Analysis of Unique Genes Reveals Potential Role of Essential Amino Acid Synthesis Pathway in *Flavobacterium covae* Virulence

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24 **ABSTRACT**

25 *Flavobacterium covae* is one of the four *Flavobacterium* species causing columnaris disease
26 among warm-water fish. *Flavobacterium covae* strain 94-081 is highly virulent in channel
27 catfish (*Ictalurus punctatus*) compared to *F. columnare* strain ATCC 49512. This study
28 focused on analyzing the unique genes present in the genome of *F. covae* strain 94-081
29 compared to the genome of *F. columnare* strain ATCC 49512. Our comparative genome
30 analysis revealed presence of 36 proteins unique to *F. covae.* Within the unique genes, nine
31 genes (*leuA*, *leuB*, *leuC*, *leuD*, *ilvA*, *ilvB*, *ilvC*, *ilvD*, and *ilvH*) were associated with the
32 branched-chain amino acids (BCAA) biosynthesis pathway and seven genes (*cysD*, *cysE*, *cysG*,
33 *cysH*, *cysI*, *cysK*, and *cysN*) were involved in the cysteine biosynthesis pathway. Among the
34 BCAA biosynthesis-related genes, *ilvC* (ketol-acid reductoisomerase) and *ilvD* (dihydroxy-acid
35 dehydratase) showed potential interaction with catfish proteins based on the host-pathogen
36 interaction database analysis. To investigate the functional significance of the BCAA pathway,
37 we generated *F. covae leuD* (*Fc*Δ*leuD*) and *ilvD* (*Fc*Δ*ilvD*) mutants. The deletion of *leuD* and
38 *ilvD* genes did not cause growth defects, but they showed reduced virulence in catfish (47.5%
39 and 70% mortality, respectively) compared to the wild-type strain (*Fc*WT) (100% mortality).
40 This is the first report indicating a potential role of the BCAA pathways in *F. covae* virulence.
41 Further studies will reveal the role of the cysteine biosynthesis pathway and other unique genes
42 on *F. covae* virulence.

43

44

45 **KEYWORDS**

46 *Flavobacterium covae*, columnaris, *leuD*, *ilvD*, *ilvC*, BCAA, virulence

47 **1 INTRODUCTION**

48 Columnaris disease affects freshwater fish species, including trout, salmon, carp, tilapia, perch,
49 and catfish (J. F. Bernardet, Grimont, P.A.D., 1989). Columnaris outbreaks in cultured channel
50 catfish often occur during the spring and autumn and can lead to substantial mortality rates,
51 particularly in stress and poor environmental conditions. The disease can manifest gradually as
52 chronic infections with a progressive increase in mortalities or rapidly cause high mortalities
53 within a few days (Austin, 2012; Wakabayashi, 1991). It causes "saddleback" lesions and
54 affects fins, skin, and gills (S. A. Bullard, McElwain, A., Arias, C.R., 2011; Decostere, 2002;
55 S. Kumru, Tekedar, H.C., Gulsoy, N., Waldbieser, G.C., Lawrence, M.L., Karsi, A., 2017).

56 The causative agent of columnaris disease was first isolated by Davis in 1922 and has
57 since been found in fresh and brackish water worldwide (J. F. Bernardet, 1997; J. F. Bernardet,
58 Segers, P., Vancanneyt, M., Berthe, F., Kersters, K., Vandamme, P., 1996; Davis, 1922;
59 Plumb, 1999). The causative agent was considered *Flavobacterium columnare*, but *F.*
60 *columnare* exhibited varying colony morphologies, genetic heterogeneity, and significant
61 variation in virulence across different fish species. Genomic analyses revealed extensive
62 genetic diversity among *F. columnare* strains, characterized by variations in average nucleotide
63 identity (ANI), DNA-DNA hybridization, and multilocus phylogenetic analysis involving 16S
64 rRNA and housekeeping genes (Dong, 2014; Kayansamruaj, 2017; S. Kumru, Tekedar, H.C.,
65 Waldbieser, G.C., Karsi, A., Lawrence, M.L., 2016; B. R. LaFrentz, Garcia, J.C., Waldbieser,
66 G.C., Evenhuis, J.P., Loch, T.P., Liles, M.R., Wong, F.S., Chang, S.F., 2018). Based on these
67 genetic results, *F. columnare* was classified into four different genetic clusters (S. Kumru,
68 Tekedar, H.C., Blom, J., Lawrence, M.L., Karsi, A., 2020; B. R. LaFrentz, Garcia, J.C.,
69 Waldbieser, G.C., Evenhuis, J.P., Loch, T.P., Liles, M.R., Wong, F.S., Chang, S.F., 2018).

70 It is now well-established that columnaris disease is caused by four different
71 *Flavobacterium* species, *F. columnare, F. covae, F. davisii,* and *F. oreochromis*, which
72 correspond to the genomovar groups I to IV (B. R. LaFrentz, Kralova, S., Burbick, C.R.,
73 Alexander, T.L., Phillips, C.W., Griffin, M.J., Waldbieser, G.C., Garcia, J.C., de Alexandre
74 Sebastiao, F., Soto, E., Loch, T.P., Liles, M.R., Snekvik, K.R., 2022). *Flavobacterium*
75 *columnare* isolates exhibit higher virulence towards cold-water fish species such as rainbow
76 trout, whereas *F. covae* is known for its heightened virulence towards warm-water fish species,
77 particularly catfish (Arias, 2004; S. A. Bullard, Mohammed, H., Arias, C.R., 2013; Darwish,
78 2005; Evenhuis, 2016; Olivares-Fuster, 2007; Shoemaker, 2008; Triyanto and Wakabayash,
79 1999). While genome sequencing and comparative genome analyses of *F. columnare* and *F.*
80 *covae* revealed their general features (S. Kumru, Tekedar, H.C., Gulsoy, N., Waldbieser, G.C.,
81 Lawrence, M.L., Karsi, A., 2017), studies on species-specific genes have not yet been
82 conducted. In this study, we determined the function-based unique genes and pathways found
83 in the *F. covae* genome and showed the potential role of essential amino acid synthesis
84 pathways in *F. covae* virulence in channel catfish.
85

86 **2 MATERIALS AND METHODS**

87 **2.1 Bacterial species and growth conditions**

88 Bacterial strains and plasmids utilized in this study are listed in Table 1. The *F. covae* strain
89 94-081 was cultivated using *F. columnare* growth medium (FCGM) agar or broth at a
90 temperature of 30°C (Farmer, 2004). *Escherichia coli* strains were cultured on Lysogeny agar
91 (LA) and in Lysogeny broth (LB) by incubating at 37°C in a shaker incubator at 200 rpm.

92 When necessary, the following antibiotics were added to the bacterial cultures: ampicillin (100

93 μg/ml), colistin (12.5-25 μg/ml), and cefoxitin (10 μg/ml).

94

95 TABLE 1 Bacterial strains and plasmids used in this study.

Bacterial strains	**Description**	**References**
Flavobacterium covae		
Strain 94-081	Wild type	(Soto, 2008)
FcΔleuD	94-081 derivative; col; Δ*leuD*	This study
FcΔilvD	94-081 derivative; col; Δ*ilvD*	This study
Escherichia coli		
BW19851λ*pir*	*RP4-2 (Km::Tn7, Tc::Mu-1), DuidA3::pir+, recA1, endA1, thi-1, hsdR17, creC510*	(Metcalf, 1994)
DH5αMCR	F− *supE44 endA thi-1* λ *recA1 gyrA96 relA1 deoR* Δ(*lacZYA-argF*)*U169 φ80lacZ*Δ*M15 mcrA* Δ(*mrr hsdRMS mcrBC*)	(Grant, 1990)
Plasmids		
pCP29	ColE1 ori; (pCP1 ori); Ap^r (Cf^r Em^r); *E. coli-F. psychrophilum* shuttle plasmid	(Kempf, 2000)

96

97 **2.2 Identification of unique genes and pathways**

98 To identify genes unique to *F. covae*, nucleotide files of *F. columnare* strain ATCC 49512

99 (accession # NC_016510.2) and *F. covae* strain 94-081 (accession # NZ_CP013992.1) were

100 obtained from the National Center for Biotechnology Information (NCBI). Subsequently, the

101 nucleotide sequence files were uploaded to the RAST (Rapid Annotation using Subsystem

102 Technology) annotation pipeline (version 2.0) (Overbeek, 2014) for annotation. Default

103 features (RAST annotation scheme: classic RAST, gene caller: RAST, FIGfam version:

104 Release70, automatically fix errors, fix frameshifts, build metabolic model, backfill gaps, turn

105 on debug, and disable replication: yes, verbose level: 0) were applied during the annotation

106 process. The SEED viewer was utilized to compare the annotated genomes of *F. columnare*

107 strain ATCC 49512 and *F. covae* strain 94-081. The SEED viewer also facilitated KEGG

108 metabolic analysis using comparative tools (Overbeek, 2014). Unique genes were compared
109 against other species using the NCBI's Basic Local Alignment Search Tool.
110
111 **2.3 Host pathogen protein-protein interactions**
112 The host-pathogen interaction database (HPIDB 3.0) was employed to investigate protein-
113 protein interactions between *F. covae* proteins and channel catfish proteins (Accession#
114 NC_030416). The host and pathogen files were downloaded from the NCBI and uploaded to
115 the HPIDB database using default features (search by homologous HPI, search options: for the
116 set of host and pathogen proteins, identify homologous interactions) (Ammari, 2016). Cellular
117 locations of interacting proteins were predicated by PSORTdb 4.0 (Lau et al., 2021).
118
119 **2.4 In-frame deletion of the *Flavobacterium covae leuD* and *ilvD* genes**
120 The nucleotide sequences of *leuD* (accession #: AWN65_RS09580) and *ilvD* (accession #:
121 AWN65_RS09745) were obtained from the *F. covae* genome available at NCBI (accession #
122 NZ_CP013992.1) (S. Kumru, Tekedar, H.C., Waldbieser, G.C., Karsi, A., Lawrence, M.L.,
123 2016). Markerless in-frame gene deletion was conducted following the previously published
124 procedures (Abdelhamed, Nho, Karsi, & Lawrence, 2021). Briefly, primers (Table 2) were
125 designed using online primer design software (Untergasser, 2012), and the upstream and
126 downstream regions of the genes were amplified separately and then combined through overlap
127 extension PCR (Horton, 1990). The resulting product and the suicide plasmid pCP29 were
128 digested with the same restriction enzymes and then ligated to the pCP29 plasmid (Kempf,
129 2000). The recombinant plasmid was isolated from *E. coli* DH5-α (Reeves, 1996) and

130 electroporated into *E. coli* BW19851 (Metcalf, 1994) for the conjugative transfer of pCP29 into

131 *F. covae*.

132

133 TABLE 2 Primer sequences for in-frame deletion.

Primers	Sequence (5' to 3')[a]	Restriction enzyme
*leuD*EF01	**atgtcgac**TTGGCACAAGTCAGGTAGCAC	SalI
*leuD*IR01	ACGAGCAGGTATAATTTGGTCTG	
*leuD*IF01	cagaccaaattatacctgctcgtGAAGCCTTTGAAAAATCAAGAAA	
*leuD*ER01	**atctgcag**TCCAACAACTTCCGTTGAATAA	PstI
*ilvD*EF01	**atgtcgac**AACATCGAACCATACATTCGTG	SalI
*ilvD*IR01	TTGGGTAATCGTTTTGCTGT	
*ilvD*IF01	acagcaaaacgattacccaaGCCTCTCAAGGATGTGTTACCG	
*ilvD*ER01	**atctgcag**TCTATCATCAAACCGCATTCC	PstI

134 [a] Lowercase bold letters show restriction enzyme recognition sequences added to primers.

135 Lowercase letters in IF01 primers indicate reverse complemented IR01 reverse primer

136 sequences.

137

138 Transformants were selected on FCGM agar supplemented with cefoxitin and colistin.

139 Confirmation of the merodiploid first recombinant event was achieved through colony PCR.

140 PCR-confirmed positive colonies were streaked onto FCGM agar supplemented with colistin to

141 screen for the second recombinant event and loss of the wild-type gene. Subsequently, these

142 colonies were re-streaked on FCGM agar plates containing colistin and FCGM agar plates

143 containing cefoxitin. Positive colonies that grew on FCGM agar with colistin but not on FCGM

144 agar with cefoxitin were selected, and PCR was performed to confirm the gene deletion and

145 absence of the wild-type gene. For validation of the *F. covae* mutants, the PCR product was

146 sequenced. The new strains were designated as *Fc*Δ*leuD* and *Fc*Δ*ilvD*.

147

148 **2.5 Bacterial growth kinetics**

149 Overnight cultures of *F. covae* mutants (*Fc*Δ*leuD* and *Fc*Δ*ilvD*) and wild-type (*Fc*WT) were
150 prepared, and the optical densities at 600 nm (OD_{600}) were measured. After density adjustment,
151 cultures were diluted 1:100 with fresh FCGM and cultivated at 30ºC. The OD_{600} values were
152 measured every hour for a duration of 72 hours. The average optical absorbance at each time
153 point was calculated and used to determine the bacterial growth rate.

154

155 **2.6 Virulence of mutants in catfish**

156 Experimental infections of catfish were conducted under an approved protocol of the
157 Mississippi State University Institutional Animal Care and Use Committee following the
158 methodology previously described (S. Kumru, Tekedar, H.C., Gulsoy, N., Waldbieser, G.C.,
159 Lawrence, M.L., Karsi, A., 2017). Specifically, specific pathogen-free catfish (10 fish per tank)
160 were transferred to 40-liter tanks, with each treatment consisting of 4 replicates. Catfish
161 fingerlings had an average weight of 14.2±2.9 grams and a length of 22.1±0.6 centimeters.
162 Tanks were supplied with flow-through dechlorinated municipal water, and water temperature
163 was maintained at 30°C throughout the experiments. For experimental infections, *FcΔleuD*,
164 *FcΔilvD*, or *FcWT* cultures were grown in a shaking incubator at 150 rpm for 18 h. After
165 adjustment of OD_{600} to 0.6, 50 ml cultures were added to assigned tanks with 10 liters of water
166 (~5 x 10^6 CFU/ml of water). The exposure lasted 1 hour, after which the water flow was
167 restored. A sham control treatment (sterile FCGM broth) was also included. Mortalities were
168 recorded daily for 8 days, and the percent and cumulative mortalities were calculated for each
169 experimental group. Kaplan-Meier survival analysis test was used to compare the mortality

170 rates of treatment and control groups. Pairwise comparisons were conducted using the Mantel-

171 Cox Log Rank test (IBM SPSS v 29 Armonk, NY). The significance threshold was $p < 0.05$.

172

173 **3 RESULTS**

174 **3.1 Unique proteins and pathways of *Flavobacterium covae***

175 We identified 36 unique protein-coding genes in *F. covae* strain 94-081 compared to *F.*

176 *columnare* strain ATCC 49512 based on gene annotations. These unique proteins were

177 categorized based on their roles (Table 3). Through pathway analysis, we identified that the

178 branched-chain amino acid (BCAA) biosynthesis pathway was present in *F. covae*, *F. davisii*,

179 and *F. oreochromis*, while not in *F. columnare*. This pathway comprises two clusters of 9

180 unique genes (*leuA*, *leuB*, *leuC*, *leuD*) and (*ilvA*, *ilvB*, *ilvC*, *ilvD*, and *ilvH*). Similarly, the

181 cysteine biosynthesis pathway was present only in *F. covae* and *F. davisii*. The valine-leucine-

182 isoleucine biosynthesis pathway of *F. columnare* and *F. covae* are depicted in Figure 1A and

183 1B, respectively.

184

185 TABLE 3 *F. covae* 94-081 unique protein-coding genes

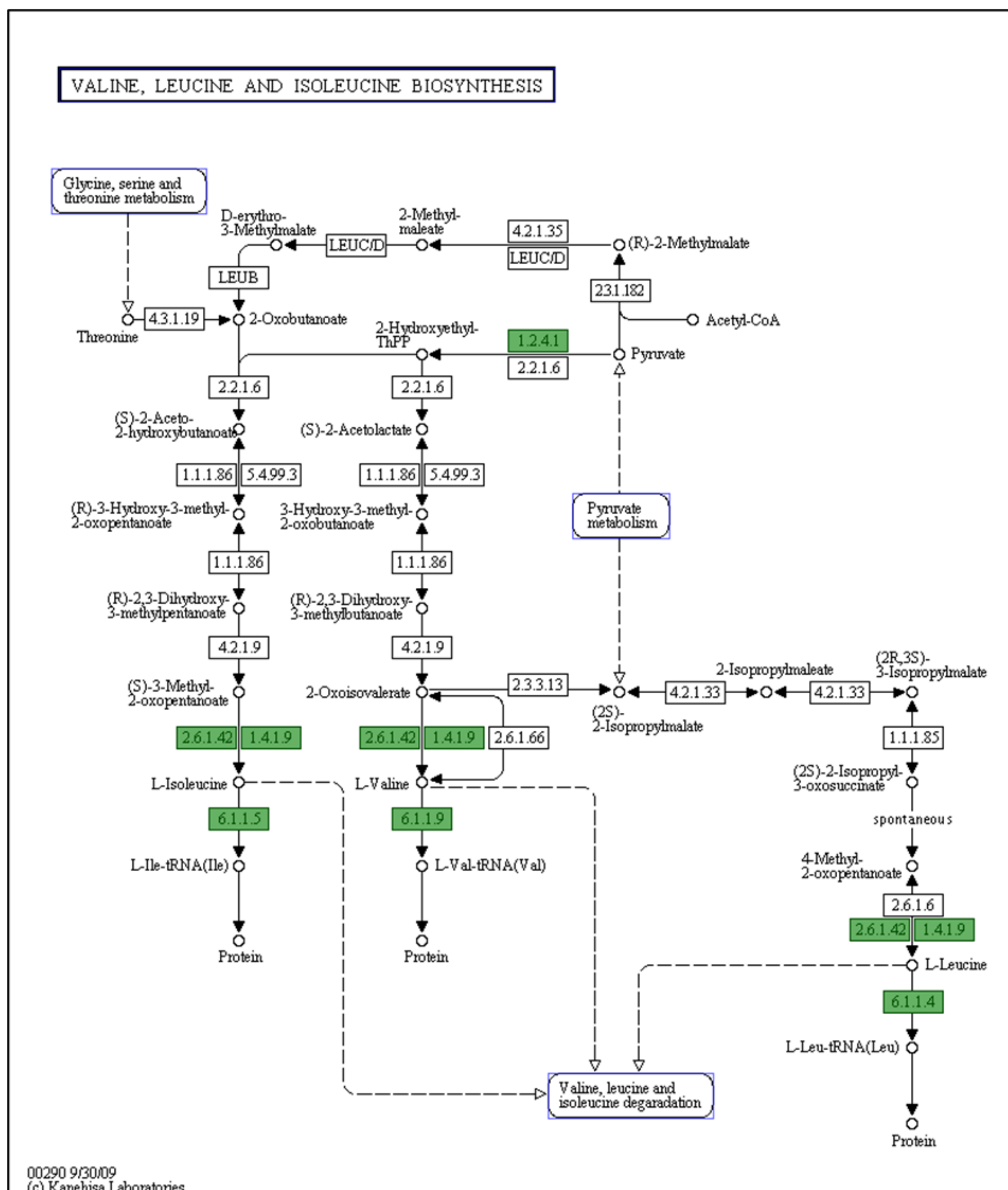
Protein_ID	Gene	RAST Annotation Subsystem	NCBI annotation
WP_060382961.1	leuA	Branched-Chain Amino Acid Biosynthesis	2-isopropylmalate synthase
WP_060382962.1	leuC	Branched-Chain Amino Acid Biosynthesis	3-isopropylmalate dehydratase large subunit
WP_060382963.1	leuD	Branched-Chain Amino Acid Biosynthesis	3-isopropylmalate dehydratase small subunit
WP_060382964.1	leuB	Branched-Chain Amino Acid Biosynthesis	3-isopropylmalate dehydrogenase
WP_060382987.1	ilvA	Branched-Chain Amino Acid Biosynthesis	Threonine dehydratase
WP_060382988.1	ilvC	Branched-Chain Amino Acid Biosynthesis	Ketol-acid reductoisomerase
WP_081078427.1	ilvH	Acetolactate synthase subunits	Acetolactate synthase small subunit
WP_060382990.1	ilvB	Acetolactate synthase subunits	Biosynthetic-type acetolactate synthase large subunit

WP_060382991.1	ilvD	Branched-Chain Amino Acid Biosynthesis	Dihydroxy-acid dehydratase
WP_060383305.1	cysI	Cysteine Biosynthesis	NADPH-dependent assimilatory sulfite reductase hemoprotein subunit
WP_060383307.1	cysG	Heme and Siroheme Biosynthesis	Uroporphyrinogen-III C-methyltransferase
WP_060383308.1	cysK	Cysteine Biosynthesis	Cysteine synthase A
WP_060383309.1	cysE	Cysteine Biosynthesis	Serine acetyltransferase
WP_060383310.1	cysN	Cysteine Biosynthesis	GTP-binding protein
WP_060383311.1	cysD	Cysteine Biosynthesis	Sulfate adenylyltransferase subunit 2
WP_060383312.1	cysH	Cysteine Biosynthesis	Phosphoadenylyl-sulfate reductase
WP_060382296.1		Phage tail fiber proteins	Hypothetical protein
WP_060382475.1		N-linked Glycosylation in Bacteria	Glycosyltransferase
WP_060382615.1		ABC transporter oligopeptide (TC 3.A.1.5.1)	Hypothetical protein
WP_060383861.1	TC.FEV.OM2	Ton and Tol transport systems	TonB-dependent receptor
WP_060382696.1		Biogenesis of c-type cytochromes	TlpA family protein disulfide reductase
WP_060381414.1		cAMP signaling in bacteria	Clp protease ClpP
WP_060382767.1		CBSS-84588.1.peg.1247	Serine hydrolase
WP_060382866.1	speG	Arginine Biosynthesis -- gjo	N-acetyltransferase
WP_060383088.1	ABC-2.LPSE.A	Rhamnose containing glycans	ABC transporter ATP-binding protein
WP_060383884.1	epsM	N-linked Glycosylation in Bacteria	Acetyltransferase
WP_060383106.1		CBSS-296591.1.peg.2330	Sugar transferase
WP_060383107.1		CBSS-296591.1.peg.2330	Glycosyltransferase family 1 protein
WP_060383303.1		Heme and Siroheme Biosynthesis	Siroheme synthase
WP_060383507.1	mazF	Phd-Doc, YdcE-YdcD toxin-antitoxin systems	Type II toxin-antitoxin system PemK/MazF family toxin
WP_060383508.1	mazE	Phd-Doc, YdcE-YdcD toxin-antitoxin systems	AbrB/MazE/SpoVT family DNA-binding domain-containing protein
WP_060381524.1	cas2	CRISPRs	CRISPR-associated endonuclease Cas2
WP_060381594.1		ABC transporter oligopeptide (TC 3.A.1.5.1)	TonB-dependent receptor
WP_060381731.1	rnz	Beta-lactamase	Peptidase
WP_060381334.1	wcaJ	CBSS-296591.1.peg.2330	Hypothetical protein
WP_060382054.1		Group II intron-associated genes	RNA-directed DNA polymerase

186

187

188 FIGURE 1A

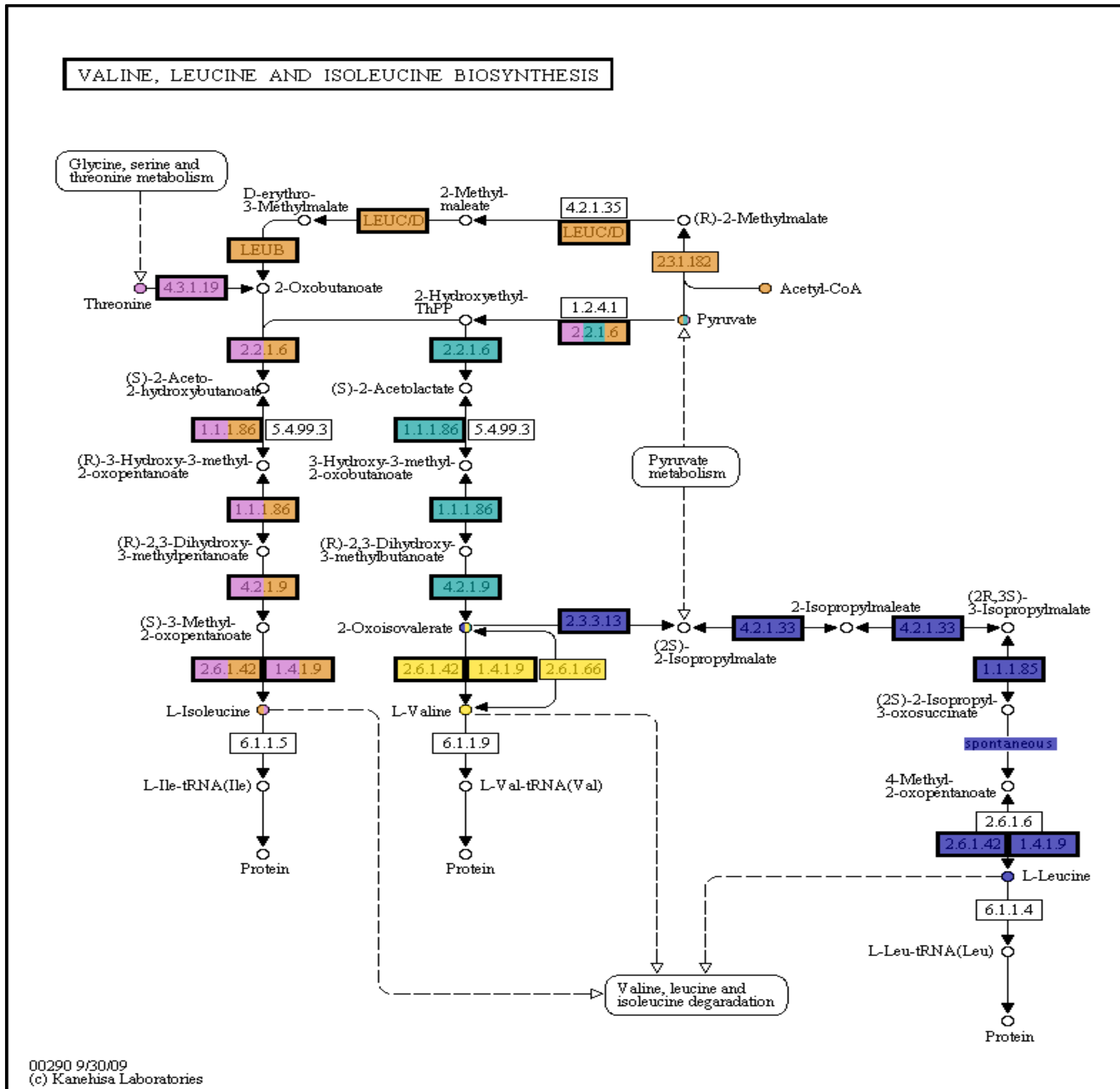


189

190

191

192 FIGURE 1B



193

Scenario	Input Compounds	Output Compounds	Status in *F. covae*
AcetylCoA and Pyruvate to Isoleucine	Acetyl-CoA Pyruvate	L-Isoleucine	incomplete
Oxoisovalerate to Leucine	3-Methyl-2-oxobutanoic acid	L-Leucine	complete
Oxoisovalerate to Valine	3-Methyl-2-oxobutanoic acid	L-Valine	complete
Pyruvate to Oxoisovalerate	Pyruvate	3-Methyl-2-oxobutanoic acid	complete
Threonine and Pyruvate to Isoleucine	L-Threonine Pyruvate	L-Isoleucine	complete

194 FIGURE 1 (A) *Flavobacterium columnare* valine-leucine-isoleucine biosynthesis pathway.

195 The green color shows the genes present in *F. columnare.* (B) *Flavobacterium covae* valine-

196 leucine-isoleucine biosynthesis pathway. The colors show the genes in *F. covae*, with different
197 colors representing pathways within the valine-leucine-isoleucine biosynthesis pathway.
198

199 **3.2 Host-pathogen protein-protein interactions**

200 The host-pathogen protein-protein interaction analysis revealed that 2 of the 36 unique proteins
201 putatively interact with catfish proteins. Specifically, the *ilvC* gene was found to potentially
202 interact with 2 catfish proteins, while the *ilvD* gene showed predicted interactions with 7
203 catfish proteins. The details of the host and pathogen proteins involved in these interactions are
204 presented in Table 4. The predicted locations of IlvC and IlvD proteins were cytoplasmic.
205

206 TABLE 4 Unique *F. covae* proteins with potential interaction with channel catfish proteins.

Gene	Protein	Protein ID	Host Protein ID	Host Protein
ilvC	ketol-acid reductoisomerase	WP_060382988.1	XP_017337580.1	Chromodomain-helicase-DNA-binding protein 4
			XP_017337579.1	Chromodomain-helicase-DNA-binding protein 4
ilvD	dihydroxy-acid dehydratase	WP_060382991.1	XP_017311531.1	Exocyst complex component 1 isoform X1
			XP_017311532.1	Exocyst complex component 1 isoform X2
			XP_017342785.1	Nascent polypeptide-associated complex subunit alpha isoform X3
			XP_017342786.1	Nascent polypeptide-associated complex subunit alpha isoform X3
			XP_017342787.1	Nascent polypeptide-associated complex subunit alpha isoform X4
			XP_017342788.1	Nascent polypeptide-associated complex subunit alpha isoform X4
			XP_017343604.1	Ras-related C3 botulinum toxin substrate 2

207

208 **3.3 *Flavobacterium covae leuD* and *ilvD* mutants**

209 We successfully performed in-frame deletions of *leuD* and *ilvD* in *F. covae*. In *Fc*Δ*leuD*, 78%
210 of the *leuD* gene was deleted, while 96% of the *ilvD* gene was eliminated in *Fc*Δ*ilvD* (Table 5).
211

212 TABLE 5. Deleted *F. covae* proteins and deleted gene properties.

Protein	Gene	GL	DR	RR	DP
3-isopropylmalate dehydratase small subunit	*leuD*	613	477	136	77.8%
Dihydroxy-acid dehydratase	*ilvD*	1674	1611	63	96.2%

213 Abbreviations: GL: Gene length, DR: deleted region, RR: Remaining region, DP: Deletion

214 percentage.

215

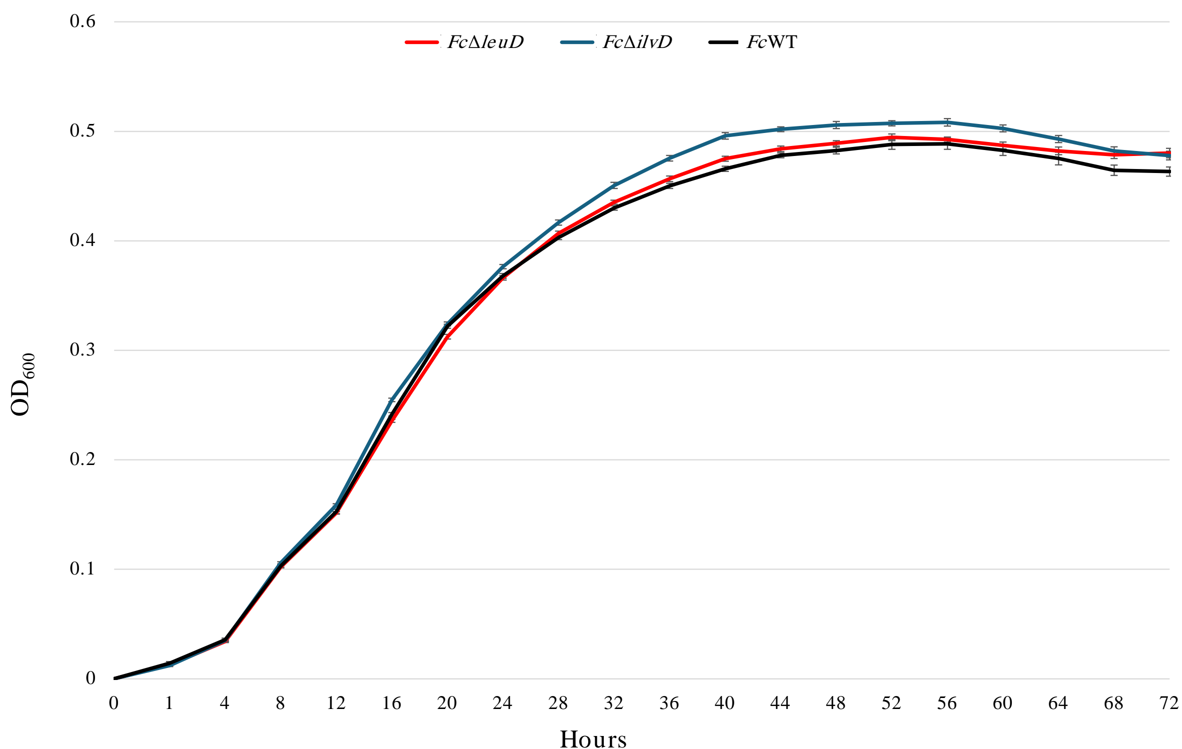
216 **3.4 Bacterial growth kinetics**

217 The growth kinetics of *FcΔleuD*, *FcΔilvD*, and *Fc*WT exhibited a similar pattern, and cultures

218 reached a plateau stage around 44 h (Figure 2).

219

220 FIGURE 2



221

222 FIGURE 2 Growth kinetics of mutants and wild-type *F. covae*.

223

224 **3.5 Virulence in catfish**

225 There was a significant difference in fish mortalities among all treatment groups ($p > 0.05$)
226 (Figure 3). Mutant strains *Fc*Δ*leuD* and *Fc*Δ*ilvD* caused 47.5% and 70% mortality in catfish,
227 respectively, while the mortality rate of *Fc*WT was 100% (Figure 3A). The progression of
228 mortalities varied among the challenge groups. Mortalities in the *Fc*Δ*leuD* treatment started to
229 manifest after two days. In contrast, mortalities in *Fc*WT and *Fc*Δ*ilvD* were observed as early
230 as one day after the challenge. Peak mortalities in all treatments occurred on the second day
231 (Figure 3B).

232

233 FIGURE 3A

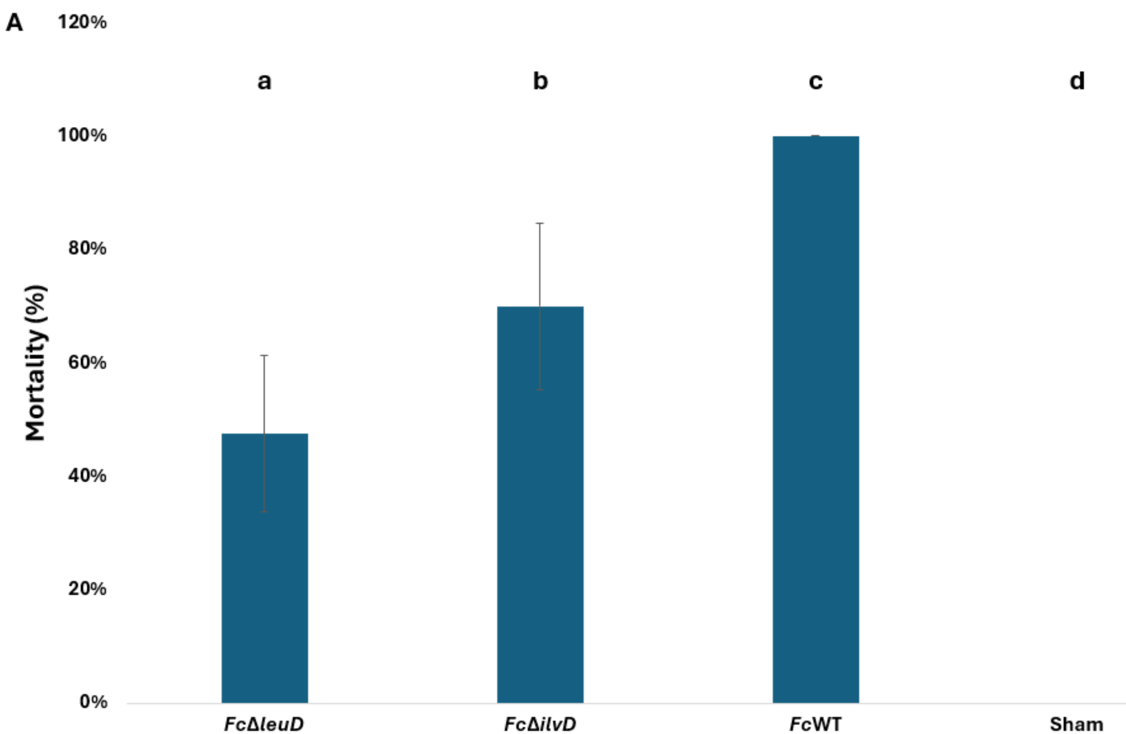


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FIGURE 3B

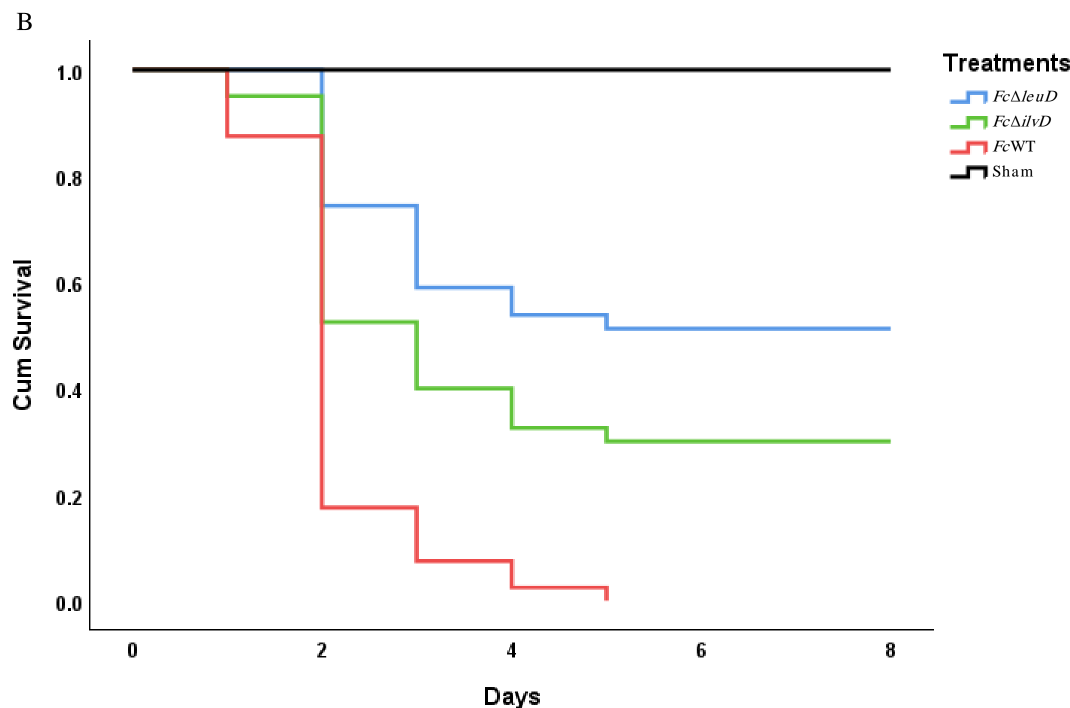

FIGURE 3 (A) Endpoint mortality and (B) cumulative survival among treatment groups.

4 DISCUSSION

In this study, our primary objective was to study unique genes of *F. covae* strain 94-081 (pathogenic to catfish) compared to *F. columnare* strain ATCC 49512 (not pathogenic in catfish). Although features of *F. covae* were previously reported (S. Kumru, Tekedar, H.C., Gulsoy, N., Waldbieser, G.C., Lawrence, M.L., Karsi, A., 2017), a detailed analysis of the unique pathways has not been performed. The pathway analysis revealed seven unique proteins involved in the cysteine biosynthesis pathway, and they were present only in *F. covae* and *F. davisii*. Cysteine serves as a precursor for the primary pathway of organic sulfur combination into cellular components and methionine biosynthesis (Rabeh, 2004; Stipanuk, 2004). The cysteine biosynthesis pathway is crucial for bacterial growth and function, detoxification, and

252 metabolic processes. Even though this pathway presents only in *F. covae* and *F. davisii*,
253 alternative pathways in *F. columnare* and *F. oreochromis* may be present, and hence, further
254 studies are needed to understand the role of cysteine biosynthesis pathway in *F. covae*
255 virulence.

256 The branched-chain amino acid (BCAA) biosynthesis pathway was present in *F. covae*,
257 *F. davisii*, and *F. oreochromis,* while not in *F. columnare*. The *F. covae* BCAA pathways
258 included 9 genes (l*euA*, *leuB*, *leuC*, *leuD*, *ilvA*, *ilvB*, *ilvC*, *ilvD*, *and ilvH*). The branched-chain
259 amino acids are synthesized in fungi, plants, and bacteria but not in animals and regulate many
260 biological activities, such as growth and protein synthesis (Dutta, Corsi, Bier, & Koehler,
261 2022; Y. Wang et al., 2021). *Listeria monocytogenes* responds to BCAA deficiency through
262 increasing virulence gene expression in mammalian cells (Brenner, Lobel, Borovok, Sigal, &
263 Herskovits, 2018). The BCAA transporters affect *Bacillus anthracis* virulence and growth
264 (Dutta et al., 2022). Interestingly, the expression of genes encoding the BCAA biosynthesis
265 pathway in *Bacillus cereus* is predominantly enriched by a quorum-sensing effector called
266 peptide-activating PlcR (PapR) (Yeo, 2014). In *Bacillus cereus*, PapR plays a crucial role as a
267 regulator of virulence factors, and it is involved in communication, adaptation, and survival in
268 various environments (Grenha, 2013; Yang, 2018; Yeo, 2014).

269 In catfish, both *leuD* and *ilvD* mutants showed attenuation, possibly due to the adverse
270 effects of mutations on bacterial adaptation in the host. However, the underlying mechanisms
271 are not yet fully understood. The *leuD* gene is a component of the PhoPR two-component
272 system and plays a crucial role in the oxidative stress response. It is also an essential enzyme
273 involved in the valine-leucine-isoleucine biosynthesis pathway in *Mycobacterium tuberculosis*.
274 Studies have shown that the *leuD* mutant exhibits enhanced protective immunity against

275 *Mycobacterium avium subsp. paratuberculosis* in mouse models, making it a potential
276 attenuated vaccine candidate (J. W. Chen, Faisal, S.M., Chandra, S., McDonough, S.P.,
277 Moreira, M.A., Scaria, J., Chang, C.F., Bannantine, J.P., Akey, B., Chang, Y.F., 2012; Faisal,
278 2013; Walters, 2006). A *leuD* mutant strain of *M. avium subsp. paratuberculosis* lost the
279 capability of utilizing several essential nutrients such as nitrogen, carbon, phosphorus, sulfur,
280 and other nutrient supplements as energy sources. Under different stress conditions, the *leuD*
281 mutant strain exhibits diverse expression patterns in over 100 genes. Furthermore, the mutant
282 strain displays a 30% reduction in fatty acid content, indicating an impact on the metabolic
283 pathway involving fatty acids due to deleting the *leuD* gene (J. W. Chen, Scaria, J., Chang,
284 Y.F., 2012). These findings highlight *LeuD*'s multifaceted role in pathogenicity and metabolic
285 adaptation in different bacterial species.

286 The *ilvD* plays a crucial role in the valine-leucine-isoleucine biosynthesis pathway,
287 exhibiting active involvement in the survival of *M. tuberculosis* under both normal and stress
288 conditions (Singh, 2011). Furthermore, *ilvD* is essential for the virulence of *Aspergillus*
289 *fumigatus* in murine infection models (Oliver, 2012). As one of the essential enzymes in
290 branched-chain amino acid (BCAA) biosynthesis, *ilvD* also plays a significant role;
291 supplementation with BCAAs promotes the aerobic growth of *Salmonella typhimurium* (Park,
292 2015). In *Bacillus anthracis*, *ilvD* has a crucial role in isoleucine production in a nutrient-
293 restrictive environment like its mammalian host (Jelinski, Cortez, Terwilliger, Clark, &
294 Maresso, 2021).

295 Understanding host-pathogen interactions is crucial for identifying potential targets and
296 developing effective disease control strategies (Ammari, 2016). In our study, we made an
297 intriguing discovery regarding the predicted interaction between unique *F. covae* proteins and

298 catfish proteins. The IlvC, which is the second enzyme in the BCAA biosynthesis, regulates
299 many functional activities in organisms (Y. Wang et al., 2021). It interacts with channel catfish
300 chromodomain helicase DNA-binding protein 4 that regulates gene expression and DNA-
301 damage responses (Silva et al., 2016). The IlvD interacts with the exocyst complex component
302 1 (EXOC1), a protein-coding gene essential for targeting exocytic vesicles to specific docking
303 sites on the plasma membrane (Sakurai-Yageta et al., 2008). The IlvD also interacts with the
304 nascent polypeptide-associated complex subunit alpha (NACα) that is involved in the innate
305 immune response to pathogens such as *V. anguillarum* and *E. tarda* in crab and fish (Li, Chen,
306 Geng, Zhan, & Sun, 2015; Li, Peng, Chen, Geng, & Sun, 2016; X. Wang, Xie, X., Liu, J.,
307 Wang, G., Qiu, D., 2020). Moreover, IlvD interacts with ras-related C3 botulinum toxin
308 substrate 2 (Rac2), which is a member of the Rho family of GTPases (Ridley, 2006). It
309 regulates various cellular activities, such as controlling cell growth and cytoskeletal
310 reorganization (Troeger & Williams, 2013). Amino acids are essential for protein synthesis,
311 metabolic physiology, and signaling in all organisms, but animals cannot synthesize several of
312 them. Microbial-synthesized essential amino acids may affect health and disease by changing
313 metabolic pathways in their hosts (McCann & Rawls, 2023). Specifically, two unique proteins,
314 IlvC and IlvD, were predicted to be located in the cytoplasm and interact with catfish proteins.

315 In summary, these findings contribute to our understanding of the molecular
316 mechanisms underlying the pathogenicity of *F. covae* in catfish and highlight potential targets
317 for future studies aimed at disease prevention and control strategies.
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320

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324

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326 None

327

328 **AUTHOR CONTRIBUTIONS**

329 **AK and ML** conceptualization, supervision, resources, and writing.

330 **SK, SWN, and HA** experiments, data analysis, and writing.

331

332 **CONFLICT OF INTEREST**

333 The authors declare no conflict of interest.

334

335 **ETHICS STATEMENT**

336 This study was carried out in strict accordance with the recommendations in the Guide for the
337 Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was
338 approved by the Institutional Animal Care and Use Committee of the Mississippi State
339 University (Protocol Number: 15-043). All efforts were made to minimize animal suffering.

340

341 **DATA AND CODE AVAILABILITY**

342 All data presented in this study are included in the article.

343

344 **DISCLAIMER**

345 This article reflects the views of its authors and does not necessarily reflect those of the U.S.

346 Food and Drug Administration. Any mention of commercial products is for clarification only

347 and is not intended as approval, endorsement, or recommendation.

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