UNIVERSIDADE FEDERAL DO PARÁ
INSTITUTO DE TECNOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIENCIA E TECNOLOGIA DE ALIMENTOS

EVELYN IVANA TRINDADE DAMASCENO

PROSPECÇÃO BACTERIANA EM PESCADO AMAZÔNICOS E SUA RESISTENCIA A SANIFICANTES

Belém
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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, do Instituto de Tecnologia da Universidade Federal do Pará, como parte dos requisitos para obtenção do título de Doutora em Ciência e Tecnologia de Alimentos

Orientador: Prof. Dr. Antônio Manoel da Cruz Rodrigues

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Por

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RESUMO

DAMASCENO, Evelyn Ivana Trindade. **Prospecção bacteriana em pescado amazônicos e sua resistencia a sanificantes**, 2015, 97f, Tese (Doutorado) – Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos. Universidade Federal do Pará, Belém.

Este estudo teve como objetivo identificar a composição da flora bacteriana, sua influência sobre o processo de deterioração microbiológica em espécies de peixes de importância econômica na região amazônica: tucunaré (*Cichla ocellaris*), piramutaba (*Brachyplatystoma vailantii)*, filhote (*Brachyplatystoma filamentosum*), dourada (*Brachyplatystoma rousseauxii*) e a resistência das bactérias isoladas pelo uso de diferentes sanificantes: hipoclorito de sódio e ácido peracético associados com ultrassom. Para este fim, a caracterização microbiológica da parte ventral dos pescado foi realizada: contagem de bactérias aeróbias mesófilas totais, bactérias psicrotróficas e coliformes a 35°C e 45°C. O isolamento das bactérias foi realizado por semeadura em superfície utilizando o ágar Violet Red Bile Glicose (VRBG), para as cepas de enterobactérias e ágar Baird-Parker com emulsão telurito-gema de ovo para espécies de estafilococos, ambas com incubação a 36°C por 48 h. As bactérias isoladas foram identificadas utilizando o kit API 20E (enterobactérias) e API Staph (estafilococos). O limite de temperatura para o crescimento dos micro-organismos foi testado com incubação a 10 e 15°C e posterior medição espectrofotométrica. Para os tratamentos com sanificantes, *S. aureus*, *S. hominis* e *P.aeruginosa* foram separadamente inoculadas em placas de aço inoxidável. Após incubação a 30ºC por 24h, as placas de aço inoxidavel foram tratadas por diferentes concentrações de hipoclorito de sódio (50, 100 e 150 mg/L) e ácido peracético (40, 60 e 80 mg/L) a 25ºC. Os tratamentos com hipoclorito de sódio (150mg/L) e ácido peracético (80 mg/L) foram também combinados com ultrassom (40Hz) por 10 minutos a 25ºC. Os resultados mostraram que as contagens de bactérias aeróbias mesófilas variaram de 5,14– 8,23 log UFC/g. Já as contagens de bactérias aeróbias psicrotróficas foram de 4,52 – 8,56 log UFC/g. Apresentaram também valores médios de 10^3 NMP/g para coliformes totais. Os micro-organismos predominantemente encontrados foram: *Staphylococcus hominis*, *Staphylococcus aureus*, *Stenotrophomonas maltophilia* e *Enterobacter intermedius* (tucunaré e piramutaba) e *Hafnia alvei*, *Pseudomonas luteola*, *Staphylococcus xylosus*, *Staphylococcus lugdunensis* (filhote e dourada). Quando submetidos sob temperaturas de 10 e 15 °C, as cepas isoladas de tucunaré e piramutaba não cresceram por 6 h ao nível de 95% de significância. Já algumas cepas isoladas de filhote e dourada se multiplicaram após 6h quando mantidas a 10 °C. Em se tratando da resistência contra os diferentes tipos sanificantes aplicados, observou-se que a melhor condição para *S. aureus*, *S hominis* e *P. aeruginosa* foi o ácido peracético (80mg/L) associado ao ultrassom. Entretanto, a aplicação do ultrassom isolado também apresentou bons resultados no controle da adesão de *P. aeruginosa.*

Palavras-chaves: Amazônia, pescado, cepas, adesão, sanificantes

ABSTRACT

DAMASCENO, Evelyn Ivana Trindade. **Bacterial exploration in Amazonian fish and their resistance to sanitizers**, 2015, 97p, Thesis (Doctoral) –Food Science and Technology Program. Federal University of Pará, Belém.

This study aimed to identify the bacterial flora composition, its influence of this process on the microbiological spoilage in economically important fish species in the Amazon region: butterfly peacock bass (*Cichla ocellaris*), piramutaba (*Brachyplatystoma vailantii)*, filhote (*Brachyplatystoma filamentosum*) and dourada (*Brachyplatystoma rousseauxii*) and the resistance of strains isolated against different sanitizers: sodium hypochlorite and peracetic acid associated with ultrasound. To this end, microbiological characterization from ventral part of fish was performed: counts of total mesophilic aerobic bacteria, psychrotrophic bacteria, and coliforms at 35°C and 45°C. Bacteria were also isolated through seeding in agar surface using Violet Red Bile Glucose (VRBG) for enterobacteria strains and Baird-Parker Agar with Egg-Yolk Tellurite for *Staphylococcus* species, both with incubation at 36°C for 48 h. The bacteria isolated were identified using the API 20E kit (Enterobacteria), and Gram-positive with API Staph (Staphylococci). The limit temperature for strain growth was tested with incubation at 10 and 15°C and subsequent spectrophotometric measurement. For analysis of sanitizers treatments, *S. aureus*, *S. hominis* and *P.aeruginosa* strains were separetely inoculated on stainless steel coupons. After incubation at 30ºC for 24h, the coupons were treated for 10 minutes by differents concentrations of sodium hypochlorite (50, 100 and 150 mg/L) and peracetic acid (40, 60 and 80 mg/L) at 25ºC. The sodium hipochlorite (150mg/L) and peracetic acid (80 mg/L) treatments were also combined to ultrassound (40Hz) for 10 minutes at 25ºC. The results showed that the mesophilic aerobic bacteria counts ranged from 5.14– 8.23 log CFU/g. To psychrotrophic aerobic bacteria the count range found were 4.52 – 8.56 log CFU/g. They also had an average score above 10^3 MPN/g for total coliforms. The most predominants microorganisms found were *Staphylococcus hominis*, *Staphylococcus aureus*, *Stenotrophomonas maltophilia*, *Enterobacter intermedius* (butterfly peacock bass and piramutaba) and *Hafnia alvei*, *Pseudomonas luteola*, *Staphylococcus xylosus*, and *Staphylococcus lugdunensis* (filhote and dourada). When subjected to temperatures of 10 and 15 °C, the strains isolated from butterfly peacock bass and piramutaba did not achieve growth for 6 h at a 95% significance level. While, some strains isolated from filhote and dourada achieve growth after 6 h at a 95% significance level. About the resistance against different types of sanitizers, it was observed the best condition for *S. aureus*, *S hominis* and *P. aeruginosa* was PAA associated with ultrasound. However, ultrasound showed good results in control of *P. aeruginosa* adhesion.

Key – words: Amazon, fish, strain, adhesion, sanitizers

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INTRODUÇÃO

O Estado do Pará com seus 21 mil km de rios, 74.780 km de igarapés e várzeas e 512 km de costa atlântica é o segundo maior produtor de pescado do Brasil com 129,9 mil toneladas (BRASIL, 2011), sendo que 97% de sua produção são provenientes da captura. Esta atividade representa uma das principais atividades econômicas do estado, sendo um importante produto de exportação. O Pará exporta para países como Estados Unidos, Japão, França, Espanha e para outros estados da Federação. Os principais recursos pesqueiros, que abastecem os mercados consumidores são: Piramutaba, Dourada, Pargo, Camarão, Tucunaré, Pescada Amarela, Mapará, Filhote, Pacu e Tambaqui.

O pescado do ponto de vista nutricional é reconhecidamente um dos alimentos mais completos, graças à disponibilidade de quantidades generosas de nutrientes essenciais, como proteína de alto valor biológico (com digestibilidade superior a 80%), vitaminas, principalmente A e D, fração lípidica, rica em ácidos graxos insaturados e baixo teor de colesterol (SARTORI e AMANCIO, 2012).

Dentre estes ácidos graxos, os pertencentes à família ômega-3, como o ácido eicosapentaenóico (EPA) e o docosahexaenóico (DHA) têm recebido maior atenção por reduzirem fatores de risco associados a doenças cardiovasculares, hipertensão, inflamações em geral, asma, artrite, psoríase e de vários tipos de câncer (VON SCHACKY, 2007). O consumo de ácidos graxos poli-insaturados (PUFAs) tem sido indicadas por serem consideradas como gordura saudável na manutenção da saúde humana (MURIUKI, PURDIE e DUMANCAS, 2012).

O valor nutritivo, no entanto, pode variar em função de numerosos fatores como espécie, idade, meio em que vive, tipo de alimentação, época de captura, peso, entre outros (RECKS e SEABORN, 2008). No entanto, o pescado é considerado um alimento altamente perecível em virtude da sua composição química favorável e por apresentar, já na captura, uma microflora natural potencialmente deterioradora (OLIVEIRA *et al*., 2008).

A higienização e o controle da temperatura de estocagem afetam fortemente a qualidade do produto final. Os procedimentos de higienização consistem, fundamentalmente, no uso de detergentes e sanificantes, sendo os compostos clorados os mais utilizados nas indústrias alimentícias. Porém os compostos clorados estão frequentemente associados a formação de triclorometanos que podem apresentar efeito cancerígeno. Outra possibilidade é a aplicação de métodos físicos como o ultrassom, pois possibilita uma boa eficiência na descontaminação e ajuda no aumento da vida-útil do produto.

OBJETIVO GERAL

Neste contexto o objetivo deste trabalho consistiu em estabelecer ações voltadas à avaliação do efeito de agentes sanificantes (ácido peracético e hipoclorito de sódio) com ou sem associação do ultrassom na microflora bacteriana de peixes capturados na região Amazônica.

CAPÍTULO I. REVISÃO DA LITERATURA

1. A MICROBIOTA CONTAMINANTE DOS PESCADO

A microbiota natural do pescado apresenta características peculiares e é influenciada pela natureza do ambiente aquático, onde a temperatura é um dos fatores seletivos. Em espécies de águas tropicais e subtropicais, os micro-organismos encontrados são predominantemente do tipo mesófilos Gram (+) como *Micrococcus* spp, *Bacillus* spp e *Corynebacterium* spp, enquanto que em peixes de águas temperadas predominam bactérias psicrotróficas, aeróbicas ou facultativas Gram (-) (LALITHA e SURENDRAN, 2006; PANTAZI *et al*., 2008).

A microbiota do pescado de água doce é composta por espécies dos gêneros *Aeromonas, Lactobacillus, Brevidumbacterium, Alcaligenes* e *Streptoccocus*, além da maioria dos gêneros encontrados em água salgada que incluem os vibrios halófilos, *Pseudomonas*, *Alteromonas*, *Flavobacterium*, *Enterococcus*, *Micrococcus* (FRAZIER; WESTHOFF, 1993).

As regiões naturalmente mais contaminadas são a pele, as guelras e o trato intestinal. No muco que recobre a pele existem bactérias do gênero *Pseudomonas*, *Alteromonas*, *Acinetobacter*, *Moraxella*, *Alcaligenes*, *Micrococcus*, *Flavobacterium*, *Corynebacterium*, *Sarcina*, *Serratia*, *Shevanella*, *Vibrio*, *Bacillus* (FRAZIER e WESTHOFF, 1993; DALGAARD, 1995), *Cytophaga* (FARBER, 1991) e *Aeromonas* (WARD e BAJ, 1989).

Todavia, a microbiota do pescado depende da carga bacteriana ambiental que sofre influência do nível de poluição da água (WARD e BAJ, 1989). Em regiões de elevada contaminação ambiental, é comum a ocorrência de micro-organismos pertencentes a família *Enterobacteriaceae*, especialmente os coliformes totais e termotolerantes, com ênfase na *Escherichia coli*. Outros patógenos pertencentes a esta família como *Salmonella* spp, *Shigella* spp e *Yersinia enterocolitica* podem ser contaminantes do pescado, constituindo grave problema de saúde pública (PELCZAR, 2004).

Apesar de serem associadas a contaminação de origem fecal, os integrantes desta família não necessariamente têm o trato intestinal de homens e animais como habitat natural, estando presentes no meio ambiente, principalmente em unidades de manipulação e beneficiamento de alimentos que apresentam higiene deficiente. Portanto, os micro-organismos da família *Enterobactericeae* são considerados indicadores da qualidade higiênico-sanitária de alimentos (HOLT, 1994).

Esta família inclui micro-organismos bastonetes retos, gram negativos, usualmente com 0,3 e 1,8 µm de comprimentos, anaeróbicos facultativos, os quais fermentam a glicose produzindo ácidos, em algumas espécies produzem gás, são oxidases negativos, usualmente catalase positivos, reduzem nitrito a nitrato, e são móveis com flagelos petríquios ou imóveis. Crescem bem a 37ºC, porém algumas espécies podem crescer também entre 25 – 30 ºC. Os gêneros mais comuns a esta família compreendem *Citrobacter*, *Enterobacter*, *Erwinia*, *Escherichia*, *Hafnia*, *Klebsiella*, *Proteus*, *Providencia*, *Salmonella*, *Serratia*, *Shigella* e *Yersinia*. Estes micro-organimos são de caráter mesófilico, porém algumas cepas psicrotróficas de Enterobacter, Hafnia e Serratia podem crescer abaixo de 0ºC (HOLT *et al*, 1994; KORNACKI e JOHNSON, 2001).

Outros micro-organismos patogênicos também podem estar presentes no pescado e se tornar problemas de saúde pública. Podem ser oriundos do próprio habitat assim como da deficiência na execução de boas práticas de higiene, como *Vibrio cholerae*, *V. vulnificus*, *V. parahaemolyticus*, *Plesiomonas shigelloides*, *Clostridium botulinum*, *Stapphylococcus aureus* (HANCKNEY e DICHARRY, 1988), *Listeria monocytogenes*, *Yersinia enterocolitica*, *Campylobacter* spp, *Salmonella* spp (WARD e BAJ, 1989) e *Aeromonas hydrophila* (TSAI e CHEN, 1996; ISONHOOD e DRAKE, 2002).

2. ADESÃO BACTERIANA E A FORMAÇÃO DE BIOFILMES

A adesão microbiana ocorre devido à deposição de micro-organismos em uma superfície de contato, onde eles se fixam e iniciam o crescimento (ZOTTOLA; SASAHARA, 1994; ZOTOLLA,1997). Dentre os fatores que contribuem para a adesão destacam-se: o tempo de contato, a temperatura, a genética, a virulência e a resistência do micro-organismo; a nutrição; a área e o material da superfície e a velocidade do fluxo de líquidos (ANDRADE, 2008).

Para a adesão ocorrer devem existir forças atrativas entre a célula e a superfície, e evidentemente essas forças devem ser mais fortes que as repulsivas. Dentre as forças atrativas relacionadas à adesão estão a atração eletrostática, força de Van der Waals, interações hidrofóbicas e ligações químicas (ANDRADE, 2008).

Do ponto de vista da segurança alimentar e da degradação de alimentos, os biofilmes são importantes devido à sua formação em alimentos, utensílios e superfícies e à dificuldade encontrada em sua remoção. Se formados em materiais da linha de produção da indústria de alimentos, podem acarretar risco à saúde do consumidor e prejuízo financeiro à indústria (FLACH et al., 2005).

Os biofilmes são tipicamente constituídos por água, micro-organismos, substâncias poliméricas extracelulares (EPS, *Extracellular Polymeric Substances*), partículas retidas e substâncias dissolvidas e adsorvidas (PEREIRA, 2001).

A água é a parte mais significativa da massa total do biofilme, variando entre 70 a 97%, ou mais, da massa total (SUTHERLAND, 2001). Já os micro-organismos representam somente uma pequena parte da massa e do volume de um biofilme (menos de 10%), embora excretem as substâncias poliméricas que representam a fração dominante da matéria orgânica seca do biofilme (PEREIRA, 2001).

O biofilme contém ainda partículas de proteínas, lipídeos, fosfolipídeos, carboidratos, sais minerais e vitaminas, entre outros, que formam uma espécie de crosta denominada matriz exopolissacarídica,

abaixo da qual, os micro-organismos continuam a crescer, formando um cultivo puro ou uma associação com outros micro-organismos, e aumentando a proteção contra agressões químicas e físicas.

A matriz exopolissacarídica é secretada para o meio externo quando se atinge o último estágio da adesão da célula à superfície, chamada de adesão irreversível e é capaz de impedir fisicamente a penetração de agentes antimicrobianos no biofilme, principalmente aqueles hidrofílicos e carregados positivamente. Foi também relatado a proteção adquirida contra radiações UV, alterações de pH, choques osmóticos e dessecação (PARIZZI et al., 2004).

3. MECANISMOS DA ADESÃO BACTERIANA

A primeira teoria foi descrita por Marshall *et al*., (1971) e ressalta que a adesão é um processo que ocorre em duas fases. Na primeira fase, o processo é ainda reversível, em função do processo de adesão do micro-organismo na superfície ocorrer por forças de Van der Walls e atração eletrostática (Figura 1). Na segunda etapa, ocorre a interação física da célula com a superfície por meio de material extracelular de natureza polissacarídica ou protéica, produzida pela bactéria, que é denominada matriz de glicocálix, que suporta a formação de biofilmes. O glicocálix é produzido após o processo de adesão superficial, e vai fornecer condições de adesão do peptideoglicano das bactérias Gram positivas e a parte externa da membrana externa das Gram negativas (CAIXETA, 2008).

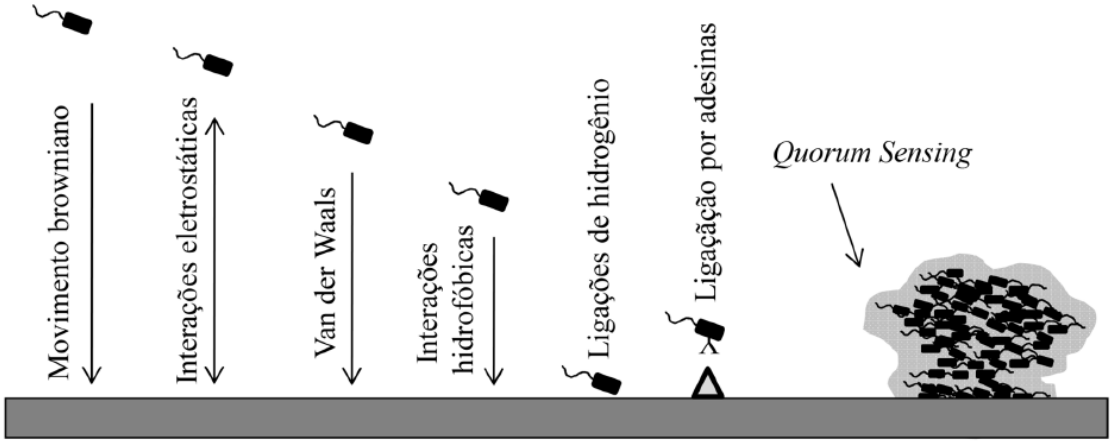

Figura 1. Interações envolvidas na adesão reversível de células bacterianas planctônicas em superfícies abióticas

Outra teoria sugere a existência de cinco etapas, diferenciadas na seguinte ordem: i) transporte de nutrientes e matéria orgânica e inorgânica para a superfície sólida; ii) formação de uma camada de nutrientes orgânicos e inorgânicos; iii) adesão dos micro-organismos à superfície e crescimento celular, iv) intensa atividade metabólica no biofilme; e v) liberação de células para o meio (CHARACKLIS; COOKSEY, 1983; ZOTTOLA, 1997).

Uma terceira teoria propõe a divisão do processo de adesão em três etapas, sendo a primeira a fixação da bactéria, seguida da consolidação da bactéria na superfície e, por último, a colonização da bactéria (NOTERMANS et al., 1991).

A consolidação é um estágio importante, pois os micro-organismos produzem, nessa fase, material extracelular que propicia a fixação das células na superfície. Nesse ponto, as células fixadas não são removidas por rinsagem com água (SCHWACH; ZOTTOLA, 1984; STONE; ZOTOLLA, 1985; GÓMEZ-SUAREZ et al., 2002), mas por ação mecânica ou química de detergentes e sanitizantes.

Durante o estágio de colonização, muitas mudanças provavelmente ocorrem entre a microcolônia e a superfície; e um complexo polissacarídico presente no glicocálix pode se ligar a íons metálicos, alterando a natureza química e física do biofilme.

Vários fatores podem influenciar a adesão de micro-organismos às superfícies, como as características do micro-organismo; do material aderente e do meio que envolve o micro-organismo (TROLLER, 1993). A espécie, o meio de cultura, a idade da cultura e a concentração do micro-organismo podem afetar o processo de adesão. Quanto ao material aderente, tanto o tipo e a forma iônica quanto o tamanho da partícula são importantes no processo de adesão. No que diz respeito ao meio, fatores como pH, concentração de sais orgânicos, compostos orgânicos, agitação, tempo e temperatura de contato são importantes nesse processo (TROLLER, 1993).

4. ASPECTOS GERAIS DA SANITIZAÇÃO

No processo de higienização de uma unidade de processamento de alimentos estão incluídas as etapas de procedimentos de pré-lavagem, usos de detergentes, enxágue e sanitização (ANDRADE, 2008). A sanitização deve ser realizada por meios físicos ou químicos empregando-se procedimentos de eficácia comprovada (CARDOSO *et al*, 2003).

Na sanitização por meios físicos pode-se empregar calor na forma de vapor e/ou água quente, radiação ultravioleta e ultrassom, enquanto que na sanitização através de agentes químicos pode-se utilizar uma série de compostos bactericidas, desde ácidos orgânicos até agentes umectantes complexos (CARDOSO *et al*, 2003).

4.1. Ultrassom

As ondas ultrassônicas podem ser produzidas através da utilização de transdutores magneto-restritivos ou pzielétricos que convertem corrente alternada de um oscilador eletrônico em ondas mecânicas no meio líquido através de uma sonda em forma de haste cilíndrica ou *sonotrodo* (CHISTI, 2003).

A aplicação do ultrassom surge como uma técnica alternativa de preservação capaz de eliminar e/ou reduzir a atividade de micro-organismos (OLIVEIRA, 2005; NASCIMENTO *et al*., 2008; CHEMAT, HUMA e KHAN, 2011; SÃO JOSÉ e VANNETTI, 2012).

A inativação de *Staphylococcos aureus, Listeria monocytogenes* e *Escherichia coli* pôde ser observada em processamento de leite UHT com a aplicação de ultrassom (CAMERON, MCMASTER e BRITZ, 2009). Além destes estudos, dados de OLIVEIRA (2005) indicaram que o ultrassom quando associado à água ozonizada pode reduzir a população de bactérias aeróbias mesófilas presentes em pescado e subprodutos, após 14 dias de tratamento. Gao et al. (2014a) observaram que o tratamento de ultrassom de alta frequência (850 kHz) puderam reduzir de 2.5 a 4.4 ciclos log de *E. aerogenes, B. subtilis* e *S.*

epidermidis. Gao et al. (2014b) observaram que o tratamento de ultrassom de baixa frequência (20 kHz) resultou em danos letais para suspensões de *E. aerogenes*.

A técnica de ultrassom de preservação de alimentos, pode ainda ser aplicada combinada com outros métodos, processo denominado de termo-ultrasonicação. Este quando combinado com tratamento térmico, por exemplo, pode ser capaz de acelerar a taxa de esterilização de determinados alimentos. Este fato permite reduzir a duração e a intensidade dos tratamentos térmicos aplicados nos alimentos, diminuindo também possiveis danos resultantes destes processos (CHEMAT *et al*., 2011). A termo-ultrasonicação, no entanto, não é indicada como método de preservação em produtos pesqueiros já que estes alimentos quando mantidos a elevadas temperaturas podem estimular a multiplicação de bactérias produtoras de histamina, assim como a perdas de alguns nutrientes importantes no pescado.

O mecanismo de inativação celular que ocorre pela aplicação do ultrassom tem sido atribuído a geração de cavitação intracelular causando ruptura da estrutura celular e de componentes funcionais até alcançar a lise das células (Figura 2) (CHEMAT, HUMA e KHAN, 2011).

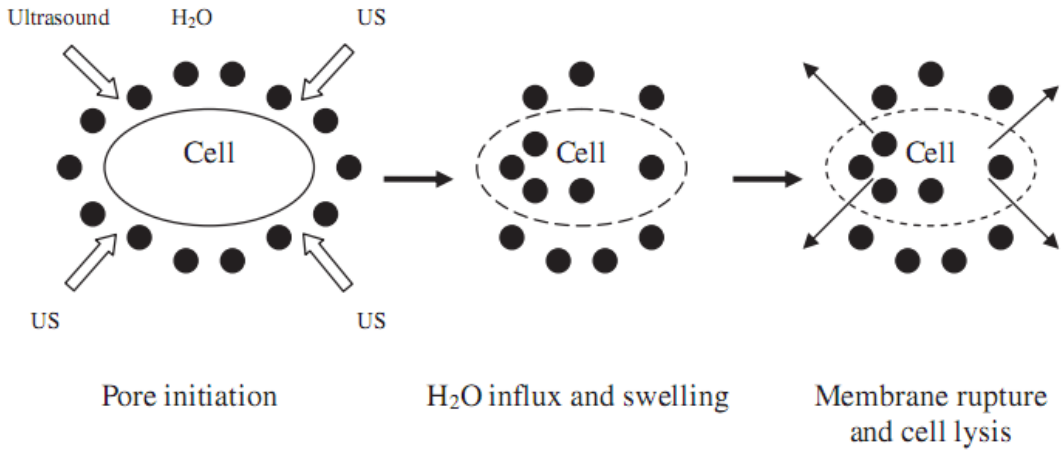

Figura 2. Mecanismo de dano celular induzido por ultrassom

Fonte: CHEMAT, HUMA e KHAN (2011)

De acordo com Mason *et al*. (2003) e Raviyan, Zhang e Feng (2005), cavitação é o processo de compressão e expansão em um meio liquido permitindo a formação, crescimento e implosão de

pequenas bolhas de gás pela passagem de ondas ultrassônicas, apresentando um efeito bactericida. A cavitação acústica pode ser dividida em dois tipos: transiente e estável. Na primeira, as bolhas da cavitação encontram-se cheias de ar ou vapor sofrendo oscilações irregulares até finalmente explodirem. Neste processo, observa-se o aumento da temperatura e pressão que são capazes de desintegrar células biológicas e/ou a desnaturação de enzimas. A cavitação estável trata-se da formação de bolhas que oscilam de forma regular por muitos ciclos acusticos (MASON *et al*., 2003).

4.2. Cloro

Os compostos clorados mais utilizados são: hipoclorito de sódio, de litio e de cálcio e dioxido de cloro, sendo todos inorgânicos. Entre os orgânicos temos as cloraminas T, dicloraminas T, dióxido ácido dicloroisocianúrico e dicloro dimetil hindatoina (ANDRADE e MACEDO, 1996; SREBERNICH, 2007).

De acordo com a Agencia Nacional de Vigilância Sanitária o limite máximo de cloro residual livre em água utilizada para consumo humano é de 2 ppm (BRASIL, 2011), o Ministério da Agricultura, Pecuária e Abastecimento recomenda valores em torno de 5ppm de cloro na água usada para lavagem de pescado (CODEX COMMITTEE ON FISH AND FISHERY PRODUCTS, 2000) enquanto que a Agência Canadense de Inspeção de Alimentos estabelece o limite de até 10 ppm para soluções que entram em contato direto com o pescado (PRINCE, 2000).

O hipoclorito de sódio em meio aquoso dissocia-se em ácido hipocloroso e hipoclorito. O poder bactericida dos compostos clorados baseia-se geralmente na liberação do ácido hipocloroso, em sua forma não-dissociada, quando em solução aquosa, com a exceção do dióxido de cloro, que não se hidrolisa em solução aquosa e a molécula inteira é considerada o agente ativo (FUJIHARA *et al*., 2003; ANDRADE, 2008).

No entanto, tem existido certa preocupação quanto ao uso do hipoclorito e dos demais sais de cloro considerados precursores na formação de trihalometanos e cloraminas orgânicas, estas prejudiciais à saúde devido ao seu alto potencial carcinogênico (ANDRADE, 2008).

Por essa razão, diversos agentes sanitizantes têm sido propostos como substitutos do hipoclorito de sódio. Uma alternativa a ser aplicada pode ser o dióxido de cloro que vem recebendo atenção especial, pois embora seja um derivado do cloro, ele gera quantidade insignifcante de subprodutos (trihalometanos) não se obtendo a formação de cloraminas, e sendo os fenóis oxidados a formas mais simples, caracterizando-se assim como um produto de baixo potencial carcinogênico (ANDRADE e MACEDO, 1996; ANDRADE, 2008).

Outro aspecto importante do dióxido de cloro é sua acentuada ação sanitizante e esporicida, que se dá em concentrações menores de cloro, e que é resultante do mesmo ser solúvel em óleos, graxas e substâncias de composição mista, como células de vírus e de bactérias, em cujas membranas penetram facilmente, ao contrário dos outros sanitizantes de natureza polar (SREBERNICH, 2007).

4.3. Ácido Peracético

O ácido peracético (APA), também chamado de peróxido de ácido acético ou ácido peroxiacético é um liquido claro, incolor e não apresenta a capacidade de formação de espuma. Apresenta um odor forte de ácido acético e apresenta pH inferior a 2 (SOLVAY INTEROX, 2002).

O ácido peracético é produzido pela reação do ácido acético com peróxido de hidrogênio, na presença de ácido sulfúrico, que tem a função de catalisador. Os produtos de decomposição são o ácido acético, o peróxido de hidrogênio e a água (BLOCK, 1991; YUAN et al., 1997; GEHR et al., 2002; SREBERNICH, 2007).

Para fins de estabilidade, o ácido peracético deve ser armazenado, de preferência, sob refrigeração (KITIS, 2004). Soluções de ácido peracético acima de 15% podem apresentar níveis iniciais de

instabilidade, combustão e reatividade (BLOCK, 1991). Soluções de ácido peracético a 12% têm sido amplamente utilizados no processamento de alimentos (DYCHDALA, 1988; KITIS, 2004).

A grande vantagem de se utilizar o ácido peracético a esta concentração consiste na perda das características de combustão apresentadas em concentrações acima de 15% (KITIS, 2004).

O mecanismo de ação como agente microbiano ainda foi totalmente elucidado (KITIS, 2004), no entanto, especula-se a ocorrência de oxidação dos componentes celulares dos micro-organismos, tendo uma rápida ação a baixas concentrações sobre um amplo espectro de micro-organismos. Desta maneira, o ácido peracético atua como esporicida em baixas temperaturas e continua efetivo na presença de material orgânico sendo, portanto, um biocida efetivo sem residual tóxico. Sua ação biocida é influenciada pela concentração, temperatura e tipo de micro-organismos (BLOCK, 1991).

Sugere-se ainda que o ácido peracético pode alterar a função quimiosmótica da lipoproteína da membrana citoplasmática assim como promover a ruptura das paredes celulares de micro-organismos (BALDRY e FRASER, 1988). Dessa forma, este composto pode ser similarmente eficaz contra as lipoproteínas da membrana externa, o que facilita sua ação contra as células de bactérias gram-negativas (LEAPER, 1984). Este comportamento como desnaturante de proteínas pode também ajudar a explicar suas características de esporicida e ovicida (BLOCK, 1991).

Além disso, o ácido peracético intracelular também pode oxidar enzimas essenciais e moléculas de DNA. Assim vias bioquímicas vitais, transporte ativo através de membranas celulares e os níveis de soluto podem acabar sendo prejudicados (TUTUMI et al., 1973; FRASER et al., 1984). Outra vantagem na utilização de ácido peracético como agente antimicrobiano é que este pode inativar a enzima catalase, responsável pela ação em radicais hidroxilas livres (BLOCK, 1991).

Além disso, o ácido peracético se degrada rapidamente em substâncias inócuas e biodegradáveis como ácido acético e oxigênio ativo, que não oferecem risco de toxicidade nem afetam o sabor e odor dos alimentos; não desenvolve resistência microbiana e não tem efeitos mutagênicos ou carcinogênicos

(HOLAH et al., 1990; HILGREN; SALVERDA, 2000; COSTA, 2007) e por este motivo tem sido amplamente utilizado no processamento de alimentos e na indústria de bebidas (DYCHDALA, 1988).

A eficiência de desinfecção por ácido peracético em micro-organismos geralmente podem ser classificados como: Bactérias > Vírus > Esporos Bacterianos > Cistos de Protozoários (RUDD e HOPKINSON, 1989; LIBERTI e NOTARNICOLA, 1999). Segundo THURMAN e GERBA (1988), MORRIS (1993) e RAJALA-MUSTONEN e colaboradores (1997), a maior parte da redução da carga microbiana ocorre durante os primeiros 10 minutos de tempo de contato apresentando uma curva de inativação dos micro-organismos, de primeira ordem.

A ação antimicrobiana do ácido peracético sob baixas temperaturas, juntamente com a ausência de resíduos tóxicos em sua produção contribui com sua vasta aplicação alimentícia (DYCHDALA, 1988; BLOCK, 1991;).

Pode ser usado como agente sanitizante de aço inoxidável, sendo este um material largamente utilizado em equipamentos de indústrias de alimentos (SOLVAY INTEROX, 2002).

Estudos realizados por HILGREN e SALVERDA (2000) mostraram que hortaliças tratadas com ácido peracético (80 ppm) reduziram a população de bactérias aeróbias mesófilas de 1.04 – 1.54 ciclos log. SILVEIRA, AGUAYO e ARTÉS (2010) observaram redução ($p< 0.05$) de 2 ciclos log na contagem total de bactérias psicrotróficas de melão galia, tratadas com ácido peracético (80 ppm). CHERRY (1999) também observaram redução de 2 ciclos log de bacterias mesófilas em frutas e vegetais minimamente processados quando tratados com ácido peracético (60ppm). Já GARMENDIA e VERO (2006) observaram um decréscimo significativo de 2 ciclos log nos esporos de *Penicillium expansum* em laranjas tratadas com ácido peracético (80 ppm). Enquanto que SREBERNICH (2007) observaram que tratamentos com ácido peracético realizados a partir de 80 ppm, em um tempo mínimo de 5 minutos, podem ser considerados suficientemente eficientes no controle de coliformes totais em cheiro verde minimamente processados. Outros estudos, no entanto, equipararam a eficiência sanitizante do ácido

peracético e hipoclorito de sódio (FARREL et al., 1998; SAPERS et al., 1999; WISNIEWSKY et al., 2000; NASCIMENTO, 2002).

Por tantas aplicações, o ácido peracético tem sido utilizado com bastante sucesso. Nos Estados Unidos, sua utilização foi aprovada pela Food and Drug Administration in the United States (FDA) em 1986 (DYCHDALA, 1988). No Brasil, o ácido peracético é autorizado pela ANVISA como esterilizante, desinfetante hospitalar para superfícies fixas, desinfetante hospitalar para artigos semi-críticos, desinfetante para indústria alimentícia e desinfetante de uso geral, através da Portaria nº122/DTN, de 29/11/1993. Também é aprovado pela ANVISA na lavagem de ovos, carcaças e/ou partes de animais de açougue, peixes e crustáceos e hortifrutícolas desde que usado em quantidade suficiente para obter o efeito desejado, sem deixar resíduos no produto final. (BRASIL, 1993; BRASIL, 2002).

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CAPÍTULO II. MICROBIOTA OF TWO SPECIES OF COMMERCIALLY IMPORTANT FISH IN THE AMAZON REGION (BELÉM-PARÁ-BRAZIL): BUTTERFLY PEACOCK BASS (*Cichla ocellaris*) AND PIRAMUTABA (*Brachyplatystoma vailantii*).

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ABSTRACT

The Amazon offers great potential for fishery activities but the fish fauna's specific microbiota is not yet known. This paper identified the bacterial flora composition and the influence of this process on the microbiological spoilage in economically important fish species in the Amazon region: butterfly peacock bass (*Cichla ocellaris*) and piramutaba (*Brachyplatystoma vailantii)*. To this end, microbiological characterization was performed: counts of total mesophilic aerobic bacteria, psychrotrophic bacteria, and coliforms at 35°C and 45°C. Bacteria were also isolated through seeding in agar surface using Violet Red Bile Glucose (VRBG) for enterobacteria strains and Baird-Parker Agar with Egg-Yolk Tellurite for *Staphylococcus* species, both with incubation at 36°C for 48 h. The bacteria isolated were identified using the API 20E kit (Enterobacteria), and Gram-positive with API Staph (Staphylococci). Finally, the limit temperature for strain growth was tested using spectrophotometry readings at 554 nm at 10 and 15°C at three different times: 0, 3, and 6 h. The mesophilic aerobic bacteria counts for fresh fish samples ranged from 6.03 – 8.23 log CFU/g for piramutaba and 4.52 – 7.24 log CFU/g for butterfly peacock bass. The count ranges of psychrotrophic aerobic bacteria found were 6.14 – 8.56 log CFU/g and 4.52 – 7.24 log CFU/g for piramutaba and butterfly peacock bass, respectively. They also had an average score above 10^3 MPN/g for total

coliforms. The most predominant strains were *Staphylococcus hominis*, *Staphylococcus aureus*, *Stenotrophomonas maltophilia*, and *Enterobacter intermedius*. When subjected to temperatures of 10 and 15 °C, the strains did not achieve growth for 6 h at a 95% significance level.

Key words: fish, *Cichla ocellaris*, *Brachyplatystoma vailantii*, microflora

1. INTRODUCTION

The coast of the state of Pará (Brazil) offers great potential for fishery activities due to the numerous rivers and estuaries that empty into the Atlantic Ocean, forming a complex aquatic environment with high biological productivity. The substantial biomass of fish species in this region is exploited by both artisanal and industrial fleets (Isaa 2c *et al*., 2009). Butterfly peacock bass (*Cichla ocellaris*) and Piramutaba (*Brachyplatystoma vailantii*) feature among these species and are two of the favorite targets of fishing in the region given their considerable importance from both an economic and nutritional perspective. However, as marine fish, freshwater fish are extremely perishable food commodities. Enzymatic and chemical reactions are usually responsible for the initial loss of freshness whereas microbial activity is responsible for the overt spoilage and thereby establishes product shelf life (Gram, 1995; Gram and Huss, 1996). In some cases, chemical changes such as auto-oxidation or enzymatic hydrolysis of the lipid fraction may result in off-flavors, while, in other cases, tissue enzyme activity can lead to unacceptable softening of the fish. The spoilage of fresh fish by microbial activity is usually due to its microbiota located mainly in the outer surfaces (skin and gills) and in the intestines of live and newly caught fish. It can also be the consequence of fish cross-contamination associated with inappropriate handling and storage (Cruz-Romero, Kelly and Kerry, 2008). The poikilotherm nature of fish allows bacteria to grow in a broad temperature range. Thus, the microbiota of temperate-water fish is dominated by psychrotrophic, aerobic, or facultative anaerobic Gram-negative, rod-shaped bacteria, and, in particular, by *Pseudomonas*, *Moraxella*, *Acinetobacter*, *Shewanella putrefaciens*,

Flavobacterium, *Cytophaga*, *Vibrio*, *Photobacterium*, and *Aeromonas* (Lalitha and Surendran, 2006; Pantazi *et al*., 2008). The microbiota in tropical freshwater fish is dominated by Gram-positive bacteria such as *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Enterobacter*, *Flavobacterium*, *Flexibacter*, *Pseudomonas*, *Psychrobacter*, *Citrobacter*, *Micrococcus*, *Staphylococcus*, *Streptococcus*, and *Moraxella* spp. (Gram and Huss, 1996; Apun *et al*., 1999; Austin, 2002; ICMSF, 2005). The specific microbiota of butterfly peacock bass and piramutaba is not yet known. During the processing of the two species, the microorganisms present in the gut and on the skin can spread to the processing equipment, the workers, and sterile flesh fillets. The objective of this paper was to identify the microbiota and study the growth temperature of isolated microorganisms to improve the refrigeration temperature conditions in two fish species from the Amazon region: butterfly peacock bass (*Cichla ocellaris*) and piramutaba (*Brachyplatystoma vailantii)*.

2. MATERIAL AND METHODS

2.1. Fish Samples

Four successive comercial-sized fish samples (n=4) (butterfly peacock bass and piramutaba) were collected between March and April 2013 at Ver-o-Peso Market (Belém, Pará, Brazil). At the time of collection, the samples were placed in sterile bags kept under refrigeration (around 10°C) and transported to the Laboratory of Food Microbiology (Federal University of Pará - UFPA) for further analysis.

2.2. Microbiological Characterization

25 g of each sample (ventral part of the filet) were aseptically collected and added with 225 mL of 0.1% sterile peptone water (SPW), thus obtaining 1:10 dilution, which were homogenized in a stomacher (STOMACHER 400 CIRCULATOR SEWARD) at 2,300 rpm for 30 seconds. Next, counts of total mesophilic aerobic bacteria, psychrotrophic bacteria, and coliform at 35°C and 45°C were

performed according to Brazil (2003). The total mesophilic aerobic and psychrotrophic bacteria counts were carried out in pour plate using plate count agar followed by incubation at 35°C/48 h for mesophilic and 7°C/10 d for psychrotrophic bacteria. Coliforms at 35 and 45°C were counted through the most probable number (MPN), with three sets of three tubes. Lauryl sulfate tryptose broth (LST) was used as a presumptive medium and incubated at 35°C for 24-48 h. The positive tubes were transferred to brilliant green bile broth 2% (GB) and EC broth. The former was incubated at 35°C/24-48 h for confirmation of total coliforms and EC broth tubes were incubated in a water bath at 45.5°C/24 h for confirmation of thermotolerant coliforms.

2.3. Bacterial Isolation

The homogenized matter used for microbiological characterization was subsequently used for bacteria isolation. The isolation to obtain pure cultures was carried out through seeding in agar surface using Violet Red Bile Glucose (VRBG) for enterobacteria strains and Baird-Parker with Egg-Yolk Tellurite for *Staphylococcus* species, both with incubation at 36°C/48 h. Next, colonies were selected to be striated in VRBG or Baird-Parker agar plates to obtain pure cultures. After another incubation at 36 °C/48 h, these colonies were transferred to BHI (brain-heart infusion) with 10% glycerol and stored in a freezer to be used in further tests.

2.4. Bacterial Strain Identification

The bacteria isolated were previously identified with Gram stain tests. Next, Gram-negative strains were identified using the API 20E kit (Enterobacteria), and Gram-positive strains with (Staphylococci). The procedure was in accordance with the manufacturer's recommended procedures (Biomérieux, France) (Harrigan, 1998).

2.5. Limit Temperature for Growth (Adapted from Bordignon-Junior *et al.*, 2012)

Strains were reactivated in BHI broth for 24 h at 36°C. After that, the isolates identified were transferred to a new BHI broth (1:15 mL) and maintained at different temperatures: 10 and 15°C during three different times: 0, 3, and 6h. Spectrophotometric readings were performed (Spectrophotometer Model Nova 2000 UV) at 554 nm.

2.6. Statistical Analysis

Tukey's test was applied to evaluate the difference of means among microorganism groups (mesophilic and psychrotrophic bacteria, total and thermo-tolerant coliforms) found in different fishes. The optical density data were subjected to ANOVA considering different groups of isolated microorganisms. The software Statistica 8.0 was employed considering a 95% level of significance.

3. RESULTS AND DISCUSSION

3.1. Microbiological Characterization

The mesophilic aerobic bacteria counts for fresh fish samples ranged from 6.03 – 8.23 log CFU/g for piramutaba and 4.52 – 7.24 log CFU/g for butterfly peacock bass (Table 1).

Table 1. Mean values of microbiological characterization in fresh fish

Samples	Mesophilic aerobic bacter (log CFU/g)	Psychrotrophi bacteria (log CFU/g)	Total Coliforms (MPN/g)	Thermotolerant Coliforms (MPN /g)
Piramutaba 01	6.23±0.03bcd	6.14±0.13c	1,100a	15c
Piramutaba 02	8.23±0.01a	8.56±0.02c	1,100a	1,100a
Piramutaba 03	6.03±0.01cd	6.46±0.00c	1,100a	1,100a
Piramutaba 04	6.61±0.65bc	7.97±0.03a	1,100a	240b
B. peacock bass 01	4.52±0.74e	6.40±0.06d	1,100a	1,100a
B. peacock bass 02	5.35±0.07de	6.66±0.06a	1,100a	1,100a
B. peacock bass 03	6.16±0.01cd	6.52±0.008c	1,100a	240b
B. peacock bass 04	7.24±0.03ab	8.33±0.04b	1,100a	1,100a

**different letters in each column means difference at 95% level of significance.*

Brazil (2001) does not establish microbiological standards for mesophilic bacteria count in fresh fish. However, the International Commission on Microbiological Specifications for Foods (ICMSF, 1986) recommends the limits for mesophilic aerobic should not exceed values of 10^7 CFU/g or cm^2 in chilled fish samples for human consumption.

Aerobic mesophilic bacteria, when present in large numbers, indicate unsanitary conditions. The high count of this microorganism in food may result from unsatisfactory storage conditions, with potential danger to health (Morton 2001, Coelho *et al*., 2010).

The fish samples analyzed had a mean score of 10^6 and 10^5 for piramutaba and butterfly peacock bass, respectively.

A similar result was observed by Fernandez and Barbosa (2010), who reported counts of $10^4 – 10^5$ CFU/g for mesophilic bacteria in sardines. Li, Li and Hu (2013) found values around 10^3 CFU/g in large yellow croaker (*Pseudosciaena crocea*) from China. Vishwanath, Lilabati and Bijen (1998) observed a total bacteria plate count range of $10^6 – 10^7$ CFU/g for *M. albus* (Manipur, India). Oku and Amakoromo (2013) found bacteria count values of $10^8 – 10^{10}$ CFU/g for fresh fish (Yenagoa metropolis, Nigeria). Thong Thi *et al*. (2013) found total mesophilic counts on raw pangasius fish around 5.1 log CFU/g.

Previous studies from Shewan (1977), Guizani *et al*. (2005), and Ercolini *et al*. (2009) have indicated that mesophilic microorganisms are dominant in tropical fish species.

The count of psychrotrophic aerobic bacteria found ranged from 6.14 to 8.56 log CFU/g for piramutaba and from 6.40 to 8.33 log CFU/g for butterfly peacock bass (Table 1).

The Brazilian legislation establishes no maximum limit for psychrotrophic microorganisms in fish for human consumption. But considering the ICMSF (1986), piramutaba and butterfly peacock bass species showed values above the maximum limit (10^7 CFU/g).

Britto *et al*. (2007) evaluated the bacteriological deterioration of whole jaraqui (*Semaprochilodus* spp.) captured in the Amazon region and found a count of psychrotrophic aerobic bacteria ranging around 2 – 3 log CFU/g. Similar results were found by Hanninen *et al*. (1997), who found freshwater fish samples contaminated by *Aeromonas* spp. Sallam *et al*. (2007) found psychrotrophic bacteria count in raw Pacific saury around 3.95 log CFU/g, while Thong Thi *et al*. (2013) found total psychrotrophic count around 4.3 log CFU/g in raw pangasius fish (*Pangasius hypophthalmus).*

The growth of psychrotrophics also indicates poor sanitary conditions. Psychrotrophic bacteria grow in foods refrigerated between 0 – 7°C, with optimum growth temperature around 20°C. Some psychrotrophics may be pathogens, such as *Aeromonas hydrophila*, some strains of *Bacillus cereus*, *Clostridium botulinum* type E, B, and F, *Listeria monocytogenes*, *Vibrio cholerae*, *Yersinia enterocolitica*, some strains of enteropathogenic *E. coli*, and other pathogens such as *Salmonella* spp. and *C. perfringens* type C. Some strains of *Bacillus cereus* and *Staphylococcus aureus*, which have a lower development temperatures between 7 – 15°C can grow if temperature abuse occurs during storage (Cousin, Vasavada and Jay, 2001).

In refrigerated fish, the psychrophilic and psychrotrophic bacteria play direct roles in fish deterioration because they multiply well in these conditions (Franco *et al*., 1996).

The butterfly peacock bass and piramutaba collected had an average score above 10^3 MPN/g for total coliforms (Table 1). Although this does not indicate the presence of pathogens, total coliforms are important indicators of potential product deterioration and its mean shelf life (Agnese *et al*. 2001).

The total and thermotolerant coliforms are indicators of hygienic quality, not representing direct contact of the product with human or animal feces, but reporting on the degree of microbial pollution to which the food has been exposed. This score thus indirectly reflects the quality of production practices.

Brazil (1997) and Brazil (2001) set the value of 10^2 MPN/g as the maximum acceptable standard for thermotolerant coliforms in fish and fishery products. However, it is observed that the average of the samples collected from piramutaba and butterfly peacock bass are above the limits established by the Brazilian legislation, indicating the possibility of water contamination as well as the existence of some source of organic matter containing animal or even human feces, which compromises the quality of the fish.

3.2. Bacterial Strain Identification

Among the 36 isolates, it was observed that 58.33% were Gram positive and the other 41.67% were Gram negative. From the results of the Gram stain test, the kits were selected to identify the strains. The data show that Gram-negative bacteria belonged to seven different species described in Tables 2 and 3. The most predominant were *Staphylococcus hominis* for butterfly peacock bass (28.57%) and piramutaba (23.81%), and *Staphylococcus aureus* (19.05%), found only in piramutaba.

Table 2. Identification of *Staphylococcus* strains isolated from piramutaba

Strains isolated	**Number of strains isolated**	**Strains isolated (%)**	**% ID**
Staphylococcus lentus	01	4.76	99.5
Staphylococcus epidermis	01	4.76	97.9
Staphylococcus aureus	04	19.04	88.4-99.6
Staphylococcus hominis	05	23.80	81.3-94.9

Table 3. Identification of *Staphylococcus* strains isolated from butterfly peacock bass.

Strains isolated	**Number of strains isolated**	**Strains isolated (%)**	**% ID**
Staphylococcus cohnii ssp. cohnii	01	4.76	98.3
Staphylococcus saprophyticus	01	4.76	80.2
Staphylococcus lentus	01	4.76	99.9
Staphylococcus xylosus	01	4.76	97.9
Staphylococcus hominis	06	28.57	81.3-92.3

Staphylococci are not part of the normal fish microbiota (Huss, 1988; Van den Broek, Mossei and Mol, 1984). This indicates that, if *Staphylococcus aureus* is found in fish, it most likely originates from human sources (Bulushi *et al*., 2010). Moreover, the presence of staphylococci in fish is an indication of (a) postharvest contamination due to poor personnel hygiene, or (b) disease in fish (Austin and Austin, 2007; Huss, 1988). In Japan, fish-borne microbes, including *Staphylococcus aureus*, are a major cause of food poisoning (Cato, 1998) both because of the very high consumption of fish and because of the common practice of eating raw fish (Huss, 1988). Other authors reported food poisoning by *Staphylococcus aureus* due to fish consumption (Cato, 1998; CDC, 2011; Huss, 1988; Iwamoto *et al*., 2010, Sokari, 1991, Ayulo, Machado and Scussel, 1994, Rodma, Satjapala and Suwanvitaya, 1991). *Staphylococcus hominis* is rarely implicated in food poisoning, because it does not multiply quickly in this environment. However, it may contaminate food since humans are carriers of microorganisms and some of these may be related to certain human infections (Pereira and Pereira, 2005; Cunha *et al*., 2006). The presence of microorganisms in fish products may also indicate the occurrence of food contamination due to poor hygiene in handling and lack of preservation techniques, since *S. hominis* is not part microbiota of these aquatic organisms.

The Gram-negative bacteria found belong to nine different species of enterobacteria as shown in Tables 4 and 5. The most prevalent were *Stenotrophomonas maltophilia* (20.0%) and *Enterobacter intermedius* (13.34%) in butterfly peacock bass and piramutaba, respectively.

Table 4. Identification of *Enterobacteriaceae* strains isolated from piramutaba

Strains isolated	**Nº of strains isolated**	**Strains isolated (%)**	**% ID**
Stenotrophomonas maltophilia	01	6.67	65.6
Enterobacter intermedius	02	13.34	72.4-92.5
Klebsiella pneumoniae ssp. pneumoniae	01	6.67	94.4
Serratia marcensces	01	6.67	71.9
Buttiauxella agrestis	01	6.67	93.6
Pantoea spp. 1	01	6.67	87.8

Table 5. Identification of *Enterobacteriaceae* strains isolated from butterfly peacock bass

Strains isolated	**Nº of strains isolated**	**Strains isolated**	**% ID**
Stenotrophomonas maltophilia	03	20.00	80.4-92.4
Enterobacter intermedius	01	6.67	93.3
Enterobacter amnigenus 2	01	6.67	82.2
Klebsiella pneumoniae ssp. pneumoniae	01	6.67	90.0
Serratia liquefasciens	01	6.67	82.6
Serratia ficaria	01	6.67	98.4

Note: % ID indicates the profile similarity of isolates with the standards established by the kits, according to the manufacturer.

Both *Stenotrophomonas maltophilia* and *Enterobacter intermedius* have not been found to cause food poisoning. However, the presence of these bacteria is worrying since they may be associated with fish deterioration.

Oku and Amakoromo (2013) obtained twelve bacterial isolates from raw tropical freshwater fish samples. The bacteria belonged to five genera identified as: *B. subtilis, Corynebacterium, Lactobacillus, Pseudomonas,* and *S. aureus.*

The specific microbiota of fresh butterfly peacock bass and piramutaba is not yet known. However, it is known that the flora in tropical fish often carries a slightly higher load of Gram-positive and enteric bacteria than fish from temperate water, but it is otherwise similar to that flora dominated by psychrotrophic Gram-negative, rod-shaped bacteria belonging to the genera *Pseudomonas, Moraxella, Acinetobacter, Shewanella, Flavobacterium, Vibrionaceae, Aeroemonadaceae,* and, to a lesser degree, *Bacillus, Micrococcus, Clostridium, Lactobacillus,* and *Corynebacterium* (Liston, 1980, Apun *et al*., 1999; Austin, 2002; ICMSF, 2005). Enterobacteriaceae family have been frequently isolated from the digestive tracts and flesh of freshwater fish (Austin, 2002; Yagoub, 2009; Gonzalez-Rodriguez *et al*., 2002; Paludan-Müller *et al*., 1998). Apun *et al*. (1999) show some Enterobacteriaceae strains such as *K. pneumoniae, E. aerogenes*, and *E. coli* have been isolated from the intestines of tropical freshwater fish. *Serratia* spp. have also been found in *Pangasius* fillets

(Thong Thi *et al*., 2013). At ambient temperature (25°C), the microbiota is dominated by mesophilic *Vibrionaceae* (Gorczyca and Pek Poh Len, 1985; Gram *et al*., 1990) and, particularly if the fish are caught in polluted waters, mesophilic Enterobacteriaceae become dominant (Gram, 1992).

3.3. Limit Temperature for Growth

The 16 strains (Figures 1 and 2) (Tables 6 and 7) from butterfly peacock bass and piramutaba, when subjected to temperatures of 10 and 15 °C, did not achieve growth for 6 h at a 95% significance level.

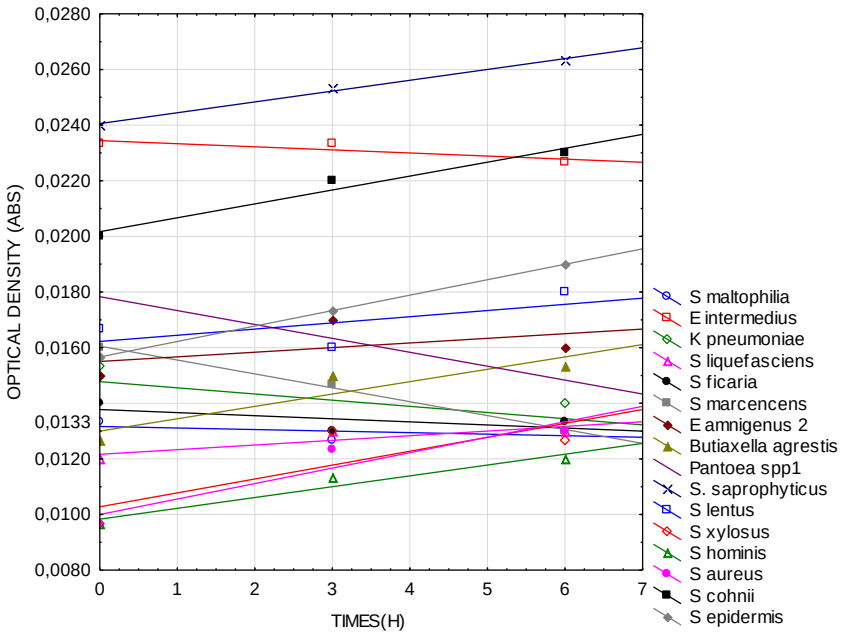

Figure 1. Optical density (absorbance) measured through spectrophotometry (554 nm) at 10°C after 6 h

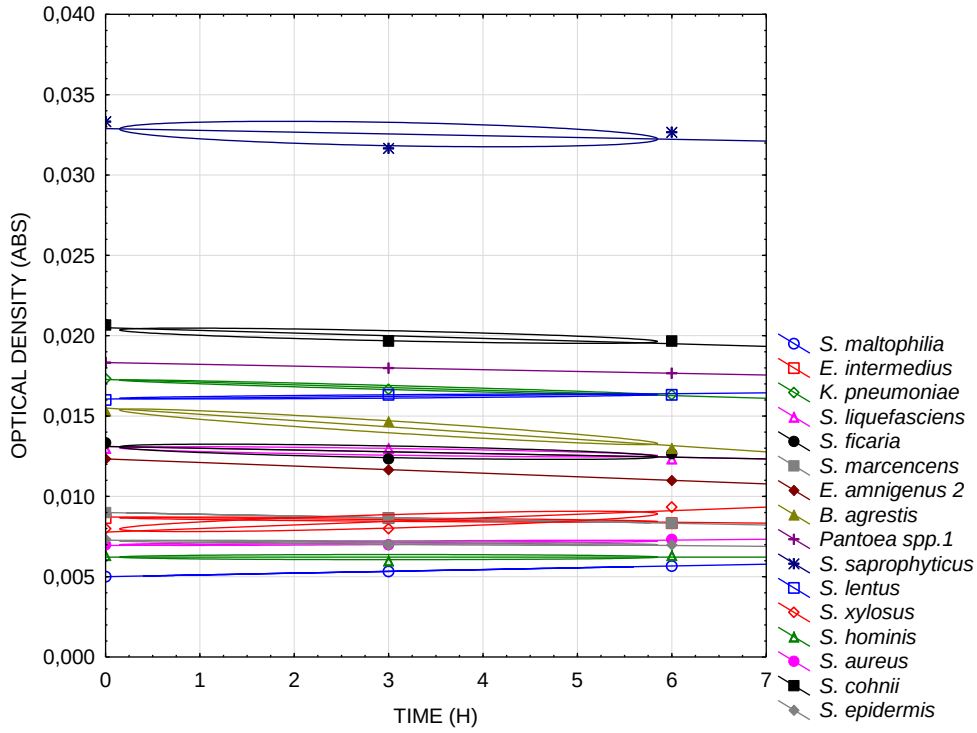

Figure 2. Optical density (absorbance) measured through spectrophotometry (554 nm) at 15°C after 6 h

Table 6. ANOVA for optical density measured through spectrophotometry (554 nm) at 10°C after 6 h.

Bacterial strains/Temperature	**F**	**p**
Staphylococcus cohnii ssp. Cohnii	1.2353	0.3554
Staphylococcus saprophyticus	0.1878	0.8335
Staphylococcus lentus	0.4179	0.6762
Staphylococcus epidermis	0.9740	0.4302
Staphylococcus xylosus	3.9565	0.0802
Staphylococcus aureus	1.7931	0.2452
Staphylococcus hominis	3.5455	0.0962
Stenotrophomonas maltophilia	0.6000	0.5787
Enterobacter intermedius	0.0125	0.9876
Enterobacter amnigenus 2	0.8182	0.4851
Klebsiella pneumoniae ssp. pneumoniae	0.6727	0.5450
Serratia liquefaciens	1.0000	0.4219
Serratia ficaria	0.1207	0.8884
Serratia marcensces	2.4400	0.1677
Buttiauxella agrestis	2.4400	0.1677
Pantoea sp.	4.0714	0.0763

Table 7. ANOVA for optical density measured through spectrophotometry (554 nm) at 15°C after 6h

Bacterial strains/Temperature	F	P
Staphylococcus cohnii ssp. cohnii	0.0234	0.9769
Staphylococcus saprophyticus	0.4220	0.6737
Staphylococcus lentus	0.0910	0.9143
Staphylococcus epidermis	0.0357	0.9651
Staphylococcus xylosus	0.6400	0.5598
Staphylococcus aureus	0.0232	0.9771
Staphylococcus hominis	0.0588	0.9434
Stenotrophomonas maltophilia	0.6000	0.5787
Enterobacter intermedius	0.3330	0.7290
Enterobacter amnigenus 2	0.3429	0.7228
Klebsiella pneumoniae ssp. pneumoniae	0.3330	0.7290
Serratia liquefaciens	0.0597	0.9426
Serratia ficaria	0.1111	0.8966
Serratia marcensces	0.6000	0.5787
Buttiauxella agrestis	0.1069	0.9003
Pantoea sp.	0.2730	0.7702

Denton & Kerr (1998) stated that *S. maltophilia* growth does not occur at temperatures lower than 5°C. However, Margesin and Schinner (1991) reported isolation of an *S. maltophilia* strain from an alpine environment capable of growth at 10°C. According to Schmitt *et al*. (1990), *S. aureus* is capable of growing in a wide range of temperatures, from 7 – 48.5°C with an optimum from 30 to 37°C. Valero *et al*. (2009) reported *S. aureus* growth can be inhibited at refrigeration temperatures (around 8°C). Schmitt, Schuler-Schmid and Schmidt-Lorenz (1990) stated the lowest temperature limit for growth was about 7°C for seven days.

Raw fish should be kept at 10°C throughout processing to inhibit the growth and toxin production of pathogenic bacteria (FDA, 2011).

However, it is important to remember that the fish studied are sourced from tropical regions, i.e., from waters with elevated temperatures. It is suggested that the strains found have adapted to moderate temperatures. Thus, refrigeration conditions are adverse for the multiplication of such microorganisms. Hence, butterfly peacock bass and piramutaba from the Amazon region could maintain their quality considering the microbiological aspects under refrigerated conditions (time and

temperature) considering the bacteria isolated in this study. However, due to the high psychrotrophic count (4.52 – 8.56 log CFU/g), greater than the 7 log CFU/g established by the ICMSF (1986), other conservation measures (besides refrigeration) are required to prevent the multiplication of these microorganisms and fish deterioration.

4. CONCLUSION

The microbiological evaluation suggests Amazonian fish species marketed at the port of Ver-o-Peso Market have high counts of total mesophilic and psychrotrophic bacteria. Furthermore, it was observed that some isolated mesophilic microorganisms did not grow under refrigeration temperatures over 6 h. However, due to the high concentrations of psychrotrophic bacteria, these fish require other conservation methods to ensure the microbiological quality.

5. CONFLICT OF INTERESTS

The authors have not declared any conflicts of interests.

6. ACKNOWLEDGEMENTS

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CAPÍTULO III. MICROBIOTA OF FRESHWATER CATFISH SPECIES FILHOTE (*BRACHYPLATYSTOMA FILAMENTOSUM*) AND DOURADA (*BRACHYPLATYSTOMA ROUSSEAUXII*) FROM THE AMAZON REGION (BELÉM-PARÁ-BRAZIL).

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ABSTRACT

This paper identified the bacterial flora composition and its influence on the microbiological spoilage process in economically important fish species in the Amazon region: Filhote (*Brachyplatystoma filamentosum*) and Dourada (*Brachyplatystoma rousseauxii*). Microbiological characterization was performed: counts of total mesophilic aerobic bacteria, psychrotrophic bacteria, and coliforms at 35 °C and 45 °C respectively. Bacteria were also isolated through seeding in agar surface using Violet Red Bile Glucose (VRBG) for enterobacteria strains and Baird-Parker Agar with Egg-Yolk Tellurite for *Staphylococcus* species, both with incubation at 36 °C for 48 h. The bacteria isolated were identified using the API 20E kit (Enterobacteria), and Gram-positive bacteria with API Staph (Staphylococci). Finally, the lag phase for strain growth was measured using spectrophotometry readings (620 nm) at different temperatures (10, 15, and 37 °C). The mesophilic aerobic bacteria counts for fresh fish samples ranged from 5.21 to 7.64. The count ranges of psychrotrophic aerobic bacteria found were 5.11 – 6.91 log CFU/g. They also had an average score above 10^3 MPN/g for total coliforms. The most predominant were *Hafnia alvei*, *Pseudomonas luteola*, *Staphylococcus xylosus*, and *Staphylococcus lugdunensis.* When subjected to temperature of 10 °C, the strains achieve growth after 6 h ($p<0.05$).

Keywords: spoilage, catfish, *Brachyplatystoma filamentosum*, *Brachyplatystoma rousseauxii.*

1. INTRODUCTION

Fish is one of the most nutritionally complete foods due to the availability of generous amounts of essential nutrients such as high-biological-value protein, vitamins – especially A and D –, lipid fraction, high unsaturated fatty acids content and low cholesterol concentration (Córser et al., 2000; Koffi-Nevri et al., 2011). These nutrients present in fish provide a good medium for microbial growth responsible for the overt spoilage and thereby establishes product shelf life (Oliveira *et al*., 2008; Gram, 1995; Gram and Huss, 1996).

The spoilage of fresh fish by microbial activity is usually due to its microbiota located mainly in the outer surfaces (skin and gills) and in the intestines of live and newly caught fish (Amaral and Freitas, 2013). It can also be the consequence of fish cross-contamination associated with inappropriate handling and storage (Cruz-Romero, Kerry and Kelly, 2008). It is known that the flora in tropical fish often carries a slightly higher load of Gram-positive and enteric bacteria than fish from temperate waters. But also it can present similar to that flora dominated by psychrotrophic Gram-negative, rod-shaped bacteria belonging to the genera Pseudomonas, Moraxella, Acinetobacter, Shewanella, Flavobacterium, Vibrionaceae, Aeroemonadaceae, and, to a lesser degree, Bacillus, Micrococcus, Clostridium, Lactobacillus, and Corynebacterium (Liston, 1980, Apun et al., 1999; Austin, 2002; ICMSF, 2005).

In the Amazon River (Brazil), fish species represent one of the most important resources and significantly contribute to the local economy, playing a vital role in the local diet as one of the primary sources of protein for the majority of the population (Angelini, Fabré and da Silva-Jr, 2006). The catfish constitute a valuable resource and are exported to several countries (Angelini, Fabré and da Silva-Jr, 2006), including filhote and dourada.

Filhote, or piraiba, (*Brachyplatystoma filamentosum*) is one of the most consumed fish species in the Amazon region. It holds great commercial value for its good taste and high yield in the filleting

process. It can reach up to 2.50 m in length and 300 kg in weight. It is also considered the largest species of catfish from South America and one of the world's largest (Petrere Junior et al., 2004). *Brachyplatystoma rousseauxii* is a large (>1.5 m) migratory catfish of the family Pimelodidae, commonly known as dorado in Bolivia and dourada in Brazil (Carvajal-Vallejos et al., 2014). It is one of the most emblematic species of the Amazon basin, owing to its economic importance and exceptional life cycle, which involves the largest known freshwater migration (Barthem and Goulding 1997; Alonso, 2002).

The specific microbiota of dourada and filhote are not yet known. The objective of this paper was to identify microbiota and to determine the lag phase of isolated microorganisms to improve the refrigeration temperature conditions in the fish species filhote (B*rachyplatystoma filamentosum*) and dourada (B*rachyplatystoma rousseauxii*) from the Amazon region.

2. MATERIAL AND METHODS

2.1. Fish Samples

Successive commercial-sized fish samples (n=4) (Filhote and Dourada) were collected between March and May 2014. At the time of collection, the samples were placed in sterile bags kept under refrigeration (around 10 °C) and transported to the Laboratory of Food Microbiology (Federal University of Pará - UFPA) for further analysis.

2.2. Microbiological analysis

To analysis, 25 g of each sample (ventral part of the filet) were aseptically collected and added with 225 mL of 0.1% sterile peptone water (SPW), thus obtaining 1:10 dilution, which were homogenized in a stomacher (STOMACHER 400 CIRCULATOR SEWARD) at 2,300 rpm for 30 seconds. Next, counts of total mesophilic aerobic bacteria, psychrotrophic bacteria, and coliform at

35 °C and 45 °C were performed according to Brazil (2003). The total mesophilic aerobic and psychrotrophic bacteria counts were carried out in pour plate using plate count agar followed by incubation at 35 °C/48 h for mesophilic and 7 °C/10 days for psychrotrophic bacteria. Coliforms at 35 °C and 45 °C were counted through the most probable number (MPN), with three sets of three tubes. Lauryl sulfate tryptose broth (LST) was used as a presumptive medium and incubated at 35 °C for 24–48 h. The positive tubes were transferred to brilliant green bile broth 2% (GB) and *Escherichia coli* (EC) broth. The former was incubated at 35 °C/24–48 h for confirmation of total coliforms and EC broth tubes were incubated in a water bath at 45.5 °C/24 h for confirmation of thermo-tolerant coliforms.

2.3. Bacteria Isolation

The homogenized matter used for microbiological characterization was subsequently used for bacteria isolation. Colonies were isolated from VRBG and Baird-Parker with Egg-Yolk Tellurite plates after incubation for 48 h at 36ºC. Next, one plate was selected for each medium and 5-10 colonies per plate were randomly chosen. The selected colonies were sub-cultured in VRBG or Baird-Parker agar plates. After incubation at 36 °C/48 h, one colony was transferred from each plate to BHI (Brain Heart Infusion) with 10% glycerol and stored in a freezer to be used in further tests.

2.4. Bacterial Strain Identification

The bacteria isolated were previously identified with Gram stain tests. Next, Gram-negative strains were identified using the API 20E kit (Enterobacteria), and Gram-positive strains with API Staph (Staphylococci). The procedure was in accordance with the manufacturer's recommendations (Biomérieux, France) (Harrigan, 1998).

2.5. Lag Phase Determination

Strains were reactivated in nutrient broth for 24 h at 36 °C. After that, the isolates identified were transferred to a new nutrient broth (1:15 mL) and maintained at different temperatures: 37, 10, and 15 °C during different times: 0, 2, 4, 6, 7, 8, and 9 h (15 and 37 °C) and 0, 3, 6, 9 and 10h (10 °C). Spectrophotometric readings were performed in triplicate (Spectrophotometer Model Nova 2000 UV) at 620 nm (Adapted from Damasceno et al., 2015)

2.6. Statistical Analysis

Tukey's test was applied to evaluate the difference of means among microorganism groups (mesophilic and psychrotrophic bacteria, total and thermotolerant coliforms) found in different fishes. The optical density (lag phase determination) data were subjected to ANOVA considering different groups of isolated microorganisms. The software Statistica 8.0 was applied considering a 95% level of significance.

3. RESULTS AND DISCUSSION

3.1. Microbiological Characterization

The mesophilic and psychrotrophic aerobic bacteria count, as well as thermotolerant coliform values, showed variation considering 95% significance level.

The mesophilic aerobic bacteria counts for fresh fish samples ranged from 5.21 to 7.49 log CFU/g for Filhote and 5.14 – 7.64 log CFU/g for Dourada (Table 1).

Table 1. Mean values of microbiological characterization in fresh fish

Samples	Mesophilic aerobic bacteria (log CFU/g)	Psychrotrophic bacteria (log CFU/g)	Total coliforms (MPN/g)	Thermo-tolerant coliforms (MPN/g)
Filhote 01	7.49 ± 0.02 a	6.91 ± 0.02 a	1,100 a	1,100 d
Filhote 02	5.27 ± 0.13 c	5.69 ± 0.05 b	1,100 a	150 b
Filhote 03	5.21 ± 0.09 c	5.54 ± 0.02 b	1,100 a	1,100 a
Filhote 04	5.43 ± 0.07 c	5.11 ± 0.05 d	1,100 a	23 e
Dourada 01	6.02± 0.11 b	5.53 ± 0.04 b	1,100 a	43 a
Dourada 02	5.18 ± 0.10 c	5.65 ± 0.03 b	1,100 a	210 c
Dourada 03	5.14 ± 0.09 c	5.33 ± 0.01 c	1,100 a	1,100 a
Dourada 04	7.64 ± 0.04 a	5.17 ± 0.08 c,d	1,100 a	93 f

**different letters in each column indicate difference at 95% level of significance.*

Brazil (2001) does not establish microbiological standards for mesophilic bacteria count in fresh fish. However, the International Commission on Microbiological Specifications for Foods (ICMSF, 1986) recommends the limits for mesophilic aerobic should not exceed values of 10^7 CFU/g or cm^2 in chilled fish samples for human consumption. Considering this limit, most of the samples were within the standards (except sample 1 for Filhote and sample 4 for Dourada).

Previous studies by Shewan (1977), Guizani et al. (2005), and Ercolini et al. (2009) indicated that mesophilic microorganisms are dominant in tropical fish species. Oku and Amakoromo (2013) found total mesophilic values $10^8 – 10^{10}$ log CFU/g in *Clarias angularis*, *Channa obscura*, and *Chrysicthtys auratus*. Thong Thi *et al*. (2013) found total mesophilic counts on raw pangasius fish around 5.1 log CFU/g. Vishwanath, Lilabati and M. Bijen (1998) observed a total mesophilic bacteria count ranging from 10^6 to 10^7 CFU/g for *M. albus* (Manipur, India). Damasceno et al. (2015) found mesophilic bacteria values ranging 4.52 to 8.23 CFU/g in Piramutaba (*Brachyplatystoma vailantii*) and Butterfly peacock bass (*Cichla ocellaris*), respectively, in tropical water.

The high count of this microorganism in food may result from unsatisfactory storage conditions, with potential danger to health (Morton, 2001, Coelho et al., 2010; Franco and Landgraf, 2005).

Thus, it appears that mesophilic bacteria are relevant to characterize the food handling conditions, so it is very important that the current legislation set limits for these microorganisms in fresh fish in order to ensure higher quality (Lopes et al., 2012).

The count of psychrotrophic aerobic bacteria ranged from 5.11 to 6.91 log CFU/g for filhote and from 5.17 to 5.65 log CFU/g for dourada (Table 1).

The Brazilian legislation establishes no maximum limit for psychrotrophic microorganisms in fish for human consumption. But considering the ICMSF (1986), Filhote and Dourada species showed values above the maximum limit (10^7 CFU/g).

In refrigerated fish, the psychrophilic and psychrotrophic bacteria play direct roles in fish deterioration because they multiply well in these conditions (Franco *et al*., 1996). Bal'a *et al*. (2000) found psychrotrophic counts around $10^3 – 10^7$ on fresh fillets of channel catfish (*Ictalurus punctatus*).

Lazarin *et al*. (2011) found psychrotrophic values of 6.54 log CFU/g in pintado (*P. coruscans*) fillets. Rodrigues *et al*. (2008), while studying the quality of tilapia (*Oreochromis niloticus*), observed heterotrophic aerobic psychrotrophic microorganism counts between 0 and 7.90 log CFU/g in skinned muscle.

Although the current legislation does not establish limits, the ability of these microorganisms to deteriorate fish through proteolytic processes, even at freezing temperatures, is widely known, which would reduce the product's shelf life (Santos *et al*., 2008).

The Filhote and Dourada collected had an average score above 1,100 MPN/g for total coliforms (Table 1). Although this does not indicate the presence of pathogens, total coliforms are important indicators of potential product deterioration and its mean shelf life (Agnese *et al*., 2001).

Brazil (1997) and Brazil (2001) set the value of 10^2 MPN/g as the maximum acceptable standard for thermotolerant coliforms in fish and fishery products.

Except for samples 4 for Filhote and 1 and 4 for Dourada, the others showed values that exceeded those established by the Brazilian legislation. Lopes *et al.* (2012) found total and thermo-tolerant coliform values ranging from 3.0 to 93 MPN/g in brackish-water grey snapper (*Cynoscion acoupa*). Araújo *et al*. (2012) observed values of 2,400 MPN/g and 11 – 150 MPN/g for total and fecal coliforms, respectively, in freshwater tambaqui (*Colossoma macropomum*). Oku and Amakoromo (2013) found values of 15 – 43 MNP/g for thermotolerant coliforms in the freshwater fish species *Clarias angularis*, *Channa obscura*, and *Chrysicthtys auratus.*

Total and thermo-tolerant coliforms are indicators of hygienic quality, not representing direct contact of the product with human or animal feces, but reporting on the degree of microbial pollution to which the food has been exposed. This score thus indirectly reflects the quality of production practices.

3.2. Bacterial Strain Identification

Among the 51 isolates, it was observed that 52.94% were Gram negative and the other 47.06% were Gram positive. From the results of the Gram stain test, the kits were selected to identify the strains.

The data show that Gram-negative bacteria belonged to 15 different species, described in Figures 1 and 2. The most predominant were *Hafnia alvei* (11.1%) for Dourada and *Pseudomonas luteola* for Filhote (11.1%).

In addition, the incidence of *Serratia marcencens*, *Klebsiella oxytoca*, *Stenotrophomonas maltophilia*, and *Aeromonas hidrophyla* was observed, which are opportunistic pathogens but can also cause fish spoilage (Holt et al., 1994).

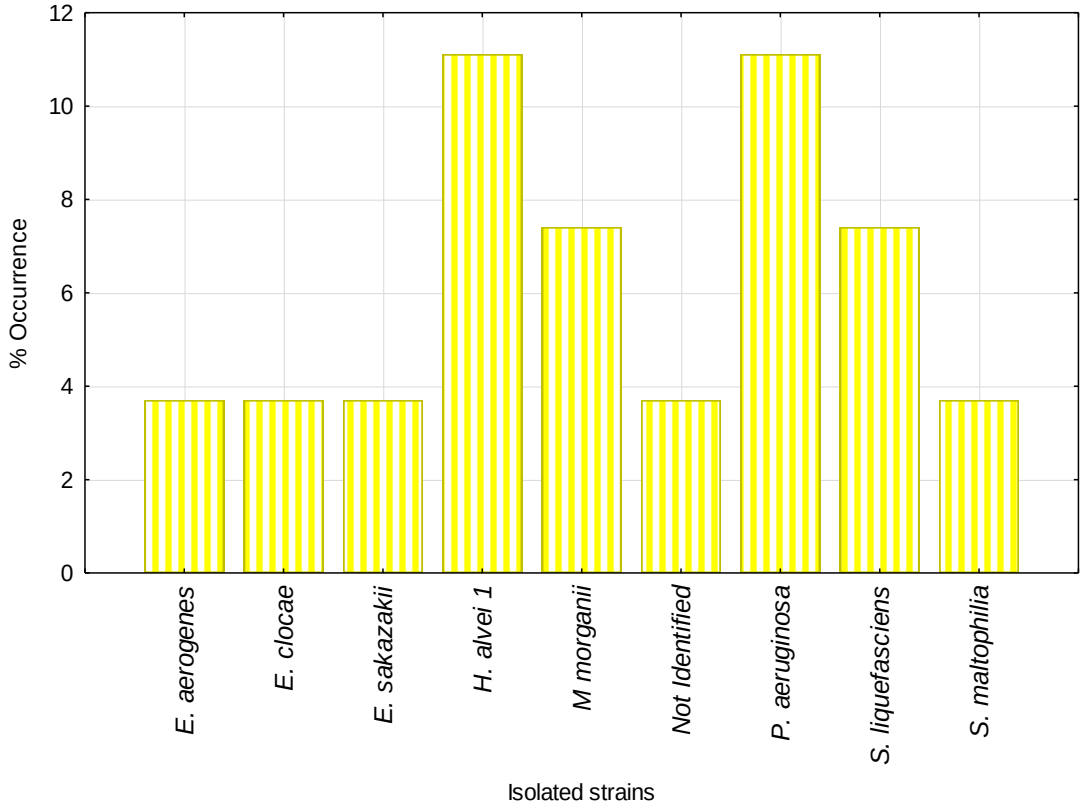

Figure 1. Percentage of occurrence (%) of gram-negative strains isolated from Dourada

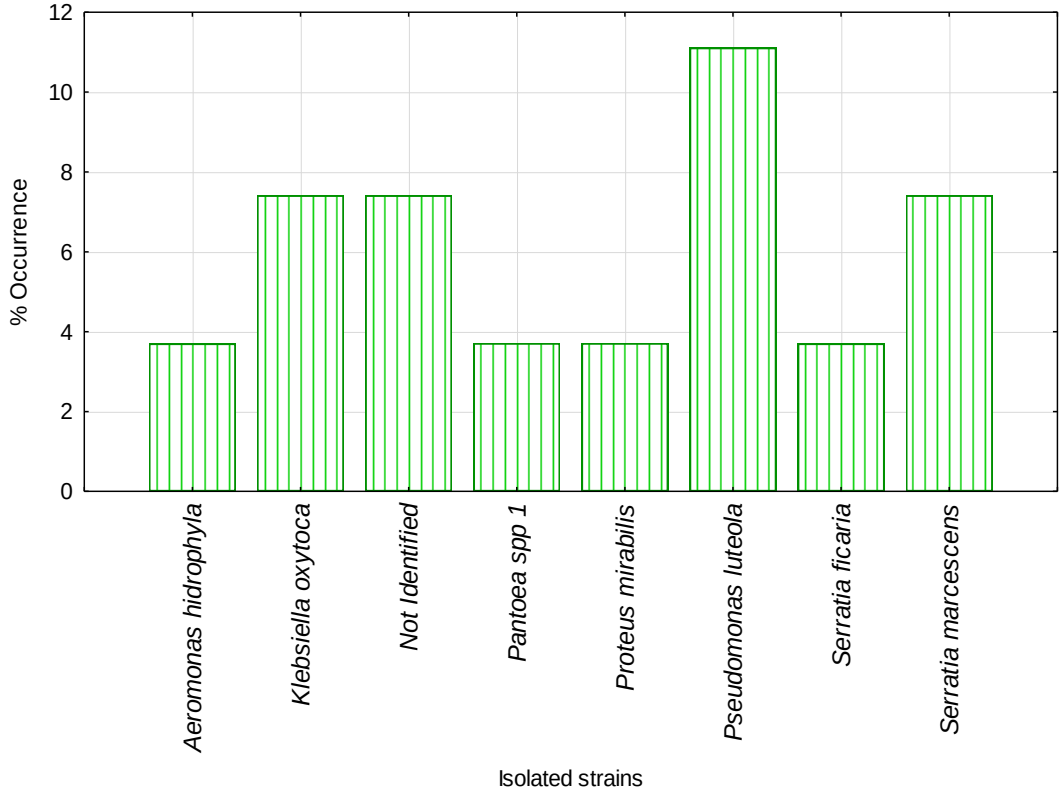

Figure 2. Percentage of occurrence (%) of gram-negative strains isolated from Filhote

H. alvei is widely distributed in nature and has been found in a variety of mammals, fish, and birds, and also in soil, sewage, freshwater, and a number of foods such as meat and dairy products (Lindberg et al., 1998, Vivas et al., 2008). The pathogenicity of *H. alvei* is important in the food industry and infection outbreaks have been reported leading to septicemia in commercial laying hens, pullets, and rainbow trout (Janda and Abbott, 2006; Liu et al., 2007; Crandall et al., 2006). In humans, *H. alvei* has been shown to be predominantly associated with several intestinal disorders, including gastroenteritis. Outbreaks or case reports of Hafnia associated with enteric infections have been chiefly reported (Hernandez-Milan and Nenendez-Rivas, 1998; Laguna et al., 1992; Orden and Franco, 1994; Reina et al., 1993; Reina and Borrell, 1991; Seral et al., 2001).

The genus Pseudomonas is known to change many protein-rich foods such as milk, eggs, meat, marine-based foods such as fish and shrimp, and vegetables. According to Franco and Landgraf (2006), pseudomonas are important in food due to their intense metabolic activity, being able to use a wide variety of organic compounds and produce water-soluble pigment and proteolytic and lipolytic enzymes.

Pseudomonas luteola has been identified as a cause of infection in patients with underlying medical disorders (Otto et al., 2013; Anzai et al., 1997; Kiska and Gilligan, 1999, Connor et al., 1987; Hawkins et al., 1991; Rohav et al., 1995). The normal habitat of *Pseudomonas luteola* is unclear; it is frequently found in soil, on plants, and in aqueous and damp environments (Freney et al., 1988; Silver et al., 1985; Hawkins et al., 1991).

Pseudomonas aeruginosa is recognized as belonging to the normal flora of plant surfaces, human skin, and animals and can form biofilms on some surfaces or substrates (Maia et al., 2009). The species *P.aeruginosa* is the most important opportunistic pathogen in humans (Massaguer, 2006; Maia et al., 2009).

The family Enterobacteriaceae has been frequently isolated from the digestive tracts and flesh of freshwater fish (Austin, 2002; Yagoub, 2009; Gonzalez-Rodriguez et al., 2002; Paludan-Müller et al.,

1998). Apun et al. (1999) showed some species of Enterobacteriaceae family such as *K. pneumoniae*, *E. aerogenes*, and *E. coli* have been isolated from the intestines of tropical freshwater fish. *Serratia* spp. have also been found in *Pangasius* fillets (Thong Thi et al., 2013). At room temperature (25 °C), the microbiota is dominated by mesophilic *Vibrionaceae* (Gorczyca and Pek Poh Len, 1985; Gram et al., 1990) and, particularly if the fish are caught in polluted waters, mesophilic Enterobacteriaceae become dominant (Gram, 1992).

The Gram-positive bacteria found belong to eight different species of staphylococcus as shown Figure 3. The most prevalent were *S. xylosus* (12.50%) and *S lugdunensis* (12.50%) in dourada and filhote, respectively.

Damasceno et al. (2015) found predominatly *S.hominis*, *S.aureus* (gram-positive), *S.maltophilia* and *E. intermedius* (gram-negative) in Piramutaba (*Brachyplatystoma vailantii*) and Butterfly peacock bass (*Cichla ocellaris*).

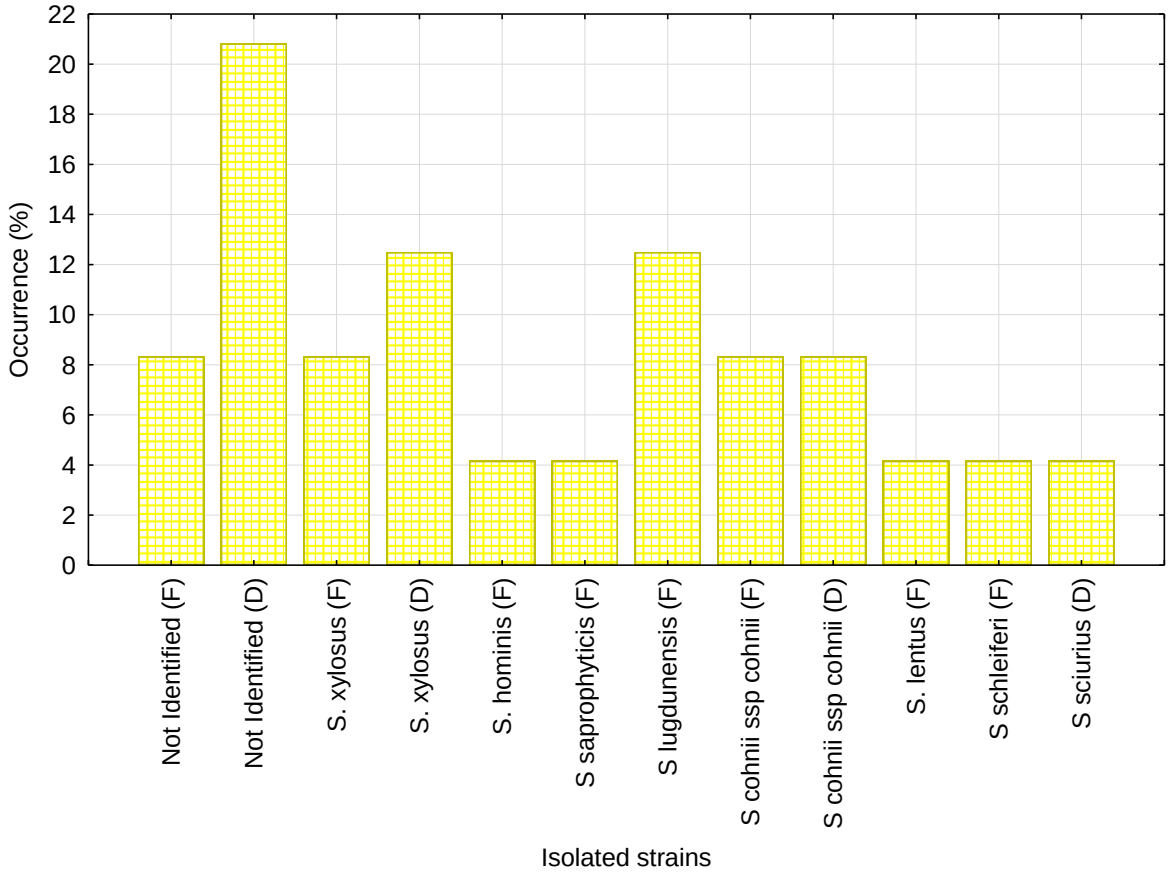

Figure 3. Percentage of occurrence (%) of gram-positive strains isolated from Filhote (F) and Dourada (D)

The genus Staphylococcus contains 41 validly described species (DSMZ, 2008) that are traditionally grouped into coagulase-positive (CPS) and coagulase-negative staphylococci (CNS). The CNS *S. xylosus* strains play a significant role in food production. They can be used as starter cultures for the production of fermented meat products such as fermented sausages (Hammes and Hertel, 1998; Chajecka-Wierzchowska et al., 2015). They can also degrade biogenic amines *in vitro* (Martuscelli et al., 2000). *S. xylosus* have been identified in high numbers in dried salted cod (Vilhelmsson et al., 1997; Doe and Heruwati, 1988) and are rarely associated with human or animal infections (Kloos and Schleifer, 1986).

S. lugdunensis is a common human skin commensal (Bellamy and Barkham, 2002; Vandenesch et al., 1995; Van der Mee-Marquet, 2003). These bacteria display pathogen characteristics, although they do not belong to this group, and exhibit pathogenicity similar to *Staphylococcus aureus*'s, with high associated morbidity

and mortality (Cercenado, 2009; Frank and Patel, 2008; Poutanen and Baron, 2001). Considering *S. lugdunensis* is not part of the normal fish microbiota, its presence in fish means there has been contamination from human sources.

3.3. Lag Phase

Among the 23 different strains isolated, 13 bacteria were selected to determine the lag phase under different temperature conditions (Figures 4, 5 and 6). There was a significant variation ($p<0.05$) in optical density of the species studied at temperatures of 37, 15, and 10 °C (Tables 2, 3, and 4).

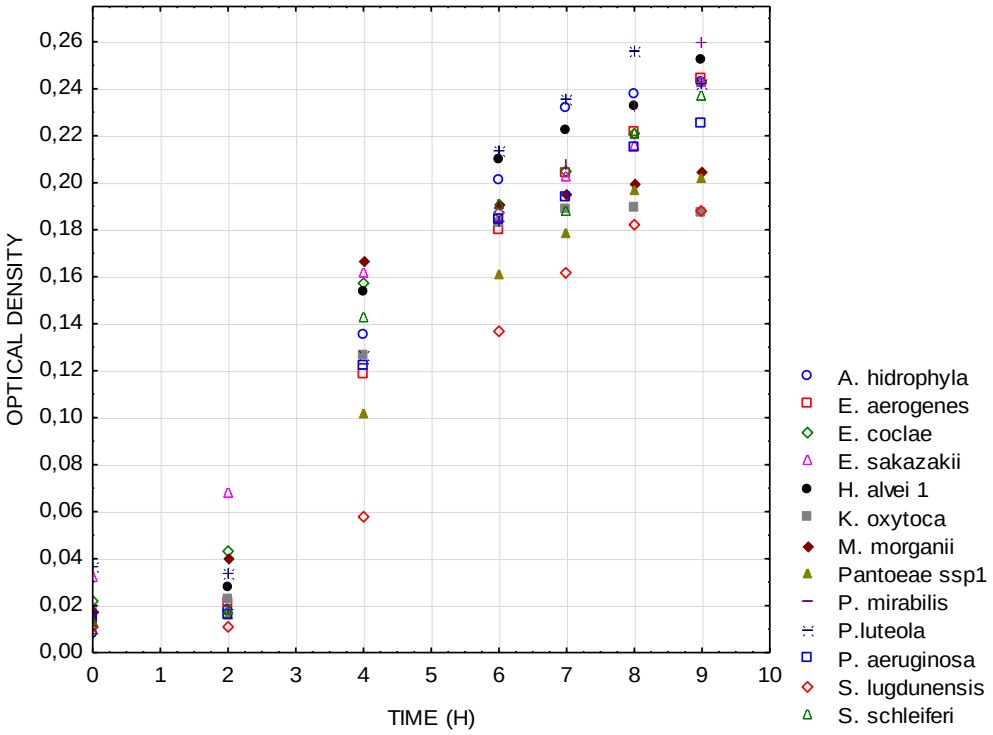

Figure 4. Lag phase measured through spectrophotometry at 37 °C

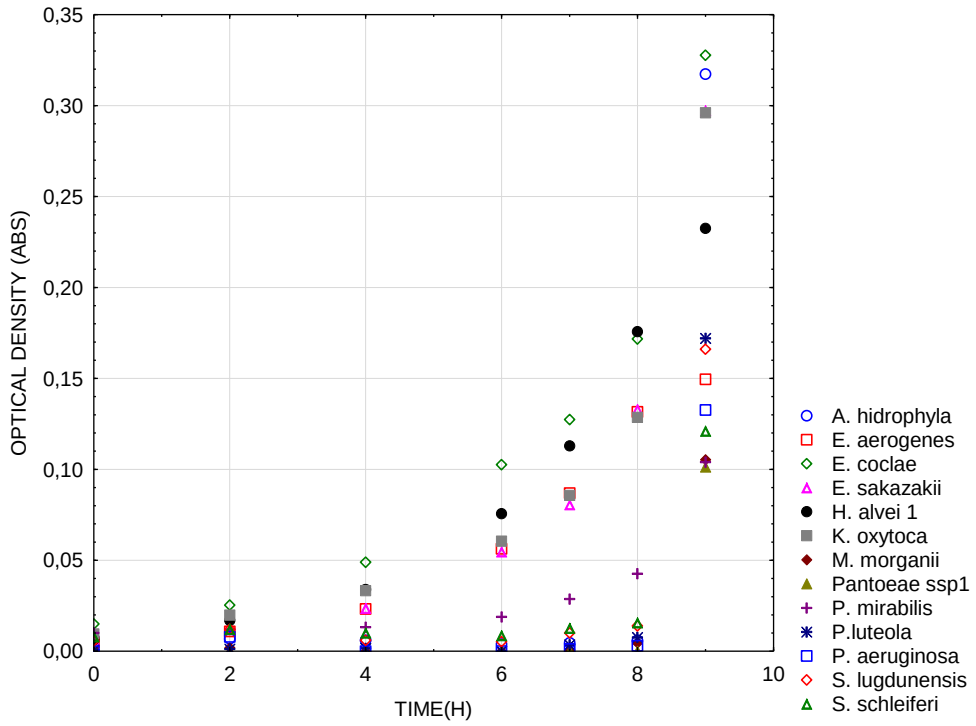

Figure 5. Lag phase measured through spectrophotometry at 15 °C

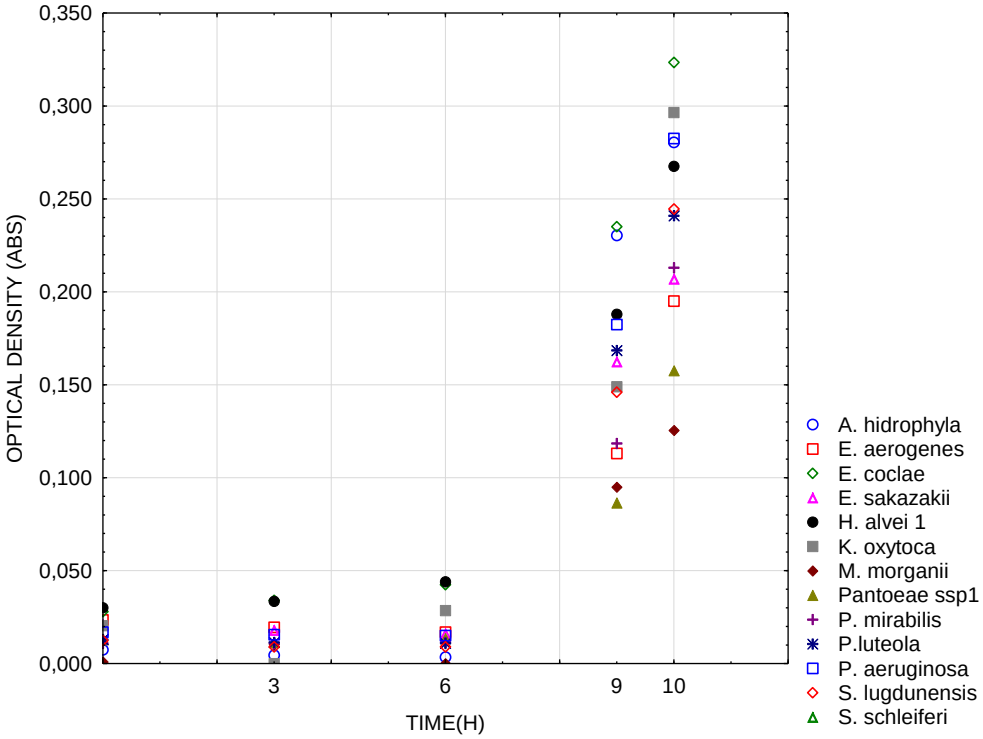

Figure 6. Lag phase measured through spectrophotometry at 10 °C

Table 2. ANOVA for optical density measured through spectrophotometry (620 nm) at 37 °C.

Bacterial strains/Temperature	**F**	**p**
Aeromonas hidrophyla	351.12	0.00
Enterobacter aerogenes	6492.10	0.00
Enterobacter clocae	1495.46	0.00
Enterobacter sakazakii	1229.58	0.00
Hafnia alvei 1	4217.81	0.00
Klebsiella oxytoca	1841.33	0.00
Morganella morganii	3786.19	0.00
Pantoea spp 1	3858.13	0.00
Proteus mirabilis	815.01	0.00
Pseudomonas luteola	1589.93	0.00
Pseudomonas aeroginosa	896.03	0.00
Staphylococcus lugdunensis	414.56	0.00
Staphylococcus schleiferi	1274.05	0.00

Table 3. ANOVA for optical density measured through spectrophotometry (620 nm) at 15 °C

Bacterial strains/Temperature	**F**	**p**
Aeromonas hidrophyla	10.78	0.00
Enterobacter aerogenes	72.31	0.00
Enterobacter clocae	26.68	0.00
Enterobacter sakazakii	117.71	0.00
Hafnia alvei 1	1080.73	0.00
Klebsiella oxytoca	312.63	0.00
Morganella morganii	1232.05	0.00
Pantoea spp 1	62.23	0.00
Proteus mirabilis	696.90	0.00
Pseudomonas luteola	8324.10	0.00
Pseudomonas aeroginosa	9304.56	0.00
Staphylococcus lugdunensis	3722.63	0.00
Staphylococcus schleiferi	2287.08	0.00

Table 4. ANOVA for optical density measured through spectrophotometry (620 nm) at 10 °C

Bacterial strains/Temperature	**F**	**p**
Aeromonas hidrophyla	505.01	0.00
Enterobacter aerogenes	164.45	0.00
Enterobacter clocae	645.99	0.00
Enterobacter sakazakii	88.74	0.00
Hafnia alvei 1	614.83	0.00
Klebsiella oxytoca	32.16	0.00
Morganella morganii	446.89	0.00
Pantoea spp 1	421.68	0.00
Proteus mirabilis	632.45	0.00

Pseudomonas luteola	1271.25	0.00
Pseudomonas aeroginosa	1184.08	0.00
Staphylococcus lugdunensis	417.88	0.00
Staphylococcus schleiferi	283.53	0.00

The lag phase of the majority of the bacteria studied (except E. *sakazakii*) at 37 °C was approximately 2 h (Figure 4). At 15 °C, the lag phase was also at least 2 hours. Since in 4 hours, *E. clocae*, E. *sakazakii e K. oxytoca* already were in log phase. While, when they were exposed to temperatures of 10 °C, the lag phase was de at least 6 h. This means that a safe condition of refrigeration temperature/time to prevent the multiplication of these microorganisms is around 10° C/6h. Damasceno *et al*. (2015) noted 16 strains isolated from Piramutaba (*Brachyplatystoma vailantii*) and Butterfly peacock bass (*Cichla ocellaris*) they did not achieve growth for 6h at 10ºC.

This result is consistent with the FDA (2011), which suggests that raw fish should be kept at 10 °C throughout processing to inhibit the growth and toxin production of pathogenic bacteria.

Thus, the adoption of correct measures in the fish industry, such as appropriate conservation through cold treatment and maintaining hygienic practices, tends to reduce the risk of transmitting the disease-causing agents and is able to produce a quality product at the end of the production chain (Lopes et al., 2012).

4. CONCLUSION

The microbiological assessment of Filhote and Dourada suggests these Amazon fish species sold in the Ver-o-Peso market have mesophilic and psychrotrophic bacteria counts within the limits set by the Brazilian legislation. The microorganisms selected after isolation underwent a significant variation ($p<0.05$) when they were subjected to different refrigeration temperatures for up to 10h, which yields the optimal refrigeration temperature/time condition (10°C/6h) to prevent the multiplication of these microorganisms.

5. CONFLICT OF INTERESTS

The authors have not declared any conflicts of interests.

6. ACKNOWLEDGEMENTS

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CAPITULO IV. EFFECT OF SANITIZERS COMBINED WITH ULTRASOUND ON ADHESION OF MICROORGANISMS ISOLATED FROM FISH FROM THE AMAZON REGION.

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ABSTRACT

Attachment of undesirable microorganisms to surfaces that contact food is a source of concern, since it can result in product contamination leading to serious economic and health problems. Bacteria aggregated to form biofilms are more resistant to environmental stress than planktonic cells. The objective of this paper was to evaluate the bactericidal effect of sodium hypochlorite and peracetic acid associated with ultrasound (40 Hz) to control the adhesion of *S. aureus*, *S. hominis,* and *P. aeruginosa* isolated from two fish species from the Amazon region: butterfly peacock (*Cichla ocellaris*) and piramutaba (*Brachyplatystoma vailantii*). After incubation at 30 °C for 24h, stainless steel coupons were treated for 10 min by different concentrations of sodium hypochlorite (50, 100, and 150 mg/L) and peracetic acid (40, 60, and 80 mg/L) at 25 °C. The sodium hipochlorite (150 mg/L) and peracetic acid (80 mg/L) treatments were also combined with ultrassound (40 Hz) for 10 min at 25 °C. The results showed that the best treatment combination both for *S. aureus* and for *S. hominis* and *P. aeruginosa* was peracetic acid associated with ultrasound. Ultrasound was not efficient in inactivating *S. aureus* and *S. hominis*, however, it had good results in controlling the adhesion of *P. aeruginosa*.

Keywords: *sanitizer, adhesion, S. aureus, S. hominis, P. aeruginosa*

1. INTRODUCTION

The surfaces that come into contact with foods are important sources of microorganism transmission in food-processing plants, which may be associated with food quality and safety (Vogel-Fonnesbech *et al*., 2001).

That happens because some pathogenic bacteria are able to adhere to food-contact surfaces and remain viable even after cleaning and disinfection (Ammor *et al*., 2004). One of the most common ways for bacteria to live is by adhering to surfaces and forming biofilms in which they are embedded in an organic, extracellular polymeric matrix (Chae and Schraft, 2000).

The surface characteristics of the microorganisms themselves and the various environmental conditions encountered in agri-food industries (organic materials, pH, temperature, water activity, etc.) influence microbial attachment to inert surfaces (Giovannacci *et al*., 2000; Gross *et al*, 2001). Adhesion of undesirable microorganisms to these surfaces is a source of concern, since it can result in product contamination leading to serious economic and health problems.

According to Costerton, Steward and Greenberg (1999), biofilms are cell aggregates embedded in an organic extracellular polymeric matrix that confers resistance to the microorganisms involved. Bacteria aggregated to form biofilms are more resistant to environmental stress than their planktonic counterparts, including sensitivity to sanitizers (Fux, Wilson and Stoodley, 2004; Spoering and Lewis, 2001).

To remove biofilm organisms, the sanitizing solution must penetrate the exopolymer matrix and gain access to the microbial cells, which causes biofilm inactivation and removal. Chlorinated products such as hypochlorite salts (Meyer, 2003; Srey *et al*., 2012) constitute the most widely used group of sanitizing compounds. However, there has been some concern regarding the use of hypochlorite and other chlorine salts considered precursors in the formation of organic chloramines, which are harmful to health due to their high carcinogenic potential (Andrade, 2008).

In order to reduce the incidence of microorganisms in foods, the industry has used several sanitizers such as chlorates, peracetic acid (PAA), and quaternary ammonium, among others.

The most widely used chlorate compounds are: sodium hypochlorite (NaClO), lithium hypochlorite, calcium hypochlorite, and chlorine dioxide (inorganic) and chloramine-T, dichloramine-T, dichloroisocyanuric acid, and dichloro dimethyl hydantoin (organic) (Srebernich, 2007).

NaClO in aqueous medium dissociates into hypochlorous acid and hypochlorite. The bactericidal power of the chlorate compounds is usually based on the release of hypochlorous acid in its non-dissociated form when in aqueous solution, except for chlorine dioxide, which does not hydrolyze in aqueous solution and the whole molecule is considered the active agent (Andrade, 2008).

The use of hypochlorite and of the other chlorine salts considered precursors in the formation of organic chloramines has raised a lot of concern since they are harmful to health due to their high carcinogenic potential (Andrade, 2008). Thus, several sanitizing agents have been proposed to replace NaClO.

The use of PAA has many advantages when compared to NaClO (Kunigk and Almeida, 2001). One important advantage is that it does not produce toxic residues when decomposed and therefore does not affect the final product or the waste treatment process. PAA can be used over a wide temperature spectrum (0 to 40 °C) in clean-in-place (CIP) processes (Leaper, 1984). PAA can also be used with hard water and protein residues do not affect its efficiency. Up until now, no microbial resistance to PAA has been reported and it is efficient over a wide pH range (3.0 to 7.5) (Block, 1991; Lenahan, 1992).

Ultrasound (US) was adopted by the electronics industry to decontaminate surfaces and its use has recently been recommended as an alternative sanitization step in the food industry (Nascimento *et al.*, 2008; Adekunte *et al.*, 2010; Cao *et al.*, 2010; Sagong *et al.*, 2011). When applied to liquids, ultrasonic waves promote cavitation, which consists on the formation, growth, and collapse of air

bubbles. These bubbles generate localized mechanical and chemical energies that are capable of inactivating microorganisms (Valero *et al*., 2007; Gogate and Kabadi, 2009; Adekunte *et al*., 2010). Bubble collapse causes pressure changes, which is considered the main cause of microbial cell disruption (Patil *et al*., 2009). US has been used to disrupt biofilm or even inactivate microorganisms. The objective of this paper was to evaluate the bactericidal effect of NaClO (50, 100, and 150 mg/L) and PAA (40, 60, and 80 mg/L) associated with US (40 Hz) to control the adhesion of *Staphylococcus aureus*, *Staphylococcus hominis*, and *Pseudomonas aeruginosa* isolated from fish species from the Amazon region.

2. MATERIAL AND METHODS

2.1. Bacterial strains

The pure cultures were isolated from fish species from the Amazon region: butterfly peacock (*Cichla ocellaris*) and piramutaba (*Brachyplatystoma vailantii*). The bacteria were isolated through seeding in agar surface using violet red bile glucose (VRBG) for *P. aeruginosa* strains and Baird-Parker with egg-yolk tellurite for *S. aureus* and *S. hominis*, both with incubation at 36 °C/48 h. Next, colonies were selected to be striated in VRBG or Baird-Parker agar plates to obtain pure cultures. After another incubation at 36 °C/48 h, these colonies were transferred to BHI (brain-heart infusion) with 10% glycerol and stored in a freezer to be used in further tests.

The bacteria isolated were previously identified with Gram stain tests. Next, *P. aeruginosa* strains were identified using the API 20E kit (Enterobacteria) while *S. aureus* and *S. hominis* strains, with the API Staph kit (Staphylococci). This procedure was in accordance with the manufacturer's recommendations (Biomérieux, France) (Harrigan, 1998).

2.2. Surface

Stainless steel coupons (6 cm^2) were used as test surfaces. The coupons were individually cleaned and sterilized according to Marques *et al.* (2007).

2.3. Adhesion to surfaces, quantification of adhered cells and sanitizers application

Strains were reactivated in nutrient broth for 72 h at 36 °C and replicated to another nutrient broth for 24 h at 36 °C. Then, 2 mL of activated contents were transferred to 300 ml of a new nutrient broth, in which the stainless steel coupons were immersed and incubated at 30 °C/24h. After this last incubation period, the population density (planktonic cells) in the bacterial suspension was estimated using nutrient agar. Next, all coupons were aseptically removed, rinsed three times with sterile distilled water to remove unattached cells, and dried in a laminar flow cabinet for 30 min.

Afterwards, the coupons were immersed in sterile distilled water at 25 °C (control group) for 10 min. Finally, microorganisms were quantified using a swab rubbed 20 times onto two coupons and then immersed in 0.1% peptone water for subsequent plating.

The remaining coupons were immersed for 10 min in sanitizer solutions at 25 °C at three different concentrations: commercial NaClO (50, 100, and 150 mg/L) and PAA (40, 60, and 80 mg/L). Two coupons were used for each sanitizer concentration. The sanitizing effect was neutralized with the aid of a 0.1% sodium thiosulfate solution. The microorganisms were quantified using the swab technique. All coupons were plated in duplicate on nutrient agar and incubated at 37 °C for 48 h. Next, the NaClO and PAA concentrations that obtained the greatest decimal reduction in the microorganism population were associated with the US treatment in an ultrasound bath (40 Hz) for 10 min following the same procedures mentioned above.

The effectiveness of the disinfectant agent expressed as germicidal effect or decimal reduction (DR), was determined by the equation below:

$$DR = \log N_i - \log N_f \quad (1)$$

where N_i is the cell count in the control group (no sanitizer treatment) (CFU/cm^2) in nutrient agar and N_f is the count after exposure to sanitizer.

2.4. Reproducibility and statistical analysis

All analyses were carried out in duplicate with three repetitions on separate occasions, and the results are expressed as the average of the assays. Counts were converted into decimal logarithmic values (log CFU/cm^2). The test results before and after sanitizer application were compared using Tukey's test. Data were analyzed using the software Statistica 7.0. A probability value $p<0.05$ was accepted as indicating significant differences.

3. RESULTS AND DISCUSSION

3.1. Bacterial adherence to surfaces

The population density of the bacterial suspensions in nutrient broth did not significantly differ ($p>0.05$) after 24 h/30 °C. However, the number of *S. hominis* cells adhering to stainless steel coupons was higher ($p<0.05$) compared to the species *S. aureus* and *P. aeruginosa*, which shows their greater adhesion capacity in the test conditions (Table 1).

Table 1. Population density of planktonic (log CFU/mL) and sessile (log CFU/cm^2) cells of *Staphylococcus aureus*, *Staphylococcus hominis*, and *Pseudomonas aeruginosa* in nutrient broth with stainless steel coupons.

Bacteria	Planktonic cells (log CFU/mL)	Sessile cells (log CFU/cm^2)
S. aureus	7.09±0.05^a	4.11±0.09^a
S. hominis	6.82±0.78^a	4.95±0.85^b
P. aeruginosa	6.99±0.17^a	4.14±0.17^a

Means followed by the same letter in the column did not differ by Tukey's test at 5% probability. Values represent the mean of three repetitions.

All species evaluated were able to adhere to stainless steel surfaces, reaching values between 4.11 and 4.95 log CFU/cm^2 (Table 1). Parizzi (2014) found results of approximately 5.0 log CFU/cm^2 for *S. aureus* on stainless steel after 12 h of contact at 30 °C. Another study showed that the adhesion of *S. aureus* reached 6.10 log CFU/cm^2 (Silva *et al*., 2009). Also, Jeromino *et al.* (2012) showed adhesion of 6.9 log CFU/cm^2 for *S. aureus* on stainless steel after 24 h at 28 °C. Krolasik *et al*. (2010) observed the adhesion of *S. hominis* in stainless steel after incubation for 4 h at 20 °C, while Vanhaecke *et al*. (1990), Cloete and Jacobs (2001), Figueiredo *et al*. (2014), and Caixeta *et al*. (2012) also observed the adhesion capacity of *P.aeruginosa* to stainless steel.

3.2. Effect of sanitizers

Counts of *S. aureus*, *S. hominis*, and *P. aeruginosa* cells adhered to stainless steel surfaces after application of PAA (40, 60, and 80 mg/L) and NaClO (50, 100, and 150 mg/L) are presented in Figures 1, 2, and 3. These results are significantly different ($p<0.05$) when submitted to analysis of variance (ANOVA) (Table 2).

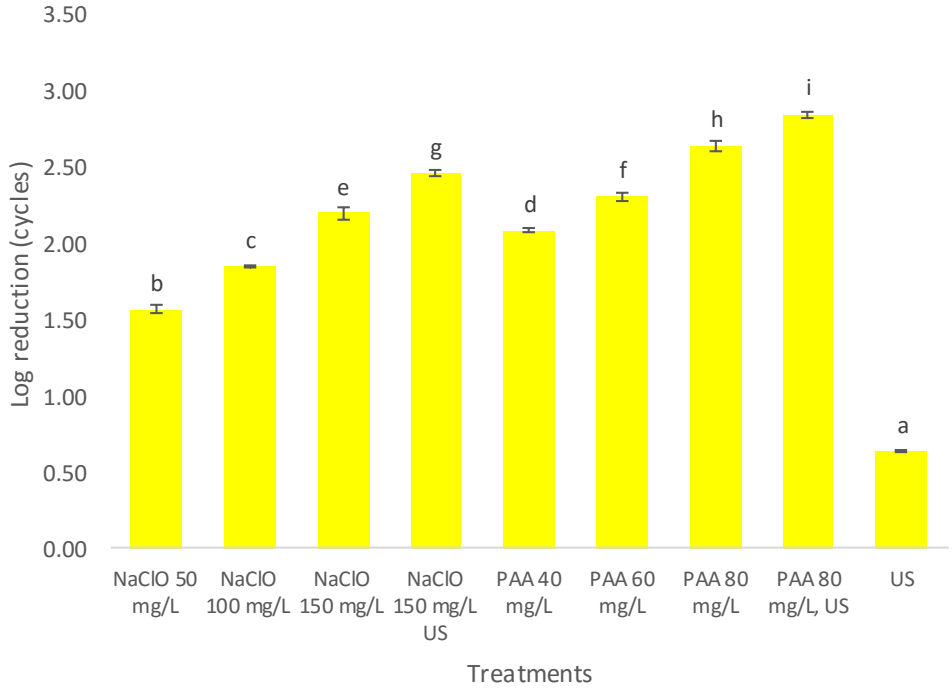

Figure 1. Effect of applying peracetic acid (PAA) and sodium hypochlorite (NaClO) combined with ultrasound (US) to control *S. aureus* adhesion. Treatments indicated with the same letter did not differ ($p>0.05$) among themselves

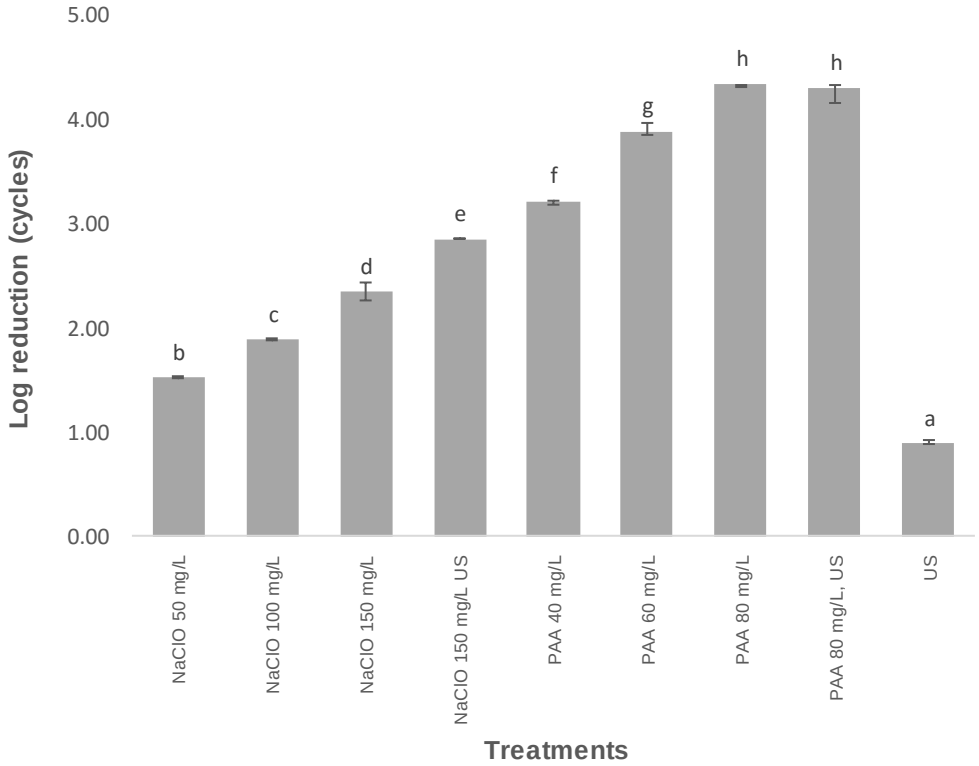

Figure 2. Effect of applying peracetic acid (PAA) and sodium hypochlorite (NaClO) combined with ultrasound (US) to control *S. hominis* adhesion. Treatments indicated with the same letter did not differ ($p>0.05$) among themselves

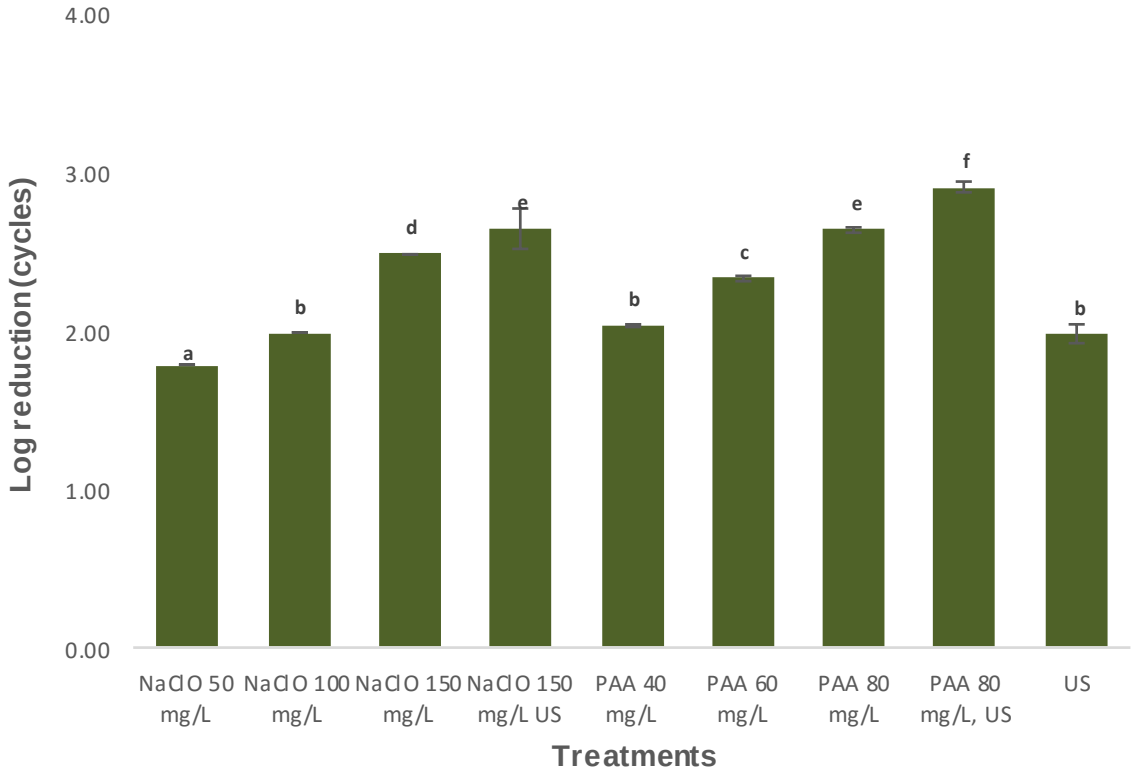

Figure 3. Effect of applying peracetic acid (PAA) and sodium hypochlorite (NaClO) combined with ultrasound (US) to control *P. aeruginosa* adhesion. Treatments indicated with the same letter did not differ ($p>0.05$) among themselves.

Table 2. ANOVA for sanitizer treatments applied to *S. aureus*, *S. hominis*, and *P. aeruginosa*

Bacteria/Treatment	SS	S	F	p
S. aureus	10.4888	1,311	1,913.50	0.00
S. hominis	36.6267	4,578	1,334.94	0.00
P. aeruginosa	3.5450	0,433	168.51	0.00

The different NaClO concentrations (50 to 150 mg/L) yielded a significant difference ($p<0.05$) when applied to adhered cells of *S. aureus* and *S. hominis*, reaching reductions between 1.57-2.20 and 1.52-2.35 log cycles, respectively. The application of PAA (40-80 mg/L) yielded reduction values between 2.09-2.64 log cycles for *S. aureus* and 3.22-4.34 for *S. hominis*. These values differed among themselves at a 95% significance level.

The United States Environmental Protection Agency's (EPA) Scientific Advisory panel has stated that any treatment which can reduce microbial contamination by 2 log cycles is significant (Michaels *et al*., 2003). That shows that PAA was more efficient than NaClO in reducing *S. aureus* and *S.*

hominis populations since lower PAA concentrations (40 mg/L) yielded population reductions equivalent to those observed for the highest NaClO concentrations (150 mg/L).

When associated with US (40Hz, 10 min, NaClO (150 mg/L) and PAA (80 mg/L) allowed the reduction of 2.46 and 2.85 cycles, respectively, of adhered *S. aureus* cells. For *S. hominis*, this association yielded reductions of 2.85 and 4.30 cycles, respectively. In other words, PAA at 80 mg/L and NaClO at 150 mg/L associated with US yield significant values (at a 95% level) in the adhesion of *S. aureus* and *S. hominis* compared to the application of these sanitizers alone.

However, US (40 Hz, 10 min) applied alone yielded a reduction of only 0.64 and 0.89 cycles for *S. aureus* and *S. hominis*, respectively. These results suggest that no synergistic or additive effect occurred between the sanitizers (NaClO and PAA) and US in the conditions studied.

Therefore, US might help aqueous sanitizers penetrate inaccessible sites (hydrophobic pockets and folds in leaf surfaces on fruits and vegetables), which makes such sanitizers more effective (Seymour *et al*., 2002, Gogate and Kabadi, 2009, Sagong *et al*., 2011)

Studies have combined US with other sanitizers such as organic acids (Sagong *et al*., 2011), hydrogen peroxide (São José and Dantas Vanetti, 2012), and chlorine dioxide (Huang *et al*., 2006) and have found an additive or even synergistic bactericidal effect compared to the individual treatments (Ding *et al*., 2015). São José and Vanetti (2015) observed no synergistic effect of applying US with sodium dichlorocyanurate (50 and 200 mg/L) and PAA (40 mg/L) to remove *Salmonella* from cherry tomato surfaces.

In fact, US is a clean technology (Rahman, Ding, & Oh, 2010) with potential to be used in bacteria inactivation. However, it is not very effective alone in killing microorganisms in food at ambient or sub-lethal temperatures (Sengül *et al*., 2011). Microorganism reduction by US is mainly due to the physical phenomenon called cavitation (Alegria *et al*., 2009; Piyasena *et al*., 2003; Seymour *et al*., 2002).

Lee, Kim and Ha (2014) suggested that the treatment with US alone may be not effective for application in the food industry. Piaysena *et al*. (2003) reported that bactericidal effects on food treated with US alone is localized and does not affect a large area.

Others studies have examined the inactivation of pathogenic bacteria by chemical disinfection treatments such as NaClO in vitro. Ha and Ha (2012) reported strong resistance of *S. aureus* against NaClO. NaClO has also been reported to be a potential antimicrobial agent against *S. aureus* in biofilm (Toté *et al*., 2010). Bodur and Cagri-Mehmetoglu (2012) noted that NaClO (250 mg/L) was not efficient in completely removing *S. aureus* cells adhered to stainless steel surfaces. Meira *et al.* (2012) found similar results when studying *S. aureus* biofilm formation on stainless steel surfaces.

Rossini and Gaylarde (2000) stated PAA has an important advantage because this compound does not pose an environmental risk and does not produce toxic compounds after reaction with organic materials. Marques *et al*. (2007) confirmed that PAA was the most effective in removing adhered *S. aureus* cells. Meira *et al*. (2012) reported that PAA (30 mg/L) was more effective than NaClO (250 mg/L) in reducing the viable cell count of *S. aureus* in the biofilm matrix. Vázquez-Sánchez *et al.* (2014) noted PAA (100-750 mg/L) was more effective against *S. aureus* biofilms and planktonic cells when compared to NaClO (500–1,000 mg/L) treatment.

Nonetheless, more studies on the inactivation of *S. hominis* by the application sanitizers are required given the scarce literature data on the subject.

The different NaClO (50 to 150 mg/L) and PAA (40-80mg/L) concentrations yielded a significant difference ($p<0.05$) when applied to adhered *P. aeruginosa* cells, reaching reductions between 1.78-2.49 and 2.04-2.64 log cycles, respectively. When associated with US, only PAA yielded reductions (2.91 log cycles) that significantly differed at a 95% level when compared to the treatment with PA alone.

The individual application of US yielded a reduction of 1.98 cycles and can be compared to the efficiency of NaClO (100 mg/L). Moreover, it can be considered an efficient treatment to control the adhesion of this microorganism according to the EPA since it alone yielded a reduction of approximately 2 log cycles.

A synergistic effect ($p<0.05$) was also observed between the treatments with NaClO (150 mg/L) and PAA (80 mg/L) when combined with US.

Herceg *et al*. (2012) noted that Gram-negative bacteria are more susceptible to the US treatment than Gram-positive ones. Gram-positive bacteria, especially *S. aureus*, usually have a thicker and more tightly adherent layer of peptidoglycan than Gram-negative bacteria, and this morphological feature did seem to be a differentiating factor in ranking the microorganisms according to the percentage of bacteria killed by US treatment.

4. CONCLUSION

The results in the present study showed that *S. hominis* is quite sensitive to the treatment with PAA and may reach reductions of up to 4 log cycles. Furthermore, the results showed that the best treatment combination both for *S. aureus* and *S. hominis* and *P. aeruginosa* was PAA at 80 mg/L associated with US. The use of US at 40 Hz to remove adherent *P. aeruginosa* can be considered efficient and has an effect comparable to that of NaClO (100 mg/L).

5. CONFLICT OF INTERESTS

The authors have not declared any conflicts of interests.

6. ACKNOWLEDGEMENTS

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CONSIDERAÇÕES FINAIS

1. Os micro-organismos mesófilos isolados de peixes amazônicos e selecionados neste trabalho não apresentaram crescimento significativo quando mantidos sob refrigeração (10º C) em curto prazo de tempo.
2. No entanto, em virtude das elevadas populações de bactérias psicrotróficas, a conservação destes pescado requer outros métodos de conservação que garantam a qualidade microbiológica.
3. O ácido peracético quando associado ao ultrassom apresentou o mais efeciente efeito sanificante no controle da adesão de *S. aureus, S. hominis* e *P. aeruginosa* podendo ser um potencial substituto do hipoclorito de sódio na sanitização de pescado.
4. O ultrassom de baixa frequência pode ser uma importante ferramenta no controle da adesão de *P. aeruginosa*, possuindo efeitos comparados ao hipoclorito de sódio.