UNIVERSIDADE FEDERAL DO RIO GRANDE - FURG
INSTITUTO DE OCEANOGRAFIA
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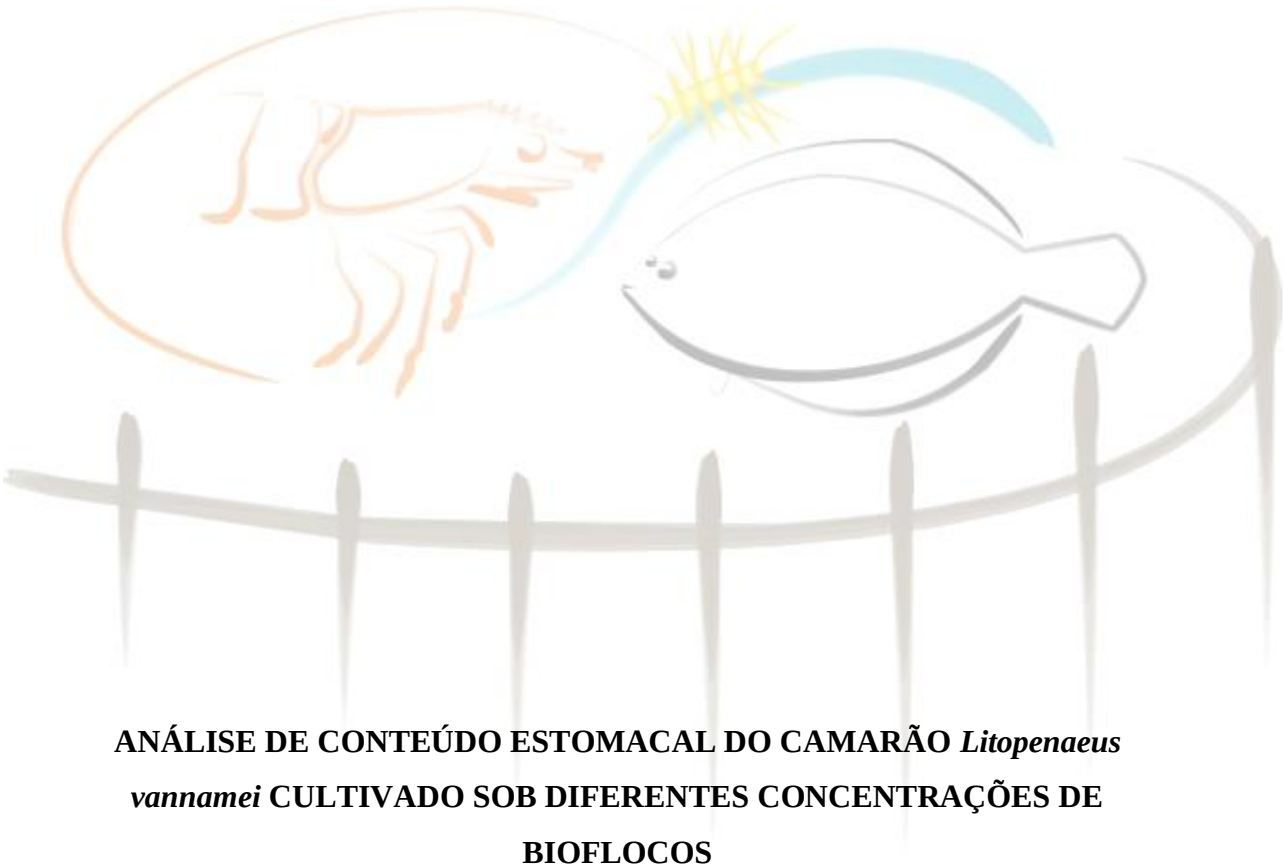

ANÁLISE DE CONTEÚDO ESTOMACAL DO CAMARÃO *Litopenaeus vannamei* CULTIVADO SOB DIFERENTES CONCENTRAÇÕES DE BIOFLOCOS

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Rio Grande, RS
2023

Universidade Federal do Rio Grande

ANÁLISE DE CONTEÚDO ESTOMACAL DO CAMARÃO *Litopenaeus vannamei* CULTIVADO SOB DIFERENTES CONCENTRAÇÕES DE BIOFLOCOS

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Dissertação apresentada como parte dos requisitos para obtenção do grau de Mestre em Aquicultura do Programa de Pós-Graduação em Aquicultura da Universidade Federal do Rio Grande.

Rio Grande, RS

Fevereiro de 2023

Ata de aprovação

Ficha catalográfica

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Dedicatória

Dedico este trabalho à minha mãe, por todo amor e força durante essa jornada.

Agradecimentos

Aos órgãos de fomento, CAPES, CNPq, FAPERGS E FINEP pela concessão da bolsa e pelos recursos que possibilitaram a execução desse trabalho.

Ao meu orientador, professor Dr. Dariano Krummenauer, por aceitar a orientação e estar presente dando o suporte necessário para a realização desse trabalho, por todo o aprendizado e disponibilidade.

Ao meu coorientador, professor Dr. Paulo Cesar Abreu, por ter sido tão grande exemplo de profissional, não apenas para mim, mas para todos que tiveram a oportunidade de conhecê-lo e ouvi-lo, por todo o ensinamento, as reuniões feitas de forma online e por toda disponibilidade.

Ao professor Dr. Luis Alberto Romano, e a Dra. Virginia Pedrosa por me auxiliarem e ensinarem durante as análises no Laboratório de Imunologia e Patologia de Organismos Aquáticos.

Ao programa de Pós-Graduação em Aquicultura da FURG, a todos os professores e pós doutores que me auxiliaram direta e indiretamente, por todo o conhecimento que foi passado através das disciplinas e ao dia-a-dia de convivência.

Aos meus amigos do laboratório de Ecologia de Microrganismos Aplicados à Aquicultura, que me ensinaram tanto e me ajudaram em todos os momentos; a amizade de vocês foi uma alegria para mim.

À minha família, por me apoiar e acreditar em mim, pelo amor a distância; vocês são a minha paz.

Ao meu namorado, que me incentivou todo o tempo, e sempre demonstrou a sua admiração pela minha trajetória, por seu amor tão grande.

Aos meus colegas que tive a oportunidade de morar durante essa jornada, por toda a troca rica de cultura e por todo companheirismo. Aos amigos que fiz em Rio Grande, por todo carinho e atenção.

Aos membros da banca examinadora, pela disponibilidade e interesse.

RESUMO

Os bioflocos são compostos por uma complexa comunidade microbiana formada não apenas por bactérias, mas também por algas, fungos, protozoários, rotíferos e outros organismos. As comunidades bacterianas do trato gastrointestinal podem diferir naturalmente entre diferentes culturas. Os objetivos deste trabalho foram analisar o conteúdo estomacal do camarão *Litopenaeus vannamei* em cultivo de bioflocos em diferentes porcentagens de inoculação e descrever quais organismos e biomoléculas são encontrados nos camarões nesses tipos de cultivo, determinando assim o melhor cultivo de acordo com o teor de microrganismos. O experimento foi conduzido na Estação Marinha de Aquacultura (EMA) do Instituto Oceanográfico da Universidade Federal do Rio Grande e foi delineado com quatro tratamentos e quatro repetições: (I) água clara (AC); (II) 25% de inóculo de biofloco (BF 25%); (III) 50% de inóculo de biofloco (BF 50%); e (IV) 100% de inóculo de biofloco (BFT 100). Em todos os casos, foram utilizados inóculos de bioflocos maduros de culturas anteriores. O desempenho zootécnico dos camarões foi monitorado semanalmente por meio de biometria, e os parâmetros físicos e químicos da água (temperatura, oxigênio dissolvido, pH, amônia e nitrito) foram medidos diariamente. A abundância da comunidade microbiana, influencia principalmente a qualidade da água nos tratamentos, podendo explicar os diferentes efeitos das condições. Os tratamentos com bioflocos alcançaram taxas de sobrevivência mais altas do que o tratamento com água limpa. Os microrganismos encontrados no estômago de camarões cultivados em sistema de bioflocos foram oocystes, ciliados, flagelados, diatomáceas, cocoides livres, filamentosas livres e aderidas, bacilos e vibrios, sendo a microbiota do estômago determinada pelo meio de cultura. Portanto, conforme os resultados obtidos no experimento, o reuso de bioflocos no percentual de 25% do volume total é eficaz em manter a qualidade da água do sistema e colonizar a microbiota do animal.

Palavras-chave: Biofloco. Microorganismos. Sistema intensivo. Bactéria. Reuso de água.

ABSTRACT

Bioflocs are composed of a complex microbial community formed not only by bacteria but also by algae, fungi, protozoa, rotifers, and other organisms. Intestinal bacterial communities can naturally differ between different cultures. The objectives of this study were to analyze the stomach contents of shrimp *Litopenaeus vannamei* in biofloc culture at different percentages of inoculation and describe which organisms and biomolecules are found in shrimp in these types of culture, thus determining the best culture according to the microorganism content. The experiment was carried out at the Marine Aquaculture Station (EMA) of the Oceanographic Institute of the Federal University of Rio Grande. It consisted of four treatments: (I) clear water (CW); (II) 25% biofloc inoculum (25% BF); (III) 50% biofloc inoculum (BF 50%); and (IV) 100% biofloc inoculum (BFT100). In all cases, inoculums of mature bioflocs from previous cultures have been used. The zootechnical performance of the shrimp was monitored weekly through weightings, and the physical and chemical parameters of the water (temperature, dissolved oxygen, pH, ammonia, and nitrite) were measured daily. The abundance of the microbial community, mainly influencing the water quality in the treatments, may explain the different effects of the conditions. Biofloc treatments achieved higher survival rates than clean water treatment. The microorganisms found in the stomach of shrimps cultivured in a biofloc system were oocystis, ciliates, flagellates, diatoms, free cocci, free and attached filaments, bacilli, and vibrios. The stomach microbiota is determined by the culture medium. The results suggested that the reuse of bioflocs in the percentage of 25% of the total volume it is effective in maintaining the water quality of the system and colonizing the animal's microbiota.

Keywords: Biofloc. Microorganisms. Intensive system. Bacteria. Water Reuse.

1 INTRODUÇÃO

O camarão branco *Litopenaeus vannamei* é o mais cultivado entre todas as espécies de camarão e em 2020 foi o mais produzido no mundo, com 5,8 milhões de toneladas (FAO, 2022). A tecnologia de bioflocos (BFT) é considerada um sistema revolucionário na aquicultura (Emerenciano et al., 2017), o BFT não agride o meio ambiente como o sistema de cultivo tradicional, pois tem a possibilidade de reaproveitar a mesma água várias vezes, evitando assim a poluição das águas costeiras (Krummenauer et al., 2014). Os bioflocos são compostos por uma complexa comunidade microbiana formada não apenas por bactérias, mas também por algas, fungos, protozoários, rotíferos e outros organismos (Emerenciano et al., 2017; Reis et al., 2023).

Existem vários tipos de produção com bioflocos. Aqueles que são expostos à luz natural, como viveiros e tanques ao ar livre, geralmente estão localizados em regiões tropicais ou subtropicais, onde há abundância de luz natural e predominância de organismos fotoautotróficos que causam uma coloração esverdeada da água (Prangnell et al., 2016). Cultivos realizados em estufas, com pouca ou nenhuma exposição à luz natural, são geralmente comuns em regiões temperadas. A cor da água é comumente marrom, e predominam os processos bacterianos que controlam a qualidade da água (Hargreaves, 2013; Samocha et al., 2017). Nesse sistema, a amônia é controlada pela adição de carbono orgânico para estimular o crescimento de bactérias heterotróficas, que metabolizam esse composto nitrogenado, transformando-o em biomassa bacteriana (Hargreaves, 2013). Já no processo quimioautotrófico, devido ao lento crescimento das bactérias nitrificantes, são produzidas pequenas quantidades de biomassa bacteriana, essas bactérias realizam a oxidação da amônia a nitrito e posteriormente a nitrato (Ebeling et al., 2006; Crab et al., 2007).

Além disso, os animais cultivados em BFT podem se alimentar dos bioflocos formados no ambiente. Existe uma relação positiva entre o tamanho de *L. vannamei* e o consumo de bioflocos, camarões alimentados com ração artificial e bioflocos apresentam melhor assimilação de nutrientes quando comparados aos alimentados apenas com ração formulada (Krummenauer et al., 2020). Isso se deve à maior quantidade de aminoácidos essenciais, ácidos graxos (PUFA e HUFA) e outros elementos nutrientes fornecidos pelos bioflocos (Tacon et al., 2002). A comunidade bacteriana que integra os bioflocos também pode atuar como um probiótico natural no controle de doenças (Emerenciano et al., 2013).

McIntosh (2000) afirma que o desenvolvimento de bioflocos microbianos é um processo demorado, que pode levar entre sete e oito semanas. Outra forma de facilitar o

processo de formação dos flocos e auxiliar na remoção mais rápida dos compostos nitrogenados do sistema seria o reaproveitamento da água de cultivos anteriores com bioflocos já desenvolvidos, esta técnica pode favorecer o rápido estabelecimento da comunidade microbiana na água, fazendo com que atue rapidamente na remoção de compostos nitrogenados das fezes e restos de ração, oferecendo assim maior estabilidade ao cultivo (Krummenauer et al., 2014; Wasielesky et al., 2022).

O trato gastrointestinal dos peneídeos é tubular, sendo dividido em intestino anterior (esôfago e estômago), intestino médio e intestino posterior (reto e ânus), e também inclui a glândula digestiva hepatopâncreas (Guillaume et al., 1999). Assim como os peixes e a maioria dos animais aquáticos, a microbiota dos camarões peneídeos pode ser determinada e influenciada pelo contato com o meio ambiente (Wu et al., 2012; Zhang et al., 2014; Cardona et al., 2016). Assim, vale ressaltar a importância da fisiologia do animal para a utilização de microrganismos pelo organismo do camarão.

O objetivo deste estudo foi analisar o conteúdo estomacal do camarão *L. vannamei* em cultivo de bioflocos em diferentes porcentagens de inoculação e descrever quais organismos e biomoléculas são encontrados no camarão nesses tipos de cultivo; caracterizar os microrganismos quanto ao seu benefício para o camarão e sua quantidade, assim determinar o melhor cultivo de acordo com o teor de microrganismos.

2 MATERIAL E MÉTODOS

O estudo foi realizado no período de 24 de janeiro a 4 de março de 2022 (40 dias) no Laboratório de Carcinicultura da Estação de Aquicultura Marinha (EMA) pertencente ao Instituto de Oceanografia da Universidade Federal do Rio Grande – FURG, localizado na cidade de Rio Grande, RS, Sul do Brasil. A espécie utilizada no estudo foi o camarão branco do Pacífico *L. vannamei*. As pós-larvas foram obtidas da Aquatec® LTDA, antes do experimento, e os camarões juvenis (13,5 ± 0,56 g) foram aclimatados em água do mar clorada por uma semana antes do experimento.

A água foi transferida para dezesseis tanques de polietileno, com volume útil de 350 L, colocados em estufa experimental. A densidade de estocagem foi de 300 camarões m^3 (105 animais/unidade). O estudo consistiu de quatro tratamentos com quatro repetições: (I) controle (AC), realizado em água clara (II) 25% do volume estocado com biofloco maduro (BFT25); (III) 50% do volume com biofloco maduro (BFT50); e (IV) 100% do volume com biofloco maduro (BFT100). Nos tratamentos II (BFT25), III (BFT50) e IV (BFT100), diferentes concentrações de reutilização de bioflocos foram utilizadas, seguindo a metodologia proposta por Krummenauer et al. (2014). O biofloco

maduro utilizado no experimento apresentou concentração média de 336 mg/L.

Figura 1: Foto das unidades experimentais. (Imagem: Natália Pereira).

O melaço de cana-de-açúcar com 25% de carbono foi utilizado nas fases iniciais do cultivo para o controle da amônia. A adição do melaço foi feita de forma a manter uma relação C:N de 6,0 g de carbono (melaço) para cada 1,0 g de amostra amoniacal total (TA-N) na água. Além disso, aplicações de probióticos comerciais (INVE® Sanolife PRO-W) foram aplicadas uma vez por semana na água usando 1ppm (Santos et al., 2019).

Os camarões foram alimentados duas vezes ao dia com ração comercial Potimar 38 active com 38% de proteína bruta produzida pela Guabi Nutrição e Saúde Animal S.A. (Brasil). A taxa de alimentação da fase de crescimento seguindo o método de Garza de Yta et al., (2004). O desempenho zootécnico dos camarões foi monitorado semanalmente por meio da aferição do peso médio de 20 animais por unidade experimental, utilizando balança digital com precisão de 0,01 (Marte® UX420H).

A temperatura da água e o oxigênio dissolvido (OD) foram monitorados duas vezes ao dia com um oxímetro (YSI, modelo Pro-20, EUA) e o pH foi medido com um pHmetro (Mettler Toledo, FEP20, Brasil). As correções de pH e alcalinidade foram realizadas para manter valores acima de 7,2 e 120 mg de $CaCO_3$ L^{-1}, respectivamente, utilizando cal hidratada [Ca(OH)2], conforme Furtado et al. (2014), com alcalinidade registrada semanalmente. A salinidade foi verificada com um refratômetro óptico (ATC, RTP-20ATC, Brasil) uma vez por semana. A concentração total de amônia foi determinada de acordo com a UNESCO (1983) e a American Public Health Association (APHA) (2012), registrada diariamente. A análise da concentração de nitrito (NO_2-N) também foi realizada diariamente e a análise de sólidos suspensos totais foi realizada semanalmente, ambas seguindo os métodos de Strikland e Parsons, (1972). As concentrações de nitrato (NO_3-N) e fosfato (PO_4 - P) foram medidas semanalmente (Aminot & Chaussepied, 1983). Os sólidos suspensos totais foram mantidos a 500 mgL^{-1} (Gaona et al. 2011).

Figura 2: Foto das análises de água no período experimental. (Imagem: Natália Pereira).

Para a quantificação dos microrganismos, amostras de água (18mL) foram coletadas uma vez por semana de cada unidade experimental para contagem. Os camarões de cada tanque foram mortos por choque térmico em banho de gelo e necropsiados assepticamente para retirada do estômago no início e no final do período experimental. As amostras foram fixadas em formaldeído a 4% e mantidas em frascos âmbar para posterior contagem e identificação dos principais grupos de microrganismos presentes.

Para determinação da abundância de bactérias, as amostras fixadas foram filtradas em filtros de membrana de policarbonato (Nuclepore, poro de 0,2 μm e 2,5 mm de diâmetro) previamente escurecidos com Irlan black e corados com laranja de acridina 1%, na concentração de 1μg/mL (Hobbie et al. outros, 1977). As bactérias foram fotografadas com câmera acoplada a microscópio de epifluorescência Axioplan-Zeiss, com aumento final de 1000X, para posterior contagem de 30 campos escolhidos aleatoriamente. Para protozoários, foi utilizado um microscópio invertido Olympus IX51 com aumento final de 200x, onde alíquotas de 2,1mL de amostra foram colocadas em câmara de sedimentação e contados 30 campos aleatórios (Utermohl, 1958). Todas as contagens foram realizadas no Laboratório de Ecologia de Microorganismos Aplicados à Aquicultura.

Para análise histológica, os camarões foram mortos por choque térmico com gelo e água. Depois disso, a solução de Davidson foi injetada nos músculos e órgãos dos animais foram armazenados por 24 horas em solução de Davidson, após este período, os animais foram transferidos para outro recipiente contendo álcool 70%. Os tecidos foram selecionados em parafina para conservação e cortes histológicos em micrótomo rotativo

(LEICA RM2245) na espessura de 5 µm. Os tecidos, por fim, foram preparados nas cores hematoxilina-eosina (HE) para análise histológica (Bell e Lightner, 1988).

Figura 3. Placa de refrigeração com amostras em cassetes à esquerda, em seguida à direita com o processo de coloração das lâminas, ambas imagens como partes do processo para análise histológica. (Imagem: Natália Pereira da Silva).

Os dados foram submetidos às análises de homocedasticidade das variâncias e normalidade das distribuições dos dados. Quando os pressupostos não foram atendidos, os dados foram submetidos a transformações estatísticas. Posteriormente, foi aplicada uma análise de variância de duas vias – ANOVA ($\alpha = 0,05$) seguida de um teste post hoc de Tukey quando foram encontradas diferenças significativas (Zar, 2010).

3 RESULTADOS

Os principais valores (± DP) dos parâmetros químicos e físicos da água durante o período experimental de 40 dias são apresentados na Tabela 1. Houve diferença significativa entre os tratamentos nos seguintes parâmetros: OD, pH, nitrito, nitrato e SST (sólidos suspensos totais). Variáveis como OD e pH foram significativamente maiores ($p < 0,05$) no tratamento controle de água limpa do que nos tratamentos com bioflocos ($p < 0,05$). O OD foi mantido em concentração superior a 5,89 mgL^{-1} e a temperatura mantida na faixa de 26-27°C. As variáveis nitrito foram significativamente menores nos tratamentos CW do que nos tratamentos com bioflocos ($p < 0,05$). O nitrogênio amoniacal total e o fosfato não apresentaram diferenças estatísticas entre os tratamentos. O nitrato apresentou menores médias nos tratamentos CW e BF 50% e houve diferença significativa entre os tratamentos CW e BF 50% para os tratamentos BF 25% e BF 100%. A salinidade apresentou concentração média acima de 35 ppt. Não houve diferença estatística para sólidos totais em suspensão entre os tratamentos com bioflocos, mas houve diferença em relação ao tratamento com água limpa ($p < 0,05$).

Tabela 1. Parâmetros físicos e químicos da água (valores médios ± desvio padrão) nos tratamentos: Água limpa (CW), 25% Biofloc (BF 25%), 50% Biofloc (BF 50%) e 100% Biofloc (BF 100 %). Letras sobrescritas diferentes seguidas indicam diferenças significativas ($P < 0,05$) entre os tratamentos.

Parâmetros	Tratamentos			
	AC	BF 25%	BF50%	BF100%
Temperatura (°C)	26,24 ± 0,86	26,60 ± 0,81	26,54 ± 0,74	26,55 ± 0,78
*OD (mg L^{-1})	6,04 ± 0,28^{a}	5,89 ± 0,19^{b}	5,91 ± 0,17^{b}	5,88 ± 0,19^{b}
pH	8,02 ± 0,08^{a}	7,73 ± 0,15^{b}	7,66 ± 0,20^{b}	7,77 ± 0,20^{b}
Salinidade	35,96 ± 0,97^{b}	37,33 ± 0,85^{ab}	38,42 ± 1,37^{a}	37,42 ± 1,20^{ab}
Alcalinidade (mg de $CaCO_3$ L^{-1})	127,64 ± 10,55	115,83 ± 29,07	107,5 ± 28,25	125,83 ± 44,85
Amônia (mg. L^{-1})	0,80 ± 0,25	1,36 ± 1,70	1,52 ± 2,28	1,29 ± 2,38
NO_2^-N (mg. L^{-1})	0,37 ± 0,28^{a}	1,44 ± 2,44^{b}	1,64 ± 2,88^{b}	1,09 ± 1,71^{b}
NO_3^-N (mg. L^{-1})	8,11 ± 4,21^{a}	104,82 ± 54,79^{b}	84,55 ± 45,23^{a}	107,11 ± 47,68^{b}
PO^3_4- P (mg. L^{-1})	0,78 ± 0,60	3,31 ± 2,05	3,60 ± 2,30	3,85 ± 1,32
*SST (mg. L^{-1})	152,25 ± 30,44^{a}	421,27±106,90^{b}	415 ± 87,89^{b}	416,95±69,56^{b}

*OD: oxigênio dissolvido; SST: sólidos suspensos totais.

A Figura 4A mostra as concentrações de Oocystes no início e no final do experimento nos estômagos dos animais. Não houve diferença significativa considerando a variável tempo ($p > 0,05$) ou entre os tratamentos, com máximo de $4,13 \times 10^1$ e mínimo de zero no início do experimento no tratamento BF 50%.

A Figura 4B mostra as concentrações de Ciliados no início e no final do experimento nos estômagos dos animais. Não houve diferença significativa considerando o início e o final do experimento ($p < 0,05$), com valor máximo de $3,12 \times 10^2$ e valor mínimo de 4,13 para BF 50%.

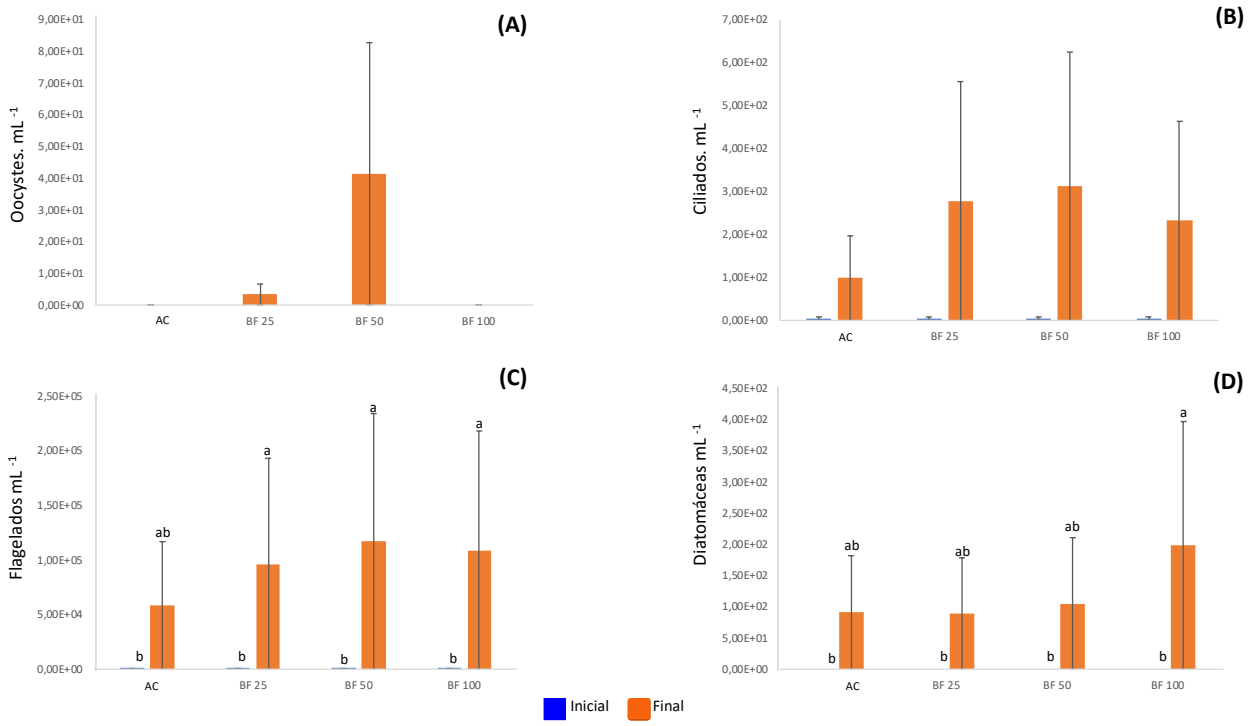

Figura 4. Valores médios (± DP) de (A) Oocystis, (B) Ciliados, (C) Flagelados e (D) Diatomáceas (mg L-1) presentes no estômago dos animais nos quatro tratamentos experimentais; água limpa (CW), biofloco 25% (BF 25%), biofloco 50% (BF 50%) e biofloco 100% (BF 100%).

Para Flagelados, houve um máximo de $1{,}17 \times 10^5$ para BF 50% e o valor mínimo foi de $3{,}39 \times 10^2$, no início do experimento, com diferenças significativas entre o tempo final e inicial do experimento (Fig. 4C).

Para Diatomáceas, observamos diferença entre os tempos ($p > 0{,}05$), onde houve um máximo de $1{,}21 \times 10^2$ e um mínimo de zero para BF 100% (Fig. 4D).

A Figura 6A mostra as concentrações de bactérias cocoides livres no início e no final do experimento nos estômagos dos animais. Houve diferença significativa considerando a variável tempo, mas não houve diferença significativa entre os tratamentos ($p > 0{,}05$), com máximo de $8{,}40 \times 10^6$ e mínimo de $1{,}85 \times 10^5$ no tratamento BF 100%.

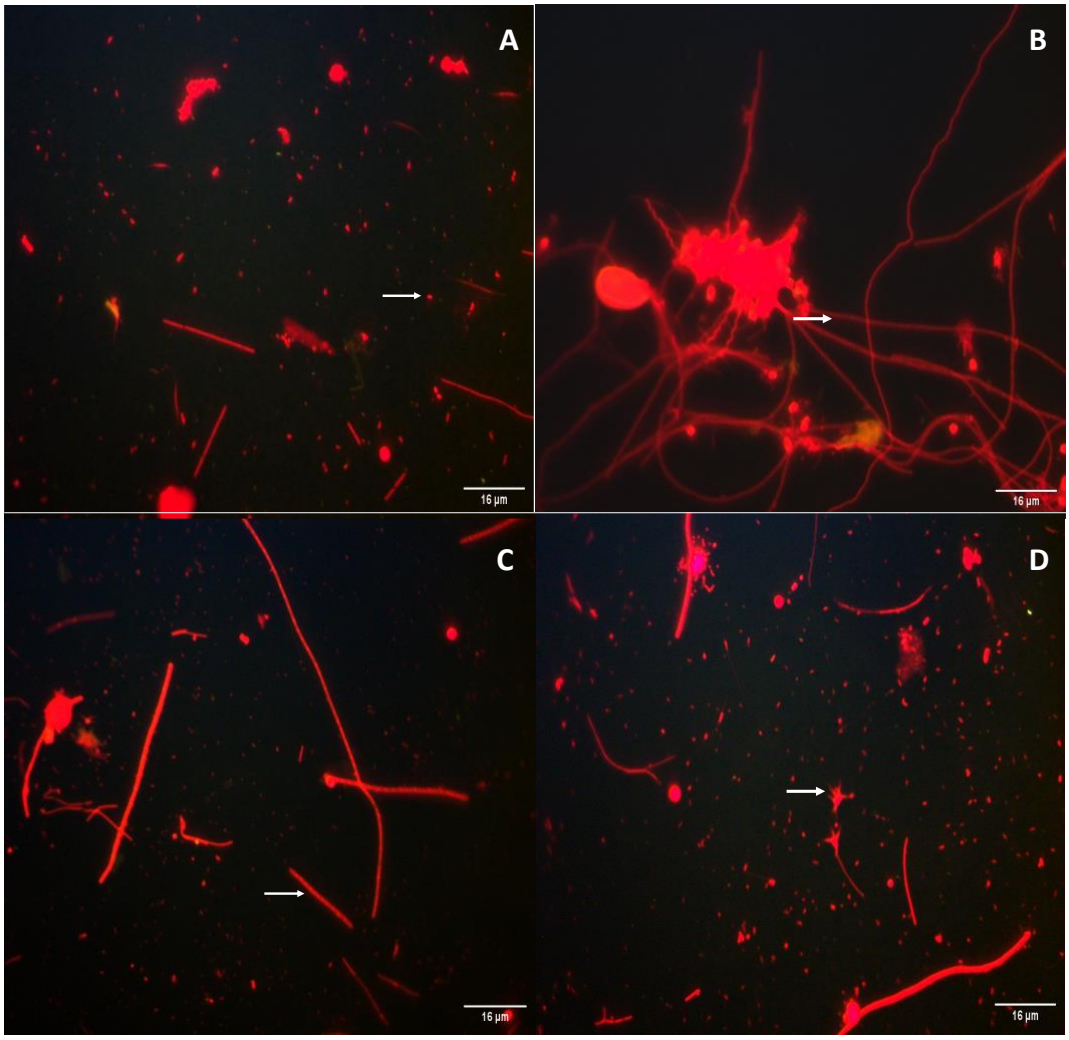

Figura 5. Abundância de bactérias e protozoários na água em quatro tratamentos: (A) cocoides no 20º dia de experimento no tratamento Água Clara; (B) bactérias filamentosas aderidas no 20º dia no tratamento com Biofloc 25%; (C) bactérias filamentosas livres no 20º dia no tratamento Biofloc 50% e ameba no 20º dia no tratamento Biofloc 100% (D). Aumento de 1000× por microscopia de epifluorescência (imagem: Natália Pereira da Silva).

A Figura 6B mostra as concentrações de bactérias filamentosas livres no início e no final do experimento no estômago dos animais. Houve diferença significativa entre o início e o final do experimento ($p < 0,05$), com máximo de $9,18 \times 10^4$ e mínimo de $9,57 \times 10^3$ para BF 50%. Para bactérias filamentosas aderidas, houve um máximo de $7,97 \times 10^2$ para BF 100% e o valor mínimo foi zero, no início do experimento. Não houve diferenças significativas ao longo do tempo e entre os tratamentos. (Fig. 6C)

Para Bacilos, observamos diferenças significativas entre os tempos ($p > 0,05$), onde houve máximo de $1,96 \times 10^4$ e mínimo de $9,11 \times 10^2$ para BF 100% (fig. 6D). A Figura 6E mostra as concentrações de Vibrio ao longo do experimento. Houve diferença significativa entre os tempos para os tratamentos água clara e biofloco 50% ($p < 0,05$),

com valor máximo de 1,14 × 10^3 para AC e valor mínimo sendo zero no início do experimento para todos os tratamentos.

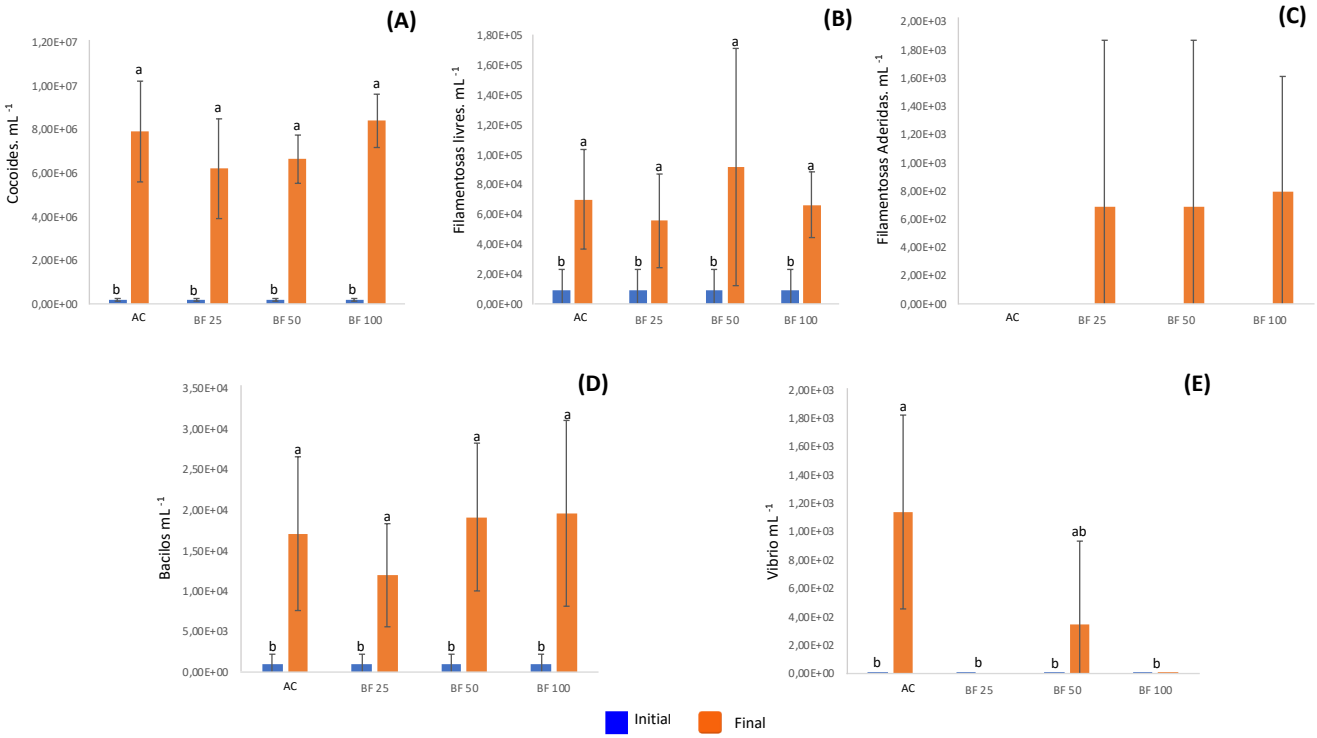

Fig 6. Valores médios (± DP) (A) de cocoides, (B) bactérias filamentosas livres, (C) bactérias filamentosas aderidas, (D) Bacillus e (E) Vibrio presentes no estômago dos animais nos quatro tratamentos experimentais ; água limpa (CW), biofloco 25% (BF 25%), biofloco 50% (BF 50%) e biofloco 100% (BF 100%).

No presente estudo, o estômago de *L. vannamei* não apresentou alterações causadas por patógenos, sendo caracterizado como saudável. Não houve diferenças significativas entre camarão cultivado em sistema bioflocos ou água limpa.

A sobrevivência dos animais foi o único parâmetro que apresentou diferença estatística entre os tratamentos, sendo o tratamento água clara o de menor valor médio. Os camarões dos quatro tratamentos testados apresentaram ganho de peso semanal com média superior a 0,50 g. Com relação aos valores de produtividade, nos quatro diferentes sistemas de produção, houve produtividade superior a 4 kg/m³ do camarão branco do Pacífico.

Tabela 2- Valores médios (± desvio padrão) do desempenho zootécnico de *L. vannamei* nos tratamentos: Água Clara (AC), 25% Biofloco (BF 25%), 50% Biofloco (BF 50%) e 100% Biofloco (BF 100%). Letras sobrescritas diferentes seguidas indicam diferenças significativas ($p < 0,05$) entre os tratamentos.

Parâmetros	Tratamentos			
	AC	BF 25%	BF 50%	BF 100%

Peso inicial (g)	13,73 ± 0,54	13,75 ± 0,32	13,49 ± 0,16	13,24 ± 0,82
Peso final (g)	17,73 ± 0,82	16,81 ± 0,63	16,12 ± 0,18	17,38 ± 0,68
Ganho de peso semanal (g)	0,80 ± 0,29	0,61 ± 0,15	0,50 ± 0,07	0,83 ± 0,22
Sobrevivência (%)	80,71 ± 10,33^{b}	92,62 ± 3,68^{ab}	94,03 ± 3,60^{a}	89,05 ± 2,96^{ab}
Produtividade (kg/m³)	4,24 ± 0,41	4,67 ± 0,22	4,56 ± 0,20	4,64 ± 0,19

4 DISCUSSÃO

Neste estudo, os parâmetros de qualidade da água, como temperatura, salinidade, oxigênio dissolvido e pH, foram mantidos dentro da faixa ideal para *L. vannamei* (Ponce-Palafox et al., 1997; Van Wyk e Scarpa, 1999). Os tratamentos com bioflocos tiveram uma OD significativamente menor do que o tratamento AC. Essa observação se deve a um aumento constante nas taxas de exercício de acúmulo de matéria orgânica e alto metabolismo bacteriano (Schveitzer et al., 2013; Taw, 2010). As diferenças significativas de pH observadas entre o tratamento controle (AC) e os tratamentos com bioflocos resultam de uma relação inversa entre as concentrações de SST devido ao maior desenvolvimento de comunidades microbianas, que consequentemente diminuem o pH (Wasielesky et al., 2006, Furtado et al., 2011; Gaona et al., 2016; Hussain et al., 2021).

A salinidade foi significativamente maior ($p<0,05$) nos tratamentos com diferentes inóculos em bioflocos, uma possível razão para isso é que durante o experimento os tanques foram preenchidos com água do mar, devido à evaporação, aumentando ainda mais a concentração de sais. Os compostos de nitrogênio, alcalinidade e concentração de sólidos (TSS) também foram considerados dentro de condições adequadas e semelhantes aos relatados na literatura (Ray et al., 2010; Samocha, 2019; Ferreira et al., 2021; Emerenciano et al., 2022). As concentrações de TAN em todos os tratamentos permaneceram abaixo do limite relatado por Lin e Chen (2001) como tóxico para o camarão.

No início do experimento ocorreram picos de amônia, mas foram rapidamente controlados, não comprometendo a sobrevivência e o crescimento dos animais. Esse processo de estabilização também foi observado por outros autores que trabalham com *L. vannamei* em sistemas com pouca ou nenhuma troca de água. Krummenauer et al. (2014) os autores demonstraram que a suplementação da água do mar com água rica em bioflocos em um nível tão baixo quanto 25% é eficaz para reutilização, que essa água ajudou a manter baixos níveis de amônia e nitrito durante todo o processo do experimento. Sobre o nitrato, estudos adicionais afirmaram que o nitrato frequentemente se acumula em

sistemas operados sem trocas de água (Kuhn et al. 2010). As concentrações de nitrato do estudo variaram entre 13,25 e 175 mg L^{-1}, portanto dentro da recomendação de vários autores, que documentaram níveis acima de 400 mg/L NO_3^-N sendo críticos nesses sistemas ao longo do ciclo de produção (Samocha et al., 2010, 2011; Krummenauer et al.,, 2011).

A concentração de sólidos tende a aumentar com o tempo. Este aumento deve-se, principalmente ao aumento da biomassa bacteriana, por meio do inóculo inserido no meio de cultura no início do experimento. Neste estudo, a concentração máxima de TSS relatada foi de 541 mg L^{-1} (25% do tratamento). Avnimelech (2009) alerta que uma concentração de TSS maior que 500 mg/L pode interferir na qualidade da água e no desempenho zootécnico do camarão e sugere manter o nível de TSS entre 200 e 500 mg L^{-1}. Diferentes métodos foram listados como possíveis abordagens para controlar os níveis de sólidos em sistemas de troca limitada ou zero de água, esses métodos incluem clarificação, filtração e troca de água (Ebeling et al., 2006; Ray et al., 2010; Gaona et al., 2011; Krummenauer 2014). No presente estudo, quando os valores registrados foram iguais ou superiores a 500 mg/L, foram realizadas trocas parciais de água, variando de 20 a 30% do volume total do tanque.

Os microrganismos têm funções importantes nos sistemas de aquicultura. Eles são responsáveis pela produção primária, ciclagem de nutrientes, também são essenciais para a manutenção da qualidade da água, controle de doenças e como mediadores do impacto ambiental de efluentes (Moriarty 1997; Decamp et al. 2002; Thompson et al., 2002). Um dos pontos alvo deste estudo é o ambiente de cultivo, pois exerce grande influência na formação e desenvolvimento da microbiota intestinal dos animais aquáticos, refletindo uma ligação entre a microbiota gastrointestinal e o meio ambiente (Tzuc et al., 2014; Hostins et al. 2017).

As diatomáceas podem ajudar a estabilizar o ecossistema em que vivem os animais, minimizando grandes flutuações na qualidade da água e evitando o acúmulo de nutrientes residuais em níveis tóxicos (Lemonnier et al., 2017, Emerenciano 2022). Ao final do experimento, foram encontradas diatomáceas nos estômagos dos camarões em todos os tratamentos, com valores ligeiramente superiores no tratamento BF 100%. Algumas espécies do gênero Oocystes possuem metabolismo heterotrófico, podendo absorver e utilizar fontes de carbono (Du et al., 2018), o que explica a presença do gênero nos tratamentos LF25% e LF 50%. Os ciliados se alimentam de algas, bactérias e fungos (Nagano et al., 2004) e são uma rica fonte de aminoácidos livres e, assim como os

flagelados, podem fornecer outras preparações orgânicas, poliinsaturadas e esteróis para o camarão. (Khanjani et al., 2022). Ao final do experimento, foram encontrados ciliados nos estômagos dos camarões em todos os tratamentos.

Os flagelados possuem uma alta relação proteína:energia, sendo também capazes de sintetizar ácidos graxos poliinsaturados (Zhukova & Kharlamenko 1999; Viau et al., 2013). A predominância de flagelados e bactérias cocoides no estômago do camarão provavelmente ocorreu devido à predominância desses microrganismos também na água. Esses resultados corroboram os trabalhos de Wu et al., 2012, Zhang et al., 2014, Cardona et al., 2016, em que os autores demonstram que a microbiota dos camarões peneídeos pode ser determinada e influenciada pelo ambiente onde os animais estão inseridos .

Embora as bactérias filamentosas não formem flocos, elas desempenham um papel na sua formação, pois formam a espinha dorsal na qual os pequenos flocos podem se tornar flocos maiores e mais densos (Jenkins et al., 2003); a presença de filamentosos foi detectada em todos os tratamentos no estômago dos animais, sendo observado um aumento ao longo do experimento. Além disso, o crescimento de bactérias filamentosas pode estar relacionado à adição de carbono orgânico dissolvido no sistema (Esteves, 1998). Apesar da presença de filamentosas, o maior domínio de bactérias em todos os tratamentos foi a bactéria cocoide, a possível explicação para isso é que devido a sua relação superfície-volume, essas bactérias assimilam melhor os nutrientes, resultados semelhantes foram obtidos por Suita (2015).

Vibrio é normalmente a flora mais abundante no sistema digestivo do camarão (Gomez-Gil et al., 1998; Moss et al., 2000; Oxley et al., 2002; Esiobu e Yamazaki 2003; Liu et al., 2011). No presente estudo, foi utilizado um probiótico comercial na água, facilitando a colonização do Bacilos no trato intestinal do animal (Nimrat et al., 2012) e promovendo resistência contra a sobrevivência pelo Vibrio, o que corroboram com os autores . (Balcazar et al., 2007; Krummenauer et al., 2014; Villaseñor et al., 2015; hostins et al., 2017). O tratamento BF 50% foi o único tratamento com bioflocos onde o Vibrio foi presente no estômago do camarão, mas em menor quantidade quando comparada à presença de Bacilos também no estômago.

Os intestinos anterior e posterior são revestidos por uma camada de quitina-proteína que se renova a cada muda (Guillaume et al., 1999) e o intestino médio é revestido por uma membrana peritrófica acelular e porosa, que permite a seleção de nutrientes para absorção (Wang et al., 2012; McGaw et al., 2013). Abbaszadeh et al., (2022) observaram diferenças na morfologia intestinal, aumento do comprimento das células epiteliais

intestinais em *L. vannamei* criados em sistemas BFT em comparação com o controle, no presente estudo, não houve alterações morfológicas no estômago do camarão em nenhum tratamento.

No presente estudo, os tratamentos com bioflocos na cultura de *L. vannamei* influenciaram positivamente o desempenho zootécnico. A abundância da comunidade microbiana, influenciou principalmente a qualidade da água nos tratamentos, podendo explicar a diferença nos tratamentos. Os bioflocos alcançaram maiores taxas de sobrevivência do que o tratamento de água limpa. No BF 50%, a sobrevivência foi significativamente maior do que na água limpa (94%). Esses resultados corroboram os de Yun et al., 2017. A diferença estatística na taxa de sobrevivência para água limpa pode ser atribuída ao estresse causado pelas trocas diárias de água dos tanques. Não houve diferença no peso final dos tratamentos com bioflocos para o tratamento com água límpida. Também não houve diferença na produtividade. Isso também foi observado pelos autores Reis et al., (2023).

5 CONCLUSÃO

O cultivo em sistema de bioflocos influência positivamente a abundância de microrganismos no estômago de *L. vannamei*, resultando em melhor desempenho animal. Os resultados do presente estudo sugerem que o reaproveitamento de bioflocos no percentual de 25% do volume total é eficaz em manter a qualidade da água do sistema e colonizar a microbiota do animal, quando comparado com os outros tratamentos.

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ANEXO 1

A dissertação está apresentada em formato de artigo científico, que segue as normas da Revista Aquaculture.

STOMACH CONTENT ANALYSIS OF SHRIMP *Litopenaeus vannamei* CULTURED IN DIFFERENT BIOFLOCS CONCENTRATIONS

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ABSTRACT

Bioflocs are composed of a complex microbial community formed not only by bacteria but also by algae, fungi, protozoa, rotifers, and other organisms. Intestinal bacterial communities can naturally differ between different cultures. The objectives of this work were to analyze the stomach contents of shrimp *Litopenaeus vannamei* in biofloc culture at different percentages of inoculation and describe which organisms and biomolecules are found in shrimp in these types of culture, thus determining the best culture according to the microorganism content. The experiment was carried out at the Marine Station of Aquaculture (EMA) of the Oceanographic Institute of the Federal University of Rio Grande – FURG. It consisted of four treatments: (I) clear water (CW); (II) 25% biofloc inoculum (25% BF); (III) 50% biofloc inoculum (BF 50%); and (IV) 100% biofloc inoculum (BFT100). In all cases, inoculums of mature bioflocs from previous cultures have been used. The zootechnical performance of the shrimp was monitored weekly through weightings, and the physical and chemical parameters of the water (temperature, dissolved oxygen, pH, ammonia, and nitrite) were measured daily. The abundance of the microbial community, mainly influencing the water quality in the treatments, may explain the different effects of the conditions. Biofloc treatments achieved higher survival rates

than clear water treatment. The microorganisms found in the stomach of shrimps cultivured in a biofloc system were oocystis, ciliates, flagellates, diatoms, free cocci, free and attached filaments, bacilli, and vibrios. The stomach microbiota is determined by the culture medium. The results suggested that the reuse of bioflocs in the percentage of 25% of the total volume is the most effective in maintaining the water quality of the system and colonizing the animal's microbiota.

Keywords: Biofloc. Microorganisms. Intensive system. Bacteria. Water Reuse.

1 INTRODUCTION

The white shrimp *Litopenaeus vannamei* is the most cultured among all shrimp species and in 2020 it was the most produced in the world, with 5.8 million tons (FAO, 2022). Among its characteristics, fast growth rate, good survival in high-density culture, and disease tolerance make it a good choice for intensive and biosafety production systems (Cuzon et al., 2004). Biofloc technology (BFT) is considered a revolutionary system in aquaculture, as the organisms help maintain water quality, reduce feed conversion rates (FCR) and increase biosecurity (Emerenciano et at., 2017). In addition, BFT system does not harm the environment like the traditional culture system, as it has the possibility of reusing the same water some times, thus avoiding the pollution of coastal waters (Krummenauer et al., 2014). Bioflocs are composed of a complex microbial community formed not only by bacteria, but also by algae, fungi, protozoa, rotifers, and other organisms (Emerenciano et al., 2017; Reis et al., 2023).

There are several types of production with bioflocs. Those that are exposed to natural light such as nurseries and outdoor tanks (intensive culture), usually are located in tropical or subtropical regions, where there is an abundance of natural light and a predominance of photoautotrophic organisms that cause a greenish water color (Prangnell et al., 2016). Cultures that are carried out in greenhouses (super-intensive systems), with little or no exposure to natural light, are generally common in temperate regions. The color of the water is commonly brown and the bacterial processes that control the quality of the water predominate (Hargreaves, 2013; Samocha et al., 2017).

Because of the increase in population density in the crops and the reduction or lack of water exchange, feed residues, detritus, and inorganic nitrogenous compounds accumulated in the water in the tanks (Burford et al., 2003). In this system, the removal of ammoniacal nitrogen is initially carried out by heterotrophic bacteria, which assimilate dissolved ammonia by manipulating the C:N ratio of the system, maintaining this ratio at

approximately 15:1. Organic carbon additions are carried out to stimulate the production of heterotrophic bacteria. In this way, ammonia is controlled by adding organic carbon to stimulate the growth of heterotrophic bacteria, which metabolize this nitrogenous compound, transforming it into bacterial biomass (Hargreaves, 2013). Already, in the chemoautotrophic process, due to the slow growth rate of nitrifying bacteria, small amounts of bacterial biomass are produced, these bacteria carry out the oxidation of ammonia to nitrite (Ammonium-oxidizing bacteria - AOB), and later to nitrate (Nitrite-oxidizing bacteria - NOB) (Ebeling et al., 2006; Crab et al., 2007).

McIntosh (2000) stated that the development of microbial bioflocs is a time-consuming process, which can take between seven and eight weeks. Another way to facilitate the process of biofloc formation and help in the faster removal of nitrogenous compounds in the system would be the reuse of water from previous cultures with already developed bioflocs, this technique can favor the rapid establishment of the microbial community in the water, making that acts quickly in the removal of nitrogenous compounds from feces and leftover feed, thus offering greater stability to culture cycles (Krummenauer et al., 2014; Wasielesky et al., 2022).

In addition, animals cultured in BFT system can feed on the bioflocs formed in the environment. There is a positive relationship between the size of *L. vannamei* and the consumption of bioflocs, since shrimp fed with artificial feed and bioflocs have better assimilation of nutrients when compared to those fed only with formulated feed. Krummenauer et al. (2020) demonstrated that in the nursery phase, bioflocs contribute to the 22-43% of carbon and the 0-43% of nitrogen in the shrimp tissue composition, while during the growth phase, carbon and nitrogen contribute with 63–100% and 35–86%, respectively. This is due to the greater amount of essential amino acids, fatty acids (PUFA and HUFA) and other nutrient elements provided by bioflocs (Tacon et al., 2002). The bacterial community that integrates the bioflocs can also act as a natural probiotic in disease control, through competition for space, substrates and nutrients with potentially pathogenic bacteria (Emerenciano et al., 2013).

The gastrointestinal tract of penaeids is tubular, being divided into the foregut (esophagus and stomach), midgut and hindgut (rectum and anus), and also includes the hepatopancreas digestive gland (Guillaume et al., 1999). The stomach is divided into an anterior portion (cardiac chamber) and a posterior portion (pyloric chamber), separated by a cardio-pyloric valve. The joi action of these parts makes it possible to macerate the food and prevent the passage of large particles into the midgut (Silveira, 2016). Like

fishes and most aquatic animals, the microbiota of penaeid shrimp can be determined and influenced by contact with the environment (Wu et al., 2012; Zhang et al., 2014; Cardona et al., 2016). The intestinal microbiota of *L. vannamei* is dominated by the Proteobacteria phylum (Xiong et al., 2015). Among the groups of Proteobacteria, the genus Vibrio spp. and Pseudoalteromonas spp., associated with the natural microbiota of the gastrointestinal tract, are the most abundant (Tzuc et al., 2014). Thus, it is worth emphasizing the importance of the animal's physiology for the use of microorganisms by the shrimp organism.

The aim of this study was to analyze the stomach contents of shrimp *L. vannamei* in biofloc culture at different percentages of inoculations and to describe which organisms and biomolecules are found in shrimp in these types of culture; characterize the microorganisms regarding their benefit to the shrimp and their quantity, thus determining the best culture according to the content of microorganisms.

2 MATERIAL AND METHODS

2.1 Culture conditions

The study was conducted between January 24 and 4 March 2022 (40 days) at the Laboratory of Marine Shrimp Culture of the Marine Station of Aquaculture (EMA) belonging to the Institute of Oceanography of the Federal University of Rio Grande – FURG, located in the city of Rio Grande, RS, Southern Brazil. The species used in the study was the Pacific white shrimp *Litopenaeus vannamei*. The post-larvae were obtained from Aquatec® LTDA, and were nursed and grown in BFT system until achieve 13.5 ± 0.56 g. Juvenile shrimp were then acclimated in clear water for one week prior to the experiment

2.2 Experimental design

The water used on experiment was previously pumped from the Cassino beach (Rio Grande, RS, Southern Brazil). Prior to the experiment, the water was chlorinated using a concentration of 10 ppm and left to act for four hours before being neutralized using ascorbic acid (vitamin C) in the proportion of one gram per thousand liters of water. The water was transferred to sixteen polyethylene 350 L-tanks were placed in an experimental greenhouse. The stocking density was 300 shrimp m^{-3} (105 shrimp/ unit).

The study consisted of four treatments with four replicates: (I) control (C), carried out in clear water (II) 25% of the volume stocked with mature biofloc (BFT25); (III) 50% of the volume with mature biofloc (BFT50); and (IV) 100% mature biofloc (BFT100). In

treatments II (BFT25), III (BFT50) and IV (BFT100), different concentrations of biofloc reuse were used, following the method proposed by Krummenauer et al. (2014). The mature biofloc used in the experiment had an average concentration of 336 mg/L.

Sugar cane molasses with 25% carbon was used in initial phases of culture for ammonia control. The addition of molasses was carried out in such a way as to maintain a C:N ratio of 6.0 g of carbon (molasses) for each 1.0 g of total ammoniacal Nitrogen sample (TA-N) in the water. In addition, commercial probiotic applications (INVE ® Sanolife PRO-W) were applied once a week in the water using 1ppm, in order to help maintain water quality in all treatments (Santos et al., 2019).

2.3 Shrimp feeding and zootechnical performance

Shrimp were fed twice a day with commercial feed Potimar 38 active with 38% crude protein produced by Guabi Healthy and Animal Nutrition S.A. (Brazil). The feeding rate of the grow-out phase following the method by Garza de Yta et al., (2004). The zootechnical performance of shrimp was monitored weekly by measuring the average weight of 20 animals per experimental unit using a digital scale with a precision of 0.01 (Marte® UX420H). Weekly, at the end of the experiment, other zootechnical performance indices were evaluated: final weight, weekly weight gain, survival, and productivity. WWG: (Final Weight – Initial Weight) / number of weeks. Weekly Weight Gain: (Average weight for the week - Average weight for the previous week). Survival Rate: (final biomass / individual average weight) / number of individuals stocked x 100. Productivity: (final biomass – initial biomass) / tank volume.

2.4 Water quality parameters

Water temperature and dissolved oxygen (DO) were monitored twice a day using an oximeter (YSI, model Pro-20, USA) and pH was measured with a pHmeter (Mettler Toledo, FEP20, Brazil). The pH and alkalinity corrections were performed to maintain values above 7.2 and 120 mg of $CaCO_3$ L^{-1}, respectively, using hydrated lime [$Ca(OH)_2$], accordingly to Furtado et al. (2014), with alkalinity recorded weekly.

The salinity was verified with an optical refractometer (ATC, RTP-20ATC, Brazil) once a week. Total ammonia Nitrogen concentration was determined according to UNESCO (1983) and American Public Health Association (APHA) (2012), recorded daily. The nitrite Nitrogen concentration analysis (NO_2-N) was also performed daily and the total suspended solids (TSS) analysis was performed weekly, both following the methods of Strikland and Parsons, (1972). The nitrate Nitrogen (NO_3^- N) and phosphate (PO_4^- P) concentrations were measured weekly (Aminot & Chaussepied, 1983). Total

suspended solids were kept below 500 mgL-1 (Gaona et al. 2017).

2.5 Microbial community assessment

For the quantification of microorganisms, water samples (18mL) were collected once a week from each experimental unit for counting. Shrimp from each tank were killed by thermal shock in an ice bath and aseptically necropsied to remove the stomach at the beginning and end of the experimental period. The samples were fixed in 4% formaldehyde and kept in amber bottles for subsequent counting and identification of the main groups of microorganisms present.

To determine the abundance of bacteria, the fixed samples were filtered through polycarbonate membrane filters (Nuclepore, 0.2 μm pore and 2.5 mm in diameter) previously darkened with Irlan black and stained with 1% acridine orange, at a concentration of 1μg/mL (Hobbie et al., 1977). The bacteria were photographed using a camera attached to an Axioplan-Zeiss epifluorescence microscope, with a final increase of 1000X, for subsequent counting of 30 randomly chosen fields. For protozoa, an inverted microscope Olympus IX51 was used with a final magnification of 200x, where aliquots of 2.1mL of sample were placed in a sedimentation chamber and 30 random fields were counted (Utermohl, 1958). All counts were performed at the Laboratory of Ecology of Microorganisms Applied to Aquaculture.

2.6 Histological analysis

For histological analysis, shrimps were euthanized by thermal shock using ice and water. After that, Davidson's solution was injected into the muscles and organs of the animals; they were stored for 24 hours in Davidson's solution, after this period, the animals were transferred to another container containing 70% alcohol. Tissues were selected in paraffin for conservation and histological sections on a rotary microtome (LEICA RM2245) to a thickness of 5 μm. The fabrics, finally, they were prepared in hematoxylin-eosin (HE) colors for histological analysis (Bell and Lightner, 1988).

2.7 Statistical analysis

The data were submitted to homoscedasticity of the variances and normality of the data distribution analyses. When the assumptions were not met, data were submitted to statistical transformations. Subsequently, a two-way analysis of variance – ANOVA ($\alpha = 0.05$) was applied followed by a post hoc Tukey's test when significant differences were found (Zar, 2010).

3 RESULTS

198 The main values (± SD) of the chemical and physical parameters of water during
199 the 40-day experiment period are shown in Table 1. There was a significant differences
200 amoung treatments in the following parameters: DO, pH, nitrite, nitrate and TSS (total
201 suspended solids). Variables such as DO and pH were significantly greater ($p < .05$) in
202 treatment control clear water than in the biofloc treatments ($p < 0.05$). The DO was
203 maintained at a concentration greater than 5.89mg/L and the temperature maintained in
204 the range of 26-27°C. The variables nitrite was significantly lower in CW than in the
205 biofloc treatments ($p < 0.05$). Total ammonia nitrogen and phosphate did not show
206 statistical differences between treatments. Nitrate showed lower averages in the CW and
207 BF 50% treatments and there was a significant difference between the CW and BF 50%
208 treatments for the BF 25% and BF 100% treatments. The salinity presented average
209 concentration above 35 ppt. There was no statistical difference for total suspended solids
210 between the biofloc treatments, but there was a difference in relation to the treatment with
211 clear water ($p < 0.05$).

212 **Table 1**. Physical and chemical parameters of water (mean values ± standard deviation)
213 in the treatments: Clear water (CW), 25% Biofloc (BF 25%), 50% Biofloc (BF 50%) and
214 100% Biofloc (BF 100%). Different superscripted letter in a row denotes significant
215 differences ($P < 0.05$) between treatments.

Parameters	Treatments			
	CW	BF 25%	BF50%	BF100%
Temperature (°C)	26.24 ± 0.86	26.60 ± 0.81	26.54 ± 0.74	26.55 ± 0.78
DO (mg L^{-1})	6.04 ± 0.28^{a}	5.89 ± 0.19^{b}	5.91 ± 0.17^{b}	5.88 ± 0.19^{b}
pH	8.02 ± 0.08^{a}	7.73 ± 0.15^{b}	7.66 ± 0.20^{b}	7.77 ± 0.20^{b}
Salinity	35.96 ± 0.97^{b}	37.33 ± 0.85^{ab}	38.42 ± 1.37^{a}	37.42 ± 1.20^{ab}
Alkalinity (mg of $CaCO_3$ L^{-1})	127.64 ± 10.55	115.83 ± 29.07	107.5 ± 28.25	125.83 ± 44.85
TA-N (mg. L^{-1})	0.80 ± 0.25	1.36 ± 1.70	1.52 ± 2.28	1.29 ± 2.38
NO_2^-N (mg. L^{-1})	0.37 ± 0.28^{a}	1.44 ± 2.44^{b}	1.64 ± 2.88^{b}	1.09 ± 1.71^{b}
NO_3^-N (mg. L^{-1})	8.11 ± 4.21^{a}	104.82 ± 54.79^{b}	84.55 ± 45.23^{a}	107.11 ± 47.68^{b}
PO^3_4- P (mg. L^{-1})	0.78 ± 0.60	3.31 ± 2.05	3.60 ± 2.30	3.85 ± 1.32
TSS (mg. L^{-1})	152.25 ± 30.44^{a}	421.27±106.90^{b}	415 ± 87.89^{b}	416.95±69.56^{b}

216 Among treatments, ammonia showed lower averages in the treatment CW and
217 higher in treatments BF 25% and BF 50%. The BF 100% treatment achieved the highest
218 average concentration of ammonia on the 4th (11.65 mg/L) day of the experiment,
219 decreasing in subsequent days. The BF 25% treatment reached the highest mean ammonia

concentration on the 4th day (10.13 mg/L), decreasing from the 5th day on (3.55 mg/L). From the 7th day until the end of the experimental period, ammonia values were more stable in all treatments (Figure 1A). Mean nitrite values were lower in the CW treatment and higher in the 25% BF and 50% BF treatments. On the 3rd day of the experiment (Figure 1B), the 50% BF treatment presented the highest mean concentration of nitrite (12.60 mg/L), decreasing in the following days, until stabilizing on the 8th day (0.9 mg/L). The highest concentration of nitrite in the 25% BF treatment occurred on the 3rd day (12.73 mg/L), stabilizing on the 9th day (0.73 mg/L). The nitrite concentration in the CW treatment remained stabilized throughout the experiment, due to daily water changes.

Mean nitrate values were higher in the 25% BF treatment and 100% lower in the CW treatment. The highest concentration of nitrate occurred at the end of the experiment in the BF 25% treatment, with an average concentration of 175 mg/L. Between days 25 to 40, the nitrate concentration in the biofloc treatments remained similar (Figure 1C). To maintain good water quality from the CW treatment, daily water changes of 80-90% of the total tank volume were required.

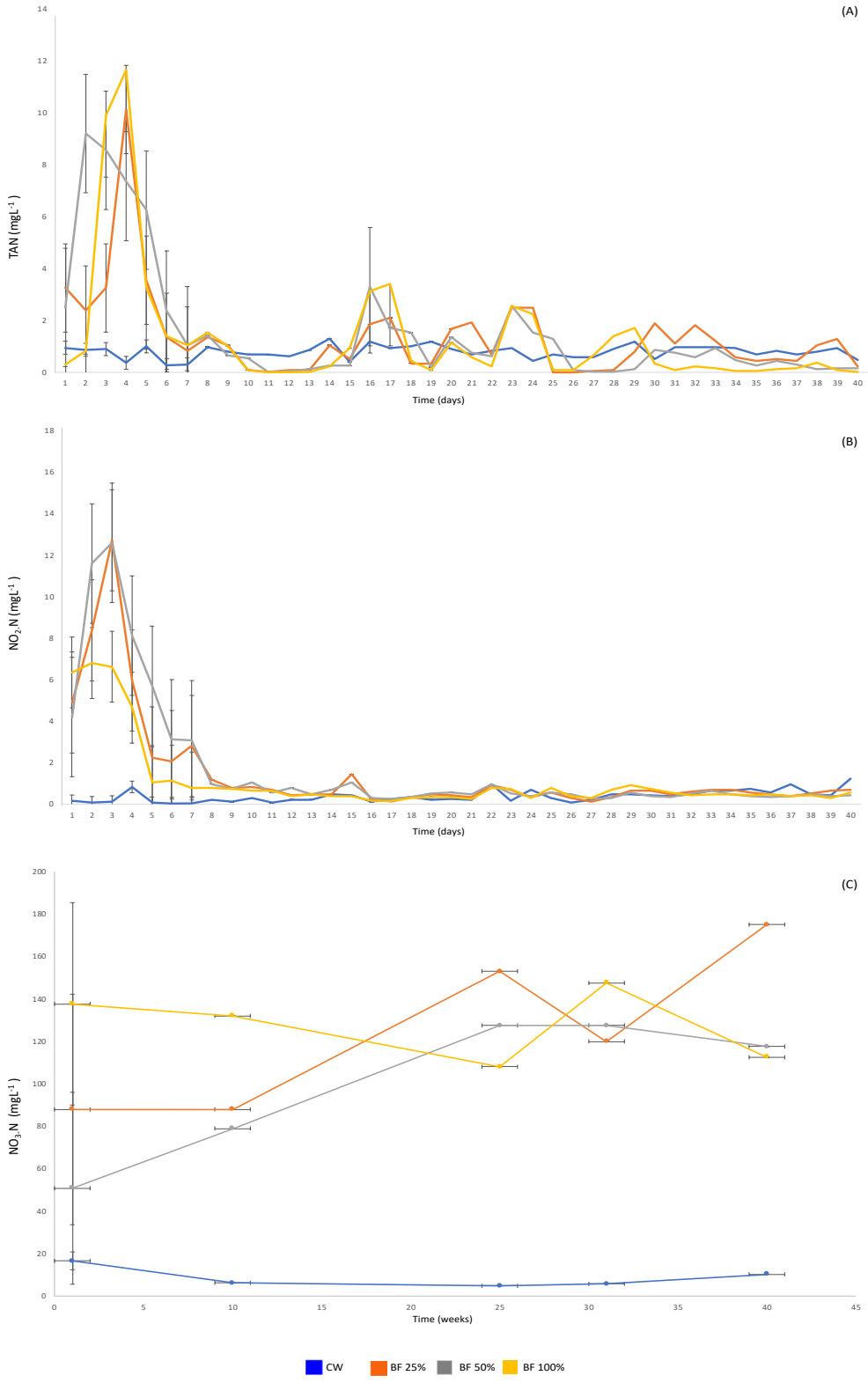

Fig 1. Mean values (± SD) of (A) total ammonia nitrogen, (B) nitrite and (C) nitrate (mg L^{-1}) in the four experimental treatments; clear water (CW), biofloc 25% (BF 25%), biofloc 50% (BF 50%) and biofloc 100% (BF 100%).

Figure 2A shows the concentrations of Oocystis at the beginning and end of the experiment in the stomachs of the animals. There was no significant difference considering the time variable ($p > 0.05$) or between treatments, with a maximum of 4.13×10^1 and a minimum of zero at the beginning of the experiment in the BF 50% treatment.

Figure 2B shows the concentrations of Ciliates at the beginning and end of the experiment in the stomachs of the animals. There was no significant difference considering the beginning and end of the experiment ($P < 0.05$), with a maximum of 3.12×10^2 and minimum value of 4.13 for BF 50%.

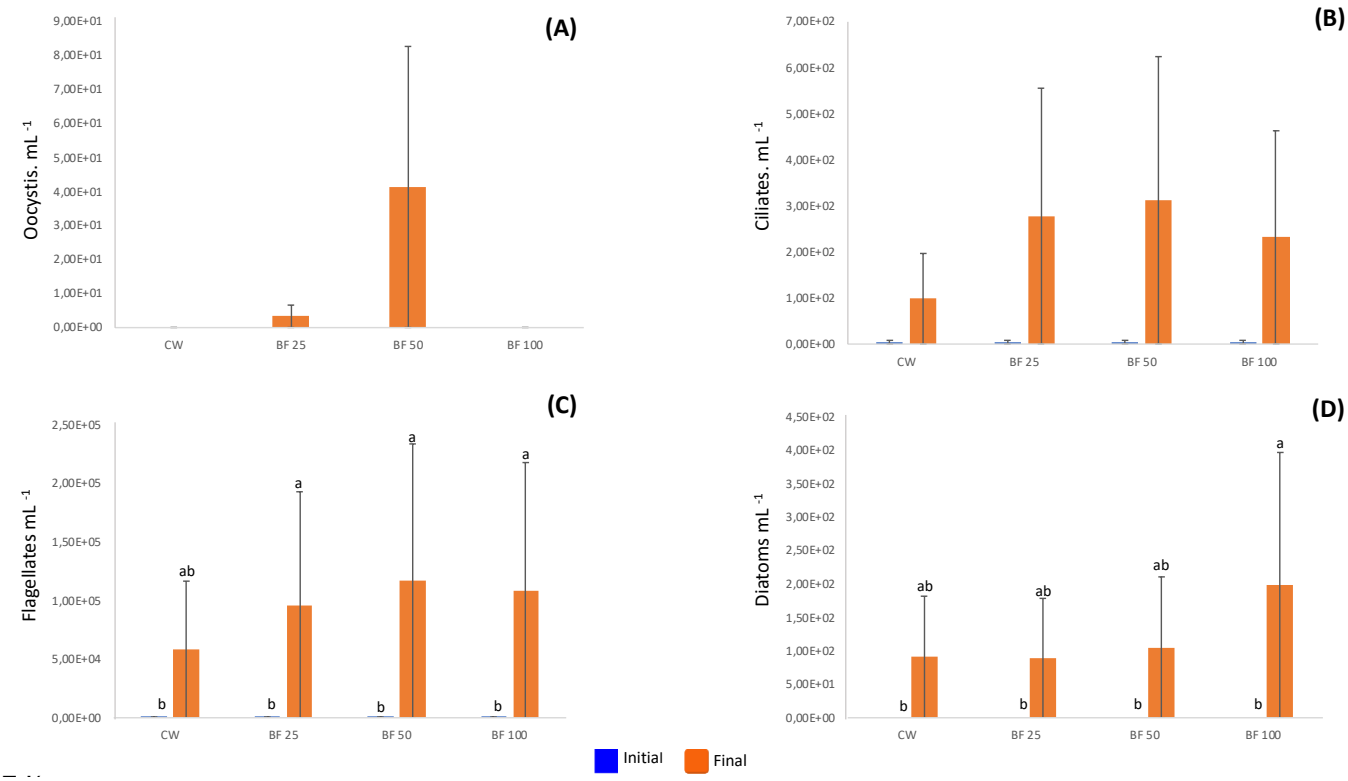

Fig 2. Mean values (± SD) of (A) Oocystis, (B) Ciliates, (C) Flagellates and (D) Diatoms (mg L^{-1}) present in the stomach of the animals in the four experimental treatments; clear water (CW), biofloc 25% (BF 25%), biofloc 50% (BF 50%) and biofloc 100% (BF 100%).

For Flagellates, there was a maximum of 1.17×10^5 for BF 50% and the minimum value was 3.39×10^2 , at the beginning of the experiment, with significant differences between the final and initial time of the experiment (Fig. 2C).

For Diatoms, we observed a difference between times ($p > 0.05$), where there was a maximum of 1.21×10^2 and a minimum of zero for BF 100% (Fig. 2D).

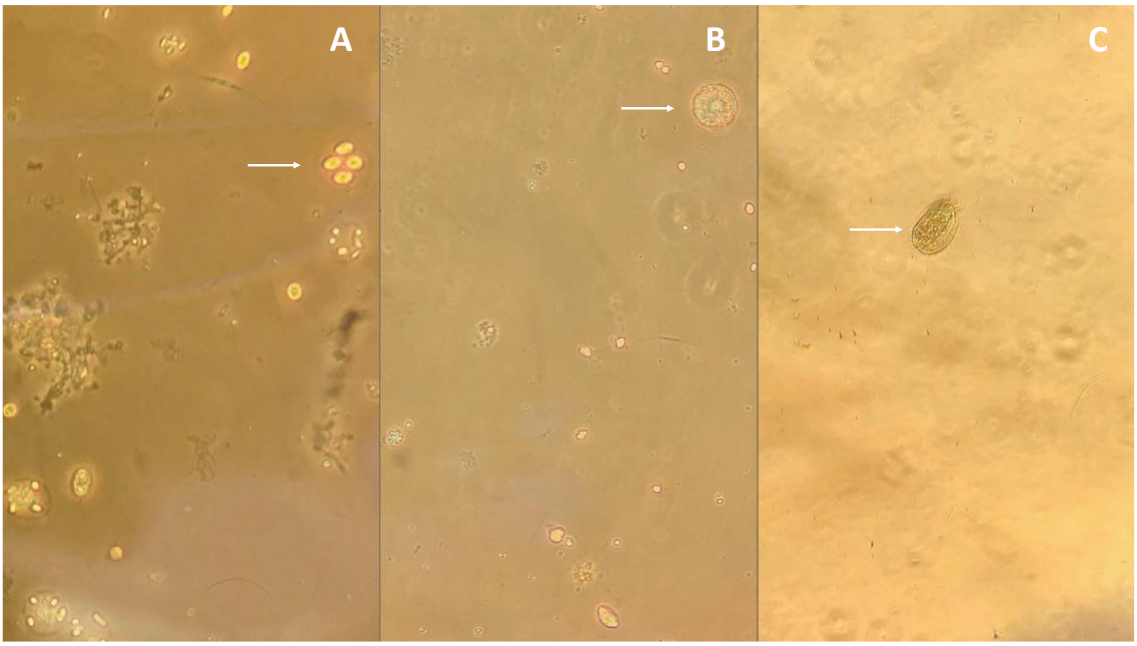

Fig. 3. Abundance of algae and protozoa in water in three treatments: (A) Oocystis on the 40th day of experiment in Biofloc 25% treatment; (B) diatom and (C) ciliate, both on the 40th day in Biofloc 50% treatment. Magnification of 200× by inverted microscope (image: Natália Pereira da Silva).

Figure 4A shows the concentrations of free coccoid bacteria at the beginning and at the end of the experiment in the animals' stomachs. There was a significant difference considering the time variable, but there was no significant difference between treatments ($p > 0.05$), with a maximum of 8.40×10^6 and a minimum of 1.85×10^5 in the BF 100% treatment. Figure 4B shows the concentrations of free filamentous bacteria at the beginning and at the end of the experiment in the stomach of the animals. There was a significant difference between the beginning and end of the experiment ($P < 0.05$), with a maximum of 9.18×10^4 and a minimum of 9.57×10^3 for BF 50%. For attached filamentous bacteria, there was a maximum of 7.97×10^2 for BF 100% and the minimum value was zero, at the beginning of the experiment. There were no significant differences over time and between treatments. (Fig 4C).

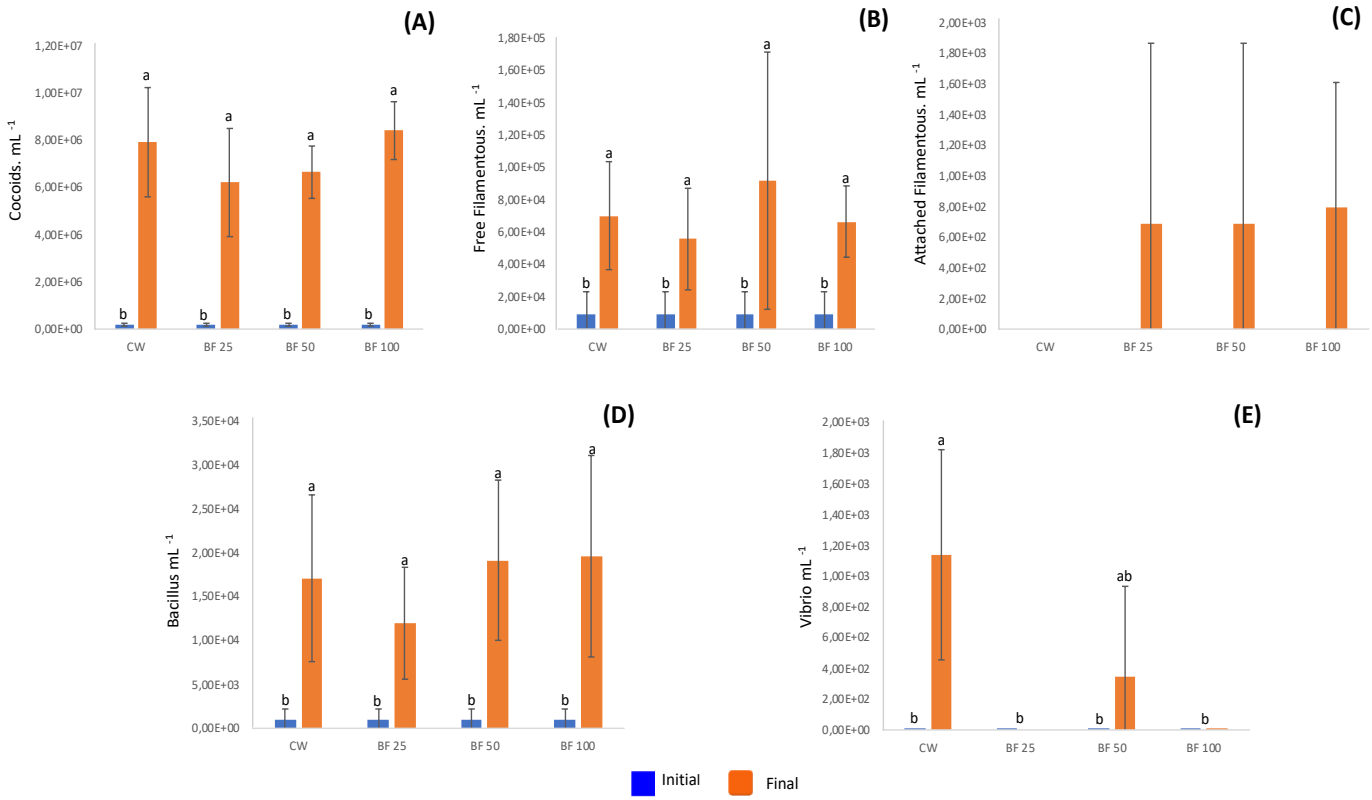

Fig 4. Mean values (± SD) (A) of coccoids, (B) free filamentous bacteria, (C) attached filamentous bacteria, (D) Bacillus and (E) Vibrio present in the stomach of the animals in the four experimental treatments; clear water (CW), biofloc 25% (BF 25%), biofloc 50% (BF 50%) and biofloc 100% (BF 100%).

For Bacillus, we observed significant differences between times ($p > 0.05$), where there was a maximum of 1.96×10^4 and a minimum of 9.11×10^2 for BF 100% (Fig. 4D). Figure 4E shows Vibrio concentrations throughout the experiment. There was a significant difference between the times for the Clear Water and Biofloc 50% treatments ($P < 0.05$), with a maximum of 1.14×10^3 for CW and a minimum value being zero at the beginning of the experiment for all treatments.

Figure 4 presents photomicrographs of the microorganisms in the four treatments at 20 days of the experiment. The biofloc treatments demonstrate a lower abundance of vibrio and a higher abundance of adhered filamentous bacteria compared to the clear water treatment. It is also possible to identify the presence of free coccoids (4A), free filamentous (4B), attached filamentous (4C) and amoeba (4D) as shown in the micrographs.

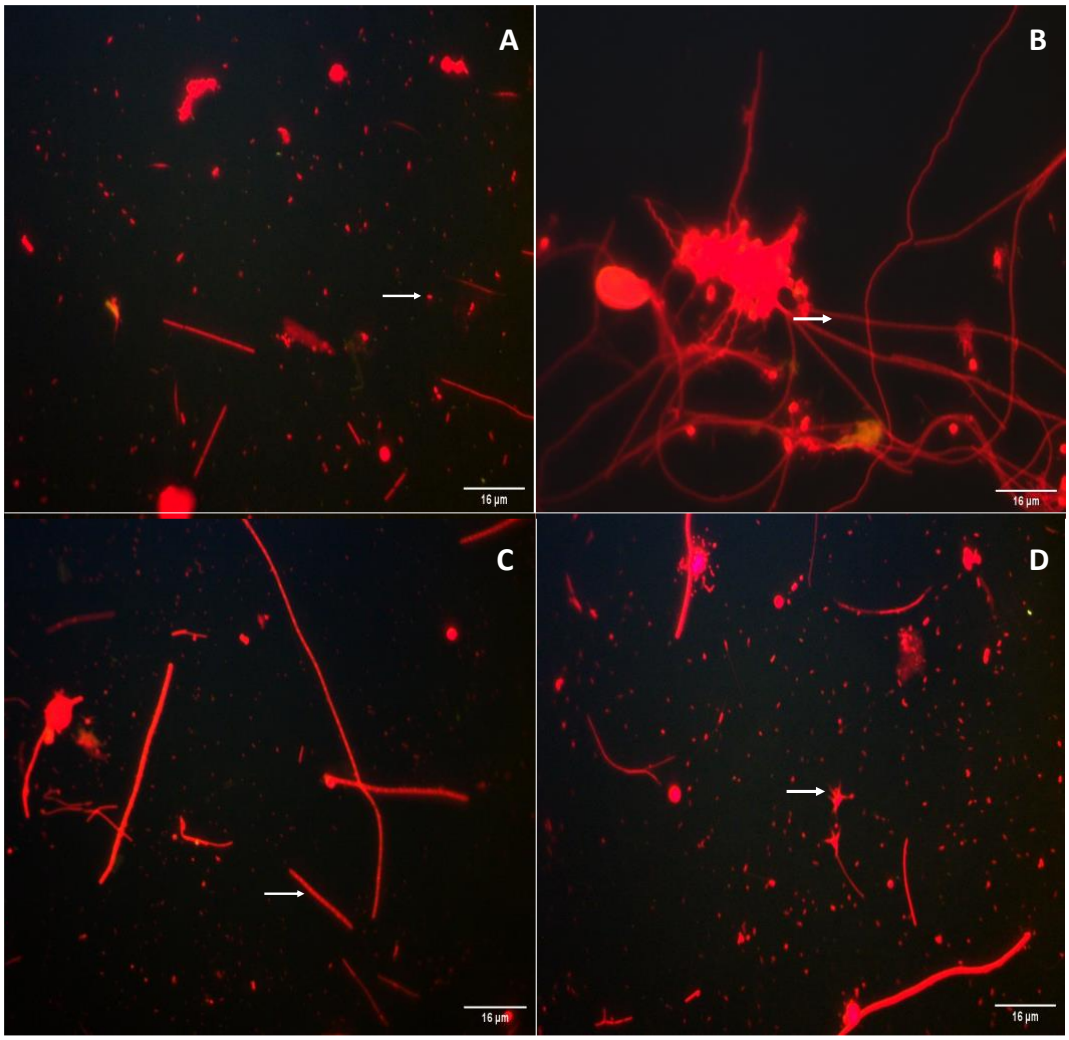

Fig. 5. Abundance of bacteria and protozoa in the water in four treatments: (A) cocoids the 20th day of experiment in Clear Water treatment; (B) attached filamentous bacteria on the 20th day in Biofloc 25% treatment; (C) free filamentous bacteria on the 20th day in Biofloc 50% treatment and amoeba on the 20th day in Biofloc 100% treatment (D). Magnification of 1000× by epifluorescence microscopy (image: Natália Pereira da Silva).

In the present study, the stomach of *Litopenaeus vannamei* did not show alterations caused by pathogens, being characterized as healthy. There were no significant differences between shrimp cultured in bioflocs system or clear water (Fig. 6).

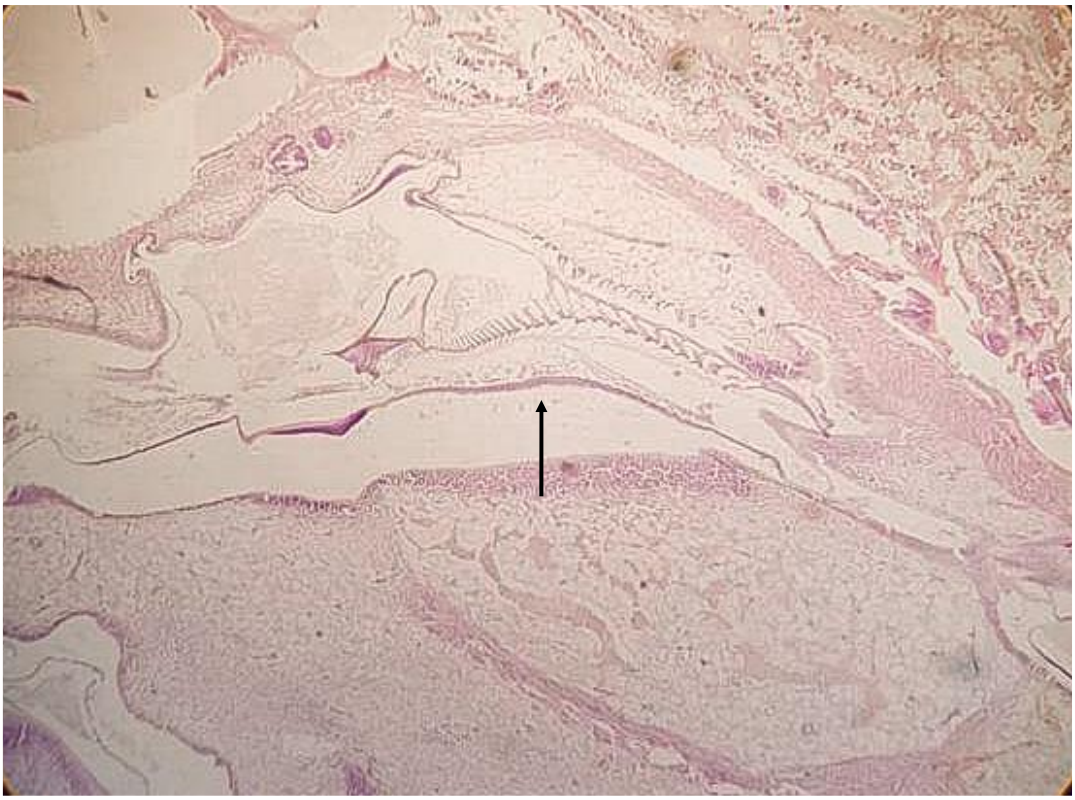

Fig. 6. Micrographs of the stomach of the shrimp *Litopenaeus vannamei* at the end of the experiment (BF 25% treatment). Magnification of 200× by inverted microscope (image: Natália Pereira da Silva).

Animal survival was the only parameter that showed statistical difference between treatments, and CW had the lower mean value. Shrimp in the four tested treatments showed a final weight gain with an average of over 3.0g. Regarding the productivity values, in the four different production systems, there was a productivity greater than 4 kg/m³ of Pacific white shrimp.

Table 2- Mean values (± standard deviation) of the zootechnical performance of *Litopenaeus vannamei* in the treatments: Clear water (CW), 25% Biofloc (BF 25%), 50% Biofloc (BF 50%) and 100% Biofloc (BF 100%). Different superscripted letter in a row denotes significant differences ($P < 0.05$) between treatments.

Parameters	Treatments			
	CW	BF 25%	BF 50%	BF 100%
Initial weight (g)	13.73 ± 0.54	13.75 ± 0.32	13.49 ± 0.16	13.24 ± 0.82
Final weight (g)	17.73 ± 0.82	16.81 ± 0.63	16.12 ± 0.18	17.38 ± 0.68
Weekly growth rate (g)	0.80 ± 0.29	0.61 ± 0.15	0.50 ± 0.07	0.83 ± 0.22

Survival (%)	80.71 ± 10.33[b]	92.62 ± 3.68[ab]	94.03 ± 3.60[a]	89.05 ± 2.96[ab]
Yield (kg/m³)	4.24 ± 0.41	4.67 ± 0.22	4.56 ± 0.20	4.64 ± 0.19

4 DISCUSSION

In this study, water quality parameters such as temperature, salinity, dissolved oxygen, and pH were kept within the optimal ranges for *L. vannamei* (Ponce-Palafox et al., 1997; Van Wyk and Scarpa, 1999). The biofloc treatments had a significantly lower OD than the CW treatment. This observation is due to a steady increase in exercise rates of organic matter accumulation and high bacterial metabolism (Schveitzer et al., 2013; Taw, 2010). The significant pH differences observed between the control treatment (CW) and the biofloc treatments result from an inverse relationship between TSS concentrations due to the greater development of microbial communities, which consequently decrease the pH (Wasielesky et al., 2006, Furtado et al., 2011; Gaona et al., 2016; Hussain et al., 2021). The salinity was significantly higher ($P<0.05$) in the treatments rich in bioflocs, a possible reason for this is that during the experiment the tanks were filled with sea water, due to evaporation, increasing even more the concentration of salts. Compounds of nitrogen, alkalinity, and solid concentration (TSS) were also considered within adequate conditions and similar to those reported in literature (Ray et al., 2010; Samocha, 2019; Ferreira et al., 2021; Emerenciano et al., 2022). TAN concentrations in all treatments remained below the threshold reported by Lin and Chen (2001) as toxic to shrimp.

At the beginning of the experiment there were high peaks of ammonia, but they were quickly controlled, not compromising the survival and growth of the animals. Rapid bacterial stabilization resulted in rapid removal of ammonia and nitrite from the culture water. This stabilization process was also observed by other researchers working with *L. vannamei* in systems with little or no water exchange. Krummenauer (2014) demonstrated that supplementing seawater with biofloc-rich water at a level as low as 25% is effective for reuse, which this water helped to maintain low levels of ammonia and nitrite throughout the experiment. About nitrate, additional studies have stated that nitrate often accumulates in systems operated without water changes (Kuhn et al. 2010). The study nitrate concentrations ranged between 13.25 and 175 mg/L, therefore within the recommendation ranges of several researchers, who documented levels above 400 mg/L NO_3−N being critical in these systems throughout the production cycle (Samocha et al. 2010, 2011; Krummenauer et al., 2011). Most of the phosphorus in the BFT systems is in dissolved or particulate form, and its accumulation is common throughout the cycle

(da Silva et al., 2013; Gaona et al., 2016). The values found in the study are within the normal range for this compound.

The concentration of solids tends to increase with time. This increase is mainly due to the increase in bacterial biomass, through the inoculum inserted in the culture medium at the beginning of the experiment. In this study, the maximum TSS concentration reported was 541 mg/L (25% of treatment). Avnimelech (2009) warns that a TSS concentration greater than 500 mg/L can interfere with the water quality and the zootechnical performance of the shrimp and suggests maintaining the TSS level between 200 and 500 mg/L. Different methods were listed as possible approaches to control solids levels in limited or zero exchange systems; these methods include clarification, filtration, and water exchange (Ebeling et al. 2006; Ray et al. 2010; Gaona et al. 2011; Krummenauer 2014). In the present study, when the recorded values were equal to or greater than 500 mg/L, partial water changes were performed, varying from 20 to 30% of the total volume of the tank.

Microorganisms have important functions in aquaculture systems. They are responsible for primary production, nutrient cycling, are also essential for maintaining water quality, disease control and as mediators of the environmental impact of effluents (Moriarty 1997; Decamp et al. 2002; Thompson et al. 2002). One of the target points of this study is the culture environment, as it exerts a great influence on the formation and development of the intestinal microbiota of aquatic animals, reflecting a link between the gastrointestinal microbiota and the environment (Tzuc et al., 2014; Hostins et al. 2017).

Diatoms (Fig. 3B) can help stabilize the ecosystem in which animals live, minimizing large fluctuations in water quality and preventing the accumulation of residual nutrients to toxic levels (Lemonnier et al., 2017, Emerenciano 2022). Diatoms also have the advantage of having different sizes within the bioflocs, in addition to being rich in fats, mainly EPA (20:5n-3) and DHA (22:6 n-3), with 5% to 35% of the total polyunsaturated fats fatty acids (Hemaiswarya et al, 2011; de Abreu., et al, 2019), providing the necessary nutrients for the growth of various microorganisms (Jiménez-Ordaz, 2021). At the end of the experiment, diatoms were found in the shrimp stomachs in all treatments, with slightly higher values in the BF 100% treatment. Some species of the genus Oocystis (Fig. 3A) have heterotrophic metabolism, being able to absorb and use carbon sources (Du et al., 2018), which explains the presence of the genus in the LF25% and LF 50% treatments. Ciliates (Fig. 3C) feed on algae, bacteria and fungi (Nagano et al., 2004) and are a rich source of free amino acids and, similar to flagellates,

can provide other organic, polyunsaturated and sterol preparations for shrimp. (Khanjani et al., 2022). At the end of the experiment, ciliates were found in the shrimp stomachs in all treatments.

Flagellates have a high protein / energy ratio, being also capable to synthesize polyunsaturated fatty acids (Zhukova & Kharlamenko 1999; Viau et al., 2013). The predominance of flagellates and coccoid bacteria in the shrimp stomach probably occurred due to the predominance of these microorganisms also in the water. These results corroborate the work by Wu et al., 2012, Zhang et al., 2014, Cardona et al., 2016, where the authors demonstrate that the microbiota of penaeid shrimp can be determined and influenced by the environment where the animals are inserted.

Although filamentous bacteria (Fig. 5C) do not form flocs, they play a role in their formation, as they form the backbone on which the small flakes can become larger and denser flakes (Jenkins et al., 2003); the presence of filamentous was detected in all treatments in the stomach of the animals, and an increase was observed throughout the experiment. Furthermore, the growth of filamentous bacteria may be related addition of organic carbon dissolved in the system (Esteves, 1998). Despite the presence of filamentous, the largest domain of bacteria in all treatments was coccoid bacteria (Fig. 5A), the possible explanation for this is that due to their surface-volume ratio, these bacteria better assimilate nutrients, similar results were obtained by Suita (2015).

Vibrio is normally the most abundant flora in the shrimp digestive system (Oxley et al. 2002; Esiobu and Yamazaki 2003; Liu et al. 2011). In the present study, a commercial probiotic was used in the water, facilitating the colonization of Bacillus in the animal's intestinal tract, extenuating the survival of Vibrio, as also occurred in previous studies. (Balcazar et al., 2007; Krummenauer et al., 2014; Villaseñor et al., 2015; Hostins et al., 2017). The BF 50% treatment was the only treatment with bioflocs where Vibrio was present in the shrimp's stomach, but in smaller amounts when compared to the presence of Bacillos also in the stomach.

The fore and hind intestines are coated with a chitin-protein layer that is renewed with each molt (Guillaume et al., 1999) and the midgut is lined with an acellular and porous peritrophic membrane, which allows the absorption nutrient selection (McGaw et al., 2013; Wang et al., 2012). Abbaszadeh et al. (2022) observed differences in gut morphology, intestinal epithelial cell length increased in *L. vannamei* reared in BFT groups compared in the control, in the present study, there were no morphological alterations in the shrimp stomach in any treatment, this is possibly due to this coating.

In the present study, the treatments of bioflocs in the culture of *L. vannamei* positively influenced the zootechnical performance. The abundance of the microbial community, mainly influencing the water quality in the treatments, can explain the difference in the treatments. The final weight in the CW treatment was higher than in the bioflocs treatment, these results also occurred in the study by Esparza-Leal (2015). But there were no statistical differences in the final weight of the treatments with bioflocs for the treatment with clear water. Biofloc treatments achieve higher survival rates than clear water treatment. In the BF 50% treatment, survival was significantly higher than in the clear water (94%). These results corroborate those of Yun et al., 2017. The statistical difference in survival rate for clear water can be attributed to the stress caused by daily tank water changes. There was no difference in the final weight of the biofloc treatments for the clear water treatment. Also, there was no difference in productivity (4.24 – 4.67 m-3). The same was observed by the authors reis et al., 2023.

5 CONCLUSION

Culturing in a biofloc system positively influences the abundance of microorganisms in the stomach of *L. vannamei*, in general resulting in better animal performance. The results of present study suggest that the reuse of bioflocs in the percentage of 25% of the total volume is effective in maintaining the water quality of the system and colonizing the microbiota of the animal under study.

ACKNOWLEDGMENTS

The authors are grateful for the financial support provided by the National Council For Scientific And Technological Development (CNPq) and Coordination for the Improvement of Higher Level Personnel (CAPES). Wasielesky, W.Jr. and Abreu, P.C.A. are a research fellow of CNPq under process number: 310652/2017-0 and 303256/2018-4 respectively. Thanks to GUABI Animal Healthy and Nutrition and AQUATEC, TREVISAN and Al Aqua for donating the experimental diets and post-larvae and Aeration system respectively.

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