HUMBERTO MOREIRA HUNGARO

ATIVIDADE DE EXTRATOS VEGETAIS SOBRE SISTEMA DE EFLUXO MULTIDROGAS EM *Campylobacter* spp. MULTIRRESISTENTES A ANTIMICROBIANOS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, para obtenção do título de *Doctor Scientiae*.

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APROVADA: 20 de fevereiro de 2014.

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A Deus, meus pais, meus irmãos, a
Brenda e
aos meus amigos ...
companheiros de todas as horas

DEDICO

"Um pouco de ciência nos afasta de
Deus. Muita, nos aproxima" (Louis Pasteur).

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RESUMO

HUNGARO, Humberto Moreira, D.Sc., Universidade Federal de Viçosa, fevereiro de 2014. **Atividade de extratos vegetais sobre sistema de efluxo multidrogas em *Campylobacter* spp. multirresistentes a antimicrobianos.** Orientador: Regina Célia Santos Mendonça. Coorientadores: Maria Aparecida Scatamburlo Moreira e João Paulo Viana Leite.

A elevada ocorrência de *Campylobacter* spp. em carne e subprodutos de frango e sua crescente resistência a antimicrobianos têm preocupado as autoridades sanitárias em todo o mundo. Os objetivos deste estudo foram avaliar a contaminação de carcaças de frango por *Campylobacter* spp. e a resistência a antimicrobianos dos isolados; bem como investigar a capacidade de extratos vegetais em modular a atividade de antimicrobianos e de inibir os sistemas de efluxo expressos por este micro-organismo. Noventa e cinco carcaças de frangos abatidos em Minas Gerais – Brasil foram analisadas por meio da técnica de Número Mais Provável (NMP) com confirmação pela reação de polimerase em cadeia (PCR). Os isolados foram submetidos ao teste de resistência a antimicrobianos por disco de difusão em ágar, e os principais genes e mecanismos de resistência foram investigados. Vinte extratos vegetais etanólicos foram avaliados quanto às atividades antimicrobiana, moduladora de resistência e de acumulação de brometo de etídeo. As principais classes de metabólitos secundários de extratos vegetais com maior atividade biológica foram analisadas por cromatografia líquida de alta eficiência (HPLC). *Campylobacter* spp. foi identificado em 16,8 % das amostras, as contagens variaram entre 60,0 - 184 NMP/carcaça, e a espécie *C. jejuni* foi prevalente (93.8 %). Os isolados foram resistentes a no mínimo cinco (31,2 %) dos antimicrobianos testados e maior resistência foi observada para cefalosporinas (75 - 100 %), quinolonas (95 %), tetraciclina (50 %) e ampicilina (45 %). A resistência a ciprofloxacina foi associada a mutações no gene *gyrA*, enquanto que 40 % da resistência à tetraciclina foi relacionada à presença do gene $tet_{(O)}$. O gene bla_{OXA-61}, que codifica uma β-lactamase, foi encontrado em isolados resistentes e suscetíveis a ampicilina. A maioria dos isolados apresentou os três genes do sistema de efluxo CmeABC. O uso de fenilalanina-arginil-naftilamida (PAβN), um inibidor de sistemas de efluxo, diminuiu a MIC de ciprofloxacina, tetraciclina, ampicilina e sais biliares entre 2 e 128 vezes, o que

confirmou a atividade deste mecanismo de resistência. Os extratos vegetais apresentaram fraca atividade antimicrobiana em comparação com os antibióticos, com a MIC variando entre 0,1 e >16,38 mg/mL. Entretanto, a maioria deles apresentou sinergismo em combinação com sais biliares quando utilizados em concentrações subinibitórias, com diminuição da MIC destes antimicrobianos entre 2 e 16 vezes. Observou-se que dez extratos vegetais aumentaram a acumulação de brometo de etídeo por *Campylobacter* spp., o que indica a inibição de sistemas de efluxo. Os extratos de *Alpinia zerumbet* e *Vernonia polyanthes* apresentaram maior efeito sinérgico com sais biliares, enquanto que os extratos de *Cyperus rotundus* e *Struthanthus flexicaulis* foram mais eficazes na inibição dos sistemas de efluxo. Grande variedade de compostos fenólicos, principalmente flavonóides, foi observada nestes extratos vegetais, os quais podem estar envolvidos nas atividades biológicas. Apesar do baixo número de carcaças de frango positivas para *Campylobacter* spp. observado neste estudo, a identificação de altos níveis de resistência a antimicrobianos é um importante problema e enfatiza a necessidade de monitoramento contínuo e de novas formas de controle deste patógeno na cadeia produtiva de aves. Os resultados aqui reportados sugerem que extratos vegetais podem ser utilizados para controlar *Campylobacter* spp. em carne de frango e subprodutos por meio da redução da colonização das aves. Contudo, existe a necessidade de trabalhos futuros para caracterização mais detalhada dos extratos vegetais, isolamento e identificação dos princípios ativos, bem como estudos para avaliar sua atividade *in vivo*.

ABSTRACT

HUNGARO, Humberto Moreira, D.Sc., Universidade Federal de Viçosa, February, 2014. **Activity of plant extracts on multidrug efflux systems from antimicrobial multi-resistant *Campylobacter* spp.** Adviser: Regina Célia Santos Mendonça. Co-advisers: Maria Aparecida Scatamburlo Moreira and João Paulo Viana Leite.

The high occurrence of *Campylobacter* spp. in chicken meat and by-products and their increasing antimicrobial resistance have worried public health authorities worldwide. The objectives of this study were to evaluate the *Campylobacter* contamination in chicken carcasses and antimicrobial resistance of the isolates, as well as to investigate the resistance-modifying and efflux inhibitory activities of plant extract against this microorganism. A total of 95 chicken carcasses from slaughterhouses from Minas Gerais – Brazil were analyzed by most probable number method (MPN) associated with subsequent confirmation by polymerase chain reaction (PCR). The isolates were screened for antimicrobial resistance by agar diffusion disk method, and the main genes and mechanisms of resistance were investigated. Twenty ethanolic extracts of plants were tested for antimicrobial, resistance-modifying and accumulation of ethidium bromide activities. The major classes of secondary metabolites in the plant extracts showing biological activities were analyzed by high performance liquid chromatography (HPLC). *Campylobacter* spp. was found in 16.8% of samples with microbial load ranging from 60.0 to 184 MPN/carcass, and *C. jejuni* was the species most prevalent (93.8 %). The isolates were resistant to at least 5 (31.2 %) of the antimicrobial agents tested, and high resistance rates were observed for cephalosporins (75 - 100 %), quinolones (95 %), tetracycline (50 %) and ampicillin (45 %). Ciprofloxacin resistance proved to be caused by mutation in the *gyrA* gene, whereas 40 % of the tetracycline resistance was explained by presence of the $tet_{(O)}$ gene. The bla_{OXA-61} gene, which encodes for a β-lactamase, was found in both resistant and susceptible isolates for ampicillin. Almost all isolates showed the three genes required to synthesize the CmeABC efflux system. The use of phenylalanine-arginine β-naphthylamide (PAβN), a well-known efflux pump inhibitor, decreased the MIC of ciprofloxacin, tetracycline, ampicillin and bile salts by 2 to 8 fold confirming the activity of this resistance mechanism. These extracts showed weak antimicrobial activity

comparing to antibiotics, with MIC values ranging from 0.1 to >16.38 mg/mL. However, most of them showed synergistic effects on combining with bile salts when used at subinhibitory concentration, decreasing 2 to 16 fold the MIC values of these antimicrobials. We observed that ten plant extracts increased the accumulation of ethidium bromide in the *Campylobacter* cells, suggesting inhibition of efflux systems. The extracts of *Alpinia zerumbet* and *Vernonia polyanthes* showed greater synergistic effect on combining with bile salts, whereas extracts of *Cyperus rotundus* and *Struthanthus flexicaulis* were more effective in inhibiting the efflux systems. Great variety of complex phenolic compounds, including flavonoids, was observed in these plant extracts, which may be involved in the biological activities. Despite low number of chicken carcasses positive for *Campylobacter* spp. observed in this study, identification of high levels of antimicrobial resistance is an important problem and emphasizes the need for continuous monitoring and new ways to control this pathogen in the poultry production chain. The results reported here suggest that plant extracts may be used to control *Campylobacter* spp. in chicken and by-products by reducing of the poultry colonization. Nevertheless, future studies are required for more detailed characterization of plant extracts, isolation and identification of active principles, as well as to evaluate activity of these plant extracts *in vivo*.

1. INTRODUÇÃO GERAL

O gênero *Campylobacter* compreende um grupo de micro-organismos patogênicos que têm causado grandes prejuízos econômicos e problemas de saúde pública em todo o mundo. As espécies deste gênero têm sido relacionadas com pelo menos um tipo de doença em seres humanos e/ou animais domésticos. *C. jejuni* e *C. coli* são os principais agentes causadores da campilobacteriose, uma doença gastrointestinal, em geral auto-limitante, que pode evoluir para graves complicações e óbito. As aves são consideradas reservatórios naturais deste patógeno e o manuseio inadequado e consumo de carne de frango contaminada constituem importantes causas de infecção em humanos. A contaminação deste tipo de alimento pode ser um reflexo de falhas no manejo de criação das aves e em condições higiênicas no abate. *Campylobacter* spp. tem sido isolado com frequência de amostras de carcaça e produtos avícolas em todo o mundo. O monitoramento da contaminação por meio de metodologias adequadas é uma ferramenta importante para dimensionar o problema, que permite adotar medidas preventivas para reduzir este patógeno na cadeia produtiva de alimentos. Medidas destinadas a reduzir a colonização das aves e a contaminação destes alimentos podem contribuir de forma efetiva na redução do risco de campilobacteriose em humanos. Nos últimos anos, o crescente aumento de resistência a antimicrobianos em *Campylobacter* spp. isolados de alimentos, especialmente frangos e produtos relacionados tem preocupado as autoridades sanitárias. Entre os diferentes mecanismos envolvidos, o sistema de efluxo multidrogas CmeABC desempenha importante papel na multirresistência deste micro-organismo a diferentes antimicrobianos, incluindo antibióticos, sais biliares, detergentes e metais pesados. Este sistema de efluxo constitui um alvo interessante para restauração da suscetibilidade aos antimicrobianos e redução da colonização intestinal das aves por *Campylobacter* spp.. Extratos vegetais têm sido importantes fontes de pesquisa de inibidores de sistemas de efluxo multidrogas (EPIs). Diversos metabólitos secundários com esta capacidade têm sido identificados e estudados a partir dessas fontes com o objetivo de prevenir a resistência e restaurar a atividade de antimicrobianos sobre várias espécies de bactérias. No entanto, poucos estudos têm sido desenvolvidos em relação à pesquisa, identificação e caracterização de compostos inibidores de sistemas de efluxo de *Campylobacter* spp.e sua aplicação em avicultura.

2. REVISÃO DE LITERATURA

2.1 *Campylobacter* spp.

O gênero *Campylobacter* pertence à fa*mília Campylobacteriaceae* e é composto por 16 espécies e seis subespécies, que se apresentam como bastonetes curvos espiralados, Gram negativos, com comprimento de 0,5 a 5 µm e largura de 0,2 a 0,8 µm, não esporogênicos, e móveis por flagelos polares (GARRITY et al., 2005). Apresentam movimento característico em "serrote" ou "saca- rolha", que pode ser observado em microscópio de contraste de fase ou de campo escuro (GUERRY, 2007). Estas bactérias são de difícil cultivo em laboratório e diferem de outros patógenos de origem alimentar devido a sua natureza fastidiosa e exigências para condições de cultivo, necessitando de meios específicos, atmosfera com baixas concentrações de oxigênio (3 – 15 %) e altas concentrações de CO_2 (3 - 5%) (PARK, 2002). Condições adversas de crescimento tais como: escassez de nutrientes, exposição a oxigênio e variações de temperatura levam à mudança da morfologia espiralada para formas esféricas (cocóides) e a um estado Viável Não Cultivável - VNC (HUDOCK et al., 2005). A temperatura ótima de crescimento varia de 30 a 42 °C, e a maioria das espécies não cresce a 25 °C. Algumas espécies do gênero são classificadas como termofílicas ou termotolerantes e apresentam temperatura de crescimento mínima de 30 °C e máxima de 46 °C, com ótimo entre 42 - 43 °C (HUMPHREY et al., 2007). Embora sejam incapazes de se multiplicarem em temperaturas de refrigeração, estas bactérias são metabolicamente ativas abaixo da temperatura ótima de crescimento (PARK, 2002). O pH ótimo de crescimento é de 6,5 -7,5, resistem ao pH máximo de 9 a 9,5 e não crescem em meios com pH de 4,7, sendo inativadas em meios com pH abaixo de 4,0. São muito sensíveis à desidratação, não crescem em presença de 2 % de NaCl e atividade de água abaixo de 0,97 (SILVA et al., 2007).

Além disso, os micro-organismos deste gênero apresentam metabolismo energético oxidativo, não utilizam carboidratos como fonte de energia, que é sempre derivada da oxidação de aminoácidos ou ácidos intermediários do ciclo do ácido tricarboxílico (JEON et al., 2010). Embora *Campylobacter* ssp. seja sensível às condições ambientais e não possua muitos dos reguladores chave de resposta ao estresse encontrados em outros micro-organismos, apresenta uma grande flexibilidade na adaptação a diferentes ambientes, incluindo o trato

gastrointestinal (PARK, 2002; COOLS et al., 2005). Essas bactérias são consideradas importantes patógenos de distribuição mundial e a maioria das espécies tem sido relacionada com pelo menos uma doença em seres humanos e/ou animais domésticos (BUTZLER, 2004). *C. jejuni* e *C. coli* são as principais espécies associadas com campilobacteriose em humanos, uma doença gastrointestinal caracterizada por cólicas, náuseas, febre, dor abdominal, diarreia sanguinolenta ou síndrome de disenteria, que geralmente é auto-limitante, com duração de 2 a 10 dias (MOORE et al., 2005). A dose infecciosa é baixa, entre 400 a 500 células e depende do sistema imunológico do hospedeiro e de fatores de virulência da estirpe envolvida (BLACK et al., 1988; MOORE et al., 2005). Em alguns casos a doença pode evoluir para síndrome de Guillain-Barré, uma complicação auto-imune, que pode ter efeitos sobre o sistema nervoso periférico causando doenças respiratórias e neurológicas graves que conduzem ao óbito (ALLOS, 1997; ZILBAUER et al., 2008). As principais características de virulência de *Campylobacter* spp. incluem motilidade, quimiotaxia, capacidade de colonização, adesão às células intestinais, invasão e translocação epitelial, sobrevivência intracelular e produção de toxinas (KETLEY, 1997). A adesão, invasão e produção de citotoxina parecem ser fatores essenciais para ocorrência de infecção (WARDAK E SZYCH, 2006). Alguns genes, tais como o gene *fla* relacionado com motilidade, os genes *cdt* responsáveis pela produção de toxinas, o gene *cad*F associado com aderência e o gene *iam* relacionado com invasão, têm sido reconhecidos como responsáveis pela expressão de patogenicidade neste micro-organismo (ROZYNEK et al., 2005; WARDAK E SZYCH, 2006; RIZAL et al., 2010). Outro fator de virulência importante é a toxina de distensão citoletal (CDT), uma toxina que causa a parada do ciclo celular e morte em células eucarióticas sensíveis (HEYWOOD et al., 2005). A síndrome de Guillain-Barré e outras neuropatias têm sido associadas com a presença de lipo-oligossacarídeos (LOS) nas células de algumas estirpes (GODSCHALK et al., 2007). Variações em manifestações clínicas e sua evolução para quadros graves podem ser associadas a uma grande diversidade genética existente entre as estirpes de *Campylobacter* spp. de origem animal e humana (DASTI et al., 2010). Esta diversidade genética pode ser atribuída a sua capacidade de adquirir DNA exógeno e integrá-lo ao cromossomo por recombinação (RICHARDSON e PARK, 1997).

2.2 Epidemiologia e ocorrência de *Campylobacter* spp. em carne de frango

Campylobacter spp. está entre os patógenos de maior relevância em surtos de doenças de origem alimentar em todo o mundo. Nos EUA, em 2010, foram estimados 845.024 casos de doenças associadas a esta bactéria, com 8.463 hospitalizações e 76 mortes (CDC, 2013). Na Europa, *Campylobacter* spp. é uma importante causa de surtos de doenças gastrointestinais em humanos desde 2005, e com crescimento significativo nos últimos anos. Em 2011, foram confirmados 220.209 casos de campilobacteriose em países membros da União Europeia (EFSA, 2013). Entretanto, a verdadeira taxa de incidência de campilobacteriose pode ser ainda maior. De acordo com Olson et al. (2008) esse patógeno pode ser responsável por 400 - 500 milhões de casos de gastroenterite por ano em todo o mundo. No Brasil, assim como em outros países em desenvolvimento, poucos casos de doenças de origem alimentar são atribuídos a *Campylobacter* spp. (SVS, 2013), principalmente por falhas no processo de notificação e pela inexistência de programas nacionais de vigilância destinados ao acompanhamento de campilobacteriose.

O consumo de carne e produtos avícolas, leite cru, água contaminada e o contato com animais domésticos são considerados importantes fatores de risco de campilobacteriose em humanos (HUMPHREY et al., 2007). Entretanto, a maior parte dos surtos desta doença é atribuída ao manuseio e consumo de carne de frango e subprodutos (HUMPHREY et al., 2007; MULLNER et al., 2009). *Campylobacter* spp. tem sido isolado com frequência destes tipos de alimentos em todo o mundo. Nos EUA, estudos demonstraram que 60 a 80 % das carcaças de frango e 91 % dos produtos avícolas estavam contaminados com *Campylobacter* spp. (ZHAO et al., 2001; SON et al., 2007). Já no Canadá, Arsenault et al. (2007) observaram prevalência deste patógeno em 35,8 % das amostras. Em alguns países da Europa, a prevalência de *Campylobacter* spp. em carne de aves foi, em média, de 31,3 % em 2011, mas variações entre 3,2 e 84,6 % foram observadas (EFSA, 2013). Alta prevalência deste patógeno, acima de 60 %, também foi observada em carne de frango e produtos avícolas de países como Japão, Nova Zelândia, Coréia e Austrália (SCHERER et al., 2006b; WHYTE et al., 2006; SALLAM, 2007; POINTON et al., 2008). Na África do sul, 32,3 % das 99 amostras de frangos frescas e congeladas analisadas foram positivas para esse micro-organismo (VAN NIEROP et al., 2005). Apesar

da legislação brasileira não exigir a pesquisa de *Campylobacter* spp. em alimentos, estudos demonstraram que carne e produtos de aves produzidos e comercializados em diferentes estados brasileiros estavam contaminados com esse patógeno*,* com variações de prevalência entre 6,7 e 100 %. (ALVES et al., 2012; DE MOURA et al., 2013; GONÇALVES et al., 2013; OLIVEIRA e OLIVEIRA, 2013). Considerando que a presença dessa bactéria tem um grande impacto sobre saúde pública e no comércio de carne, é provável que seus padrões microbiológicos sejam definidos semelhantes àquelas para agentes patogênicos tais como, *Salmonella* spp. e *Listeria monocytogenes*, que exigem a ausência destes micro-organismos em 25 g de amostras, avaliada por meio de métodos de presença/ausência (BRASIL, 2001). Entretanto, a quantificação de *Campylobacter* spp. nas amostras também é importante para realização de estudos de avaliação de risco e representa o principal obstáculo para essa abordagem nos países em desenvolvimento (FAO/WHO, 2002). Nos últimos anos, muita atenção tem sido dada à enumeração de *Campylobacter* spp. em amostras de frango, a fim de se conhecer o nível de contaminação, os principais pontos de controle e estabelecer os riscos na cadeia produtiva (NAUTA et al., 2005; UYTTENDAELE et al., 2006). Grandes variações na contagem de *Campylobacter* spp. neste tipo de alimento têm sido observadas em diferentes estudos em todo o mundo. Por exemplo, em pesquisa realizada por Berrang et al. (2007) em 20 abatedouros dos EUA, a contaminação das carcaças com esse patógeno foi em média de 2,66 $\log_{10}$ UFC por mL de líquido de rinsagem. Enquanto Allen et al. (2007) encontraram maior contaminação das amostras no Reino Unido, com média de contagem de 5,3 $\log_{10}$ UFC e variação entre 1,3 e 8,0 $\log_{10}$ UFC. Altas contagens de *Campylobacter* spp. em carcaças de frango também foram encontradas na Nova Zelândia, Noruega e Chile, cujas variações estavam entre 2,6 a 7,7 $\log_{10}$ UFC/carcaça (JOHANNESSEN et al., 2007; CHRYSTAL et al., 2008; FIGUEROA et al., 2009). Em contrapartida, estudos realizados por Tang et al. (2010), Wong et al. (2007) e Scherer et al. (2006b), utilizando a metodologia de número mais provável, revelaram uma baixa contaminação das amostras, com variações de <100 MPN/g^{-1}, <0,3 a 10 MPN/g^{-1}, e <0,3 MPN/g^{-1}, respectivamente. Nos poucos estudos sobre quantificação de *Campylobacter* spp. em alimentos realizados no Brasil, Kuana et al. (2008a) observaram contaminações médias de carcaças de frango de 5,15 $\log_{10}$ UFC/carcaça, enquanto Oliveira e Oliveira

(2013) observaram valores menores, variando de 1,67 a 2,69 $\log_{10}$ UFC/carcaça. As variações observadas tanto nas taxas de prevalência quanto na contagem de *Campylobacter* spp. nestes tipos de amostra podem ser atribuídas a diferentes fatores tais como, os métodos de manejo de criação das aves, falhas em práticas higiênicas na cadeia produtiva, técnicas de amostragem e as diferenças entre os métodos analíticos (STERN e LINE, 1992; DASKALOV e MARAMSKI, 2012).

De forma geral, a alta correlação entre os produtos de aves e a ocorrência de infecções por *Campylobacter* spp. está relacionada com a rápida disseminação do micro-organismo na criação e alto grau de colonização das aves, em torno de 5 a 9 $\log_{10}$ UFC/g de conteúdo intestinal, com ausência de sintomas aparentes (ROSENQUIST et al., 2006; HORROCKS et al., 2009); contaminação das carcaças no abate, principalmente por contato com o conteúdo intestinal das aves durante o processo de evisceração (SMITH et al., 2007a; BOYSEN e ROSENQUIST, 2009), condições higiênico-sanitárias inadequadas e contaminação cruzada entre carne crua e cozida no preparo dos alimentos (TANG et al., 2011). O controle da campilobacteriose é, portanto, geralmente focado na redução da ocorrência de *Campylobacter* spp. em carne de frango. A maioria dos trabalhos sobre este tema tem enfatizado a necessidade de reduzir a prevalência e os números deste patógeno dentro da cadeia produtiva de aves. De fato, estima-se que uma redução de 2 $\log_{10}$ de *Campylobacter* spp. em fezes de aves pode causar uma redução de até 30 vezes na incidência de campilobacteriose em humanos (ROSENQUIST et al., 2003). Em estudo sobre avaliação de risco microbiológico quantitativo foi demonstrado que reduções de 60 %, 78 % e 97 % dos casos desta doença podem ser atingidas por meio da redução da contaminação de carcaças de frango em 1, 2 e 3 $\log_{10}$ UFC, respectivamente (MESSENS et al., 2007). Embora o controle da contaminação de carcaças de frango nos abatedouros seja possível, os procedimentos disponíveis para essa finalidade são limitados pela praticidade, legalidade ou aceitabilidade dos consumidores (HUE et al., 2010). Assim, as intervenções realizadas no início da cadeia produtiva para controlar e evitar a colonização das aves com *Campylobacter* spp. são fundamentais para redução da contaminação e consequente redução dos casos de campilobacteriose em humanos (HERMANS et al., 2011). Varias formas de redução desse patógeno na criação de aves tais como, uso de

probióticos, bacteriófagos, antimicrobianos naturais e outros compostos vêm sendo pesquisadas, especialmente após a eliminação do uso de antibióticos como promotores de crescimento (GANAN et al., 2012).

2.3 Metodologias analíticas

O isolamento e a enumeração de *Campylobacter* spp. em amostras de alimentos constituem desafios frequentes aos microbiologistas, pelo fato de serem micro-organismos fastidiosos, exigirem condições específicas para o crescimento e serem facilmente inibidos pela microbiota competidora (JASSON et al., 2009). A microbiota contaminante presente nos alimentos pode afetar a detecção de *Campylobacter* spp., principalmente, em amostras com baixo grau de contaminação (OAKLEY et al., 2012). A adição de um ou mais antibióticos aos meios de cultivo é uma alternativa para resolução deste problema, entretanto, reduz a possibilidade de recuperação do micro-organismo alvo (OYARZABAL et al., 2005). Os agentes seletivos mais comumente utilizados são cefoperazona, ciclohexamida, trimetoprima, rifampicina, vancomicina e polimixina B (CORRY et al., 1995). Entretanto, estudos têm demonstrado que micro-organismos presentes em carne de frango tais como, *Escherichia coli*, *Pseudomonas* spp., *Proteus* spp. e *Acinetobacter* sp. são resistentes a estes antibióticos, e podem crescer em meios de cultura seletivos interferindo na detecção e enumeração de *Campylobacter* spp. (HABIB et al., 2008; JASSON et al., 2009). Outras condições importantes para o cultivo deste micro-organismo são a temperatura de incubação, que pode variar entre 37°C e 42 °C e a atmosfera microaerófila com a presença de pequenas concentrações de oxigênio e gás carbônico (5 % O_2, 10 % CO_2 e 85 % N_2) (KIM et al., 2009; WILLIAMS et al., 2009). Em função da sensibilidade de *Campylobacter* spp. ao oxigênio, os meios de cultivo também são adicionados de sangue, carvão e/ou substâncias redutoras, capazes de neutralizar o efeito de compostos oxidantes tóxicos e favorecer o crescimento microbiano (CORRY et al., 1995). Os principais meios usados no cultivo de *Campylobacter* spp. são os caldos de enriquecimento *Brucella*, Bolton e Preston, e os ágares Campy-Cefex, Carvão Cefoperazona Desoxicolato modificado (mCCDA), Karmali, CampyFood ID (CFA), Campy-Line (CLA), Campy FDA, e Campy-CVA (CVA) (CORRY et al., 1995; OYARZABAL et al., 2005; HABIB et al., 2008; RODGERS et al., 2010). A maioria destes meios de cultivo não apresenta propriedades diferenciais que

favoreçam a distinção entre colônias típicas e atípicas, o que constitui um problema metodológico na detecção do patógeno (AHMED et al., 2012). Além disso, em função da sua motilidade, é comum que as colônias de *Campylobacter* spp. espalhem-se e aglomerem-se sobre a superfície do ágar, o que dificulta a quantificação com precisão (STERN, 2001).

Várias metodologias têm sido desenvolvidas e avaliadas para o isolamento e enumeração de *Campylobacter* spp. em amostras de alimentos, em virtude do grande número de meios de cultivo, e condições de incubação e preparo de amostras disponíveis. Os métodos preconizados pela norma ISO 10272:2006 são utilizados com frequência para amostras de carne de frango. A avaliação de presença/ausência por meio dessa metodologia consiste em uma etapa de enriquecimento seletivo em caldo Bolton, seguida de isolamento em dois meios sólidos, sendo mCCDA e um outro qualquer, confirmação morfológica das colônias ao microscópio e testes complementares. Já a enumeração é realizada por meio de plaqueamento direto em ágar mCCDA após a diluição da amostra, e posterior contagem e confirmação das colônias (ISO, 2006). Outras metodologias utilizadas para essas finalidades são recomendadas pelo Departamento de Agricultura dos EUA (USDA) e pelo *Food and Drug Administration* (FDA), as quais diferem entre si e das metodologias ISO 10272:2006 nas etapas de preparo da amostra, alíquotas analisadas, meio de cultivo seletivo (ágar Campy-Cefex e Abeyta-Hunt-Bark) e confirmação das colônias (USDA, 2013; HUNT et al., 2001). Outra opção analítica interessante é o método conhecido como "*Cape Town*" que explora a motilidade natural de *Campylobacter* spp., por meio da incorporação de uma membrana utilizada para filtrar a amostra sobre a superfície de meios de cultura, a qual atua como barreira seletiva contra os micro-organismos não-móveis e competidores (LE ROUX e LASTOVICA, 1998). Esta metodologia possibilita a eliminação dos antibióticos nos meios de cultivo, maior recuperação de espécies fastidiosas e sensíveis; entretanto, partículas do alimento podem reduzir a passagem da amostra através da membrana, e as bactérias móveis contaminantes podem interferir no isolamento do patógeno (LYNCH et al., 2011).

A técnica do número mais provável (NMP), também denominada técnica dos tubos múltiplos, é outra maneira muito utilizada pelos laboratórios de microbiologia de alimentos para estimar a contagem de alguns tipos de micro-organismos. A técnica de NMP é aplicável para detecção micro-organismos em

amostras pouco contaminadas por apresentar baixo limite de detecção, e foi utilizada em estudos para enumeração de *Campylobacter* spp. em amostras de alimentos e água (SAVILL et al., 2001; WONG et al., 2007; TAN et al., 2008). O princípio deste método baseia-se na distribuição de alíquotas da amostra em séries de tubos contendo caldo de enriquecimento, e posterior confirmação da presença de *Campylobacter* spp. por técnicas de cultivo tradicional ou métodos moleculares (SCHERER et al., 2006a; TANG et al., 2010).

Os métodos de identificação e enumeração de *Campylobacter* spp. baseados em cultivo microbiano são trabalhosos, apresentam falhas na detecção de algumas espécies, dependem de condições de microaerofilia e demandam tempo, entre 4 a 5 dias para identificação completa (COX et al., 2007). Por estas razões, tem aumentado o interesse pelo desenvolvimento de ferramentas moleculares que permitam a detecção rápida e inequívoca de *Campylobacter* spp. em alimentos. Alguns métodos moleculares foram desenvolvidos nos últimos anos para detecção desse patógeno e disponibilizados comercialmente, embora geralmente não sejam utilizados em testes de rotina em laboratórios (WILLIAMS et al., 2009). Os métodos baseados em reação de polimerase em cadeia (PCR) são direcionados à amplificação de pequenos fragmentos do genoma que podem ser visualizados por eletroforese em gel de agarose. Esses métodos podem ser utilizados para detectar gênero ou grupos de espécies, tais como *Campylobacter* termofílicos, ou diferenciar várias espécies de *Campylobacter* spp. presentes em uma mesma amostra (BONJOCH et al., 2010). Vários genes alvo tais como *omp*50, 16S rRNA, 23S rRNA, *hipO*, *mapA*, gene da aspartokinase, *cadF* e subunidades de oxidoredutase têm sido utilizados para identificação de *Campylobacter* spp. (ON e JORDAN, 2003; DEDIEU et al., 2004; NAYAK et al., 2005). Métodos baseados em PCR são rápidos, sensíveis, fáceis de utilizar, apresentam um custo relativo baixo, e têm o potencial de serem totalmente automatizados (WANG et al., 2002). Entretanto, existem desvantagens como a não distinção entre células viáveis e não viáveis, alguns componentes do meio de cultivo ou amostra podem inibir a enzima DNA polimerase, e os limites de detecção são altos (10^3 a 10^4 UFC/g), o que significa que as etapas de enriquecimento ainda são necessárias para amostras com baixo grau de contaminação (WISESSOMBAT et al., 2009; EL-ADAWY et al., 2012).

Alguns métodos baseados em reação antígeno/anticorpo (EIA) também são utilizados para detecção de *Campylobacter* spp. em diferentes tipos de amostras (BAILEY et al., 2008; LIU et al., 2009; CHON et al., 2011). Embora esses métodos sejam rápidos e automatizáveis, ainda existem algumas limitações quanto à especificidade relacionada com a alta reatividade cruzada dos anticorpos, baixa sensibilidade, e necessidade de equipamentos e reagentes específicos (TRAM et al., 2012).

A detecção e quantificação de *Campylobacter* spp. em carcaças de frango é uma ferramenta importante para dimensionar o problema, permitindo tomar medidas preventivas para reduzir esse patógeno na cadeia produtiva de alimentos e a identificação da fonte dos surtos associados com o consumo de carne de aves. Apesar de todos os esforços e variedade de opções analíticas, ainda não existem metodologias ou meios de cultivo considerados ideais para a recuperação e identificação de *Campylobacter* spp. em amostras de alimentos. A escolha de uma metodologia em particular é pautada no tipo de amostra, grau de contaminação esperado, microbiota competidora e objetivo da análise, e é fundamental para o sucesso do isolamento ou enumeração desse patógeno.

2.4 Resistência a antimicrobianos

A resistência a antimicrobianos em bactérias provenientes de alimentos de origem animal, incluindo *Campylobacter* spp., tornou-se nos últimos anos um importante problema de saúde pública. Muitas dessas bactérias são resistentes a mais de um tipo de antimicrobiano (multirresistentes – MDR), o que causa redução considerável do arsenal terapêutico e a necessidade constante de desenvolvimento de novas formas de tratamento das infecções (MOŽINA et al., 2011). Os principais fatores que influenciam a resistência bacteriana são o uso indiscriminado de antimicrobianos no tratamento de infecções em humanos e sua aplicação excessiva em medicina veterinária e como promotores de crescimento em produção animal (ALFREDSON e KOROLIK, 2007; MOR-MUR e YUSTE, 2010). A utilização desses agentes em aves pode causar a seleção de estirpes de *Campylobacter* resistentes e à sua transmissão para os seres humanos via alimentos contaminados (MAĆKIW et al., 2012). Na maioria dos casos a campilobacteriose é auto-limitante, mas algumas infecções graves ou crônicas requerem tratamento com

antimicrobianos, e o aumento da resistência a esses agentes dificulta as intervenções terapêuticas (ALFREDSON e KOROLIK, 2007; BLASER et al., 2008). Alto grau de resistência a antibióticos como quinolonas, tetraciclina e ampicilina em *Campylobacter* spp. isolados de frango e subprodutos tem sido relatado em vários estudos em todo o mundo. A resistência à tetraciclina e quinolonas em *C. jejuni* isolados de carne de frango em países membros da União Europeia em 2010 foi em média 22 % e 50 %, respectivamente. Enquanto para *C. coli* maiores valores foram reportados, 62 % para tetraciclinas e 72 % para quinolonas (EFSA, 2012). Nos EUA, em 2011, a resistência à tetracilina, e quinolonas em *C. jejuni* e *C. coli* isolados de carne de frango foi de 48 – 49 % e 18 – 22 %, respectivamente (NARMS, 2011). Em estudo com isolados de frango na Austrália, Obeng et al. (2012) observaram alta resistência a ampicilina (33,3 - 60,2 %) e a licomicina (51 - 100 %). Alta resistência à ampicilina também foi observada por Griggs et al. (2009) no Reino Unido, 67,4 % para *C. jejuni* e 47,5 % para *C. coli*. Em comparação com os antimicrobianos descritos anteriormente, a resistência à macrolídeos, especificamente a eritromicina é, em geral menor, entre 0 e 33 % na Europa (EFSA, 2012) e entre 0,7 e 6,9 % nos EUA (NARMS, 2011). Entretanto, os isolados resistentes a eritromicina são mais frequentes e, em geral multirresistentes (MOŽINA et al., 2011).

Três principais mecanismos estão envolvidos na resistência bacteriana a antimicrobianos: produção de enzimas que inativam o antibiótico; modificações ou alterações estruturais do sítio alvo; e alterações na membrana plasmática, que incluem modificações de permeabilidade e a expressão de transportadores (IOVINE, 2013). *Campylobacter* spp. apresenta vários desses mecanismos que lhe conferem resistência às principais classes de antimicrobianos incluindo macrolídeos, quinolonas, tetraciclinas, β-lactâmicos e aminoglicosídeos (WIECZOREK e OSEK, 2013). A resistência a macrolídeos e quinolonas em *Campylobacter* spp. é atribuída, principalmente, a mutações no domínio V do gene 23S rRNA e na subunidade *gyr*A da enzima DNA girase, respectivamente, que alteram o sítio alvo destes antimicrobianos (PIDDOCK et al., 2003; PAYOT et al., 2004). Em relação à tetraciclina, o principal mecanismo de resistência envolve a expressão de uma proteína de proteção ribossomal (PPR) codificada pelo gene $tet_{(O)}$, que está amplamente presente em *C. jejuni* e *C. coli*. Essa proteína reconhece uma abertura no sítio A do ribossomo e se liga

de tal forma a induzir uma alteração conformacional que resulta na liberação da molécula de tetraciclina ligada ao ribossomo (CONNELL et al., 2003). Já a resistência a β-lactâmicos é, em sua maioria, mediada por enzimas denominadas de β-lactamases que degradam a estrutura desses agentes (GRIGGS et al., 2009). Em geral, com exceção de alguns carbapenêmicos, a maioria das estirpes de *Campylobacter* é considerada resistente a β-lactâmicos, especialmente penicilinas e algumas cefalosporinas (WIECZOREK e OSEK, 2013).

A resistência a aminoglicosídeos é menos frequente em *Campylobacter* spp., mas também tem sido causada por enzimas modificadoras, geralmente codificadas por plasmídeos (TENOVER et al., 1992). Além dos mecanismos citados, a resistência a diferentes classes de antimicrobianos pode ser conferida por meio de sistemas de efluxo, que limitam o acesso desses agentes aos alvos devido ao bombeamento de forma ativa para fora da célula bacteriana, o que impede seu acúmulo intracelular necessário para a letalidade (KURINČIČ et al., 2012a). Os sistemas de efluxo em bactérias podem ser divididos em cinco grandes famílias, com base em homologia de sequência de aminoácidos: (1) subfamília de resistência a multidrogas (SMR), (2) transportadores de múltiplos antimicrobianos e extrusão de toxinas (MATE), (3) família de resistência-nodulação-divisão (RND), (4) superfamília dos facilitadores principais (MFS) e (5) superfamília de transportadores tipo ABC (PUTMAN et al., 2000; LI e NIKAIDO, 2004). Estas classes de sistemas de efluxo dependem de energia para o transporte ativo de substâncias, que pode ser proveniente de força protomotiva por meio de antiporte com H^+ (RND, SMR, e MFS) ou Na^+ (MATE) ou por hidrólise de ATP (ABC) (PIDDOCK, 2006; MISRA e BAVRO, 2009). Em *Campylobacter* spp. pelo menos 14 sistemas de efluxo estão presentes, incluindo três da família RND (CmeB, CmeD, e Cj1373), quatro da superfamília MFS, quatro da subfamília SMR e um da superfamília de transportadores ABC (ZHANG e PLUMMER, 2008). Entretanto, o sistema de efluxo CmeABC (RND) é o principal responsável pela resistência intrínseca dessa bactéria a macrolídeos, fluoroquinolonas, tetraciclinas, sais biliares, corantes e detergentes (LIN et al., 2002; GIBREEL et al., 2007). É constituído por três componentes, CmeB, que é a proteína localizada na membrana citoplasmática, CmeC, uma proteína que forma um canal na membrana externa, e CmeA, uma proteína de fusão periplasmática adaptadora

(LIN et al., 2002). Estes componentes do sistema de efluxo são codificados por um operon de três genes localizados no cromossomo e regulado por CmeR, um repressor transcricional (LIN et al., 2005).

Vários estudos demonstraram a contribuição significativa desse sistema de efluxo na resistência a antimicrobianos, e também sua atuação sinérgica com outros mecanismos de resistência à quinolonas, macrolídeos, tetraciclina e ampicilina presentes em *Campylobacter* spp. (LIN et al., 2002; PUMBWE e PIDDOCK, 2002; PAYOT et al., 2004; GIBREEL et al., 2007). Além disso, o sistema de efluxo CmeABC também tem papel chave na colonização de frangos por este patógeno, pois é responsável pelo aumento de resistência a sais biliares (LIN et al., 2003). Notavelmente, esse sistema de efluxo pode ser induzido por sais biliares, o que pode facilitar a adaptação de *Campylobacter* spp. rapidamente às condições de estresse e sua adesão aos sítios alvo intestinais. Os sais biliares interagem com o regulador transcricional CmeR, impedem sua ligação à região promotora e induzem a expressão do sistema de efluxo CmeABC (LIN et al., 2005).

Devido à contribuição evidente de sistemas de efluxo do tipo RND no aumento contínuo da multirresistência bacteriana, estes são considerados alvos terapêuticos chave no desenvolvimento de estratégias para restaurar a atividade de antimicrobianos e reduzir a capacidade das bactérias em colonizar o hospedeiro ou até mesmo de provocar infecções (LOMOVSKAYA e BOSTIAN, 2006; PAGÈS e AMARAL, 2009). Portanto, o sistema de efluxo CmeABC presente em *Campylobacter* spp. é um alvo interessante para o desenvolvimento de estratégias de intervenção contra infecções causadas por este patógeno em humanos e colonização de animais destinados à produção de alimentos.

2.6 Extratos vegetais moduladores de resistência

Plantas e compostos naturais sempre atraíram a atenção da comunidade científica e farmacêutica, como uma alternativa para a descoberta de novos antimicrobianos capazes de atuar sobre as bactérias MDR (SAVOIA, 2012). As plantas são ricas em uma grande variedade de metabólitos secundários, tais como taninos, terpenóides, alcalóides e flavonóides, que podem apresentar propriedades antimicrobianas ou sinergismo com agentes terapêuticos existentes e utilizados em clínica médica e veterinária (GIBBONS, 2005;

LEWIS e AUSUBEL, 2006). Já foi demonstrado que extratos brutos de algumas plantas medicinais e alguns de seus compostos purificados podem potencializar a atividade de antimicrobianos *in vitro* (LORENZI et al., 2009; OLAJUYIGBE e AFOLAYAN, 2012). Um dos fatores que contribuem para esta atividade de modulação é a inibição de sistemas de efluxo presentes nas bactérias. Um exemplo dessa capacidade pode ser observado na planta *Berberis* que produz o composto antimicrobiano berberina e um inibidor de efluxo (EPI), 5'-metoxihidnocarpina (5'-MHC), que atuam em sinergismo (STERMITZ et al., 2000). Nos últimos anos, extratos vegetais que atuam como inibidores de sistemas de efluxo têm sido estudados e caracterizados para aplicação em várias espécies de bactérias (Tabela 1). Porém, poucos estudos nesta linha têm sido desenvolvidos em relação à *Campylobacter* spp. multirresistentes a antimicrobianos (KLANČNIK et al., 2012a; KLANČNIK et al., 2012b; KURINČIČ et al., 2012b).

Os extratos vegetais, dependendo do solvente utilizado, podem apresentar composições complexas com estruturas químicas variadas (TIWARI et al., 2011), que dificultam a identificação de princípios ativos e mecanismos de inibição. A diversidade de estruturas químicas de compostos naturais que apresentam atividade sobre sistemas de efluxo sugere que diferentes mecanismos de atuação possam estar envolvidos na inibição do efluxo (STAVRI et al., 2007). Além disso, os mecanismos de inibição dependem do tipo de sistema de efluxo envolvido (MCKEEGAN et al., 2004). Os compostos com atividade sobre sistemas de efluxo do tipo RND tais como CmeABC de *Campylobacter* spp. podem atuar em diferentes sítios de inibição, que incluem alteração nas etapas de regulação, associação dos componentes, bloqueio da proteína de membrana externa, dissipação da energia, competição pelos sítios ativos das bombas e bloqueio do canal de transporte (PAGÈS e AMARAL, 2009). Poucos estudos encontrados na literatura descrevem os mecanismos de ação de compostos derivados de extratos vegetais (FADLI et al., 2011; FIAMEGOS et al., 2011; OJEDA-SANA et al., 2013). Alguns compostos químicos tais como, fenil-arginil-β-naftilamina (PAβN), carbonil cianeto m-clorofenilhidrazona (CCCP) e 1-(1-naftilmetil)-piperazina (NMP) têm capacidade de inibir os sistemas de efluxo presentes em várias bactérias e restaurar a suscetibilidade de diferentes classes de antimicrobianos (PAGES et al., 2010). Além disso, estes compostos são utilizados como inibidores de efluxo padrão

em estudos científicos, e alguns apresentam os mecanismos de ação elucidados (BOHNERT E KERN, 2005; LOMOVSKAYA et al., 2007). PAβN inibe o efluxo de antimicrobianos por competir com estes agentes pelos sítios específicos de ligação presentes na proteína de membrana plasmática de sistemas de efluxo responsável pelo bombeamento de compostos (LOMOVSKAYA e BOSTIAN, 2006). Modificações na estrutura deste EPI têm sido realizadas a fim de melhorar sua estabilidade em fluidos biológicos e os efeitos de inibição do efluxo (RENAU et al., 2001). Muitos inibidores competitivos como PAβN tem efeito limitado a apenas uma classe ou sub-grupo específico de antimicrobianos e sua baixa afinidade pelos sistemas de efluxo exige o uso de doses elevadas e tempo de exposição longo para obter eficiência (YU et al., 2003).

A principal vantagem deste tipo de inibidor de efluxo é que o desenvolvimento de resistência por meio de mutações nos sítios de ligação também pode afetar o transporte do antimicrobiano (POOLE e LOMOVSKAYA, 2006). CCCP é outro EPI muito utilizado em laboratório para suprimir o efluxo de várias moléculas, atua por meio da dissipação da força protomotiva da membrana citoplasmática bacteriana e impede o transporte ativo por falta de energia (PAGES et al., 2005). NMP e compostos relacionados são capazes de inibir sistemas de efluxo do tipo RND de várias bactérias e sua atividade é modulada por substituições halogênicas no anel de benzeno e pelo comprimento da cadeia carbônica entre o anel benzênico e o anel piperazina, mas o mecanismo de ação destes compostos ainda não foi elucidado (BOLLA et al., 2011). Alguns fatores como a toxicidade, estabilidade, seletividade e biodisponibilidade das moléculas de inibidores de efluxo disponíveis ainda limitam a sua utilização clínica (LOMOVSKAYA e BOSTIAN, 2006; PAGÈS e AMARAL, 2009). Até o presente momento, nenhum inibidor de sistema de efluxo, seja ele sintético ou natural, foi registrado para ser comercializado e aplicado no tratamento de infecções bacterianas em medicina humana ou veterinária. Com a restrição do uso de antibióticos como promotores de crescimento na produção animal, muitos estudos têm sido realizados com o objetivo de utilizar compostos antimicrobianos naturais com essa finalidade, inclusive na cadeia produtiva de aves (GANAN et al., 2012; VENKITANARAYANAN et al., 2013).

Tabela 1. Plantas e compostos derivados de plantas com atividade modificadora de resistência e de inibição de sistemas de efluxo de bactérias.

Plantas	Micro-organismos	Material vegetal	Solvente/Tipo de extrato	Referências
Aframomum melegueta	*Mycobacterium smegmatis*	Sementes	Hexano	Gröblacher et al. (2012)
Alpinia galanga	*Mycobacterium smegmatis*	Rizomas	Hexano e clorofórmio	Roy et al. (2012)
Alpinia katsumadai	*Campylobacter* spp.	Sementes	EtOH, CH_2Cl_2, MetOH e hexano	Klancnik et al. (2012a)
Alpinia officinarum	*S. aureus*	Rizomas	Composto purificado	Eumkeb et al. (2010)
Berberis aetnensis	*S. aureus*	Raízes e folhas	EtOH, $CHCl_3$ e éter etílico	Musumeci et al. (2003)
Camellia sinensis	*Campylobacter* spp.	Partes aéreas	Composto purificado	Kurincic et al. (2012b)
Cymbopogon citratus	*Salmonella* spp.	Folhas	Óleo essencial	Shin (2005)
Helichrysum italicum	*E. coli*, *P. aeruginosa*, *A. baumanii* e *E. aerogenes*	Folhas	Óleo essencial	Lorenzi et al. (2009)
Humulus lupulus	Gram positivos e negativos	Produto comercial	Composto purificado	Natarajan et al. (2008)
Ipomoea murucoides	*S. aureus*	Flores	$CHCl_3$	Chérigo et al. (2009)
Mentha arvensis	*E. coli*	Folhas	EtOH	Coutinho et al. (2008)
Momordica balsamina	*S. aureus* e *E. faecalis, E. coli, Salmonella* spp.	Partes aéreas	Composto purificado	Ramalhete et al. (2011)
Persea lingue	*S. aureus*	Folhas	Composto purificado	Holler et al. (2012)
Piper gaudichaudianum	Gram positivos e negativos	Folhas	Composto purificado	Puhl et al. (2011)
Piper nigrum	*S. aureus*	Produto comercial	Composto purificado	Khan et al. (2006)
Rhus coriaria	*P. aeruginosa*	Sementes	EtOH	Adwan et al. (2010)
Sophora alopecuroides	*E. coli*	Produto comercial	Composto purificado	Zhou et al. (2012)
Thymus broussonetii	Gram negativos	Partes aéreas	Óleo essencial	Fadli et al. (2011)
Thymus maroccanus	Gram negativos	Partes aéreas	Óleo essencial	Fadli et al. (2011)
Turnera ulmifolia	*E. coli*	Folhas	EtOH	Coutinho et al. (2010)
Vernonia anthelmintica	*E. coli, P. aeruginosa* e *S. aureus*	Sementes	MetOH	Ravula et al. (2012)

EtOH – etanol, MetOH – metanol, $CHCl_3$ – clorofórmio, CH_2Cl_2 – diclorometano

Estudos *in vitro* têm demonstrado a eficácia de diferentes substâncias naturais tais como, os óleos essenciais e compostos fenólicos, como antimicrobianos contra algumas estirpes de *Campylobacter* spp. (FRIEDMAN et al., 2002; GAÑAN et al., 2009; NANNAPANENI et al., 2009). A incorporação de compostos obtidos de fontes naturais tais como, eugenol, carvacrol, timol, e cinamaldeído, na ração animal também tem sido avaliada como opção para redução de patógenos na cadeia produtiva de aves (VENKITANARAYANAN et al., 2013). No entanto, nenhum desses compostos controlou de forma eficiente a contaminação das aves por *Campylobacter* spp.. A inclusão de substâncias antimicrobianas naturais ou de componentes que atuam de forma sinérgica com antimicrobianos contra *Campylobacter* spp. e outros patógenos alimentares é uma estratégia interessante na luta contra as doenças bacterianas de origem alimentar.

3. OBJETIVOS

Objetivo geral

Realizar uma triagem de extratos vegetais com potencial para modular a atividade de antimicrobianos e atuar sobre os sistemas de efluxo de *Campylobacter* spp. multirresistentes isolados de carcaças de frango, visando fornecer uma alternativa para redução deste patógeno na cadeia produtiva de aves.

Objetivos específicos

- ✓ Enumerar, isolar e identificar espécies de *Campylobacter* spp. de carcaça de frango.
- ✓ Avaliar a resistência dos isolados de *Campylobacter* spp. a antimicrobianos comumente utilizados em terapia humana e animal.
- ✓ Determinar o efeito de L-fenilalanina-L-arginil-β-naftilamida (PAβN), um inibidor de sistemas de efluxo, sobre a resistência a antimicrobianos em *Campylobacter* spp..
- ✓ Identificar a presença de genes de resistência a antimicrobianos e do sistema de efluxo CmeABC em *Campylobacter* spp..
- ✓ Obter extratos vegetais etanólicos, determinar sua concentração mínima inibitória (MIC) e efeito sinérgico em combinação com antimicrobianos sobre *Campylobacter* spp..
- ✓ Confirmar a atividade de extratos vegetais sobre sistemas de efluxo de *Campylobacter* spp. por meio de ensaio de acumulação de brometo de etídio.
- ✓ Realizar uma análise fitoquímica exploratória dos extratos vegetais que apresentarem efeitos sinérgicos e de acumulação de brometo de etídeo.

4. CAPÍTULO I

PREVALENCE, ENUMERATION AND ANTIMICROBIAL RESISTANCE OF *Campylobacter* spp. ISOLATED FROM CHICKEN CARCASSES IN BRAZIL

ABSTRACT

Campylobacter spp. is one of the most important foodborne pathogens, widely present in chicken meat and by-products, and its resistance to antimicrobials is increasing worldwide. Here we investigated *Campylobacter* spp. contamination in chicken carcasses, antimicrobial resistance of the isolates and its molecular basis. A total of 95 chicken carcasses were collected from 19 slaughterhouses from Minas Gerais - Brazil, and analyzed by MPN method associated with PCR confirmation. *Campylobacter* spp. was found in 16.8% of samples with microbial load ranging from 60.0 to 184 MPN/carcass. *C. jejuni* was the species most frequently isolated (93.8%). All isolates were resistant to at least 5 (31.2%) of the antimicrobial agents screened using the disk diffusion method. The highest resistance rates were noted for cephalosporins (75-100%), quinolones (95%), tetracycline (50%) and ampicillin (45%). Great variations in bile salts resistance (0.390 and >25 mg/mL) were observed among *Campylobacter* isolates. Ciprofloxacin resistance proved to be caused by mutation in the *gyrA* gene, whereas only 40% of the tetracycline resistance was explained by presence of the $tet_{(O)}$ gene. Curiously, the bla_{OXA-61} gene that confers resistance to ampicillin was found in both resistant and susceptible isolates. Almost all isolates showed the three genes required to synthesize the CmeABC efflux system. The use of PAβN decreased the MIC of ciprofloxacin, tetracycline and ampicillin by 2 - 8 fold, whereas for bile salts the reduction ranged from 2 to 128-fold. The results reported here indicate low prevalence and numbers of *Campylobacter* spp. in chicken carcass samples; nonetheless, the identification of high-levels of antimicrobial resistance and multidrug-resistant isolates is an important problem and emphasizes the need for continuous monitoring of this pathogen in the poultry production chain.

Keywords: *Campylobacter* spp., antimicrobial resistance, CmeABC efflux system.

1. INTRODUCTION

Campylobacter spp. have been recognized as the major cause of foodborne disease worldwide. In 2011 the European Food Safety Authority (EFSA, 2013) reported that the total number of campylobacteriosis cases was 220,209 in the European Union, and it has followed a significant increasing trend in the last years. In the United States, this pathogen was the second most common infection with 14.3 cases reported per 100,000 population in FoodNet in 2012 (CDC, 2013). Although it is generally recognized that there are many contamination sources of this pathogen, chicken meat and by-products are the most important vehicles for human campylobacteriosis (HUMPHREY et al., 2007). Poultry are considered natural reservoirs for this pathogen, and carcass contamination occurs more frequently via crop leakage and intestinal rupture during the evisceration stage in slaughterhouses (SMITH et al., 2007a). Several studies have reported *Campylobacter* contamination in chicken carcasses around the world. (ARSENAULT et al., 2007; SON et al., 2007; POINTON et al., 2008; FIGUEROA et al., 2009; EFSA, 2013; OLIVEIRA and OLIVEIRA, 2013). However, great variations in the findings have been observed, which can be attributed mainly to differences in sampling and analytical methodologies (SCHERER et al., 2006a). Although *Campylobacter* spp. are recognized as an important foodborne pathogen, the epidemiological and risk assessment studies for this microorganism are recent in Brazil, and there are still no legal standards established for its presence or counts in foods (BIASI et al., 2011).

More recently, concern regarding *Campylobacter* contamination in foods has increased because of high levels of antimicrobial resistance, including the frequent isolation of multidrug-resistant strains (MOŽINA et al., 2011). Antimicrobial resistance among bacterial pathogens isolated from foods has been attributed to the indiscriminate use of antimicrobials in animal husbandry for preventive or therapeutic purposes as well as for increasing the growth rate of animals (ALFREDSON and KOROLIK, 2007). The dissemination of resistant *Campylobacter* spp. to other environments via livestock and foods of animal origin may contribute to the emergence of antimicrobial resistance in humans and complicate effective clinical treatment of campylobacteriosis (AARESTRUP and ENGBERG, 2001). Multiple mechanisms for antibiotic resistance are present in *Campylobacter* spp., which may be associated with mutational

alteration or modification of target molecules, repression of uptake systems, activation of efflux pumps, and inactivation of the antibiotic (IOVINE, 2013; WIECZOREK and OSEK, 2013). Among these, the CmeABC efflux system plays a key role in resistance to a wide variety of compounds including antibiotics, bile salts, dyes and detergents, and has been broadly studied and characterized as a potential target to reduce antimicrobial resistance of *Campylobacter* spp. (LIN et al., 2002; GIBREEL et al., 2007).

Resistance of *Campylobacter* isolates from chicken and by-products to antibiotics such as quinolones, macrolides, tetracycline and ampicillin as well as the molecular mechanisms of this resistance have been reported in several studies (PRATT and KOROLIK, 2005; GRIGGS et al., 2009; MAĆKIW et al., 2012; OBENG et al., 2012). However, there is a paucity of information regarding the molecular basis of antimicrobial resistance of *Campylobacter* isolates in Brazil. Most studies have focused on the prevalence of *Campylobacter* spp. in food and animals and its phenotypic profiles of antimicrobial resistance (KUANA et al., 2008b; BIASI et al., 2011; DE MOURA et al., 2013). Therefore, to our knowledge, there are no published data about genotypic resistance in *Campylobacter* isolates from chicken carcasses in Brazil. The objectives of this study were to investigate the prevalence and numbers of *Campylobacter* spp. in chicken carcasses in Minas Gerais State (Brazil) as well as antimicrobial resistance of the isolates and its molecular basis, including the CmeABC efflux system.

2. MATERIALS AND METHODS

2.1 Sample collection

A total of 95 chicken carcasses were collected from 19 slaughterhouses localized in Minas Gerais, Brazil, during January and February 2012. These establishments were previously selected by simple random sampling, representing 56% of the total number of federally inspected slaughterhouses in this brazilian state. Five samples were randomly selected from each slaughterhouse after the chilling stage. Each carcass was aseptically packed in a sterile plastic bag and transported to the laboratory under cooled conditions. Upon arrival at the laboratory, the carcass samples were weighed and submitted to *Campylobacter* enumeration.

2.2 Enumeration of *Campylobacter* spp.

Previously, each carcass was aseptically transferred into a new sterile plastic bag, to which was added 200 mL of 0.1% buffered peptone water – BPW (Himedia, Mumbai, India), and vigorously shaken by hand for 1min. This chicken carcass rinse was serially diluted (10-fold) using BPW and analyzed by the Most Probable Number Method (MPN) as described by Scherer et al. (2006a) with some alterations. Briefly, aliquots of 1 mL from each dilution (direct homogenate, 10^{-1} and 10^{-2}) were brought into 3 MPN-tubes containing 9 mL Preston Enrichment Broth Base (Himedia,Mumbai, India) containing 0.4% charcoal and supplemented with antibiotics (20 mg/L of trimethoprim lactate, 15 mg/L of cephalothin, 20 mg/L of vancomycin, 2.500 UI/L polymyxin B and 2 mg/L of amphotericin B – Sigma-Aldrich, Saint Louis, USA) and 5% reductive solution FBP (0.5% of ferrous sulfate, sodium metabisulfite, and sodium pyruvate - Vetec, Rio de Janeiro, Brazil). The MPN-tubes were incubated under microaerobic conditions (approximately 2% H_2, 5% O_2, 10% CO_2 and 83% N_2) at 42 °C for 24 h using an anaerobic chamber (Forma 1025 Thermo Scientific, USA). Then, 10 µL from each tube were streaked onto *Campylobacter* Agar Base (Himedia, Mumbai, India) supplemented as previously described for Preston broth. After an incubation time of 48 h at 42 °C under microaerobic conditions, plates were examined for presence of presumptive colonies. At least five colonies per plate were selected, Gram stained and examined by phase-contrast microscopy for typical *Campylobacter* morphology. One colony per tube-NMP showing typical cellular morphology was isolated and confirmed using polymerase chain reaction (PCR). After molecular confirmation of these colonies, the number of positive tubes at each dilution was determined and the *Campylobacter* spp. counts present in the rinse fluid and, consequently, in the chicken carcass samples were computed from the statistical MPN table of de Man (1983) (95% confidence interval).

The isolates were coded based on source and sampling data. The letter represents the slaughterhouse code followed by the sample number and the collection period (e.g., A52013).

2.3 Confirmation and species identification

The isolates were confirmed and identified using genus- and species-specific PCR assays. DNA extraction was carried out using a boiled-cell method. Fresh cultures of isolates were suspended in 500 µL of sterile ultra-pure water and boiled at 95 °C for 10 min. After centrifugation at 13,000 x *g* for 2 min, the supernatants were collected and stored at –20 C until use as template DNA in the PCR reactions. Primer sequences for the 16S rRNA gene were used to confirm isolates as belonging to the *Campylobacter* genus. The species were identified using primer sequences to detect hippuricase gene (*hipO*) specific for *C. jejuni*, and siderophore transport gene (*ceuE*) specific for *C. coli*. Amplifications were carried out in 25 µL reactions containing 2.5 µL of 10× PCR buffer; 0.2mM of deoxynucleoside triphosphate mix (ATP, GTP, TTP and CTP); 0.4 µM of each primer; 2.5 U Taq PCR polymerase (Promega, Madison, USA) and 2 µL of DNA preparation. PCR reaction mixtures were heated at 96 °C for 2 min as an initial denaturation step followed by 30 cycles of denaturation at 95 °C for 30 s, annealing for 45 s and extension 72 °C for 60 s, and a final extension at 72 °C for 5 min. The sequence of primers and annealing temperatures are listed in Table 1. The amplified products were electrophoresed in 1.5 % agarose gel containing ethidium bromide (0.5 µg/mL) at 90 V for 40 min and visualized using a UV gel documentation system (Quantum ST4 1100/26MX - Vilber Lourmat). A 100-bp DNA ladder (New England Biolabs, MA, USA) was used as a standard for molecular size determinations.

2.4 Antibiotic susceptibility screening

The *Campylobacter* isolates were analysed for antimicrobial resistance using the agar disk diffusion method (CLSI, 2012). The bacterial suspension was prepared in sterile saline solution (0.85% NaCl wt/v) from colonies grown on Müller-Hinton agar containing 5% (v/v) sheep blood (M-H blood) at 42 °C for 24 h under microaerobic conditions, and adjusted to an absorbance of 0.13 at 625 nm equivalent to 10^8 CFU/mL, as described by Wiegand et al. (2008).

Table 1. Primers and annealing temperature used for PCR in this study and the expected amplicon sizes.

Target gene	Primer Sequence (5′–3′)	Amplicon size (bp)	Annealing temperature	Reference
16S rRNA	ATCTAATGGCTTAACCATTAAAC GGACGGTAACTAGTTTAGTAT	857	59 °C	Denis et al. (1999)
hipO	GAAGAGGGTTTGGGTGGTG AGCTAGCTTCGCATAATAACTTG	735	66 °C	Linton et al. (1997)
ceuE	TGATTTTATTATTTGTAGCAGCG AATTGAAAATTGCTCCAACTATG	462	59 °C	Denis et al. (1999)
tet(O)	GCGTTTTGTTTATGTGCG ATGGACAACCCGACAGAAG	559	54 °C	Pratt and Korolik (2005)
bla_{OXA-61}	AGAGTATAATACAAGCG TAGTGAGTTGTCAAGCC	372	54 °C	Obeng et al. (2012)
gyrA (Thr-86-Ile)	TTTTTAGCAAAGATTCTGAT CAAAGCATCATAAACTGCAA	265	48 °C	Zirnstein et al. (1999)
cmeA	TTTGGATCCTTGATGGCTAAGGCAACTTTC CTCCAATTTCTTAAGCTTCGCTACCAA	771	50 °C	Lin et al. (2002)
cmeB Cj	GGTACAGATCCTGATCAAGCC AGGAATAAGTGTTGCACGGAAATT	820	50 °C	Lin et al. (2002)
cmeB Cc	TCCTAGCAGCACAATATG AGCTTCGATAGCTGCATC	241	54 °C	Obeng et al. (2012)
cmeC	GCTTGGATCCTTATCTTGGGAAAAA TTTTTAAAGCTTTAAGGTAATTTTCTT	624	50 °C	Lin et al. (2002)

Cj – *C. jejuni*; Cc – *C. coli.*

Sterile cotton-tipped swabs were used to transfer the inoculum onto M-H blood plates to produce a confluent lawn of bacterial growth. After the inoculum the plates were dried, the antibiotic disks were distributed over the inoculated plates and these were incubated at 42 °C for 48 h under microaerobic conditions. The standard disks (Laborclin, Brazil) containing the following antibiotics were used in this assay: ciprofloxacin (5 µg); nalidixic acid (30 µg); chloramphenicol (30 µg); tetracycline (30 µg); gentamicin (10 µg); imipenem (10 µg); ampicillin (10 µg); amoxicillin-clavulanic acid (10 µg); cephalothin (30 µg); cefoxitin (30 µg); cefuroxime (30 µg); cefotaxime (30 µg); ceftazidime (30 µg); cefepime (30 µg); trimethoprim-sulfamethoxazole (23.75/1.25 µg); and erythromycin (15 µg). The inhibition zone diameter obtained for these antibiotics was interpreted according to the breakpoints of the Clinical and Laboratory Standards Institute for *Enterobacteriaceae* (CLSI, 2012), except for erythromycin, which followed recommendations from The European Committee on Antimicrobial Susceptibility Testing – EUCAST (http://www.eucast.org). The multiple antibiotic resistance (MAR) index was calculated and interpreted according to the method described by Krumperman (1983) using the formula: a/b,where 'a' represents the number of antibiotics to which a particular isolates was resistant and 'b' the total number of antibiotics tested.

2.5 Detection of antibiotic resistance genes

All the *Campylobacter* spp. were tested for the presence of $tet_{(O)}$, bla_{OXA-61}, *gyrA* (Thr-86-Ile mutation), *cmeA*, *cmeB*, and *cmeC* genes, representing resistance to tetracycline, ampicillin and quinolones, and CmeABC efflux system components, respectively. Each amplification reaction consisted of 2.5 µL of 10× PCR buffer; 0.2 mM of deoxynucleoside triphosphate mix (ATP, GTP, TTP and CTP); 0.4 µM of each primer; 2.5 U Taq PCR polymerase (Promega, Madison, USA) and 2 µL of DNA preparation in a total reaction volume of 25 µL. Simplex PCR assays for each gene were performed using the following protocol: initial denaturation step of 96 °C for 1 min, followed by 30 cycles of 94 °C for 30 s, specific annealing temperature for 45 s, and 72 °C for 1 min, and a final extension step at 72 °C for 5 min. The sequence of primers and annealing temperatures are listed in Table 1. The various amplicons were electrophoresed and visualized as previously described.

2.6 Effect of efflux pump inhibitor on antimicrobial resistance

To investigate the contribution of efflux systems to resistance to ampicillin, tetracycline, ciprofloxacin (Sigma–Aldrich, Saint Louis, USA) and bile salts (50% sodium cholate and 50% sodium deoxycholate) (Becton Dickinson, Sparks, USA), the minimum inhibitory concentrations (MIC) of these antimicrobials were determined in all isolates using broth microdilution method in the presence and absence of phenylalanine-arginine β-naphthylamide (PAβN) (Sigma–Aldrich, Saint Louis, USA). The resistance assays were carried out in the 96-well microtiter plates containing a final volume of 100 µL per well of Mueller Hinton broth (Himedia, Mumbai, India), with addition of testing agents and final inoculum of 10^5 CFU/mL as described by Wiegand et al. (2008). Two-fold serial dilutions of antibiotics and bile salts were performed in the 96-well plates for concentrations from 0.125 to 512 µg/mL and 0.05 to 25 mg/mL, respectively. The PAβN was used to a final concentration of 5 µg/mL defined by a preliminary microdilution assay, and that had no inhibitory effects on bacterial growth for any of the isolates. All of the MIC measurements were carried out in triplicate, and positive and negative control wells were included. The microtiter plates were incubated at 42 °C for 48 h under microaerobic conditions. Antibacterial activity was detected using a colorimetric method by adding 30 µL of resazurin staining (0.01%) aqueous solution in each well at the end of the incubation period (at least 4 hours before). The MICs were defined as the lowest concentration of an antimicrobial where no metabolic activity is seen after the incubation period, and they were determined by visual observation on the basis of change in resazurin staining (living cells – red; dead cells – blue) (SALVAT et al., 2001).

3. RESULTS

A total number of 95 chicken carcasses from 19 slaughterhouses were analyzed for the presence of *Campylobacter* spp. by the Most Probable Number Method (MPN) associated with PCR confirmation. Sixteen samples from nine slaughterhouses were contaminated with microbial load ranging from 60.0 to 184 MPN/carcass (Table 2).

Table 2. *Campylobacter* spp. enumeration in chicken carcass samples using MPN technique.

Slaughterhouses	Sample Code	Positive Tubes (Dilution)	MPN/carcass
		D-10^{-1}-10^{-2}	
A	A52013	0-1-0	60
B	B12013	1-0-0	72
C	C42013	1-0-0	72
D	D22013	2-0-0	184
	D32013	1-0-0	72
	D42013	1-1-0	148
E	E22013	1-0-0	72
	E32013	2-0-0	184
	E52013	1-0-0	72
F	F12013	2-0-0	184
	F22013	1-0-0	72
	F32013	1-0-0	72
G	G12013	1-0-0	72
	G52013	1-0-0	72
H	H42013	1-0-0	72
I	I52013	0-1-0	72

Note: Remaining samples showed *Campylobacter* spp. < 60.0 MPN/carcass. Inoculum of direct homogenate (D) and 10-fold dilutions (10^{-1} and 10^{-2}).

The overall contamination of chicken carcasses with *Campylobacter* spp. was 16.8%, while the variation in positive samples between the slaughterhouses was 20 to 60%. *C. jejuni* was the species most frequently isolated, representing 93.8% of the positive samples, whereas *C. coli* was present in only one sample. A total of 20 *Campylobacter* isolates made up of 18 *C. jejuni* and 2 *C. coli* isolates were obtained during sampling of typical colonies grown on *Campylobacter* agar, corresponding to one isolate per positive MPN-tube (Figure 1).

An initial screening was performed to identify the antimicrobial susceptibility of 20 *Campylobacter* strains against 16 antimicrobial agents by using the disk diffusion test. All isolates were resistant to at least 5 (31.2%) of the antimicrobial agents tested (Table 3). The highest resistance rates were noted for cephalosporins (75-100%), ciprofloxacin (95%), nalidixic acid (95%) and trimethoprim-sulfamethoxazole (100%). A similar level of resistance was found for tetracycline (50%) and ampicillin (45%), whereas a lower frequency of resistance was observed toward amoxicillin-clavulanic acid (10%).

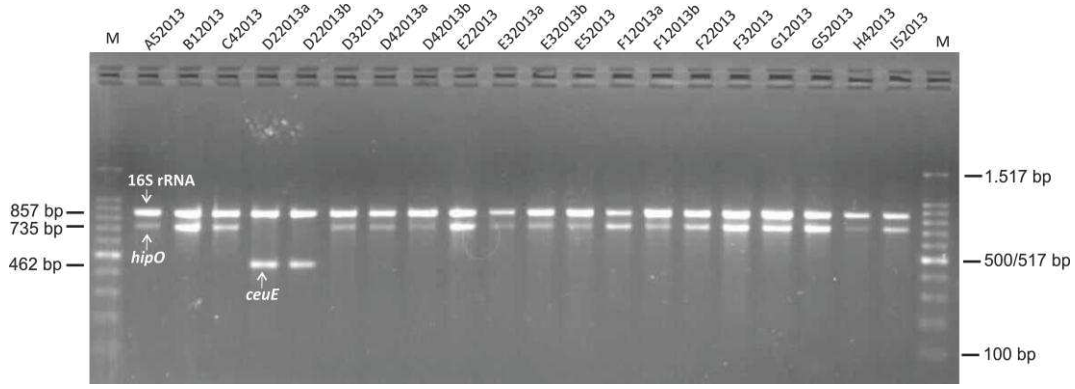

Figure 1. PCR assay of bacterial isolates from chicken carcasses using 16S rRNA, *hipO* gene and *ceuE* gene for identification of *Campylobacter* spp., *C. jejuni* and *C. coli*, respectively. Lanes M - Molecular size marker (100pb DNA Ladder, New England Biolabs, MA, USA). Other lanes refer to bacterial isolate code.

On the other hand, no resistance to chloramphenicol, gentamicin, imipenem, cefepime or erythromycin was observed in any of the isolates. From these data, we selected three antibiotics (ciprofloxacin, tetracycline and ampicillin) and bile salts for an investigation of the MICs and molecular mechanisms contributing to antimicrobial resistance. Eight isolated strains (40%) showed multi-drug resistance to ampicillin, tetracycline and ciprofloxacin, and only one was sensitive to the three antibiotics evaluated using the broth microdilution method (Table 4). The MIC values of bile salts were highly variable, with resistance rates between 0.390 and >25 mg/mL.

The results of phenotypic and genetic analysis of ciprofloxacin susceptibility were sufficiently compatible, which was not confirmed for tetracycline and ampicillin. All ciprofloxacin-resistant isolates had a point mutation Thr-86-Ile in *gyr*A gene of DNA gyrase, except for one. In contrast, ten isolates were resistant to tetracycline, but only four showed the $tet_{(O)}$ gene responsible for the synthesis of a ribosomal protection protein that confers resistance to the inhibitory effect of tetracycline on protein synthesis. Eight ampicillin-resistant isolates had a β-lactamase gene (bla_{OXA-61}), and this fact was also observed in ten sensitive isolates.

Table 3. Antibiotic resistance profile and multiple antibiotic resistance (MAR) index of *Campylobacter* spp. isolates from chicken carcasses.

Strain	Antibiotic resistant profile	MAR index
A52013	CipNalCphCfoCrxCtxCazSut	0.50
B12013	CipNalTetAmpCfphCfoCrxCtxCazSut	0.63
C42013	CipNalTetCphCfoCrxCtxCazSut	0.56
D22013a	CipNalTetAmpAmcCphCfoCrxCtxCazSut	0.69
D22013b	CipNalTetAmpAmcCphCfoCrxCtxCazSut	0.69
D32013	CipNalAmpCphCfoCrxCtxCazSut	0.56
D42013a	CipNalCphCfoCrxCtxCazSut	0.50
D42013b	CipNalTetCphCfoCrxCtxCazSut	0.56
E22013	CipNalCphCfoCrxCtxCazSut	0.50
E32013a	CipNalCphCfoCrxCazSut	0.44
E32013b	CipNalCphCfoCrxCtxCazSut	0.50
E52013	CipNalTetCphCfoCrxCtxCazSut	0.56
F12013a	CipNalTetAmpCphCfoCrxCtxCazSut	0.63
F12013b	CipNalTetAmpCphCfoCrxCtxCazSut	0.63
F22013	CipNalTetAmpCphCfoCrxCtxCazSut	0.63
F32013	CipNalTetAmpCphCfoCrxCtxCazSut	0.63
G12013	CipNalCphCfoCrxSut	0.38
G52013	CipNalCphCfoCazSut	0.38
H42013	AmpCphCfoCrxSut	0.31
I52013	CipNalCphCfoCrxCazSut	0.44

Ciprofloxacin (Cip) 5 µg; Nalidixic acid (Nal) 30 µg; Chloramphenicol (Clo) 30 µg; Tetracycline (Tet) 30 µg; Gentamicin (Gen) 10 µg; Imipenem (Imp) 10 µg; Ampicillin (Amp) 10 µg; Amoxicillin-clavulanic acid (Amc) 10 µg; Cephalothin (Cfl) 30 µg; Cefoxitin (Cfo) 30 µg; Cefuroxime (Crx) 30 µg; Cefotaxime (Ctx) 30 µg; Ceftazidime (Caz) 30 µg; Cefepime (Cpm) 30 µg; Trimethoprim-sulfamethoxazole (Sut) 23.75/1.25 µg; Erythromycin (15 µg).

Moreover, we also investigated the presence of the three genes that encode the CmeABC efflux pump in *Campylobacter* spp. These three genes, named *cmeA*, *cmeB* and *cmeC,* are arranged in an operon, and encode for a periplasmic fusion protein, an inner membrane drug transporter and an outer membrane protein, respectively. The *cmeB* gene was found in all isolates, whereas *cmeA* was absent in two isolates, one of which also showed no *cmeC*. Representative gel of size of the PCR amplicons of resistance and efflux system genes from *Campylobacter* spp. isolates from chicken carcasses is shown in Figure 2.

Table 4. Minimum inhibitory concentrations (MICs) of antimicrobials in the absence and presence of the efflux pump inhibitor (EPI) against *Campylobacter* isolates and research on resistance genes.

Strain	MIC of antibiotics (mg/L)								Resistance Genes					
	Ciprofloxacin		Tetracycline		Ampicillin		Bile salts		*gyrA*	*tet(O)*	*bla*$_{OXA-61}$	*cmeA*	*cmeB*	*cmeC*
	−PAβN	PAβN	−PAβN	PAβN	−PAβN	PAβN	−PAβN	PAβN						
A52013	4 (R)	**0.5**	<0.5 (S)	<0.5	4 (S)	**2**	6250	**390.6**						
B12013	8 (R)	8	128 (R)	**64**	32 (R)	**8**	25000	**390.6**						
C42013	8 (R)	**4**	256 (R)	256	2 (S)	**1**	3125	**390.6**						
D22013a	8 (R)	**4**	64 (R)	**32**	128 (R)	**64**	12500	**390.6**						
D22013b	32 (R)	**16**	256 (R)	256	512 (R)	**256**	25000	**781.3**						
D32013	8 (R)	8	<0.125 (S)	<0.125	2 (S)	2	3125	**195.3**						
D42013a	32 (R)	**16**	<0.125 (S)	<0.125	8 (S)	8	12500	**1562.5**						
D42013b	8 (R)	**4**	64 (R)	**16**	128 (R)	**64**	3125	**390.6**						
E22013	4 (R)	4	<0.125 (S)	<0.125	1 (S)	1	1562.5	**781.25**						
E32013a	8 (R)	**4**	<0.125 (S)	<0.125	8 (S)	**4**	>25000	>25000						
E32013b	16 (R)	**8**	<0.125 (S)	<0.125	8 (S)	**4**	>25000	>25000						
E52013	8 (R)	8	64 (R)	**32**	8 (S)	**2**	12500	**390.6**						
F12013a	4 (R)	4	128 (R)	128	128 (R)	128	390.6	**97.65**						
F12013b	8 (R)	**4**	512 (R)	**256**	256 (R)	128	25000	**781.3**						
F22013	8 (R)	8	128 (R)	128	64 (R)	64	12500	**195.3**						
F32013	8 (R)	8	64 (R)	64	64 (R)	64	12500	**390.6**						
G12013	8 (R)	**4**	<0.125 (S)	<0.125	4 (S)	4	1562.5	**781.3**						
G52013	8 (R)	**4**	<0.125 (S)	<0.125	4 (S)	4	1562.5	**781.3**						
H42013	0.5 (S)	0.5	0.5 (S)	0.5	8 (S)	8	3125	**1562.5**						
I52013	4 (R)	4	<0.125 (S)	<0.125	4 (S)	**2**	25000	**195.3**						

* R – resistant; S – sensitive; dark gray squares – presence of genes; and light gray squares – absence of genes. Breakpoint - ciprofloxacin ≥ 4 mg/L (Cip); tetracycline ≥16 mg/L (Tet); and ampicillin ≥ 32 mg/L (Amp) (CLSI, 2012). –PAβN: absence of phenylalanine-arginine β-naphthylamide; PAβN: presence of phenylalanine-arginine β-naphthylamide; Values highlighted in bold indicate difference in the MIC in presence of the EPI.

We used phenylalanine-arginine β-naphthylamide (PAβN) to inhibit the CmeABC efflux system from *Campylobacter* isolates in order to evaluate its effect on transport and resistance to antimicrobials. A correlation between this efflux system and resistance to bile salts was observed for almost all isolates, whereas with other antibiotics it was not so evident. Approximately 90, 55, 50 and 25 % of the isolates had transport of bile salts, ciprofloxacin, ampicillin and tetracycline affected by PAβN, respectively. In combination with PAβN, the MIC of ciprofloxacin, tetracycline and ampicillin decreased from 2 to 8-fold for at least one of the isolates evaluated, whereas for bile salts the decrease ranged from 2 to 128-fold (Table 4). In addition, our results revealed that the absence of *cmeA* and *cmeC* genes in two isolates did not influence the resistance or transport of antimicrobials.

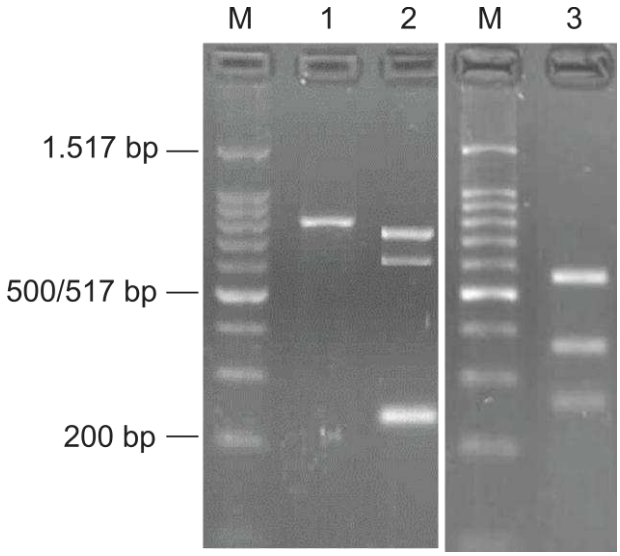

Figure 2. Representative amplicons obtained by PCR assay for identification of resistance and efflux system CmeABC genes from *Campylobacter* spp. isolates from chicken carcasses. Lanes M - Molecular size markers (100pb DNA Ladder, New England Biolabs, MA, USA). Lane 1 – *cmeB* gene (820 bp) specific to *C. jejuni* ; Lane 2 – *cmeA* (771 bp), *cmeC* (624 bp), and *cmeB* (241 bp) specific to *C. coli*, respectively; Lane 3 – *tet(O)* gene (559 bp, tetracycline resistance), bla_{OXA-61} gene (372 bp, ampicilin resistance); *gyrA* gene (265 bp, ciprofloxacin resistance), respectively.

4. DISCUSSION

Our results revealed low prevalence (16.8%) and low numbers (60 – 184 MPN/carcass) of *Campylobacter* spp. in chicken carcasses obtained from slaughterhouses from Minas Gerais State, (Brazil). However, high resistance levels were observed in isolates for most of the antibiotics, which proved to be caused by different molecular mechanisms.

Campylobacter contamination rates in this type of food vary widely among countries. In a study of 13 European Union member states performed in 2011, the chicken carcass contamination rate was 31.3% on average, but ranged from 3.2% to 84.6% (EFSA, 2013). Higher incidences of *Campylobacter* contamination in chicken carcass (>60%) were reported in other studies worldwide, including the USA (SON et al., 2007), Australia (POINTON et al., 2008), China (SHIH, 2000) and Chile (FIGUEROA et al., 2009). On the other hand, a lower number of *Campylobacter* positive samples (<40%) were observed in chicken carcasses from Canada (ARSENAULT et al., 2007), South Africa (VAN NIEROP et al., 2005) and Mexico (CASTILLO-AYALA et al., 1993). A literature survey of studies performed in Brazil also showed that the occurrence of *Campylobacter* spp. in chickens and by-products is widespread and ranges from 6.7 to 100% (SCARCELLI et al., 2005; FRANCHIN et al., 2007; ALVES et al., 2012; DE MOURA et al., 2013; GONÇALVES et al., 2013; OLIVEIRA and OLIVEIRA, 2013). Recently, Oliveira and Oliveira (2013) analyzed chicken carcasses from Minas Gerais, Brazil, and they reported a greater prevalence of *Campylobacter* spp. (44%), compared to results obtained in our study. These variations in the prevalence rates of *Campylobacter* may be attributed to poultry breeding methods, hygienic practices during slaughter, sampling strategies and differences in analytical methods (DASKALOV and MARAMSKI, 2012).

Although many studies have confirmed the prevalence of *Campylobacter* spp. in chicken carcasses, a quantitative risk assessment of human campylobacteriosis depends on quantitative data, which has been highlighted as a major hindrance in carrying out such an assessment in developing countries (FAO/WHO, 2002). In recent years, much attention has been paid to the enumeration of *Campylobacter* spp. in chicken samples in order to know the level of contamination that is required for the risk assessment studies (NAUTA

et al., 2005; UYTTENDAELE et al., 2006). However, there are many methods for identification and enumeration of *Campylobacter* spp. in chicken meat and by-products, which present great variations responsible for differences in the results (SCHERER et al., 2006a; STERN et al., 2007). In previous assays to select the enumeration methodology, we observed that the agar-selective *Campylobacter* plates used in direct plating were always overgrown by non-*Campylobacter* spp., some of which were confirmed to be *Proteus mirabilis* and *E. coli* by sequencing of fragments (1.400 bp) of the 16S rDNA gene (data not shown). These non-*Campylobacter* spp. strains grew faster than the target bacteria and made it impossible to count typical colonies correctly. Therefore, we chose to use the Most Probable Number Method (MPN) associated with identification of *Campylobacter* spp. by using a species-specific PCR technique, which proved to be more suitable for enumeration of this pathogen in chicken carcasses. This method was also used and adapted by other authors to enumerate *Campylobacter* spp. in a variety of samples (SCHERER et al., 2006b; WONG et al., 2007; TAN et al., 2008; TANG et al., 2010; CHENU et al., 2013). The MPN is a method that has been studied extensively and widely accepted, which allows the detection of small numbers of bacteria because of its low detection limit (SCHERER et al., 2006a; CHENU et al., 2013).

The present study showed that the concentration of *Campylobacter* spp. in chicken carcasses was low and similar to results reported by Tang et al. (2010), Wong et al. (2007) and Scherer et al. (2006b), who found the contamination of most samples ranging from <100 MPN/g^{-1}, <0.3 to 10 MPN/g^{-1}, and <0.3 MPN/g^{-1}, respectively. However, even a low contamination of chicken carcasses may be of concern to food safety authorities as because *Campylobacter* strains are highly infectious, with the infective dose being as low as 800 cells (BLACK et al., 1988).

A number of authors have quantified *Campylobacter* spp. on chicken carcasses using enumeration methods other than MPN, and they observed that counts ranged from <1.0 to 8.0 $\log_{10}$ CFU/carcass (DUFRENNE et al., 2001; JOHANNESSEN et al., 2007; STERN et al., 2007; CHRYSTAL et al., 2008; KUANA et al., 2008a; FIGUEROA et al., 2009). However, it is difficult to compare results across studies, owing to the use of different materials and enumeration methods, which consequently impedes the risk assessment

studies (SCHERER et al., 2006a). For that reason, there is a need to adapt and standardize methodologies for isolation and enumeration of *Campylobacter* spp. in food.

In the present study, the most prevalent species was *Campylobacter jejuni* detected in 93.8% of positive findings, similar to what has been observed in other studies researching prevalence of this pathogen in chicken and by-products (TANG et al., 2010; ZHAO et al., 2010; GARIN et al., 2012). Besides *C. jejuni* and *C. coli*, other *Campylobacter* species were not detected by our study. However, other species such as *C. lari*, *C. upsaliensis*, *C. concisus* and *C. fetus* have also been isolated from chicken and by-products in other studies, even if at a lower frequency (LYNCH et al., 2011; GARIN et al., 2012). The higher rate of detection of *C. jejuni* in several studies may be a reflection of the methods used, rather than the actual incidence in food (LYNCH et al., 2011).

Our study also represents one of the few reports on antibiotic susceptibility of *Campylobacter* spp. in chicken carcasses from Minas Gerais, Brazil. We used the disk diffusion test to perform an initial screening in order to investigate antimicrobial resistance of 20 *Campylobacter* isolates against 16 antimicrobials. Although the agar dilution method has been classified as the standard test to investigate antimicrobial resistance in *Campylobacter* spp. (MCDERMOTT et al., 2004), the disk diffusion method has provided results comparable with this reference method having greater convenience of measurement (LUANGTONGKUM et al., 2007). All *Campylobacter* spp. evaluated in this study were resistant to at least five antimicrobials, thus characterized as multidrug-resistant isolates. The percentage of antibiotics to which a particular isolate was resistant ranged from 31% to 69%. We observed a generalized resistance to first- and second-generation cephalosporin, namely cephalothin, cefoxitin and cefuroxime, whereas some isolates were susceptible to third-generation cephalosporin, cefotaxime and ceftazidime. Surprisingly, all isolates were susceptible to cefepime, a fourth-generation cephalosporin. Similar results were observed by Tajada et al. (1996), who demonstrated that cefepime and cefotaxime were active against 88 and 32% of the isolates, respectively. In another study performed by Gritchina et al. (2005), they observed high resistance of *Campylobacter* spp. to the first-generation cephalosporins, and general susceptibility to cefepime. Individual differences among members of this

family of antibiotics, such as the amount and variety of side chains, may explain the variation in antimicrobial resistance (IOVINE, 2013).

In addition, high resistance of *Campylobacter* spp. to β-lactams may be a consequence of selective pressure caused by isolation and quantification methods. The selectivity of culture media is provided by adding antibiotics, and cefoperazone, a third-generation cephalosporin, is used as a selective agent in some of them (International Standards Organization, 2006). The presence of such agents in culture media is required to inhibit enteric flora contaminants, but also decreases the recovery of sensitive *Campylobacter* strains (CORRY et al., 1995; JACOB et al., 2011). In this study, we used trimethoprim lactate, cephalothin, vancomycin, polymyxin B and amphotericin B as selective agents for both broth and *Campylobacter* agar. This fact may in part explain the high incidence of *Campylobacter* resistant to first- and second-generation cephalosporins. Moreover, all *Campylobacter* spp. isolated in this study were resistant to trimethoprim-sulfamethoxazole, and this finding is comparable to that reported by Nobile et al. (2013). Intrinsic resistance in *C. jejuni* and *C. coli* has been described against penicillins and most of the cephalosporins, as well as trimethoprim, sulfamethoxazole, rifampicin, and vancomycin (FLIEGELMAN et al., 1985; MCNULTY, 1987).

The cephalosporins belong to a diverse class of compounds named β-lactam antibiotics, which also include penicillins, carbapenems and monobactams, all of which contain the β-lactam ring required for antimicrobial activity (IOVINE, 2013). Resistance to a large number of β-lactam antimicrobial agents is becoming more widespread in foodborne bacteria, including *Campylobacter* spp. (LI et al., 2007; CANTÓN et al., 2008). However, extended-spectrum penicillins and cephalosporins are not used for therapy of *Campylobacter* infections in humans, and are therefore considered to be of low priority for surveillance purposes (MEVIUS, 2008). Besides cephalosporins, we evaluated the antimicrobial resistance of *Campylobacter* spp. to other β-lactams, including ampicillin, amoxicillin-clavulanic acid and imipenem. We observed a high level of resistance to ampicillin, which was similar to that reported in other studies (MIFLIN et al., 2007; GRIGGS et al., 2009; OBENG et al., 2012). In contrast, all isolates were susceptible to imipenem, and most of them also to amoxicillin-clavulanic acid. In previous studies, these antimicrobial

groups have also shown excellent in vitro activities against *Campylobacter* spp. (HAKANEN et al., 2003; GRIGGS et al., 2009). Our results suggest that these agents might be candidates for clinical trials in enteritis caused by multi-drug resistant *Campylobacter* spp.. Nevertheless, there are few data on the clinical efficacy of β-lactams for the treatment of infections of this nature (HAKANEN et al., 2003).

Campylobacter spp. isolated in the present study were also susceptible to gentamicin, erythromycin and chloramphenicol. Results similar to ours were reported by other authors, who observed that *Campylobacter* spp. isolated from chicken were highly sensitive to these three antibiotics (MIFLIN et al., 2007; ZHAO et al., 2010; NARMS, 2011; EFSA, 2012). In contrast, higher resistance to gentamicin (93.7%), erythromycin (68.7%) and chloramphenicol (37.5%) were observed by De Moura et al. (2013), who evaluated the antimicrobial susceptibility of *Campylobacter* spp. isolated from chicken carcasses in Brazil. In another study performed by Nobile et al. (2013) in Italy, the levels of resistance to these three antibiotics were also higher than the results obtained in our study, at 19%, 57.1% and 28.6%, respectively.

Alarmingly, we observed high resistance to quinolones (ciprofloxacin and nalidixic acid) and tetracycline. High levels of *Campylobacter* spp. resistant to these antibiotics have also been reported by other authors (EFSA, 2012; MAĆKIW et al., 2012). The rates of resistance to quinolones and tetracycline vary worldwide, and in the last years the high prevalence of *Campylobacter* spp. resistant to these drug classes has been increasingly frequent and worrying (GE et al., 2005; ALFREDSON AND KOROLIK, 2007; EFSA, 2012; MAĆKIW et al., 2012). These concerns have become more severe because fluoroquinolones and tetracyclines, along with macrolides, are used for treating human infections (MAĆKIW et al., 2012; OBENG et al., 2012).

Overall, by comparing our results for antimicrobial resistance with those reported by other authors, we observed both similarities and differences amongst them. These variations in resistance profiles of *Campylobacter* spp. might be expected, and occur due to the variations in the use of antibiotics among countries, the origin and type of the samples, the geographical distribution of resistant species and the methodology applied to evaluate susceptibility (WILSON, 2003; WASSENAAR et al., 2009; ZHAO et al., 2010).

This hampers effective comparison of antibiotic resistance among these studies performed in various countries and different conditions (ADZITEY et al., 2012). The occurrence of high resistance rates to some of the antibiotics investigated in this study could be explained by their use as growth promoters in animal feed and veterinary medicine (IOVINE and BLASER, 2004), and reflects the extent to which these antimicrobial agents are used in Brazil. Although the use of antibiotics as growth promoters has been banned or restricted in some countries, including Brazil (CASTANON, 2007; BRAZIL, 2009), high antimicrobial resistance is still a reflection of its indiscriminate use over the past few years.

In order to investigate the molecular basis of antimicrobial resistance of *Campylobacter* isolates, we evaluated the presence of resistance and efflux system genes as well as the minimum inhibitory concentrations in the absence and presence of the efflux pump inhibitor for three antibiotics selected and bile salts. Bile salts were included in this last assay because their resistance in *Campylobacter* spp. has proved to be mediated by CmeABC efflux system (LIN et al., 2003; LIN et al., 2005). We observed that there was a strong agreement between the resistance results previously obtained by disc diffusion method and the MIC for all three antibiotics tested. The high resistance to ciprofloxacin observed in this study was strongly correlated with the Thr-86-Ile modification of GyrA. This mutation is the main mechanism of resistance to fluoroquinolones in *Campylobacter* spp., and has been frequently reported in other studies (ZIRNSTEIN et al., 1999; GRIGGS et al., 2005; MAĆKIW et al., 2012; WIECZOREK et al., 2013). In our study only one isolate was resistant to ciprofloxacin and did not show the Thr-86-Ile mutation, and therefore its resistance may be associated with other mechanisms. Other mutations in the *gyrA*, and efflux via multidrug efflux systems, have also been reported to cause fluoroquinolone resistance in *Campylobacter* spp. (LIN et al., 2002; LUO et al., 2003). On the other hand, a relatively low percentage (40%) of the tetracycline-resistant isolates showed the *tet*$_{(O)}$ gene. This gene encodes the Tet$_{(O)}$ protein that protects the ribosome from the inhibitory effect of tetracycline (CONNELL et al., 2003), and has been found either chromosomally or in transferable plasmids (DASTI et al., 2007). Low correlation between tetracycline resistance and the *tet*$_{(O)}$ gene was also observed by Obeng et al. (2012). Whilst, in other studies

the high levels of phenotypic resistance to tetracycline were attributed almost solely to presence of this gene (MAĆKIW et al., 2012; WIECZOREK et al., 2013). Beyond the *tet*$_{(O)}$ gene, nonspecific efflux systems such as CmeABC multidrug efflux pump may decrease susceptibility to tetracycline (GIBREEL et al., 2007; IOVINE, 2013). This other mechanism could explain the high resistance to tetracycline of the *Campylobacter* isolates, in which the *tet*$_{(O)}$ gene was not detected. Concerning the genetic basis of ampicillin resistance, all resistant isolates showed the *bla*$_{OXA-61}$ gene, which is associated with β-lactamase production (ALFREDSON and KOROLIK, 2005). Griggs et al. (2009) observed that 91% of the ampicillin-resistant isolates carried the *bla*$_{OXA-61}$ gene. However, it is worth noting that in our study many susceptible isolates also showed this ampicillin resistance gene. These results were in agreement with a previous observation by Obeng et al. (2012). They reported that 10 of the ampicillin-susceptible *Campylobacter* isolates from chicken were able to encode the *bla*$_{OXA-61}$ gene. Such findings indicate that the *bla*$_{OXA-61}$ gene in these *Campylobacter* isolates may have been inactivated or is not being expressed. The inactivation of *bla*$_{OXA-61}$ has been noted to enable an increase in susceptibilities to ampicillin in both ampicillin-sensitive and ampicillin-resistant strains (GRIGGS et al., 2009). Beyond synthesis of β-lactamases, β-lactam resistance may also be conferred by reduced uptake due to alterations in outer membrane porins and efflux systems (IOVINE, 2013).

Great variations in bile salts resistance were observed among *Campylobacter* isolates, which was consistent with the findings of the previous studies. Dzieciol et al. (2011) reported that the resistance to sodium salts of cholic acid and taurocholic acid ranged from 2.5 to 40 mg/mL and 25 to 200 mg/mL, respectively. Mavri and Smole Mozina (2013) observed a variation in resistance to bile salts between 1 and 128 mg/mL in *Campylobacter* strains from different sources. The CmeABC efflux system plays a key role in mediating bile salts resistance and is essential for *Campylobacter* growth in bile-containing media such as the animal intestinal tract (LIN et al., 2003). The observed difference in bile salts resistance in our study could not be explained by failures in the CmeABC efflux system, since most of the isolates showed the three genes (*cmeA*, *cmeB* and *cmeC*) required for its synthesis, and even the absence of some of them did not influence the levels of resistance. Therefore,

other mechanisms of resistance to bile salts are probably involved in these results, perhaps even a high genetic diversity between the isolates. In Gram-negative bacteria, bile salts can pass the outer membrane through porins (QUINN et al., 2007), and two of them (Omp50 and PorA) have been identified in *Campylobacter* spp. (BOLLA et al., 1995; BOLLA et al., 2000). In addition, other efflux systems such as CmeDEF and CmeG may contribute to antimicrobial resistance, including bile salts (AKIBA et al., 2006; JEON et al., 2011).

The efflux inhibitor (PAβN) slightly decreased the MIC of ciprofloxacin, tetracycline and ampicillin, but did not restore the susceptibility to either of them in the *Campylobacter* isolates. The effects of PAβN were mostly of 2-4 fold MIC reduction for these antibiotics. The activity of PAβN on quinolone resistance in *C. jejuni* and *C. coli* is controversial. Most studies have demonstrated that PAβN did not produce a significant decrease on the resistance to quinolones in *Campylobacter* spp. (MAMELLI et al., 2003; CORCORAN et al., 2005; HANNULA and HÄNNINEN, 2008). However, significant decreases in the MIC of fluoroquinolones and restoration of susceptibility in *Campylobacter* isolates were reported by Payot et al. (2004) and by Kurincic et al. (2012a). Although our data, as well as those from other studies, demonstrate a lack of efficacy of PAβN in reversing fluoroquinolone resistance, *cmeB* mutants of *Campylobacter* spp. have showed a significant decrease in their resistance to this antibiotics class (LUO et al., 2003; GE et al., 2005). These authors have also shown that the CmeABC system acts synergistically with *gyrA* mutations to confer fluoroquinolone resistance. With regards to tetracycline, Kurincic et al. (2012a) demonstrated that PAβN caused MIC reductions of at least 2-fold to 64-fold, and this EPI also restored susceptibility to some tetracycline-resistant isolates. In contrast, lower effects of PAβN on tetracycline MIC (1.3 - 2.6-fold decrease) were observed by Gibreel et al. (2007) and by Hannula and Hänninen (2008). As previously reported by Lin et al. (2002), the *cmeB* mutant of *Campylobacter* that carried the $tet_{(O)}$ gene had an 8-fold decrease in the MIC of tetracycline, suggesting that the CmeABC system acts synergistically with $tet_{(O)}$ to contribute to tetracycline resistance. In addition, our results showing the effect of PAβN on ampicillin resistance were similar to those reported by Martinez and Lin (2006), who observed that the MIC of β-lactams (cefotaxime and ampicillin) decreased

2-fold using this EPI. In another study performed by Griggs et al. (2009) the presence of PAβN had no effect on the MICs of β-lactams, including ampicillin, for the *Campylobacter* isolates or their respective bla_{OXA-61} mutants. On the other hand, PAβN had a strong effect on bile salts resistance in almost all isolates in our study, causing MIC reductions of up to 128-fold. Other studies performed by Lin and Martinez (2006) and Mavri and Smole Mozina (2013) also demonstrated that the MICs of bile salts were dramatically decreased in the presence of PAβN, around 16–512 fold and 2–64 fold, respectively. The different effect of PAβN on susceptibility of antimicrobials indicates that the inhibition occurs via binding site specific on the CmeABC system and in a competitive manner, as suggested in previous studies (YU et al., 2005; LOMOVSKAYA and BOSTIAN, 2006). It has been proposed that inhibition of multidrug efflux systems by EPIs is a novel approach to enhance drug accumulation inside the bacterial cell, thereby increasing bacterial susceptibility to antimicrobials (LOMOVSKAYA and WATKINS, 2001). In recent years, many studies have been developed in order to find out new compounds which exhibit such properties (STAVRI et al., 2007; KLANČNIK et al., 2012a; KURINČIČ et al., 2012b).

In conclusion, this work provides a baseline study on *Campylobacter* contamination and its antimicrobial resistance and molecular basis in chicken carcasses in Brazil. Despite the low prevalence and the low concentration of *Campylobacter* spp. in chicken carcass samples, the identification of high levels of antimicrobial resistance and multidrug-resistant isolates makes this issue even more serious. Our results point to the need for more frequent monitoring of the prevalence and antimicrobial resistance of *Campylobacter* spp. in order to provide support for actions directed at reducing this pathogen in the food chain. In addition, we also suggest that efflux pump inhibitors may be an important tool to reduce the antimicrobial resistance and colonization of *Campylobacter* spp. in animals raised for food purposes.

5. CAPÍTULO II

MEDICINAL PLANT EXTRACTS WITH RESISTANCE-MODIFYING AND EFFLUX INHIBITORY ACTIVITIES AGAINST *Campylobacter* spp.

ABSTRACT

Campylobacter spp. is main cause of foodborne diseases around the world. The increasing antimicrobial resistance among strains from food origin has become a worrying public health problem. Here we hypothesized that plant extracts used in Brazilian folk medicine may act synergistically with antimicrobials by inhibiting the efflux systems of *Campylobacter* spp. A total of 20 ethanolic plant extracts were produced and evaluated for their antimicrobial and synergistic activities against eight isolates using broth microdilution. These extracts showed weak antimicrobial activity with MIC values mostly very high and ranging from 0.1 to >16.38 mg/mL. However, the most of them decreased 2 to 16 fold the MIC values of bile salts when used at a subinhibitory concentration, indicating synergistic effects on combining with these antimicrobials. *A. zerumbet* and *V. polyanthes* showed the most interesting synergistic results against almost all isolates. In contrast, the association of the different extracts and ciprofloxacin had no significant synergistic effect. Ethidium bromide accumulation assays showed that ten plant extracts inhibited the efflux systems of *Campylobacter* spp., of which *C. rotundus* and *S. flexicaulis* were the more effective. The phytochemical analysis of plant extracts by HPLC fingerprint revealed a great variety of complex phenolic compounds, including flavonoids, which may be involved in the biological activities, reported in this study. Our findings suggest that these plant extracts may be used to control *Campylobacter* spp. in food chain by reducing of the poultry colonization, as well as in the clinical treatment of campylobacteriosis as therapeutic adjuvants.

Keywords: plant extract, efflux system, poultry, bile salts, synergism

1. INTRODUCTION

Multidrug-resistant (MDR) bacteria are an important public health problem involving the ineffectiveness of antibiotics and reduction of therapeutic options. In recent years, antimicrobial resistance of *Campylobacter* spp. and its impact on human health have drawn much attention worldwide (MOŽINA et al., 2011). This foodborne pathogen is main cause of gastrointestinal diseases related to the consumption of food of animal origin, especially chicken meat and by-products (HUMPHREY et al., 2007; PIRES et al., 2010). Additionally, high levels of antimicrobial resistance have been reported in *Campylobacter* spp. isolated from food and food-producing animals, including multi-resistant strains, which thus compromises the effectiveness of its control in the food chain as well as antibiotic treatment of campylobacteriosis (LUANGTONGKUM et al., 2009; MAĆKIW et al., 2012; OBENG et al., 2012). New approaches to reduce the prevalence and antimicrobial resistance of *Campylobacter* spp. in food chain have been investigated, searching to understand the resistance mechanisms and alternative ways of overcoming them (MOŽINA et al., 2011; GANAN et al., 2012). Amongst the multiple mechanisms that may be involved in bacterial resistance, the efflux systems that decrease the intracellular concentration of the antimicrobial are considered as one of the most important contributors to multidrug resistance of *Campylobacter* spp. (ZHANG and PLUMMER, 2008; WIECZOREK and OSEK, 2013). The CmeABC efflux system belonging to the resistance-nodulation-cell division (RND) superfamily is the main mechanism of nonspecific resistance identified in this foodborne pathogen and allows the extrusion a wide variety of compounds, including antibiotics, bile salts, dyes, heavy metals, and detergents (LIN et al., 2002). This efflux system also plays a key role in facilitating *Campylobacter* colonization of the animal intestinal tract by mediating bile salts resistance (LIN et al., 2003). Therefore, the inhibition of efflux systems in this pathogen is a promising approach to their control in the food chain as well as restores the activity of antibiotics contributing for the campylobacteriosis treatments.

Plants are known to produce a great variety of secondary metabolites classes, which are valuable sources of new and biologically active molecules possessing antimicrobial properties (ABDALLAH, 2011). The antimicrobial

activity of plants and plant-derived have been investigated in several fields, including the control of bacterial growth in food and reduction of pathogen colonization in the animal husbandry (NEGI, 2012; VENKITANARAYANAN et al., 2013). The interest in using natural antibacterial compounds for preventive or therapeutic purposes in the animal husbandry has increased due to the banning the use of growth-promoting antibiotics in some countries (SERRATOSA et al., 2006; CASTANON, 2007, BRAZIL, 2009). Many studies have also demonstrated synergistic interactions of plants and plant-derived with antimicrobials, which decreasing the antimicrobial resistance in several bacteria, including Gram-negative and Gram positive (ADWAN et al., 2010; EUMKEB et al., 2010; FADLI et al., 2011; WEERAKKODY et al., 2011; LACMATA et al., 2012). These effects have been attributed to presence of compounds able to inhibit the bacterial efflux systems increasing the intracellular concentration of antimicrobials (STAVRI et al., 2007). Several molecules have been characterized for their efflux inhibitory ability, but some issues such as toxicity, stability, selectivity and bioavailability still hamper their use in human and animals (LOMOVSKAYA and BOSTIAN, 2006). Despite the advances in this area, few studies have investigated the synergistic and efflux inhibitory activities of plants and natural compounds against *Campylobacter* ssp. (KLANČNIK et al., 2012a; KLANČNIK et al., 2012b; KURINČIČ et al., 2012b). Therefore, the objectives of this study were to evaluate the antimicrobial and resistance-modifying activities of extracts from plants used in Brazilian folk medicine against *Campylobacter* spp. isolated from chicken, and to investigate their capability blocking the drug efflux systems expressed by this microorganism.

2. MATERIAL AND METHODS

2.1 Bacterial strains

Eight *Campylobacter* strains isolated from chicken carcasses were used in the present study (Table 1). They were previously selected from a total of 20 isolates based on phenotypic and genotypic resistance profiles to ampicillin, tetracycline, ciprofloxacin and bile salts (CHAPTER I).

Table 1. *Campylobacter* strains used in present study and their antimicrobial resistance profiles.

Strain *	MIC of antibiotics (mg/L)				Resistance genes					
	Cip	Tet	Amp	Bile salts	*gyrA*	*tet(o)*	bla_{oxa-61}	*cmeB*	*cmeA*	*cmeC*
A52013 *C.j*	4 (R)	<0.5 (S)	4 (S)	6,250	■	□	■	■	■	■
B12013 *C.j*	8 (R)	128 (R)	32 (R)	25,000	■	■	■	■	■	■
D22013 *C.c*	8 (R)	64 (R)	128 (R)	12,500	■	■	■	■	■	■
E52013 *C.j*	8 (R)	64 (R)	8 (S)	12,500	■	□	■	■	■	■
F22013 *C.j*	8 (R)	128 (R)	64 (R)	12,500	■	□	■	■	■	■
I52013 *C.j*	4 (R)	<0.125 (S)	4 (S)	25,000	■	□	■	■	■	■
44991 *C.c*	4 (R)	16 (R)	2 (S)	25,000	■	■	□	■	□	□
45662 *C.j*	8 (R)	1 (S)	2 (S)	3,125	■	□	□	■	■	■

*The isolates were coded based on source, sampling data and species. The letter represents the slaughterhouse code followed by the sample number and the collection period, *C.j* – *C. jejuni* and *C.c* – *C. coli*. Cip – ciprofloxacin; Tet – tetracycline; and Amp – ampicillin; R – resistant; S – sensitive, Breakpoint (CLSI, 2012); *gyrA* (mutation Thr-86-Ile, ciprofloxacin resistance), *tet(O)* (ribosomal protection protein, tetracycline resistance) bla_{OXA-61} gene (β-lactamase, ampicillin resistance), *cmeA*, *cmeB*, and *cmeC* (components of efflux pump CmeABC); gray squares – presence of genes; and dark light gray squares – absence of genes.

The cultures were stored at - 20°C in cryopreservation medium (1% peptone – Himedia, Mumbai, India; 0.5% NaCl and 25% glycerol - Vetec, Rio de Janeiro, Brazil) until required. The isolates were subcultured on Columbia agar (Himedia, Mumbai, India), supplemented with 0.4% charcoal and 5% reductive solution FBP (0.5% of ferrous sulfate, sodium metabisulfite, and sodium pyruvate - Vetec, Rio de Janeiro, Brazil) at 42°C for 24 - 48 h under microaerobic conditions (2% H_2, 5% O_2, 10% CO_2 and 83% N_2).

2.2 Pulsed-field gel electrophoresis (PFGE)

The genotypic diversity of these isolates was evaluated by macrorestriction analysis of genomic DNA using *SmaI* and *KpnI* restriction enzymes and pulsed-field gel electrophoresis (PFGE). In situ DNA extraction, restriction endonuclease digestion and PFGE were performed as described previously (RIVOAL et al., 2005; RADULOVIC et al., 2013). The *SmaI* and *KpnI* macrorestriction patterns were analysed using BioNumerics 3.5 (Applied Maths, Sint-Martens-Latem, Belgium); and similarities between the profiles, based on band positions, were derived by using the Person correlation coefficient. Dendrograms were constructed to reflect the similarities between the strains in

the matrix, and the unweighted pair-group method using arithmetic averages (UPGMA) was used to cluster patterns.

2.3 Plant Material

Twenty plants used in Brazilian folk medicine (Table 2) were selected based on results of previous studies about resistance-modifying and efflux inhibitory activities against other bacteria, as well as random criteria. Aerial parts of these plants were collected in January and February 2013 from specimens cultivated in medicinal gardens of the Federal University of Juiz de Fora, and Federal University of Viçosa, in Minas Gerais, Brazil. Plants were identified by Dr. Fátima Regina Gonçalves Salimena and Dr. Rosane Maria de Aguiar Euclydes, and voucher specimens were deposited at the Herbarium Leolpoldo Krieger (CESJ), in Juiz de Fora - MG, and at the Herbarium VIC-UFV, in Viçosa, MG, Brazil. The plant materials (aerial parts) were dried in an air flow oven (40 °C for 48 h), powdered (40 mesh) and maintained in hermetic packages until use.

2.4 Extract Preparation

Crude plant extracts were prepared using the ultrasound method as described by Holler et al. (2012). Briefly, dried plant material (30 g) were ultrasonicated three times with 96% ethanol (Vetec, Rio de Janeiro, Brazil) 1:10 (mass to volume) for 30 min in an ultrasound bath (Cristófoli, Paraná, Brazil) with 90w of potency and 42 kHz of frequency. After the extraction, the extracts were filtered through a filter paper (Whatman n° 2, Sigma-Aldrich, Saint Louis, USA), and concentrated under reduced pressure using rotatory evaporator (Heidolph Laborota 4000, Schwabach, Germany) at 40°C until a small volume (5 – 10 mL). The concentrated extracts were collected in glass vials and dried at 40 °C for 1 -2 h. After drying, the samples were weighted to calculate yield and stored at -20 °C.

2.5 Antimicrobial and synergistic activities

Plant extracts were tested for their ability to inhibit the growth of *Campylobacter* isolates and their synergistic effects in combination with ciprofloxacin and bile salts using broth microdilution method and the

determination of the minimum inhibitory concentrations (MIC). Each dried plant extract was resuspended in 2 mL of dimethyl sulfoxide (DMSO, LGC Biotecnologia, São Paulo, Brazil) and added of ultra-pure water to give a stock solution of 65.5 mg/mL in a final volume of 20 mL. These stock solutions were centrifuged at 3,000 × *g* for 3 min and filtered through a 0.22-µm-pore-size cellulose acetate membrane (Nalgene syringe filter Thermo scientific, Massachusetts, USA).

The antimicrobial activity assays were performed in 96-well microtiter plates in a final volume of 150 µL. The plant extracts were serially diluted (2-fold) in Mueller Hinton broth (Himedia, Mumbai, India) for final concentrations from 0.032 to 16.38 mg/mL, and then added of bacterial inoculum. The highest concentration of DMSO in a test well was 2.5% (v/v), which did not significantly affect the growth of isolates tested. The bacterial inoculum was prepared from an overnight culture by suspending a loopful of colonies in Mueller Hinton broth and adjusting to an absorbance of 0.13 at 625 nm equivalent to 10^8 CFU/mL, as described by Wiegand et al. (2008). The cell suspension was then diluted in Mueller Hinton broth and added to give a final inoculum of 10^5 CFU/mL per well. The density of inoculum was also confirmed by plating on Columbia agar supplemented with 0.4% charcoal and 5% FBP and counting colony forming units. Each plate also included controls: negative control containing DMSO and bacterial inoculum; sterility control including broth media and DMSO; extract control containing plant extract and broth; and a positive control containing broth and bacterial inoculum.

In order to evaluate the synergistic activity of the plant extracts were used ciprofloxacin (Sigma–Aldrich, Saint Louis, USA) and bile salts (50% sodium cholate and 50% sodium deoxycholate - Becton Dickinson, Sparks, USA) in the concentrations ranging from 0.125 to 512 µg/mL and 0.05 to 25 mg/mL, respectively. These antimicrobials were serially diluted (2-fold) in 96-well microtiter plates containing Mueller Hinton broth added of plant extract at a final concentration of ¼ MIC (subinhibitory), previously determined. The PAβN, a well-known efflux pump inhibitor (Pagès and Amaral, 2009), was included in this assay to serve as a standard for comparison with the extract effects. This EPI was used to a final concentration of 5 µg/mL defined by a preliminary microdilution assay, and that had no inhibitory effects on bacterial growth for

any of the isolates. The bacterial inoculum was prepared as described above and positive (broth, antimicrobial and bacterial inoculum), negative (broth, extract or PAβN and bacterial inoculum) and sterility (broth and extract or PAβN) controls were included.

All of the MIC measurements for both assays were carried out in triplicate. The microtiter plates were incubated at 42 °C for 48 h under microaerobic conditions. Antibacterial activity was detected using a colorimetric method by adding 30 µL of resazurin staining (0.01%) aqueous solution in each well at the end of the incubation period (at least 4 hours before). The MICs were defined as the lowest concentration of plant extract or antimicrobials where no metabolic activity is seen after the incubation period, and they were determined by visual observation on the basis of change resazurin staining (living cells – red; dead cells – blue) (SALVAT et al., 2001). The synergistic effect was attributed to the combination between extract and antimicrobial that reduced the MIC when compared to activity of the individual antimicrobial (OLAJUYIGBE and AFOLAYAN, 2012).

2.6 Ethidium bromide accumulation assay

The ethidium bromide accumulation was used to investigate the capacity of the plant extracts to inhibit the efflux systems expressed in *Campylobacter* spp. This assay were performed as described by Lin et al. (2002) with some modifications. Briefly, the bacteria were grown on Columbia agar supplemented with charcoal and FBP at 42 °C for 24 h. The colonies were harvested in 15 mM PBS (8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4 and 0.24 g/L KH_2PO_4, pH 7.2 - Sigma–Aldrich, Saint Louis, USA), washed once, and resuspended in PBS (pH 7.2) to an absorbance of 0.2 at 625 nm. Aliquots of 55 µL of cell suspension were distributed to replica sets wells in 96-well microtiter plates and incubated at 37°C for 10 min. Then, ethidium bromide - EtBr (Sigma–Aldrich, Saint Louis, USA) was added to the cell suspension at a final concentration of 2 µg/mL, which was re-incubated at 37 °C for 7 min. After, the cell suspension containing EtBr were added of the plant extracts to a final concentration of ¼ MIC, and the fluorescence was measured at 1-min intervals for 30 min using a microplate reader (Bio tek Synergy HT, Bio tek, Vermont, USA) at excitation and emission wavelengths of 530 and 600 nm, respectively. Fluorescence of the cell

suspension was used as an indicator of the amount of EtBr taken up by the cells and its increase indicates the efflux pump inhibition (STAVRI et al., 2007). Each experiment was repeated three times. In each accumulation assay were included a positive control of the efflux inhibition using PAβN at a final concentration of 5 µg/mL, and a negative control containing only cell suspension and EtBr.

From the fluorescence data over a 30-min period of EtBr accumulation, the slope of the accumulation–time curve was calculated by linear regression analysis (Microsoft Excel; Microsoft Corp., Redmond, Washington, EUA). The significant differences in slope coefficients between extract and control curves for each isolate tested were determined by one-way analysis of variance (ANOVA) and Student T-test at a 5% level of significance. The greater the difference in the slope between the extract-containing assay and its relative control (without extract) was indicative of the activity of the extract to inhibit the efflux of EtBr.

2.7 Phytochemical analysis of extracts

A phytochemical screening of the plant extracts that showed better synergistic and accumulation activities was performed in order to characterize the main compound classes. Initially, the crude ethanolic extracts were resuspended in methanol HPLC grade (Merck, Darmstadt, Germany) for concentration of 5 mg/mL, filtered through a 0.45-µm-pore-size cellulose membrane, and injection volume of 15 µL was analyzed. High-performance liquid chromatography analysis was performed using Shimadzu UFLC system equipped with LC-20AD (Tokyo, Japan) binary pump, SPD-M20A diode array detector, SIL-A0AHT auto sampler, CBM-20A system communications bus module and CTO-20A column oven. Chromatographic separation was achieved on a Shim-pack VP-ODS (150 x 4.6 mm i.d.; 4.6 µm particle size) from Shimadzu with a guard column (10 x 4.6 mm). The mobile phase consisted of solvent A: pure water and solvent B: acetonitrile (Merck, Darmstadt, Germany); starting with linear gradient, initial percentage of A (95 – 5%) and B (5 – 95%) from 0 – 65 min; isocratic elution A (5%) and B (95%) from 65 – 70 min; linear gradient, A (5 – 95%) and B (95 – 5%) from 70 – 72 min; column temperature 40 °C, flow rate at 1.0 mL/min and UV-diode array detection at 254 nm. The

UV-Vis absorption spectra of the majority peaks of each extract were performed by scanning at 200 – 700 nm. The software Shimadzu Labsolutions was used to analyze the data.

3. RESULTS

High levels of similarity (77 to 100 %) were observed between the macrorestriction profiles of genomic DNA of the eight isolates using *SmaI* and *KpnI* restriction enzymes (Figure 1). The A52013 and E52013 isolates showed 100 % similarity for both restriction enzymes. All other isolates showed differences between macrorestriction patterns of *SmaI*-digested and *KpnI*-digested DNA. Despite of this genetic similarity between the isolates, preliminary tests showed differences in the phenotypic and genotypic profiles of antimicrobial resistance (Table 1).

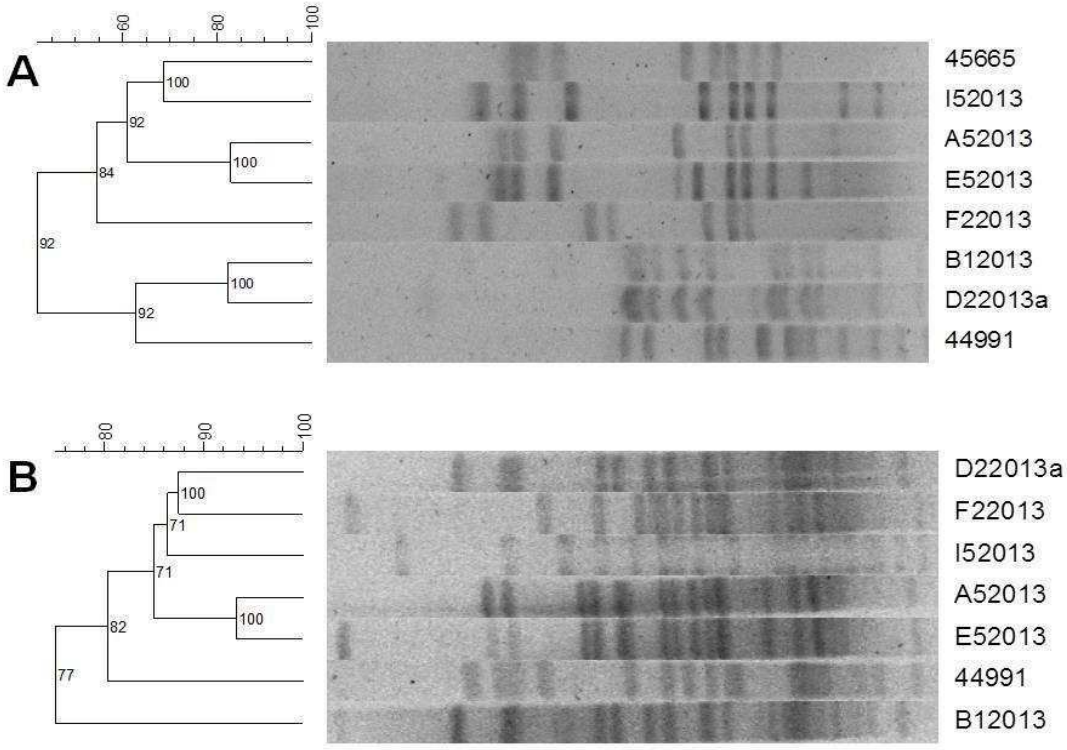

Figure 1. PFGE dendrograms based on band patterns of *SmaI*-digested DNA (A) and *KpnI*-digested DNA (B) from *Campylobacter* spp. isolated from chicken carcasses.

Note: The 45665 isolate was excluded of the dendrogram B (*KpnI*-digested DNA) because was not observed DNA digestion by this restriction enzyme.

A total of 20 ethanolic extracts of plants used in Brazilian folk medicine were produced and their yields ranged from 3 to 17.2 % g/g dry weight of plant (Table 2).

Table 2. Identification of plant species and yields of ethanolic extracts produced by ultrasonic method.

Family	Plants	Popular name	Voucher number	Yield (%)
Zingiberaceae	*Alpinia zerumbet*	Pacová	CESJ 49199	7.93
Annonaceae	*Annona muricata*	Graviola	CESJ 48236	6.90
Annonaceae	*Annona squamosa*	Conde	CESJ 1797	4.33
Asteraceae	*Baccharis dracunculifolia*	Alecrim	CESJ 47482	12.57
Fabaceae	*Bauhinia forticata*	Pata de Vaca	VIC 41898	11.57
Poaceae	*Cymbopogon citratus*	Capim limão	CESJ 50052	7.00
Poaceae	*Cymbopogon winterianus*	Citronella	CESJ 55790	9.47
Cyperaceae	*Cyperus rotundus*	Tiririca	VIC 39499	4.17
Ebenaceae	*Diospyros kaki*	Caqui	CESJ 7469	5.17
Fabaceae	*Glycine max*	Soja	VIC 39503	7.40
Malvaceae	*Malva sylvestris*	Malva	VIC 39504	7.67
Lamiaceae	*Mentha arvensis*	Menta	CESJ 57544	3.60
Cucurbitaceae	*Momordica charantia*	Melão São Caetano	VIC 38569	8.33
Lamiaceae	*Ocimum gratissimum*	Alfavaca	CESJ 48248	3.33
Lauraceae	*Persea americana*	Abacate	CESJ 58465	5.37
Piperaceae	*Piper aduncum*	Jaborandi	CESJ 59018	10.00
Plantaginaceae	*Plantago major*	Tranchagem	VIC 38568	17.20
Loranthaceae	*Struthanthus flexicaulis*	Erva de passarinho	*	3.17
Asteraceae	*Vernonia condensata*	Necroton	*	3.03
Asteraceae	*Vernonia polyanthes*	Assapeixe	VIC 41474	4.80

VIC-UFV; CESJ - Herbarium Leopoldo Krieger; * Cataloging in progress.

All extracts were initially tested for antimicrobial activity against *Campylobacter* strains using the broth microdilution assay. These extracts showed weak antimicrobial activity against all isolates evaluated. The MICs were mostly very high and ranged from 0.1 to >16.38 mg/mL, with only two plant extracts (*A. zerumbet* and *M. sylvestris)* showing inhibitory concentrations of ≤ 4.10 mg/mL (Table 3). However, we observed synergic antimicrobial effects between the most of these plant extracts and bile salts. The MIC values of bile salts were decreased 2 - 16 fold to at least one of *Campylobacter* evaluated using subinhibitory concentrations of plant extracts (¼ MIC) (Table 4).

Table 3. Antimicrobial activity of ethanolic extracts of plant used in Brazilian folk medicine against *Campylobacter* spp..

Plants	Minimum inhibitory concentration (MIC - mg/mL) *Campylobacter* strains							
	A52013	**B12013**	**D22013a**	**E52013**	**F22013**	**I52013**	**45662**	**44991**
A. zerumbet	4.10	4.10	2.05	1.02	1.02	4.10	4.10	2.05
A. muricata	8.19	16.38	16.38	8.19	8.19	> 16.38	16.38	> 16.38
A. squamosa	8.19	16.38	> 16.38	> 16.38	16.38	> 16.38	16.38	> 16.38
B. dracunculifolia	8.19	16.38	16.38	> 16.38	4.10	> 16.38	16.38	16.38
B. forticata	4.10	8.19	16.38	4.10	8.19	8.19	16.38	16.38
C. citratus	4.10	16.38	16.38	2.05	4.10	16.38	8.19	8.19
C. winterianus	8.19	16.38	8.19	1.02	4.10	8.19	16.38	4.10
C. rotundus	> 16.38	> 16.38	> 16.38	16.38	16.38	> 16.38	8.19	> 16.38
D. kaki	16.38	> 16.38	> 16.38	8.19	16.38	> 16.38	8.19	> 16.38
G. max	4.10	16.38	8.19	16.38	0.26	> 16.38	4.10	16.38
M. sylvestris	0.26	4.10	4.10	0.1	0.26	2.05	0.51	1.02
M. arvensis	4.10	16.38	16.38	> 16.38	> 16.38	> 16.38	16.38	> 16.38
M. charantia	16.38	> 16.38	16.38	> 16.38	> 16.38	16.38	0.51	> 16.38
O. gratissimum	4.10	> 16.38	16.38	1.02	4.10	8.19	16.38	16.38
P. americana	4.10	4.10	4.10	8.19	8.19	16.38	16.38	8.19
P. aduncum	8.19	16.38	16.38	4.10	8.19	16.38	2.05	16.38
P. major	1.02	> 16.38	4.10	1.02	4.10	4.10	> 16.38	4.10
S. flexicaulis	> 16.38	> 16.38	> 16.38	> 16.38	16.38	> 16.38	> 16.38	16.38
V. condensata	4.10	8.19	8.19	1.02	4.10	4.10	4.10	8.19
V. polyanthes	8.19	16.38	16.38	2.05	8.19	16.38	4.10	8.19

Extract concentration used: 0.032 to 16.38 mg/mL.

The E52013 and 44991 isolates were affected by a larger number of extracts. The most interesting results were observed with *A. zerumbet* and *V. polyanthes*, which showed higher decreases in MIC for almost all isolates. However, none of these plant extracts showed better activity comparing to the efflux pump inhibitor (PAβN), which reduced the MIC of bile salts by up to 128-fold. Among the 20 plant extracts evaluated, only four (*A. squamosa*, *B. dracunculifolia*, *M. sylvestris* and *P. americana*) did not show synergism when combined with bile salts. In contrast a limited synergic effect was observed combining the plant extracts and ciprofloxacin. Only 2-fold MIC reductions were observed for all isolates evaluated, and this effect was comparable to those obtained with PAβN (Table 5).

Table 4. Synergic effect between bile salts and ethanolic extracts of plants used in Brazilian folk medicine against *Campylobacter* spp..

Plants	Minimum inhibitory concentration (MIC - mg/mL) *Campylobacter* strains							
	A52013	**B12013**	**D22013a**	**E52013**	**F22013**	**I52013**	**45662**	**44991**
Positive control	6.25	25	12.5	12.5	12.5	25	3.12	25
PAβN	0.39 (16x)	0.39 (64x)	0.39 (32x)	0.39 (32x)	0.195 (64x)	0.195 (128x)	0.195 (16x)	0.39 (64x)
A. zerumbet	0.78 (8x)	3.12 (8x)	3.12 (4x)	1.56 (8x)	3.12 (4x)	12.5 (2)	3.12	12.5 (2x)
A. muricata	6.25	25	12.5	1.56 (8x)	12.5	3.12 (8x)	1.56 (2x)	3.12 (8x)
A. squamosa	6.25	25	12.5	12.5	12.5	25	3.12	25
B. dracunculifolia	6.25	25	12.5	12.5	12.5	25	3.12	25
B. foticata	6.25	25	3.12 (4x)	6.25 (2x)	12.5	12.5 (2x)	3.12	25
C. citratus	1.56 (4x)	25	12.5	6.25 (2x)	6.25 (2x)	12.5 (2x)	1.56 (2x)	12.5 (2x)
C. winterianus	6.25	12.5 (2x)	12.5	6.25 (2x)	12.5	3.12 (8x)	1.56 (2x)	12.5 (2x)
C. rotundus	6.25	25	3.12 (4x)	6.25 (2x)	12.5	25	3.12	25
D. kaki	6.25	12.5 (2x)	12.5	12.5	12.5	25	3.12	12.5 (2x)
G. max	6.25	25	3.12 (4x)	6.25 (2x)	6.25 (2x)	25	3.12	6.25 (4x)
M. sylvestris	6.25	25	12.5	12.5	12.5	25	3.12	25
M. arvensis	6.25	25	12.5	12.5	12.5	12.5 (2x)	1.56 (2x)	3.12 (8x)
M. charantia	6.25	12.5 (2x)	6.25 (2x)	6.25 (2x)	3.12 (4x)	25	3.12	25
O. gratissimum	6.25	12.5 (2x)	3.12 (4x)	12.5	6.25 (2x)	25	3.12	6.25 (4x)
P. americana	6.25	25	12.5	12.5	12.5	25	3.12	25
P. aduncum	6.25	25	12.5	1.56 (8x)	12.5	1.56 (16x)	3.12	12.5 (2x)
P. major	3.12 (2x)	25	12.5	3.12 (4x)	12.5	12.5 (2x)	3.12	3.12 (8x)
S. flexicaulis	0.78 (8x)	12.5 (2x)	12.5	6.25 (2x)	6.25 (2x)	12.5 (2x)	1.56 (2x)	12.5 (2x)
V. condensata	6.25	25	3.12 (4x)	6.25 (2x)	6.25 (2x)	25	3.12	12.5 (2x)
V. polyanthes	0.78 (8x)	6.25 (4x)	3.12 (4x)	6.25 (2x)	12.5	25	3.12	3.12 (8x)

***** - The values in brackets represent the fold-reduction of the MIC when comparing with positive control. Concentrations used: bile salts - 0.05 to 25 mg/mL and extracts – ¼ MIC.

Table 5. Synergic effects between ciprofloxacin and ethanolic extracts of plants used in Brazilian folk medicine against *Campylobacter* spp..

Plants	Minimum inhibitory concentration (MIC - µg/mL)							
	A52013	**B12013**	**D22013a**	**E52013**	**F22013**	**I52013**	**45662**	**44991**
Positive control	4	8	8	8	8	4	8	4
PAβN	0.5 (8x)	8	4 (2x)	8	8	4	4 (2x)	2 (2x)
A. zerumbet	4	8	8	8	8	4	8	4
A. muricata	4	8	4 (2x)	8	8	4	8	2 (2x)
A. squamosa	2 (2x)	8	4 (2x)	8	4 (2x)	4	8	4
B. dracunculifolia	4	4 (2x)	8	8	4 (2x)	4	8	4
B. foticata	4	8	8	8	8	4	8	4
C. citratus	2 (2x)	8	4 (2x)	8	4 (2x)	4	4 (2x)	2 (2x)
C. winterianus	4	8	8	8	8	4	8	4
C. rotundus	4	8	8	8	8	4	8	4
D. kaki	4	8	8	8	8	4	8	4
G. max	4	8	8	8	8	4	8	4
M. sylvestris	4	8	8	8	8	2 (2x)	8	4
M. arvensis	4	8	4 (2x)	8	8	4	8	2 (2x)
M. charantia	4	4 (2x)	8	8	8	4	8	4
O. gratissimum	4	8	8	8	8	4	8	4
P. americana	4	8	8	8	8	4	4 (2x)	4
P. aduncum	4	8	8	4 (2x)	8	4	8	4
P. major	4	8	8	8	8	4	8	4
S. flexicaulis	2 (2x)	8	8	8	8	4	8	4
V. condensata	2 (2x)	8	8	4 (2x)	8	4	8	4
V. polyanthes	4	8	8	8	8	4	8	4

***** - The values in brackets represent the fold-reduction of the MIC when comparing with control. Concentrations used: ciprofloxacin - 0.125 to 512 µg/mL and extracts – ¼ MIC.

No antagonistic effect was observed between extracts and antimicrobials (bile salts and ciprofloxacin). In order to investigate the activity of the plant extracts against efflux pump systems of *Campylobacter* spp., we performed an ethidium bromide accumulation assay in the absence and presence of the synergistic extracts. Ten plant extracts showed effect on ethidium bromide accumulation in *Campylobacter* cells based on the slopes of the curves obtained by linear regression analysis of the results over assay time (Table 6). The *C. rotundus* and *S. flexicaulis* extracts were the more active against almost all isolates (Figure 2). In this assay, the PAβN also showed higher activity to inhibit efflux systems than the plant extracts.

Table 6. Regression coefficients obtained by linear regression analysis of the results of the ethidium bromide accumulation in *Campylobacter* cells.

Plants	Regression coefficient							
	A52013	**B12013**	**D22013**	**E52013**	**F22013**	**I52013**	**45662**	**44991**
PAβN	0.6736	0.4145	0.4442	0.5028	0.4181	0.2671	0.5536	0.4002
Control	0.1319	0.0561	0.0298	0.1669	0.0005	0.0035	0.0334	0.1141
A. zerumbet	-	0.1319	-	-	-	-	-	-
B. foticata	-	-	0.0592	-	0.2005	-	-	-
C. citratus	-	-	-	0.3784	-	-	-	-
C. winterianus	-	-	-	0.3569	-	-	-	-
C. rotundus	0.4257	0.2833	0.1696	-	0.2178	0.0785	0.2408	0.1306
D. kaki	-	-	0.1071	-	-	0.1055	0.2213	-
G. max	0.2533	-	0.0954	-	0.1828	-	0.2157	-
M. arvensis	0.4768	-	0.1265	-	-	-	-	-
S. flexicaulis	0.3667	0.1907	0.1081	-	-	0.1378	0.1519	-
V. condensata	-	0.1853	-	0.2692	0.3162	-	0.2455	-

(-) - No significant differences were noticed between regression coefficients of the extracts and control using the Student's t-test ($p > 0.05$).

The ethanolic extracts that had the best effects on antimicrobial synergism or ethidium bromide accumulation were analyzed by high performance liquid chromatography using UV-VIS photodiode array detector. The exploratory analysis of chromatographic fingerprints demonstrated great diversity of compounds in ethanolic extracts of *A. zerumbet, V. polyanthes, C. rotundus* and *S. flexicaulis* (Figure 3). The detection of intense peaks at wavelength of 254 nm and 360 nm suggests the extracts contain a great variety of complex phenolic compounds. These findings become more apparent when analysing absorption spectra of the peaks of *A. zerumbet* and *S. flexicaulis* extracts, which indicate the presence of flavonoids. The typical UV-Vis spectra of flavonoids include two absorbance bands, which are characterized for absorbing in the ranges 310 - 385 nm (Band A) and 250-295 nm (Band B) (Bohm, 1998). The strong presence of high polarity compounds with overlaying of peaks in the time retention less than 20 min was observed in *V. polyanthes, C. rotundus* and *S. flexicaulis* extracts.

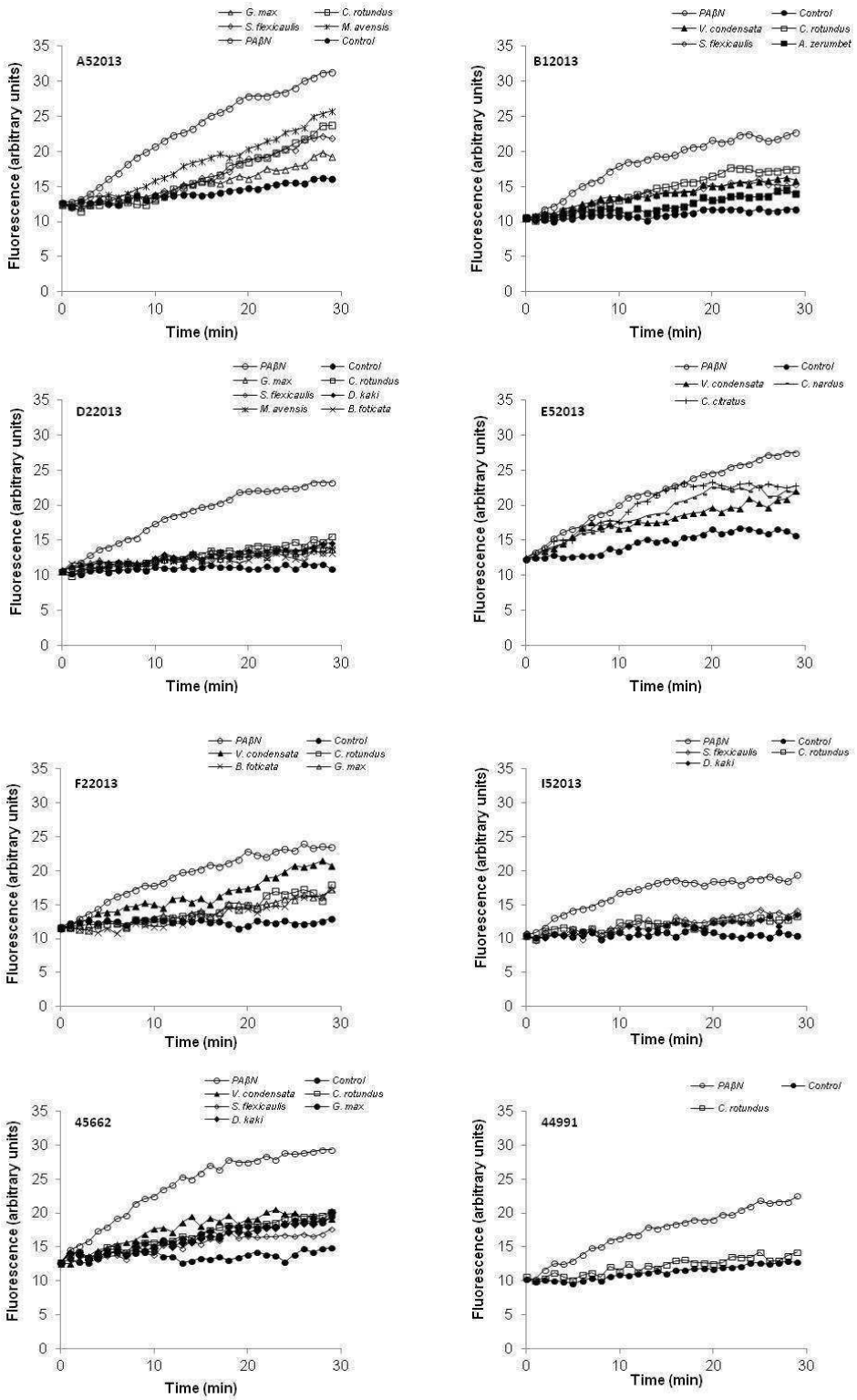

Figure 2. Effect of plant extracts on the accumulation of ethidium bromide (EtBr) (2 µg/mL) by *Campylobacter* spp. isolated from chicken carcasses.

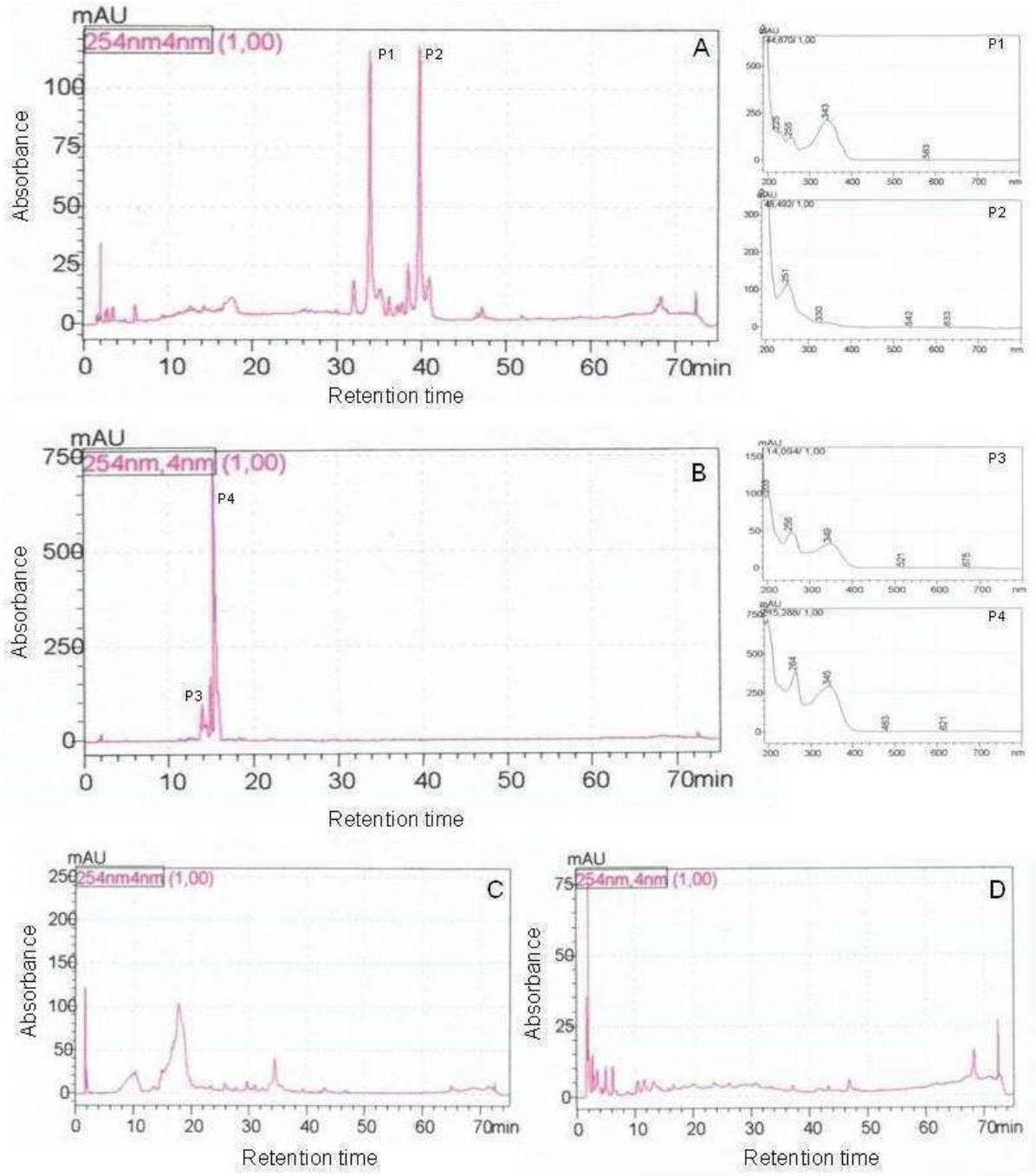

Figure 3. Analysis of ethanolic plant extracts by HPLC-PDA. Chromatographic fingerprint of *A. zerumbet* (A); *S. flexicaulis* (B); *V. polyanthes* (C) and *C. rotundus* (D) at 254 nm. UV-VIS spectra of major components (peaks) of *A. zerumbet* (P1 and P2), and *S. flexicaulis* (P3 and P4).

4. DISCUSSION

In the present study, we conducted a comprehensive evaluation of the antimicrobial and resistance-modifying activities of the ethanolic extracts from plants used in folk medicine in Brazil using *Campylobacter* spp. isolated from chicken carcasses. Overall, the used extracts had not significant antibacterial effects against the isolates tested. However, our results demonstrated synergistic effect of some extracts and bile salts and increase of ethidium bromide accumulation, which indicate the possible presence of efflux pump inhibitors.

The antimicrobial activity of several plant extracts and their derivatives against *Campylobacter* spp. has been documented by others authors (LEE et al., 2004; ASLIM and YUCEL, 2008; SAMIE et al., 2009; SUDJANA et al., 2009; DHOLVITAYAKHUN et al., 2011; ANDERSON et al., 2012; KUREKCI et al., 2012). However, the most plants used in this study had not yet been evaluated against this foodborne pathogen. Although previous studies reported effective antimicrobial activity of many of them against various bacteria, our results demonstrated little effect on *Campylobacter* spp. (OKUNADE et al., 1997; SAMUELSEN, 2000; JEONG et al., 2009; GONÇALVES et al., 2010; NAIK et al., 2010; KILANI-JAZIRI et al., 2011; PIDUGU and ARUN, 2012; SILVA et al., 2012, RAJALAKSHMI et al., 2013; SUPRAJA and USHA, 2013). The extracts exhibiting the greatest activity were obtained from *A. zerumbet* and *M. sylvestris*. These plants are widely used in folk medicine and contain various secondary metabolites including flavonoids and terpenoids, which could be responsible for their antibacterial activity (COWAN, 1999; CORREA et al., 2010, GASPARETTO et al., 2012). Antibacterial effectiveness of a particular plant species are reported to vary according to the geographical area of the plant, plant part, extraction method, composition of secondary metabolites, types of microbial strains and assay methods (COWAN, 1999; NOSTRO et al., 2000; ÖZKAN et al., 2009, METINER et al., 2012). In addition, the bacterial resistance to natural products such as plant extracts and derivates is frequent and may be attributed to efflux mechanisms (LORENZI et al., 2009; FADLI et al., 2011; KUETE et al., 2011). According to Klancnik et al. (2012b) these mechanisms are present in *Campylobacter* spp. and contribute significantly to its resistance

to the plant phenolic compounds. The majority of the efflux systems in bacteria are non-drug-specific proteins that can recognize and export a broad range of chemically and structurally unrelated compounds from bacteria without drug alteration or degradation (KUMAR and SCHWEIZER, 2005). The CmeABC efflux pump is the main responsible to resistance of *Campylobacter* spp. to great variety of antimicrobials, including antibiotics, bile salts and detergents (LIN et al., 2002). Given the great contribution of these efflux systems in the bacterial resistance, the inhibition of this mechanism appears to be a promising strategy for restoring the activity of existing antimicrobials (ZECHINI and VERSACE, 2009). In the last years, much attention has been paid on the search of natural compounds able to inhibit efflux systems of several bacteria (CHÉRIGO et al., 2009; FADLI et al., 2011; RAMALHETE et al., 2011, ROY et al., 2012). However, few studies have investigated natural sources of efflux pump inhibitors against *Campylobacter* spp. (KLANČNIK et al., 2012a; KLANČNIK et al., 2012b; KURINČIČ et al., 2012b). Thus, we performed synergy assays to evaluate the resistance-modifying activity of the ethanolic extracts in combination with bile salts and ciprofloxacin. Our results demonstrated that the most of ethanolic extracts increased the antibacterial activity of bile salts against *Campylobacter* spp., although their activities were variable and closely related to isolates tested. *A. zerumbet* and *V. polyanthes* extracts had the greatest synergistic effects. These data agree with several recent studies that have reported the potential of plant extracts and derivates to improve the effectiveness of antimicrobials against resistant Gram-negative bacteria (LORENZI et al., 2009; ADWAN et al., 2010; FADLI et al., 2011; LACMATA et al., 2012). The synergistic activity of some species and genera of plants evaluated in this study had already been described in the literature. For example, *M. charantia*, *M. arvensis* and *C. citratus* showed synergistic effects with antimicrobials against methicilin-resistant *Staphylococcus aureus*, *Escherichia coli* and *Salmonella* Typhimurium, respectively (SHIN, 2005; COUTINHO et al., 2008; COUTINHO et al., 2010). Some species of the *Alpinia, Vernonia,* and *Piper* genera also demonstrated synergism with antimicrobials against Gram-negative and Gram-positive bacteria (PUHL et al., 2011; WEERAKKODY et al., 2011; EUMKEB et al., 2010; EUMKEB et al., 2012; RAVULA et al., 2012). Our results suggest possible presence of compounds

that can acts as efflux pump inhibitors in the plant extracts presenting synergism with bile salts. The reduction of bile salts MIC in the presence of PAβN demonstrated that the efflux systems were active in all isolates, being involved in the resistance to these antimicrobials. Moreover, all isolates tested showed the three genes required to synthesis of CmeABC efflux pump, except for one of them (44991 number). The different effects of each extract on the isolates may be attributed to the diverse intrinsic characteristics of resistance existing between them. Although these isolates showed high genetic similarity as observed in the macrorestriction profiles of genomic DNA, some differences in the phenotypic and genotypic resistance to antimicrobials were observed. Regarding the interaction between ciprofloxacin and plant extract, no significant synergistic effect against the isolates was observed for any of the extracts evaluated. The same result occurred when PAβN was used instead of the extracts indicating that others mechanisms than the efflux systems are involved in ciprofloxacin resistance. In fact, all isolates used in this study showed a point mutation Thr-86-Ile in *gyr*A gene of DNA gyrase, which can confer resistance to ciprofloxacin (PIDDOCK et al., 2003). However, this resistance mechanism acts synergistically with CmeABC efflux pump raising levels of resistance to quinolones (GE et al., 2005, LUO et al., 2003). Therefore, the lack of synergistic activity between PAβN and ciprofloxacin may be attributed to the competition to drug efflux, in which ciprofloxacin should preferably be transported. Previous studies have demonstrated that the action mechanism of PAβN is through competitive inhibition with antibiotics on the efflux pump resulting in increased intracellular concentration of antibiotic (LOMOVSKAYA and BOSTIAN, 2006; ASKOURA et al., 2011). The preferential efflux of a compound instead of other may also has been the reason for the variation of synergistic effects between extracts and bile salts or ciprofloxacin.

The preliminary results have led to investigate whether the efflux pumps inhibition could be involved in the synergistic effect between bile salts and extracts. For this purpose, we performed an ethidium bromide accumulation assay and the results demonstrated that of the 20 plant extracts evaluated, 10 extracts showed activity against at least one *Campylobacter* isolate. *C. rotundus* and *S. flexicaulis* extracts were the more active against almost all isolates. These results suggest that these extracts contain compounds able to inhibit the

efflux systems of *Campylobacter* spp.. Many studies have showed that plants are a source of secondary metabolites for inhibiting of efflux systems of several bacteria (RAMALHETE et al., 2011; GRÖBLACHER et al., 2012; HOLLER et al., 2012; KURINČIČ et al., 2012b; ROY et al., 2012). However, this study represents the first evidence that *C. rotundus* and *S. flexicaulis* extracts may inhibit the efflux systems in *Campylobacter* spp. We observed that there was a strong agreement between the results of synergism and accumulation obtained from *S. flexicaulis* extract indicating that the increasing of bile salts susceptibility caused by this extract occurs via inhibition of efflux pump systems. Curiously, the *A. zerumbet* and *V. polyanthes* extracts that showed the better synergistic effects had no activity on ethidium bromide accumulation. These results suggest that the synergist effect observed between these extracts and bile salts occurs via other mechanisms than efflux systems. According to some authors the synergic effects between plant secondary metabolites and antimicrobials may occur through several mechanisms including the inactivation of drug-modifying enzymes, increasing of membrane permeability, and blocking drug efflux (HEMAISWARYA et al., 2008; WAGNER and ULRICH-MERZENICH, 2009; RADULOVIC et al., 2013). Here, we supposed that the synergistic effect of *A. zerumbet* and *V. polyanthes* extracts occurred due to increasing of membrane permeability.

The crude plant extracts contain a great diversity of secondary metabolites including alkaloids, flavonoids, terpenoids, tannins, and many others, which are responsible by their biological activities (BUCHANAN et al., 2000). In this study we performed a HPLC fingerprint to define the phytochemical profiles of the extracts that showed the better synergistic and EtBr accumulation results. We observed different chromatographic profiles for each extract evaluated. These results suggest that the biological activities identified in each plant extract could be attributed to different secondary metabolites. A wide variety of plant compounds have shown inhibitory activity of efflux pumps, particularly, those belonging to flavonoid and terpenoid classes (SMITH et al., 2007b; KUETE et al., 2010; RAMALHETE et al., 2011; HOLLER et al., 2012; KLANČNIK et al., 2012a; KURINČIČ et al., 2012b; ROY et al., 2012). The evaluation of chromatograms and UV-Vis absorption spectra indicate the presence of phenolic compounds in the extracts evaluated, mostly flavonoids, which could

be involved in the synergistic effect and blocking of EtBr efflux. This compound class was also reported by other authors as responsible for antimicrobial, resistance-modifying, and efflux-inhibiting activities of plant extracts against *Campylobacter* spp. (KLANČNIK et al., 2012a; KLANČNIK et al., 2012b; KURINČIČ et al., 2012b).

In conclusion, we demonstrate that the ethanolic extracts of plant used in Brazilian folk medicine show synergistic effect in combination with bile salts and act in the inhibiting of efflux systems of *Campylobacter* spp. Our findings suggest that these extracts may have potential to reduce the poultry colonization by *Campylobacter* spp. controlling its incidence in food production. In addition, they may act as therapeutics alternative in combination with antibiotics for clinical treatment of campylobacteriosis. These possibilities await investigations in future studies.

6. CONCLUSÃO GERAL

A prevalência e os níveis de contaminação de *Campylobacter* spp. em carcaças de frangos abatidos em Minas Gerais – Brasil foram baixos em comparação com outros estudos disponíveis na literatura. Entretanto, a maioria das estirpes isoladas apresentou alto índice de resistência a antimicrobianos, incluindo agentes utilizados no tratamento de campilobacteriose humana. Estes dados evidenciam a necessidade de um monitoramento frequente da prevalência e resistência antimicrobiana deste patógeno em carne de frango e subprodutos comercializados no Brasil por serem considerados os principais veículos de transmissão. Diferentes mecanismos foram identificados e confirmaram a resistência dos isolados, entre eles destacam-se a presença de mutações e modificações do sítio alvo, enzimas modificadoras de antimicrobianos e sistemas de efluxo. O uso de inibidor de sistemas de efluxo gerou uma redução limitada da resistência e não restaurou a atividade de alguns antimicrobianos avaliados, o que sugere que o efluxo ocorre de forma competitiva e múltiplos mecanismos estão envolvidos na resistência. Em contrapartida, os sistemas de efluxo tiveram uma considerável contribuição na resistência a sais biliares, o que indica sua importância na colonização das aves por *Campylobacter* spp.. Este mecanismo mostrou-e um alvo interessante para o controle deste patógeno na cadeia produtiva de aves. Neste sentido, propôs-se a investigação de extratos de plantas usadas na medicina popular brasileira em relação à capacidade de modulação da resistência a antimicrobianos por meio da inibição dos sistemas de efluxo expressos por *Campylobacter* spp. A maioria destes extratos apresentou efeito sinérgico em combinação com sais biliares, e alguns deles inibiram os sistemas de efluxo. Estes resultados sugerem que estes extratos de planta podem ser uma alternativa para reduzir a colonização de *Campylobacter* spp. no intestino das aves e, por consequência, sua incidência em alimentos. Além disso, eles apresentam potencial para uso como adjuvantes terapêuticos no tratamento clínico de campilobacteriose. Contudo, existe a necessidade de trabalhos futuros para caracterização mais detalhada dos extratos vegetais, isolamento e identificação dos princípios ativos, bem como estudos para avaliar sua atividade *in vivo*, a fim de desenvolver um produto que seja efetivo e possa ser utilizado de forma segura nas aplicações pretendidas.

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