Reducing pathobiomes could slow arms race between man and microbes

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The Koch's postulates that defined true pathogens are the cornerstones of the twentieth century microbiology. Because medical microbiology was focused on the isolation of true pathogens in pure culture from a patient, the rationale of treatment and prevention was also based on the protection and the fight against the true pathogens. This rationale succeeded since antimicrobial agents have changed the prognosis of most infectious diseases and vaccines have eradicated smallpox or decreased the incidence of other human plagues. However, the end of the 20th century saw the emergence and the spread of multiresistant opportunistic bacteria that threaten to end the antibiotic era. The recent proposal to include antibiotics in the UNESCO list of cultural heritage is almost like an admission of failure. The time has come for new concepts on microbial pathogens that could provide new approaches for the battle against infections.

The « omics » era raised awareness on the huge web of interactions established among living beings and particularly among microorganisms. Considering an individual microbe, whether a true or an opportunistic pathogen, without its associated community or its niche hides most parts of its living cycle. The concept of pathobiome is born in the context of microbial community interactions. It aims at considering the pathogenic agent within its biotic environment but also the community, which harbors accomplished or developing pathogens and can promote their transmission, emergence, pathogenicity or antibioresistance. The pathobiome concept provides a new conceptual frame for very promising developments from fundamental microbiology to infectiology. In this chapter, different tracks to achieve pathobiome reduction or closure will be discussed in the domains of use of antimicrobial agents, management of chronic infections and healthcare-associated infection control. In infectiology, the shift of paradigm from armful specific pathogens to adapted pathobiomes as cause of infectious diseases should lead to a shift of battles against pathogens from specific weapons to biological/ecological control.

Keywords: pathobiome; niche reduction; antimicrobial agents; bacterial communities; hospital environments

1. From bacterial species to community, from pathogen to pathobiome

1.1 Koch and Winogradsky

The basic concept underlying Koch's postulates [1], and their re-interpretations by molecular microbiologists [2], is the essentialist concept of *monomorphism* that considers the bacterial world as multiple separately evolving species each displaying a restricted range of variations. François Jacob and Elie Wollman in 1961 said "General acceptance of the concept of monomorphism was the first essential step for the establishment, not only of bacterial genetics, but also of bacteriology as a science." They recognized the "vital services" provided by monomorphism but they tempered as follows: "…it (monomorphism) was illegitimately extended to assert the constancy of bacterial form and function, and to deny to bacterial species all possibility of variation" [3]. Authors accepted the dynamics of bacterial species but remained essentialists, the bacterial species and its variations remaining the main object of study for bacteriologists.

Alternatives to essentialism and to Kochian vision are also old but they gave less conspicuous traditions. The masterful Russian soil microbiologist, Sergeï Winogradsky (1856-1953), developed the interactive microbiology (or microbial ecology) methodologically based on mixed cultures, for instance on the famous and the ever up to date Winogradsky column [4]. In the early 1930s, he described the soil as "…a living environment, a collective entity that possessed the characteristic functions of a living organism" [5]. It is probably the first presentation of microbial communities as global and irreducible biological entities, the first step towards the new microbial ontology based on metagenomic thinking and community systems biology.

However, the disciplinary division of science fields confined the Winogradsky's tradition to environmental microbiology and the Koch's tradition to medical microbiology without serious debates along the twentieth century. The advent of metagenomics at the beginning of the twenty-first century appears as a reunification around the interactive vision of Sergeï Winogradsky but, old traditions die hard, the first advances in metagenomics have been promoted by soil microbiologists [6].

1.2 Metagenomics: barcoding species by genomes and defining the genome of the community

Culture methods that had rendered "vital services" [3] to the bacteriology became, at the end of the twentieth century, a technological limitation for the inventory of complex communities mainly because of the very diverse growth requirements of bacteria or the unculturability of lot of them. In the early 2000s, the necessity to develop new methods in bacteriology met the emergence of new generation sequencing technologies [7], this synchrony leading to the advent of metagenomics as a new technology and further as a new discipline.

Metagenomics, also called environmental genomics, consists of the genome-based analysis of entire communities of interacting micro-organisms in diverse ecological contexts. Initially, in 1998, Jo Handelsman and his collaborators defined the metagenome as the collective genome of the total microbial community of a specific environment [8]. Today, most of the metagenomic studies provide a barcoding of the microbial diversity rather than the holistic vision of a whole community genome. Therefore, a more pragmatic definition of metagenomics is the direct genetic analysis of genomes contained within an environmental sample without the prior need for cultivation.

The repertory of species in an ecosystem is generally performed by marker gene amplification metagenomics (i.e., 16S ribosomal RNA gene) [9] and may be more accurately named "meta-genetics" [10]. By contrast, full shotgun metagenomics gives the complete sequences for theoretically all genomes within a community and thereby associates community diversity repertory to functional analysis [11]. For example, the community of microbial organisms living on the skin and mucous membranes of human beings are now extensively characterized by both barcoding and functional analysis [12].

1.3 The human microbiota is a supplementary organ

Microbes on inner and outer human body surfaces are ten times more frequent than the total human cells in the body. Moreover, the microbiota gene repertoire has been estimated to be about 150-times greater than that of the human host [13]. The human microbiota associates bacterial but also archaeal, fungal [14], protozoal and viral populations [15].

Infants are axenic before the birth but as soon as they are *ex utero*, bacteria from mother, family and environment will colonize their skin and mucous surfaces [16] and then evolve by steps until stabilization at the adulthood [17]. In adults, microbiota appears spatially organized particularly on the skin surface [18] and along the gut [19]. The microbiota composition also physiologically varies during the adult life, as it can be seen during elderly [20]. Inter-individual and intra-individual variations are particularly marked concerning taxonomic barcoding but surprisingly the human microbiota is rather constant in terms of functions in spite of the variation in species repertory [21].

Well-known microbiota functions are barrier protection, self and non-self-recognition, nutrition [22] and xenobiotic metabolism [23]. This is in accordance with microbiota location at the interface between human body and outside world. Conversely, changes in environmental conditions such as diet [24–26], lifestyle (stress, smoking, …) or drug treatments disturb the microbiota and the effect lasts for years in the case of antibiotics consumption [27,28]. Relationships between microbiota disequilibrium and human diseases are more and more described, such as in immune (Crohn's disease, Inflammatory Bowel Disease (IBD), ulcerative colitis, psoriasis…) [29–31], metabolic (diabetes, obesity…) [32,33] or infectious diseases (*Clostridium difficile* infections, vaginosis) [34–36].

Finally, the properties of human microbiota such as ontogenesis, highly conserved functions and disequilibrium associated to local and systemic human diseases suggest that microbiota acts as a supplementary organ involved in highly regulated physiological functions. Therefore, human beings (and other metazoans) could be considered as supra-organisms or meta-organisms.

1.4 Pathobiome concept

A recent study showed that *Homo sapiens* is not at the top of the food chain as are apex predators (crocodiles, wolves, etc) but rather in the middle of the chain beside anchovies and pigs [37]. Because of ancient and efficient cultural protections against apex predators, the main life-threatening living organisms for humankind are now pathogenic microbes (WHO data). Microbes are also constitutive of the human body but microbial world is highly diverse and human pathogens generally differ from commensal and mutualistic microbes. Unfortunately, separation between pathogen and commensal/mutualistic microorganisms is fuzzy and versatile mainly because horizontal gene transfer (HGT) can change commensal/mutualistic bacteria to pathogens [38]. The case of *Escherichia coli* pathotypes that acquired specific virulence genes and thereby change their lifestyle towards strict human pathogen is particularly emblematic [38]. If we also consider that host deficiencies can change relationships from commensalism or mutualism to pathogenicity or virulence, the frontier between beneficial microbes and bugs become even more moving.

Environmental bacteria can also behave as opportunistic pathogens [39]. In environmental ecosystems, potentially opportunistic pathogens display community lifestyle associated with various other bacteria but also with shelter organisms [40]. They are sheltered by invertebrates, plants and protozoa, which are recognized as hotspots for genetic exchanges and for the emergence of pathogens [40,41]. Given the current and ancient predominance of protozoa, plants and invertebrates, it is likely that bacterial interactions with such organisms have shaped bacterial evolution [42]. Therefore, sheltered bacteria are equipped with factors that overcome the innate defenses of their hosts. Thanks to the

continuity between environmental communities and human microbiota, these factors might secondarily be useful for the adaptation in human beings and might also spread into existing mutualistic or pathogenic bacterial populations [42].

Beside host adaptation and virulence, exchangeable genes frequently encode antimicrobial or multidrug resistance. If antibiotics resistance genes (ARGs) were selected after HGT by antimicrobial selective pressure, resistant clones emerged and currently encounter epidemiological successes leading to therapeutic dead-ends considered as a major threat to global public health [43].

Even if a pathogen fulfills the Koch's postulates and is "pathogen by itself", its dynamics of emergence depends on other members of its associated community and on the gene flow inside and among communities. Moreover, a recent study on the true pathogen *Salmonella enterica* serotype Typhimurium, suggests a general principle by which abiotic conditions such as antibiotic treatment can promote cooperative virulence during within-host evolution, increase duration of transmissibility, and thereby enhance the spread of an infectious disease [44]. The role of associated communities and abiotic conditions in the emergence of opportunistic pathogens is even more salient because, for these agents, exposition, transmission and pathogeneicity are the results of complex favorable conditions and interactions rather than specific virulence mechanism [39].

Metagenomic approaches contribute to the pathobiome concept that considers the pathogen within its microbial community context. The pathobiome designs the complex biological entity involved in infection and provides a broader view on infectious diseases than do the pathogen alone. In this chapter, the pathobiome concept will be confronted to 3 examples of current concerns in infectiology and public health: i) reservoirs and transmission of resistant bacteria or resistance and virulence genes involved in water-related infections, ii) acute healthcare-associated infections secondary to microbiota disequilibrium, iii) altered microbiota in chronic infectious diseases such as cystic fibrosis. Different tracks to achieve pathobiome stabilization or reduction will be discussed in the domains of use of antimicrobial agents and management of infections control and of chronic infections.

2. Control the making of superbugs in aquatic environments

2.1 Context

Water ecosystems contain complex bacterial communities mixing autochtonous stable communities and allochtonous species [45]. They are also favorable environments for microbial interactions and adaptation to changing conditions (Fig. 1). Indeed, water ensures a continuum among hydrogeological (underground and superficial systems), technological (domestic, hospital, industrial and waste waters) and living compartments (vital resource for all living beings). In aquatic niches, bacteria from contrasted origin (soil, water, polluted environment, animal and human microbiota) have direct connections with humans through the wide range of water usages. Therefore, waterborne infectious diseases represent a terrible threat to human health: in 2015, the WHO estimates that more than 1000 children under five die daily from diarrheal diseases due to poor sanitation or unsafe drinking water (http://www.who.int/fr/). In developed countries, the water networks are generally free of fecal microbes, which are involved in diarrheal diseases in developing countries but autochtonous bacteria belonging to water networks communities (*Pseudomonas aeruginosa*, *Legionella pneumophila, Stenotrophomonas maltophilia*, nontuberculous mycobacteria or *Aeromonas* sp., etc.) caused opportunistic infections particularly when the host presents altered immune response or other underlying conditions. This group of bacteria is currently so-called opportunistic premise plumbing pathogens [46]. Another concern for human health is the richness in ARGs in aquatic natural reservoir, either carried by antibiotics resistant bacteria (ARB) or exchanged between microorganisms thanks to mobile genetic elements (MGE) [47]. The aquatic resistome can provide genes to all members of the microbial community including allochtonous human pathogens, and potentially lead to the emergence of superbugs associating resistance, virulence and close contacts with human through water usages.

Unlike traditional fecal pathogens considered as allochtonous contaminants, opportunistic pathogens and ARBs are part of the resident water microbial community and therefore require new paradigms for their control.

2.2 Water-related resistomes

Aquatic environments are submitted to biotic and abiotic constraints, for instance human-mediated constraints like antibiotics and biocides. These compounds used in human and veterinary medicine can enter the environment via wastewater treatment plant (WWTP) effluents, hospital effluents, application of agricultural waste to fields, and leakage from waste-storage containers and landfills [48]. Effluent from WWTP is one of the major sources of ARGs in aquatic ecosystems and a source for bacterial community composition changes [49]. A study of plasmid metagenomics detected 140 clinically relevant ARGs in a WWTP [50], others showed that ARGs could persist in effluents sludges of a variety of full-scale WWTPs even after disinfection [51] but also in river sediment [52].

Beside antibiotics and biocides, other compounds such as heavy metals can act as co-selectors for ARBs. These co-selection mechanisms include co-resistance (different resistance determinants present on the same genetic element) and cross-resistance (the same genetic determinant responsible for resistance to antibiotics and metals) [53]. Moreover,

environmental selective pressures other than antibiotics, such as metals, detergents, and even nanomaterials, can facilitate the transmission of ARGs by HGT [54].

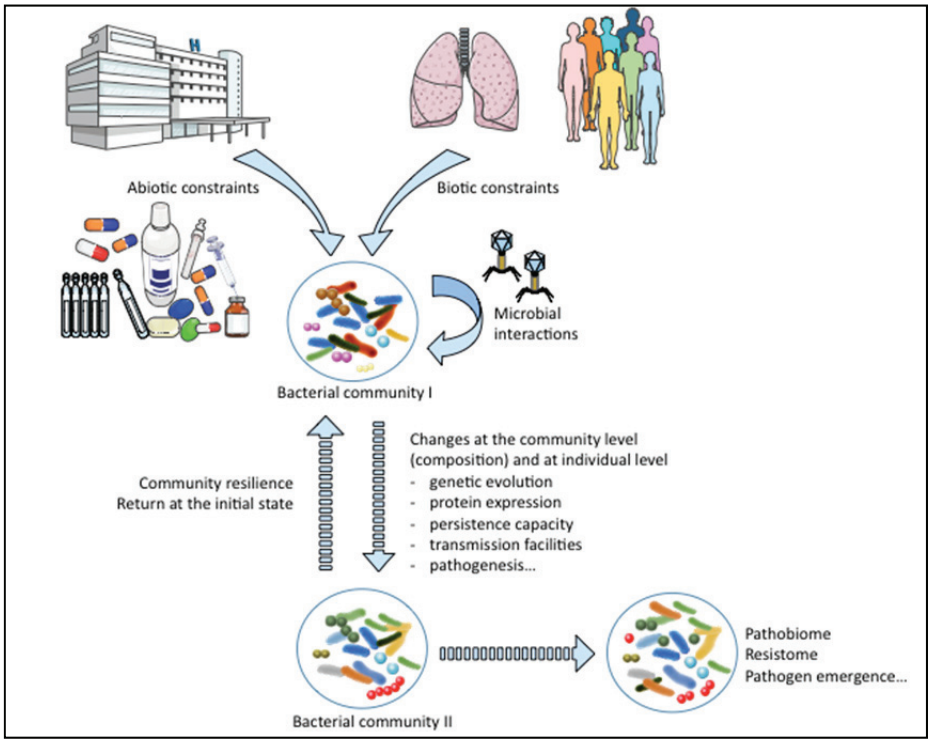

Fig. 1 Microbial community interactions, host environment and human activities, that can play a fundamental role in host adaptation and pathogen emergence.

Finally, bacteria living in polluted environments are frequently ARBs. For instance, potentially virulent and antibiotic resistant strains of *P. aeruginosa* [55] or other opportunistic pathogens [56] are often among the best biodegraders of environmental pollutants. Because of the large occurrence of genetic movements, aquatic ecosystems can be considered as reactors for emergence and spread of ARGs among microbial communities [57,58].

The above data suggest that water ecosystems are training places where bacteria evolve toward antibiotic tolerance or resistance. However, the question of the actual impact of the environmental resistome on the epidemiology of ARB infections remains open even if epidemiological links have been revealed between clinical and environmental species [59,60]. Of recent concern is the detection of the NDM-1 gene encoding resistance to most betalactams in polluted surface waters and chlorinated tap water in India [61]. NDM-1, first found in *E. coli*, is highly mobile and is spreading worldwide [62] in multiple waterborne pathogens, including *Vibrio cholerae* [61].

Hospital and domestic water systems, and more generally premise plumbing systems, are particular ecosystems where microbial communities, in planktonic stage or in biofilm, enter in close contact with humans. Their study would give insights about emergence of resistant clones among clinically relevant bacterial species. Epidemiological link between water networks and ARBs in healthcare-associated infections (HAIs) is demonstrated in numerous studies because hospital is an amplificator of the global infectious risk in developed countries. Within the European Union (EU), it is estimated that 2 million patients every year acquire infections caused by multiresistant bacteria accounting for 175,000 deaths per year in hospitals.

Among environmental ARBs, *P. aeruginosa* is known to cause redoubtable HAI and hospital outbreaks, particularly in patients hospitalized in intensive care units (ICUs). Many studies have demonstrated the transmission of *P. aeruginosa* from water networks and outlets to patients [63,64]. The main reasons for the success of *P. aeruginosa* in hospital water networks are i) the presence of numerous technological niches (osmosis systems, softeners, biomedical devices) where *P. aeruginosa* can develop in biofilm, ii) the use of biocides and antimicrobial agents that can select *P. aeruginosa*, which is particularly resistant, iii) the capacity of *P. aeruginosa* to act as a shuttle between patient microbiota and water community particularly thanks to interface zone such as faucets, U-bend siphons or biomedical devices (ICU ventilators, dialysis machines, endoscope reprocessors, etc.). The hospital water networks and outlets could therefore represent hot spots for pathogenic ARB emergence [65].

Very few studies have investigated resistant bacterial community in the drinking water distribution systems out of hospital. Indeed, in many countries, bacterial parameters defining drinking water quality are based on the quantification of fecal indicators like *E. coli* or *Enterococcus* sp. Resistant bacteria or resistome are nowadays poorly investigated in natural reservoir of drinking water while aquifers, particularly karstic ones, are very vulnerable to pollutants related to

anthropic activities (WWTP, agricultural lands, industries, urban areas…). In a study of Ribeiro and colleagues, the occurrence of antibiotic-resistant *Pseudomonas* sp. in drinking water has been evaluated. They showed that *E. coli* and *Pseudomonas* sp. densities in the water resource varied depending on climatic conditions impacting on water turbidity. Whatever the initial turbidity, *E. coli* was totally eliminated from drinking water while *Pseudomonas* sp. was able to survive to treatments used for drinking water production probably due to aggregations of suspended matters or to intrinsic tolerance to biocides [66]. Through further investigations on *Pseudomonas* strains recovered in water distribution system, authors showed that all presented phenotypic resistance to at least one antibiotic [66].

2.3 The hidden effects of antibiotics in the environment

Water-related resistomes are currently more and more described [67] but there are limited evidences to say whether environmental concentrations of antibiotics enhance the development and spread of resistance in the environment [68]. Such evidences have been provided in outstanding conditions with excessive concentrations of antibiotics only. For instance, streams receiving effluents from a drug-manufacturing site in India displayed huge ciprofloxacin concentration (1mg per g of organic matter) and concurrent high levels of resistance [52]. Authors concluded that antibiotic contamination plays a role in the selection of ARGs and their mobilization from environmental microbes to other species [52].

However, in more regular conditions, the link between antibiotic concentration in environment and resistance is not so clear. Consequently, current guidelines on the environmental risk assessment in the EU, for example, do not explicitly address the effect of antibiotics on the prevalence of antibiotic resistance in the environment [69]. One step toward a better evaluation of the ecological and epidemiological impacts of antibiotics in environment is defining indicators and parameters. Classically, antibiotic activity is explored *in vitro* by the determination of the minimal inhibitory concentration (MIC) leading to the characterization of the pathogenic strains as resistant, intermediate or sensitive to an antimicrobial agent. MIC is thought to be predictive for *in vivo* therapeutic effect. However, MIC definition is unique and doesn't take into account the heterogeneity of drug distribution compartments whether in the human body or in diverse environmental niches where the antimicrobial agent concentrations could be markedly lower. These sub-MIC have biological effects [70] that remain hidden if MIC determination is used as the sole parameter for activity prediction. Therefore, another parameter, so-called minimum selective concentration (MSC) is now taken into consideration. The MCS is reached when its reducing effect on growth of a susceptible strain balances the fitness cost of the resistance in a resistant strain. In other words, it is the lowest antibiotic concentration that allows selection for resistant mutants [71]. It is noteworthy that the selection with sub-MIC not only enriches low-level resistance mutants but also those with clinically relevant levels of resistance. This phenomenon occurs at concentrations several hundred-fold below the lethal concentrations for susceptible microorganisms [71].

While the deleterious effects of pharmaceuticals used at supra-MIC on human microbiota are indisputable and thoroughly studied [72], the effects of sub-MIC on microbiota or environmental communities are poorly documented. Measures of antibiotic concentrations in aquatic environment showed, in general, ng or lower µg-per-liter ranging concentrations [73,74] depending on the antibiotic and nature of water (WWTP effluent, surface water or ground water). These levels are in the same order of magnitude than MSC; so they could provoke enrichment in clinically relevant resistant bacteria, particularly if the mutations leading to resistance have low fitness cost [75]. It also appears that resistant mutants selected at sub-MIC are generally fitter than those selected at high concentrations suggesting that, in the absence of antibiotic pressure, these mutants will be more persistent in bacterial populations than those selected by high antibiotic concentration and having higher fitness cost [71].

Moreover, antibiotic residues not only allow the selection of resistant communities but also promote genetic adaptation, for instance through integron recombinations [76]. Antibiotics should therefore not only be considered as selectors for ARBs but also as resistance promoters.

2.4 Control antibiotics and resistant bacteria in aquatic environments

For most authors, limiting the use of antibiotics in animal production is the most direct route of controlling antibiotic release into the environment, and likely also antibiotic resistance. For instance, multidrug resistance rates of *Enterococcus faecium* in U.S. poultry declined from 84% to 17% following a conversion to organic feeding [77]. However, both organic and conventional cattle lagoon water contain average ARG levels about three times greater than detected in "pristine" river sediments [78]. This indicates that even under minimal antibiotic use conditions such as organic animal husbandry, there is a potential for environmental release of resistance genes. Composting, long-term storage and containment of manures associated or not to on-farm methanogenic biogas facilities could greatly reduce antibiotics and ARG concentrations in aquatic systems [79]. Of course, keeping animals healthy and animal welfare by better management practices is important to reduce antibiotics use [80], but active prevention by vaccines could be also considered. A 99% reduction in the use of antimicrobial agents in Norwegian salmon and rainbow trout aquaculture was achieved from 1987 to 2007 thanks to vaccination, despite a massive increase in fish production [81].

Sanitation and sewage treatment are other hotspots for resistance emergence, as described above for the NDM-1 gene in India, and its worldwide spread. No doubt that sewage collection and treatment protect human if we consider that it is

among the 2.6 billion people lacking access to sanitation that the higher children mortality by diarrheal diseases is observed (WHO data). Traditional WWTP are designed to remove conventional pollutants, including suspended solids, nitrogen, phosphorus, organic matter, and, to some extent, pathogens. However, WWTPs do not remove antibiotics or ARGs whom may either decreased via bacterial death or increased via HGT and/or selective enrichment. Some new technologies appear promising such as thermophilic anaerobic sludge digestion [82] and membrane separation that could be applied to retain microbial cells and their DNA [83]. Source separation sanitation is another strategy that allows separation of waste streams with specific characteristics, for instance hospital wastewater that contains high concentrations of pharmaceuticals and ARBs. The separation of urines that contain toxic compounds and solid that contain ARGs and ARBs in hospital wastewater could also be a valuable strategy [59,73]. Membrane bioreactors can partially remove antibiotics and ARBs before discharging hospital wastewaters into public sewer systems [84]. Advances in the development of such technologies are particularly required because wastewater reuse is becoming a promising strategy for water sustainability.

Control hot spots of antibiotic pollution and antibiotic resistance near hospitals and drug factories is of major concern [84] both locally and globally due to the high spread capacity of ARBs and ARGs [62]. Removing antibiotics from hospital wastewater would be easily regulated in developed countries but decisions and policy measures at the state level are needed and are pending. The effluents of antibiotic manufacturing sites should also be urgently regulated because the levels of antibiotics can reach milligram-per-liter concentrations in studied cases [52]. This is largely more than the amount of any pharmaceuticals excreted in the environment after human or animal usage. It is also urgent to collect exhaustive data because publically available data on antibiotic effluents from drug manufacturing are still very partial. This inventory would serve as basis for policy measures and for the inclusion of environmental consideration in good pharmaceutical practices.

The use of disinfectants to inactivate microbial pathogens in drinking and hospital waters has played a central role in reducing the incidence of waterborne diseases. This strategy of infection control is considered to be among the most successful interventions for preserving and promoting public health. Chlorine-based disinfectants are the most commonly used disinfectants because they are cheap and easy to use. Comparative metagenomics of drinking water treated with either free-chlorine or monochloramine showed differences in microbial community structure [86]. Concerning pathogens, *Legionella*-like genes were more abundant in the free-chlorine-treated samples while mycobacterial genes were more abundant in monochloramine-treated samples. In addition, sequences linked to antibiotic resistance mechanisms were observed in both microbial communities [86]. These data showed that biocide treatments besides killing indicator pathogens could enrich drinking water in autochtonous pathogens and in ARGs. As alternatives to chlorine and chloramines, disinfectants such as ozone, UV and nanotechnologies are gaining popularity [86]. If their efficiency to kill fecal microbes and pathogens is studied and validated, their impact on the autochtonous community and resistome is generally less evaluated. Ozone has been proposed to kill ARBs and destroy ARGs but also to performe oxidative destruction of pharmaceuticals including antibiotics [87].

In order to limit and prevent the emergence of ARBs, evaluation should be a pre-requisite before the wide use of new compounds for water treatment or for other applications leading to their bulk emission into the aquatic environment. For instance, ionic liquids considered as an "environmentally friendly" replacements for organic solvents are widely applied in modern industry and were shown to enhance HGT of ARGs in freshwater microcosms [54].

Several studies showed that chemical treatment of hospital water systems lead to a decrease of point-of-use water contamination but pathogenic bacteria such as *Legionella* sp. and *Pseudomonas* sp. re-grew after chemical shock and persisted during long-term treatment [88]. Moreover, chemical shocks performed for reducing bacterial load in water systems, such as dental care unit waterlines, could select and promote colonization by tolerant pathogens such as *P. aeruginosa* and *Achromobacter* sp. [89]. In comparison, 0.22 μm point-of-use filters limit water contamination in all cases [88]. Moreover, several studies clearly showed that point-of-use filters decrease the incidence of infection and outbreaks caused by water-associated bacteria [90,91]. This suggests that niche closure is more efficient than chemical treatments to limit the proliferation of water autochtonous pathogens.

Consequently, other more innovating strategies are proposed for pathogen niche reduction and driving ecological interactions such as competition, antagonism or predation might be efficient for the control of opportunistic pathogens in water systems [46]. As in human therapeutics, a probiotic approach for water treatment is defined as intentional inoculation of beneficial microbes or choosing conditions that select for a desirable community, which is generally the indigenous non-pathogen community. Competition could be achieved by the production of water with very low carbonaceous nutrients concentrations that allow the development of autochthonous microbial community acting as “placeholders” to close the niche for pathogen growth even after disinfection [92]. For instance, dual sand filters used for drinking water treatment processes harbored a stable bacterial community that could seed the distribution system. Control and modification of the filter community could reduce or close the niches for pathogens, i.e. the hydric pathobiome [93]. Micropredators such as bacteriophages, *Bdelovibrio* and like organisms (BALO) and amoeba as well as their association are also proposed for the control of pathogens in drinking and waste water [94].

Finally, innovating strategies integrating diverse approaches could be used as proposed by Wang et al. (2013): i) use biofilter for controlled seeding of indigenous community; ii) stop massive disinfection to protect indigenous

community; iii) apply nutrient limitation; iv) smartly design water networks; v) incorporate extreme selective predators for pathogens such as bacteriophages.

3. Respect or restore the microbiota equilibrium

3.1 Microbiota and health

The supra-organism constituted by the human body and its microbiota is governed by complex interactions between human cells and microorganisms [95,96]. Microbiota installation is concomitant with the acquisition of the adaptive immunity, the bacterial adaptation to the intestinal niche being a key stage in the maturation of the immunity because it conditions the self-tolerance, the tolerance to mutualistic bacteria and the recognition of pathogens [17,97–99].

The gut gathers both the biggest bacterial community of the human body and a major component of the immune system. For these reasons, gut microbiota equilibrium is a predictive key point for future health [100]. Many authors characterized the role of bacterial components of this community in immune, inflammatory and metabolic systems [101]. Several mechanisms of crosstalk between microbiota and immunity cells were described, with direct impacts on inflammatory regulation but also indirect correlations with many central nervous system (CNS) functions. Based on these discoveries, the description of gut-brain axis has been proposed, and brought new insights in pathophysiology of several diseases [100,102]. This axis schematizes the different interactions between microbiota, immunity, metabolic pathways and nervous system (Fig. 2). Hence, commensal bacteria and probiotics develop functional crosstalk with human cells (epithelial cells, immune cells…). Then, the gut microbiota regulates neural, endocrine, metabolic and immune pathways by upward and downward controls on Hypothalamus-Pituitary Axis (HPA) [102]. On the same principle, the skin microbiota has an influence on local immunity and acts as the first barrier against pathogens colonization [103]. Moreover, it seems that some crosstalk could exist between skin and gut microbiota, and the CNS, leading to the description of the skin-joint-gut-brain axis [104]. These intricate communications between microbiota and immunity cells is considered to be true within all human niches, such as gut, skin, respiratory tract and urogenital tract[105,106].

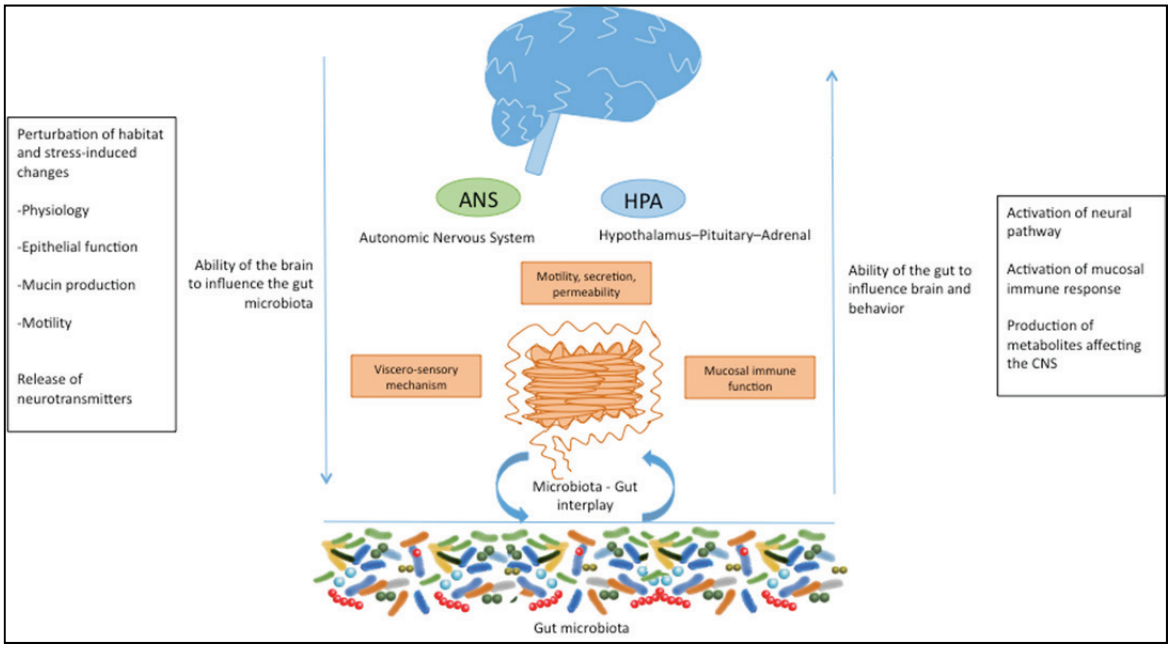

Fig. 2 Gut-brain-axis and crosstalk between microbiota and human cells.

The qualification of "supplementary organ" is particularly attributed to the gut microbiota because it plays a central role in diverse metabolic functions (carbohydrate metabolism, vitamins assimilation…). It is thus essential for several vital functions of the human body. Therefore, the gut microbiota illustrates perfectly the concept of "living together" corresponding to the symbiosis.

The precarious balance, which characterizes microbiota, is subjected to constant reorganization and regulation contributing to its equilibrium [107]. Some endogenous and exogenous factors are important to consider for maintaining this balance. Certain factors can perturb it and promote the occurrence of dysbiosis, which can lead to several pathologies such as inflammatory deregulations, metabolic disturbances or infectious diseases [95,96,107–110].

3.2 Microbiota disequilibrium and consequences

The infectiology paradigm is shifting from a classical concept in which one infection is caused by one bacterium, to a modern concept in which infection emerges from the complex phenomenon of dysbiosis. This shift comes from recent discoveries on the human-associated microbiota and increased knowledge of their composition and roles in health and diseases [21,108]. Among human-associated microbiota, gut microbiota is the more studied one. The gene catalogue for this microbiota references about 10^7 bacterial genes from 1267 subjects [111]. The gut microbiota differs among individuals by bacterial genes content, species and ecology. Three major enterotypes are described: *Bacteroidetes*, *Ruminococcaceae*/*Methanobrevibacter* and *Prevotella*, according to the predominant taxa present [112]. Numerous studies also characterized different microbial patterns and their link with certain pathologies such as Crohn's disease, IBD, or CNS disorders. [24,101,102,112–114]. For example, 7 species seem to be powerful markers of liver cirrhosis [115]. Moreover, 28 rarely dominant species in healthy subjects become dominant according to the disease severity in cirrhotic patients. The gene diversity and richness of the gut microbiome can be used as a health indicator for risk detection or prediction in metabolic perturbations [116]. Indeed, the subjects harboring a low gene count in their gut microbiota have higher co-morbidities (adiposity, insulin resistance, dyslipidemia and inflammation) [117]. Moreover, the relative abundance of some species can also be an health indicator, for instance a low proportion of *Faecalibacterium prausnitzii* in the gut microbiota is a dysbiosis signature because of the anti-inflammatory properties of this species [118].

Regarding the skin microbiota, few studies have shown correlations between dysbiosis and pathologies such as psoriasis, atopic dermatitis or acne vulgaris. However, the causative links between them are not demonstrated [119]. As an example, a recent study identifies a specific 'cutaneotype' involved in psoriatic lesions. It presents higher richness in *Firmicutes* and *Actinobacteria* [120] associated to a lower overall diversity compared with healthy skin microbiota. Moreover, the global diversity of the skin microbiota in psoriasis seems decreased compare to healthy skin, to the benefit of these Gram-positive phyla [120,121]. In atopic dermatitis, a high rate of *Staphylococcus aureus* carriers is found (about 90% of the patients), especially during exacerbation periods, which seems to modify the barrier effect of the skin and to promote immunity deregulations [122,123]. *S. aureus* presence is associated with a decrease of the global cutaneous microbiota diversity, a significant increase in the proportion of *Staphylococcus epidermidis* [123], and a skin colonization by Gram-negative bacilli such as *Pantoea agglomerans*, *Enterobacter cloacae*, *Chryseobacterium indologenes* and *Acinetobacter lwoffii* [124].

Even if these microbiota disequilibria are more and more documented, they need more studies in order to argue causative links with pathologies and to open new avenues in the management of immune, metabolic, neurologic or infectious pathologies.

3.3 Medical constraints, hospital environment and consequences on microbiota balance

Modern medecine and particularly, therapeutics, intensive care and surgery have increased markedly the life expectancy. The HAIs that cause significant increases of morbidity and mortality for hospitalized patients appear as the price of the modern medecine progress. One major reason for HAIs emergence is that hospital constitutes a particular environment, where unusual conditions for microbiota are present. The consequences of such unusual conditions can lead to microbiota perturbations at both individual (patients and healthcare professionals) and collective levels (unit ecosystems). Among these conditions, the organization of certain units, with pair rooms and several beds rooms exerts a demographic pressure on the patients' microbiota and ecosystems of the unit [125–128]. These conditions are particularly marked in long-stay wards such as gerontology units, or in always-booked units such as in neonatology wards or ICUs [125,127,128]. The physical conditions such as temperature or bed rest are also unusual pressures exerted on microbiota. Moreover, the barrier disruption and the physical aggressions provided by invasive medical devices injured the local conditions of the microbiota and perturb their balance. In the particular case of the surgery, other unusual conditions can perturb microbiota, such as the preoperative length of stay, the stress regarding the surgical intervention and the intensive cares, the use of medical devices and preoperative preparation and antimicrobial prophylaxis [129–132].

3.4 To control HAIs while avoiding microbial resistance

The battle against HAIs is a priority to ensure safety of patients. Since the 1980's, there are 2 major axes for HAIs control, which were the use of efficient antimicrobial agents and the prevention by implementation of hygiene measures [133].

Round 1: antimicrobial agents to treat HAIs and microbes counter-attack

The use of antimicrobial agents such as antibiotic and antiseptic uses, or disinfecting cleaners led to selective conditions that promote the development of bacterial mechanisms of resistance and favor the constitution of resistome within the hospital/patient ecosystems [125–128,130,134–138]. Then, the major consequences of antibiotic administration are a decrease in the bacterial diversity, an increase in the bacterial resistances to antibiotic molecules, and a potential selective effect on pathogenic bacteria. Because hospital acts as an epidemiology amplificator, resistant pathogens emerge in hospital and then diffuse in the community. Consequently, MDR bacteria considerably increased

in past few years, with the emergence of hospital-acquired and community-acquired methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant *Enterococcus* (VRE), extended-spectrum β-lactamase (ESBL)- and carbapenemase-producing enterobacteria (CPE)… For instances, among Gram-negative species, *E. coli* isolates are frequently ESBL producers, *Klebsiella pneumoniae* strains are more and more resistant to carbapenems, antibiotic molecules that are usually active against MDR bacterial infections, and about 10% of *P. aeruginosa* are carbapenem-resistant, and usually MDR. [133].

Round 2: hygiene measures to prevent HAI and microbes counter-attack

The second strategy of battle against HAI is the prevention. Hygiene measures are based on the careful application of best medical and nursing practices in a safe healthcare environment. However, best hygiene practices are often incompatible with hospital overbooking and daily constraints faced by healthcare workers. To help for the application of hygiene measure, more and more agents that are aggressive for microbiota and ecosystems are used. One example among many, repeated hand hygiene and glove wearing by healthcare workers modify their hand physiological conditions with higher dryness and decrease of lipid secretion. These practices linked to the care activities decrease the barrier effects of the skin with irritation and micro wounds, and lead to a modification of the skin microbiota and a possible colonization by more resistant bacteria from exogenous sources [139,140]. Another example, recent clinical studies are controversial concerning the efficiency of chlorhexidine for daily bathing of ICU patients compared to water-and-soap bathing to prevent HAIs [141–143]. Chlorhexidine bathing leads to a shift of skin microbiota with a reduced rate of gram-negative bacteria compared to water-and-soap bathing [143]. The decrease of gram-negative bacteria such as enterobacteria and nonfermenting bacilli could explain the decrease of HAI incidence after chlorhexidine bathing [143]. However, gram-negative bacteria are also major resident of the normal skin microbiota and are probably involved in the fine-tuning of the skin homeostasy [18]. Consequently, the shift of microbiota caused by chlorhexidine could lead to functional disequilibrium of the skin barrier. Last example, the consequences of the overuse of biocides in hospital water networks have been evoked in the paragraph 2.

Round 3: towards strategies of HAIs control that limit microbes counter-attacks

Curative and preventive use of antibiotics and biocides constitute stresses on microbiota contributing to disturbances and disequilibrium, themselves promoting the emergence of multidrug resistance phenotypes [144,145]. Consequently, more conservative management preserving the microbiota equilibrium are in progress as probiotic and prebiotic use. An effort consisting in a more adapted and targeted utilization of antibiotics and biocides contributes to the commensal microbial communities respect [146]. For examples, recently, some hygiene surgery practices have changed, taking away antiseptic shower replaced by a simple soap shower.

Negative impacts of medicine practices on microbial ecology are more and more studied and understood. Consequently, conserving mutualist communities is now considered in the management of patients to preserve or restore the structure and functions of the patients' microbiota and to prevent the risk of emergence of resistant strains. With this in mind, the old concept of microbiotherapy, i.e., the use of microorganisms to improve host health, has renewed interest. One example is the widely use of probiotics, prebiotics and symbiotics in anti-infective indications, as well as for restoration of immunity disorders [147]. Another alternative approach is the modification of abnormal gut microbiota by faecal transplantation. This strategy is only indicated for *C. difficile* recurrent infections [148]. Because microbiota has a critical position in the transition towards chronic immune diseases, stool transplantation could also contribute to correct immunity imbalance or be beneficial in some diseases (IBD, Crohn's disease, obesity, liver cirrhosis, side effects of antitumor chemotherapy…), but such practices still need more approval standardization [149].
Prebiotics, probiotics and symbiotics act as modulators of the microbiota but other biotherapy agents are predators for bacteria and can be used directly in the infectious site, such infected wounds. "*Bdellovibrio* and like organisms" (BALO) are small gram-negative bacteria that are predators against other gram-negative bacteria species such as *E. coli* and *P. aeruginosa* [150]. Some patents are currently deposited for treatment and prevention of infections by BALO attack. In addition to BALO, bacteriophages are included in the treatment regimen for synergistic effects [151]. As also described for CF treatments (see paragraphe 4.4), the use of bacteriophages in phagotherapy strategy is more and more evoked to replace or complement antimicrobial chemotherapy [152]. The use of phagotherapy is particularly suggested for treatment of infected burn wounds and other HAI caused mainly by *P. aeruginosa*, *S. aureus* and *A. baummanii* [152]. Amoeba are natural grazer of bacteria that act similarly to human phagocytes for destroying bacteria. Certain species such as *Dictyostelium discoideum* has been proposed in treatment of infections caused by bacteria, even MDR strains [151]. It is supposed that contrarily to antibiotics, which kill bacteria but leave toxic bacterial compounds such as endotoxin in the infection site, amoeba consume totally the bacteria and promote healing.

4. Pathobiome of chronic pulmonary infections in cystic fibrosis: a new concept for new weapons

4.1 Context

In the context of increasing investigation of the role of human microbiota in health and diseases, the majority of studies focused on the gut microbiota and several types of disequilibrium have been demonstrated in pathologic situations like

IBD [153,154]. On the other hand, studies showing that systemic inflammation could be abrogated with the administration of broad-spectrum antibiotics underlined the link between microorganisms and inflammatory processes in such chronic diseases [155]. Regarding the respiratory tract, composition and diversity of the airway microbiota has received less attention. However, several studies suggest that observations similar to those made at the gut level could be valuable at the respiratory tract level during chronic respiratory diseases, particularly that dysbiosis and perturbed host-microbe interactions may have a critical role in controlling respiratory immune homeostasis and ultimately airway chronic inflammation and disease progression [105,156]. Studies were mainly conducted in Cystic Fibrosis (CF), chronic obstructive pulmonary disease and asthmatic patients [157–159]. We choose hereafter the CF disease as a model of chronic infection with complex pathogenesis warranting new therapeutic strategies designed to target altered microbiota and its implications.

4.2 CF respiratory tract infection, a chronic polymicrobial disease with complex pathophysiology

CF is a potentially letal genetic disease with multiorgan pathology. CF respiratory tract (CFRT) is mainly affected with production of thick and sticky mucus in the patient airways, immune local inflammation and abnormal microbial colonization. Direct exposure to environmental microorganisms and to endogenous microbiota from gut and oropharynx is thought to represent the source for initial establishment and further maintain of lung microbiome [158]. Disease evolution is punctuated by repeated pulmonary exacerbations associated with progressive decline in lung function and ultimately death. A modest number of bacterial pathogens, mainly from environmental origin and acting as opportunistic pathogens correlates with pulmonary function decline [160]. Among them, *Pseudomonas aeruginosa* and the *Burkholderia cepacia* complex are the most studied. However, several other non-fermentative Gram-negative bacilli such as *Achromobacter* species, *Burkholderia gladioli*, *Ralstonia mannitolilytica*, *Stenotrophomonas maltophilia*, and *Pandoraea* species are problematic emerging pathogens, cumulatively infecting approximately 25% of people with CF and being especially resistant to therapy [161–163]. These pathogens also display ability to chronically colonize the CFRT, to form biofilm and to generate inflammation level similar to that induced by *P. aeruginosa* [161]. Moreover, both cultivation-based and -independent methods evidence that CF airway infection is rather a polymicrobial disease [164]. Altered composition of the CFRT microbiota encompasses a reduced overall diversity, an increased rate of *Proteobacteria* and *Firmicutes*, and a decrease of *Bacteroidetes* [158]. On the other hand, specific and/or unexpected sequences as well as atypical microorganisms are found in the CFRT [164–166]. In this context, bacterial richness has been correlated to lung function and significant difference in the microbiota diversity between acute exacerbation and clinical stability has been demonstrated [167]. Certain genera / species are suspected to have important roles in driving change in airway bacterial community composition at exacerbation like members of the genus *Gemella* or of the *Streptococcus milleri* group [168–170].

4.3 CF respiratory tract infection, a valuable model to be considered in light of the pathobiome concept

It is however highly probable that more complex perturbations than those of the microbiota occur in the CF airways that represent a particular niche where multiple biotic but also abiotic selective pressures drive bacterial selection and adaptation. Among complex and intricate constraints in the CFRT niche, the most notable are the altered osmotic pressure, immunological defenses and inflammation, and the repeated antimicrobial treatments. Indeed, patients receive various combinations of oral, inhaled and intravenous antibiotics either directed towards specific pathogens or as regular long-term antibiotics in an attempt to prevent infections or to lower bacterial load. Under such a panel of selective pressures, it has been proposed that transition from stable disease to exacerbated status are occasions of dysbiosis and that dysbiosis provokes a dysregulated host immune response which in turn alters growth conditions for microbes in airways promoting further dysbiosis and perpetuating a cycle of inflammation and disordered microbiota [171]. A community study suggests that exacerbations may not necessarily be associated with specific changes in airway bacterial communities but may be associated with changes in only a few operational taxonomic units (OTUs) and/or very minor changes in several OTUs, or may result primarily from host-specific factors such as the nature and degree of inflammatory response to some as yet undefined stimuli [172]. Another study postulates the existence of two major functional communities; a virulent attack community consisting of transient viral and microbial populations that induces strong innate immune response creating microenvironments that facilitate the establishment of a climax community that is slower-growing and inherently resistant to antibiotic therapy [173]. Acute pulmonary exacerbations can then result from aggressively growing revertants derived from slowly growing, endogenous microbial populations, or from a change in bacterial gene expression eliciting stronger immune responses [173].

In such episodes of dysbiosis, perturbed microbes-microbes but also microbes-host interactions may play a crucial role in the disease progression [174]. Studies conducted in animal lung infection model or in *Drosophila* suggested an important contribution of the host microbiota to *P. aeruginosa* infection. Regarding oropharyngeal microbiota usually considered as commensal or mutualistic but avirulent, interactions with pathogenic *P. aeruginosa* isolated from sputum samples of CF patients enhanced lung damage compared to *P. aeruginosa* alone, *P. aeruginosa* virulence gene expression being modulated by host microbiota through interspecies communication mediated by autoinducer-2 [168], [175]. A similar observation has been made for the association of two species of the oropharyngeal microbiota,

Streptococcus constellatus (*S. milleri* group) and *Prevotella intermedia* that induced a 6-fold higher mortality rate than that obtained by each microorganism [176]. Over time, antimicrobial therapy became ineffective in pathogen eradication independently of the suitability of antibiotics and antimicrobial susceptibility patterns of the pathogen identified by standard microbiological cultivation-based diagnosis. This could be attributed to altered drug pharmacokinetics in these patients and to several adaptive and protective features developed by the pathogens such as biofilm and small variant colony formation.

As a global illustration of such a complex evolution of the CF respiratory pathobiome, Figure 3 presents the evolution of cultivable microbial community in terms of species diversity, load and genotype in successive sputum samples of a CF patient with chronic colonization by *Achromobacter xylosoxidans* according to clinical status and antibiotic courses. A highly dynamic community is demonstrated despite low impact of antimicrobial agents against dominant species and major changes in both diversity and load of pathogens are observed over time according to clinical stability or pulmonary exacerbation.

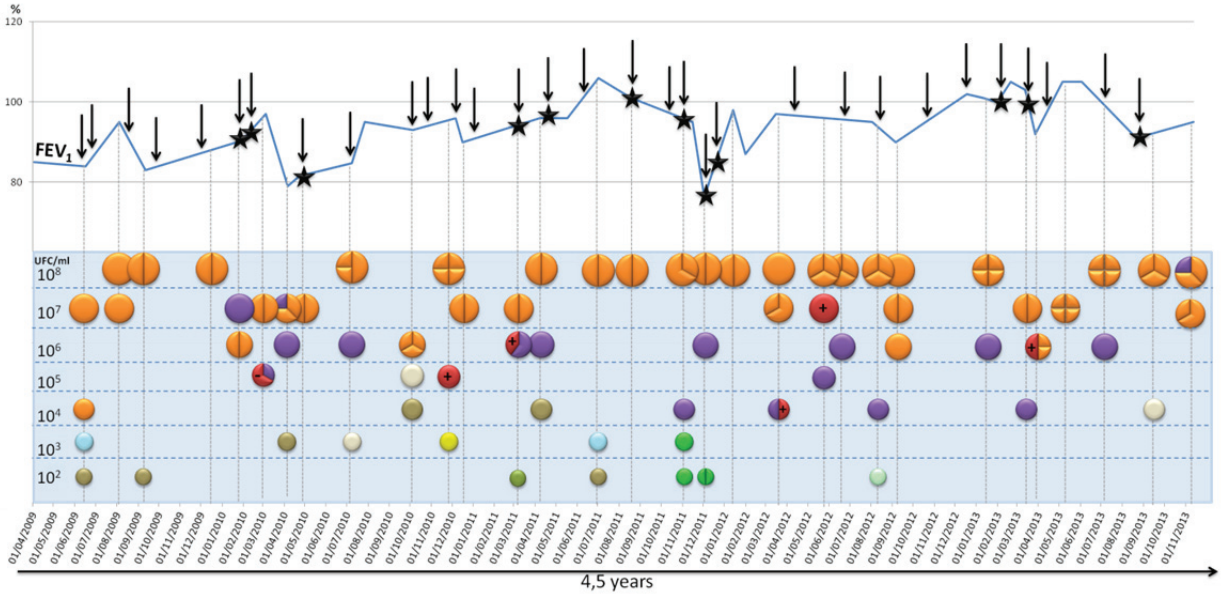

Fig. 3 Community composition and evolution in sputum according to forced expiratory volume in 1 second (FEV_1) for a 13 year-old patient chronically colonized by *A. xylosoxidans*. Data are represented for each sputum vertically: sampling date is indicated at the bottom of the figure. Pathogens are indicated according to color code below and load (left scale in colony forming unit (CFU)/ml.). Circles separated in different parts indicate different species (bicolor) or different colonial morphotypes (unicolor). +/- represents the presence or absence of ß-lactamase production for *Haemophilus influenzae*.

4.4 Battle against CF respiratory tract infection, a multifaceted challenge

Current strategies include antimicrobial therapy administered orally, by inhalation and/or intravenously aiming at eradicate pathogen in early stages of the disease and then at decrease bacterial load and nebulization to favor mucociliary clearance always associated to physiotherapy. Specific drugs are used for their anti-inflammatory effects like azithromycin that interferes with quorum-sensing-dependent virulence factor production, biofilm formation, and oxidative stress resistance in *P. aeruginosa* [177]. Such strategies disrupting coordinated bacterial behavior have a real benefit for the patients and the potential to increase the efficacy of currently available antibiotic drugs [178]. Despite sputum sensitivities may be discordant with the outcome of antibiotic treatment in the patient, once more highlighting the high complexity of the infectious processes in CF, these strategies led to an overall increase in life expectancy during the past decades [179].

However, more recent knowledge on CF respiratory tract infections, notably showing differences in the composition of baseline microbiota and microbiota at pulmonary exacerbation, suggests that novel targets could be considered for therapeutic intervention. News strategies aiming at modulate local inflammation and/or at restore a balanced airway microbiota and/or at counteract altered microorganism interactions have to be considered in this context [180]. For this purpose, a direct approach by using inhaled probiotics [181] or an indirect approach considering interactions between gut and respiratory microbiota via modulation of gut microbiota by ingested probiotics [182] appear promising. Indeed, several studies show the impact of diet on airway microbiota dysbiosis and the modulation of gut microbiota on the frequency of pulmonary exacerbations [166]. Simultaneously, a renewed interest in phage therapy as an alternative or

complement to conventional antibiotic therapy has emerged based on efficacy of bacteriophage therapy in a model of pulmonary infection against CF pathogens such as life-threatening opportunistic bacterial pathogen *Burkholderia cenocepacia* [184].

Finally, unexpected antimicrobial effect has been recently demonstrated for hypertonic saline as yet though to act through mucus rehydration, mucociliary clearance improvement, anti-inflammatory activity and indirect effects by activation of antimicrobial peptides. Beneficial effects of hypertonic saline nebulization on lung function in reducing exacerbations or on the perception of effectiveness of chest physiotherapy may also at least in part be linked to bacterial growth inhibition or killing, biofilm formation inhibition or biofilm disruption, and motility inhibition demonstrated on *P. aeruginosa* isolates behind the concentration used in therapeutics [185].

4.5 Conclusion

Complexity of CF respiratory tract infection pathogenesis confers meaning to the concept of pathobiome and the CFTR microbiome could be considered as a pathogenic complex or a "metapathogen" in this context. This methapathogen thus represents a new target for adapted anti-infectious strategies. New therapeutic strategies that probably will have to be used as complementary weapons against disease have to be designed to target complex modifications of the diversity of the respiratory tract microbiota during airway diseases, to normalize perturbed interactions between microorganisms and between microbes and host cells, and to directly or indirectly modulate airway inflammation. However, if bacterial community has been extensively studied, more global studies are still needed because virobiota and fungal communities have received less attention, as it is also the case for antimicrobial host defenses.

5. Concluding remarks

The three examples of pathobiome developed in this chapter were chosen for their importance in human health but also because they illustrated the shift to the ecological vision of infectious diseases. These examples show that environmental and clinical microbiologies should fuse in a continuum of disciplines because, today, they share concepts, approaches and issues.

The major research concern is now to understand the ecological and evolutionary pressures that shape microbial communities. It is critically important for the optimal use of current anti-infectious agents and for the development of the new strategies evoked herein. New strategies could be gathered with the following objective: changing conditions across the entire environmental and human microbiome continuum to promote the maintenance or the re-establishment of optimal communities, i.e., reduce or close pathobiomes. This is no less than a refundation of applied and clinical microbiology in the actual complexity of the microbial life. Today, metagenomic data invade scientific databanks but integration of these data in experimental, medical or applicative approaches face barriers, which are the lacks of indicators, parameters and models easily applicable to interpret data and predict risks. This is particularly true for epidemiological surveillances in environment or hospital, and for improving the rationale of infection prevention and patient treatment. Future effort is recommended to demonstrate the feasibility of the pathobiome reduction strategies by the development of effective, practical, and safe protocols.

As for other major global issues of the 21th century, the programmed end of antibiotics is not only a scientific concern but also a cultural and political issue. The example of phage therapy in the 20th century is particularly emblematic. Phage therapy has been used for decades in USSR but subjected to multiple hindrances in USA and Western Europe, countries where the chemical/pharmaceutical lobby is powerful. Consequently, since the 'rediscovery' of phages by western scientist 20 years ago, not a single phase III clinical trial has taken place. The battle against infectious diseases and antimicrobial resistance is now a global ecological challenge and thereby it needs to adopt a global plan that should surpass individual and national priorities. Our predicted defeat should force us to slow and stop the arm race against microbes. The consideration of microbes in their ecological dimension in order to reduce the pathobiomes (or to avoid their construction) is a research avenue that deserves urgent developments. Paradoxically, this global challenge will probably go through the development of the personalized medicine, which will consider each patient's genome, microbiome and environmental conditions for prevention and treatment of infectious diseases and more largely of all other diseases involving dysbiotic microbiome.

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