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PÓS-GRADUAÇÃO EM ALIMENTOS E NUTRIÇÃO
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Tese apresentada ao Programa de Pós-Graduação em Alimentos e Nutrição, área de Ciências dos Alimentos, da Faculdade de Ciências Farmacêuticas - Universidade Estadual Paulista "Júlio de Mesquita Filho" como requisito parcial para a obtenção do título de doutor.

Orientador: Prof. Dr. Adalberto Farache Filho

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Maria Laura

A minha irmã,
Helga

Aos meus sobrinhos,
Sila, Matheus, Igor e Sarah

"Perdoem a cara amarrada
Perdoem a falta de abraço
Perdoem a falta de espaço
Os dias eram assim"

(Ivan Lins & Vitor Matins)

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"Pedras no caminho?
Guardo todas,
um dia vou construir um castelo..."

Fernando Pessoa

Resumo

O uso da contagem de heterotróficos em placa (CHP) como um parâmetro de qualidade para águas minerais engarrafadas ainda requer atenção e a variação quantitativa das bactérias durante a estocagem dessas águas precisa ser melhor entendida. Além disso, a hipótese da água mineral representar uma fonte de bactérias resistentes a antibióticos, dada a possibilidade de transmissão direta aos humanos, é preocupante. Este trabalho foi dividido em três capítulos; no primeiro capítulo temos uma introdução geral e revisão de literatura e no segundo e terceiro capítulos apresentamos os artigos resultantes das pesquisas realizadas. No segundo capítulo nosso trabalho teve como objetivo detectar variações quantitativas na CHP e na presença de microrganismos indicadores durante o período de validade de águas minerais brasileiras. Nenhuma variação foi detectada na presença de microrganismos indicadores (*E. coli*, coliformes totais, *P. aeruginosa* e enterococos), mas variações na CHP foram observadas em algumas marcas, o que sugere que a qualidade dessas águas podem estar sofrendo alterações. Embora nenhum limite seja estabelecido para CHP em água mineral, este estudo se baseou no limite de 500 unidades formadoras de colônias por mL de amostra (UFC/mL) e setenta e duas garrafas (22,22%) apresentaram níveis acima deste limite com valores de até 560.000 UFC/mL. Este estudo mostrou que o controle da CHP (<500 UFC/mL) em embalagem não retornável parece ser adequado para garantir a qualidade da água mineral durante o armazenamento. Os elevados valores de CHP e suas variações detectadas durante o armazenamento parecem justificar a necessidade de uma reavaliação do uso da CHP na gestão da qualidade da água mineral. Além disso, estudos mais detalhados sobre o risco potencial à saúde de alta CHP e de suas variações na água mineral também são necessários. No terceiro capítulo, a hipótese de que a água mineral pode representar uma fonte de bactérias resistentes a antibióticos para os seres humanos foi testada. Os fenótipos de resistência a antibióticos de bactérias cultiváveis presentes em nove lotes de duas marcas portuguesas e de uma marca francesa de águas minerais engarrafadas foram examinadas. Entre os 238 isolados bacterianos recuperados, a maioria das bactérias foram identificadas (com base na análise da sequência do gene 16S rRNA) como Proteobactérias das divisões Beta, Gama e Alpha. Bactérias resistentes a mais de três classes distintas de antibióticos foram detectadas em todos os lotes das três marcas de água em contagens de até 10^2 UFC/mL. Em todo o conjunto de isolados, observou-se resistência a todos os 22 antimicrobianos testados (ATB, BioMerieux e difusão em disco),

com a maioria dos isolados mostrando resistência a três ou mais classes de antibióticos. Bactérias com os mais altos índices de multirresistência eram membros do gênero *Variovorax*, *Bosea*, *Ralstonia*, *Curvibacter*, *Afipia* e *Pedobacter*. A presumível aquisição de resistência pelas bactérias das águas minerais é sugerida pela observação de bactérias da mesma espécie, mas isoladas de marcas diferentes, exibindo distintos perfis de resistência a antibióticos. A água mineral engarrafada foi confirmada como uma possível fonte de bactérias resistentes a antibióticos, com potencial de ser transmitidas para os humanos.

Palavras-chave: Água mineral. Contagem em placa de heterotróficos. Qualidade da água. Água potável. Bactérias resistentes a antibióticos.

Abstract

The use of heterotrophic plate count (HPC) as a quality parameter for bottled mineral water still requires attention and the variation of the bacteria during storage of these waters must be better understood. Furthermore the hypothesis that bottled mineral waters may represent a source of antibiotic resistant bacteria, given the possibility of direct transmission of bacteria to humans is worrying. This work was divided into three chapters, the first chapter we have a general introduction and review of literature and the second and third chapters present the articles. In the second chapter of our work aimed to detect quantitative variations in the HPC and in the presence of indicator microorganisms during their shelf life of Brazilian mineral water. No variations were identified in the presence of indicator microorganisms (*E. coli*, coliformes totais, *P. aeruginosa* and enterococos), but variations in HPC were observed in some brands, which suggests that changes may be occurring in the water quality during storage. Although no limit is set for HPC in mineral water, this study relies on the limit of 500 colony-forming units per mL of sample (CFU/mL) and seventy-two bottles presented levels above this limit and up to 560,000 CFU/mL. This study showed that the control of HPC (< 500 CFU/mL) for non-returnable packaging seems to be adequate to ensure the quality of mineral water during storage. The high values of HPC and the variations detected during storage seem to fully justify the need for a reevaluation of the use of HPC in bottled mineral water quality management. Furthermore, more detailed studies on the potential health risk of HPC and its variations in mineral water are also needed. In the third chapter, the hypothesis that the mineral water can be a source of antibiotic resistant bacteria to humans was tested. The antibiotic resistance phenotypes of the cultivable bacteria present in nine batches of two Portuguese and one French brands were examined. Among the 238 isolates recovered most were identified (based on 16S rRNA gene sequence analysis) as Proteobacteria of the divisions Beta, Gamma and Alpha. Bacteria resistant to more than three distinct classes of antibiotics were detected in the all the batches of the three water brands at densities in counts up to 102 CFU/mL. In the whole set of isolates, it was observed resistance against all the 22 antimicrobials tested (ATB, bBioMéerieux and disc diffusion), with most of the species isolates showing resistance to three or more classes of antibiotics. Bacteria with the highest multi-resistance indices were members of the genera *Variovorax*, *Bosea*, *Ralstonia*, *Curvibacter*, *Afipia* and *Pedobacter*. Presumable acquired resistance may be suggested by the

observation of bacteria taxonomically related but isolated from different brands, exhibiting distinct antibiotic resistance profiles. Bottled mineral water was confirmed as a possible source of antibiotic resistant bacteria, with the potential to be transmitted to humans.

Keywords: Mineral water. Heterotrophic plate count. Water quality. Drinking water. Antibiotic resistance.

SUMÁRIO

CAPÍTULO 1

1 INTRODUÇÃO

A água mineral natural é considerada como a água obtida diretamente de fontes naturais ou por extração de águas subterrâneas. Ela pode ser claramente distinguida das outras águas pela sua natureza, que é caracterizada pelo conteúdo definido e constante de determinados sais minerais, oligoelementos e outros constituintes considerando as flutuações naturais, como também pelo fato de que não pode ser tratada, preservando as qualidades originais da água subterrânea, que deve ser protegida da poluição (BRASIL, 2006; CAC, 2001).

O crescimento nas vendas de água mineral engarrafada tem mostrado um aumento consistente e substancial durante a última década. Acompanhando este aumento vieram mudanças significativas na legislação e nos regulamentos que regem a produção dessas águas e também na opinião pública que impulsiona o mercado. Orientações também têm sido desenvolvidas para garantir que o produto mantenha os mais altos padrões possíveis exigidos pelo consumidor (LECLERC; DA COSTA, 2005).

O maior motivo do aumento no consumo de água mineral é a dúvida em relação a qualidade da água de abastecimento público. Entretanto, pode não ser verdadeira a afirmação de que a água mineral apresenta qualidade microbiológica superior a das águas de abastecimento público, apesar de sua origem em mananciais subterrâneos (HUNTER, 1993). A água mineral engarrafada já foi implicada em surtos de cólera (BLAKE et al., 1977), incidências relatadas de “diarréia dos viajantes” e febre tifóide (PAVIA, 1987; ROSENBERG, 2003). Existem também relatos de potenciais patógenos como bactérias entéricas (SCHINDLER, 1994), protozoários (RIVERA et al., 1981; SALAZAR; MOURA; RAMOS, 1982), bactérias acido láticas (CAROLI et al., 1985; PAPAPETROPOULOU; TSINTZOU; VANTARAKIS, 1997) e ameba (ROSENBERG, 2003) sendo detectados em água mineral engarrafada.

A microbiologia das águas minerais engarrafadas depende das propriedades geoquímicas e hidrogeológicas do aquífero e também da ecologia microbiana das águas subterrâneas. As populações bacterianas que são, de longe, os membros mais abundantes da comunidade das águas subterrâneas, vivem com poucos nutrientes e assim se tornam mais resistentes ao estresse ambiental. Além disso, estas bactérias não requerem fatores de crescimento tais como vitaminas, aminoácidos ou nucleotídeos (LECLERC; DA COSTA, 2005).

Como as águas minerais não podem ser submetidas a nenhum tratamento que modifique seus componentes biológicos é esperado que nela encontrem-se bactérias autóctones. Após o engarrafamento, estas bactérias autóctones se multiplicam rapidamente (LECLERC; MOREAU, 2002; ROSENBERG, 2003). Devido a isto, as legislações específicas para águas minerais como o "Code of Hygienic Practice for collecting, processing and marketing of natural mineral Waters" (CAC, 1985) e a legislação brasileira RDC n° 275 (BRASIL, 2005) não estabelecem limites para a CHP durante a comercialização dessas águas. Várias pesquisas realizadas não conseguiram identificar nenhum risco microbiológico em relação a presença de elevada CHP em água potável (EDBERG; ALLEN, 2004; OTTERHOLT; CHARNOCK, 2011; VARGA, 2011). Entretanto algumas espécies presentes na CHP podem causar doenças em indivíduos imunodeficientes (OTTERHOLT; CHARNOCK, 2011; VARGA, 2011). Também se sabe que altos níveis de crescimento microbiano podem afetar o sabor e odor da água além de indicar a presença de nutrientes e biofilmes (SARTORY, 2004). Além disso, é conhecido que o longo tempo de estocagem da água mineral engarrafada tem um profundo efeito na variação da população bacteriana, o que pode indicar alterações na qualidade da água (LECLERC; MOREAU, 2002; MORAIS; DA COSTA, 1990). Assim o uso da contagem de heterotróficos em placa (CHP) como um parâmetro de qualidade para águas minerais ainda requer atenção e a variação da população bacteriana durante a estocagem precisa ser melhor entendida.

Outro problema em relação à alta CHP em água mineral engarrafada é a presença das bactérias resistentes a múltiplos antibióticos, o que já foi detectado por alguns pesquisadores como Mary et al. (2000); Massa et al. (1995); Messi et al. (2005); Rosenberg e Hernandez Duquino (1989). Diferentes autores acreditam que o uso intensivo de antimicrobianos contribuiu para um importante aumento de bactérias resistentes a antibióticos observado em ambientes aquáticos (BAQUERO et al, 2008; MESSI et al, 2005). Estas bactérias representam um reservatório de determinantes de resistência, bem como um meio para a propagação e evolução de genes de resistência e dos seus vectores (BAQUERO et al., 2008).

Este trabalho teve como objetivo detectar variação na contagem de heterotróficos em placa e na presença de microrganismos indicadores durante o período de validade das águas minerais engarrafadas e testar se a água mineral engarrafada pode representar uma fonte de bactérias resistentes a antibióticos, dada a possibilidade de transmissão direta das bactérias aos humanos. Este trabalho foi dividido em três Capítulos. No capítulo 1 estamos apresentando uma introdução geral e uma revisão de literatura dos principais assuntos abordados. O capítulo 2 apresenta a variação na contagem de heterotróficos em placa e na

presença de microrganismos indicadores durante o período de validade de diversas marcas de águas minerais brasileiras em garrafas de 0.5, 1.5 e 20 litros. A qualidade microbiológica dessas águas e o limite da CHP também são avaliados e discutidos. O capítulo 3 caracteriza a diversidade de bactérias cultiváveis e o seu fenótipo de resistência à antibióticos em duas marcas de águas minerais portuguesas e uma marca francesa.

2 REVISÃO BIBLIOGRÁFICA

2.1 A água mineral

A cultura de utilização das águas minerais teve origem na Roma antiga com os banhos medicinais. No entanto, o comércio de águas minerais foi regulamentado na França no século XVII mas foi ao longo do século XIX que nasceu realmente a indústria de envasamento de água mineral (FINLAYSON, 2005; MACÊDO, 2007). Inicialmente sua venda era feita em farmácias pois, essencialmente, sua função era medicinal; a igreja reconhecia as qualidades terapêuticas "milagrosas" das águas minerais e colocava as fontes sob a proteção de um santo, o que justifica a maioria do nome das fontes (MACÊDO, 2007).

No século XX, até o ano de 1968, a produção brasileira de água engarrafada manteve-se estável; a partir daí iniciou-se uma nova fase no mercado com o lançamento do garrafão de vidro de 20 litros que possibilitou sua ampliação. Em 1970, outra novidade da indústria de águas minerais que conquistou o consumidor foi a garrafinha plástica de polietileno de baixa densidade - PEBD. Em 1979, o crescimento do mercado aumentou ainda mais com a introdução do garrafão de 20 litros de Policarbonato (MACÊDO, 2007).

Nos últimos anos ocorreu um grande aumento no consumo de água mineral em todo o mundo. Durante 1994 a 2002, o mercado mundial de água engarrafada cresceu de 58 para 144 bilhões de litros (FINLAYSON, 2005). Em 2007, foram consumidos 206 bilhões de litros e a estimativa de consumo para 2011 era de 250 bilhões de litros. A água mineral está substituindo a água de abastecimento público nas casas, escritórios e escolas, entre outros. Acompanhando essa tendência mundial o mercado brasileiro de águas minerais continua em franco crescimento; no ranking dos dez maiores produtores o Brasil ficou na 7ª posição, com 10 bilhões de litros (ABINAM, 2010).

No entanto, os brasileiros ainda consomem menos água mineral que em muitos outros países, com média de 20 litros de água mineral por ano; na Argentina essa média é de 70 litros, na Itália 150 e na França são 160 litros. Essa falta de hábito do brasileiro em consumir água mineral é, provavelmente, o principal motivo do interesse das multinacionais em nosso país que, por meio de campanhas publicitárias, tentam incentivar o consumo com o apelo dos benefícios que a água pode trazer à saúde. A restrição ao uso das garrafas plásticas nos EUA e

na Europa também faz com que as multinacionais optem pelo Brasil (REVISTA GUIA DE MERCADO DE ÁGUAS, 2010).

Existem duas teorias clássicas sobre a origem das águas minerais, a teoria da origem meteórica que admite ser a água mineral proveniente da água das chuvas infiltradas a grandes profundidades e a teoria da origem magmática que explica a origem dessas águas a partir de fenômenos magmáticos como o vulcanismo. Com os conhecimentos que se tem sobre a distribuição da água no planeta, a primeira teoria é a mais aceita (VAITSMAN; VAITSMAN, 2005). Segundo a teoria meteórica, as águas minerais e as águas subterrâneas têm a mesma origem; são águas de superfície que infiltram no subsolo e cujo conteúdo em sais guarda uma relação direta com o calor, pois a capacidade de dissolverem minerais e incorporarem solutos aumentam com a temperatura. Considera-se como água mineral aquela que conseguiu atingir profundidades maiores e, dessa forma, se enriqueceu em sais, adquirindo novas características físico-químicas (RIEDEL, 1992). As águas minerais provêm, principalmente, de aquíferos intermediários situados a aproximadamente 300 m de profundidade, separados dos aquíferos superficiais por camadas limitantes. Neles, a água mineral fica represada em uma área bem definida e protegida, cuja composição química, temperatura e taxa de vazão são geralmente estáveis (MAIER; PEPPER, 2000). Dependendo da qualidade dos sais que possui a água pode ser benéfica para o tratamento de determinados problemas de saúde. Porém, na grande maioria dos casos, essas águas têm apenas discreto poder diurético ou alcalinizante e as campanhas promocionais é que enaltecem seu poder medicinal (RIEDEL, 1992).

As águas minerais podem aflorar na superfície terrestre sob a forma de fontes naturais ou, mais comumente, são captadas artificialmente através de perfuração de poços tubulares (VAITSMAN; VAITSMAN, 2005). A exploração de água mineral no Brasil obedece ao Código de Mineração e ao Código de Águas Minerais que constituem os instrumentos básicos legais reguladores da pesquisa e da lavra dessas águas no território nacional (DNPM, 2005). A coleta deve ser realizada sob condições que garantam a manutenção das características originais da água no poço ou fonte; essas águas devem permanecer estáveis dentro dos limites de flutuação, sem influência direta de águas superficiais (BRASIL, 2006; CAC 1985).

Além da implementação de várias medidas de higiene durante a captura e embalagem da água mineral tais como saneamento dos equipamentos e cuidados no armazenamento dos materiais de embalagem, cuidados especiais devem ser tomados durante o armazenamento e transporte do produto final. As garrafas cheias devem ser armazenadas e transportadas em condições que excluam a possibilidade de contaminação e proliferação microbiana e protejam o produto e sua embalagem de danos e de deterioração (BRASIL, 2006; CAC, 1985).

2.2 Bactérias autóctones e sua multiplicação após o engarrafamento das águas minerais

A água mineral ao atravessar superfícies de rochas tem suas moléculas orgânicas filtradas. O resultado são águas com baixo conteúdo de nutrientes orgânicos que são necessários para a multiplicação dos microrganismos. Nesse processo elas também perdem apenas parte de suas bactérias (STICKLER, 1989).

A presença de bactérias autóctones na água mineral tem dado origem a uma série de questões sobre seus efeitos a saúde, principalmente porque estas bactérias ainda não estão muito bem caracterizadas (LECLERC; DA COSTA, 2005).

As características geoquímicas e hidrogeológicas do aquífero influenciam a abundância e diversidade da população bacteriana das águas minerais (MORAIS; DA COSTA, 1990). As bactérias frequentemente isoladas das águas minerais são os heterotróficos aeróbios pertencentes principalmente a classe Gammaproteobactéria, mas também as classes Alpha- e Betaproteobactérias como as *Pseudomonas fluorescens*, *Pseudomonas stutzeri*, *Brevundimonas spp.*, *Burkholderia cepacia*, *Stenotrophomonas maltophilia*, *Ralstonia pickettii*, *Comamonas spp.*, *Sphingomonas paucimobilis*, *Acinetobacter spp.* e *Alcaligenes spp.* (LECLERC; DA COSTA, 2005). Ao contrário do que foi encontrado por Loy et al. (2005) onde as espécies dominantes eram as betaproteobacterias pertencentes aos gêneros *Hydrogenophaga*, *Aquabacterium*, *Polaromonas*, *Rhodoferax* e *Limnobacter* (todas da ordem Burkholderiales) e as alphaproteobacterias do gênero *Caulobacter* e *Bradyrhizobium*. Otterholt e Charnock (2011) também encontraram que a populaçao bacteriana era dominada por Beta- e Alphaproteobacterias e todas as betaproteobacterias eram da ordem Burkholderiales. Em outros estudos a população bacteriana prevalente consistia de *Pseudomonas*, juntamente com espécies de *Acinetobacter*, *Achromobacter* e *Flavobacterium* (MESSI et al., 2005; ROSENBERG; HEMANDEZ-DUQUINO, 1989; URMENETA et al., 2000).

A contagem de microrganismos autóctones, que embora se encontrem num nível baixo enquanto a água está em seu ambiente natural, logo após o engarrafamento começam a se multiplicar rapidamente (LECLERC; MOREAU, 2002; ROSENBERG, 2003; SCHMIDT-LORENZ, 1976; STICKLER, 1989).

Alguns autores estudaram as causas da multiplicação dos microrganismos após o engarrafamento das águas minerais. Para Schimdt-Lorenz (1976), este fenômeno pode ser devido ao aumento da oxigenação durante as operações de engarrafamento e também devido

ao contato com compostos orgânicos que podem estar presentes em tubulações, reservatórios, embalagens e tampas. Para outros autores essa multiplicação é causada pelo aumento da superfície devido ao engarrafamento, eles acreditam que os nutrientes presentes em baixas concentrações na água mineral são adsorvidos e concentrados na superfície da garrafa, estando mais disponíveis para as bactérias. (BISCHOFBERGER et al., 1990; JAYASEKARA et al., 1998; SCHIMDT-LORENZ, 1976; ZOBELL; ANDERSON, 1936). Para Jones et al. (1999) a rugosidade da superfície do frasco é muito importante na determinação da adesão da bactéria à superfície e assim influencia na multiplicação bacteriana. A temperatura de estocagem também é um fator muito importante para a multiplicação. (SCHIMDT-LORENZ, 1976). Todos estes fatores podem ser fomentados devido ao longo tempo que as garrafas ficam estocadas. Uma vez a garrafa preenchida e selada, a água engarrafada pode ficar nas prateleiras dos mercados ou estocadas em casa por meses e até um ano. (ROSENBERG, 2003).

A multiplicação bacteriana em águas engarrafadas caracteriza-se por uma alternância de aumentos e diminuições da população cuja razão não se conhece exatamente. Para explicar este fato existem algumas teorias que consideram que os produtos da autólise das células mortas são utilizados como nutrientes para as novas populações que são compostas por espécies quase sempre diferentes (SCHIMDT-LORENZ, 1976). Por outro lado, alguns autores estão pesquisando se essa multiplicação das bactérias na água depois do engarrafamento é devido à reativação de um grande número de células não cultiváveis presente na fonte de água ou no sistema de engarrafamento, ou se ela é resultado da divisão celular e multiplicação de poucas células cultiváveis inicialmente presentes (LECLERC; MOREAU, 2002).

Bischofberger et al. (1990) concluíram que, após uma semana de engarrafamento, ocorreu uma mudança na composição da população bacteriana da água mineral. O longo tempo de estocagem da água mineral tem um profundo efeito na variação da população bacteriana o que pode refletir mudanças nos nutrientes disponíveis, um decréscimo na viabilidade de algumas cepas, competição, antagonismo ou outros fatores ainda não identificados. Os produtores e as agências reguladoras normalmente avaliam a qualidade da água mineral em termos da presença de indicadores e patógenos. Portanto, mudanças na população bacteriana após o engarrafamento ou durante a estocagem podem também indicar alterações na qualidade da água mineral. Os produtores deveriam examinar e caracterizar a população bacteriana na fonte e durante a estocagem da água engarrafada como uma adição ao controle do processo (MORAIS; DA COSTA, 1990).

2.3 Padrões de qualidade e a contagem de heterotróficos em placa

O "Codex Alimentarius Comission (CAC)", que regulamenta a qualidade microbiológica de alimentos mundialmente, estabelece em seu regulamento específico para águas minerais "Code of Hygienic Practice for collecting, processing and marketing of natural mineral Waters" limites para os microrganismos indicadores: coliformes totais, *Escherichia coli* (*E. coli)*, *Pseudomonas aeruginosa* (*P. aeruginosa)*, *Clostridium perfringens* e enterococos (CAC, 1985), o mesmo ocorre na legislação brasileira para águas minerais que se baseia nele (BRASIL, 2005).

O grupo dos coliformes totais é um indicador das condições higiênicas do processo e sua enumeração é muito utilizada em indústrias alimentícias, indicando contaminação pós-sanitização ou pós-processo, evidenciando práticas de higiene e sanificação aquém dos padrões requeridos para o processamento de alimentos (SILVA; JUNQUEIRA; SILVEIRA, 1997). A ocorrência de *E. coli* é considerada um indicador específico de contaminação fecal e indica a possível presença de patógenos entéricos (APHA, 1998; STICKLER, 1989; WHO, 2011).

P. aeruginosa é capaz de crescer abundantemente até em águas puras e sua ocorrência em água mineral deve ser evitada por duas razões; de um lado, como indicadora da vulnerabilidade ou controle deficiente do ambiente de envase e, de outro, pelo fato de ser um patógeno oportunista (LECLERC; MOREAU 2002). No período de armazenamento, a multiplicação dessas bactérias pode conduzir a níveis altos de *P. aeruginosa* na água envasada, representando grande risco para os consumidores (LEGNANI et al. 1999) e ela é freqüentemente associada com alterações no sabor, odor e turbidez da água (STIKCLER, 1989; WHO, 2011). Além disso, muitas *Pseudomonas spp*. recuperadas de águas potáveis são resistentes a múltiplos antibóticos (HERNANDEZ-DUQUINO; ROSENBERG, 1987).

Os enterococos são encontrados em fezes humanas e de animais mas em menor número que os coliformes totais ou fecais, no entanto, são mais resistentes que estes (APHA, 1998; WHO, 2011). Em águas não tratadas, são usados como indicadores de patógenos que sobrevivem mais que a *E. coli* (WHO, 2011).

As bactérias heterotróficas são aquelas que utilizam a matéria orgânica ou compostos orgânicos como fonte de carbono para seu crescimento e para a síntese de material celular (TORTORA et al., 2004). A contagem em placas é utilizada para se estimar a população de bactérias heterotróficas aeróbias e anaeróbias facultativas presentes na água, que têm a

capacidade de se desenvolver nas condições de nutrição, temperatura e tempo de incubação definidos para o teste (LECLERC; DA COSTA, 2005).

Legislações específicas para águas minerais como o "Code of Hygienic Practice for Collecting, Processing and Marketing of Natural Mineral Waters" (CAC, 1985) e a legislação brasileira RDC n° 275 (BRASIL, 2005) não estabelecem limites para a CHP durante a comercialização dessas águas, diferentemente do que ocorre com a legislação para água destinada ao consumo humano proveniente de abastecimento público que estabelece um limite de 500 UFC/mL para este grupo de bactérias (BRASIL, 2004; US EPA, 2009). Acredita-se que a presença de elevado número de bactérias heterotróficas em águas minerais engarrafadas pode ser decorrente da microbiota natural da fonte que como vimos anteriormente pode se multiplicar após o envase. A ausência de um desinfetante residual, como o cloro e períodos longos de armazenamento à temperatura ambiente ou mais alta, podem elevar ainda mais o número destas bactérias até o consumo.

Algumas das bactérias heterotróficas encontradas nas águas minerais produzem fatores de virulência e podem agir como patógenos oportunistas (PAVLOV et al., 2004). Entretanto, as evidências clínicas e epidemiológicas são insuficientes para concluir que altas CHP em água oferece risco à saúde dos consumidores (ALLEN; EDBERG; REASONER, 2004; EDBERG; ALLEN, 2004; STELMA JR. et al., 2004). O fato é que altos níveis de multiplicação microbiana podem afetar o sabor e odor da água e podem indicar a presença de nutrientes e biofilme (SARTORY, 2004). Portanto, acredita-se que este grupo de bactérias deve ser usado como um indicador de qualidade da produção da água mineral e não como um indicador de risco a saúde (ALLEN; EDBERG; REASONER, 2004). Além disso, a CHP em água mineral ajuda a garantir que, a partir da fonte até o produto acabado, nenhuma mudança quantitativa ocorreu na população bacteriana da água pois a variação das contagens pode dar um aviso prévio de alteração da qualidade (LECLERC; MOREAU, 2002).

2.4 Bactérias resistentes a antibióticos

A propagação aparentemente sem controle de múltipla resistência a antibióticos em microrganismos patogênicos clinicamente relevantes é alarmante (DANTAS et al., 2008). A seleção de cepas resistentes aos antibióticos tem sido acelerada pela prescrição imprudente e uso excessivo dos quimioterápicos, particularmente agravada pelo uso generalizado de

quantidades sub-terapêuticas de antibióticos como promotores de crescimento em animais. Junto com a descoberta dessas múltiplas resistências, nos últimos anos, um conjunto diversificado de mecanismos moleculares da resistência aos antibióticos microbianos foi elucidado junto com fortes provas da transferência destes mecanismos entre diferentes microrganismos (SOMMER et al., 2009).

Os principais mecanismos de resistência a antibióticos são: 1 - Redução da permeabilidade da membrana, algumas bactérias modificam a abertura da membrana plasmática (porinas) de modo que os antibióticos são incapazes de entrar no espaço periplasmático. 2 - Modificação no alvo do antibiótico, pequenas modificações neste alvo podem neutralizar os efeitos dos antibióticos sem significativamente afetar a função celular. 3- Remoção ativa de antibióticos de dentro da célula, mecanismo este chamado de bombas de efluxo. As bombas de efluxo fazem com que o antibiótico seja rapidamente bombeado para fora da célula, diminuindo significativamente a eficácia do antibiótico. 4- Produção de enzimas que degradam a composição química dos medicamentos, assim o microrganismo pode ser capaz de modificar o antibiótico, que passa a apresentar uma forma inativa. (TORTORA et al., 2004).

A resistência bacteriana pode ser natural ou ser adquirida. A resistência natural ou intrínseca faz parte das características biológicas primitivas dos microrganismos e por ser previsível, uma vez identificada, tem menor relevância clínica na atualidade considerando-se a multiplicidade de substâncias antimicrobianas disponíveis para o tratamento de infecções bacterianas. A resistência adquirida a um determinado antibiótico é aquela que surge em uma bactéria primitivamente sensível a esse mesmo antibiótico. Refere-se, portanto, ao aparecimento em um determinado momento, de exemplares de uma espécie bacteriana que não mais sofrem a ação de medicamentos que se mostraram efetivos contra a população original dessa bactéria. Esse tipo de resistência é mais importante que a anterior devido à crescente participação de microrganismos com resistência adquirida nos quadros clínicos infecciosos em humanos e animais (PRESCOTT; BAGGOT, 1988).

Os genes que codificam os mecanismos de resistência aos antibióticos estão localizados no cromossomo bacteriano ou nos plasmídeos extracromossomais. Estes genes são transmitidos para as próximas gerações (transferência vertical do gene) como também podem ser trocados entre bactérias de diferentes associações taxonômicas (transferência horizontal do gene) (DAVIDSON, 1999).

A transferência horizontal é muito comum na natureza; ela ocorre quando um dado microrganismo recebe material genético de outro microrganismo, passando a expressar a

característica contida no gene recentemente adquirido. Esse material genético pode ser transferido por transformação, transdução ou conjugação (SUMMERS, 2006).

A transformação é um processo de incorporação de DNA exógeno proveniente, por exemplo, da lise de determinado microrganismo com liberação do seu material genético, ficando este disponível para que outra bactéria o incorpore no seu genoma. Esse DNA pode ser originário do cromossoma, de plasmídeos ou ainda de bacteriófagos e para que o processo ocorra a bactéria receptora tem de estar apta a receber esse material, no estado de competência, quando sintetiza proteínas de superfície que estabelecem a ligação ao DNA (SUMMERS, 2006).

A transdução envolve a incorporação acidental do DNA bacteriano cromossómico ou plasmídico por um bacteriófago durante o processo de infecção celular. Após a lise celular, o bacteriófago atua como um vetor e, ao infectar uma célula nova, pode introduzir o DNA contendo o(s) gene(s) de resistência, tornando-a resistente a determinado antibiótico. Teoricamente ocorre somente entre bactérias da mesma espécie (SUMMERS, 2006).

A conjugação é mediada por um tipo de plasmídeo (que possui o gene de resistência), um pedaço circular de DNA que se replica de forma independente do cromossomo e que são transmitidos de uma célula a outra durante a conjugação. A conjugação difere da transformação principalmente porque requer contato direto célula a célula. (TORTORA et AL., 2004). A conjugação é um mecanismo de transferência de gene mais comumente utilizado pelas bactérias (DAVIDSON, 1999) e pode desempenhar um importante papel na disseminação de resistência a drogas em condições naturais (BAQUERO et al., 2008).

Recentemente, o uso desenfreado de antibióticos tem levado ao monitoramento dos resíduos desses no meio ambiente, devido ao fato dessa substância ser frequentemente encontrada em esgotos que podem atingir e contaminar os recursos hídricos e também devido ao fato de que alguns microrganismos presentes no ambiente criam resistência a esses fármacos (KÜMMERER et al., 2004).

Na medicina veterinária, os antibióticos podem ser utilizados como promotores de crescimento, no tratamento terapêutico na bovinocultura, utilizados também na produção avícola e intensivamente usados como aditivos de alimento de peixe na aquicultura e na criação de suínos. Sendo assim, podem contaminar o solo, águas superficiais e águas subterrâneas. A presença de antibióticos no ambiente aquático, oriundos da utilização pela medicina humana, ocorre através da disposição direta da droga excedente no esgoto, como também na excreção das drogas nas fezes e na urina que, na maioria das vezes, são

encaminhados para um sistema de tratamento de esgoto. (HALLING-SORENSEN et al., 1998)

As estações de tratamento de esgotos têm sido demonstradas como importante fonte de bactérias resistentes a antibióticos (NOVO; MANAIA, 2010). Dessa forma, bactérias resistentes a antibióticos são constantemente lançadas com as águas residuais nos recursos hídricos. Muitos destes organismos conduzem genes de resistência a antibióticos, eventualmente, inserida em plataformas genéticas móveis (plasmídeos, transposons e integrons) que são capazes de se espalhar entre a comunidade bacteriana da água e do solo. Portanto, a água constitui não só uma forma de disseminação de organismos resistentes aos antibióticos entre populações humana e animal, mas também o caminho pelo qual genes de resistência são introduzidos em ecossistemas bacterianos naturais. Em tais sistemas, bactérias não patogênicas podem servir como um reservatório de genes de resistência (BAQUERO et al., 2008).

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CAPÍTULO 2

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TITLE: Variations in heterotrophic plate count and in the presence of indicator microorganisms in bottled mineral water.

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Running title: Variations in bottled mineral water

ABSTRACT

Quantitative variations in heterotrophic plate count (HPC) and in the presence of indicator microorganisms in 0.5, 1.5 and 20-L bottles of different brands of Brazilian mineral water were analyzed during their shelf life. No variations were identified in the presence of indicator microorganisms, but quantitative variations in HPC were observed in some brands, which suggests that changes may be occurring in the water quality during storage. This study objectived to evaluate the quality of the bottled mineral waters and the presence of enterococci and *Pseudomonas aeruginosa* were verified in six and two bottles, respectively, which is in disagreement with the microbiological quality criteria established in the current legislation. Although no limit is set for HPC in mineral water, this study relies on the limit of 500 colony-forming units per mL of sample (CFU/mL). Seventy-two bottles presented levels above 500 CFU/mL and up to 560,000 CFU/mL. This study showed that the control of HPC (< 500 CFU/mL) for non-returnable packaging seems to be adequate to ensure the quality of mineral water during storage. The high values of HPC and its variations detected during storage seem to fully justify the need for a reevaluation of the use of HPC in bottled mineral water quality management. More detailed studies on the potential health risk of HPC and its variations in mineral water are also needed.

Keywords: Heterotrophic plate count; mineral water; water quality.

1. INTRODUCTION

Natural mineral water means microbiologically wholesome water, originating in an underground water table or deposit and emerging from a spring tapped at one or more natural or bore exits. It may not be subjected to any treatment aside from the separation of unstable constituents and the elimination, introduction or reintroduction of carbon dioxide (Codex, 2001). Sales of bottled mineral water have been increasing all over the world. Dissatisfaction with the odor and taste of tap water due to chlorine, greater consumer concern about the safety of tap water and the use of bottled mineral water as a substitute for other beverages may have contributed to this increase (Warburton, 2000). The successful promotion of bottled mineral water as clean, pure, safe and especially suitable for infants may also have increased its consumption (Bharath et al., 2003).

However, natural mineral waters are not sterile environments, but complex ecosystems with a high phenotypic and genetic diversity of autochthonous bacteria (Manaia et al., 1990; Rosenberg, 2003). The autochthonous bacteria remain low in terms of population while the water is in its natural environment, but soon after bottling they begin to multiply rapidly (Leclerc and Moreau, 2002; Rosenberg, 2003) and can reach counts of 10^4 to 10^5 colony-forming units per mL of sample (CFU/ml) within a few days (Leclerc and Moreau, 2002). However, despite this multiplication being considered normal (Rosenberg, 2003), the long storage time of mineral water has a profound effect on the variation of the bacterial population, which may also indicate changes in water quality (Leclerc and Moreau, 2002; Morais and Da Costa, 1990). The reasons for this multiplication after bottling are still a matter of debate. The influence of materials, the relative contributions of attached (e.g., biofilms) to unattached microbes (Bischofberger et al., 1990; Jayasekara et al., 1998; Schimdt-Lorenz, 1976; Zobell and Anderson, 1936), and the regrowth (from small initial populations) as opposed to resuscitation of existing microbes (Leclerc and Moreau, 2002) show the complexity of the issue. For these reasons, the study of variations of bacteria in bottled mineral water during storage can provide important data for excluding the possibility of microbial growth, thus protecting the product from deterioration and ensuring its quality to the consumer.

Because of the normal multiplication of autochthonous bacteria after bottling, the heterotrophic plate count (HPC) is not used as a quality parameter for bottled mineral water during marketing (ANVISA, 2005; Codex, 1985). Moreover, there is insufficient clinical and epidemiological evidence to conclude that high HPC in drinking water pose a risk to

consumer health (Edberg and Allen, 2004; Otterholt and Charnock, 2011; Varga, 2011). However, members of some species that may be part of the mineral water microbiota can cause diseases, mainly in vulnerable individuals, i.e., the very young, the elderly, the immune suppressed population, and pregnant women (Otterholt and Charnock, 2011; Varga, 2011). In addition, multiple antibiotic-resistant bacteria were isolated from bottled mineral water using HPC procedures (Mary et al., 2000; Massa et al., 1995; Messi et al., 2005; Rosenberg and Hernandez Duquino, 1989). It is also known that high levels of microbial growth can affect the taste and odor of drinking water and may well indicate the presence of nutrients and biofilms (Sartory, 2004). Therefore, HPC procedures are always recommended, although there is still much discussion about its importance to health (Rosenberg, 2003). It is believed that HPC should be used as an indicator in bottled water production, as it is in public water supply (Allen et al., 2004).

All those studies suggest that variations in bacteria counts in bottled mineral water may occur during storage, which may indicate changes in water quality, and that the use of HPC as a quality parameter for bottled mineral water still requires attention. Thus, the aim of the current study was to detect quantitative variations in HPC and in the presence of indicator microorganisms during the shelf life of bottled mineral waters originated from the State of São Paulo, Brazil. Their microbiological quality and the HPC limit were also evaluated and discussed.

2. MATERIALS AND METHODS

2.1. Sampling

Four brands for each type of packaging (0.5, 1.5 and 20-liter) of non-carbonated natural mineral water were analyzed. These waters were distributed as: 0.5-liter (L) bottles – brands A, B, C and D; 1.5-L bottles – brands A, B, C and D and 20-L bottles – brands A, D, E and F (Table 1). Thirty-three 0.5 and 1.5-L bottles of the same batch were acquired for each brand and were analyzed during their shelf life (12 months); fifteen 20-L bottles of the same batch were also acquired for each brand and analyzed during their shelf life (2 months). Three bottles (same batch) of each brand were analyzed on each day of analysis, and all analyses were made in triplicate. Prior to the investigation, the bottles were stored at room temperature (22 to 25°C) and kept away from the sun. All these mineral waters were purchased from retail outlet in Araraquara city (State of São Paulo, Brazil)

2.2. Microbiological analysis

2.2.1. Determination of total coliforms and *Escherichia coli* (*E. coli)*

To determine the presence of total coliforms and *E. coli*, the Enzyme Substrate Test was used as described in the standard methods for the examination of water and wastewater (APHA, 2005). The dehydrated Colilert® medium (IDEXX, Maine, USA) was added to 100 mL of each sample in a disposable sterile bottle, and incubated at 35°C for 24 h. Following incubation, the samples were read for yellow color, which indicates coliform β-galactosidase activity from total coliforms and fluorescence as a result of the action of β-glucuronidase from *E. coli*. An ultraviolet (UV) lamp (at 365 nm) was used to identify the fluorescence.

2.2.2. Determination of *Pseudomonas aeruginosa* (*P. aeruginosa)*

The presence of *P. aeruginosa* was determined by the multiple tube method as described in the standard methods for the examination of water and wastewater (APHA, 2005). For the presumptive test, 10-mL aliquots of sample were introduced into each of ten tubes with 10 mL double Asparagine broth (VETEC, Rio de Janeiro, Brazil) and incubated at 35°C for 48 h. The production of pyoverdin in the asparagine broth, detected by fluorescence under a UV lamp (at 365 nm), meant that the presumptive test was positive. The confirmatory test was carried out by transferring 0.1-mL inocula from the positive asparagine broth tubes into Acetamide broth (VETEC, Rio de Janeiro, Brazil) and incubating the tubes at 35°C for 24-36 h. Tubes that developed an alkaline reaction (purple coloration) were considered positive for the confirmatory test.

2.2.3. Determination of Enterococci

To determine the presence of enterococci, the multiple tube method was used as described in the standard methods for the examination of water and wastewater (APHA, 2005). For the presumptive test, 10-mL aliquots of sample were introduced into each of ten tubes with 10-mL double Dextrose azide broth (MERCK, Darmstadt, Germany) and incubated at 35°C for 48 h. The turbidity of the culture medium was considered positive for the presumptive test. The confirmatory test was carried out by transferring 0.1-mL inocula from the positive Dextrose azide broth tubes into Ethyl violet azide broth (MERCK, Darmstadt, Germany) and incubating the tubes at 35°C for 48 h. Tubes that developed purple pigmentation were considered positive for the confirmatory test.

2.2.4. Determination of HPC

The pour-plate technique was used to determine HPC as described in the standard methods for the examination of water and wastewater (APHA, 2005). Petri dishes were inoculated with 1 mL of sample or decimal dilutions thereof and then the medium (Plate Count Agar) (PCA, MERCK, Darmstadt, Germany), previously melted and brought to 44°C, was added. All plates were incubated at 35°C for 48 h. All colonies formed were enumerated using a colony counter (CP600 Plus, Phoenix, São Paulo, Brazil) and the result was expressed in CFU/mL.

2.2.5. Statistical analysis

Data on the presence of indicator microorganisms from the different days of analysis for each brand were compared using the Kruskal-Wallis test (SPSS 17.0 for Windows). Data on CFU/mL for bottles of the same brand and for brands from each day of analysis were also compared. Finally, data on CFU/mL for each brand on each day of analysis were compared using the analysis of variance and the post-hoc test of Tukey (SPSS 17.0 for Windows).

3. RESULTS

Three bottles of the same batch of each water brand were examined on each day of analysis. Neither total coliforms nor *E. Coli* were found in any bottle (Table 2). However, the presence of enterococci was verified in 6 bottles: 0.5 and 1.5-L bottles of brands A and D and in 0.5-L bottles of brand C. It is worth noting that only in 0.5-L bottles of brand A enterococci were found on two days of analysis (one bottle on each day, 60 and 90 days following bottling). The presence of *P. aeruginosa* was demonstrated in two bottles of one brand on two consecutive days of analysis (one bottle on each day). The presence of both enterococci and *P. aeruginosa* were not significantly different during storage.

In respect to quantitative variations in HPC during storage, three bottles of the same batch of each brand were examined on each day of analysis. There were cases of significant variations in the counts of different bottles, but those were of the same batch and same brand, and the variations occurred on the same day (Table 3 and 4). Nevertheless, there were also significant differences between brands on the same day. First, regarding 0.5 and 1.5-L bottles, brand C of 1.5-L showed significantly higher values of CFU/mL than other brands on all days of analysis, except for 360 days following bottling (Table 3). In what refers to 20-L bottles,

only on the 15th and 30th days following bottling the CFU/mL values were not significantly different in the four brands examined (Table 4). Significant differences in the HPC values between the 0.5 and 1.5-L bottles of same brand were observed only in brand C (Table 3). Significant differences between the HPC values and the days of analysis were also found in some brands. For the 0.5 and 1.5-L bottles, brand C of 1.5-L was the one that presented more significant differences regarding the CFU/mL values on the days of analysis (Table 3). Nevertheless, for the 20-L bottles brand D was the one that presented more significant differences in the values of CFU/mL on the days of analysis (Table 4).

The mean of the CFU/mL values of three bottles of each brand for each day of analysis was intended to make it possible to perform the curve of HPC variations during the shelf life (Figure 1). Except for 1.5-L bottles of brand C and 20-L bottles of brand A, all brands showed low HPC on the second day following bottling. 0.5-L bottles of brand C showed alternating periods of increase and decrease of HPC (Figure 1a) and the same occurred with 1.5-L bottles of the same brand (Figure 1c). 1.5-L bottles of brands A and D showed variations only after 180 days following bottling (Figure 1b). Regarding 20-L bottles, brands D, E and F showed a large increase of HPC until 30 days following bottling; after that period, there was a decrease of HPC in brands E and F, whereas in brand D it continued to increase until 60 days following bottling. On the other hand, brand A showed a large decrease of HPC until 15 days following bottling (Figure 1d and 1e).

The results obtained for each bottle were compared with the Resolution RDC 275/2005 of the Brazilian Health Surveillance Agency (ANVISA, 2005), which establishes the absence of total coliforms, *E. coli*, enterococci and *P. aeruginosa* in 100 mL of sample. Although the Brazilian legislation does not mention any standard for HPC, this microbial group was included in the analysis because its presence could indicate poor hygiene conditions during the processing of the mineral water (Allen et al., 2004) and because high counts of these bacteria could include opportunistic pathogens, especially multiresistant forms, which could mean a health risk to immunologically compromised individuals (Varga, 2011). Thus, we rely on the established limit of 500 CFU/mL for drinking water delivered by public water supply in the United States (U.S.EPA, 2002). This limit is also followed in Brazil for public water supply (ANVISA, 2004), and is used in other studies on bottled mineral water (e.g., Da Silva et al., 2008) and by some bottlers in Brazil.

Four (1.23%) 0.5-L bottles and two (0.62%) 1.5-L bottles, in a total of six bottles (1.85%), were in disagreement with the standards for Enterococci (Table 2). Two (0.62%) 20-L bottles were in disagreement with the standards for *P. aeruginosa*. The number of bottles

with HPC over the maximum level (500 CFU/mL) was three (0.92%) of 0.5 L, 29 (8.95%) of 1.5 L (of only one brand) and 40 (12.35%) of 20 L (Table 2). HPC ranged from $<10^{-2}$ to 560,000 CFU/mL in the 324 mineral water bottles examined (Table 3 and 4), including 72 bottles (22.22%) with levels above 500 CFU/mL (Table 2). In 0.5 and 1.5-L bottles of all brands with counts below 500 CFU/mL on the second day following bottling there was a growth of such bacteria along the days, but it did not exceed 500 CFU/mL (except in 0.5-L bottles of brand C 15 and 90 days following bottling) (Table 3). That situation did not occur with 20-L bottles (Table 4).

4. DISCUSSION

This study was based on the hypotheses that variations in bacteria counts in bottled mineral water may occur during storage, which may indicate changes in water quality, and that the use of HPC as a quality parameter for bottled mineral water still requires attention. The experimental planning consisted of the detection of quantitative variations in HPC and in the presence of indicator microorganisms during the shelf life of the bottled mineral waters. The presence of indicator microorganisms not presented variations during shelf life of mineral waters. However, quantitative variations in HPC during shelf life were found in some brands. Variations in HPC after bottling may occur, because the bottling process does not change the natural composition of mineral water, therefore, large bacterial populations usually develop from small initial populations present in the source (Leclerc and Moreau, 2002; Loy et al., 2005). In most brands that showed low HPC on the second day following bottling there was a subsequent increase of HPC. However, variations in the counts of bacteria after bottling and/or during storage may also indicate changes in the quality of mineral water, and such alterations may reflect changes in available nutrients, a decrease in the viability of some strains, competition or other factors not yet identified (Morais and Da Costa, 1990). Therefore, samples of bottled mineral waters analyzed in this study may be undergoing changes in their quality during storage because of HPC variations. In view of the fact that the characteristics of the water source influence the bacterial population of mineral water (Morais and Da Costa, 1990; Rosenberg, 2003), in this study it was expected that significant differences in HPC values would be verified between brands, but not between bottles of 0.5 and 1.5 L of the same brand.

The reasons for the bacterial multiplication after bottling are still a matter of debate, however there are some explanations for this. Microbial development in bottled mineral water can be characterized by alternating increases and decreases in population (Schmidt-Lorenz, 1976), which was observed in HPC during storage in some brands analyzed in this study. To explain this fact, there are some theories that consider that each new population is composed of several species that grow at the expense of organic matter from dead cells of the former population (Schmidt-Lorenz, 1976). Moreover, investigations have been carried out in order to found if this multiplication of bacteria in water after bottling is due to the reactivation of a large number of non-cultivable cells present in the source water or bottling system, or if it is a result of growth and multiplication of a few cultivable cells initially present (Leclerc and Moreau, 2002).

Is also know that, any bacteria present will attach to the sides and bottom of the container and multiply at the expense of whatever organic matter is present in the water. This matter may vary between brands depending on the source. Thus, water containing few organisms when bottled may show a logarithmic increase in the numbers of bacteria in a relatively short time. This increase continues in typical growth curve fashion until the organic material in the water has been depleted (Rosenberg, 2003) as noted in the brands D, E and F of 20-L bottles (Figure 1). And these brands showed large amounts of bicarbonate and total dissolved solids in features of your source (Table 1), which may have served as material for the multiplication of these bacteria. Photodegradation of dissolved organic matter is a common phenomenon. Thus the exposure time of organic substances in water samples to daylight may again stimulate the growth of microorganisms since complexes substances may become bioavailable (Leclerc e Moreau, 2002) which may explain the peaks with high counts observed in some brands.

This study also sought to determine the quality of the bottled mineral waters with respect to microbiological quality criteria established in the current legislation (ANVISA, 2005). The absence of *E. coli* and total coliforms verified in this study is in agreement with the standards, but the presence of the indicator microorganisms enterococci and *P. aeruginosa* in some bottles is not. Total coliforms are traditionally used as indicators of water quality (Sartory, 2004), but the specific presence of *E. coli* indicates fecal contamination and possible occurrence of enteric pathogens (Bharath et al., 2003). Furthermore, enterococci are an indicator of fecal pollution, serving as a marker for fecal pathogens that survive longer in water than *E. coli* (WHO, 2011). The importance of the presence of *P. aeruginosa* in mineral water is related to its potential as an opportunistic pathogen (Warburton, 1993), the resistance

to antibiotics by some of its strains (Rosenberg and Hernandez Duquino, 1989), and its potential to contribute to the production of off-flavors in water (Stickler, 1989; WHO, 2011). Therefore, bottled mineral waters must be free from these indicator microorganisms because their presence constitutes a health risk to the general public.

Seventy-two bottles analyzed in this study presented HPC values above of limit 500 CFU/mL and besides, reached levels of up to 560,000 CFU/mL. Given such high values, it is important to point out for the consumer the health significance, if any, of the particular bacteria present in the water. No limit is set for HPC of bottled mineral waters which develop after bottling, and little is known about its composition and the numbers and types of carbon sources in the water supporting its development (Otterholt and Charnock, 2011). It is now generally accepted that HPC alone does not directly relate to health risks for the population in general. However, the infection risk to immunocompromised patients remains unclear, as there is insufficient data for its evaluation (Pavlov et al., 2004). It is also known that these bacteria, when present in high concentrations, can cause deterioration of the bottled water, and alterations in the product's odor and flavor (Sartory, 2004). In addition, HPC in bottled mineral water helps ensure that, from source to consumption, no change has occurred in the microbial quality of water (Leclerc and Moreau, 2002).

This study also tried to evaluate the limit of 500 CFU/mL established for drinking water delivered by public water supply (U.S.EPA, 2002; ANVISA, 2004). It was observed that in 0.5 and 1.5-L bottles of all brands that showed counts below 500 CFU/mL on the second day following bottling the growth of HPC did not exceed the limit of the 500 CFU/mL during storage. This showed that for non-returnable packaging the control of HPC (< 500 CFU/mL) of mineral water until 48 h following bottling can keep these scores low during the product shelf life and can also exclude the possibility that quantitative variations occur in the bacterial population during storage. Thus, the limit of 500 CFU/mL for HPC seems to be adequate to ensure the quality of mineral water during storage. The same did not occur for the 20-L bottles, probably on account of the reuse of bottles, often without appropriate cleaning and disinfection (Kokkinakis et al., 2008; Marzano et al., 2011; WHO, 2011). In addition, 20-L bottles were the only packaging in which four brands presented HPC above 500 CFU/ml. This is a cause for concern, because the market of this type of packaging is very common in Brazil and should be a target of policies to improve public health.

A curious fact observed in this study is that two brands showed high HPC on the first day of analysis and then HPC decreased, which probably occurred because those brands had contaminant bacteria. In addition, they continued to show HPC above 500 CFU/mL during all

their shelf life. This data showed once more the importance of the control of HPC in mineral waters, as high scores soon after bottling can remain high throughout storage, which could impair the quality of water that reaches the consumer. In most cases, the source of these contaminants has been attributed to the bottling plant: pumps, pipes, caps and bottles (Warburton, 1993). Nevertheless, the potential contamination of the sources with pathogenic microorganisms should not be underestimated (Casanovas-Massana et al., 2012).

5. CONCLUSIONS

The presence of the indicator microorganisms enterococci (six bottles) and *P. aeruginosa* (two bottles) were in disagreement with the microbiological quality criteria established in the current legislation.

Seventy-two bottles (22.22%) analyzed in this study presented HPC values above 500 CFU/mL, reaching up to 560,000 CFU/mL.

The limit of 500 CFU/mL for HPC seems to be adequate to ensure the quality of bottled mineral water during storage in non-returnable packaging.

The 20-L bottles (returnable) showed high HPC, which is a cause for concern, because the market of this type of packaging is very common in Brazil and should be a target of policies to improve public health.

Alterations in HPC during the period of validity were observed in some of the mineral water brands analyzed.

The high values of HPC and its variation during storage of the mineral waters detected in this study seem to fully justify the need for reevaluation of the current microbiological water quality guidelines in Brazil regarding the use of HPC. More detailed studies on the potential health risk of HPC and its variations in mineral water are also needed.

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Table 1: Features of the mineral waters examined *

Composition	Brands					
	A	**B**	**C**	**D**	**E**	**F**
pH	5.34	6.79	5.25	5.30	6.20	6.49
Barium (mg/L)	0.013	0.040	0.043	0.053	0.112	-
Strontium (mg/L)	0.007	0.054	-	-	0.018	0.067
Phosphate (mg/L)	-	-	-	-	-	0.09
Bicarbonate (mg/L)	5.62	78.46	0.68	9.86	37.47	105.31
Chloride (mg/L)	0.14	1.04	0.30	0.35	0.92	0.79
Sulfate (mg/L)	-	0.2	0.1	0.1	1.2	5.64
Sodium (mg/L)	0.60	3.21	0.37	1.05	1.92	8.74
Calcium (mg/L)	0.70	12.19	0.56	0.32	5.95	17.48
Potassium (mg/L)	0.50	1.53	0.51	3.01	2.38	3.50
Fluoride (mg/L)	0.02	0.02	0.02	0.03	0.06	0.191
Nitrate (mg/L)	0.30	3.30	4.30	3.10	3.30	0.47
Magnesium (mg/L)	0.33	7.10	0.42	0.39	3.38	6.54
Total dissolved solids (mg/L)	10.30	94.61	16.16	33.01	56.10	132.16
Temperature at the source (˚C)	25.3	22.0	23.0	23.8	21.0	21.5
Conductivity (μS/cm)	11.8	123.2	16.2	23.8	78.1	18.4
Radioactivity (ME)	-	5.10	-	-	13.67	45.56

*Information as provided in the bottle label.

Table 2: Number of positive bottles for each indicator microorganism in 324 mineral water bottles

	N° positive bottles (%)				
Brand	**CT**	**EC**	**PA**	**E**	**HPC**
Packaging 0.5 L					
A	-	-	-	2 (0.62)	-
B	-	-	-	-	-
C	-	-	-	1 (0.31)	3 (0.92)
D	-	-	-	1 (0.31)	-
Total	-	-	-	4 (1.23)	3 (0.92)
Packaging 1.5 L					
A	-	-	-	1 (0.31)	-
B	-	-	-	-	-
C	-	-	-	-	29 (8.95)
D	-	-	-	1 (0.31)	-
Total	-	-	-	2 (0.62)	29 (8.95)
Packaging 20 L					
A	-	-	-	-	10 (3.09)
D	-	-	-	-	10 (3.09)
E	-	-	2 (0.62)	-	12 (3.70)
F	-	-	-	-	8 (2.47)
Total	-	-	2 (0.62)	-	40 (12.35)

CT: total coliform

EC: *Escherichia coli*

PA: *Pseudomonas aeruginosa*

E: enterococci

HPC: heterotrophic plate count

Table 3: Colony-forming units (CFU) of heterotrophic plate count per mL of bottled mineral waters in packaging of 0.5 and 1.5-L during their shelf life

Brand	Volume (L)	Bottle	Mean ± SD (CFU/ml)																									
			Days																									
			2		7		15		30		60		90		120		180		240		300		360					
A	0.5	1	$7.4x10^{1} \pm 2.9x10^{1}$	a	< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1					
		2	$2.0x10^{0} \pm 1.4x10^{0}$	b	< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1					
		3	< 1	b	< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1					
		Total	$2.5x10^{1} \pm 3.9x10^{1}$	1,*	< 1	2,*	< 1	2,*	< 1	2,*	< 1	2, *	< 1	2, *	< 1	2,*	< 1	2,*	< 1	2,*	< 1	2,*	< 1	2,*				
B	0.5	1	< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1					
		2	< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1					
		3	< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1					
		Total	< 1	*	< 1	*	< 1	*	< 1	*	< 1	*	< 1	*	< 1	*	< 1	*	< 1	*	< 1	*	< 1	*				
C	0.5	1	$1.4x10^{1} \pm 3.5x10^{0}$	a	$1.2x10^{1} \pm 9.9x10^{0}$	a	$5.9x10^{2} \pm 9.2x10^{1}$	a,b	$1.9x10^{1} \pm 2.8x10^{0}$	a	$5.9x10^{1} \pm 3.5x10^{1}$	a	$6.4x10^{2} \pm 1.6x10^{2}$	a	$5.2x10^{1} \pm 9.2x10^{0}$	a	$1.3x10^{2} \pm 1.4x10^{1}$	a	$7.2x10^{1} \pm 1.5x10^{1}$	a	$5.7x10^{1} \pm 8.5x10^{0}$	a	$8.6x10^{1} \pm 4.5x10^{1}$	a				
		2	$4.5x10^{0} \pm 2.1x10^{0}$	a,b	$1.5x10^{2} \pm 2.9x10^{1}$	b	$1.4x10^{3} \pm 3.6x10^{2}$	b	$6.5x10^{0} \pm 3.5x10^{0}$	a	$1.3x10^{2} \pm 2.9x10^{1}$	a	$1.6x10^{2} \pm 3.1x10^{1}$	b	$2.1x10^{2} \pm 1.8x10^{1}$	b	$2.9x10^{1} \pm 9.2x10^{0}$	b	$2.4x10^{1} \pm 7.8x10^{0}$	b	$6.9x10^{1} \pm 2.1x10^{0}$	a	$2.5x10^{2} \pm 2.8x10^{1}$	b				
		3	< 1	b	$1.9x101 \pm 0.0x10^{0}$	a	$4.8x10^{1} \pm 7.1x10^{0}$	a	$6.2x10^{1} \pm 1.3x10^{1}$	b	$1.4x10^{2} \pm 1.7x10^{1}$	a	$1.9x10^{2} \pm 6.4x10^{1}$	b	$1.3x10^{2} \pm 2.4x10^{1}$	b	$2.1x10^{2} \pm 2.8x10^{0}$	c	$1.7x10^{1} \pm 4.2x10^{0}$	b	$7.6x10^{1} \pm 1.7x10^{1}$	a	$3.2x10^{1} \pm 6.6x10^{0}$	a				
		Total	$6.0x10^{0} \pm 6.4x10^{0}$	1,*	$6.0x10^{1} \pm 7.0x10^{1}$	1,f	$6.8x10^{2} \pm 6.4x10^{2}$	2,f	$2.9x10^{1} \pm 2.6x10^{1}$	1,f	$1.1x10^{2} \pm 4.5x10^{1}$	1, *	$3.7x10^{2} \pm 2.7x10^{2}$	1, 2, *	$1.3x10^{2} \pm 7.3x10^{1}$	1,*	$1.2x10^{2} \pm 8.2x10^{1}$	1,*	$3.8x10^{1} \pm 2.8x10^{1}$	1,*	$6.8x10^{1} \pm 1.2x10^{1}$	1,*	$1.4x10^{2} \pm 1.0x10^{2}$	1,*,f				
D	0.5	1	< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1					
		2	< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1					
		3	< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1					
		Total	< 1	*	< 1	*	< 1	*	< 1	*	< 1	*	< 1	*	< 1	*	< 1	*	< 1	*	< 1	*	< 1	*				
A	1.5	1	$2.1x10^{1} \pm 2.9x10^{1}$	a	< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1		$5.0x10^{-1} \pm 7.0x10^{-1}$	a				
		2	$5.0x10^{1} \pm 1.4x10^{0}$	a	< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1		$3.3x10^{2} \pm 1.0x10^{1}$	b				
		3	$3.5x10^{0} \pm$	a	< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1		$7.4x10^{1} \pm 2.3x10^{1}$	c				
		Total	$2.5x10^{1} \pm 2.5x10^{1}$	1, *	< 1	1,*	< 1	1,*	< 1	1,*	< 1	1,*	< 1	1,*	< 1	1,*	< 1	1,*	< 1	1,*	< 1	1,*	$1.3x10^{2} \pm 1.5x10^{2}$	2,*, f				
B	1.5	1	< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1					
		2	< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1					
		3	< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1		< 1					
		Total	< 1	*	< 1	*	< 1	*	< 1	*	< 1	*	< 1	*	< 1	*	< 1	*	< 1	*	< 1	*	< 1	*				
C	1.5	1	> $5.6x10^{5}$		> $5.6x10^{5}$		> $5.6x10^{5}$		> $5.6x10^{5}$		$2.3x10^{4} \pm 2.3x10^{3}$	a	$1.3x10^{4} \pm 6.6x10^{3}$	a	$1.1x10^{4} \pm 1.3x10^{3}$	a	$1.4x10^{3} \pm 5.7x10^{1}$	a	$1.9x10^{4} \pm 7.8x10^{3}$	a	$1.0x10^{3} \pm 7.1x10^{0}$	a	$2.3x10^{2} \pm 4.7x10^{1}$	a				
		2	> $5.6x10^{5}$		> $5.6x10^{5}$		> $5.6x10^{5}$		> $5.6x10^{5}$		$6.4x10^{3} \pm 2.0x10^{3}$	b	$1.3x10^{4} \pm$	a	$1.0x10^{4} \pm 8.5x10^{2}$	a	$7.1x10^{3} \pm 4.2x10^{2}$	b	$1.1x10^{4} \pm 2.9x10^{3}$	a	$9.8x10^{2} \pm 1.7x10^{2}$	a	$1.2x10^{3} \pm 7.1x10^{1}$	b				
		3	> $5.6x10^{5}$		> $5.6x10^{5}$		> $5.6x10^{5}$		> $5.6x10^{5}$		$6.5x10^{3} \pm 7.8x10^{2}$	b	$2.1x10^{3} \pm 9.2x10^{1}$	a	$1.0x10^{4} \pm 2.7x10^{3}$	a	$5.1x10^{2} \pm 1.6x10^{2}$	a	$1.9x10^{4} \pm 5.4x10^{3}$	a	$9.3x10^{2} \pm 3.1x10^{2}$	a	$8.9x10^{1} \pm 2.1x10^{0}$	a				
		Total	> $5.6x10^{5}$	1,f	> $5.6x10^{5}$	1, e	> $5.6x10^{5}$	1,e	> $5.6x10^{5}$	1, e	$1.2x10^{4} \pm 8.6x10^{3}$	2, f	$9.3x103 \pm 6.3x10^{3}$	3,f	$1.0x10^{4} \pm 1.4x10^{3}$	2,f	$3.0x10^{3} \pm 3.2x10^{3}$	3,4,f	$1.6x10^{4} \pm 6.0x10^{3}$	2,f	$9.8x10^{2} \pm 1.7x10^{2}$	4,f	$4.9x10^{2} \pm 5.2x10^{2}$	4,f				
D	1.5	1	< 1		< 1		< 1		< 1		< 1		< 1		$3.3x10^{1} \pm 8.5x10^{0}$	a	< 1	a	< 1		$7.5x10^{0} \pm 9.2x10^{0}$	a	< 1	a				
		2	< 1		< 1		< 1		< 1		< 1		< 1		< 1	b	$2.4x10^{2} \pm 8.3x10^{1}$	b	< 1		$4.5x10^{0} \pm 4.9x10^{0}$	a	< 1	a				
		3	< 1		< 1		< 1		< 1		< 1		< 1		< 1	b	< 1	a	< 1		$5.0x10^{0} \pm 4.2x10^{0}$	a	$2.0x10^{1} \pm 1.7x10^{1}$	b				
		Total	< 1	1, *	< 1	1,*	< 1	1,*	< 1	1,*	< 1	1, *	< 1	1,*	$1.1x10^{1} \pm 1.7x10^{1}$	1,2,*	$7.9x10^{1} \pm 1.3x10^{2}$	2,*	< 1	1,*	$5.7x10^{0} \pm 5.2x10^{0}$	1,2,*	$6.7x10^{0} \pm 1.3x10^{1}$	1,2,*				

SD, standard deviation; values are mean of three determinations;
Tukey test:
Letters represent significant (P<0.05) differences between bottles of the same brand and batches for each day of analysis
Symbols represent significant (P<0.05) differences between brands on each day of analysis
Numbers represent significant (P<0.05) differences between HPC and the day of analysis of each brand

Table 4: Colony-forming units (CFU) of heterotrophic plate count per mL of bottled mineral waters in packaging of 20-L during their shelf life

Brand	Volume (L)	Bottle	Mean ± SD (CFU/ml)									
			Days									
			2		7		15		30		60	
A	20	1	$5.2x10^3 \pm 5.9x10^3$	a	$8.1x10^2 \pm 1.7x10^1$	a	$7.4x10^2 \pm 8.4x10^2$	a	$1.2x10^1 \pm 1.4x10^0$	a	$1.5x10^3 \pm 7.9x10^2$	a
		2	$1.4x10^3 \pm 7.0x10^1$	a	$7.7x10^2 \pm 3.4x10^2$	a	$1.4x10^2 \pm 0.7x10^0$	a	$1.8x10^3 \pm 7.8x10^1$	b	$5.9x10^2 \pm 2.9x10^2$	a
		3	$5.6x10^3 \pm 5.9x10^3$	a	$7.1x10^2 \pm 7.0x10^1$	a	$6.5x10^1 \pm 4.9x10^0$	a	$1.5x10^0 \pm 0.7x10^0$	a	$1.2x10^2 \pm 1.3x10^2$	a
		Total	$4.1x10^3 \pm 4.3x10^3$	1,*	$7.7x10^2 \pm 5.7x10^1$	1,2,*	$3.2x10^2 \pm 4.9x10^2$	2,*	$6.1x10^2 \pm 9.4x10^2$	2, *	$7.2x10^2 \pm 7.1x10^2$	1,2,*
D	20	1	$< 10^{-2}$		$1.4x10^1 \pm 1.4x10^0$	a	$5.7x10^3 \pm 3.5x10^2$	a	$5.5x10^3 \pm 4.9x10^2$	a	$4.0x10^1 \pm 0.0x10^0$	a
		2	$< 10^{-2}$		$7.5x10^2 \pm 4.9x10^1$	b	$2.1x10^4 \pm 1.4x10^3$	a	$2.5x10^4 \pm 6.2x10^3$	a	$6.3x10^4 \pm 3.9x10^3$	b
		3	$< 10^{-2}$		$6.5x10^3 \pm 0.0x10^0$	c	$6.6x10^4 \pm 1.0x10^4$	b	$1.4x10^5 \pm 5.7x10^4$	a	$9.7x10^4 \pm 5.8x10^3$	c
		Total	$< 10^{-2}$	1,f	$2.4x10^3 \pm 3.1x10^3$	1, *	$3.1x10^4 \pm 2.8x10^4$	2,*	$5.8x10^4 \pm 7.1x10^4$	2, *	$5.3x10^4 \pm 4.4x10^4$	2, f
E	20	1	$4.5x10^0 \pm 0.7x10^0$	a	$6.2x10^3 \pm 8.5x10^2$	a	$1.1x10^4 \pm 2.2x10^3$	a	$1.6x10^3 \pm 7.1x10^1$	a	$1.5x10^3 \pm 4.9x10^2$	a
		2	$7.5x10^0 \pm 3.5x10^0$	a	$9.4x10^3 \pm 1.8x10^3$	a,b	$1.2x10^4 \pm 2.5x10^3$	a	$7.0x10^3 \pm 1.6x10^3$	b	$1.9x10^3 \pm 2.8x10^2$	a
		3	$4.0x10^1 \pm 0.7x10^0$	b	$1.7x10^4 \pm 2.5x10^3$	b	$9.5x10^4 \pm 4.7x10^3$	a	$1.3x10^4 \pm 9.9x10^2$	c	$9.9x10^3 \pm 1.4x10^3$	b
		Total	$1.7x10^1 \pm 1.7x10^1$	1,f	$1.1x10^4 \pm 4.9x10^3$	1,2,f	$3.9x10^4 \pm 4.8x10^4$	2,*	$7.3x10^3 \pm 5.4x10^3$	1,2,*	$4.4x10^3 \pm 4.3x10^3$	1,2,*
F	20	1	$< 10^{-2}$		$1.1x10^2 \pm 7.2x10^1$	a	$1.7x10^3 \pm 2.8x10^2$	a,b	$7.5x10^2 \pm 1.8x10^2$	a	$< 10^{-2}$	
		2	$< 10^{-2}$		$2.0x10^3 \pm 4.7x10^2$	b	$2.4x10^3 \pm 2.5x10^2$	b	$7.2x10^2 \pm 5.7x10^1$	a	$< 10^{-2}$	
		3	$< 10^{-2}$		$1.4x10^3 \pm 8.0x10^1$	b	$7.7x10^2 \pm 1.9x10^2$	a	$7.1x10^2 \pm 4.6x10^1$	a	$< 10^{-2}$	
		Total	$< 10^{-2}$	1,f	$1.2x10^3 \pm 8.8x10^2$	2,3,*	$1.6x10^3 \pm 7.4x10^2$	3,*	$7.3x10^2 \pm 9.0x10^1$	1,2,*	$< 10^{-2}$	1, *

SD, standard deviation; values are mean of three determinations;
Tukey test:
Letters represent significant (P<0.05) differences between bottles of the same brand and batches for each day of analysis
Symbols represent significant (P<0.05) differences between brands on each day of analysis
Numbers represent significant (P<0.05) differences between HPC and the day of analysis of each brand

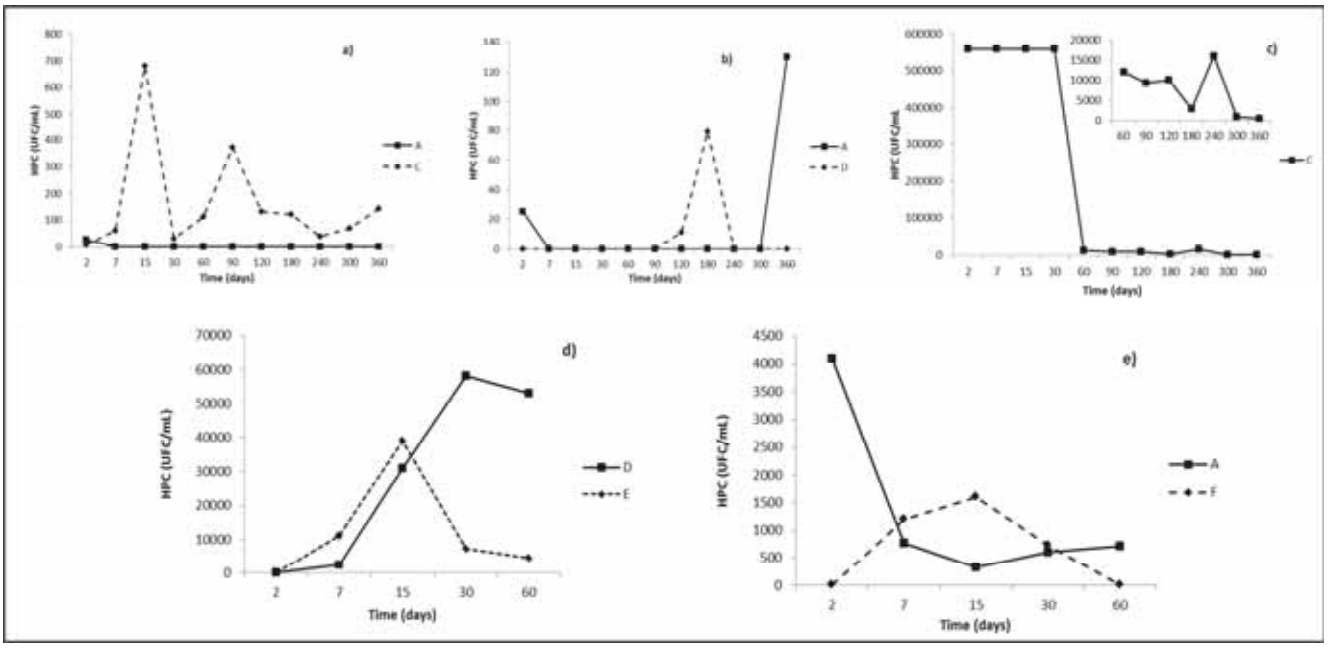

Figure 1: Variations in heterotrophic plate count in bottled mineral waters during their shelf life.
a) Packaging of 0.5 L. b) Packaging of 1.5 L. c) Packaging of 1.5 L. d) Packaging of 20 L. e) Packaging of 20 L. Only for brands that showed bacterial growth.

CAPÍTULO 3

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TITLE: Bottled mineral water as a potential source of antibiotic resistant bacteria

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Running title: *Antibiotic resistance in bottled mineral water*

ABSTRACT

The antibiotic resistance phenotypes of the cultivable bacteria present in nine batches of two Portuguese and one French brands of commercially available mineral waters were examined. Most of the 238 isolates recovered on R2A, *Pseudomonas* Isolation agar or on these culture media supplemented with amoxicillin or ciprofloxacin, were identified (based on 16S rRNA gene sequence analysis) as *Proteobacteria* of the divisions *Beta*, *Gamma* and *Alpha*. Bacteria resistant to more than three distinct classes of antibiotics were detected in all the batches of the three water brands in counts up to 10^2 CFU/ml. In the whole set of isolates, it was observed resistance against all the 22 antimicrobials tested (ATB, bioMérieux and disc diffusion), with most of the bacteria showing resistance to three or more classes of antibiotics. Bacteria with the highest multi-resistance indices were members of the genera *Variovorax*, *Bosea*, *Ralstonia*, *Curvibacter*, *Afipia* and *Pedobacter*. Some of these bacteria are related with confirmed or suspected nosocomial agents. Presumable acquired resistance may be suggested by the observation of bacteria taxonomically related but isolated from different brands, exhibiting distinct antibiotic resistance profiles. Bottled mineral water was confirmed as a possible source of antibiotic resistant bacteria, with the potential to be transmitted to humans.

Keywords: Antibiotic resistance; drinking water; mineral water;

1. INTRODUCTION

Natural mineral water means microbiologically wholesome water, originating in an underground water table or deposit and emerging from a spring tapped at one or more natural or bore exits. This type of water cannot be sterilized, pasteurized or otherwise treated to remove or destroy microorganisms (European Parliament & Council of the European Union, 2009).

Mineral water has been marketed as ideal for infant formula preparation and nursery drinking water, reconstitution of foods and as drinking water, particularly for the immunosuppressed people (Warburton, 1993). Many, if not most, people believe that bottled water is superior to tap water in that it contains no microorganisms (Rosenberg, 2003). However, this is not the case because spring waters typically contain a characteristic bacterial flora (Manaia et al., 1990; Ferreira et al., 1996; Rosenberg, 2003). Natural mineral waters are not free of bacteria, and counts of 10^4–10^5 CFU/ml can be reached within a few days after bottling (Leclerc and Da Costa, 1998). According to numerous studies, the microorganisms most frequently found in bottled natural mineral water are aerobic heterotrophs belonging mainly to the *Alpha*-, *Beta*- and *Gammaproteobacteria* (Rosenberg and Hernandez Duquino, 1989; Leclerc and Da Costa, 1998; Urmeneta et al., 2000; Leclerc and Moreau, 2002; Loy et al. 2005; Messi et al., 2005; Otterholt and Charnock, 2011).

Different authors believe that the intensive use of antimicrobials contributed for the important increase of antibiotic resistant bacteria observed in aquatic environments (Witte, 2000; Messi et al., 2005; Baquero et al., 2008). These bacteria represent a reservoir of resistance determinants as well as a means for the spread and evolution of resistance genes and their vectors (Baquero et al., 2008; Cantón, 2009). The genes encoding antibiotic resistance mechanisms can be located on the bacterial chromosome or on extrachromosomal genetic elements (plasmids or phages), and be transmitted to the next generation (vertical

gene transfer) or be exchanged among bacteria, even of distinct taxonomic groups (horizontal gene transfer) (Baquero et al., 2008; Cantón, 2009). The mechanisms involved in resistance acquisition and spreading in drinking waters are not very well understood, in part due to low bacterial loads that colonize these environments. Nevertheless, the occurrence of antibiotic resistant bacteria and/or genes in disinfected drinking waters has been demonstrated (Schwartz et al., 2003; Faria et al., 2009; Vaz-Moreira et al., 2011). In the same way, some studies have shown antibiotic resistant bacteria in bottled mineral water (Rosenberg and Hernandez Duquino, 1989; Massa et al., 1995; Mary et al., 2000; Messi et al., 2005). Although these studies are valuable contributes to evidence the occurrence of antibiotic resistant bacteria in water, their comparison is hampered by the use of different techniques and culture media for isolation, different methods of phenotypic characterization, and different temperatures for antibiotic resistance testing (Mary et al., 2000).

It has been suggested that bacteria inhabiting natural habitats are potential sources of antibiotic resistance genes which can be, somehow, transmitted to human commensal and pathogenic bacteria. For instance, indigenous soil microbiota was demonstrated as an important reservoir of resistance determinants which can be mobilized into the microbial community (D'Costa et al., 2006; Dantas et al. 2008; D'Costa et al., 2011). Based on these studies it is possible to hypothesize that bacteria indigenous in spring waters can also harbor antibiotic resistance determinants. The current study was designed to test such hypothesis, since bottled mineral waters may represent a particularly important source of antibiotic resistance, given the possibility of direct transmission of bacteria to humans. Based on this hypothesis, the diversity of culturable bacteria and of antibiotic resistance phenotypes were characterized in three bottled mineral water brands, originated from different regions.

2. MATERIALS AND METHODS

2.1. Sampling

Three brands (herein named A, B and C) of noncarbonated natural mineral water were analyzed (Table 1). These waters were contained in 1.5 liter polyethylene terephthalate (PET) bottles and were purchased from retail outlet in Portugal. Two of these waters were produced in Portugal (A and B) and one in France (C). Mineral water bottles were stored at room temperature (20 to 22 °C) prior to investigation. For each brand were analyzed three bottles of different batches.

2.2. Enumeration of Total Cultivable and Antibiotic-Tolerant Bacteria

Bacteriological analyses were performed using the membrane filtration method based on the procedure described before (Novo and Manaia, 2010). Two culture media widely used for microbiological quality control of drinking water were employed: R2A (Difco) and *Pseudomonas* isolation agar (PIA; Difco). R2A agar is a nonselective culture medium recommended for the examination of total heterotrophic bacteria and PIA is recommended for the enumeration of *Pseudomonas* spp. and related bacteria (Eaton et al., 2005). The respective antimicrobial-tolerant subpopulations, i.e. bacteria able to grow in the presence of antimicrobial agents, were enumerated on the same media supplemented with 32 mg/l amoxicillin (AML) or 4 mg/l ciprofloxacin (CIP). These and other antimicrobial compounds will be referred to as “antibiotics”, i.e. organic molecules able to inhibit or kill bacteria by specific interactions with cell targets, according to the definition proposed by Davies and Davies (2010). With the addition of those two antibiotics (AML and CIP) it was expected to

enrich the cultures in resistant bacteria, using two reference compounds – a beta-lactam against which drinking water bacteria are frequently resistant and a fluoroquinolone for which resistance is rarer, although observed in drinking water (Massa et al., 1995; Mary et al., 2000; Messi et al., 2005; Xi et al., 2009; Vaz-Moreira et al., 2011). The antibiotic concentrations used were determined in previous studies, as adequate to recover antibiotic-resistant bacteria (Watkinson et al., 2007). Volumes of 1-100 ml (culture medium with antibiotic) and of 1-10 ml of water samples or decimal dilutions thereof (culture medium without antibiotic) were filtered through cellulose nitrate membranes (0.45 μm pore size, 47 mm diameter, Albet), which were placed onto the different culture media described above and incubated for 48 h at 30 °C. All analyses were made in triplicate. After the incubation period, the number of colony forming units (CFU) was registered on the basis of filtering membranes containing between 10 and 100 colonies. Values of CFU/ml were registered for each culture medium.

2.3. Bacterial isolation and preliminary characterization

Bacteria were isolated after the visual examination of the triplicates of culture plates which evidenced a countable number of CFU's after 48 h of incubation. Culture plates on which no growth was observed within this period were incubated until 7 days. During the incubation period, plates were examined and bacteria were isolated and purified. Representatives of all colony types were selected for further culture purification, according to the following criterion: all colonies of morphotypes represented by up to two colonies, 50-75% colonies of morphotypes represented between three and nine colonies, and five colonies with morphotypes represented by more than nine colonies. Cultures were purified by sub-culturing on R2A or on PCA (Plate Count Agar, Pronadisa), used whenever poor or very slow growth was observed on R2A. Pure cultures were preserved at -20 and -80 ºC in nutritive

broth supplemented with 15 % (v/v) glycerol. Colony and cellular morphologies, Gram staining, cytochrome *c* oxidase and catalase tests were performed as described by Smibert and Krieg (1994).

2.4. Bacterial identification and typing

In order to detect possible repetitions (the same bacterium isolated on the same filtering membrane), the bacterial isolates were genotyped using random amplified polymorphic DNA (RAPD) analysis as described by Ferreira da Silva et al. (2006). The RAPD genotypes were analyzed visually and whenever two isolates from the same filtering membrane presented an identical profile were considered repetitions and one of those was excluded from the further identification process. All non-repetitive bacterial isolates were identified based on the analysis of the 16S rRNA gene sequence, using the primers 27F and 1492R (Lane, 1991). The nucleotide sequences with about 1400 bp were used to query the EzTaxon library (Chun et al., 2007), in order to achieve the closest type strain and thus attain a species affiliation and possible identification to the level. In order to compare the 16S rRNA gene sequences of isolates from different samples, nucleotide sequences were aligned using ClustalW from MEGA software, version 5.05 (Tamura et al., 2011), sequence relatedness was estimated based on the model of Jukes and Cantor (1969), and dendrograms were created using the neighbor-joining method. The methods maximum parsimony and maximum likelihood were also used to assess the tree stability. The type strains of the closest neighbors of each isolate were added to the dendrogram in order to support the identification of the isolates under study. A total of 1065 nucleotide positions were included in the analysis of the 16S rRNA gene sequences. Non-homologous and ambiguous nucleotide positions were excluded from the calculations. The 16S rRNA gene sequences were deposited in the

GenBank with the accession numbers JQ317794-JQ31795, JQ317807-JQ317808, JQ317810-JQ317814 and JQ689172- JQ689199.

2.5. Determination of antibiotic resistance phenotypes

Antibiotic resistance phenotypes were determined using the ATB PSE EU (08) panel (bioMérieux) according to the manufacturer's instructions. This panel enables the determination of the antibiotic susceptibility of non-fermenting Gram-negative rods in a semi-solid medium under conditions similar to the reference methods for agar dilution or micro-dilution, following the recommendations of the EUCAST committee (2008) and CASFM committee (Cavallo et al., 2008). The antibiotics tested were: beta-lactams - ticarcillin (16 mg/l), ticarcillin plus clavulanic acid (16 plus 2 mg/l, respectively), piperacillin (16 mg/l), piperacillin plus tazobactam (16 plus 4 mg/l, respectively), imipenem (2 and 8 mg/l), meropenem (2 and 8 mg/l), ceftazidime (4 and 8 mg/l), and cefepime (4 and 8 mg/l); aminoglycosides - amikacin (8 and 16 mg/l), gentamicin (4 mg/l), and tobramycin (4 mg/l); fluoroquinolones - ciprofloxacin (1 and 2 mg/l), levofloxacin (1 and 2 mg/l); polymyxin - colistin (2 mg/l); sulfonamides - cotrimoxazol (4 mg/l trimethoprim and 76 mg/l sulphamethoxazole); fosfomycin (32 mg/l); rifampicin (4 and 16 mg/l). Phenotypes were classified as resistant, intermediary or susceptible according to the manufacturer's instructions. For the antibiotics nalidixic acid, tetracycline, cephalothin and streptomycin, not included in the ATB panel, and for the Gram-positive isolates, antibiotic resistance phenotypes were determined using the agar diffusion method (CLSI, 2007). The antibiotics tested were: beta-lactams ticarcillin (75 µg), cephalothin (30 µg), ceftazidime (30 µg), meropenem (10 µg); aminoglycosides- streptomycin (10 µg), gentamicin (10 µg); quinolones - nalidixic acid (30 µg), ciprofloxacin (5 µg); polymyxin - colistin sulphate (50 µg); sulfonamides -sulphamethoxazole (25 µg), sulphamethoxazole/trimethoprim (1.25 µg

trimethoprim and 23.75 µg sulphamethoxazole); tetracycline (30 µg). For the antibiotic colistin, which is not included in the CLSI list, was used the following criteria: S≥ 10/R < 10. Diameters larger than R but smaller than S were referred to as intermediary. The strains *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* DSM 1117 (=ATCC 27853) were included as quality controls. For the Gram-positive isolates, minimum inhibitory concentrations (MIC's) were determined for the antibiotics ceftazidime (0.016-256 µg/ml, bioMérieux), meropenem (0.002-32 µg/ml, Oxoid) and ciprofloxacin (0.002-32 µg/ml, Oxoid) using the disk diffusion method, based on the Etest (bioMérieux) and M.I.C. Evaluator (Oxoid), according to the manufacturer instructions.

2.6. Statistical analysis

Data on Log (CFU per milliliter) within each culture medium were compared between batches of the same brand and between the three brands, and data on CFU per milliliter for each brand were compared between culture medium with antibiotic and the same culture medium without antibiotic, through the analysis of variance and the post hoc test of Tukey (SPSS 17.0 for Windows), at a significance level (P) of 0.05.

3. RESULTS

3.1. Total Cultivable and Antibiotic-Tolerant Bacteria

Two culture media were used in order to recover total heterotrophs (R2A) and *Pseudomonas* and related bacteria (PIA), reported as highly prevalent in this type of waters. Additionally, given the expected low abundance of antibiotic resistant bacteria in mineral

waters, culture media was supplemented with amoxicillin or ciprofloxacin in order to facilitate the isolation of beta-lactam or quinolone resistant bacteria.

Three batches of each brand of water were examined in this study. In every case were observed significant variations on the counts determined for different batches of the same brand, on both culture media (Table 2). Nevertheless, such variations did not hamper the observation of significant differences between the three brands. On the culture medium R2A and on the same medium supplemented with AML, brands A and C presented significantly ($P<0.05$) lower values of CFU/ml than brand B. In contrast, on the culture medium PIA without antibiotic the values of CFU/mL were non-significantly different ($P<0.05$) in the three brands examined. On the culture medium PIA supplemented with AML, brand A presented significantly ($P<0.05$) lower values of CFU/mL than brand B, although brand C did not differ significantly from those. In the absence of antibiotics, cultivable bacteria ranged $4.3x10^{0}$ - $7.7x10^{3}$ CFU/ml after 48 h at 30 ºC, presenting lower values on antibiotic supplemented media. For all brands, the values of CFU/ml were significantly higher ($P<0.05$) on R2A than on the same medium supplemented with AML. In the presence of the beta-lactam amoxicillin the average values for the counts of R2A cultivable bacteria ranged $2.3x10^{-1}$ – $4.9x10^{2}$ CFU/ml. PIA supplemented with AML supported also lower counts than PIA, for the Portuguese brand B and the French brand C. In contrast, for the Portuguese water brand A the counts on PIA or and PIA-AML were non-significantly different ($P>0.05$). The addition of ciprofloxacin to the culture medium caused a more pronounced inhibitory effect on bacterial growth than amoxicillin, since only samples of the brand A exhibited growth in CIP-supplemented media, after 48 h of incubation (Table 2). In brand A, PIA-CIP counts were non-significantly ($P>0.05$) different from those on PIA or on PIA-AML, although R2A-CIP counts were inferior to those on R2A. Although in brands B and C growth on CIP-

containing culture media was also observed, it only occurred after a longer period of incubation (3 to 7 days).

In total, 238 bacterial isolates were recovered during this study. From brand A, were collected 93 isolates (17 on R2A, 13 on R2A-AML, 19 on R2A-CIP, 16 on PIA, 13 on PIA-AML and 15 on PIA-CIP); from brand B were recovered 95 isolates (18 on R2A, 17 on R2A-AML, 10 on R2A-CIP, 12 on PIA, 12 on PIA-AML and 26 on PIA-CIP), and from brand C were recovered 50 isolates (12 on R2A, 10 on R2A-AML, 3 on R2A-CIP, 12 on PIA, 10 on PIA-AML and 3 on PIA-CIP). The 16S rRNA gene sequence analysis of these isolates showed similarity values of at least 98 % with type strains of validly named species (Chun et al., 2007), of the phyla *Firmicutes*, *Actinobacteria*, *Bacteroidetes* and *Proteobacteria* (*Alpha*-, *Beta*- and *Gamma*- divisions). Based on the 16S rRNA based analysis and EzTaxon database (Chun et al., 2007), the isolates could be affiliated to 31 species - 12 in brand A, 11 in brand B and 11 in brand C, with the same taxa present in more than one water brand (Figure 1). Considering the whole set of isolates, the species affiliated to the *Proteobacteria* prevailed. Nevertheless, the pattern of taxa differed for each water brand.

Isolates affiliated to the species *Arthrobacter nitroguajacolicus* predominated in brand A. These isolates were abundant on both culture media R2A and PIA, and on these media supplemented with ciprofloxacin, and were detected in the three batches examined (Figure 1). *Acidovorax delafieldii* was also abundant in this water brand, but it was recovered from a single batch, on culture media without antibiotic or supplemented with amoxicillin. In brand B, the isolates affiliated to the species *Ralstonia picketii* and *Curvibacter lanceolatus* predominated, showing the highest abundance on the culture media PIA and R2A without antibiotic, respectively. These species were detected in the three batches examined. In brand C, predominated isolates affiliated to the species *Acidovorax facilis* and *Hydrogenophaga taeniospiralis*. The isolates related to *Hydrogenophaga taeniospiralis* were recovered only on

culture medium without antibiotic and those to the species *Acidovorax facilis* were recovered also on culture medium supplemented with AML.

3.2. Antibiotic resistance phenotypes

Considering the whole set of isolates and the three water brands were observed resistance phenotypes to all the antibiotics tested (Table 3). Nevertheless, some resistance phenotypes were absent in each water brand. In brand B, although it was observed resistance to every antibiotic class, none of the isolates resisted the carbapenem imipenem. In contrast, C was the only brand in which was observed imipenem resistance, although no colistin, nalidixic acid, tetracycline or streptomycin resistance was detected. In brand A, none of the isolates presented resistance to imipenem and meropenem (carbapenems) or to gentamicin and amikacin (aminoglycosides). In all brands, most of the isolates showed resistance to three or more classes of antibiotics, and thus can be considered multiple antibiotic resistant (MAR, Table 3). Nine MAR phenotypes were observed in brands A and B and five in brand C. Resistance patterns were classified as MAR(n), with n indicating the number of different classes of antibiotics to which was observed resistance. Patterns MAR(3) to MAR(7) were observed, with brand A exhibiting the highest frequency of MAR (7, 6 and 5) phenotypes (Table 3). In general, isolates affiliated to the beta-*Proteobacteria* were those with the highest multi-resistance indices. Among the Gram-positive bacteria, *Actinobacteria* exhibited resistance against more antibiotics than *Firmicutes*.

All isolates of the same species (100 % 16S rRNA gene sequence similarity) that were found in different batches of the same brand showed identical antibiotic resistance phenotypes (data not shown). This suggests that the resistance phenotype is fairly stable for each species in a brand. However, isolates affiliated to the same species found in different brands,

exhibited distinct antibiotic resistance profiles. For instance, isolates affiliated to the species *Variovorax paradoxus* were detected in brands A and B, shared 99.7 % similarity, and presented different antibiotic resistance phenotypes for example, for beta-lactams and quinolones (Table 3). Also isolates affiliated to the species *Variovorax boronicumulans* that were found in brands B and C, shared 98.6 % similarity and presented different resistance phenotypes. The Portuguese isolates affiliated to *Variovorax boronicumulans* were resistant to tetracycline, cephalothin and streptomycin, while the French isolates were resistant to imipenem and meropenem. Curiously, carbapenem resistance was observed in *Hydrogenophaga taeniospiralis*, other *Betaproteobacteria* in the French water brand. Similarly, streptomycin resistance, detected only in the Portuguese water brands, was also observed in other *Betaproteobacteria* in the same water brand (Table 3). Isolates affiliated to the species *Bacillus nanhaiensis* were found in brands B and C and, shared 98.2 % similarity, which suggest may represent distinct species, but in both cases, were susceptible to all the antibiotics tested. Members of the genus *Pseudomonas* were found in the brands A and C and in all cases presented resistance to ticarcillin, ticarcillin with clavulanic acid, cotrimoxazole and cephalothin, intrinsic resistance phenotypes in this genus.

The *Actinobacteria* isolated during this study presented high resistance levels to some of the antibiotics tested. For example isolates affiliated to *Microbacterium pumilum*, detected in brand B, were able to grow in the presence of 256 µg/ml of ceftazidime and 32 µg/ml of meropenem, the highest concentrations of the antibiotic in the strip used to determine the MIC. On the other hand isolates related with the species *Microbacterium trichothecenolyticum* and *Microbacterium arthrosphaerae*, both from brand A and isolated from R2A supplemented with ciprofloxacin, were able to grow on the maximum concentration of ciprofloxacin tested (32 µg/ml), on 8 µg/ml of ceftazidime, and on 0.2 and 0.5 µg/ml of meropenem, respectively. The other *Actinobacteria*, the *Arthrobacter*

nitroguajacolicus, also from brand A, presented MIC values of 12 µg/ml for ceftazidime and ciprofloxacin, and 0.5 µg/ml for meropenem. The other Gram-positive isolates, affiliated to the species *Bacillus nanhaiensis*, *Staphylococcus pasteuri* and *Lysinibacillus sphaericus,* within the phylum *Firmicutes*, presented lower MIC values. For ciprofloxacin and meropenem the MIC values were below 0.09 µg/ml and for ceftazidime were 1-1.75, 2 and 12 µg/ml, respectively.

4. DISCUSSION

This study was based on the hypothesis that bottled mineral waters may represent a particularly important source of antibiotic resistant bacteria, given the possibility of occurring transmission of bacteria to humans. If antibiotic resistant, bacteria can have an aggravated potential to cause disease, mainly in immune-compromised people. On the other hand, these antibiotic resistant bacteria may have the potential to transfer resistance to commensal and pathogenic bacteria. The experimental planning comprised the detection and characterization of antibiotic resistant cultivable bacteria in mineral water.

In respect to bacteria enumeration, the highest counts of heterotrophic bacteria were observed on R2A and on this culture medium supplemented with the beta-lactam amoxicillin, with values of $7.7x10^3$ and $4.9x10^2$ CFU/ml, respectively (Log values in Table 2). These values are within the range of heterotrophic counts reported for this type of water, some weeks after bottling (Mary et al., 2000; Leclerc and Moreau, 2002). Although some variations on the number of CFU may occur, the bottling process does not change the natural composition of the mineral water, therefore large bacterial populations usually develop from small initial populations present in the source (Leclerc and Moreau, 2002; Loy et al., 2005). There are insufficient clinical and epidemiological evidence to conclude that the high

heterotrophic counts in drinking water pose a risk to consumer's health (Edberg and Allen, 2004; Otterholt and Charnock, 2011; Varga, 2011). However, members of some species that may be part of the mineral water microbiota can cause disease, mainly in vulnerable individuals, i.e., the very young, the elderly, the immune suppressed population, and pregnant women (Szewzyk et al., 2000; Otterholt and Charnock, 2011).

In respect to diversity, it was observed that in the different water brands, the bacterial isolates were affiliated to distinct species, although distributed by the same phyla (Figure 1). It is known that the characteristics of the water source influence the bacterial population of a mineral water (e.g. Morais and Da Costa, 1990; Rosenberg, 2003). Nevertheless, some bacterial groups, such as *Proteobacteria*, *Actinobacteria* and *Firmicutes*, are commonly detected in these habitats (Leclerc and Moreau, 2002; Casanovas-Massana and Blanch, 2012). Besides the natural properties of the waters and its influence on the microbiota, also the screening, isolation and identification methods may contribute to find distinct prevailing populations. The studies of Loy et al. (2005) and of Otterholt and Charnock (2011) demonstrated the predominance of *Beta*- and *Alphaproteobacteria* in bottled mineral waters in Germany and Norway, respectively. Results from both studies, which relied, respectively, upon isolation on R2A and on culture independent 16S rRNA gene sequence-based identifications, are apparently in contrast to many previous ones, supported by isolation on nutrient-rich culture media (eg. PCA) and identifications based on biochemical traits. Indeed, these studies detected mainly *Gammaproteobacteria* (Rosenberg and Hernandez-Duquino, 1989; Armas and Sutherland, 1999; Mary et al., 2000; Urmeneta et al., 2000; Messi et al., 2005). In the current study it was observed the predominance of *Betaproteobacteria* followed by *Gammaproteobacteria* and *Alphaproteobacteria* (Figure 1). As suggested by other authors, the culture medium can influence the patterns of bacteria isolated. For instance, in the current study, most of the *Betaproteobacteria* were isolated on R2A (nine out of 11 species groups),

whereas most of the *Alphaproteobacteria* were recovered on PIA (five out of six species groups). Curiously, *Gammaproteobacteria* were not detected in brand B. Gram-positive bacteria were mainly represented by *Actinobacteria* of the genera *Arthrobacter* and *Microbacterium*, detected only in the Portuguese water brands. *Firmicutes* and *Bacteroidetes* were minor representatives.

Considering the three water brands, it was observed resistance against all the antibiotics tested, which comprised eight different classes. The detection of counts as high as 10^2 CFU/ml, of (multi)-antibiotic resistant bacteria in mineral waters deserves attention. The bacterial isolates with the highest multi-resistance indices were *Betaproteobacteria*, members of the genera *Variovorax*, *Curvibacter*, *Ralstonia* or *Alphaproteobacteria* members of the genera *Bosea* or *Afipia*. The only *Bacteroidetes*, member of the genus *Pedobacter*, was also among the isolates with the highest multi-resistance index. Two major concerns arise from the observation of multi-resistant bacteria in mineral water. The first is related with the observation that some of these bacteria are confirmed or suspected nosocomial or opportunistic pathogens. For instance, *Ralstonia pickettii* and *Ralstonia mannitolilytica* are recognized persistent nosocomial agents associated with bacteremia and septicemia (Daxboeck et al., 2005; Ryan et al., 2006). *Alphaproteobacteria* as those of the genera *Bosea* and *Afipia* are also suspected nosocomial agents, against which antibiotherapy may be problematic (LaScola et al., 2003; Berger et al., 2006). Being multi-antibiotic resistant, the proliferation of these bacteria is favored under antibiotherapy conditions and the chances of a successful therapy are reduced. The second concern is related with the possibility of resistance transfer to human pathogens. Some of the resistance phenotypes observed in this study may be part of the natural or wild phenotype. The clearest example of this is given by members of the species *Bosea vestrisii*, closely related to isolates found in brand B, and which are resistant to almost all classes of antibiotics (LaScola et al., 2003). However, even if

natural or wild in the bacteria, resistance determinants can be transferred to other bacteria. Indeed, it has been largely argued that most of the resistance genes found in multi-resistant clinical isolates had origin both in antibiotic producing bacteria and in environmental isolates not recognized as antibiotic producers (Cantón, 2009; Allen et al., 2010; Wright, 2010; D' Costa et al., 2011).

Members of the genera found in this study are widely distributed in nature, polluted environments, clinical samples and occasionally are associated with beneficial roles (e.g. Scola et al., 2003; Daxboeck et al., 2005; Ryan et al., 2006; Parschat et al., 2007; Han et al., 2011). The information of the antibiotic resistance on most of the bacterial groups detected in this study is really scarce. This gap limits both the distinction between wild and acquired resistance in the isolates examined and the search for genetic elements associated with the observed phenotypes. As a consequence, it is also difficult to estimate the contributions of bacteria from bottled mineral water to enrich the human antibiotic resistome. In a study on the fate of drinking water in the gastrointestinal tract, Lee et al. (2010) demonstrated a correlation between the microbiota of drinking water and its presence in the gastrointestinal tract of germ-free mice. Also Sommer et al. (2009) demonstrated that most of the resistance genes identified in human gut and saliva are evolutionarily distant from known resistance genes, suggesting that numerous bacteria ingested by humans, and harboring unknown resistance determinants can contribute to enrich the human resistome. Given that the microbiota of mineral waters is driven from the natural aquifer, this study represents a further confirmation of the widespread distribution of antibiotic resistant bacteria in nature (D'Costa et al., 2011). However, in regard to public health this is also a warning that mineral water can represent a source of antibiotic resistant bacteria, which can either behave as opportunistic pathogens or transfer resistance determinants to human commensal and pathogenic bacteria.

It has been argued that antibiotic resistance in food-related bacteria may reflect the resistance situation in bacteria from the various environments from where food for human consumption is produced and handled (Sørum and L'Abée-Lund, 2002). In this respect, mineral water represents a particular situation, as the manipulation of this natural resource is minimal. In the current study were observed identical resistance phenotypes for members of the same species isolated from the same water brand, even if were different batches. However, isolates affiliated to the same species but found in different brands, exhibited distinct antibiotic resistance profiles, suggesting that some antibiotic resistance phenotypes may have been acquired. For instance, the type strain of *Variovorax paradoxus* is resistant to penicillins and susceptible to streptomycin and tetracycline, suggesting that these and other resistance phenotypes may be acquired in the strains examined in the current study (Yoon et al., 2006). For *Ralstonia pickettii* it is reported that resistance to ciprofloxacin, sulphamethoxazole-trimethoprim, piperacillin-tazobactam, imipenem and ceftazidime may occur (Stelzmueller et al. 2006). Comparing *Ralstonia pickettii* isolated from different types of water it was observed that different populations harbor distinct resistance phenotypes, suggesting the occurrence of resistance acquisition (our data, unpublished). Also in clinical isolates of *Ralstonia mannitolilytica* resistance to aminoglycosides and cephalosporins is common, as in the current study (Daxboeck et al., 2005). Nevertheless, it is difficult to identify in these environmental bacteria which are the natural and the acquired resistance phenotypes. Based on these considerations, it is possible to hypothesize the observation of acquired resistance in the present study. However, the environmental niches where it occurred and the modes of genetic modification towards the development of a resistance phenotype hardly can be proposed based on the current state of the art. Further studies on the genetic determinants responsible for the observed resistance phenotypes may bring additional clues to the discussion of resistance acquisition and development by these bacteria.

5. CONCLUSIONS

The predominant cultivable bacteria detected in the water samples examined were *Proteobacteria* of the *Beta*-, *Gamma*- and *Alpha* divisions.

Bacteria resistant to more than three distinct classes of antibiotics were detected in the three water brands in counts up to 10^2 CFU/ml. Bacteria with the highest multi-resistance indices were members of the genera *Variovorax*, *Bosea*, *Ralstonia*, *Curvibacter*, *Afipia* and *Pedobacter*. Some of these are confirmed or suspected nosocomial agents.

The observation of distinct antibiotic resistance phenotypes in isolates from different water brands, but affiliated to the same species, supports the hypothesis of resistance acquisition. However, the modes of resistance acquisition cannot be proposed at the current state of the art.

Bottled mineral water was confirmed as a potential source of antibiotic resistant bacteria, and a possible transfer to human commensal or pathogenic bacteria should not be disregarded.

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Table 1. Characteristics of the mineral waters examined in this study*

Composition	Brands		
	A	B	C
	08. 2012	03. 2012	15.12.2012
Expiration date	08. 2012	07. 2012	05.01.2013
	09. 2012	08. 2012	26.01.2013
pH	5.8	5.64 ± 0.13	7.2
Silica (mg/l)	9.6 ± 2	13.0 ± 0.6	15
Bicarbonate (mg/l)	8.0 ± 0.8	10.8 ± 1.5	360
Chloride (mg/l)	4.2 ± 0.4	9.3 ± 0.2	6.8
Sulfate (mg/l)	1.0 ± 0.2	—	12.6
Sodium (mg/l)	4.1 ± 0.4	6.9 ± 0.4	6.5
Calcium (mg/l)	1.3 ± 0.3	0.74 ± 0.07	80
Potassium (mg/l)	0.6 ± 0.1	—	1
Fluoride (mg/l)	—	< 0.13	—
Nitrate (mg/l)	—	1.67 ± 0.05	3.7
Magnesium (mg/l)	—	1.64 ± 0.11	26
Total dissolved solids (mg/l)	25.8 ± 4	46.3 ± 2.4	309

*Information as supplied in the bottled label.

Table 2. Colony forming units (CFU) of culturable bacteria *per* 100 ml of bottled mineral water, recovered on R2A and PIA and in the same culture media supplemented with amoxicillin (AML, 32 mg/l) or ciprofloxacin (CIP, 4mg/l).

Brand	Batch	Mean ± SD (Log CFU/100ml)											
		R2A ⚭		R2A AML		R2A CIP		PIA ⚭		PIA AML		PIA CIP	
A	1	4.08 ± 0.07	a	1.32 ± 0.27	a	1.54 ± 0.49	a	2.63 ± 0.04	a	1.46 ± 0.28	a	2.43 ± 1.90	a
	2	3.90 ± 0.01	b	1.52 ± 0.13	a	3.66 ± 0.46	b	3.41 ± 0.04	b	1.31 ± 0.13	a	3.69 ± 2.90	b
	3	3.64 ± 0.07	c	3.57 ± 0.76	b	0.36 ± 0.10	c	3.49 ± 0.08	b	3.46 ± 0.70	b	0.47 ± 0.00	c
	Total	3.88 ± 0.20	1, *	2.13 ± 1.09	2,*	1.85 ± 1.45	2	3.15 ± 0.43	1,*	2.08 ± 1.05	1,*	2.20 ± 0.47	1
B	1	4.53 ± 0.42	a	3.76 ± 0.45	a	< 0		3.62 ± 0.72	a	3.11 ± 0.72	a	< 0	
	2	5.89 ± 0.40	b	4.69 ± 0.76	b	< 0		3.11 ± 0.05	b	2.88 ± 0.74	b	< 0	
	3	4.40 ± 0.55	a	3.75 ± 0.04	a	< 0		4.75 ± 0.26	c	3.40 ± 0.08	c	< 0	
	Total	4.99 ± 0.75	1,f	4.06 ± 0.47	2,f	< 0		3.83 ± 0.72	1,*	3.10 ± 0.25	2, f	< 0	
C	1	4.08 ± 0.08	a	3.54 ± 0.06	a	< 0		3.81 ± 0.07	a	3.38 ± 0.08	a	< 0	
	2	4.76 ± 0.25	b	1.86 ± 0.03	b	< 0		4.75 ± 0.03	b	1.68 ± 0.07	b	< 0	
	3	3.28 ± 0.28	c	3.23 ± 0.09	c	< 0		3.08 ± 0.08	c	3.28 ± 0.02	a	< 0	
	Total	4.14 ± 0.62	1,*	2.88 ± 0.77	2,*	< 0		3.87 ± 0.73	1,*	2.78 ± 0.83	2,*,f	< 0	

SD, standard deviation; values are mean of three determinations;
Letters indicate significant (P<0.05) differences between batches of the same brand for each culture medium;
Numbers indicate significant (P<0.05) differences between culture medium with antibiotic and the same culture medium without antibiotic for each brand;
Symbols indicate significant differences (P<0.05) between the brands for each culture medium.

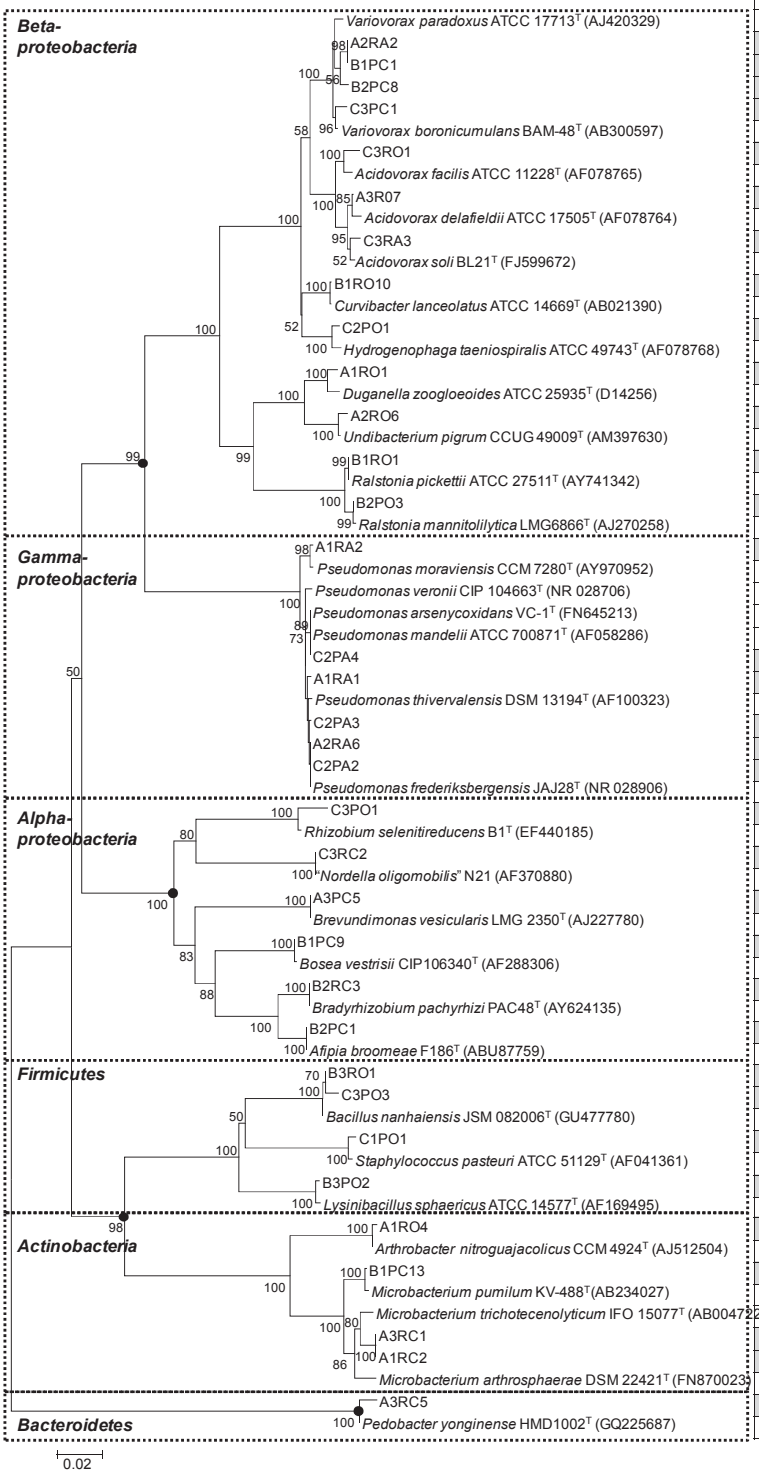
Table 3. Antibiotic resistance patterns observed for each species, corresponding to a total of 238 bacterial isolates from mineral waters.

Brand	Species	Tax. ID	Nº isolates	Antibiotics																				MAR (nº of resistance classes/nº of classes tested)
				Beta-lactams									Aminoglycosides				Quinolones			Polymyxin			Sulfonamides		Tetracycline	
				1	2	3	4	5	6	7*	8	9	10	11	12	13*	14	15	16*	17	18	19	20	21*	22*	
A	*Variovorax paradoxus*	BP	1	R	R	S	S	S	S	R	S	S	S	S	S	R	R	R	S	S	R	R	R	S	R	MAR (7/8)
	Acidovorax delafieldii	BP	15	R	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	R	S	R	S	S	S	MAR (3/8)
	Duganella zoogloeoides	BP	4	R*	-	-	-	R*	-	R	-	-	S*	-	-	S	-	S*	S	S*	-	-	S*	R	S	-
	Undibacterium pigrum	BP	1	S	S	S	S	R	R	S	S	S	S	R	S	S	R	R	R	R	R	S	S	S	S	MAR (5/8)
	Pseudomonas moraviensis	GP	14	R	R	S	S	R	I	R	S	S	S	S	S	I	S	S	I	S	R	I	R	S	S	MAR (3/8)
	Pseudomonas veronii	GP	3	R	R	S	S	S	S	R	S	S	S	S	S	S	S	S	S	R	S	I	R	S	S	MAR (3/8)
	Pseudomonas arsenicoxydans	GP	2	R	R	S	S	S	S	R	S	S	S	S	S	S	S	S	S	R	S	I	R	S	S	MAR (3/8)
	Brevundimonas vesicularis	AP	4	S	S	R	R	R	I	S	S	S	S	S	S	S	R	R	R	R	S	S	R	R	S	MAR (4/8)
	*Arthrobacter nitroguajacolicus**	A	39	S*	-	-	-	I*	-	S	-	S*	S*	-	-	S	-	R*	R	R*	-	-	S*	S	S	-
	*Microbacterium trichothecenolyticum**	A	7	S*	-	-	-	R*	-	S	-	S*	S*	-	-	S	-	R*	R	R*	-	-	S*	S	S	MAR (3/6)
	*Microbacterium arthrosphaerae**	A	2	S*	-	-	-	I*	-	S	-	S*	S*	-	-	S	-	R*	R	S*	-	-	S*	S	S	-
	Pedobacter yonginense	B	1	R	S	R	S	R	S	S	S	S	S	R	I	I	R	R	I	R	R	S	S	S	S	MAR (5/6)
B	*Variovorax paradoxus*	BP	12	R	R	R	R	R	R	R	S	S	S	S	S	R	S	I	S	S	R	R	S	S	I	MAR (4/8)
	Variovorax boronicumulans	BP	4	R	R	R	R	R	R	R	S	S	S	S	S	R	R	R	S	S	R	R	S	S	R	MAR (5/8)
	Curvibacter lanceolatus	BP	20	R	R	S	S	R	R	S	S	R	R	R	R	S	R	R	R	R	R	S	S	S	S	MAR (5/8)
	Ralstonia pickettii	BP	26	R	R	R	R	I	S	S	I	R	R	R	R	R	S	S	S	R	R	S	S	S	S	MAR (4/8)
	Ralstonia mannitolilytica	BP	8	R	R	S	S	R	S	S	I	R	R	R	R	R	S	S	S	R	R	S	S	S	S	MAR (4/8)
	Bosea vestrisii	AP	10	R	S	R	R	R	R	-	S	S	R	R	S	-	R	R	-	R	R	S	R	-	-	MAR (6/8)
	Bradyrhizobium pachyrhizi	AP	5	R	S	R	S	I	S	-	S	S	S	S	S	-	S	S	-	R	S	S	S	-	-	-
	Afipia broomeae	AP	4	R	R	R	R	R	I	-	S	R	S	S	S	-	R	R	-	R	R	S	R	-	-	MAR (5/8)
	Bacillus nanhaiensis *	F	1	S*	-	-	-	S*	-	S	-	S*	I*	-	-	S	-	S*	S	S*	-	-	S*	S	S	-
	*Lysinibacillus sphaericus**	F	1	S*	-	-	-	R*	-	S	-	S*	S*	-	-	R	-	S*	R	S*	-	-	S*	S	S	MAR (3/8)
	Microbacterium pumilum *	A	4	S*	-	-	-	R*	-	S	-	R*	S*	-	-	S	-	S*	R	R*	-	-	S*	R	S	MAR (4/6)
C	*Variovorax boronicumulans*	BP	3	R	R	R	R	R	R	S	R	R	S	S	S	S	R	R	S	S	R	R	S	S	S	MAR (4/8)
	Acidovorax facilis	BP	22	R	S	R	S	R	S	S	S	S	S	S	S	S	S	S	S	S	R	I	R	S	S	MAR (3/8)
	Acidovorax soli	BP	3	R	S	R	R	R	R	-	S	S	S	S	S	-	S	S	-	S	R	I	S	-	-	-
	Hydrogenophaga taeniospiralis	BP	10	R	R	R	S	R	R	-	R	R	S	S	S	-	S	S	-	S	R	S	S	-	-	-
	Pseudomonas mandelii	GP	4	R	R	S	S	I	S	R	S	S	S	S	S	S	S	S	S	S	S	I	R	S	S	-
	Pseudomonas thivervalensis	GP	1	R	R	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	R	I	R	S	S	MAR (3/8)
	Pseudomonas frederiksbergensis	GP	1	R	R	S	S	I	S	R	S	S	S	S	S	S	S	S	I	S	R	I	R	R	S	MAR (3/8)
	Rhizobium selenitireducens	AP	1	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	R	S	S	-
	Nordella oligomobilis	AP	3	S	S	S	S	S	S	-	S	S	R	R	R	-	R	R	-	S	R	S	S	-	-	MAR (3/8)
	*Bacillus nanhaiensis**	F	1	S*	-	-	-	S*	-	S	-	S*	S*	-	-	S	-	S*	S	S*	-	-	S*	S	S	-
	Staphylococcus pasteuri *	F	1	S*	-	-	-	S*	-	S	-	S*	S*	-	-	S	-	S*	I	S*	-	-	S*	S	S	-

Taxonomical identification: AP, *Alphaproteobacteria*; BP, *Betaproteobacteria*; GP, *Gammaproteobacteria*; B, *Bacteroidetes*; A, *Actinobacteria*; F, *Firmicutes*.

MAR (multiple antibiotic resistant), represents species resistant to 3 or more distinct antibiotic classes. * tested by disk diffusion method

Antibiotics: 1, ticarcillin (16 mg/l or *75 µg); 2, ticarcillin-clavulanic acid (16/2 mg/l); 3, piperacillin (16 mg/l); 4, piperacillin plus tazobactam (16/4 mg/l), 5, ceftazidime (4 and 8 mg/l or *30 µg); 6, cefepime (4 and 8 mg/l); 7, cephalothin (*30 µg); 8, imipenem (2 and 8 mg/l); 9, meropenem (2 and 8 mg/l or *10 µg); 10, gentamicin (4 mg/l or *10 µg); 11, tobramycin (4 mg/l); 12, amikacin (8 and 16 mg/l); 13, streptomycin (*10 µg); 14, levofloxacin (1 and 2 mg/l); 15, ciprofloxacin (1 and 2 mg/l or *5 µg); 16, nalidixic acid (*30 µg); 17, colistin (2 mg/l or *50 µg); 18, fosfomycin (32 mg/l); 19, rifampicin (4 and 16 mg/l); 20, cotrimoxazol (4/76 mg/l or *25 µg); 21, sulphamethoxazole (*25 µg); 22, tetracycline (*30 µg).



No. isolates	Abundance log CFU/100mL (min./max.)						Batch
	$R2A_{Ø}$	$R2A_{AML}$	$R2A_{CIP}$	$PIA_{Ø}$	PIA_{AML}	PIA_{CIP}	
1		0.0					2
12	3.9				3.1	0.0/1.6	1, 3
4						0.9	2
3						0.0	3
22	3.1/4.1	3.2/3.5		2.5/3.8	3.3/3.4		1, 3
15	3.6	3.6		3.3	3.5		3
3		0.0/2.0			2.0		2, 3
20	2.9/5.8	3.5/4.7					1, 2, 3
10	3.0/4.8			2.5/4.7			1, 2, 3
5	2.0/3.0						1, 2, 3
1	2.0						2
26	3.9	3.4/3.7		2.5/4.5	2.2/3.1		1, 2, 3
8				3.0	2.7/3.1		2, 3
14		1.4/1.5			0.0/2.0		1, 2, 3
4		1.8			1.6		2
3		0.0/1.4		1.4			1, 2
1					0.0		2
2		0.0		2.0			1, 2
1					0.0		2
1				2.5			3
3						0.0	3
4			0.0			0.0	3
10			1.5			1.3	1
5			4.5				2
4						1.6	2
1	3.9						3
1				2.5			3
1				2.3			1
1				4.3			3
39	3.9/4.0		2.6/3.7	2.6/3.5		2.4/3.7	1, 2, 3
4						1.1	1
7			0.0			0.0	3
2			1.0				1
1			0.0				3

Figure 1. Dendrogram representative of the diversity of species isolated from mineral waters, constructed based on the 16S rRNA gene sequences, and indication of the culture medium of isolation and abundance in different water brands and batches. The designations of the isolates are of the type InMAi, in which I is the water brand (A, B or C); n, the batch from which the isolate included in the dendrogram was isolated; M, the culture medium on which it was isolated (R, for R2A or P, for PIA) and A, indicates the supplementation with antibiotic (O, none, A, amoxicillin, C, ciprofloxacin).