Betaproteobacteria are predominant in drinking water: are there reasons for concern?

Abstract

Betaproteobacteria include some of the most abundant and ubiquitous bacterial genera that can be found in drinking water, including mineral water. The combination of physiology and ecology traits place some *Betaproteobacteria* in the list of potential, yet sometimes neglected, opportunistic pathogens that can be transmitted by water or aqueous solutions. Indeed, some drinking water *Betaproteobacteria* with intrinsic and sometimes acquired antibiotic resistance, harboring virulence factors and often found in biofilm structures, can persist after water disinfection and reach the consumer.

This literature review summarizes and discusses the current knowledge about the occurrence and implications of *Betaproteobacteria* in drinking water. Although the sparse knowledge on the ecology and physiology of *Betaproteobacteria* thriving in tap or bottled natural mineral/spring drinking water (DW) is an evidence of this review, it is demonstrated that DW holds a high diversity of *Betaproteobacteria*, whose presence may not be innocuous. Frequently belonging to genera also found in humans, DW *Betaproteobacteria* are ubiquitous in different habitats, have the potential to resist antibiotics either due to intrinsic or acquired mechanisms, and hold different virulence factors. The combination of these factors place DW *Betaproteobacteria* in the list of candidates of emerging opportunistic pathogens. Improved bacterial identification of clinical isolates associated with opportunistic infections and

additional genomic and physiological studies may contribute to elucidate the potential impact of these bacteria.

Keywords:

Microbiological hazard; autochthonous bacteria; intrinsic antimicrobial resistance; virulence factors

Introduction

The access to safe drinking water (DW) is defined as one of the Sustainable Development Goals and an important human right (https://www.un.org/sustainabledevelopment/sustainable-development-goals/). By definition, DW is suitable for human consumption, washing/showering and domestic food preparation (EuropeanComission 1998; Bartram et al. 2003; WHO 2011). DW comprises i) tap water originating from a surface water (river, lagoons, alluvial wells) or groundwater source that, when necessary may be subjected to treatment before distribution to the consumer, and ii) the bottled natural mineral or spring water originating from a groundwater table or deposit that emerges from a spring or borehole exit (Barrell et al. 2000). While the so-called tap-water needs treatment in most world regions, due to the widespread contamination of water sources, the natural mineral or spring water is "microbiologically wholesome" and must not receive any treatment capable of changing the original chemical and microbiological composition (EuropeanComission 2009). Mineral and spring waters are commonly bottled before distribution to the consumer.

The natural mineral and spring waters microbiomes comprise the autochthonous bacterial community, although the structure of that bacterial community may change after bottling and storage (Flemming et al. 2016). Otherwise, the tap water microbiome occurring in the water that reaches the consumer does not necessarily mirror that thriving in the water source. This is due to the successive alterations that take place from the source to the tap, shaped mainly by a complex interplay between treatment, reactivation, and piping (Norton and LeChevallier 2000; Hoefel et al. 2005; Eichler et al. 2006; Lautenschlager et al. 2010; Vaz-Moreira et al. 2013; Lautenschlager et al. 2014). Indeed, the bacterial diversity of tap water results from

the persistence of some autochthonous bacterial community members that survive the treatment (e.g. chlorination, ozonation or UV irradiation), together with potential intrusions of bacteria throughout the system from the source to the tap. The properties of water and specific physicochemical factors, such as total organic content or hydrodynamic regime, the conditions of the pipes, the range of temperature and pH, the residence time, among others, may influence the shape of the bacterial community (Pepper et al. 2004; Lautenschlager et al. 2010; Pinto et al. 2012; Douterelo et al. 2013; Lautenschlager et al. 2014). Another important driver of the tap water bacterial community composition and structure is the formation of biofilms along the distribution systems, which may rule the release of biofilm bacteria into the circulating water (Batté et al. 2003). Despite the specificities of each water source, piping and treatment conditions, *Proteobacteria* (mainly of the classes *Alpha*, *Beta* and *Gamma*) are among the predominant populations in DW, tap or mineral/spring, worldwide (Leclerc and Moreau 2002; Hoefel et al. 2005; Loy et al. 2005; Eichler et al. 2006; Poitelon et al. 2009; Revetta et al. 2010; Pinto et al. 2012; Vaz-Moreira et al. 2014). Dias et al. (2019) recently described that the *Proteobacteria* profile changes from the distribution system to tap water, with *Alphaproteobacteria* being dominant in the distribution system (92% *vs*. 65% in tap waters), whereas *Betaproteobacteria* prevalence in tap water was higher than in the distribution system (18% *vs*. 2%). This variation was attributed to the higher chlorine tolerance observed in members of the class *Alphaproteobacteria* when compared to members of the class *Betaproteobacteria* (Williams et al. 2004; McCoy and VanBriesen 2012; Dias VCF et al. 2019).

Although water *Alphaproteobacteria*, and mainly *Gammaproteobacteria*, that include some well-known pathogens (e.g. the *Alphaproteobacteria Rickettsia* and *Bartonella* spp.; or the

Gammaproteobacteria Legionella, Escherichia coli, Vibrio spp., *Salmonella, Acinetobacter baumannii* and *Klebsiella pneumoniae*) have been frequently discussed, *Betaproteobacteria* are, comparatively, a neglected group. This gap of information was a major motivation to bring forward the current review, focused on DW *Betaproteobacteria*.

DW is an important source for the dissemination and transmission of microbial agents to humans, meaning that the DW microbiome may pose important potential risks for human health. In a previous study, Vaz-Moreira and colleagues (2017) observed that *Proteobacteria* genera can persist after DW treatment, being ubiquitous along the DW source-treatment-distribution-tap thread. In that study, the ubiquity of *Betaproteobacteria* in the DW system was evidenced, confirming previous studies conducted in other clean environments, such as filtered water, antiseptics or disinfectants (Hahn 2004; Weber et al. 2007). These results are also in line with data reported in studies about bottled natural mineral water, which identify *Betaproteobacteria* among the predominant bacterial groups (Leclerc and Moreau 2002; Loy et al. 2005; França et al. 2015). The remarkable capacity to form biofilm in freshwater habitats (Manz et al. 1999; Araya et al. 2003) and the survival to disinfectants and disinfection processes (Mi et al. 2015; Becerra-Castro et al. 2016) are probably part of the explanation for the observed ubiquity of *Betaproteobacteria* in DW. These evidences claim for the attention of the scientific community mainly because some of the DW *Betaproteobacteria* genera may comprise opportunistic pathogens and/or drug resistant bacteria. In this review, we were interested in overviewing what is known about *Betaproteobacteria* ecology, intrinsic or acquired antibiotic resistance and virulence factors, as background information for discussing potential human health implications and, if justified, identifying relevant knowledge gaps.

Context and approach

Based mainly on phylogenetic evidence, recently Parks et al. (2018) proposed that the class *Betaproteobacteria* would be better reclassified into the order *Betaproteobacteriales*, within the class *Gammaproteobacteria*. For practical reasons, this review followed the NCBI Taxonomy database (https://www.ncbi.nlm.nih.gov/Taxonomy/), in which the class *Betaproteobacteria* comprises 23 families and a large group of unclassified *Betaproteobacteria*, including some groups with *Candidatus* statute (accessed in https://www.ncbi.nlm.nih.gov/Taxonomy/ in August 2019). Most of these 23 families (17) have been reported in DW habitats (Figure 1). This is not surprising, given the ubiquity of *Betaproteobacteria*, whose colonized habitats include soil and rhizosphere, plants, foods, clinical samples, among other (Garrity et al. 2005), as well as aquatic environments, particularly DW (Leclerc and Moreau 2002; Hoefel et al. 2005; Loy et al. 2005; Eichler et al. 2006; Poitelon et al. 2009; Revetta et al. 2010; Pinto et al. 2012; Vaz-Moreira et al. 2014).

For this review were selected studies that approach the bacterial diversity in water destined for human consumption, both treated tap water and bottled natural mineral/spring water. This selection included also the bacterial diversity of treated drinking water biofilms, since biofilms are known to strongly influence and result from the tap water bacterial diversity (Berry et al. 2006; Srinivasan et al. 2008). For the review were selected papers published after 1998, most of which based on culture-independent methods, although some relied also on culture-dependent methods. Were excluded the studies in which bacterial identification relied exclusively on phenotypic methods. Because human health implications may result

from a transient or resident bacterial colonization, we also explored if the *Betaproteobacteria* genera detected in DW have been reported in the human microbiome. These analyses were based on the Human Microbiome (https://hmpdacc.org/catalog/) and Human Oral microbiome (http://www.homd.org/) catalogs, and the NCBI database (www.ncbi.nlm.nih.gov) filtering by "Host: *Homo sapiens*", accessed in June 2018. Our rationale was that closely related bacteria, as are the members of the same genera or species, tend to share an important part of the core genome, including housekeeping functions that may also serve for colonization and infection in a host (Wu HJ et al. 2008; Linz et al. 2016; Wu Y et al. 2018). In contrast, the gain or loss of some functions and genes may be part of the adaptation process to a given environment and may be the basis of the speciation transformation (Lawrence 2002). In this process, it is observed that some traits may be even strain specific (Bentley 2009; D'Auria et al. 2010). However, the demonstration that in a given bacterial group some traits can be observed, is a good indication of the potential occurrence in the whole species or genus. This is particularly relevant in ubiquitous bacterial groups, the focus of this review, in which adaptation and speciation may be hindered or at least shaped by a permanently changing environment.

The filters used led to a list of 24 *Betaproteobacteria* genera that were detected both in tap and bottled natural mineral/spring water and whose association with humans was also reported. Members of these genera were examined for their potential as carriers/disseminators of virulence or of antimicrobial resistance determinants. The virulence factors were compiled from the literature available and from the Virulence Factors Database (VFDB, http://www.mgc.ac.cn/VFs/), accessed in July 2018. Intrinsic and acquired antimicrobial resistance was compiled from the literature available.

Betaproteobacteria in drinking water

As mentioned above, a total of 17 *Betaproteobacteria* families, belonging to six orders, were reported in DW habitats. The most commonly reported families (*Comamonadaceae*, *Oxalobacteraceae*, *Burkholderiaceae*, *Alcaligenaceae*, and unclassified *Burkholderiales*), represented by 54 out of 83 genera, belong to the order *Burkholderiales* (Figure 1). A total of 63 bacterial genera were identified in bottled natural mineral/spring water and 55 in tap DW. Among those, 36 genera were reported in both mineral/spring and tap DW. These bacteria were members of 5 of the 6 orders of *Betaproteobacteria* described in DW: *Burkholderiales* (25 genera), *Rhodocyclales* (5 genera), *Neisseriales* (2 genera), *Nitrosomonadales* (2 genera), *Hydrogenophilales* (1 genus), and *Methylophilales* (1 genus) (Figure 1). This distribution suggests the endemic character of bacteria of these orders to DW, independently of being tap or bottled mineral/spring water. In contrast, some *Betaproteobacteria* were only reported in bottled mineral water habitats, and, to our knowledge, were never reported in treated tap DW (e.g. *Pseudorhodoferax*, *Brachymonas*, *Ottowia*, *Caenimonas*, *Alicycliphilus*, *Ramlibacter*, *Diaphorobacter*, *Xenophilus*, *Xylophilus*, *Leptothrix*, *Piscinibacter*, *Tepidimonas*, *Oxalobacter*, *Telluria*, *Paucimonas*, *Derxia*, *Alcaligenes*, *Methylobacillus*, *Sulfuritalea*, *Azoarcus*, *Deefgea*, and *Ferritrophicum*) (Figure 1). This may suggest the influence of physiologic and metabolic properties of these bacteria and/or their susceptibility to water treatment.

As expected, most of the bacterial genera observed in treated DW biofilms were also observed in the tap water (27 out of 33 genera), being the exception the genera *Sutterella*,

Undibacterium, *Neisseria*, *Methylibium*, *Methylotenera*, and *Methylovorus*. Most of the genera observed to be ubiquitous in DW were also reported in association with humans (24 out of the 36: *Achromobacter*, *Ralstonia*, *Limnobacter*, *Burkholderia*, *Cupriavidus*, *Acidovorax*, *Delftia*, *Polaromonas*, *Curvibacter*, *Variovorax*, *Comamonas*, *Pelomonas*, *Malikia*, *Herminiimonas*, *Janthinobacterium*, *Herbaspirillum*, *Massilia*, *Aquabacterium*, *Ideonella*, *Chromobacterium*, *Methylophilus*, *Dechloromonas*, *Propionivibrio*, and *Azospira*) (Figure 1). Members of these genera represent candidates for possible interaction with the human microbiome, leading to the eventual resident colonization or transfer of acquired traits, such as virulence or resistance to antibiotics. However, the possible risks to human health are obviously dose dependent, and therefore any risk discussion should rely also on quantitative analyses rather than only on qualitative diversity assessments. However, the use of diverse sampling and analyses methods in the supporting literature seriously limit the possibility of doing accurate quantitative comparisons. Not much is known about the influence of DW bacteria in the human gut and in what conditions DW bacteria can represent a risk for human health. The importance of DW as a vehicle of *Betaproteobacteria* was highlighted by Lee *et al*. (2010), who used germ-free mice to demonstrate a correlation between the bacterial communities originating in the DW and those present in the gastrointestinal tract, with the *Betaproteobacteria Ralstonia* representing one of the bacterial genera transported to the gastrointestinal tract via DW. Recently, Dias *et al*. (2018) studied the response of the mouse gut bacterial community to the ingestion of different types of DW. After 23 days of water consumption, it was observed a significant increase in feces of the relative abundance of *Firmicutes* for the different types of water, and of *Acinetobacter* and *Staphylococcus* spp. for treated tap water.

Survival strategies

Betaproteobacteria comprise bacteria with the capacity to survive disinfectants or disinfection processes (Williams et al. 2004; Garrity et al. 2005; Mi et al. 2015), which facilitate the persistence of these bacteria in DW treatment systems. Although the mechanisms responsible for this increased survival capacity are not fully understood, they are supposed to result from the complex interplay of different physiological and structural properties, such as the oligotrophic and auxotrophic character, detoxification, efficient stress responses or charity mechanisms among community members (Chapman 2003; Davin-Regli and Pages 2012; Mi et al. 2015). For example, detoxification is hinted by the capacity of some *Betaproteobacteria* (e.g. *Burkholderia cepacia*, *Ralstonia* spp., and *Delftia* spp.) to biodegrade disinfection byproducts (Field and Sierra-Alvarez 2004; Miyake-Nakayama et al. 2006; Bull et al. 2011). These properties may explain the *Betaproteobacteria* dominance in treated DW, and their fitness to survive the water treatment, becoming the largest proteobacterial class in treated water and associated biofilms (Kalmbach et al. 2000; Mi et al. 2015). In a study aiming to identify the microorganisms and genes involved in the biodegradation of benzalkonium chlorides and quaternary ammonium compounds, Ertekin et al. (2016) highlighted the capacity of *Proteobacteria*, with *Achromobacter* spp. (members of the class *Betaproteobacteria*) among the most abundant species, to survive and degrade benzalkonium chlorides. Interestingly, such a capacity was associated with multidrug resistance (mainly multidrug resistance efflux proteins), oxidative stress response (e.g. glutathione S-transferases), gene expression regulation (e.g. members of the LysR, LysE, MerR, rpiR, AraC and AsnC families of transcriptional regulators), catabolic reactions

(mainly dehydrogenases and FAD dependent oxidoreductases), protein metabolism, outer cell structure modification, and transport (Ertekin et al. 2016; Duangurai et al. 2018). The exposure to sub-inhibitory concentrations of quaternary ammonium compounds, as well as to other antimicrobials, creates (oxidative) stress. The response to that stress may boost gene transfer and recombination events via prophages, transposons, integrons and integrative-conjugative elements (ICEs) (Tezel and Pavlostathis 2015). Those mobile genetic elements are frequently described in *Betaproteobacteria* (Riccio et al. 2001; Shin et al. 2005; Ryan et al. 2009; Rhodes and Schweizer 2016). These mechanisms have also implications in the microbial community charity. In addition, the oligotrophic and/or auxotrophic character, as well as, the efficient stress response of some of these bacteria are related with the resilience of *Betaproteobacteria*, demonstrated to occur as contaminants of sterile solutions or of disinfectant solutions (Weber et al. 2007). For example, *Ralstonia* spp. are often reported as contaminants in blood culture medium, sterile saline solution or other medical solutions (Gardner and Shulman 1984; McNeil et al. 1985; Roberts et al. 1990; Lacey and Want 1991; Maki et al. 1991; Luk 1996; Labarca et al. 1999; Maroye et al. 2000; Boutros et al. 2002; Gröbner et al. 2007). Also, *Burkholderia* spp. (Magalhaes et al. 2003; Doit et al. 2004; Nasser et al. 2004; Estivariz et al. 2006; Held et al. 2006; Ko et al. 2015), and *Achromobacter* spp. (Vu-Thien et al. 1998; Tena et al. 2005; Turgutalp et al. 2012; Hugon et al. 2015) have been reported as contaminants of disinfectants solutions and medications. This capacity to survive disinfectants or disinfection processes may explain the high diversity of *Betaproteobacteria* observed in treated tap water (Figure 1).

Associated with the capacity to survive treatment processes (e.g. disinfectants, toxic metals, antibiotics), the capacity of *Betaproteobacteria* to form biofilms is frequently described (Mah

and O'Toole 2001; Emtiazi et al. 2004; Schwering et al. 2013; Ertekin et al. 2016; Flemming et al. 2016; Ferro et al. 2019). The association between both characteristics may have two explanations: i) the bacteria with increased fitness to survive antimicrobial agents are those able to form or incorporate biofilm structures, or ii) the biofilm provides an increased protection against external attacks (e.g. disinfectants) working as a kind of shield by inhibiting the antimicrobial diffusion by the extracellular polymeric substance (EPS) molecules or by a direct consequence of the slow growth state of the biofilm cells avoiding drugs that target metabolic processes occurring during growth (Lewis 2001; Berry et al. 2006; Anderson and O'Toole 2008; Dufour et al. 2010; Schwering et al. 2013; Flemming et al. 2016). Indeed, both mechanisms are probably combined, as is reported for example for *Ralstonia pickettii*, able to survive disinfectant solutions and form biofilm in industrial and pharmaceutical high-purity water systems (Kulakov et al. 2002; Adley et al. 2005; Ryan et al. 2011). In DW, it was observed that most of the bacterial genera reported in biofilms were also reported in tap water (e.g. *Ralstonia*, *Limnobacter*, *Burkholderia*, *Cupriavidus*, *Acidovorax*, *Delftia*, *Polaromonas*, *Curvibacter*, *Variovorax*, *Janthinobacterium*, *Herbaspirillum*, *Aquabacterium*, *Dechloromonas*), suggesting that these bacteria exist in a dynamic equilibrium between the planktonic and biofilm state. However, some genera, described mainly in biofilms rather than in the planktonic state in DW, such as *Sutterella*, *Undibacterium*, *Neisseria*, *Methylibium*, *Methylotenera*, and *Methylovorus*, may benefit from the protective biofilm structure (Figure 1). That protective effect was demonstrated for instance in *Neisseria gonorrhoeae* observed to be more resistant to non-thermal atmospheric pressure plasma treatment in the biofilm-resident state than in the planktonic form (Xu et al. 2011). Also UV disinfection may enhance the biofilm metabolic activity (Schwartz et al. 2003).

Other mechanisms, such as the association with free-living amoebas, may also explain the good fitness of the *Betaproteobacteria* in DW. The free-living amoebas can easily resist the DW treatment and are important in the bacterial community modulation since they feed on bacteria, by phagocytosis (Delafont et al. 2016). However, some bacteria developed mechanisms of amoeba-digestion resistance, and instead of dying when internalized by amoeba, they survive and multiply, being later released back to the environment. Among the bacterial characteristics described as relevant for their increased survival to amoeba grazing are features as the cell surface properties, the production of bioactive metabolites, the swimming speed, the microcolony formation or the cell-to-cell communication (Matz and Kjelleberg 2005). As happens with other taxa, *Betaproteobacteria* comprise amoeba-resistant members, as for example the genera *Achromobacter*, *Burkholderia*, *Chromobacterium*, *Delftia*, and *Ralstonia* (Thomas et al. 2010). Curiously, all of these genera have been reported in both tap and bottled mineral DW as well as in the human microbiome (Figure 1).

DW *Betaproteobacteria* as potential carriers of virulence factors

Virulence factors are molecules that enable a microorganism to establish itself on or within a host and enhance its potential to cause disease. The virulence of a pathogen depends on its ability to accomplish the different steps required to cause infection: adhesion, colonization, invasion, immune response inhibition and/or production of toxins. In general, the success of the pathogen relies, among other factors, on the diversity and sophistication of the invasion,

proliferation and defense mechanisms. With modest virulence machinery, opportunistic pathogens are commensal or environmental bacteria, often innocuous for a healthy individual. However, these bacteria, have the potential to cause disease in individuals with diminished defenses (e.g., disease, wound, medication, prior infection, immunodeficiency, ageing), due to the presence of virulence factors that facilitate invasion and or proliferation in the host (Brown et al. 2012). Some of the *Betaproteobacteria* found in DW have a distinct array of virulence factors and, therefore, meet the criterion of opportunistic pathogens (Table 1).

Virulence factors or homologous genes have been described in 11 out of the 24 *Betaproteobacteria* genera detected in both DW (tap and mineral) and in the human microbiome (Table 1). The fact that only these 11 genera were reported as potential carriers of virulence factors suggests a major knowledge gap about ubiquitous and potentially hazardous microbial groups. Curiously, not even for species associated with outbreaks, as *Ralstonia pickettii* and *R. mannitolilytica*, were described virulence factors (Labarca et al. 1999; Maroye et al. 2000; Daxboeck et al. 2005; Gröbner et al. 2007; Coman et al. 2017).

Virulence factors may be divided into membrane proteins, capsule, secretory proteins, and others (Table 1). The membrane proteins are mainly associated with the increased capacity of adhesion of the bacteria to the host cells (Wu HJ et al. 2008). Specifically, type IV secretion systems (T4SS), only described in Gram-negative bacteria and common among these bacteria, were frequently reported in DW *Betaproteobacteria*, in six different genera (Table 1). The presence of a capsule, a key virulence determinant that can mediate resistance to both phagocytosis and complement-mediated killing (Reckseidler-Zenteno et al. 2005; Abreu and Barbosa 2017), was described in *Burkholderia* species. The secretory proteins

include the systems of transport of toxins, the toxins, and immune response inhibitors, as well as other siderophores or proteins, all of them observed in DW *Betaproteobacteria* (Table 1). Secretion systems (SS) are used by bacteria to secrete virulence factors from the cytosol into host cells or the host environment, and can span the inner and outer membrane (e.g. RND efflux systems, T1SS, T2SS, T3SS, T4SS, T6SS) or only the outer membrane (e.g. T5SS) (Costa et al. 2015). In human-associated DW *Betaproteobacteria*, the most common secretion systems seem to be T2SS, T3SS, and T6SS (Table 1). One of those, the T3SS, also known as "injectisome", has an important role in the proteins export from the bacterial cytoplasm into the host eukaryotic cells (Cornelis 2006; Puhar and Sansonetti 2014), being the mechanism used by *B. pseudomallei* to cause melioidosis in mammals or *R. solanacearum* to cause plant bacterial wilt (Stevens et al. 2002; Valls et al. 2006; Puhar and Sansonetti 2014). The multidrug RND (resistance nodulation cell division) efflux pumps, described for *B. pseudomallei* (Table 1), may be responsible for intrinsic resistance to several antimicrobials (Munita and Arias 2016; Rhodes and Schweizer 2016). T4SS, only described in *B. cenocepacia* and *A. xylosoxidans* (Table 1), allow the transport of DNA and may have an important role in the transfer of genetic material (Cascales and Christie 2003; Green and Mecsas 2016). Toxin production is described in members of the genera *Burkholderia*, *Chromobacterium*, and *Achromobacter* (Table 1).

Quorum-sensing (QS) rules a bacterial cell-to-cell communication process, based on auto-inducer signaling, enabling bacteria to adjust the cell density and gene expression, regulating activities such as bioluminescence, sporulation, competence, antibiotic production, biofilm formation, or virulence factor secretion (Rutherford and Bassler 2012). QS is important in biofilm formation and also for the activation of virulence factors (Dufour et al. 2010; Soto

2013). These communication processes have been described in *Burkholderia* spp. and *Chromobacterium violaceum*, *Ralstonia solanacearum*, or *Polaromonas* spp. (Table 1).

This review on virulence factors reveals that the machinery for host colonization, invasion and infection, typical of opportunistic pathogens, is available in DW *Betaproteobacteria* that can also be associated with the human microbiome. Potential virulence may not be eliminated by disinfection as was demonstrated by previous studies that showed that chlorination may promote the increase of the relative abundance of virulence proteins in drinking water (e.g. translocases, transposons, Clp proteases, and flagellar motor switch proteins) (Huang et al. 2014). Potential virulence combined with disinfection resilience put DW *Betaproteobacteria* among the potentially relevant safety biomarkers.

Antimicrobial resistance in DW *Betaproteobacteria*

In addition to the ubiquitous character and virulence potential, some *Betaproteobacteria* exhibit resistance to different antibiotics (Vaz-Moreira et al. 2014; Khan et al. 2016; Vaz-Moreira et al. 2017), which may increase the risk associated with their presence in DW. Jia et al. (2015) demonstrated that the relative abundance of antibiotic resistance genes (ARGs) increased after DW chlorination, being *Betaproteobacteria Acidovorax* spp. among the bacterial groups that most contributed to that shift. Also in natural mineral/spring water, not subjected to any kind of treatment, the presence of *Betaproteobacteria* yielding antibiotic resistance phenotypes has been reported (Messi et al. 2005; Falcone-Dias et al. 2012). These evidences suggest the important contribution of *Betaproteobacteria* to the DW resistome.

Although most of the antimicrobial resistance mechanisms detected in the environment can be intrinsic, meaning they are a phenotypic expression of a gene that is common to all members of a given species or genus, they can still contribute to the failure of antibiotic therapy (Cox and Wright 2013; Perry et al. 2014). A well-known example of intrinsic resistance is the presence of the outer membrane (OM) in Gram-negative bacteria that may modify their porin channels to confer impermeability to different molecules or the presence of efflux pumps that allow the reduction of the intracellular concentration of a given drug contributing to multidrug resistance (MDR) phenotype (Cox and Wright 2013; Perry et al. 2014; Pothula et al. 2016). The intrinsic resistance is inherited vertically, from one generation to the next.

Different intrinsic antimicrobial resistance mechanisms are described in *Betaproteobacteria* species, although this information is available for a reduced number of species, specifically for six out of the 36 genera reported in both tap and bottled mineral water (Table 2). This information scarcity is also related with the limited attention that has been given to this group of bacteria, with the exception of a few species that are considered of high clinical relevance (e.g. *Achromobacter xylosoxidans* and *Burkholderia cepacia*). The DW *Betaproteobacteria* intrinsic resistance is frequently against penicillins and cephalosporins, as well as to other antimicrobial agents, as fosfomycin (Table 2). It is important to note that some of the species related to the bacterial genera commonly found in DW habitats present intrinsic resistance to some drugs that are considered last-resort drugs, being only used in clinical settings. For example, the colistin (polymyxin E) is the only clinically approved therapeutic agent that inhibits the OM and efflux systems (Cox and Wright 2013). However, some *Burkholderia* spp., *Chromobacterium violaceum* and *Janthinobacterium lividum* are described as being

intrinsically resistant to colistin (Table 2), and are also reported as infectious agents (Patjanasoontorn et al. 1992; Jones et al. 2001; Sirinavin et al. 2005; Yuan et al. 2006; Kennedy et al. 2007; Yang and Li 2011; Hu C-h and Wang 2012). Also beta-lactams are frequently used as front-line treatments in combinations antibiotic/beta-lactamase inhibitor (e.g. sulbactam, clavulanate, tazobactam) (Cox and Wright 2013). However, also to these combinations were detected intrinsic resistance phenotypes in *Achromobacter xylosoxidans* and *Burkholderia cepacia* (Table 2). Aminoglycosides resistance, described in *Burkholderia* spp. or *A. xylosoxidans* (Table 2), is supposedly intrinsic and may be associated to the presence of RND multidrug efflux pumps (e.g. BpeAB-OprB, AmrAB-OprA or AxyXY-OprZ) (Buroni et al. 2009; Bador et al. 2013). This is particularly relevant when some studies show that the occurrence of the RND efflux systems increases in DW after chlorination (Jia et al. 2015). The association of these efflux systems to an increased tolerance or resistance to aminoglycosides is curious because previous studies have shown a higher prevalence of resistance to aminoglycosides after DW treatment (Armstrong et al. 1982; Vaz-Moreira et al. 2011; Vaz-Moreira et al. 2012; Narciso-da-Rocha et al. 2013; Ma et al. 2017). Although intrinsic resistance has a low potential to be transferred to other bacteria, it may jeopardize the treatment of infections caused by these bacteria.

In addition, some of the described *Betaproteobacteria* characteristics may contribute to their capacity to acquire new resistance to antibiotics, as the capacity to form biofilms and the presence of type 4 secretion systems (T4SS) (Table 1). While the T4SS allows the transport of DNA, the biofilm formation allows a close proximity between cells, facilitating both the dissemination of resistance genes between cells by horizontal gene transfer (HGT) (Cascales and Christie 2003; Flemming et al. 2016; Green and Mecsas 2016). Król *et al*. (2013)

observed that conjugation can be up to 700-fold more efficient in biofilms than in free-living bacterial cells. Described examples are the *A. xylosoxidans* acquired resistance to ciprofloxacin, ceftazidime and carbapenems, in clinical isolates from cystic fibrosis patients (Amoureux et al. 2013) and the acquisition of new genetic elements associated to mobile genetic elements (Riccio et al. 2001; Iyobe et al. 2002; Shin et al. 2005; Neuwirth et al. 2006; El Salabi et al. 2012; Yamamoto et al. 2012; Hu Y et al. 2014), or the *Burkholderia* spp. acquired antibiotic resistance to fluoroquinolones, trimethoprim among others (Pitt et al. 1996; Thibault et al. 2004; Rhodes and Schweizer 2016). Apart from these two genera, based on our literature search, no information is available for possible acquired antibiotic resistance mechanisms.

Of special interest in *Betaproteobacteria*, are the processes of co-resistance or cross-resistance. While co-resistance is mainly due to genetic linkage (e.g. antibiotic and metal resistance in the same genetic element), cross-resistance is due to broad spectrum resistance mechanisms (e.g. MDR efflux pumps). In both cases, resistance to the exposure to a specific agent (e.g. antibiotics, metals, disinfectants) may facilitate the selection of populations resistant to different antimicrobial agents (Chapman 2003; Baker-Austin et al. 2006).

Concluding remarks and future research challenges

Water quality is a central issue for human health and wellbeing. On average, an adult ingests about 1 L of water per day, every day. This makes of water the food product ingested at the highest amounts during a person lifetime. Simultaneously, water is also an important way of dissemination of bacteria and chemical compounds, including contaminants (WHO 2012).

For these reasons, DW microbiome may play an important role in human health and wellbeing, with relevant implications of the major populations, such as *Betaproteobacteria*. While some DW bacteria may be beneficial or innocuous, others may represent a risk for human health. The latter may be due to some DW *Betaproteobacteria.*

Betaproteobacteria are abundant and diverse in DW or DW biofilms, being some of them ubiquitous to tap and bottled natural mineral/spring water (Figure 1). Moreover, some DW *Betaproteobacteria* are also reported in humans. The human health risk posed by DW *Betaproteobacteria* can be inferred from their resistance to disinfection, the presence of virulence factors and intrinsic antibiotic resistance. Some of the virulence factors described in *Betaproteobacteria*, such as adherence factors or the capacity to form biofilms, may contribute to explain the ability of these bacteria to survive in water habitats. Hypothetically, all these are factors that may increase the probability of causing opportunistic infections, being here highlighted in the need for further research in this field.

From this literature review, three bacteria genera seem to stood out: *Achromobacter*, *Burkholderia*, and *Ralstonia*. Members of these genera were also those previously associated with infection outbreaks. Given the phylogenetic and physiologic proximity, other *Betaproteobacteria* genera might share similar properties still unknown, given the scarcity of information. This was, indeed, a major conclusion of this review. Bacteria that are not considered primary pathogens are, most of the times, not screened in routine monitoring analyses in clinical situations. For example, *Ralstonia* spp. occasionally associated with infection episodes, may be a misidentified opportunistic pathogen, if it is not included in the screened pathogen database (Daxboeck et al. 2005; Ryan et al. 2006; Ryan and Adley 2014; Coman et al. 2017).

The first step to improve the current knowledge is to have a good overview of the *Betaproteobacteria* diversity in DW and their possible association with humans, virulence, adaption potential, and genome dynamics for antimicrobial resistance or virulence acquisition. This review is a first step to fill in this gap. Because some of those characteristics will be better understood based on culture methods, additional investment in culturomic approaches are most welcome in the DW microbiology field (Greub 2012; Lagier et al. 2012).

Although DW is considered important for human health and well-being, many questions are still requiring our attention. It is important to understand how/if the DW microbiota, including the *Betaproteobacteria* group, focused in this review, may direct or indirectly influence human health.

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1109

1110 Table 1. Described virulence factors or homologous genes (*) in *Betaproteobacteria* genera observed in tap and bottled mineral
1111 drinking water and described as human-associated bacteria

Classification	Sub-classification	Examples	Drinking-water associated bacteria	References
Membrane proteins	Adhesion	*Burkholderia* oligomeric coiled-coil adhesin A (*BoaA*) and b (*BoaB*).	*Burkholderia pseudomallei*	(Balder et al. 2010)
		Pilus structural proteins (Type IV pili)	*B. pseudomallei*; *Burkholderia cenocepacia*; *Acidovorax avenae subsp. avenae*; *Acidovorax citrulli*; *Ralstonia solanacearum*; *Limnobacter thiooxidans* (*); *Chromobacterium violaceum* (*)	(Liu et al. 2001; Kang et al. 2002; Alves de Brito et al. 2004; Essex-Lopresti et al. 2005; Bahar et al. 2009; Holden M. T. et al. 2009; Burdman and Walcott 2012; Ibrahim et al. 2012; Stone et al. 2014; Har et al. 2015)
		Chaperone-usher type fimbriae	*B. cenocepacia*	(Holden M. T. et al. 2009)
		Flp-type pili	*B. cenocepacia*; *Cupriavidus taiwanensis* (*)	(Amadou et al. 2008; Holden M. T. et al. 2009)
		Hemagglutinin/hemolysin related	*B. pseudomallei* (*); *L. thiooxidans* (*); *Achromobacter xylosoxidans* (*)	(Dowling et al. 2010; Li et al. 2013; Har et al. 2015)
		Mannose-fucose binding lectin (LecM)	*R. solanacearum*	(Meng et al. 2015)
		22-Kda adhesion protein AdhA	*B. cenocepacia*	(Holden M. T. et al. 2009)
		BuHA family of proteins	*B. cenocepacia*	(Holden M. T. et al. 2009)
		BcaA autotransporter protein	*B. pseudomallei*	(Campos et al. 2013; Stone et al. 2014)
		poly-β-1,6-N-acetyl-D-glucosamin (*pga* operon)	*A. xylosoxidans* (*)	(Jakobsen et al. 2013)
		Outer Membrane Protein (Omp21)	*Delftia acidovorans*	(Baldermann et al. 1998)
	Actin-based intracellular motility	*Burkholderia* intracellular motility A (BimA)	*B. pseudomallei*, *Burkholderia mallei; Burkholderia thailandensis*	(Stevens et al. 2005; Sitthidet et al. 2010; Sitthidet et al. 2011)
	Invasion and colonization	Polar flagella	*B. pseudomallei*; *B. cenocepacia*; *A. citrulli*	(Chua et al. 2003; Inglis et al. 2003; Urban et al. 2004; Burdman and Walcott 2012)
		BuHA family of autotransporting membrane proteins	*B. cenocepacia*	(Holden M. T. et al. 2009)

	Surface components	LPS core oligosaccharide	*B. cenocepacia*; *A. xylosoxidans* (*); *C. violaceum* (*)	(Alves de Brito et al. 2004; Loutet and Valvano 2010; Li et al. 2013)
		EPS (extracellular polysaccharide)	*R. solanacearum*	(Genin and Denny 2012)
	Others	HtrA protease	*B. cenocepacia*	(Flannagan et al. 2007)
		cbb3-Type Cytochrome c Oxidase	*R. solanacearum*	(Colburn-Clifford and Allen 2010)
Capsule	Antiphagocytosis	Type I O-polysaccaharide (capsule I)	*B. pseudomallei*	(DeShazer et al. 1998; Reckseidler-Zenteno et al. 2005; Wikraiphat et al. 2009)
		Cepacian polysaccharide	*B. cenocepacia*	(Holden M. T. et al. 2009)
		Capsular polysaccharides (CPS)	*B. pseudomallei*, *B. thailandensis*	(Reckseidler-Zenteno et al. 2005; Cuccui et al. 2012; Marchetti et al. 2015)
Secretory proteins	Immune response inhibitors	Mip-like (macrophage infectivity potentiator)	*C. taiwanensis* (*)	(Amadou et al. 2008)
		Proteases	*B. pseudomallei* (*)	(Dowling et al. 2010)
		Phospholipases	*B. pseudomallei* (*)	(Dowling et al. 2010)
		TssM (BPSS1512) deubiquitinase	*B. pseudomallei*	(Tan et al. 2010)
	Toxins	HicA toxin	*B. pseudomallei*	(Butt et al. 2014)
		Bcc toxin	*Burkholderia cepacia* complex	(Thomson and Dennis 2012)
		Burkholderia Lethal Factor 1 (BLF1)	*B. pseudomallei*	(Cruz-Migoni et al. 2011)
		Hemolysin	*B. cepacia*; *B. pseudomallei* (*); *C. violaceum* (*)	(Hutchison et al. 1998; Alves de Brito et al. 2004; Dowling et al. 2010)
		RTX toxin	*A. xylosoxidans* (*)	(Li et al. 2013)
		Colicin V and exoenzyme regulatory protein (AepA)	*A. xylosoxidans* (*); *C. violaceum* (*)	(Alves de Brito et al. 2004; Jakobsen et al. 2013)
	Transport of toxins	RND efflux pump (e.g. BpeAB-OprB)	*B. pseudomallei*	(Chan and Chua 2005; Mima and Schweizer 2010)
		Type I secretion system (T1SS)	*B. pseudomallei*; *B. cenocepacia*; *C. violaceum* (*)	(Alves de Brito et al. 2004; Holden Matthew TG et al. 2004; Holden M. T. et al. 2009)

	Type II secretion system (T2SS)	*B. pseudomallei*; *B. mallei*; *B. cenocepacia; R. solanacearum*; *A. avenae subsp. avenae* (*)*; A. citrulli* (*)*; C. taiwanensis* (*)*; L. thiooxidans* (*); *C. violaceum* (*); *A. xylosoxidans* (*)	(Holden Matthew TG et al. 2004; Amadou et al. 2008; Holden M. T. et al. 2009; Persson et al. 2009; Poueymiro and Genin 2009; Burdman and Walcott 2012; Ibrahim et al. 2012; Har et al. 2015)
	Type III secretion system (e.g. Bsa T3SS)	*B. pseudomallei*; *B. mallei*; *B. thailandensis*; *B. cenocepacia; R. solanacearum*; *A. citrulli; Herbaspirillum rubrisubalbicans*; *A. avenae subsp. avenae* (*); *C. taiwanensis* (*); *Limnobacter sp.* (*); *C. violaceum* (*); *A. xylosoxidans* (*)	(Stevens et al. 2003; Alves de Brito et al. 2004; Holden Matthew TG et al. 2004; Genin et al. 2005; Amadou et al. 2008; Cullinane et al. 2008; Whitlock et al. 2008; Holden M. T. et al. 2009; Poueymiro and Genin 2009; Muangman et al. 2011; Ibrahim et al. 2012; Schmidt et al. 2012; Jakobsen et al. 2013; Li et al. 2013; Kondo et al. 2017)
	Type IV secretion system (T4SS)	*B. cenocepacia*; *A. xylosoxidans* (*)	(Engledow et al. 2004; Li et al. 2013)
	Type V secretion system (T5SS)	*B. pseudomallei*; *B. mallei*; *B. cenocepacia*; *Limnobacter* sp. (*)	(Holden Matthew TG et al. 2004; Holden M. T. et al. 2009; Persson et al. 2009)
	Type VI secretion system (e.g. T6SS-5)	*B. pseudomallei*; *B. mallei*; *B. cenocepacia*; *B. thailandensis*; *A. avenae subsp. avenae*; *A. citrulli*; *C. taiwanensis* (*); *L. thiooxidans* (*); *Limnobacter* sp. (*); *A. xylosoxidans* (*)	(Amadou et al. 2008; Schell et al. 2008; Holden M. T. et al. 2009; Persson et al. 2009; Schwarz et al. 2010; Ibrahim et al. 2012; Jakobsen et al. 2013; Burtnick et al. 2014; Har et al. 2015; Tian et al. 2015)
Other	Zinc metalloproteases ZmpA and ZmpB	*B. cenocepacia*	(Holden M. T. et al. 2009)
	Phospholipases C	*B. cenocepacia*	(Holden M. T. et al. 2009)
	Siderophores (e.g. ornibactin, salicylic acid, pyochelin, staphyloferrin B, micacocidin)	*B. cenocepacia; R. solanacearum*; *L. thiooxidans* (*)	(Sokol et al. 1999; Bhatt and Denny 2004; Holden M. T. et al. 2009; Kreutzer et al. 2011; Har et al. 2015)
	bipB, bipC and bipD proteins	*B. pseudomallei*	(Stone et al. 2014; Vander Broek and Stevens 2017)
	Malleipeptin A and malleipeptin B	*B. pseudomallei*	(Biggins et al. 2014)

		MprA serine metalloprotease	*B. pseudomallei*	(Valade et al. 2004; Burtnick et al. 2014)
		MgtC protein	*B. cenocepacia*	(Rang et al. 2007)
Others	Biofilm production	FixLJ system	*B. cepacia* complex	(Schaefers et al. 2017)
		Lys-R type regulator	*B. cenocepacia*; *R. solanacearum*	(Brumbley et al. 1993; Schell 2000; Bernier et al. 2008)
		Mannose-fucose binding lectin (LecM)	*R. solanacearum*	(Meng et al. 2015)
	Phenylacetic acid catabolic pathway		*B. cenocepacia*	(Law et al. 2008)
	Denitrification	Nitrate reduction (e.g. Nos system, NirV)	*A. xylosoxidans* (*)	(Jakobsen et al. 2013)
	Signalling	c-di-GMP-specific phosphodiesterase (CdpA)	*B. pseudomallei*	(Lee HS et al. 2010)
		CepIR Quorum-sensing system	most *Burkholderia* spp.	(Lewenza et al. 1999; Ulrich et al. 2004; Chan and Chua 2005; Song et al. 2005; Subsin et al. 2007; Holden M. T. et al. 2009; Subramoni and Sokol 2012)
		CciIR Quorum-sensing system	*B. cenocepacia*	(Baldwin et al. 2004)
		BDSF, nonhomoserine lactone signal molecule	*B. cenocepacia*	(Boon et al. 2008)
		BviIR Quorum-sensing system	*B. vietnamiensis*	(Malott and Sokol 2007)
		PmlI-PmlR Quorum-Sensing System	*B. pseudomallei*	(Valade et al. 2004)
		Violacein (CviI/R AHL QS system)	*C. violaceum*	(Steindler and Venturi 2007)
		other Quorum sensing systems	*A. citrulli; R. solanacearum*; *Polaromonas* spp. (*)	(Spirig et al. 2008; Johnson and Walcott 2013; Meng et al. 2015; Wang et al. 2016)

1112

1113 Table 2. Described intrinsic antimicrobial resistance in *Betaproteobacteria* species belonging to bacterial genera detected in both tap
1114 and bottled natural mineral/spring drinking water.

Species	Beta-lactams				Aminogly-cosides	Polype-ptides	Quinolones	Sulfonamides	Tetracyclines	Others	References
	Penicillins	**Cephalosporins**	**Carbapenems**	**Monobactam**							
Achromobacter xylosoxidans	Ampicillin, Amoxicillin-clavulanate,	Cefazolin, Cefotaxime, Ceftriaxone, Cefepime	Ertapenem	Aztreonam	+	n.i.	n.i.	n.i.	n.i.	Trimethoprim, Fosfomycin	(Almuzara et al. 2010; Bador et al. 2013; Leclercq et al. 2013; Abbott and Peleg 2015)
Burkholderia cepacia	Ampicillin, Amoxicillin, Piperacillin, Ticarcillin, Ampicillin-sulbactam, Amoxicillin-clavulanate, Piperacillin-tazobactam, Ticarcillin-clavulanate	Cefotaxime, Ceftriaxone, Ceftazidime, Cefepime, Cefsulodin Cefazolin.	Imipenem, Meropenem, Ertapenem	Aztreonam	+	Colistin	Ciprofloxacin	Trimethoprim-sulfamethoxazole	Tetracyclines	Tigecycline, Trimethoprim, Fosfomycin, Chloramphenicol	(Baxter et al. 1997; Palleroni 2005; Leclercq et al. 2013; Abbott and Peleg 2015; CLSI 2015)
Burkholderia gladioli	Ticarcillin, Ticarcillin-clavulanate	Cefsulodin	Imipenem	n.i.	+	Colistin	n.i.	n.i.	n.i.	Fosfomycin	(Baxter et al. 1997; Palleroni 2005)
Burkholderia mallei	Ticarcillin	n.i.	n.i.	n.i.	n.i.	n.i.	Norfloxacin	n.i.	n.i.	Fosfomycin, Clindamycin	(Thibault et al. 2004)
Burkholderia pseudomallei	Ticarcillin	Cefoxitin	n.i.	n.i.	Gentamicin, Streptomycin, Erythromycin	n.i.	Norfloxacin	n.i.	n.i.	Fosfomycin, Clindamycin	(Thibault et al. 2004; Buroni et al. 2009)
Chromobacterium violaceum	Penicillin, Ampicillin	Cephaloridine	n.i.	n.i.	n.i.	Colistin	n.i.	Sulfafurazole	n.i.	Vibriostatic agent O/129	(Gillis and Logan 2005a)
Herbaspirillum seropedicae and H. rubrisubalbicans	Pennicilin	n.i.	n.i.	n.i.	n.i.	n.i.	Nalidixic acid	n.i.	n.i.	Novobiocin, Rifampicin	(Baldani et al. 2005)
Janthinobacterium agaricidamnosum	Pennicilin	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	Vancomycin	(Lincoln et al. 1999; Gillis and Logan 2005b)

Janthinobacterium lividum	Pennicilin	n.i.	n.i.	n.i.	n.i.	Colistin	n.i.	n.i.	n.i.	Nitrofurantoin, Vibriostatic agent O/129	(Gillis and Logan 2005b)
Variovorax paradoxus	Ampicillin, Methicillin	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	Novobiocin	(Willems et al. 2005)

1115 +, described intrinsic resistance; n.i., no information available.

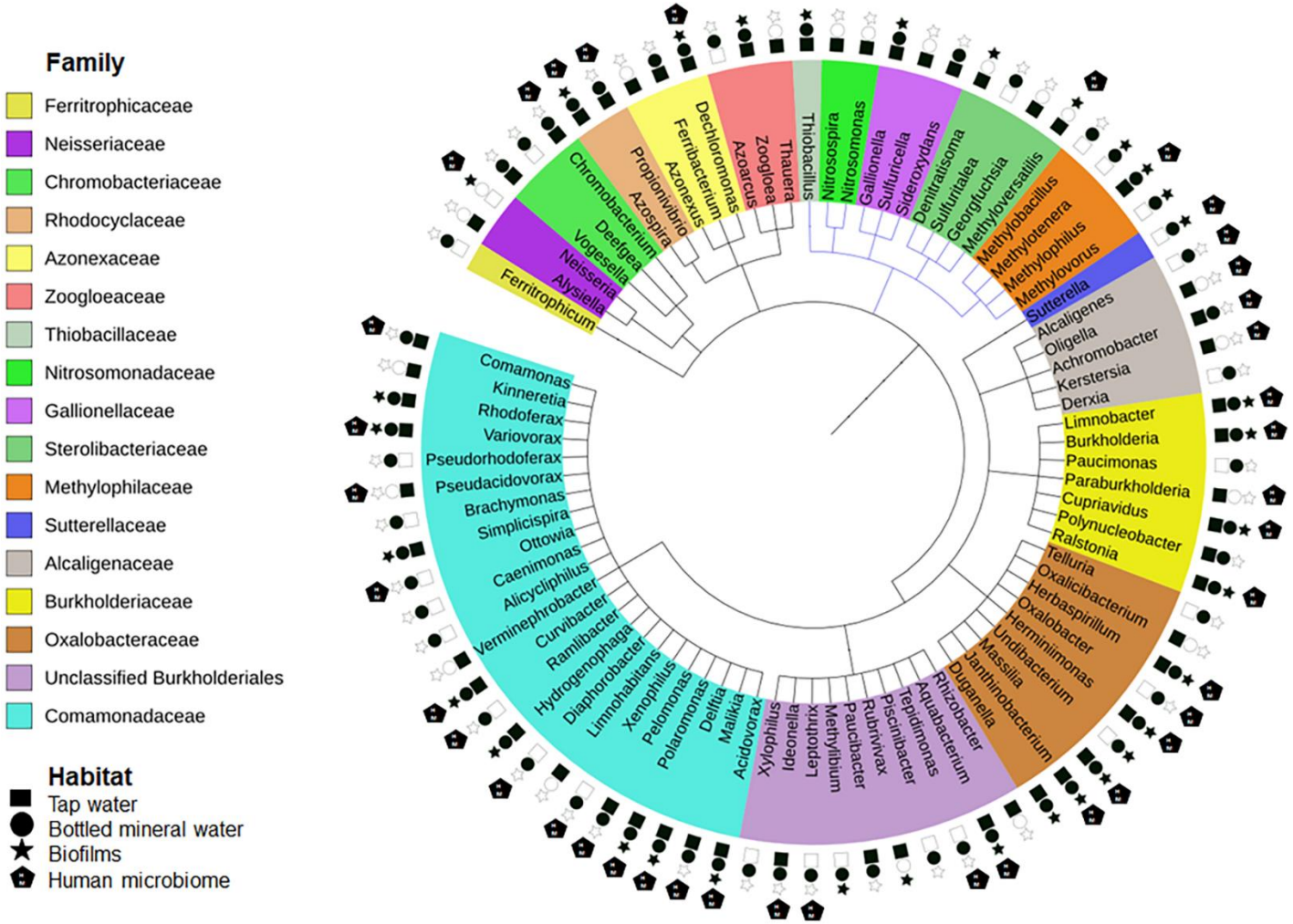

Figure 1. Diversity of *Betaproteobacteria* in drinking water habitats and in the Human microbiome. The black symbol means "detected", the white "non-detected". The dendrogram was constructed with the iTOL – interactive tree of life (Letunic and Bork 2016), based on the taxon ID codes.