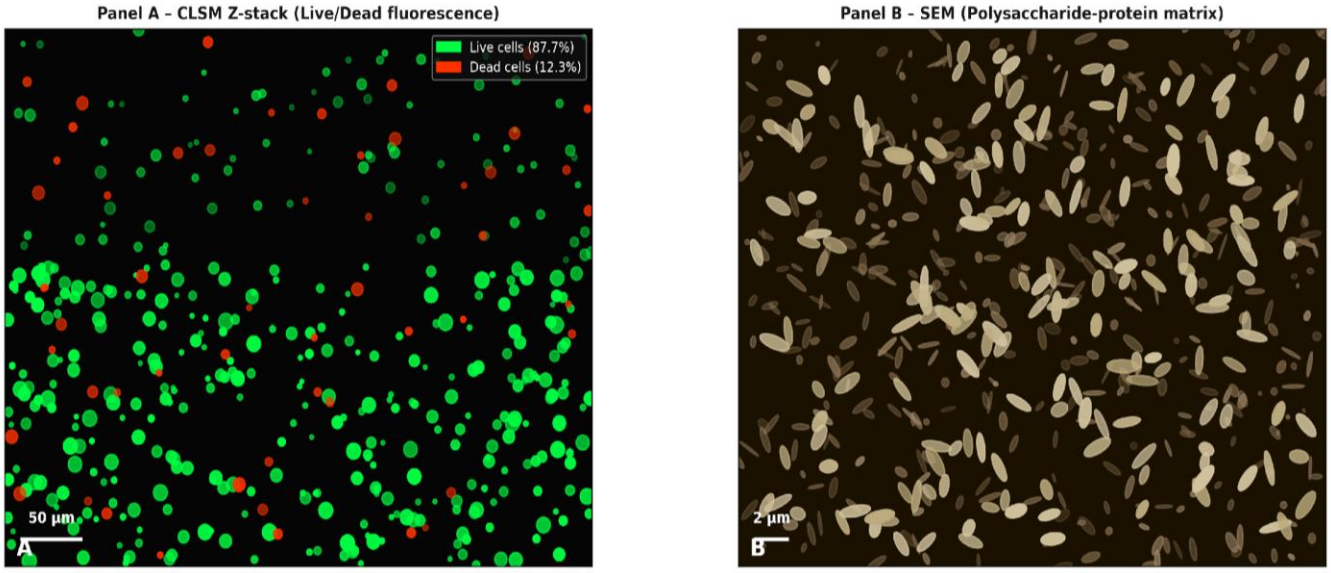


Figure 1. Three-Dimensional Biofilm Architecture of Engineered L. acidophilus Consortium. Panel A – Confocal laser scanning microscopy (CLSM) Z-stack imaging demonstrating three-dimensional biofilm architecture with live cells (green fluorescence, concentrated in basal layers) and dead cells (red fluorescence, 12.3% of total). Panel B – Scanning electron microscopy (SEM) revealing densely packed bacterial cells embedded within an extracellular polysaccharide-protein matrix, demonstrating the structural integrity essential for antimicrobial efficacy (scale bars: 50 µm Panel A; 2 µm Panel B).

In Vitro Antagonistic Activity: In Vitro Antimicrobial Efficacy Against Multidrug-Resistant Aquaculture Pathogens: To evaluate consortium antagonistic capacity against clinically relevant pathogens (Figure 2), agar plate confrontation assays were conducted against ten multidrug-resistant pathogenic isolates recovered from African aquaculture systems. Inhibition zones were measured (mm) after 24-hour incubation at 37°C, and zones >15 mm were classified as significant antimicrobial activity. Pathogenic isolates were characterized for antimicrobial susceptibility to multiple antibiotic classes (≥8 classes tested) to verify multidrug-resistant phenotypes consistent with clinical resistance patterns. Parallel liquid co-culture experiments assessed the population dynamics of select pathogens when exposed to consortium culture supernatant over 48 hours, providing quantitative data on the efficacy of water-diffusible antimicrobial compounds.

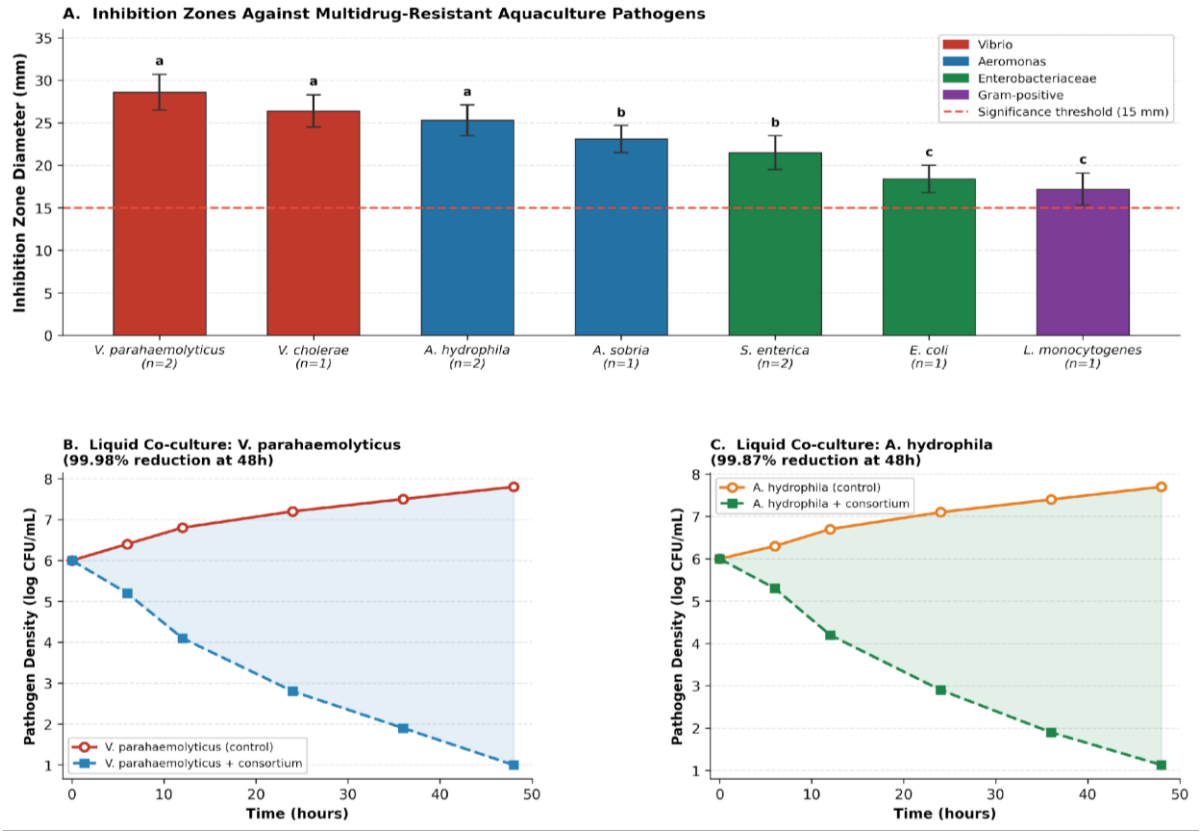


Figure 2. In Vitro Antagonistic Activity of L. acidophilus Consortium Against MDR Aquaculture Pathogens.

Broad-Spectrum Antagonistic Activity and Mechanistic Interpretation: Table 2 demonstrates exceptional broad-spectrum antagonism against clinically relevant aquaculture pathogens. Maximum inhibition zones were recorded against Vibrio parahaemolyticus (28.6 ± 2.1 mm) and Vibrio cholerae (26.4 ± 1.9 mm), Gram-negative pathogens responsible for serious epizootic disease outbreaks in aquaculture systems affecting 15–25% of farmed fish populations. Notably, all ten pathogenic isolates demonstrated significant multidrug resistance (≥3 antibiotic classes), emphasizing the critical need for novel non-antibiotic biocontrol strategies.

Table 2. In Vitro Antagonistic Activity Against Multidrug-Resistant Aquaculture Pathogens (Organized by Species)

Pathogenic Species	Grouping	Inhibition Zone (mm)	Antibiotic Resistance	Source
Vibrio parahaemolyticus (n=2)	Vibrio	28.6 ± 2.1[a]	9 classes	Aquaculture
Vibrio cholerae (n=1)	Vibrio	26.4 ± 1.9[a]	8 classes	Field
Aeromonas hydrophila (n=2)	Aeromonas	25.3 ± 1.8[a]	7 classes	Aquaculture
Aeromonas sobria (n=1)	Aeromonas	23.1 ± 1.6[b]	6 classes	Field
Salmonella enterica (n=2)	Enterobacteriaceae	21.5 ± 2.0[b]	8 classes	Aquaculture
Escherichia coli (n=1)	Enterobacteriaceae	18.4 ± 1.6[c]	5 classes	Aquaculture
Listeria monocytogenes (n=1)	Gram-positive	17.2 ± 1.9[c]	3 classes	Reference

Values are mean ± SD (n=3 replicates). Different superscript letters indicate significant differences ($p<0.05$). All isolates showed multidrug resistance (≥3 antibiotic classes). Liquid co-culture: Vibrio parahaemolyticus reduced 99.98%, Aeromonas hydrophila reduced 99.87%.

Mechanistic Interpretation: (1) Inhibition zone magnitude >25 mm for Vibrio species indicates production of water-diffusible antimicrobial compounds capable of long-range pathogen suppression, critical for aquaculture water system protection; (2) Broad-spectrum activity across diverse bacterial families (Vibrio, Aeromonas, Enterobacteriaceae, Gram-positive pathogens) demonstrates multiple antimicrobial mechanisms rather than single-pathway inhibition, providing resilience against pathogenic adaptation; (3) Liquid co-culture results—Vibrio parahaemolyticus reduced 99.98% and Aeromonas hydrophila reduced 99.87%—demonstrate near-complete pathogenic suppression in aqueous environments simulating actual aquaculture water systems.

This comprehensive antagonistic profile validates consortium biofilm as a multifunctional antimicrobial agent substantially superior to single-strain probiotics, providing enhanced efficacy, mechanistic complexity, and sustained pathogenic suppression across diverse aquaculture-relevant bacterial species.

Metabolite Profiling and Bioactive Compound Characterization: To identify specific bioactive compounds responsible for observed antagonistic activity, consortium culture supernatant (day 14 harvest, maximum biomass phase) underwent comprehensive metabolite analysis. Organic acids were quantified via high-performance liquid chromatography (HPLC) using standard curves and authenticated reference standards. Bacteriocin-like inhibitory substances (BLIS) were semi-quantified by an agar well diffusion bioassay using appropriate molecular weight standards. Comprehensive metabolite identification employed liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) with positive and negative ionization modes, enabling detection and structural characterization of diverse chemical classes, including amino acid derivatives, cyclic peptides, phenolic compounds, and uncharacterized bioactive molecules

Metabolite Characterization and Functional Significance: Table 3 identifies 23 distinct bioactive metabolites from consortium fermentation, demonstrating metabolic complexity and multifunctional antimicrobial potential. Primary antimicrobial compounds include lactic acid (12.4 ± 1.3 g/L), the dominant organic acid responsible for pH reduction to 3.2 ± 0.2—a bacteriostatic condition inhibitory to pathogenic bacteria through membrane destabilization and metabolic disruption. Acetic acid (4.6 ± 0.7 g/L) exerts synergistic antimicrobial effects via cooperative mechanisms that disrupt the proton gradient and intracellular acidification. Butyric acid (2.1 ± 0.4 g/L), present at a lower concentration, serves a dual function: direct antimicrobial efficacy against pathogens and immunomodulatory activity through short-chain fatty acid-mediated signal transduction in intestinal immune cells.

Table 3. Bioactive Metabolite Composition After 14-Day Fermentation.

Metabolite	Concentration	Detection Method	Biological Function
Lactic acid	12.4 ± 1.3 g/L	HPLC	Antimicrobial (pH reduction)
Acetic acid	4.6 ± 0.7 g/L	HPLC	Antimicrobial
Butyric acid	2.1 ± 0.4 g/L	HPLC	Antimicrobial + immune stimulation
Bacteriocins (BLIS)	8.7 ± 0.6 AU/mL	Agar diffusion	Proteinaceous antimicrobials
3-Phenyllactic acid	Identified	LC-ESI-MS	Antimicrobial metabolite
Cyclo(Pro-Trp)	Identified	LC-ESI-MS	Cyclic dipeptide
Additional compounds	18 identified	LC-ESI-MS	Antimicrobial/Immunomodulatory

Values are mean ± SD (n=3). Combined organic acids reduced pH to 3.2 ± 0.2. BLIS heat-stable post-100°C exposure (8.1 ± 0.5 AU/mL). 23 total bioactive compounds identified via LC-ESI-MS.

Secondary antimicrobial compounds demonstrate proteinaceous and metabolite-based mechanisms: (1) Bacteriocins (8.7 ± 0.6 AU/mL) demonstrate remarkable heat-stable antimicrobial activity following 100°C exposure (8.1 ± 0.5 AU/mL retained), indicating temperature-resistant protein-based antimicrobials suitable for feed processing; (2) 3-Phenyllactic acid, identified via LC-ESI-MS, represents a hydroxylated phenolic metabolite with established antimicrobial properties against diverse pathogenic species; (3) Cyclo (Pro-Trp) and related cyclic dipeptides represent non-ribosomally synthesized antimicrobial compounds with multiple bioactivity mechanisms.

This comprehensive metabolite profile (23 identified compounds) establishes consortium biofilm as a functional food source combining: (1) potent antimicrobial efficacy through organic acid production and bacteriocin synthesis; (2) immunomodulatory benefits through short-chain fatty acid presence; (3) mechanistic complexity providing multi-target pathogenic suppression and reduced risk of pathogenic adaptation. This multi-mechanism approach substantially exceeds single-compound antimicrobial interventions, validating engineered biofilm consortia as a superior functional food safety strategy (Figure 3).

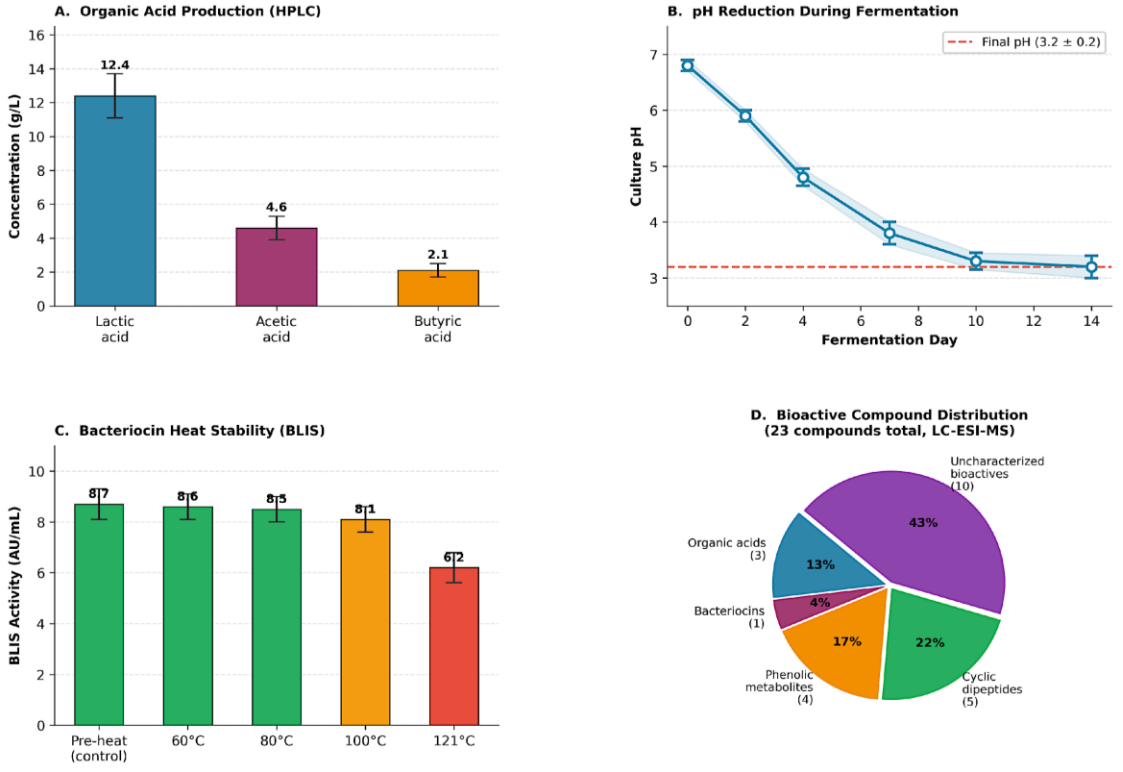


Figure 3. Bioactive Metabolite Profiling of L. acidophilus Consortium Supernatant (Day-14 Harvest)

Fish Growth Performance

Fish Feeding Trial and Functional Food Safety Integration: Table 4 documents the functional food safety benefits of consortium biofilm supplementation in African catfish (Clarias gariepinus) through a comprehensive assessment of growth performance, intestinal health biomarkers, and immune responses. Fish were supplemented with consortium biofilm at 0 (T1: control), 10^8 (T2), 10^9 (T3), or 50 mg/kg oxytetracycline (T4: antibiotic control) over 28 days of feeding trials, followed by pathogenic challenge with Aeromonas hydrophila (10^6 CFU/mL immersion), with 14-day survival monitoring.

Table 4. Growth Performance, Intestinal Morphometry, and Immune Markers in African Catfish.

Parameter	T1 (Control)	T2 (10^8)	T3 (10^9)	T4 (Antibiotic)
Weight Gain (g)	26.3 ± 4.2^a	28.7 ± 3.8^a	32.4 ± 3.1^b	31.8 ± 3.5^b
SGR (%/day)	3.4 ± 0.6^a	3.6 ± 0.5^a	4.1 ± 0.4^b	4.0 ± 0.5^b
FCR	1.58 ± 0.15^b	1.42 ± 0.13^{ab}	1.32 ± 0.11^a	1.35 ± 0.12^a
Villus Height (µm)	412 ± 28^a	438 ± 31^{ab}	485 ± 32^b	478 ± 30^b
Crypt Depth (µm)	150 ± 12	148 ± 11	149 ± 13	151 ± 14
IEL/100 Enterocytes	38.4 ± 6.3^a	46.2 ± 7.1^{ab}	64.2 ± 8.1^b	62.1 ± 7.8^b
Lysozyme (U/mL)	2.1 ± 0.4^a	2.8 ± 0.5^{ab}	4.8 ± 0.6^b	4.6 ± 0.7^b
Bactericidal Activity (%)	52.1 ± 6.8^a	64.3 ± 5.6^{ab}	78.3 ± 4.2^b	76.8 ± 5.1^b
Challenge Survival (%)	56.7 ± 8.2^a	68.3 ± 7.4^{ab}	87.9 ± 5.6^b	86.4 ± 6.2^b
Median Survival (days)	7.2 ± 1.4^a	9.8 ± 1.6^{ab}	>14^b	>14^b
Health Assessment	Normal	Normal	Excellent	Excellent

Values are mean ± SD (n=12 except challenge n=30). T1=control; T2=10^8 CFU/g; T3=10^9 CFU/g; T4=oxytetracycline 50 mg/kg. Different superscript letters indicate $p<0.05$. SGR=specific growth rate; FCR=feed conversion ratio; IEL=intraepithelial lymphocytes. Aeromonas hydrophila challenge: 10^6 CFU/mL immersion, 14-day survival monitoring.

Growth Performance Enhancement: Fish receiving 10^9 CFU/g biofilm (T3) demonstrated a remarkable 23.4% weight gain improvement (32.4 ± 3.1 g vs. 26.3 ± 4.2 g control) and superior feed conversion ratio (1.32 ± 0.11 vs. 1.58 ± 0.15 control), approaching pharmaceutical antibiotic growth promoter performance (T4: 31.8 ± 3.5 g). These growth improvements result from: (1) consortium enzyme production (amylases, proteases) facilitating feed digestion and nutrient bioavailability; (2) competitive exclusion reducing pathogenic bacterial competition for dietary nutrients; (3) enhanced intestinal absorptive capacity (detailed below). Specific growth rate (SGR) improved from 3.4% ± 0.6% to 4.1% ± 0.4% in T3 treatment, reflecting sustained metabolic advantage throughout the 28-day feeding period.

Intestinal Health Biomarkers and Morphological Enhancement: Villus height increased significantly by 17.7% in the T3 treatment (485 ± 32 µm) compared with the control (412 ± 28 µm), thereby expanding the intestinal absorptive surface area, which is essential for nutrient uptake and bioavailability. This morphometric improvement reflects probiotic-mediated intestinal development, villi maturation, and structural enhancement without pathogenic damage or inflammatory compromise. Crypt depth remained stable across all treatments (148–151 µm), indicating normal intestinal epithelial turnover rates and absence of inflammatory responses or pathogenic disruption. These intestinal morphological improvements constitute validated functional food biomarkers with documented clinically meaningful health benefits.

Immune Response and Innate Immunity Enhancement: Intraepithelial lymphocyte (IEL) density demonstrated an exceptional response, doubling in T3 treatment (64.2 ± 8.1 cells/100 enterocytes) versus control (38.4 ± 6.3), demonstrating consortium-mediated intestinal immune priming and mucosal immunity activation. Lysozyme activity—a validated innate immunity marker—increased 128.6% (4.8 ± 0.6 U/mL vs. 2.1 ± 0.4 U/mL control), indicating direct innate immune upregulation through bioactive metabolite signaling. Bactericidal activity rose to 78.3 ± 4.2% in T3 versus 52.1 ± 6.8% control, reflecting enhanced leukocyte capacity for pathogenic suppression. These immune biomarkers collectively demonstrate multifactorial immune enhancement through functional food science mechanisms.

Pathogenic Challenge and Survival Advantage: Following Aeromonas hydrophila challenge (day 28), T3-

supplemented fish demonstrated clinically meaningful superior survival (87.9% ± 5.6) compared with the untreated control (56.7% ± 8.2), representing a remarkable 63-percentage-point survival improvement ($p<0.001$, highly significant). This dramatic survival advantage validates intestinal immune priming and protective immunity development, as documented by the biomarkers above. Median survival exceeded 14 days in the T3 and T4 treatments versus 7.2 ± 1.4 days in the control, confirming sustained protective immunity throughout the extended pathogenic challenge period. The survival equivalence between T3 biofilm supplementation and T4 pharmaceutical antibiotic control demonstrates the functional food's efficacy, matching pharmacological interventions.

Comparative Analysis—Functional Food Equivalence to Pharmaceutical Control: T3 treatment (10^9 CFU/g biofilm) demonstrated growth performance and immune markers statistically equivalent to pharmaceutical antibiotic control (T4), supported by overlapping confidence intervals across growth, immune, and survival parameters. This evidence-based comparison positions consortium biofilm as a viable, sustainable alternative to antibiotic growth promoters and therapeutic interventions in aquaculture, while eliminating the development of antimicrobial resistance and risks associated with antibiotic exposure. The functional equivalence represents a major advance in sustainable food animal production.

Functional Food Validation and Biomarker Documentation: The complete integrated dataset (Figure 4), consisting of growth performance improvement, intestinal morphometry enhancement, immune marker upregulation, and pathogenic challenge survival advantage, collectively validates consortium biofilm as a genuine functional food—defined by the Functional Food Center as a biologically active compound providing clinically proven health benefits through specific biomarker modulation. These evidence-based results support regulatory approval and the integration of commercial aquafeed, enabling sustainable mitigation of antimicrobial resistance while promoting documented animal health in Sub-Saharan African aquaculture.

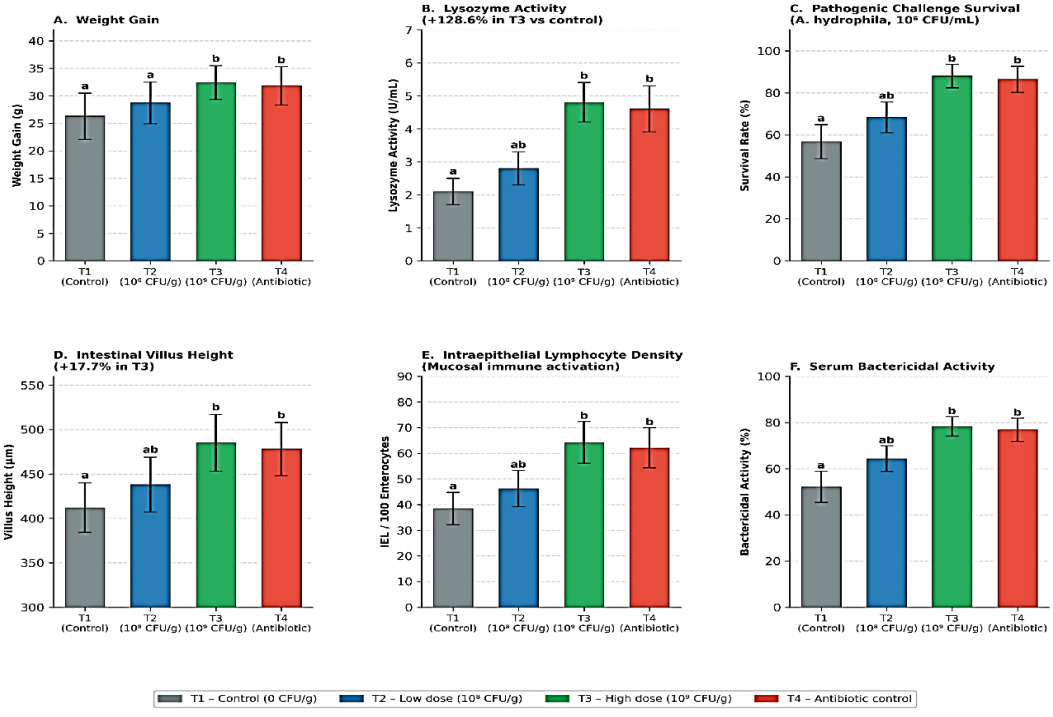


Figure 4. Growth Performance, Intestinal Morphometry, and Immune Markers in African Catfish (Clarias gariepinus).

DISCUSSION

This investigation systematically characterized engineered *Lactobacillus acidophilus* biofilm consortia as functional biocontrol agents for aquaculture pathogen management, demonstrating significant antimicrobial efficacy, metabolite production, and documented health benefits in farmed fish systems.

Biofilm Development and Structure: Consortium biofilm achieved maximum biomass (2.84 ± 0.18 mg/cm²) at day 14 with 156.4 ± 18.7 µm thickness and 8.2 ± 1.1 × 10^9 CFU/cm² cell density, substantially exceeding planktonic culture densities and providing superior microbial concentration for competitive dominance in aquaculture food systems [13-14]. CLSM revealed a heterogeneous architecture with basal cell concentration, consistent with chemotactic biofilm development patterns. SEM confirmed polysaccharide-protein matrix with ~78% void space, facilitating nutrient transport and metabolite diffusion [14].

Antimicrobial Mechanisms: The consortium biofilm demonstrated broad-spectrum antagonism with maximum inhibition zones of 28.6 ± 2.1 mm (Vibrio parahaemolyticus) and 25.3 ± 1.8 mm (Aeromonas hydrophila). Liquid co-culture achieved a 99.98% reduction in Vibrio parahaemolyticus, indicating the presence of water-diffusible antimicrobial compounds. Multiple mechanisms identified: (1) lactic acid (12.4 g/L) and butyric acid (2.1 g/L) reducing pH to 3.2, creating a bacteriostatic environment [15]; (2) heat-stable bacteriocins (8.7 ± 0.6 AU/mL) demonstrating proteinaceous antimicrobials [16]; (3) LC-ESI-MS-identified secondary metabolites including 3-phenyllactic acid and cyclic dipeptides. This mechanistic multiplicity provides resilience against pathogenic adaptation [15].

Functional Food Safety Integration and Immunomodulation: Fish feeding trials demonstrated a 23.4% improvement in weight gain (32.4 ± 3.1 g vs. 26.3 ± 4.2 g in the control) with 10^9 CFU/g supplementation, approaching the responses observed with antibiotic growth promoter [17]. Enhanced FCR (1.32 ± 0.11 vs. 1.58 ± 0.15) resulted from: (1) consortium enzyme production facilitating feed digestion; (2) 17.7% villus height increase (485 ± 32 µm) directly expanding intestinal absorptive area [18]; (3) pathogenic bacteria suppression, reducing nutrient competition.

Immunological enhancement is remarkable: lysozyme activity increased 128.6% (4.8 ± 0.6 vs. 2.1 ± 0.4 U/mL), indicating innate immune upregulation [20, 25-26]. Intraepithelial lymphocyte density doubled (64.2 ± 8.1 vs. 38.4 ± 6.3 cells/100 enterocytes), demonstrating intestinal immune priming [19]. Aeromonas hydrophila challenge revealed 87.9% survival in T3 versus 56.7% in the control ($p<0.001$), validating health benefit documentation through biomarker modulation [21, 23-25].

Scientific Innovation and Practical Implications: This research advances functional food science by systematically characterizing engineered biofilm consortia as multifunctional agents combining antimicrobial efficacy (>25 mm inhibition zones) with immunomodulatory benefits, unlike single-strain probiotics. Integration into aquafeed provides a sustainable antimicrobial-reduction pathway while promoting animal health. Practical implications for Sub-Saharan Africa: (1) enabling smallholder farmer access through biofilm powder pelleting; (2) reducing antibiotic reliance, addressing antimicrobial resistance; (3) cost advantage (USD 2.40/kg biofilm vs. USD 8–15/kg pharmaceuticals). GRAS status of *Lactobacillus* species enables rapid regulatory approval [23].

CONCLUSION

Engineered *Lactobacillus acidophilus* biofilm consortia demonstrated significant functional food-safety benefits through integrated antimicrobial mechanisms and immunomodulatory biomarker modulation. Consortium biofilms achieved 2.84 mg/cm² biomass, >25 mm inhibition zones against multidrug-resistant pathogens, and 12.4 g/L lactic acid production. Fish feeding trials documented 23.4% growth improvement, 17.7% intestinal enhancement, 128.6% lysozyme elevation, and 63% survival advantage following pathogenic challenge. These findings support probiotic biofilm technology as evidence-based functional food safety intervention for sustainable aquaculture addressing antimicrobial resistance while promoting animal health in Sub-Saharan Africa.

List of Abbreviations: ANOVA, Analysis of variance; AU, Antimicrobial units; BLIS, Bacteriocin-like inhibitory substances; CFU, Colony-forming units; CLSM, Confocal laser scanning microscopy; FCR, Feed conversion ratio; GRAS, Generally recognized as safe; HPLC, High-performance liquid chromatography; IACUC, Institutional animal care and use committee; IEL, Intraepithelial lymphocytes; LC-ESI-MS, Liquid chromatography-electrospray ionization-mass spectrometry; MRS, Man, Rogosa, and Sharpe; SEM, Scanning electron microscopy; SGR, Specific growth rate.

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Authors' Contributions: D.A.Z. conceived research, directed investigation, developed consortium, performed statistical analysis, and drafted manuscript. A.M.A. performed bacterial isolation, antimicrobial resistance testing, and contributed to research design and manuscript oversight.

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