FREDERICO AUGUSTO DE ALCÂNTARA COSTA

INFECÇÃO POR *Weissella* sp. EM TRUTAS ARCO-ÍRIS (*Oncorhynchus mykiss*): CARACTERIZAÇÃO DA DOENÇA, VIAS DE TRANSMISSÃO E PERFIL DE RESISTÊNCIA AOS ANTIMICROBIANOS

LAVRAS - MG

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Dissertação apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Ciências Veterinárias, área de concentração em Sanidade em Animais de Produção, para a obtenção do título de Mestre.

Orientador

Dr. Henrique César Pereira Figueiredo

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Ao amigo eterno Lamartine (in memorian), onde quer que esteja, com todo o carinho.

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RESUMO

Nos últimos anos, surtos de uma doença septicêmica hemorrágica vêm ocorrendo nas fazendas que cultivam trutas arco-íris (*Oncorhyncus mykiss*) em diferentes estados do Brasil (SP, MG e RJ). Durante os anos de 2008 e 2009, foram acompanhados surtos desta enfermidade em três truticulturas no país. Quarenta e um peixes doentes foram submetidos à avaliação bacteriológica. Os isolados foram caracterizados por testes bioquímicos, PCR *Weissella* gênero-específica, seqüenciamento do gene 16S rRNA e análise filogenética. Para avaliar as potenciais rotas de infecção da bactéria, juvenis de trutas arco-íris foram infectados experimentalmente por via intraperitoneal, imersão e por cohabitação entre peixes saudáveis e doentes. Adicionalmente, o perfil de resistência antimicrobiana para cinco antibióticos foi determinado e os pontos de corte epidemiológicos provisórios (PCEP) calculados pela interpretação normalizada de resistência (INR). Setenta e sete isolados caracterizados como cocos, Gram positivos, oxidase negativo e catalase negativos foram obtidos. Esses apresentaram resultados positivos na PCR *Weissella* gênero-específica. As seqüências de 16S rRNA das amostras brasileiras apresentaram 100% de similaridade com as seqüências de *Weissella* sp. isoladas de trutas doentes na China no primeiro relato de infecção por uma bactéria deste gênero em peixes. A doença foi reproduzida com sucesso em condições laboratoriais por todas as vias testadas. A cohabitação com peixes doentes, sem contato direto, ocasionou a doença em peixes saudáveis. Todas as amostras foram resistentes a sulfonamida e, de acordo com os PCEP, determinados pela análise de INR, todos os isolados foram classificados como selvagens (WT) quanto à sensibilidade ao florfenicol. Respectivamente, um, dois e três isolados foram classificados como não selvagens (NWT) para eritromicina, oxitetraciclina e norfloxacina. Esta é a primeira descrição de múltiplos casos de infecção por *Weissella* sp. em fazendas de trutas arco-íris no mundo. Assim como, as vias de infecção da doença e pontos de corte epidemiológicos provisórios para esta bactéria frente à quatro antibióticos.

Palavras-chave: Septicemia hemorrágica. Truticultura. Patogênese. Antibiograma.

ABSTRACT

Hemorrhagic septicemia outbreaks in commercial rainbow trout (*Oncorhynchus mykiss*) farms have been occurred, over the last years, in different Brazilian states (SP, RJ and MG). During 2008 and 2009, outbreaks in three commercial rainbow trout farms in Brazil were accompanied. Seventy-seven Gram positive isolates were obtained from diseased fish from distinct farms. The bacterial species was identified at genus levels by biochemical tests, *Weissella* genus-specific PCR and 16S rRNA sequencing. To address the potential infectious route of the bacteria, the rainbow trout juveniles were experimentally infected by different via. In addition, the antibiotic resistance profiles for five antibiotics were determined and provisional epidemiological cut-off values calculated by normalized resistance interpretation (NRI). All isolates presented similar phenotypic profiles and positive reactions for *Weissella* genus-specific PCR. The 16S rRNA sequences of Brazilian strains showed 100% of similarity with sequences of Chinese isolates identified as the first *Weissella* sp. infection in fish. The disease was successfully reproduced in lab conditions by intraperitoneal injection, immersion and cohabitation between diseased and healthy fish with no direct contact, through the water. All isolates were resistant to sulfonamide and based in NRI analysis, none, one, two and three isolates were classified as non-wildtype (NWT) for florfenicol, erytromycin, oxytetracycline and norfloxacyn. This is the first description of multiple cases of *Weissella* sp. infection rainbow trout farms, as well as infectious routes for disease and provisional epidemiological cut-off values for these bacteria to four antibiotics.

Keywords: Hemorrhagic septicemia. Rainbow trout farm. Pathogenesis. Antibiogram.

SUMÁRIO

PRIMEIRA PARTE

1 INTRODUÇÃO

Seguindo a tendência mundial da atividade, a aquicultura nacional vem apresentando forte crescimento nas últimas décadas. Tal expansão tem sido impulsionada, principalmente, pela maior demanda de proteína de origem animal e por uma mudança nos hábitos alimentares da população. A piscicultura no Brasil é representada pela produção de espécies nativas e exóticas. Tilápias, carpas e a truta arco-íris (*Oncorhynchus mykiss*) são as principais espécies exóticas cultivadas no país atualmente. Pertencente à família dos salmonídeos, a truta arco-íris é originária de regiões de clima temperado, com faixa de conforto térmico entre 9°C e 15°C. Por esse motivo, as truticulturas nacionais estão localizadas em áreas restritas, geralmente regiões de altitudes elevadas no Sul e Sudeste do Brasil. Nessas, o clima e a temperatura da água são mais frios.

Desde o ano de 2008, surtos de uma doença infecciosa, caracterizados por um quadro de septicemia hemorrágica, vêm sendo acompanhados em fazendas de trutas arco-íris no Brasil. O agente etiológico da doença foi identificado como uma bactéria do gênero *Weissella*. Primeiramente descrita na China em 2009 (LIU et al., 2009), as infecções por *Weissella* sp. em truticulturas são ainda pouco caracterizadas. A enfermidade, que ocorre na forma de surtos, com altos índices de mortalidade, tem causado perdas econômicas consideráveis.

A antibioticoterapia pode ser uma eficiente forma de controle a ser adotada frente a um problema sanitário na aquicultura. Por outro lado, o uso inadequado e contínuo de agentes antimicrobianos pode aumentar a freqüência de bactérias resistentes, causando impacto negativo nas terapias subseqüentes.

Atualmente, não existem estudos sobre a identificação e caracterização do agente etiológico dos surtos de septicemia hemorrágica nas fazendas de trutas arco-íris e do perfil de resistência dos isolados frente aos antimicrobianos.

2 REFERENCIAL TEÓRICO

O aumento da população mundial e a busca por uma alimentação mais saudável impulsionou o consumo por peixes e outros organismos aquáticos nas últimas décadas. A aquicultura atende a uma parcela cada vez maior desta demanda. O aumento significativo na produção -- de menos de um milhão de toneladas no início da década de 1950 para aproximadamente 68,5 milhões em 2008 -- destaca a aquicultura como o setor de produção animal que mais cresceu nos últimos anos segundo dados da Food and Agriculture Organization - FAO (2009) (FAO, 2010).

Seguindo a tendência mundial da atividade, a aquicultura nacional vem apresentando forte crescimento. Enquanto a pesca no país manteve-se consideravelmente estável entre os anos de 2003 e 2009, o incremento da aquicultura foi de 49% neste mesmo período (BRASIL, 2010b). Devido à grande disponibilidade hídrica, o potencial para a expansão da produção aquícola no Brasil é ainda maior, podendo superar o déficit da balança comercial nacional de pescado que, em 2009, foi de US$ 519 milhões segundo dados do Ministério do Desenvolvimento, Indústria e Comércio Exterior - MDIC (BRASIL, 2010b). Além disso, o consumo "*per capita*" de pescado no Brasil (9 kg/ano) ainda está abaixo da média mundial (16 kg/ano) e do consumo mínimo (12 kg/ano) recomendado pela Organização Mundial de Saúde - OMS (FAO, 2009).

Em 2009, a piscicultura representou 27% da produção total de pescado do Brasil (BRASIL, 2010a). A atividade se caracteriza pela produção de peixes nativos e exóticos. Dentre as espécies exóticas, tilápias, carpas e a truta arco-íris, se destacam na piscicultura nacional atualmente. A truta arco-íris -- *Oncorhynchus mykiss* (Walbaum 1792), da ordem Salmoniformes, família Salmonidae -- é uma das espécies de peixe mais cultivadas comercialmente em

todo o mundo. Originária dos rios da América do Norte, essa foi introduzida para fins recreativos e para aquicultura em todos os continentes, com exceção da Antártica. No Brasil, a importação da espécie ocorreu no ano de 1949, quando os primeiros ovos embrionados provenientes da Dinamarca foram trazidos pelo Ministério da Agricultura (BRASIL, 2001). Desde então as truticulturas apresentam uma estreita relação com o turismo nas regiões serranas do país. Essas têm na pesca esportiva, na culinária e no ecoturismo estratégias que viabilizam a atividade pela agregação de valor ao produto. Além disso, a atividade tem importância econômica para o país devido ao alto valor comercial das trutas arco-íris, sendo responsável por uma movimentação financeira superior a R$ 30 milhões/ano (INSTITUTO BRASILEIRO DO MEIO AMNBIENTE DOS RECURSOS RENOVÁVEIS - IBAMA, 2008).

Do total proveniente da produção de peixes no mundo, o cultivo de truta arco-íris representa a sexta maior receita (FAO, 2010). Atualmente, os problemas sanitários estão entre os principais entraves na cadeia produtiva desta espécie, sendo a maioria de seus patógenos e doenças já foi descrita pela literatura. Diversos agentes infecciosos foram identificados como causadores de doença septicêmica hemorrágica em trutas arco-íris. A Septicemia Hemorrágica Viral e a Necrose Hematopoiética Infecciosa, ambas causadas por vírus da família Rhabdoviridae, são as enfermidades de etiologia viral de maior impacto para o cultivo de salmonídeos em água doce e a notificação de sua ocorrência à Organização Mundial de Saúde Animal é obrigatória (WORLD ORGANISATION FOR ANIMAL HEALTH – OIE, 2010).

Entre as doenças de etiologia bacteriana que cursam com quadro de septicemia hemorrágica em trutas arco-íris, as de maior relevância são: a Doença da Boca Vermelha (do inglês “Red Mouth Disease”), causada pela bactéria *Yersinia ruckeri*; as Streptococoses, cujo principal agente causador de doenças, nas truticulturas, pertencente ao grupo é o *Lactococcus garvieae* e a Doença

Renal Bacteriana, causada pela bactéria *Renibacterium salmoninarum* (CHAMBERS; GARDINER; PEELER, 2008; FOUZ; ZARGA; AMARO, 2006; VENDRELL et al., 2006). No entanto, não existem relatos da ocorrência dos patógenos supracitados em fazendas de truta arco-íris no Brasil. Diferente dos animais terrestres, em peixes, a maioria das doenças não promove sinais patognomônicos. Assim, as enfermidades dificilmente podem ser caracterizadas somente pelos sinais clínicos, sendo necessária a realização de exames laboratoriais para o diagnóstico correto do agente etiológico da doença (NOGA, 2000).

Nos últimos anos, as fazendas brasileiras de truta arco-íris (*Onchorhynchus mykiss*) vêm enfrentando um problema sanitário que ocorre sob a forma de surtos e ocasiona altos índices de mortalidade. Esses acontecem principalmente durante o verão, quando a temperatura da água atinge os valores mais elevados do ano. A enfermidade, caracterizada por um quadro de septicemia hemorrágica, tem causado perdas econômicas consideráveis nas truticulturas nacionais. Condições adversas dentro do sistema de cultivo intensivo, como altas densidades populacionais e manejo intenso reduzem significativamente a imunidade dos peixes, favorecendo o estabelecimento e a disseminação de agentes infecciosos (PULKKINEN et al., 2010). Tais condições têm favorecido a ocorrência dos surtos no país.

Segundo análise realizada pelo Intergovernamental Panel on Climate Change – IPCC, o fenômeno do aquecimento global tem provocado efeitos adversos diretos sobre os recursos hídricos dulcícolas (BATES et al., 2008). Os peixes, animais heterotérmicos, têm a fisiologia afetada, incluindo o sistema imune, quando a temperatura da água se encontra fora da faixa de conforto térmico. De forma similar, os patógenos também têm temperatura ótima de crescimento e diversas enfermidades em salmonídeos apresentam a elevação da temperatura como fator determinante, para que ocorra a infecção e as altas taxas

de mortalidade (MARCOS-LÓPEZ et al., 2010). Além disso, a solubilidade do oxigênio diminui com o aumento da temperatura da água. Fator que pode ser impactante para a criação de trutas arco-íris, espécie que tem alta demanda de oxigênio dissolvido na água, acima de 5 mg/L (WEDEMEYER, 1996).

O desenvolvimento de medidas de controle e prevenção na aqüicultura em resposta a uma emergência sanitária depende inicialmente da identificação do(s) agente(s) etiológico(s) (MOHAN et al., 2008). Estudos relacionados à etiologia e patogenia de uma determinada enfermidade são fundamentais nas decisões dos programas de biossegurança, na implementação de tratamentos eficazes e nas demais intervenções estratégicas a serem adotadas dentro do sistema de produção para evitar maiores impactos decorrentes dos surtos (GONZALEZ, 2009).

A antibioticoterapia utilizada de forma apropriada na aquicultura tem sido uma das mais efetivas formas de controle nos casos de emergência associada a surtos por doenças infecciosas bacterianas. No entanto, o uso indiscriminado destes agentes tem o potencial de aumentar a freqüência de bactérias resistentes, com impacto negativo nas terapias subseqüentes. Vários estudos têm mostrado que a medicação direta aos peixes pode levar a um aumento no nível de resistência bacteriana aos antibióticos, seja pelo uso incorreto, seja pela permanência do produto na água (MIRANDA; ZEMELMAN, 2002; PETERSEN; DALSGAARD, 2003). Além disso, a disseminação de bactérias resistentes provenientes da aquicultura pode ter um impacto negativo no uso de antimicrobianos na medicina humana e em outros animais terrestres (SMITH, 2008). Em relatório publicado pelas instituições internacionais FAO/OIE/WHO (WORLD HEALTH ORGAZINATION - WHO, 2006), a respeito da utilização de antibióticos na aquicultura, foi recomendado o desenvolvimento de medidas para prevenir os riscos da disseminação da resistência antimicrobiana. Uma das principais medidas a serem adotadas é a

implementação de programas de monitoramento de resistência aos antibióticos de patógenos isolados de animais aquáticos cultivados.

A análise do perfil de resistência antimicrobiana de patógenos na aquicultura ainda é limitada devido à escassez de dados disponíveis e à variação dos métodos utilizados para esta avaliação. No caso de um agente emergente, a classificação dos isolados como sensíveis ou resistentes a um antibiótico testado é ainda mais limitada. No entanto, uma possibilidade de interpretação diferenciada dos resultados obtidos do teste de antibiograma tem sido aplicada em estudos de bactérias isoladas em ambiente aquático (DOUGLAS et al., 2007; RUANE et al., 2007). O método de interpretação normalizada de resistência (INR) permite que valores de corte epidemiológicos sejam determinados apenas com os dados obtidos dentro de um laboratório. A INR tem a vantagem de conseguir detectar amostras com baixos níveis de resistência. Entretanto, para isso, é necessário um elevado número de isolados de uma mesma espécie (SMITH et al., 2007).

Assim, esse trabalho teve como objetivos identificar o agente etiológico dos surtos de septicemia hemorrágica nas fazendas de trutas arco-íris, caracterizar as vias de transmissão do patógeno, bem como o perfil de resistência dos isolados frente aos antimicrobianos.

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SEGUNDA PARTE - ARTIGOS

ARTIGO 1

Weissella sp. outbreaks in commercial rainbow trout (*Oncorhynchus mykiss*) farms

(Artigo preparado de acordo com as normas da revista "Veterinary Microbiology")

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ABSTRACT

The genus *Weissella* is comprised by fourteen bacterial species, usually found in nutrient rich enviroment and fermented foods and beverages. During 2008 and 2009, outbreaks of hemorrhagic septicemia in three commercial rainbow trout farms in Brazil were accompanied. Seventy-seven Gram positive isolates were obtained from diseased fish from distinct farms. The bacterial species was identified at genus levels by biochemical tests, *Weissella* genus-specific PCR and 16S rRNA sequencing. To address the potential infectious route of bacteria, rainbow trout juveniles were experimentally infected by different via. In addition, the antibiotic resistance profiles for five antibiotics were determined and provisional epidemiological cut-off values calculated by normalized resistance interpretation (NRI). All isolates presented similar phenotypic profiles and positive reactions for *Weissella* genus-specific PCR. The 16S rRNA sequences of Brazilian strains showed 100% of similarity with sequences of Chinese isolates identified as the first case of *Weissella* sp. infection in fish. The disease was successfully reproduced in lab conditions by intraperitoneal injection, immersion and cohabitation between diseased and healthy fish with no direct contact, through the water. All isolates were resistant to sulfonamide and based in NRI analysis, none, one, two and three isolates were classified as non-wildtype (NWT) for florfenicol, erytromycin, oxytetracycline and norfloxacyn. This is the first description of multiple cases of *Weissella* sp. infection rainbow trout farms, as well as infectious routes for disease and provisional epidemiological cut-off values for these bacteria to four antibiotics.

1. Introduction

The genus *Weissella* constitutes together with *Leuconostoc* and *Oenococcus* a line of descent of lactic acid bacteria (Chelo et al., 2007). Previously known as *Leuconostoc*-like organisms, the phylogeny of this genus was clarified in 1990

based in the sequence data of 16S and 23S rRNA (Bjorkroth et al., 2002). Currently, the genus *Weissella* is comprised by fourteen bacterial species (Bruyne et al., 2008; Bruyne et al., 2010). The bacteria belonging to this genus are Gram-positive, catalase-negative, non-spore-forming, heterofermentative, non-motile, coccoid or rod-shaped organisms (Collins et al., 1993). *Weissella* strains have been isolated from a variety of sources, being usually found in nutrient-rich environments such as milk, meat, vegetable products and fermented drinks. Some species have been identified in intestinal contents of healthy human, dogs, chickens, ducks and fish (Cai et al., 1998; Kurzak et al., 1998; Walter et al., 2001; Beasly et al., 2006; Chelo et al., 2007; Wise and Sirugusa, 2007; Sirirat et al., 2008) and from vaginal tract of healthy women (Nam et al., 2007). Therefore, the potential probiotic activities of these bacteria have been speculated and addressed (Cai et al., 1998; Lee, 2005; Manninen et al., 2006). In spite of this, previous reports described fatal cases of bacteremia and endocarditis in humans and septicemia in primate (*Cercopithecus mona*) caused by *W. confusa*, as well as otitis in dogs associated to *W. cibaria* and *W. confusa* infections (Olano et al., 2001; Bjorkroth et al., 2002; Flahert et al., 2003; Vela et al., 2003; Shin et al., 2007; Svec et al., 2007).

Recently, Liu and coworkers (2009) performed the first description of *Weissella* sp. infection in a cultivated fish species. They accompanied sequential outbreaks in one commercial rainbow trout (*Oncorhynchus mykiss*) facility in China. Until now, there is no additional report about other cases of *Weissella* infection in different farms and outside of China.

The pattern of antibiotic resistance for *Weissella* species is poorly understood nowadays. *W. confusa* is recognized as a vancomycin-resistant bacterium (Flaherty et al., 2003; Vela et al., 2003) and some human isolates showed to be resistant to cotrimoxazol, metronidazole, teicoplanin (Svec et al., 2007), and trimethoprim-sulfamethoxazole (Olano et al., 2001). Multi-resistance profiles

were also observed in fish isolates of *Weissella* sp. (Liu et al., 2009), however, only six strains were evaluated.

Herein, we described the occurrence of outbreaks of hemorrhagic septicemia caused by *Weissella* sp. in multiple commercial farms of rainbow trout (*Oncorhynchus mykiss*) located in different Brazilian states. In addition, we performed the first study to address the potential infectious routes of these bacteria in fish, as well as, the description of the antibiotic resistance profile, and laboratory-specific epidemiological cut-off values calculated by normalized resistance interpretation.

2. Material and Methods

2.1. Outbreaks

Three outbreaks of hemorrhagic septicemia in commercial farms of rainbow trout (*Oncorhynchus mykiss*) were accompanied. The farms were located in mountain regions of three Brazilian states, Rio de Janeiro (farm 1), Minas Gerais (farm 2) and São Paulo (farm 3). The outbreaks occurred in March 2008, February 2009 and March 2009, respectively in the farms 1, 2 and 3. In the first two farms, the fish were reared in circular ponds, and concrete "raceways" was the culture system adopted in farm 3. The cases occurred in the summer season, when water temperature in these farms usually changes over of 17°C. The main clinical signs verified in diseased fish were anorexia, lethargy, exophtalmia, ascite and hemorrhages in the mouth, oral cavity, tongue and eyes.

During the outbreaks, diseased rainbow trout were sampled, stored at 4ºC in ice boxes and immediately carried to the laboratory for bacteriological analysis. A total of 41 sick fish were collected, being ten (adult) from farm 1, six (adult) from farm 2 and twenty five (12 fingerlings, 8 juveniles and 5 adult) from farm 3.

2.2. Isolation and Biochemical characterization

For bacterial isolation, swabs of brain, kidney, liver and ascitic fluid of diseased fish were aseptically sampled, streaked onto sheep blood agar and incubated at 25º during 72 hours. Pure colonies were submitted to Gram stain, catalase test, oxidase test, verification of the presence or absence of haemolysis, growth under different temperatures (20, 25, 30, 37 and 45°C) and on McConkey Agar. The strains were maintained at -80ºC in brain heart infusion broth (BHI) with 15% of glycerol until the use. Before the biochemical and molecular tests, the isolates were grown on Man Rogosa Sharpe (MRS) agar (Sigma-Aldrich, USA) at 25°C for 24 h. Biochemical characterization was achieved by using commercial kit Rapid ID32 Strep (BioMerieux, France).

2.3. Molecular analysis and phylogenetic tree construction

Amplification and sequencing of the 16S rRNA gene were performed for nine isolates (WS-06, WS-08, WS-14, WS-30, WS-32, WS-56, WS-65, WS-71 AND WS-72) randomly selected from the total amount of strains. Total DNA was extracted using commercial DNeasy kit (Qiagen, Germany). 16S rRNA was amplified by PCR with the universal primers C70 (5´-AGA GTT TGA TYM TGG C-3´) and B37 (5´-TAC GGY TAC CTT GTT ACG A-3´), according to the method described by Fox et al. (1995). PCR products were purified using a Wizard PCR Preps kit (Promega, USA) and sequenced using forward and reverse primers. Sequencing reactions were performed using a BigDye™ Terminator Cycle sequencing kit (Applied Biosystems, USA) and run on an ABI 3730XL genetic analyzer (Applied Biosystems). Sequences were then compared to sequences from the NCBI database using the BLASTn algorithm. The limit fixed for identification of a bacterial species was 98% nucleotide identity for the 16S rRNA gene.

The phylogenetic relationship of the isolates was determined by comparative 16S rRNA gene sequence analysis. The sequences of the isolates were aligned in

BioEdit using CLUSTALW (Thompson et al., 1994) with sequences of the following bacterial species: *Weissella paramesenteroides* NRIC 1542 (AB023238), *Weissella thailandensis* FS61-1 (AB023838), *Weissella hellenica* NCFB 2973 (X95981), *Weissella confusa* JCM 1093 (AB023241), *Weissella cibaria* LMG 17699 (AB362617), *Weissella viridensces* NRIC 1536 (AB023236), *Weissella minor* NRIC 1625 (AB022920), *Weissella halotolerans* NRIC 1627 (AB022926), *Weissella kandleri* NCFB 2753 (X52570), *Weissella koreensis* S-5623 (AY035891), *Weissella soli* LMG 20113 (AY028260), *Weissella beninensis* LMG 25373T (EU439435), *Weissella ghanensis* LMG 24286 (AM882997), *Weissella fabaria* 257T (FM179678.1), *Pediococcus damnosus* (AJ318414); and the fish strain *Weissella* sp. JZ-1L (EU869289). The genetic distances matrix was obtained using Kimura's two-parameter model (Kimura, 1980) and an evolutionary tree was created using the neighbor joining method (Saitou and Nei, 1987) with Mega4 (Tamura et al., 2007). Bootstrap values from 1000 replicates are displayed as percentages.

To unequivocally bacterial identification, *Weissella* genus-specific PCR were performed to all isolates using the primers WeiF (5' CGTGGGAAACCTACCTCTTA 3') and WeiR (5' CCCTCAAACATCTAGCAC 3') according to described by Jang et al. (2002) with some modifications. Briefly, the amplification was carried out with a pre-cycle denaturation at 95°C for 5 min followed by 30 serial cycles of 95°C for 30° s, 55°C for 45 s and 72°C for 1 min, with a final extension at 72°C for 7 min.

2.4. Antimicrobial susceptibility testing

The resistance pattern for five of the most regular used antibiotics in world aquaculture (Serrano, 2005) was determined to all isolates by disc diffusion assay. Disks containing the antimicrobials (Oxoid, UK), florfenicol (FLO) (30µg), erythromycin (ERY) (15 µg), norfloxacin (NOR) (10 µg),

oxytetracycline (OXY) (30 µg) and sulfonamide (SUL) (25 µg) were used. The tests were carried out in accordance with the protocols specified in M42-A guidelines (CLSI, 2006), with some adaptations. All tests were performed in Muller-Hinton Agar (Difco, USA) enriched with 5% of defibrinated sheep blood; the suspensions made in sterile saline, and the plates were incubated at 28°C for 24-28h. The diameter of the inhibition zone was measured with a ruler and rounded to the nearest millimeter.

Normalised Resistance Interpretation (NRI) was carried out using the method developed by Kronvall (2003) and Kronvall et al. (2003), according to the adaptation performed by Smith et al. (2007). In the present work the epidemiological cut-off values for defining fully susceptible strains was set at 2.5 standard deviations below the normalized mean for these strains (Smith et al., 2007). Strains generating equal or larger zones than the NRI threshold for susceptible strains were classified as fully susceptible (WT), those generating smaller zones were classified as not fully susceptible (NWT).

2.5. Fish

Two fish species were used in the *in vivo* assays presented here; rainbow trout juveniles (*Oncorhynchus mykiss*), gently provided by Campos do Jordão Research and Development Unit of São Paulo Agency for Agrobusiness Technology (APTA, Brazil); and Nile tilapia (*Oreochromis niloticus*) fingerlings acquired from a commercial hatchery. Before introduction, a random sample of three fish of each species were collected and submitted to bacteriological analysis and to *Weissella* genus-specific PCR, shown to be negative for bacterial infections. Each experimental group was individually kept in glass aquaria and comprised six fish. Rainbow trout juveniles, with average weight of 80.74 g (±16.27 g), were maintained in 120 L aquarium supplied with flow-throughout dechlorinated water (2L h^{-1}) at a temperature ranging from 16 to 18°C. Nile tilapia fingerlings (50.94g±16.24) were kept in 57 L under the same water

renovation level and at a water temperature of 20°C. Fish were maintained on a 12:12h light/dark period and were fed with NUTRIPEIXE TC40 (Purina, Brazil) four times a day until apparent satiation. Fish were acclimated for a period of 15 days.

2.6. Challenge assays

In order to fulfill Koch's postulate, juveniles of albino rainbow trout were experimentally infected with a *Weissella* sp. isolate. Besides, to clarify some infection routes of this bacterium in fish and its capacity to cause disease in different hosts, rainbow trout were challenged by different via and Nile tilapia fingerlings were intraperitoneally infected, respectively. The *Weissella* sp. strain WS-08, isolated from the first outbreak, was used in all *in vivo* assays. The isolate was thawed, streaked onto MRS agar (Sigma-Aldrich) and incubated at 25°C for 24h. One colony was picked up, inoculated in MRS broth (Sigma-Aldrich) and incubated at 25°C for 18h under low agitation (150 rpm). Afterwards, the bacterial suspension was centrifugated (3000 x *g* for 20 min), washed three times and resuspended in sterile phosphate buffered saline (PBS). Before all challenges, fish were anesthetized by immersion in a bath containing 10 mg/L benzocaine (Sigma-Aldrich).

Three infection routes were tested in rainbow trout: intraperitoneal, immersion and a co-habitation assay. Groups A and B were intraperitoneally infected with 0.2 mL of bacterial innoculum at dosage of 3.4 $x10^4$ (low) and 2 $x10^8$ (high) cfu $fish^{-1}$, respectively. For immersion trial, 100 mL of bacterial inoculums at 3 x 10^{10} cfu mL^{-1} were diluted in a 10 L bucket containing 9 900 mL of sterile water, reaching a final concentration of 3 x 10^8 cfu mL^{-1}. After that, group C was immersed, and 15 min later returned to the 120 L aquarium with uncontaminated water. Co-habitation challenge assay was performed as follows: two rainbow trout were infected by intraperitoneal injection at dosage of 1.6 $x10^8$ cfu $fish^{-1}$ as previously described; when first clinical signs appeared, these two diseased fish

were placed in a static plastic cage (40 cm^3) located inside of the 120 L aquarium which contained six healthy fish (Group D); there was no direct contact (fish:fish) among diseased and healthy rainbow trout. Two control groups, E and F, were submitted to the same experimental procedures applied to groups B and C, except to the use of sterile PBS instead of bacterial inoculums. In addition, during the experimental period a group of six fish were not manipulated and maintained under same physical situation (experimental conditions control). Two groups of six Nile tilapia fingerlings were used in challenge assay. The group I was infected by intraperitoneal injection with 0.2 mL of *Weissella* sp. inoculums at dosage of 1.2 x 10^8 cfu $fish^{-1}$. The group II was challenged intraperitoneally with 0.2 mL of sterile PBS. An extra group was maintained under the same circumstances to address the potential alterations caused by experimental conditions. All challenge assays were performed in duplicate, and the results are presented as an average of each replicate data.

Fish were monitored four times a day. The experimental period comprised 21 days and all dead fish were submitted to bacteriological analyses and histopathological evaluation. At the end of the experiments, all survival fish were killed by benzocaine overdose and submitted to the same exams to address the potential asymptomatic carrier status of those animals and microscopical lesions. All *in vivo* experiments were carried out in accordance with animal welfare standards and approved by the Ethics Committee in Animal Experimentation (CETEA/UFMG, Brazil).

2.7. Bacteriology and pathological examination

Samples of brain, kidney, liver, spleen, heart, eye and intestine were aseptically collected from all dead and the survival fish at the end of experimental period. After that, the strains were streaked onto MRS agar and sheep blood agar for bacterial reisolation. Additionally, bacterial load per gram of tissue was determined in three organs, kidney, brain and intestine. Briefly, in sterile

conditions, a small amount of each tissue was collected, weighted, physically disrupted and suspended in germ free PBS. The tissue suspensions were 10-fold serially diluted in sterile PBS and 100 µL of each dilution were streaked onto MRS. The plates were incubated at 25°C for 48h. The data were recorded and converted in cfu per gram of tissue according to the bacterial counting in serial dilution. The bacterial identification of the reisolated strains was confirmed by genus *Weissella*-specific PCR.

Pathological examination was performed for same the organs. The tissues were fixed in Bouin's solution (picric acid 0.9%, formaldehyde 9% and acetic acid 5%), embedded in paraffin wax and processed by routine methods. Sections were stained with haematoxylin and eosin (HE).

3. Results

3.1. Outbreaks data

During the period from March 2008 to March 2009, three contemporaneous outbreaks of hemorrhagic septicemia were followed in distinct commercial farms of rainbow trout in Brazil. Located in three different states (Rio de Janeiro, Minas Gerais and São Paulo), the farms were geographically isolated and had no recent history of animal acquisition, transit or introduction of biological materials, such as fry, fingerlings, embryonated eggs etc. In three cases the field presentation and evolution of disease was similar. The outbreaks were preceded by a period of anorexia, followed by the observation of lethargic and sick fish in the culture systems. Exophtalmia, ascite and hemorrhage in the mouth, tongue and eyes were main clinical signs verified. High mortality rates, varying from 50 to 80%, were verified 4-5 days after the detection of first diseased fish.

Adult rainbow trout in pre-slaughter phase (average weight of 250 g) were the principal life stage affected by disease. In the third case (farm 3) characteristic

clinical signs of the infection were detected in fingerlings, juveniles, adults and dams (data not shown).

3.2. Phenotypic profiles

A total of 77 isolates were obtained from the three outbreaks (Supplementary Table 1). All strains showed to be Gram-positive cocci, catalase negative, oxidase negative, and non-haemolytic or alfa haemolytic strains were verified among the isolates. Growth was observed at all temperatures tested and the strains did not grow on McConkey agar. In the Rapid ID32 Strep kit, all isolates presented acid production from $_{D}$-ribose, $_{D}$-trehalose, pullulan, $_{D}$-maltose, but did not produced acid from $_{D}$-manitol, $_{D}$-sorbitol, $_{D}$-lactose, $_{D}$-saccharose, $_{D}$-arabitol, α-cyclodextrin, glycogen, $_{D}$-melibiose, $_{D}$-melezitose, methyl-β_{D}-glucopyrasonide and $_{D}$-tagatose. Positive results were obtained for Voges-Proskauer test and esculin. Variable results were verified for $_{L}$-arginine (hydrolysis), 4-nitrophenyl-α_{D}-galactopyranoside-2-CHA and sodium hippurate (hydrolysis). Negative results were observed in the other tests.

3.3. Molecular analysis

Amplification of the 16S rRNA fragments from the nine selected isolates yielded a product of approximately 1500 bp. Blast analysis of the sequences revealed that those strains showed at least 98% of similarity to a previously reported sequence of 16S ribosomal RNA gene of *Weissella* sp. strain RT-2L (accession number EU869293.1) isolated in China (Liu et al., 2009). Positive *Weissella* genus-specific PCR reactions were verified for the 77 isolates, with characteristic fragments of 798 bp.

Phylogenetic analysis of 16S rRNA gene sequences resulted in the neighbour-joining tree presented in the figure 1. The Brazilian strains (WS-06, WS-08, WS-14, WS-30, WS-32, WS-56, WS-65, WS-71 and WS-72) showed bootstrap percentage (based on 1000 replicates) of 100% with strain *Weissella* sp. JZ-1L

(accession number EU869289) previously isolated from diseased rainbow trout in China (Liu et al., 2009).

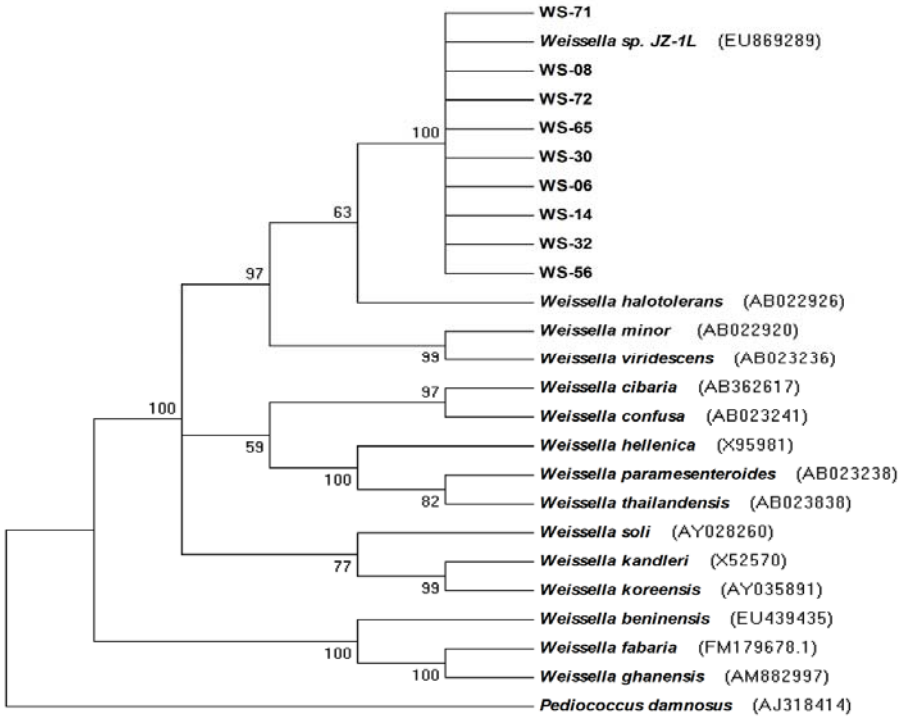

Figure 1 Phylogenetic neighbour-joining tree, based on 16S rRNA gene sequences, showing the phylogenetic relationships of Brazilian strains (WS-06, WS-08, WS-14, WS-30, WS-32, WS-56, WS-65, WS-71 and WS-72) with respect to members of the genus *Weissella*. Bootstrap percentages (based on 1000 replications) are shown at branch points

3.4. NIR analysis and epidemiological cut-offs

The application of NRI analysis permitted the estimate of mean and standard deviation for susceptible strains against four of five antibiotics tested. The inhibition zones for the five antibiomicrobials are presented in supplementary table 1. All 77 isolates were resistant to sulfonamide. For FLO, the 77 strains

presented zones between 18 mm and 31 mm, and might represent just one modal group. NIR analysis of these data resulted in an epidemiological cut-off value for fully susceptible WT (CO_{WT}) strains of ≥ 16 mm, with mean zone size (MZS) of 24.4 mm and standard deviation (SD) of 3.1. Therefore, all isolates were classified as WT. CO_{WT} values for ERY, OXY and NOR were calculated to be of ≥ 21 mm (MZS: 28.4 mm; SD: 2.6 mm; 76 WT strains), ≥ 10 mm (MZS: 15.8 mm; SD: 2.1 mm; 75 WT strains) and ≥ 7 mm (MZS: 13.3 mm; SD: 2.3 mm; 74 WT strains), respectively. The use of these limit values allowed the classification of one NWT strain for ERY (WS-70, 20 mm), two for OXY (WS-09, ≤ 6 mm; and WS-66, 8 mm) and three for NOR (WS-09, WS-67 and WS-71, all with inhibition zone ≤ 6 mm). There was an overall similarity in distribution data for the three agents, with at least 96% of strains classified as WT and belonged in one modal group. Except to sulfonamide (since all isolates were resistant), only the strain WS-09 presented to be resistant to more than one antibiotic (OXY and NOR).

3.5. Challenge study and bacteriology

The disease was successfully reproduced in experimental conditions by the different tested via and in both fish species. Neither clinical signs nor mortalities were observed in the fish of control groups (E, F and II) during the experimental period. The results are presented in Table 1. The main clinical signs observed in diseased rainbow trout were initially anorexia and lethargy followed by ascite, exophthalmia, erratic swimming, rectal prolapse, hyperemia and hemorrhage in the eye, pectoral fin, lateral fin, and anal region. Intraperitoneally challenged fish with high (Group B) and low (Group A) doses presented first clinical signs 22 and 48 hours post-infection (h.p.i.), respectively; the mortalities were verified 48 h.p.i. and at 16° day, respectively. The group C (immersion) presented initial clinical signs 24 h.p.i., and the only dead 48 h.p.i. In the co-habitation assay (Group D), the two fish infected by intraperitoneal injection presented signs of

sick 22 h.p.i., and die 16 and 48 hours after introduction in the cage. The healthy fish of Group D when co-habited with diseased fish without direct contact were infected by bacteria. In those, first clinical signs were observed three days after cohabitation, and mortalities occurred at sixth, eighth and 14th day. Episodes of clinical signs manifestation followed by recovery of fish were verified during experimental period. It was observed mainly in fish of groups which presented low mortality rates (A and C). Positive bacterial isolation was obtained from samples of the different organs of all rainbow trout that dead and those that showed clinical signs and recovery. Darkness, anorexia, lethargy and exophtalmia (only one fish) were the clinical signs observed in ill Nile tilapia fingerlings. The asymptomatic tilapia presented negative results in bacteriology, in contrast to positive reisolation of bacteria in the three diseased fish.

Bacterial populations above 10^4 cfu g^{-1} were verified in the different tissues of dead and recovered rainbow trout and in dead Nile tilapia fingerlings. No direct relation was observed between the average bacterial load in the organs (brain, kidney and intestine) and mortalities (Table 1). Until 21th day after infection, challenged rainbow trout (recovered fish) presented high bacterial load in the brain, kidney and intestine (Table 1).

3.6. Pathological evaluation

The most consistently observed microscopical changes in infected fish that died during experimental period were severe septicemia. Inflammatory mononuclear infiltrate were predominantly localized in the brain, heart and retrobulbar region of eyes. Multifocal necrosis in brain and heart were verified mainly in chronic infected and recovered fish. There was no relation between frequency and intensity of histopathological alterations and the different infection via tested. No microscopical changes were observed in the fish from the control groups.

Table 1 Results of mortality observed in the different experimentally challenged groups according to fish species, infection route and dosage, as well as the bacterial load in different tissues of diseased, recovered (got back to infection) and healthy fish

Species	Group	Infection Route	Dosage (cfu)	Mort. (%)	Recovered Fish‡ (%)	Healthy Fish+ (%)	Average Bacterial Load in Tissues (cfu g $^{-1}$)					
							Dead			Recovered/Healthy		
							Brain	Kidney	Intest.	Brain	Kidney	Intest.
Oncorhynchus	A	i.p.	3.4 x10^4*	17	83	0	2.2 x 10^7	6.8 x 10^4	2.8 x 10^5	1.1 x 10^5	5.75 x 10^6	LBC
Mykiss	B	i.p.	2 x10^8*	100	0	0	1.81 x10^7	1.7 x 10^7	4.48 x 10^6	-	-	-
	C	Immersion	3 x10^8**	17	83	0	3.5 x 10^8	6 x 10^7	2.02 x 10^6	2.26 x 10^7	5 x 10^4	3.3 x 10^7
	D	Cohabitation	1.6 x $10^{8\dagger}$	100	0	0	9 x 10^7	2.62 x 10^8	3.4 x 10^6	-	-	-
	E	i.p	PBS	0	0	100	-	-	-	0	0	0
	F	Immersion	PBS	0	0	100	-	-	-	0	0	0
Oreochromis niloticus	I	i.p.	1 x 10^9*	50	0	50	3.74 x 10^6	2.23 x 10^7	6.7 x 10^7	0	0	0
	II	i.p.	PBS	0	0	100	-	-	-	0	0	0

-i.p.: intraperitoneal; *cfu fish^{-1}; **cfu mL of water; † dosage administered in the two fish i.p. challenged and after put in the cage inside the aquarium; PBS: phosphate buffered saline;‡fish clinically affected by disease which recovered from infection and did not die; $^{+}$ healthy fish that did not present clinical signs and negative results were obtained in bacteriological evaluation; LBC: low bacterial counting (< 10^1)

4. Discussion

The genus *Weissella* is comprised by several bacterial species generally found in fermented beverages, foods and animal microbiota (Jang et al., 2002). Recently associated with infectious in human and animals, the first report of *Weissella* sp. infection in fish was performed in 2009 (Liu et al., 2009). Since then, there is no additional description of diseases caused by members of this genus in aquatic animals. During the period of March 2008 to March 2009, three outbreaks of hemorrhagic septicemia caused by *Weissella* sp. were recognized in commercial farms of rainbow trout, located in distinct Brazilian states. This is the first report of multiple cases of *Weissella* sp. infection in fish farms and outside of China.

During the outbreaks the clinical presentation of disease was similar to previously described in rainbow trout facilities in Asia (Liu et al., 2009). However, higher mortality rates were observed. The main predisposal factor associated with the disease verified was the elevation of water temperature (17°C or more). Although firstly observed in adult fish, in field conditions *Weissella* sp. can also cause disease in rainbow trout fry and fingerlings. Therefore, the disease is a potential problem for hatcheries and commercial farms.

The Brazilian isolates showed a homogeneous phenotypic pattern, but, some discrepancies were observed when compared to biochemical profiles of the six Chinese *Weissella* sp. strains. Those presented growth at 45°C, variable hydrolysis of arginine and positive esculin hydrolysis, in contrast to verified for the previously isolated and characterized fish isolates (Liu et al., 2009). Traditionally, the identification of lactic acid bacteria is performed using phenotypic criteria. But, the use of this classical methodology does not allow the unequivocal discrimination of new isolates of *Weissella* (Schillinger et al., 2008). To avoid misidentification, the isolates were submitted to molecular analysis. *Weissella* genus-specific PCR confirmed the genus, and 16S rRNA

sequencing followed by BLAST analysis demonstrated that Brazilian isolates shown high similarity degree with the sequences of *Weissella* sp. isolated from diseased rainbow trout (Liu et al., 2009). In the phylogenetic tree based on 16S rRNA sequences, the sequences of nine *Weissella* sp. isolates from Brazil formed a single cluster with Chinese isolates (100% of bootstrap percentage), implying that those strains might be the same bacterial species. In addition, these *Weissella* sp. isolates were belonged in a phylogenetic lineage closely related to *W. halotolerans*. However, based on phenotypic and molecular results, there are no suitable data to classify them as the same bacterial species (Liu et al., 2009). Additional studies have to be performed to characterize at species level the *Weissella* sp. associated with infections in fish.

The disk diffusion test continues to be the most versatile, broadly accurate and reproducible antibiotic sensibility test used in laboratories of clinical microbiology (Kronvall, 2003). However, several problems with data interpretation have been verified (Smith and Christofilogiannis, 2007). A unified basis for improved interpretative criteria currently has been investigated; it involves various attempts to generate valid epidemiological cut-off values. This allows the characterization of the isolates in wild-type and non-wild type, according the antibiotic resistance pattern (Smith et al., 2009). Recently, a method called normalised resistance interpretation (NRI) has been developed (Kronvall, 2003) and used to determine epidemiological cut-offs for fish bacterial pathogens (Smith and Christofilogiannis, 2007; Smith et al., 2009). Previous reports determined high level of antibiotic resistance of *Weissella* sp. strains isolated from diseased rainbow trout (Liu et al., 2009). However, the methodology and criteria used to determine the resistance and sensitivity of the isolates were not clear. The NRI analysis performed for FLO, ERY, NOR, OXY and SUL resistance data of 77 Brazilian isolates permitted the characterization of the bacterial populations. Additionally to be recognized as vancomycin-

resistant bacterium, the members of *Weissella* genus seem to be naturally resistant to sulfonamide, according to verified here and early (Olano et al., 2001; Liu et al., 2009). The results of NIR analysis for the four antibiotics allowed the classification of the majority of strains as WT, excepting one isolate for ERY, two for OXY and three for NOR. These were classified as NWT, and should be considered as manifesting resistance (Smith and Christofilogiannis, 2007). The NRI analysis demonstrated to be an applicable method for detection of *Weissella* sp. strains with reduction in sensibility or resistance to the antibiotics.

The disease was successfully reproduced in lab conditions, fulfilling the Koch's postulate. Furthermore, some advices about infectious routes of bacteria were obtained, since clinical disease caused *Weissella* sp. could be promoted in rainbow trout fingerlings by non-invasive via. The bath challenge demonstrated that the bacteria can actively infect health fish when they are immersed in water containing viable microorganisms. Moreover, cohabitation assay showed that this pathogen can be transmitted to diseased for health fish through the water without direct contact between then animal. High bacterial counting was observed in the different organs of challenged fish independent of infectious route, innolucum concentration, and time after infection. The data presented here shown that bacteria are able to infect, proliferate, survive, and can be transmitted for health fish under normal conditions. These results argue that *Weissella* sp. is a specialized pathogen not an opportunistic one as suggested previously (Liu et al., 2009). In addition, the characteristics presented above could explain the fast dissemination of disease in the farms as observed during the outbreaks (data not shown). Similar of reported for crucian carp (*Carassius auratus*) (Liu et al., 2009), in experimental conditions Nile tilapia fingerlings were susceptible to disease, showing that bacteria could have a larger broad of susceptive hosts.

In conclusion, we described the occurrence of multiple cases of outbreaks caused by *Weissella* sp. in commercial rainbow trout farms outside of China.

NRI analysis showed to be a valuable method to determine antibiotic resistance profile of these bacteria. Additionally, the disease could be reproduced in experimental conditions by non-invasive via and we demonstrated that bacteria can be transmitted through the water.

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Competing interest statement

The authors declare no competing financial interests.

4 CONCLUSÃO

Weissella sp. foi o agente etiológico dos três surtos de septicemia hemorrágica acompanhados nas diferentes truticulturas brasileiras. A doença foi reproduzida em condições experimentais por todas as rotas testadas, inclusive vias não invasivas. A transmissão do patógeno, de um peixe para outro, pode ocorrer de forma indireta via água.

O perfil de resistência dos isolados de *Weissella* sp. frente à quatro antimicrobianos foi determinado pela análise de INR.

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ANEXO

Supplementary Table Results of antimicrobial susceptibility test of *Weissella* sp. strains isolated from *Oncorhynchus mykiss*, in different stage life, used in this study

Isolation					Antimicrobial susceptibility testing [b]				
Strain	Life	Date	Farm [a]	Source	Florfenicol	Eritromycin	Oxytetracyclin	Norfloxacin	Sulfonamide
WS-01	Adult	05/05/08	A	Brain	28	32	19	13	≤ 6
WS-02	Adult	05/05/08	A	Kidney	28	32	17	14	≤ 6
WS-03	Adult	05/05/08	A	Kidney	23	27	13	12	≤ 6
WS-04	Adult	05/05/08	A	Ascitic fluid	24	30	15	11	≤ 6
WS-05	Adult	05/05/08	A	Liver	23	27	17	15	≤ 6
WS-06	Adult	05/05/08	A	Kidney	23	29	16	12	≤ 6
WS-07	Adult	05/05/08	A	Liver	26	28	16	11	≤ 6
WS-08	Adult	05/05/08	A	Brain	24	26	14	12	≤ 6
WS-09	Adult	05/05/08	A	Kidney	26	27	R	0	≤ 6
WS-10	Adult	05/05/08	A	Liver	31	35	21	12	≤ 6
WS-11	Adult	05/05/08	A	Brain	25	29	16	13	≤ 6
WS-12	Adult	05/05/08	A	Kidney	26	29	14	12	≤ 6
WS-13	Adult	05/05/08	A	Ascitic fluid	28	31	15	12	≤ 6
WS-14	Adult	05/05/08	A	Brain	22	25	13	13	≤ 6
WS-15	Adult	05/05/08	A	Kidney	28	33	16	13	≤ 6

Supplementary Table, continuation

WS-16	Adult	05/05/08	A	Liver	26	28	15	12	≤ 6
WS-17	Adult	05/05/08	A	Brain	28	31	18	11	≤ 6
WS-18	Adult	05/05/08	A	Kidney	29	33	18	13	≤ 6
WS-19	Adult	05/05/08	A	Liver	28	29	18	12	≤ 6
WS-20	Adult	05/05/08	A	Brain	30	34	22	12	≤ 6
WS-21	Adult	05/05/08	A	Kidney	25	25	17	11	≤ 6
WS-22	Adult	05/05/08	A	Liver	25	27	16	13	≤ 6
WS-23	Adult	05/05/08	A	Brain	23	25	17	11	≤ 6
WS-24	Adult	05/05/08	A	Kidney	24	26	16	12	≤ 6
WS-25	Adult	05/05/08	A	Liver	22	23	17	12	≤ 6
WS-26	Adult	05/05/08	A	Eye	26	29	19	10	≤ 6
WS-27	Adult	05/05/08	A	Ascitic fluid	24	28	17	13	≤ 6
WS-28	Adult	05/05/08	A	Brain	23	26	18	12	≤ 6
WS-29	Adult	05/05/08	A	Kidney	28	29	21	12	≤ 6
WS-30	Adult	05/05/08	A	Eye	27	29	20	11	≤ 6
WS-31	Fingerli	16/03/09	B	Brain	27	30	17	12	≤ 6
WS-32	Fingerli	16/03/09	B	Brain	25	24	15	16	≤ 6
WS-33	Fingerli	16/03/09	B	Kidney	27	30	16	19	≤ 6
WS-34	Fingerli	16/03/09	B	Brain	27	31	17	14	≤ 6

Supplementary Table, continuation

WS-35	Fingerli	16/03/09	B	Kidney	25	31	18	15	≤ 6
WS-36	Fingerli	16/03/09	B	Brain	27	32	17	14	≤ 6
WS-37	Fingerli	16/03/09	B	Kidney	25	31	16	16	≤ 6
WS-38	Fingerli	16/03/09	B	Brain	28	29	16	17	≤ 6
WS-39	Fingerli	16/03/09	B	Kidney	25	28	17	10	≤ 6
WS-40	Fingerli	16/03/09	B	Brain	24	29	17	14	≤ 6
WS-41	Fingerli	16/03/09	B	Kidney	24	29	17	15	≤ 6
WS-42	Fingerli	16/03/09	B	Brain	24	27	15	16	≤ 6
WS-43	Fingerli	16/03/09	B	Kidney	25	29	16	15	≤ 6
WS-44	Fingerli	16/03/09	B	Brain	22	28	13	12	≤ 6
WS-45	Fingerli	16/03/09	B	Kidney	24	28	17	19	≤ 6
WS-46	Fingerli	16/03/09	B	Brain	26	31	18	14	≤ 6
WS-47	Fingerli	16/03/09	B	Kidney	23	28	14	11	≤ 6
WS-48	Fingerli	16/03/09	B	Brain	25	27	14	12	≤ 6
WS-49	Fingerli	16/03/09	B	Kidney	22	24	13	11	≤ 6
WS-50	Fingerli	16/03/09	B	Brain	25	29	16	12	≤ 6
WS-51	Fingerli	16/03/09	B	Kidney	23	30	14	11	≤ 6
WS-52	Juvenile	16/03/09	B	Brain	22	28	16	13	≤ 6
WS-53	Juvenile	16/03/09	B	Kidney	23	25	17	12	≤ 6

Supplementary Table, continuation

WS-54	Juvenile	16/03/09	B	Brain	23	28	16	14	≤ 6
WS-55	Juvenile	16/03/09	B	Kidney	23	28	15	14	≤ 6
WS-56	Juvenile	16/03/09	B	Brain	24	29	17	14	≤ 6
WS-57	Juvenile	16/03/09	B	Kidney	23	29	17	16	≤ 6
WS-58	Juvenile	16/03/09	B	Brain	23	26	14	12	≤ 6
WS-59	Juvenile	16/03/09	B	Kidney	22	24	12	13	≤ 6
WS-60	Juvenile	16/03/09	B	Brain	24	27	15	15	≤ 6
WS-61	Juvenile	16/03/09	B	Kidney	24	24	13	16	≤ 6
WS-62	Juvenile	16/03/09	B	Brain	25	29	13	17	≤ 6
WS-63	Juvenile	16/03/09	B	Kidney	23	26	13	14	≤ 6
WS-64	Juvenile	16/03/09	B	Brain	22	25	13	13	≤ 6
WS-65	Juvenile	16/03/09	B	Kidney	25	26	15	14	≤ 6
WS-66	Adult	16/03/09	B	Brain	20	24	8	15	≤ 6
WS-67	Adult	16/03/09	B	Kidney	25	28	13	0	≤ 6
WS-68	Adult	16/03/09	B	Brain	26	30	14	12	≤ 6
WS-69	Adult	16/03/09	B	Kidney	18	23	11	13	≤ 6
WS-60	Adult	16/03/09	B	Kidney	19	20	12	12	≤ 6
WS-71	Adult	16/03/09	B	Brain	22	24	12	0	≤ 6
WS-72	Adult	16/03/09	B	Kidney	23	27	14	12	≤ 6

Supplementary Table, continuation

WS-73	Adult	16/03/09	B	Brain	22	24	13	13	≤ 6
WS-74	Adult	17/02/09	C	Brain	18	24	12	13	≤ 6
WS-75	Adult	17/02/09	C	Liver	23	25	14	12	≤ 6
WS-76	Adult	17/02/09	C	Kidney	21	23	14	11	≤ 6
WS-77	Adult	17/02/09	C	Liver	19	24	13	12	≤ 6

a The location of the farms in Brazilian states are presented follow: RJ – Rio de Janeiro (A); SP – São Paulo (B); MG - Minas Gerais (C);
b Measure of the diameter of the inhibition zone, in millimeter, found in the disc diffusion assay.