Victor Pylro · Luiz Roesch *Editors*

The Brazilian Microbiome

Current Status and Perspectives

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ISBN 978-3-319-59995-3 ISBN 978-3-319-59997-7 (eBook)
DOI 10.1007/978-3-319-59997-7

Library of Congress Control Number: 2017948867

Printed on acid-free paper

This Springer imprint is published by Springer Nature
The registered company is Springer International Publishing AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Preface

The relationship between humans and microbes has attracted our interest since the creation of the first single-lens microscope by Antonie van Leeuwenhoek and the discovery of tiny living beings, which he called "animalcules" (now known as microbes/microorganisms) in 1674. For the past 100 years we have been trying to remove microbes from our lives, as they have been closely associated with diseases. However, we are starting to realize that some microbes are fundamental to our health and to the maintenance of environmental homeostasis. Our thoughts about how to deal with microbes are changing in an unprecedented way. In recent years intriguing works revealing the multiple facets of microbial life have flooded the scientific literature. Thanks to new molecular tools, mainly those based on next-generation sequencing, new evidence of microbial interactions has been revealed in several environments and hosts. Our ability to detect microbes in nature has radically improved and our appreciation of the importance of microbes has completely changed. We are now living in the age of microbiomes. New microbiome reports and discoveries appear daily, describing the vast and diverse microbial communities in innumerable biomes, organisms, surfaces, and in any other imaginable place. So much has happened around the world and much more is still to come.

Brazilian Microbiome: Current Status and Perspectives unites a set of distinguished investigators conducting microbiome research and builds a comprehensive reference book with up-to-date information regarding Brazilian microbiome studies and trends. It covers terrestrial-, plant-, and host-associated microbiomes, unveiling biological and technical aspects of research. This book is devoted to students and professionals interested in learning about and better understanding the biology of microorganisms in nature, with an emphasis on Brazilian microbiomes.

This book is supported by the Brazilian Microbiome Project (http://www.brmicrobiome.org) and the Brazilian Institute of Science and Technology on Microbiomes (http://www.inct-microbiome.org).

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The Brazilian Microbiome Project

Victor Pylro and Luiz Roesch

Abstract Brazil harbors about 20% of global macro-biodiversity, but despite the well-accepted tenet that microbes are essential for ecosystem maintenance and although microbes represent a fundamental resource for Brazil's economic and technological development, knowledge of Brazil's microbial diversity is still largely neglected. This might be partially explained by our inefficiency in detecting microbes directly from the environment. However, recent advances in biomolecule extraction/purification procedures, next-generation sequencing (NGS) technologies, and computational biology and modeling are now changing this scenario. Important discoveries and advances have recently been made, but such advances have not been as enlightening as expected. We argue that the success of microbiome studies is tied to appropriate integration with the scientific community, and only integrated research models will be able to reveal the full microbial potential to benefit local communities and citizens, as well as ongoing conservation efforts. In this chapter we introduce the Brazilian Microbiome Project, a local initiative that aims to coordinate national microbiome research, enabling appropriate integration with international initiatives to better decipher Brazilian microbial diversity and its dynamics and environmental interrelationships.

Microorganisms play an essential role in all ecosystems, from nutrient cycling to maintaining human health. In 1988 Whipps et al. [1] defined the term microbiome as "a characteristic microbial community occupying a reasonably well-defined habitat, which has distinct physicochemical properties". They emphasized that the term

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V. Pylro, L. Roesch (eds.), *The Brazilian Microbiome*,
DOI 10.1007/978-3-319-59997-7_1

"not only refers to the microorganisms but also encompasses their theatres of activity". More recently [2], the microbiome was described as "the entire habitat, including the microbes (bacteria, archaea, lower and higher eukaryotes, and viruses), their genomes (i.e., genes), and the surrounding environmental conditions". As we can see, the term microbiome is not as simple as we usually think. Nowadays, it is easy to find various works just describing microbial communities in different habitats being incorrectly characterized as "microbiome studies". However, the complete analysis of a given microbiome is very complex, and should include input from different fields, such as microbiology, biochemistry, genetics, molecular biology, ecology, environmental engineering, bioinformatics, and others.

The study of microbial communities and their relationship with the host and/or environment is essential for the understanding of ecosystem dynamics. Currently, scientific and technological advances, which have revolutionized the traditional approaches used to study biological resources, have also fundamentally boosted microbiome studies. Recent advances in biomolecule extraction/purification procedures, next-generation sequencing (NGS) technologies, computational biology and modeling, metagenomics, metatranscriptomics, and all other "omics" are now allowing us to perform a variety of comparative analyses of diversity, abundance, and important ecosystem functional genes of whole microbial communities at far greater depths than ever before.

Microorganisms, with their vast diversity, are an important biological resource not only because of the environmental services they provide, but also because of their biotechnological potential and their application in the development of new tools for sustainable ecosystem management. In this scenario, Brazil stands out by harboring around 20% of all macro-biodiversity on earth, being one of 17 countries that, together, house around 70% of all catalogued animal and plant species [3]. Some recent efforts have affirmed the Brazilian government's commitment to making biodiversity information widely available, such as Brazil joining the Global Biodiversity Information Facility (GBIF; http://www.gbif.org) as an associate member in 2012, and the creation of the "Brazilian Biodiversity Portal" (http://portaldabiodiversidade.icmbio.gov.br), by the Ministry of the Environment and its related institutions, in 2015. Although these steps are valuable for increasing international cooperation and consolidating the knowledge of Brazilian biodiversity, microbial diversity is ignored [4], despite the well-accepted tenet that microbes are essential for ecosystem maintenance, and the principle that they represent a fundamental resource for Brazil's economic and technological development.

In a broader view, Dubilier et al. [5] proposed the creation of an International Microbiome Initiative. Based on the Unified Microbiome Initiative [6] the authors added that "…microbiome research will require a coordinated effort across the international community". However, microbial diversity and functioning are strongly tied to geographic features [7]; therefore, strategies to deal with these peculiarities are essential. Intellectual property, publishing, and national policies for biodiversity protection/use are fundamental requirements to enable a nation's development of technology and bioscience. Accordingly, a global initiative also needs local leaderships [8, 9]. The Brazilian Microbiome Project (BMP) [10]

(http://www.brmicrobiome.org) is a local initiative that aims to fill this gap by organizing national microbiome research to enable appropriate integration with international initiatives. Since its creation at the end of 2012, the BMP has expanded the knowledge and the visibility of Brazil's microbial diversity resources (e.g., [11]), besides providing user-friendly open source bioinformatics tools and human resources training for microbiome data analyses [12].

Several Brazilian research groups are studying microbial diversity in various biomes – e.g., Caatinga [13, 14]; Cerrado [15, 16]; Amazon [17, 18]; Pampa [19, 20]; environments such as oceanic islands [21, 22], seas, oceans, and coral reefs [23–29]; mangroves [30]; ruminant animals [31, 32]; plants [33]; and arthropods [34]. Furthermore, studies focused on microbial greenhouse gas emissions are also being performed [35, 36]. However, little or no interaction among study groups has been achieved until now. Also, although several of these studies use a common currency (DNA) to profile microbial biodiversity, the lack of standardized methods and metadata collection precludes robust inter-study comparisons, limiting the value of these precious resources [37, 38]. Expanding bioinformatics capacity is still critical because the current bottleneck for biosciences is how to deal with "big data" [39]. In-depth analysis of the growing number of completely sequenced microbial genomes and metagenomes in public databases is providing fascinating contributions to our understanding of how these genomes are genetically tailored to the microbial lifestyles. The BMP has established the Brazilian Institute of Science and Technology for Microbiome Studies (INCT-Microbiome; http://www.inct-microbiome.org; see [8]), which has fostered the integration of research groups by subject of interest, and the development and dissemination of uniform standards for 16S rRNA (bacteria/archaea) and ITS (Internal Transcribed Spacer – fungi) microbial community profiling, and the associated data analysis, aiming to make them comparable [see http://www.brmicrobiome.org/standardsandprotocols] [40].

The BMP has become inherently collaborative, with coordination between six committees that represent specific scientific research domains, and two strategic committees that focus on training and the transfer of knowledge and technology. The research domain committees are thematic, focused on microbial diversity and processes in (a) plants, (b) animals, (c) soils, (d) aquatic environments, and (e) humans, and (f) focused on bioprospecting. Each theme considers research drivers, horizon scanning, and paths to translate research into socioeconomic relevance. Promising translational areas include the effects of pollution and land use change, water treatment, management of water resources, animal breeding, and microbial effects on human health. The two strategic committees are responsible for identifying paths for transferring knowledge (Knowledge Transfer) and data analysis resources (Bioinformatics). In summary, this consortium aims to increase the understanding of Brazil's microbial resources with the goal of developing strategies to (a) mitigate environmental greenhouse gas emissions; (b) increase the activity of beneficial microorganisms from humans to soils (e.g., by supporting sustainable agriculture); (c) suppress pathogenic microorganisms in plants and humans; (d) understand the impact of pollutants in aquatic environments; and (e) create a rapid and efficient strategy for scientific and technological bioprospecting.

A comprehensive catalogue of Brazilian microbiomes has yet to be developed. We argue that only a broad-scale survey that brings together multiple investigators from different areas of expertise will be able to decipher Brazilian microbial diversity, dynamics, and environmental interrelationships. This interdisciplinary approach will be made feasible only by strengthening collaborations and defining a standard core of practices for the field. The BMP is working to ensure appropriate project alignment with other international efforts [5, 6].

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Plant Microbiome: Composition and Functions in Plant Compartments

Maike Rossmann, Stalin Wladimir Sarango-Flores, Josiane Barros Chiaramonte, Maria Carolina Pezzo Kmit, and Rodrigo Mendes

Abstract Knowledge of the vastness of microbial diversity associated with plants is still limited. Plant microbiome structure and functions are shaped by several factors, including host genotype and developmental stage, the presence or absence of diseases, and environmental conditions. These factors may lead to distinct microbial communities in the rhizosphere, endosphere, and phyllosphere. Studies directed to microbial interactions in plant compartments are fundamental for understanding the microbial ecology of phytobiomes, enabling the development of microbiome-based technologies in the search for sustainable agriculture. In this chapter, we describe plant compartments, i.e., the rhizosphere, phyllosphere and endosphere, and the more common bacterial composition of each compartment. We also discuss manipulation of the plant microbiome toward improved plant health. Advances in this field will lead to strategies where the manipulation of the plant microbiome will allow the reduction of pesticide and fertilizer use in field crops, paving the way to a more sustainable agriculture.

Introduction

The concept of the microbiome was described for the first time as the "ecological community of commensal microorganisms, symbionts or pathogens, which literally occupy a space in our body" [1]. Recently, this term has been used for different environments inhabited by microorganisms [2–4]. This term has also been used in the plant context as "an environment, which consists of the plant and all microbes associated with it" [3].

The relationship between plants and their surroundings, especially those plant-microbe interactions with a beneficial output, has been the center of attention of various studies [5]. Traditionally, many researchers have tried to understand these

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V. Pylro, L. Roesch (eds.), *The Brazilian Microbiome*,
DOI 10.1007/978-3-319-59997-7_2

interactions, looking to individual plant-microbe relationships, i.e., a one vs. one approach, but these interactions are much more complex, as they involve a vast diversity of microbes and environmental factors [6].

Plant, soil, soil-borne microbes, and environmental factors together influence the various changes that cooperate to create plant health and productivity. Recent advances in "-omics" research have shed light on microbiome compositions and interactions with the environment [7]. These advances have contributed to the development of novel approaches that seek to improve plant fitness through the artificial selection of microbes with specific effects on host performance. The selection of microbial communities occurs indirectly through host traits that have coevolved together with the microorganisms and influence the microbiomes [8].

In this chapter, firstly, we define each plant compartment, i.e., the rhizosphere, phyllosphere, and endosphere, and within each compartment we describe "who" is there (microbiome structure), "what" they are doing (microbiome functions), and what are the major drivers shaping the assembly of the microbiome. Finally, we discuss the advances in microbiome manipulation and the possibilities of using such manipulation to improve and optimize crop productivity.

The Rhizosphere Ecosystem

The term rhizosphere was coined by the soil bacteriologist Lorenz Hiltner in 1904 [9]. This term is derived from the Greek word *rhiza* (root) and the Latin word *sphaera* (sphere), referring to an environment or compartment that encloses the inhabited "microbial world" on the plant roots. The rhizosphere is the narrow zone of soil surrounding the root system where plants and microorganisms interact [10–14] (Fig. 1) and it is characterized by a chemical, biological, and physical gradient that changes radially and longitudinally along the roots [15].

The idea of microbial colonization of the rhizosphere seems to be supported by the niche theory of species diversity, which is driven by various abiotic and biotic factors, such as plant genotype and soil [5, 13, 16–19]. Changes in the rhizosphere microbial community begin when the soil microbiota is exposed to rhizodeposits, which are influenced by the plant genotype, including glucose, amino acids, organic acids, polysaccharides, and proteins [10, 13]. Rhizodeposition increases the microbial populations in the rhizosphere, known as the "rhizosphere effect" [11–13, 16]. Later, the plant genotype selects and assembles a closely associated microbial community in the rhizoplane and within the plant roots [13, 16, 20]. It has been hypothesized that each plant species selects specific microbial populations as a result of the high degree of host specificity in the coevolution of plants and microbes [5, 13, 21].

Plants release exudates into their direct surroundings to attract, stimulate, or repel microorganisms on the roots. The amount and composition of the rhizodeposits, which structure and modulate the rhizosphere microbial community throughout the plant life cycle, may vary among different plant species [22] and throughout their growth [23], as well as in different stages of root development [5]. Microbial succession starts with the release of carbon from seeds during the

germination stage, and microorganisms in the rhizosphere are distributed according to root type and zones, as well as according to their movement through the soil during root growth [13]. In the early stages of plant development (seedlings), alcohol and sugars are released, while in the later stages, amino acids and phenolic compounds predominate [23]. This phenomenon suggests the attraction of a large diversity of microorganisms in the early stages of plant development, while later the release of specific substrates selects certain microorganisms in the rhizosphere [5, 21, 23].

The number of microorganisms in the rhizosphere is higher than that in bulk soil, due to the carbon availability in the rhizosphere. Generally, gram-negative bacteria are stimulated by rhizodeposition, whereas gram-positive bacteria are inhibited [10]. Proteobacteria (α, β, γ), Firmicutes, Actinobacteria, Bacteroidetes, Crenarchaeota, Acidobacteria, Ascomycota, and Glomeromycota, and also unclassified bacteria, represent relatively large groups detected in the rhizosphere [5, 12, 13] (Fig. 1).

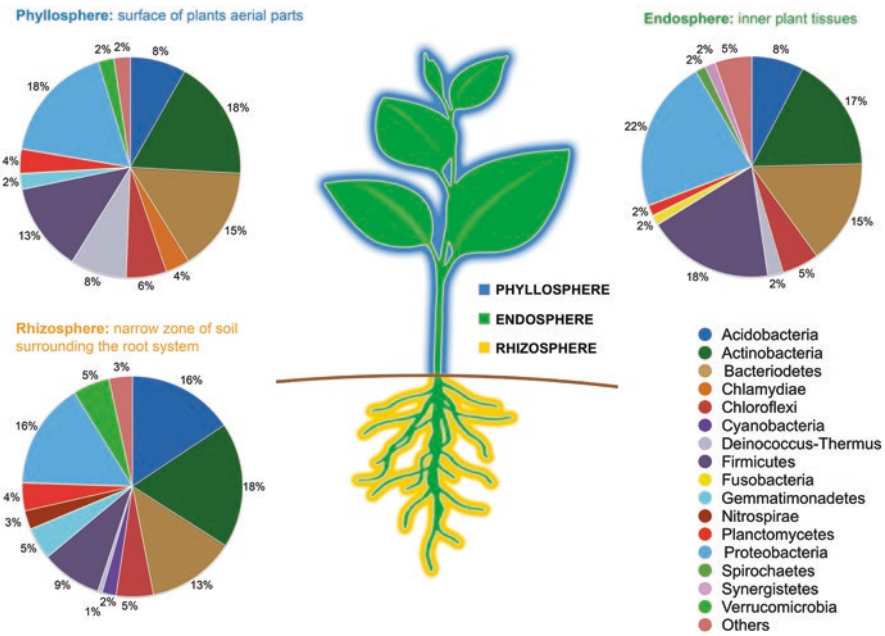

Fig. 1 Schematic representation of plant microbiome compartments and frequency of studies describing bacterial phyla in each compartment, i.e., phyllosphere, endosphere, and rhizosphere. Each pie graph shows the frequency of studies reporting bacterial phyla per plant compartment. For example, 18% of 15 studies on the phyllosphere detected Actinobacteria in the bacterial community. Seventy-one studies were surveyed, 15 for the phyllosphere, 29 for the endosphere, and 27 for the rhizosphere. Searches were performed in the Scopus database between February 03, 2016 and March 15, 2016. The search used a combination of words describing plant compartments ("rhizosphere", "phyllosphere", "endophytic", "endosphere") and investigative techniques ("sequencing", "metagenomic", "next-generation sequencing"). Studies using cultivation-dependent approaches were not included in the survey. Phyla cited in only one manuscript were included in the "Others" category

The microorganisms found in the rhizosphere can have beneficial or deleterious effects on the growth and health of the plant [13]. The beneficial microbes, among others, include mycorrhizal fungi and rhizobia, which provide phosphorus and nitrogen; siderophore-producing bacteria, which facilitate iron acquisition; and plant-growth-promoting rhizobacteria (PGPR), which promote plant growth [12, 14, 24]. PGPR can suppress disease by mechanisms such as competition for nutrients and microsites, parasitism and antibiosis, or by inducing systemic resistance to pathogens in the plant [13]. There are some examples of microorganisms that promote plant adaptation to abiotic stresses such as drought, flooding, saline stress, temperature or pH extremes, and high concentrations of toxic compounds, and these cases reveal complex associations of microorganisms with plants as a result of coevolution in their native habitats [13, 25]. Biotic stress includes the presence of phytopathogenic microorganisms such as nematodes, fungi, and oomycetes, which have agronomic importance because they reduce the yields of food, feed, fiber, and fuel crops [12].

Given that root exudates are strongly linked to the recruitment of the microorganisms that comprise the rhizosphere microbial community, it can be seen that the rhizosphere is closely involved with plant health and growth; therefore, the understanding of rhizosphere functioning and ecology is key to increasing crop yield.

The Phyllosphere

The second compartment of the plant microbiome is the phyllosphere, or aerial plant surface, which is characterized as being nutrient poor when compared with the rhizosphere [26]. The phyllosphere is composed of microbial cells that are able to colonize the aerial plant surfaces [27, 28] that are dominated by the leaves, although the term phyllosphere can be used for any aerial part of the plant [29] (Fig. 1).

The microbial habitat on the surfaces of leaves may be one of the largest microbial habitats on earth, with the terrestrial leaf surface area estimated to exceed 10^8 km^2 globally [30]. The phyllosphere microbiome is composed of viruses, bacteria, filamentous fungi, yeasts, algae, and, occasionally, protozoa and nematodes [26]. Bacteria are the most abundant of the cellular organisms in the phyllosphere community, present in numbers between 10^6 and 10^7 cells cm^{-2} of leaf tissue [26, 29]. Fungi and archaea are apparently less abundant; however, their population has not been estimated yet [26, 30, 31].

Overall, species richness in phyllosphere communities is high [32]; however, the bacterial community diversity is lower than the diversity of the communities in the rhizosphere or bulk soil [31, 33]. Advances in sequencing technologies have vastly expanded our understanding of plant microbiome structure, including that in the phyllosphere [34]. At the phylum level, the phyllosphere bacterial communities are composed mainly of Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria [35], with a predominance of the classes Alphaproteobacteria and Gammaproteobacteria [36, 37] (Fig. 1). Further analysis of community composition

at the genus level suggests that *Pseudomonas*, *Sphingomonas*, *Methylobacterium*, *Bacillus*, *Massilia*, *Arthrobacter*, and *Pantoea* are consistently found as part of the phyllosphere microbiome across a wide range of plant species [35].

The colonization of plant leaf surfaces, in large part, occurs through the immigration of bacteria, fungi, and other microorganisms from air, soil, water, seeds, or through animal sources [29]. Furthermore, studies have shown that some of these microorganisms of the foliar microbiome can be transferred not only through environmental exchange, but also vertically, through generations of plants [38]. Neighboring environmental ecosystems can also randomly contribute to the assembly of the foliar microbiome [39]. Even after the stabilization of phyllosphere microbial communities, variations may occur, caused by nutritional heterogeneity in different regions on the leaf surface, where the carbon sources (e.g., glucose, fructose, and sucrose) are spatially heterogeneous, leading to distinct microbial assemblages on the leaf veins, which are regions near the stomata and surface appendages [26, 29]. Large fluxes in temperature, moisture, and radiation throughout the day and night also cause changes in the phyllosphere microbiome structure [26, 29, 40]. In some cases, this spatial heterogeneity is promoted by the organization of microbial cells into biofilms, which are a common feature of organisms in the phyllosphere, acting as aggregators and protectors of the microbial cells under the frequently inhospitable conditions [26, 41].

The microbial communities found in the phyllosphere may perform key processes related to plant development; for example, nitrogen fixation [42, 43], protection from invading pathogens [44], modification of metabolites, and the biosynthesis of phytohormones [45]. Metagenomic and metaproteomic studies showed that microbes in the phyllosphere could produce proteins that promote substrate uptake, via porins and ABC transporters; resistance to stresses, including reactive oxygen species (ROS); and nutrient cycling [31]. Methylobacteria are involved in methanotrophy and are often detected in phyllosphere communities [46, 47].

The interactions between the plant and the phyllosphere microbial communities, and the variations in their distinct environmental factors, modulate the assemblage of these microbial communities in the phyllosphere and contribute to the heterogeneity in their abundance and structure in distinct plant species. New molecular technologies have shown the importance of microbial functions in the phyllosphere and have provided new insights into the major drivers of microbial community composition. The combination of multiple "omics" technologies will lead us to a system-level understanding of the phyllosphere microbial communities and their physiological potential.

The Endosphere

The endosphere consists of the inner plant tissues, inhabited by microorganisms intimately interacting with the host plant [28, 48, 49]. This compartment is composed of the internal root tissue (endorhizosphere), internal shoot and leaf tissue

(endophyllosphere), internal plant reproductive tissue, and the internal seed tissue [50–55]. Endophytic microorganisms are organisms that reside internally in plant tissues for at least part of their life cycle [48] without causing visible disease symptoms [56] and they can be accessed from the plant after surface disinfection by cultivation-dependent and/or molecular approaches [57–59] (Fig. 1). Although this concept is one of the most commonly accepted ones and is currently applied, it is important to note that there are niches on the surfaces of aerial parts and roots where microorganisms may remain protected from the action of the chemical products usually used for surface disinfection. Recent studies have used sonication to remove surface layers of the plant tissue and to access the endophytic microorganisms on the remaining tissue [17, 20].

Endophytes are beneficial or commensal, and they can shift between parasitic and mutualistic life strategies [60, 61]. Their beneficial role in plant development and health can be mediated and is characterized by metabolic interactions, including the production of plant growth hormones [62–64], antibiotics, and toxicants [65, 66]; the improvement of nutrient uptake; and/or increasing the plant tolerance to biotic and abiotic stresses [62, 67, 68]. In addition to these characteristics, the lifestyle of endophytes can also involve altering/inducing the gene expression of plants' defense and metabolic pathways [66, 69, 70], and, depending on the type of interaction, members of the endosphere microbiome can induce both local and systemic alterations in the host [71]. As an example of these alterations, genome analysis of *Bacillus pumilus* INR7, an endophytic bacterium that promotes plant growth and induces systemic resistance against several plant patogens, revealed the presence of non-ribosomal peptide synthetase gene clusters for the production of antibacterial compounds such as surfactin, bacillibactin, and bacilysin, as well as genes for the biosynthesis of growth promoters such as indole-3-acetaldehyde and 2,3-butanediol [72].

The endosphere microbiome structure is driven by soil type, host phylogeny, and/or microbes. The soil traits that affect microbial recruitment from bulk soil are soil type [20, 53, 73], soil pH [53, 74], local edaphic conditions [75], and anthropogenic management factors, such as fertilizer and pesticide application and soil preparation [76, 77] The endosphere microbiome structure is also variously affected by plant species [78], plant life stage [77, 79], and plant health, as a result of the differences in root architecture and types of exudates [16]. Finally, the capacity of microbes to reach inner plant tissues and establish themselves there also affects the microbial composition of the endosphere. Endophytes need to have the capacity to reach the root surface, and to express genes for the invasion of plant tissue and the colonization of a niche within the plant tissue [80]. Studies have shown that the endosphere is mainly composed of bacterial phyla, such as Proteobacteria, Actinobacteria, Bacteriodetes, and Firmicutes [17, 20, 77, 81], and fungi, including Ascomycota and Basidiomycota [35, 82–84] (Fig. 1).

Endophytes are classified as systemic/true and transient/nonsystemic [56] or as obligate and facultative [48]. Systemic or obligate endophytes are dependent on the plant metabolism, and are disseminated among plants by vertical transmission or by vector activity [48]. In addition, systemic endophytes do not produce any visible symptoms of disease in the host at any life stage [56]. Because they live in a low-

competition and low-predation environment, obligate endophytes have evolved to produce specific metabolites that support their interaction with the host [85]. In contrast, facultative or transient endophytes live inside plant tissues for at least part of their life cycle, without producing any apparent disease symptoms in the plants, but they become pathogenic when the host plant faces resource-limited conditions [86]. Transient endophytes vary both in diversity and abundance, depending on changes in the environment [83] and they face high levels of competition in the rhizosphere before entering the plant [80], therefore producing many metabolites that are involved in both their defense and in interactions with the plant [85].

The microbiomes associated with above-ground (phyllosphere), below-ground (rhizosphere), and internal (endosphere) tissues are distinct, especially considering that the endosphere is where specific metabolic capacities are required to survive. Endophytes have a significant effect on the host plant by modulating its health, growth, and development. Naveed et al. [87] observed that *Enterobacter* sp. strain FD17 promoted the growth and health of maize grown under natural conditions, increasing grain yield by 42% and reducing the time until flowering. Mendes et al. [62] reported that the endophytic *Burkholderia* spp. showed ability to control the growth of the sugarcane pathogen *Fusarium moniliforme*. Khan et al. [88] have shown that tomato plants inoculated with endophytic *Sphingomonas* sp. LK11 showed increases in shoot length, chlorophyll content, and shoot and root dry weights, indicating that the phyto-hormones produced by this strain may help in increasing crop growth. Although there are still gaps in our knowledge of endophytes, the investigation of these microbes as a bioresource for plant growth-promoting regulators and as biocontrol agents for disease and pest management represents opportunities for improving crop yield and health in a sustainable way.

Manipulation of the Plant Microbiome Toward Improved Plant Health

According to the latest United Nations projections, the world population will exceed ten billion by 2100 [89]. In order to meet the demand for food, both the land area used by agriculture and productivity must increase in the near future. In this scenario, intentional manipulation of the plant microbiome may be an alternative way to improve agriculture sustainability. This would be done by exploiting rhizosphere microorganisms with beneficial traits to, for example, make nutrients more available for plants or increase plant tolerance to biotic and abiotic stresses, consequently decreasing the dependence on chemical input in agriculture.

Manipulating the plant microbiome can be achieved simply by promoting good management of soil. Crop rotations increase the diversity of microorganisms in soil, promoting high resilience to plant pathogens [90]. Bakker et al. [91] showed that where resource changes altered the bulk soil microbial community, the effects were observed in the rhizosphere of two different cultivars of corn, suggesting that rhizosphere microbial communities are altered depending on the site history and selective events.

The stimulation of certain microorganisms or the introduction of inoculants is another strategy for plant microbiome manipulation. This approach aims to establish a beneficial community that competitively excludes plant pathogens. Reducing the time of niche exploration is crucial for enhancing microbial root-colonizing capacity [80, 92]; this can be achieved by the co-inoculation of several beneficial strains, including endophytes. The inoculation of a bacterial consortium might also promote the release of antimicrobial compounds [93] that improve the suppression of soil-borne pathogens [94].

The inoculation of microorganisms also has the potential to improve plant nutritional status. *Rhizobium* spp. are some of the most common microorganisms used as inoculants in legumes and their use can supply almost all of the nitrogen required by legume crops [95]. Phosphorus-solubilizing microorganisms can also be applied as inoculants, either alone or in association with rock phosphate [96]. A limitation in the use of inoculants is that the densities of the inoculated microorganisms are subject to decline over time, and the inoculants have to be able to survive under different field conditions. It is also important to consider that inoculants must be free of metabolites that are hazardous for humans, animals, and plants [97].

The plant genotype, in interaction with environmental conditions, is responsible for regulating the release of exudates in the rhizosphere soil, and this exudate release is one of the main drivers of the microbiome structure. In this context, the microbiome may be manipulated by changing the amount and quality of root exudates through plant breeding or genetic modification [98–100]. However, it is important to note that this strategy is limited in many ways: (a) the methods are very time-consuming and are restricted to the target species/cultivar; (b) traditionally, breeding programs do not consider the interaction among plants and microorganisms when new cultivars are being developed [101]; and (c) the quantity and quality of the exudates vary tremendously among soil types and physiological conditions of plants, making the exudates difficult to manipulate [102].

Although less commonly studied, manipulation of the microbiome of aerial plant parts can also be a strategy for improving plant growth and health. Falk et al. [103] suggested that it is possible to reduce the severity of powdery mildew infections caused by *Uncinula necator* on grapevines by releasing the conidia of the mycoparasite fungus *Ampelomyces quisqualis*. Several pesticides applied in agriculture have the potential to affect the natural occurrence of a microbial community [104, 105], while it has already been shown that the natural leaf microbiome is beneficial to the plant. Perazzolli et al. [106] showed that the naturally occurring microbiomes of grapevine leaves could reduce signs of powdery mildew on the surfaces of the leaves under controlled conditions.

Optimizing plant-microbiome interactions through microbiome manipulation has the potential to improve crop sustainability, reducing the impacts of traditional agricultural practices. Although many efforts have been made to understand the factors controlling microbiome assemblage, manipulating the microbiome is still a challenge to be addressed.

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The Brazilian Soil Microbiome

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Abstract Brazil, where several biomes occur with an extraordinary exuberance of flora and fauna, is recognized worldwide as an important hotspot for biodiversity. However, a key but yet unexplored component of this biodiversity is represented by the microbial life that permeates Brazilian soils. This chapter aims to summarize the characteristics and knowledge of microbial life in Brazilian soils—the soil microbiome. Our summary will encompass soils occurring in pristine conditions, such as those from the Amazonia, Caatinga, Atlantic rainforest, Pantanal, and Pampa biomes, in combination with commentaries about soils used for agriculture in Brazilian territory. The chapter provides information about the occurrence and functionality of microbes in soils. Here, we aim to link the occurrence of microbial groups with soil characteristics. A great part of the information on this issue is recent, as promoted by the adoption of culture-independent analyses. We hope to provide here information compiled for people interested in soil microbiology. Possibly, this compilation will constitute the first step toward the inclusion of microbial life in the Brazilian inventory of biodiversity.

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V. Pylro, L. Roesch (eds.), *The Brazilian Microbiome*,
DOI 10.1007/978-3-319-59997-7_3

Introduction

The field of microbial ecology has been revolutionized with the use of culture-independent methods that avoid the bias imposed by the cultivation of microbes as a source of information about microbial communities, and these methods have revealed an unexpected microbial diversity. In complex systems such as soils, the vast majority of microbes are not suitable for culture, as they mostly require specific conditions and the occurrence of particular biological interactions to trigger cell multiplication. The complexity of soil microbial communities increases according to species richness and abundance, which makes the soil system the most challenging environment for the study of microbial ecology. But there are many challenges in the study of this field, with the description of groups comprising the microbial communities in Brazilian soils not yet having been completely deciphered. Despite the growing number of studies in these environments, little is known about the extent of the diversity and the functional role of the microbiomes in the distinct soils of Brazilian biomes.

Part of this challenge can be attributed to the singularities attributed to these microbiomes, which are determined by the peculiar environments encountered in our country. These peculiarities are promoted by climatic and geological variations, which are determining factors in the process of soil formation, and consequently in the life forms (mainly plants) that make up the biomes. In all these environments, the microbial community constitutes the base of the food chain, providing nutrients to plants and influencing the biogeochemical and geomorphological processes that occur in the soils that sustain them. In addition, we have, in our territory, very different soils compared with those where microbial communities are more widely studied, such as in temperate regions. Another factor that may lead to the occurrence of a unique selective process in Brazil is the use of specific agricultural practices, such as conservation tillage, that are compatible with our climate and soil types.

Our study examined a heterogeneous, fast-moving (with innovations promoted by the development of different techniques for microbial analysis), challenging (because of its complexity), important, and promising (because of the peculiarities of Brazilian soils) scenario. This document was developed with the aim of reporting the advances achieved and the ongoing studies focusing on deciphering the structure and functionality of the microbiome in Brazilian soils.

Characteristics and Particularities of Brazilian Soils and Biomes

Brazil harbors several biomes within its continental territory (Fig. 1a), which together account for about one-third of the pristine areas on Earth (http://brazilbiodiversity.org/), making Brazil one of the largest reserves of terrestrial biodiversity. The importance of the biodiversity in Brazilian biomes is inestimable, especially because of their potential for human and environmental benefit, and their promotion

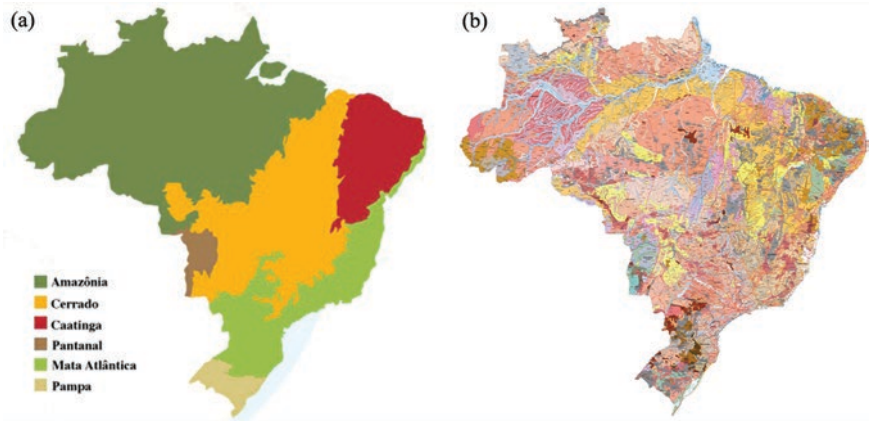

Fig. 1 Map of Brazilian territory highlighting biomes (**a**) and soil types (**b**). The names of the soil types are omitted because of their large number, and because the main goal of this chapter is not the examination of soil types. Source: http://mapas.ibge.gov.br/tematicos/solos; http://www.portalbrasil.net/brasil_solo.htm

of sustainable agriculture and livestock farming. This large biodiversity and variety of biomes throughout the national territory harbors a multitude of distinct ecosystems, which are present under particular environmental conditions and are supported by different types of soil.

The biomes found in Brazil are the Amazon, the Caatinga, the Cerrado, the Pantanal, the Atlantic forest, and the Pampa (Fig. 1a). The Amazon is the largest rainforest in the world, occupying an area of 5,500,000 km^2, shared between nine countries (of which Brazil hosts the largest part). This biome is under threat of constant deforestation, mainly caused by the illegal exploitation of this area for the timber industry and the expansion of agricultural frontiers [1].

Caatinga is the biome only occuring in Brazil, comprising a total area of 850,000 km^2 (approximately 10% of the country) [2], distributed throughout the semi-arid region of northeastern of Brazil, and spanning several Brazilian states [3]. This biome has had its area diminished in recent years, mainly due to a desertification process [1], which makes the need for research on the microbiota associated with such an environment unquestionable, especially research that focuses on soil microorganisms that survive under high water stress conditions, high temperatures, and high levels of solar radiation.

The Cerrado occupies an area of approximately 2,050,000 km^2 (distributed among eight Brazilian states), and is considered, together with the Caatinga, a tropical savannah biome . With the extensive agricultural expansion in recent decades, mainly caused by intensive farming practices, the Cerrado biome has been constantly modified for agricultural use. This biome was not considered arable until the 1960s; however, since this period, there has been a steady increase in the use of this area for national agricultural production, making the savannah the great Brazilian

green belt at present [4]. This revolution in use was made possible by the adoption of new management practices, among which the practice of adding gypsum stands out. The addition of gypsum reduces the amount of aluminum, a naturally occurring mineral in the soils of this region. Along with this practice, the use of liming, which corrects the soil pH, was also adopted, as well as the use of tillage, which promotes increases in the amount of organic matter [5, 6].

The Pantanal, a biome adjacent to the Cerrado, is characterized by the flooding of large parts of the land at certain times of the year. This biome has an area of about 250,000 km^2, distributed between Brazil, Paraguay, and Bolivia [7, 8]. Variations in water levels in this region, caused by periods of flood and ebb, characterize this environment, which is still underexplored in relation to microbial diversity and function, and untouched with regard to the exploitation of its natural resources. The most prominent human activity in the Pantanal area is extensive livestock farming [9].

The Atlantic forest is the biome that hosts the greatest diversity of animals and plants. Thus, studies of the structure and function of this biome are particularly relevant, considering that the remaining areas of native vegetation are embedded in a matrix that has been greatly altered by human action [1, 10]. The great biological diversity present in the soil of this biome is caused by, among other factors, the north-south distribution of this forest and the existence of considerable geological and altitude differences. Also, the great changes that the region has undergone as a result of intense climate changes that have occurred in different geological periods also play a role in the area's soil biological diversity [11]. Within the Atlantic forest biome there are mangrove ecosystems, similar to those distributed worldwide, covering about 60–75% of the tropical and subtropical coastline. The importance of these ecosystem lie in their high biological productivity, with a great diversity of fish, crustaceans, molluscs, birds, reptiles, and mammals [12, 13].

The Pampa is a prairie biome, located in southern Brazil, Uruguay, and Argentina (which houses the largest area). This biome has unique characteristics because of its location in a temperate region. The area of the Pampa is 750,000 km^2, and approximately 15% of the area is located in Brazilian national territory [1, 14]. It is also worth mentioning that one of the most widely explored biomes in Brazil is of anthropogenic origin, and occurs over most different soil types that are found in the other biomes. The agricultural biome, which is present in a fractional and differentiated manner throughout the country, currently occupies about 70 million hectares, which corresponds to approximately 8.2% of the country. This biome originated as a result of changes in physical, chemical, and biological soil properties, and its inclusion is very important in approaches that seek to understand the functioning and the characteristics of Brazilian soils. Therefore, knowing the factors that modulate microbial diversity in the agricultural biome and their influence on plant development constitutes an important strategy to bring agricultural production to a high level of sustainability.

The predominant class of soils in Brazil is the oxisols, which are widely distributed throughout the country, and upon which many of the Brazilian biomes are developed (Fig. 1a). This soil type is extremely abundant in the Central West

region of Brazil and constitutes the savannah areas, where most Brazilian agricultural production is concentrated (Fig. 1b). These soils are highly weathered, with low cation exchange capacity; they are dystrophic, acidic, and well drained and mainly composed of mineral type 1:1 (kaolinite, for example) and iron and aluminum oxides and hydroxides, and therefore show low natural fertility [15]. They have physical properties that favor good soil structure, such as microaggregation, which facilitates water percolation and retention, making the soil crispy when wet, and allowing the penetration of crop roots. Therefore, these soils are highly responsive to management focused on mechanization and increasing their fertility. As well as latosols, large areas of Brazil are covered by soils classified as Arcgis loamy soils (Fig. 1b), whose main characteristic is the presence of a diagnostic textural B horizon, arising from subsurface clay accumulation, with variable depth and drainage [16].

The relationship between the type of soil and the ecosystem it maintains can be easily seen on some occasions. The hydromorphic cambisols, fluvisols, and gleysols are typical soils in the Pantanal biome, where water fluctuation and poor drainage result in a system highly subject to periods of anaerobiosis and the accumulation of silt and grayish sediments [17, 18]. Similar characteristics are also observed for histosols, which are predominantly formed in floodplains and coastlines under river flood, such as in mangroves (an ecosystem of the Atlantic forest biome) [19, 20]. Because these soils are usually rich in organic matter and are anaerobic, they have a greater potential for occupation, and, thus, lower plant diversity.

As well as the soils, another very important environmental factor in Brazil, in regard to biomass distribution, is the climate, which is determined by the average temperature and precipitation regime of the region. In most of the country, which is dominated by tropical and subtropical regions with high temperatures, high moisture levels, and good soil drainage, the process of weathering is favored [17, 18]. In the Northeast region, this process is slower because of water scarcity, leading to the predominant formation of slightly weathered soils such as neossols, or soils with a clay mineral ratio of 2:1 (smectite, for example), such as vertisols [17, 18, 21] . The formation of clay 2:1 also occurs in temperate regions, specifically in the south of Brazil, where the formation of montmorillonite predominates, along with the slow decomposition of organic material, leading to soils with high CEC (capacity to exchange cations), such as chernossols, luvisols, and cambisols [17, 18, 21].

Soil Biology

The organisms that inhabit the soil form an essential part of the system and they have very important functions, which are even more essential than previously thought. Among the functions performed by soil organisms are those that are widely known, such as the degradation of organic compounds and nutrient cycling [22–24], and those that are more specific, such as biological nitrogen fixation [25, 26], and assistance in plant nutrient uptake [23, 27]. However, before a more specific

discussion of these functions is undertaken, it is necessary to describe the groups of organisms that encompass the live fraction of soil, because they are extremely diverse [28], ranging from prokaryotic organisms, such as bacteria and archaea (which represent two of the three domains of life), to eukaryotic organisms, where fungi are important. Insects, nematodes, protozoa, algae, oligochaetes (worms), and even viruses are also present, and these play a still largely unexplored role in this environment [28].

The different classes of organisms are sometimes studied separately and are selectively named soil fauna (higher organisms), and soil microfauna (smaller organisms) [22]. Among the functions assigned to components of the soil fauna the initial degradation of organic compounds (development and grinding) and their role in soil structuring stand out [22]. Soil fauna are also used as parameters of soil quality, depending on their presence or abundance [29, 30]. The attributed functions of the soil microfauna are much more numerous, mainly because of the higher metabolic diversity found in bacteria, archaea, and fungi compared with that in other soil organisms. This higher diversity of the soil microfauna is directly related to their genetic variability, which arises from their origin and evolution, making them an essential component of soil system metabolism. This essentiality is shown by the functions performed exclusively by microorganisms and their numerical dominance over other soil organisms [22]. Therefore, for the complete understanding of the soil system, study of the organization and functioning of these communities is very important.

In general terms, two microbial groups are the greatest examples of how microorganisms can benefit plant development: those related to biological nitrogen fixation and those able to form mycorrhizae [25, 27, 31]. These interactions have been widely studied, and many details of these types of symbiosis are described in the literature. However, as the microbial diversity in soils is huge, many other processes may be essential in maintaining the soil system, influencing the development of plants. Therefore, the great challenge is to describe and manipulate these processes, thus obtaining higher energy efficiency in crop production. The idea of vast microbial diversity is still recent, as this diversity was only elucidated with the use of culture-independent methods. Thus, new technologies have allowed us to access and understand more deeply the biological complexity of the soil system.

The Microbiome

The term ‘microbiome’ was used for the first time by Joshua Lederberg [32], who, in referring to the human microbiome, defined it as “an ecological community of comensalists, symbionts or pathogenic microorganisms, which literally occupy the space in our body.” In 2002, this definition was simplified as “microorganisms associated with humans” [33–35]. Nowadays it is known that the human microbiome consists of a 1:1 ratio of microbial to human cells [36, 37]. In regard to the number of genes, this proportion is even higher, with one human gene for 100 microbial

genes. This huge diversity of organisms and functions has been referred to as being a living organ, which we depend on to perform several vital functions, such as the regulation of certain physiological processes, aiding in digestion and nutrient absorption and resistance to pathogens, among others [35, 38]. Some examples of the functionality of this "microbial body" have revealed that 36% of the molecules found in our blood are produced by microorganisms associated with our gut [39]. Another study showed the phenotypic response of mice inoculated with microbiomes derived from either obese or lean individuals, where it was observed that, in the recipients, the phenotype of the donor organism replaced their own microorganism phenotype [40].

Currently the term 'microbiome' is used to describe the set of microorganisms that live in a particular host, or which jointly occupy an environment [41, 42]. Boon et al. [41] propose that the best definition of microbiome would be related to the set of genes found in association with organisms colonizing a particular environment. This definition would be structured in order to eliminate variations that occur when only taxonomic inferences are used to characterize microbiomes. Taxonomic information is the most commonly used source of information in this kind of study; however, it is known that complex microbial communities have high rates of transfer of genetic material, resulting in ecological and metabolic functions being performed by distinct organisms (i.e., metabolic redundancy), making the taxonomic description dependent on the functional depiction. Therefore, Boon et al. suggest that the best way to describe a microbiome is based on a robust description of the genes comprising it, as well as being based on a description of the functions that can be performed by the microbiota associated with a particular host or environment.

Within this broader scope and in contrast with the examples of the human microbiome, we study the soil microbiome, which is extremely challenging, mainly because of the heterogeneity of soil, which leads to a great diversity of life forms. A better understanding of the soil microbiome is essential for and potentially constitutes the foundation of future revolutions in agriculture and land use. An example of this potential is the allocation of the suppressive characteristics of soils to plant pathogens in their respective microbiomes [43], with the microorganisms being the agents that inhibit the occurrence of plant diseases even in the presence of pathogens [44]. Despite the enormous progress in access to microbiological information, made through technological innovations, no method is robust enough to allow the study of the complete soil microbiome [45]. Therefore new methodologies are necessary to elucidate the changes that occur in soil systems on a temporal scale; however, the development of new methods is still limited by the costs of analysis and the desired sampling coverage.

Soils present similar microbial community structures when analyzed at a high taxonomic level [46–48], meaning that a core microbiome is observed in most soils. The core bacterial community of soils mainly consists of the phyla Acidobacteria, Actinobacteria, Proteobacteria, Verrucomicrobia, Bacteroidetes, Firmicutes, and Planctomycetes [47]. The composition is relatively stable within the taxonomic concept of the microbial community, but differences can be distinguished by the functions performed by members of the community. These differences result from

the high rate of gene transfer in complex environments, as described by Dini-Andreote et al. [49]. In their review, these authors propose that the genomic organization of bacteria is the result of their interaction with the environment. Therefore, organisms that are taxonomically different may have different functions, according to the environment they come from. The concept of the microbiome should thus be better applied to soil, as proposed by the initiative called Terragenoma (http://www.terragenome.org/) [45], which aims to organize information on the soil microbiome, generating a complete description of the microbial genetic material present in one gram of soil. Based on this initiave, we expect to gain a better understanding of the microbial interactions governing the soil ecosystem.

This type of study is particularly necessary for Brazilian soils, because the soils and their microbiomes sustain the biodiversity of the biomes and the biodiversity of agricultural areas with high productivity and economic importance. Yet it is possible to extend the concept of the microbiome, considering it not only as a group of organisms present in a distinct area, but also as a group of organisms associated with different soils where the same crop is grown, or associated with areas that show the same landscape, thus, the concept of biogeography can be added to the definition of the microbiome [50–52]. The Brazilian Microbiome Project (http://www.brmicrobiome.org/) aims to describe the microbiomes associated with several Brazilian environments. This group has published its first paper, which presents a detailed review of the studies carried out with this aim in many different Brazilian environments [53], in which the soil system is highlighted and explored in different areas of the national territory. The members of this initiative work in collaboration with a global initiative called Earth Microbiome (http://www.earthmicrobiome.org/), and this should facilitate the integration of data on Brazilian biomes in a global scenario. The Brazilian biomes may then be compared with other environments, supporting the comparison and elucidation of the high biodiversity in the Brazilian biomes and the high biodiversity of their microbial communities.

Soil Microbial Diversity in Brazilian Biomes

The living fraction of soils is now seen as essential, being responsible for many processes that govern the maintenance and functionality of soils. However, similar functions in different soils can be performed either by the same group or by different organisms, leading to the need for understanding the composition and the metabolic functioning of the soil microbiomes that support the Brazilian biomes. Considering the natural areas, we still know very little about the microbiology of the main Brazilian biomes, mainly because of the extent of the country; this creates the need for large sampling efforts, which are sometimes limited by restricted access to remote areas. Few studies have accessed the microorganisms present in the Caatinga. One such study was conducted by Gorlach-Lira and Coutinho [54], who investigated the population dynamics of bacteria present in the rhizosphere of *Aristida adscensionis* (Poaceae). These authors observed the prevalence of heterotrophic

mesophilic spore-forming bacteria and actinomycetes in this environment, suggesting the development of special microbial adaptations to environmental conditions in a similar way to that observed for plants and animals. More recently, Kavamura et al. [55] reported the prevalence of the rhizosphere effect in savannah plants, such as mandacaru, in the rainy season, suggesting that certain microbial groups change according to variations in the life cycles of plants in these regions.

Within the Amazon biome, the most studied topic is the effect of deforestation on the diversity and structure of the soil microbial communities and associated plants. In this regard, a recent study showed homogenization of the microbiota in forest areas converted to pasture, indicating that the removal of forest decreases the beta diversity[1] of this ecosystem [56]. This effect occurs possibly because of the physical disruption of the soil, resulting in a greater exposure of nutrients and consequently more niches to be occupied by the microflora. Studies of soil microbiology are still scarce in the Atlantic forest biome. Santos et al. [10] demonstrated high spatial variability in the composition of microbial communities within the same sample area. In this biome, a description of the bacterial communities of plant phyllospheres revealed the hitherto unknown vast microbial diversity that occurs in a specific system depending on the plant species inhabiting this system [57]. Diverse ecosystems can be found within the Atlantic forest, in which there are mangroves, an ecosystem that links terrestrial and marine environments. Mangrove microbial communities have been widely described, revealing their taxonomy [58–61] and functionality [62–65]. Several of these studies indicate the occurrence of genes and organisms that are possibly endemic, i.e., unique to a defined geographic region, which may result from a particular combination of selection factors that occurs in this environment, characterizing an ecotone.

In agricultural biomes, the main focus is studying the effects of changes in land use on the microbial communities and the possibilities of using these communities to increase agricultural productivity. Several studies have used areas of agricultural expansion as a model of land use changes [66, 67]. One of these studies accessed the soil bacterial community in natural areas of the Pampa, and compared it with the community found in the same soil under different types of land use [68]. The authors found that changes in the land use had led to changes in the taxonomy, but not the functionality of the soils. Rodrigues et al. [56] revealed the homogenization of the soil microflora when land use was converted from a native to a pasture area. Mendes et al. [69] reported a deterministic effect of the soy rhizosphere on the microbial community in soils in the Amazon. These studies suggest that plant cultivation leads to the selection of certain microbial groups, thus explaining soil homogenization and the consequent reduction of beta diversity (a characteristic of the natural biomass) in agricultural areas.

[1] Beta diversity: diversity between distinct locations, revealing spatial or temporal heterogeneity in the structuring of communities.

Accessing Microbial Diversity: Culture-Independent Methods

The diversity of life forms in soil is quite wide, and is governed by the great heterogeneity of this environment. Although a soil may appear homogeneous, this environment is composed of a wide variety of niches, with each one of them consisting of a combination of different environmental factors, making soil a highly heterogeneous environment for microorganisms. In addition to this spatial heterogeneity there is also temporal heterogeneity, such as fluctuations in temperature, which occur in Cerrado soils during the day, and in Pampa soil throughout the year. Fluctuations in temperature result in alterations in the soil atmosphere and the pH thereof, directly influencing the soil microbial communities [70]. In soils we have perfect environmental conditions, so that, in the long run, a huge diversity of life forms is maintained, and fractions of this total diversity reap benefits for every millimeter and every minute in the soil in which they are found.

Considering the huge diversity of organisms and considering that the adaptation of different organisms takes place under different conditions, it seems obvious that only a minority can easily be cultured in laboratory conditions [71, 72]. In a culture plate, the nutritional and physical conditions are constant and homogeneous, so we can easily understand why we cannot represent soil microbial communities with colonies obtained in culture media [72]. Recent studies focused on descriptions of soil bacterial groups that are difficult to culture have revealed the evolutionary strategy of these organisms, such as their compact genome organization, which leads to higher efficiency of cellular multiplication, which is, however, connected to a high dependence on interaction with other organisms to complete their life cycle [49, 73]. Thus, the proper understanding of soil microbial communities is very difficult to achieve with the use of culture-dependent methods only, mainly because of the distinct environmental and nutritional conditions required by the different organisms, and because of our anthropic view of obtaining the components of microbial communities in soil in an isolated manner.

Following this line of thought, the application of so-called culture-independent techniques, based on the detection and analysis of the diversity of nucleic acids (i.e., DNA or RNA) in environmental samples, is essential for studying the microbial diversity of soil, allowing a more accurate analysis of the structure of these communities [74, 75]. Among these methods, there are some single-gene analyses (based on the amplification of the target gene by polymerase chain reaction; PCR), and analyses that comprise all the genes together (metagenomics and metatranscriptomics). These analyses are now highly automated, facilitated by the evolution in methodologies and reductions in DNA sequencing costs. This has made it possible to work with a great number of samples, accessing enormous numbers of individual organisms in each sample, providing great robustness to the inferences made. These analyses are essential in ecology because they allow the sampling of a huge number of individuals within communities that consist of a large number of taxonomic groups, thus generating the necessary ecological coverage to infer the composition and the responses of these communities under different environmental conditions.

A pioneering example of such analysis has enumerated differences in the compositions of soil microbiomes in different countries by analyzing a large number of 16S rRNA gene sequences [76].

Most studies based on a single gene refer to the taxonomy of microbial groups. This is achieved by analyzing sequences of the ribosomal operons (the 16S rRNA gene for bacteria and archaea and the 18S rRNA gene and the internal transcribed spacer regions for fungi) [77, 78]. The amplification of these genes from DNA or cDNA (translated from RNA) obtained from soil samples supports further analysis, generating information on the structure of the target microbial communities (fingerprinting methods) and the abundance (quantification) or taxonomic composition of organisms present in these communities (sequencing methods) [75]. However, to obtain information about the role of microbial groups in soils, other genes have been used in molecular microbiology studies, especially those genes related to specific steps within biogeochemical cycles. Among these genes, the most widely used are those related to nitrogen cycling (nifH–biological nitrogen fixation; amoA–nitrification; nirK, nirS, and nosZ–denitrification), sulfur (dsrB–sulfate reduction, aprA–reduction and oxidation of sulfur), or carbon (mcrA–methanogenesis, pmoA–methanotrophy) [79–83]. Other functions can also be studied. The only limiting factor is determination of the relationship between gene presence and the desired phenotype in organisms that host the DNA sequence in the environment.

Considering broader analyses, we should first think about metagenomics, which constitute a great alternative for describing the microbial diversity of soils, providing taxonomic and functional information about the community in a single analysis. The term "metagenome" was coined in 1998 to represent the complete genomes of microbes found in a community [84]. The metagenomic strategy offers an alternative for exploiting the metabolic potential of microorganisms that are not recovered by culture-dependent methods. The strategy initially consisted of cloning large DNA fragments (40–100 kb), obtained from environmental samples, in bacterial artificial chromosome vectors or cosmids, followed by analysis of the resulting libraries and a search for new phenotypic expression in *Escherichia coli* host strains [84]. However, today, with high-throughput sequencing technologies, it is possible to gain broad genetic information from soil samples, excluding the cloning step. These technologies are quite interesting for their ability to describe, in a representative manner, the functional and taxonomic genes jointly, in a single analysis, allowing better inferences to be made about the relationship between the structure and function of soil organisms.

In the first study using metagenomics, the authors were able to reconstruct bacterial genomes by directly sequencing the DNA extracted from samples of an acid mine environment, where only a few microbial groups comprised the microbiome [85]. In another example, the phylogenetic and functional diversity of the microbial community in glacial ice cores was described [86], and results showed part of the microbial metabolism in this environment, highlighting the presence of genes adaptive to *Pseudomonas psicrophylia*. This type of analysis has been widely used in soils, with one of the first studies carried out to elucidate the microbiota and its features and biotechnological potential, based on the sequenc-

ing of DNA obtained directly from the soil [87]. More recently, this type of analysis has been employed to describe novel enzymes and to identify the response of the soil microbiota to contamination events [87, 88]. Regarding the Brazilian biomes, this approach was used to analyze the microbiome of mangroves [63], where key organisms were identified and the main metabolic changes involved in the nitrogen, carbon, and sulfur cycles were described. Other Brazilian biomes have been explored using this approach, and results are summarized in a recently published article by Pylro et al. [53].

It is worth mentioning the varying numbers of DNA sequences obtained in metagenomic analyses that have low or no similarity to those found in databases. This finding demonstrates the potential of these analyses to describe new genes or new genomic arrangements, distinct from those already found in the literature. The non-afiliated sequences were initially treated as less important; however they have recently attracted considerable attention, as a source of possible new functions or taxonomical groups represented by these molecules [89]. In a similar vein, there is the possibility of sequencing the functional part of the microbiome using RNA molecules as a template, in an approach called metatranscriptomics. In this context, metatranscriptomics appears to be a powerful approach for determining patterns of gene expression in microbial communities [90]. In contrast to metagenomics, which provides an analysis of the genetic structure of the community, metatranscriptomics identifies which of these genes are being actively transcribed in the studied environment [91, 92]. Analyzing samples of marine microbial communities, Gilbert et al. [91] described the high efficiency of this methodology, highlighting the possibility of detecting genes belonging to many families that have never been previously described using DNA-based analyses. Some soil studies have used this methodology to describe eukaryotic genes expressed under various conditions, such as forest soils [93], or to determine genes related to heavy metal resistance [94].

The initial focus on eukaryotes arose from the method of separation of mRNA from the total RNA. Because the vast majority of the obtained RNA is of ribosomal origin, more efficient separation is obtained by purification in polyT columns, where the mRNA, which has a poly(A) tail, is retained. However, this process identifies only a fraction of eukaryotic communities. Access to bacteria and archaea transcripts is done through sequencing of the total RNA, or by the separation of mRNA using hybridization probes to remove rRNA, as described by He et al. [95]. There is still the possibility of sequencing the entire extracted RNA, thus using the sequences of ribosomal genes for a taxonomic analysis of the groups with active metabolism, whereas mRNA sequences, even though lower in number, are used to analyze active functions in the sample. This sequencing of the entire extracted RNA was done in one of the first metatranscriptomic studies in soils, where a simultaneous analysis of the taxonomy and microbiome functionality of soils was conducted in a conservation area in Germany [96]. A recent review lists the studies performed using this technique, and discusses the variables present in metatranscriptomic studies in soils [97]. Metatranscriptomics represents a tool with great potential for the description of the microbial activity in different Brazilian soils, leading to the

description of active groups in different environments and under different conservational conditions and land use.

It is important to mention that there is no perfect methodology that provides a complete set of information on soil microbial communities, although there are appropriate approaches to address the different questions generated when studying soil microbiomes. In a comparative analysis, it is possible to see the advantage of methodologies based on PCR, which allow a better description of the target group (better coverage, for instance), while the "omics" techniques generate more complete data on taxonomy and microbial functions, although the coverage of the community is smaller (usually representing the most abundant groups). We do need to mention the costs of data collection and analysis, which require specific skills, and in most cases, extensive computational resources related to processing capability and analysis time.

Future Perspectives and Final Considerations

The luxuriance of Brazilian macrobiodiversity is supported by its microbial biodiversity, which acts in the cycling of chemical elements and sustains plant (and therefore animal) growth in the different Brazilian biomes. Determining the organisms responsible for these processes is itself a challenge, and the use of this ubiquitous natural resource still sounds utopian in the scenario of a highly competitive global agricultural market. The results presented in this line of research, where the organisms constituting the respective microbiomes, as well as their function and structure, are described, are of great importance. These results can be achieved either by studying the genes related to specific metabolism or by more complete analyses, or by sequencing the genetic material obtained directly from the soil. However, these results have quite an exploratory character, with the main study objectives being the description of microbial groups present in the different soils, or the existence of colonization patterns in the explored areas. A more technological view of the processes can be glimpsed when the taxonomic and functional components of the soils of the studied environment are compared under different states of conservation, or under different contamination conditions. One possibility, perhaps still utopian, would be agricultural management that promotes the development and growth of beneficial microbial groups or microbial groups with functions related to improvements in plant development.

However, despite all the advances made in the study of microbial communities, there are still some limitations and challenges to be overcome. One of these issues is the relationship between taxonomy and microbial functionality, which assumes great importance in soil environments, because of the high functional redundancy in soil microbial communities, and also because of the high genomic plasticity of some organisms. The elucidation of these relationships, perhaps, and the biggest challenge in microbial ecology studies, is the determination of the intrinsic characteristics of the soil microorganisms that constitute these communities. Both the genomic organi-

zation of these organisms and the rules that regulate their metabolism are sometimes seen by researchers as being similar to those observed for animals and plants. However, from an evolutionary point of view, these organisms have very distinct structures. More clearly, this difficulty in regard to research views of microbial characteristics arises from a lack of understanding of the occurrence and behavior of these microbial organisms when more detailed levels of taxonomy, such as genera and "species", are considered, which are not always constant, as they are in other groups of organisms. It is known, for instance, that microbial organisms allocated within the same species may have very different metabolic characteristics depending on the environment from which they were obtained. This observation justifies the use of the word "species" in quotation marks throughout this document in order to clarify the vision of the present authors about this concept applied to microbial ecology. Thus, it is extremely important to consider the "gene flow" that exists between the components of complex microbial communities, which will surely lead studies of microbial ecology to the next level in the coming years.

Applying this concept to studies of soil biology creates a huge possibility for new studies, which will be highly challenging but equally promising in generating innovative results on the structure of microbial communities and microbial genomes present in the soil. However, all these studies depend on our ability to efficiently access the genetic information contained in the microbial cells. At this point, despite the advances in sequencing methodologies, it is extremely important to return to the starting point of the revolution in microbial ecology, and to again use cultured organisms as models for the study of evolution and genomic modulation. Methodologies are developing rapidly, creating a scenario of "everything is possible", giving support to great progress in scientific advances, as well as enhancing the ability of researchers to become creative and ask the appropriate questions of their data sets. We believe the intense scientific development in the study of environmental microbial communities, including those present in soil, is extremely worthy. It is also worth noting that both in situations of technological limitations and in contrasting situations, science makes its greatest steps through innovative ideas and holistic views of processes, characteristics which are shown only by the human brain.

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Microbiomes Associated with Animals: Implications for Livestock and Animal Production

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Abstract The Brazilian fauna is very diverse and domestic ruminants (cattle, sheep, goats, and buffalos) are particularly important to Brazil's economy. Ruminants have developed a symbiotic relationship with anaerobic microorganisms, being able to convert fibrous plant materials into food products useful for human consumption, such as meat and milk. Analysis of the animal gut microbiome using next-generation sequencing studies suggests that the diversity and composition of the microbial communities co-diversified with their hosts, being influenced by diet composition, host genetics, geographical location, and environmental factors. Here we present an overview of the microbiome studies performed in the ruminant livestock of Brazil and discuss how the symbiotic relationship between ruminants and their microbes can affect the host productivity.

Introduction

Brazil is considered to be one of the most biodiverse countries in the world, with most of its animal species mainly distributed in six distinct biomes (Amazon, Pantanal, Cerrado, Caatinga, Atlantic forest, and the Pampa) and in the coastal and marine zone, with Brazil having one of the longest coastlines in the world [1]. Previous reports indicate that Brazil's fauna is quite diverse, with more than 104,000 species of animals and approximately 700 species of mammals [2].

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V. Pylro, L. Roesch (eds.), *The Brazilian Microbiome*,
DOI 10.1007/978-3-319-59997-7_4

Among these, the domestic ruminants (cattle, sheep, goats, and buffalos) are particularly important to Brazil's economy. In 2014, the number of head of cattle was 212.34 million, followed by sheep (17.61 million), goats (8.85 million), and buffalos (1.31 million) [3].

Ruminants have developed a symbiotic relationship with microorganisms to utilize a range of abundant lignocellulosic substrates. The rumen has ideal conditions for the growth of the anaerobic microbiota involved in polymer degradation and pre-gastric fermentation, and several species of ruminants have been domesticated and used commercially to convert fibrous plant materials into food products useful for human consumption (e.g., meat and milk).

Brazil has the largest commercial cattle herd in the world and approximately 80% of Brazilian beef cattle are zebuine breeds (*Bos taurus indicus*), with the Nelore breed representing 90% of the animals distributed in different regions of the country [4]. Cattle raised for beef production in Brazil are kept predominantly on pasture and consume grasses for the greater part of their life cycle. Because of their economic relevance, many efforts have been made to improve the management practices, health, nutrition, and the genetic selection of these animals to promote productivity [5, 6]. However, recent studies have demonstrated that the ruminant microbiome can also influence several aspects of the animal physiology that affect productivity, including the health and efficiency of feed utilization of the host [7–9]. Here we present an overview of the microbiome studies performed in ruminant livestock in Brazil and an exploration of some aspects of the symbiotic relationship between ruminants and their microbes, highlighting: (1) the main forces driving microbial diversity in the animal gut, (2) examples of advances in the field, and (3) areas where microbiome research is needed in production animals in the tropics.

Drivers of Microbial Diversity in the Animal Gut

The intestinal tract of animals is a specialized tube that differentiates into anatomically defined regions adapted for the utilization of the ingested foods [10]. These regions represent a nutrient-rich environment often colonized with a highly dense (~ 10^{13} bacteria) and diverse community of symbiotic microbes. Previous estimates of the number of microbes in the human microbiome were that the number was at least ten times higher than the number of cells in the human body, which immediately implied an important role of these microbes in human physiology. However, revised estimates of these calculations recently showed that the ratio of bacteria to human cells in the adult body is approximately 1.3:1 [11]. The diversity and abundance of gut microorganisms also vary considerably with host development and anatomical location, mainly because of varying physical-chemical conditions (e.g., pH, redox potential, O_2), the availability of nutrients and sites for adhesion, host secretions (mucins), and exposure to exogenous compounds that cause disturbance in the ecosystem (e.g., antibiotics, diet changes, pathogens) [12–14].

The intestinal microbiota is considered to be the most complex of the human biotas, with more than 1000 species identified to date [15]. In other mammals, microbes can colonize the foregut (in animals that developed fermentation in a segment that precedes the gastric stomach), midgut (small intestine), and hindgut (large intestine). The term "hologenome" was introduced to describe the sum of the genetic information of the host and its diverse symbiotic microorganisms (including bacteria, fungi, Archaea, protozoa, and viruses). The "holobiont-hologenome" theory of evolution postulated that a host and its microbiome represented a "superorganism" that evolved as a single cooperative unit, driving the evolution of animals and plants [16, 17]. However, this concept has been challenged, based on the idea that there are multiple levels of selection, and that a broad range of ecological relationships often exists between microbes and their hosts; also it is unlikely that high partner fidelity associations will occur between a host and its entire microbiome [18]. Therefore, alternative terminologies such as "symbiome" and "symgenome" have been proposed to define the host-microbiome community and their combined genomes, respectively [18].

Analyses of the animal microbiome suggest that the diversity and composition of the microbial communities in the gut co-diversified with their hosts, being particularly influenced by host diet and phylogeny [19, 20]. Mammalian species can be grouped according to their eating habits as carnivores, herbivores, and omnivores, and experiments measuring stable isotope ratios of carbon and nitrogen demonstrated that these isotopes were enriched more effectively in carnivores and less so in omnivores, with the lowest ratios of $^{13}C/^{12}C$ and $^{15}N/^{14}N$ found in herbivores [19]. Additionally, phylogenetic analysis based on 16S rRNA gene sequences indicated that herbivores contained the largest number of bacterial phyla in their microbiota, followed by omnivores and carnivores, with the latter having the least diverse microbiota [19].

There are many possible explanations for the diversity observed in the animal gut. Although mammals vary in their feeding strategies and digestive physiology, the composition of the feeds ingested is complex, containing carbohydrates, proteins, lipids, nucleic acids, vitamins, minerals, non-nutritional factors, and several organic compounds. Substrate affinities and nutrient use efficiency vary among microbial populations and these traits can influence the competition for niche occupation based on the accessibility and concentration of the available resources (nutrients) [21–23]. Competition for the available resources is a key factor determining the establishment and maintenance of species distribution and biodiversity in complex microbial communities such as those in the animal gut. Some species are considered to be niche specialists and are highly adapted to metabolize a few of the substrates presented in the diet to produce energy and biomass, while some species are generalists, being able of obtaining the free energy (ΔG) and the carbon atoms needed for growth from a wide range of substrates [24–27]. Beneficial ecological relationships between autochthonous members of the microbiota are ubiquitous in stable microbial communities and the environmental conditions in complex ecosystems such as the gastrointestinal tract (GIT) select microbial mutualisms and cooperative

phenotypes that play a very important role in the symbiotic relationships between the microbes and their animal host [10, 28].

Mammals usually do not produce the enzymes needed for the breakdown of complex substrates (e.g., structural polysaccharides, starch, mucins), and so several symbiotic microbial species coexisting in the gut act synergistically and synthesize degradative enzymes that allow the utilization of these polymers as a source of energy by the host [29, 30]. The complexity of dietary substrates and the metabolic cross-feeding of partially oxidized substrates and fermentation products in these symbiotic associations provide great niche differentiation, which selects the individuals most fitted for occupying an empty niche and for converting foods into microbial cells [27]. In the anaerobic microbial communities of the animal gut, fermentation is the prevalent pathway to harvesting the catabolic free energy in the feed. The thermodynamic efficiency of microbial growth is influenced by both the source of electron donors and electron acceptors, and the catabolic free energy can be correlated with carbon chain length and the degree of reduction of the electron donors [31]. The extent to which feed components are utilized by the microbial community and the host depends, among other factors, on the surface area, digestion kinetics, and the passage rate of the feed through the GIT [32, 33]. Organisms showing higher growth rates under these conditions have an ecological advantage in niche colonization and become permanent residents. Microbial species that cannot maintain their growth with the flow of digesta (solids or liquids) are usually removed from the system and replaced by more adapted organisms.

The spectrum and the levels of interaction between microbial species living in a community are broad and many negative or antagonistic ecological relationships exist between organisms that occupy a specific environment. These interactions vary from direct growth inhibition mediated by chemical warfare among individuals coexisting in the same environment and to cell lysis caused by hydrolytic enzymes, bacteriophages, or predators [34–36]. Although the extent to which these relationships affect microbial community structure in the animal gut has not yet been studied systematically, several reports indicate that these interactions play a major role in the microbial diversity of several ecosystems. For example, predator-prey interactions have been documented between protozoa/bacteria, viruses/bacteria, and bacteria/bacteria [37–39]. Obligate predators such as *Bdellovibrio*, *Bacteriovorax*, and *Vampirovibrio* can influence the abundance and distribution of their prey species, potentially shaping community structure and diversity [38, 40]. Bacteriophages are well known for promoting horizontal gene transfer between bacterial cells, but computer models and simulation experiments have indicated that phage infection can decrease the rate of bacterial speciation and affect the evolution of microbial communities [36]. In addition, several intrinsic mechanisms promote genetic heterogeneity in the gut microbiome, and this affects microbial diversity, including adaptive mutations, horizontal gene transfer, non-homologous recombination, and clustered regularly interspaced short palindromic repeats-mediated immunity [41–43].

These observations support the idea that microbial diversity in the GIT is determined by selection forces operating at the microbial level (bottom-up) and at the

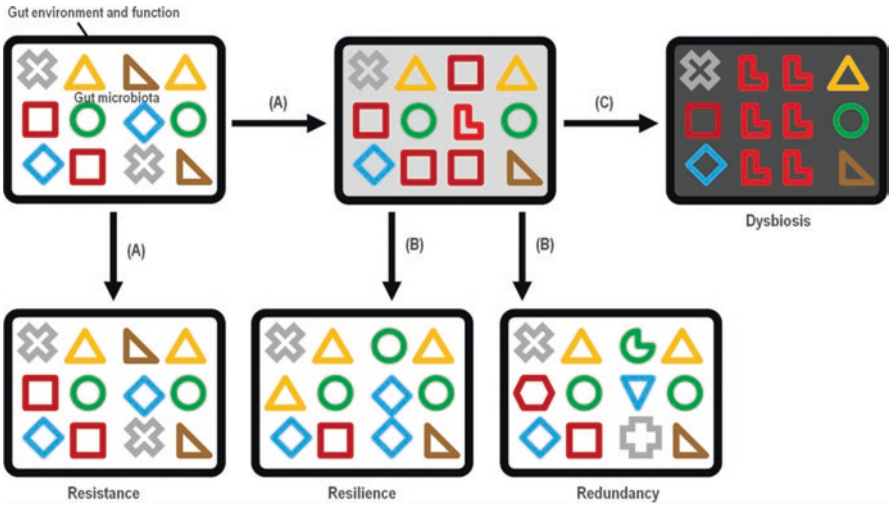

Fig. 1 Ecological properties of the animal gut microbiome. The gut microbiota may be subjected to several external forces (e.g., diet composition, exposure to antibiotics and additives, changes in pH or redox potential, and the presence of alternative electron acceptors, among others) causing disturbance (*A*) of the community structure and function of the gut ecosystem. If these external forces are mild or if the microbiota is resistant to the perturbation, changes in composition and function of the microbiota may not be observed (resistance). The perturbation can, however, affect the microbial community, changing the gut environment and its function. Depending on the nature, intensity, frequency, and duration of the disturbance, the initial community can re-establish (*B*) with various degrees of diversity and abundance compared with the original microbiota (resilience). Alternatively, multiple microbial species (generalists and specialists) with overlapping physiological capabilities can occupy the perturbed niches and restore the function of the gut ecosystem (redundancy). Dysbiosis may occur if the disturbance causes irreversible changes (*C*) in the taxonomic composition and function of the microbial community, often changing the gut environment and affecting the health of the host

host level (top-down) [44]. Ecological properties of the gut microbiome resulting from these selection forces include functional redundancy, resistance, resilience, and host specificity (Fig. 1) [12, 45]. The gut microbiota continuously responds to a variable environment with potentially stressful conditions imposed by nutrient availability, host secretions, antimicrobials, and the innate and adaptive immune systems. In addition, it has been recognized that unrelated microbial species can express proteins and synthesize enzymes and metabolites that perform similar functions in the community, and this can also affect host physiology [12]. These observations indicate that convergent evolution of the host and its microbes allows the gut ecosystem to maintain a dynamic equilibrium while conferring the characteristic stability of gut function [46].

Microbial Diversity in the Rumen Ecosystem

Colonization and Establishment of the Ruminal Microbial Ecosystem

Ruminants are herbivorous mammals that do not synthesize hydrolytic enzymes required for the degradation of the fibrous components (cellulose, hemicellulose, and lignin) of their diet. However, the establishment of symbiotic relationships with anaerobic microorganisms that can ferment a range of substrates (e.g., soluble and insoluble carbohydrates, proteins, and lipids) allows the host to harvest the energy stored in feed from different sources, especially grasses [47]. When ruminants are fed forage, the ingested feed particles are rapidly colonized [48]. Experiments performed with perennial grass incubated *in sacco* indicated that successive changes in the diversity of the attached bacterial community occurred within 4 h after the feed entered the rumen [49]. After colonizing the feed, ruminal microorganisms degrade the forage cell wall and ferment plant cell components and other dietary constituents into organic acids, ammonia, amino acids, and vitamins that supply energy, nitrogen, and essential nutrients to the host [50].

At birth, ruminants are rapidly colonized by an abundant and diverse microbial community [48]. The first contact with microorganisms occurs during the passage of the calf through the vaginal canal, where it is exposed to pioneer colonizing species, such as members of the genus *Lactobacillus*. The contact of the newborn with other animals (cows naturally lick their calves) and with the bedding materials (contaminated by feces and rotting organic matter), as well as the ingestion of colostrum and milk and physical contact with the cow udder during breast-feeding, contribute to the transfer of the microbial communities that will become the primary colonizers of the newborn animal [48, 51]. Food intake provides a continuous inoculum of microorganisms that can potentially colonize the rumen of young animals, contributing to the establishment of the rumen microbial ecosystem [52]. Initially, the rumen of newborn calves is colonized by a large number of facultative aerobic and anaerobic bacteria; however, the introduction of solid foods in the diet increases the diversity of the microbial community, providing substrates and suitable conditions for the establishment of the anaerobic microorganisms found in the ruminal microbiota of adult animals [47, 53].

Pyrosequencing analysis of 16S rRNA amplicons indicated that the bacterial community in the rumen of 2-day-old calves was composed mainly of members of the phyla Proteobacteria (70%) and Bacteroidetes (14%), while the *Pasteurellaceae* was the most prevalent family (58%) [51]. Changes in bacterial community composition were observed between day 2 and day 3 after birth and the phylum Bacteriodetes represented more than 55% of the ruminal bacterial community at day 12, while the phylum Proteobacteria represented only 17% of the total population.

The early colonization of the rumen by cellulolytic bacteria was demonstrated in 3-day-old calves by studies that were based both on culture-dependent [47, 52] and culture-independent techniques [51, 54, 55]. In calves fed milk and concentrate,

species of *Prevotella* appeared to be the most abundant populations in the rumen after 2 weeks of age [51, 54, 55], and this genus has also been reported as the predominant bacterial group in adult animals [56]. Furthermore, the presence of representative microorganisms belonging to the major functional groups typical of adult animals in the rumen of 42-day-old calves reinforces the idea of the early colonization of the "keystone species" in the rumen of cattle [54]. This core bacterial community is defined as the community comprising the microbial groups shared by all samples. These observations indicate that the rumen is colonized very early after birth by a less diverse and heterogeneous microbial community which gradually become more diverse, specific and homogeneous between different animals in the mature rumen.

Major Groups of Rumen Microbes

The rumen ecosystem contains a dense and genetically complex microbiota, represented by various species of bacteria, protozoa, fungi, archaea, and viruses. Bacteria are the most abundant and diverse group of organisms in the rumen, both in terms of number of species and in metabolic capacity [57]. According to Wu et al. [58], the ruminal microbiota comprises at least 8 phyla, 11 classes, 15 families, and 17 genera of bacteria. Enumeration of rumen bacterial populations showed greater than 10^{10} colony-forming units /ml of rumen contents, with most species being strict anaerobes [59, 60]. The rumen is a stratified environment where microorganisms can be found forming biofilms on feed particles, attached to the rumen wall, or growing on soluble substrates available in the liquid phase [61].

Rumen bacteria can be classified into nutritional groups according to the type of substrate used for fermentation. The species that ferment structural carbohydrates hydrolyze cellulose or hemicelluloses by enzymatic complexes and produce mainly acetate, propionate, butyrate, succinate, formate, CO_2, and H_2 [62]. Some of the cultured species include *Fibrobacter succinogenes*, *Ruminococcus albus*, *Ruminococcus flavefaciens*, and *Butyrivibrio fibrisolvens* [63], but metagenomic analysis of biomass-degrading genes and genomes from the microbiota associated with plant fiber (switchgrass; *Panicum virgatum*) incubated in the cow rumen revealed at least 15 uncultured ruminal microbial genomes involved in plant biomass degradation [64]. Many species that metabolize nonstructural carbohydrates have also been isolated and starch utilization appears to be a common trait among species of the genus *Prevotella* and strains of *Streptococcus bovis*, *Ruminobacter amilophilus*, and *Succinomonas amylolytica* [65].

Proteolytic species, represented by the *Prevotella* genus, hydrolyze the rumen-degradable proteins and produce succinate, acetate, formate, and propionate as the main end products [66, 67]. These bacteria provide peptides and amino acids to obligate amino acid fermenters that often have specific deamination activity that is at least one order of magnitude higher than that of the mixed ruminal bacteria [68, 69]. The "classical" hyper-ammonia-producing bacteria (HAB), represented

by *Peptostreptococcus anaerobius* C, *Clostridium sticklandii* SR, and *Clostridium aminophilum* F were originally identified through dilution series experiments, but culture-independent experiments have indicated that these strains are found in only small numbers in the rumen [70]. More recently, HAB that could also ferment carbohydrates were isolated from Nelore cattle fed tropical forages and supplemented with casein, indicating that the ecological niche for this group of bacteria could be broader than predicted by earlier studies [71]. In addition to the species involved in carbohydrate and nitrogen metabolism in the rumen, several other bacterial groups play a fundamental role in ruminal fermentation, being primarily responsible for the utilization of lactate, pectin, and maltodextrins, among other dietary substrates [72, 73].

Recently, a global rumen census was performed to evaluate the composition of the rumen and foregut microbiota from a range of ruminant and camelid species, diets, and geographical regions [74]. Samples were collected from 742 individual animals from 32 animal species and 35 countries and the patterns of community composition and relative abundance of bacteria, archaea, and protozoa were characterized across hosts and diets [74]. Results indicated that *Prevotella* (22.0%), unclassified *Clostridiales* (15.3%), unclassified *Ruminococcaceae* (7.9%), and unclassified *Lachnospiraceae* (6.3%) were the most abundant and prevalent rumen bacterial groups at the genus level or higher, while the hydrogenotrophic methanogens of the *Methanobrevibacter gottschalkii* clade (46.9%) and *Methanobrevibacter ruminantium* clade (27.1%) were the most abundant archaeal species-level groups [74]. Other studies have also indicated a core rumen microbiome in cattle fed forage and concentrate-based diets [9, 75].

Petri et al. [75] combined quantitative real-time polymerase chain reaction (PCR), PCR-denaturing gradient gel electrophoresis (DGGE), and pyrosequencing analysis to investigate taxa abundance and bacterial community composition in solid and liquid samples obtained from the rumens of eight Angus heifers used to study the impact of an acidotic challenge on rumen function. Dietary treatments (forage, mixed forage, high grain, post-acidotic challenge and recovery) affected the abundance of several bacterial targets, but only the *Fibrobacter succinogenes* population differed between the liquid and solid samples. A rumen core microbiome was described based on comparisons of all solid and liquid samples obtained from all heifers across all dietary treatments. The core microbiome was represented by 32 operational taxonomic units (OTUs) from ten distinct bacterial taxa, including members of the phyla Bacteroidetes (32.8%), Firmicutes (43.2%), and Proteobacteria (14.3%). Although bacterial populations dominate the rumen environment, other groups of microorganisms participate actively in rumen ecology and function.

The protozoa represent 40–60% of the rumen microbial biomass, and rumen ciliates play an active role in the degradation of starch and structural carbohydrates, as well as in the maintenance of ruminal pH (buffering effect) [76]. The genera *Entodinium* and *Epidinium* are the most prevalent in the rumen, occurring in more than 90% of the 592 rumen samples obtained from different ruminant species and representing 54.7% of the protozoal sequence data analyzed by Henderson et al. [74].

Anaerobic fungi found in the rumen are also thought to play important roles in fiber degradation. Most fungal species found in the animal gut belong to the phyla Chytridiomycota and *Neocallimastigomycota* and appear to have a monophyletic origin. Strains of *Neocallimastix*, *Piromyces*, and *Caecomyces* degrade structural carbohydrates and are capable of degrading lignified vascular tissues by the production of extracellular hydrolytic enzymes, but further structural and genetic characterization of these enzymes is needed [77–79]. There is evidence that fungi actively participate in the separation of fibers through the formation of rhizoids during forage colonization [80]. However, there is a lack of information about the interaction of ruminal fungi with other ruminal microbes and their potentially cooperative activities toward fiber degradation.

Bacteriophages occur in large numbers in the ruminal ecosystem and infect specific bacterial groups, but the relevance of the viral population to the rumen ecology has been underexplored. The lysis of ruminal bacteria as a consequence of phage infection appears to be an important factor contributing to the turnover of microbial mass in the rumen [81]. It has been proposed that the specificity of bacteriophages may be exploited to reduce undesirable bacteria, such as *Streptococcus bovis* and methanogenic archaea, in the ruminal ecosystem [82, 83]. However, until now these early expectations have not yet been met. Analysis of ruminal virome diversity in 13 lactating Australian Holstein dairy cattle by deep sequencing revealed that the average number of species per sample was 435,304, with a range varying between 3370 and 4,126,756 species per sample [84]. A large amount of sequence variation was observed for animals housed separately, but the results indicated that the functional characteristics of the rumen virome were conserved between animals [84]. Additional work will be needed to further address the effect of diet, host, and farming practices on the rumen virome.

The Goat and Sheep Rumen Microbiome

In 2014, the population of goats and sheep in Brazil was estimated to be 8.85 million and 16.61 million head, respectively, which represents an increase of 0.8% and 1.9% compared with the population in 2013 [85]. Most of the goat herds (91.6%) are located in the Northeast Region of Brazil, while the sheep population is distributed in the Northeast Region (57.5%) and the South Region of the country (29.3%). Commercial products include meat, milk, and leather, but wool production is also economically relevant for sheep farmers, especially in the Southern states.

The main breeds of goats and sheep raised for commercial purposes in Brazil are adapted to the semi-arid regions in the northeastern states [86]. Goats and sheep are considered generalist herbivores that select grasses, shrubs, herbs, or leaf litter as part of their diets [87, 88]. It is considered that the ruminal microbiota of Brazilian goats and sheep might reveal unique features imposed by climate adaptation and diet that could be useful for designing strategies to improve animal productivity [86, 89, 90].

Studies using culture-independent techniques for evaluating the rumen microbial community in goats are scarce [91–93]. The first work using 16S rRNA gene libraries to characterize the bacterial and archaeal communities present in the liquid and solid-associated fractions of the rumen from free-ranging Moxotó goats was published by a Brazilian group [86]. The Moxotó breed is well adapted to challenging conditions such as the droughts and limited grazing areas that are common in the Northeast Region of Brazil. The composition of the bacterial community found in the rumens of Moxotó goats showed a predominance of the phyla Firmicutes and Bacteroidetes in both the liquid- and solid-associated fractions [57, 94]. The most abundant sequences found in the liquid and solid fractions of the goat rumen belonged to the classes Bacteroidia (27.4% and 22.9% of liquid- and solid-associated sequences) and *Clostridia* (54.2% and 37.8% of liquid- and solid-associated sequences). *Methanobrevibacter* was the dominant genus of Euryarchaeota and corresponded to 69.7% and 86.7% of the total number of archaeal sequences from liquid- and solid-associated fractions, respectively. Liquid and solid fractions shared some OTUs that could be assigned to the phyla Firmicutes (8.6%) and Bacteroidetes (21.5%), but some sequences were only observed in the liquid (Proteobacteria) or solid fraction (Verrucomicrobia, Actinobacteria, Sphingobacteria, and Lentisphaerae). The sequences that could be assigned to genus level were mainly related to the genera *Olsenella*, *Prevotella*, *Mogibacterium*, *Succiniclasticum*, *Selenomonas*, *Coprococcus*, *Butyrivibrio*, *Ruminococcus*, and *Oscillibacter*. Some of these genera (*Prevotella, Butyrivibrio, Ruminococcus*) are well known to produce hydrolytic enzymes involved in the degradation of plant structural polysaccharides, such as cellulases, xylanases, and beta-glucanases, and these bacterial groups might also be relevant for fiber degradation in the goat ruminal ecosystem [86, 95].

Phylogenetic profiling of 16S rRNA genes and shotgun metagenomic sequencing were used to investigate the bacterial community composition and fiber-degrading activity of the rumen microbiome of four male sheep of the Santa Inês breed (*Ovis aries*) [89]. Although the animals were reared under similar conditions and were fed the same diet, the number of OTUs determined by 16S rRNA sequencing ranged from 6415 to 9559 at the 97% identity level and varied among individuals, with 1633 OTUs being shared by the four sheep. The phyla Bacteroidetes and Firmicutes were the most abundant in the four animals sampled, with an average relative abundance of 39.46% and 32.97%, respectively [89]. The Prevotellaceae family dominated the sheep bacterial community and the genus *Prevotella* accounted for 99.8% of the bacterial phylotypes of this family. Other bacterial families found in high abundance in the sheep rumen microbiome included Succinivibrionaceae (~23%), Veillonellaceae (~16%), Ruminococcaceae (~8%), and Lachnospiraceae (~6%). However, when the phylogenetic profile was based on shotgun sequencing, some differences in the relative abundance of the major phyla were observed, indicating a greater prevalence of Bacteroidetes (72.18%), while Firmicutes (15.87%) and Proteobacteria (3.04%) were less abundant compared with the 16S rRNA gene sequencing data.

Functions associated with lower methane emissions (e.g., consumption of hydrogen through succinate production) and cellulose degradation could be identified and were related to taxa from the Succinivibrionaceae and Ruminococcaceae families. Additionally, functional profiling revealed that amino acid and carbohydrate metabolism were highly represented in the sheep metagenome, with 59 potential carbohydrate-active enzymes being identified in sheep of the Santa Inês breed [89]. These observations indicate that the sheep rumen represents an underexplored source of biomass-degrading enzymes. Considering the lack of information available in the literature and the economic relevance of these ruminants, more studies will be needed to characterize the rumen microbiomes of different breeds of Brazilian goats and sheep, focusing on biotechnological applications of their hydrolytic enzymes and the development of strategies to manipulate the ruminal microbiota to improve feed-conversion efficiency.

The Nelore Microbiome

The Nelore (*Bos indicus*) breed of cattle, an indicine species that has been genetically improved through selection, represents over 72% of the bovine herds in Brazil and is the top exported beef cattle in the world [96]. Similarly to studies in other breeds of cattle, several studies focusing on the Nelore ruminal microbiota have been carried out in Brazil to evaluate the effect of different diets on rumen parameters, microbiota, and animal efficiency [97–99]. Besides these studies, other studies have demonstrated the microbial diversity associated with Nelore cattle in different parts of the animal's body, giving a more detailed view of the associated microbiome that can also affect the health and efficiency of the animal [100, 101].

A comprehensive analysis of the bacterial communities associated with the GIT of Nelore cattle applied 16S rRNA pyrosequencing to ten different GIT compartments (rumen, reticulum, omasum, abomasum, duodenum, jejunum, ileum, cecum, colon, and feces) of a Nelore steer in order to determine how the microbiota is structured along the entire GIT [100]. The sampling strategy comprised all three GIT segments (forestomach, small and large intestine), allowing a comparison of how the ruminal and fecal microbial communities are related to the microbial communities in other sections of the GIT.

In total, 20 phyla were recovered from the entire GIT, with two predominant phyla, Firmicutes and Bacteroidetes, which have been shown to be ubiquitous in bovines [60, 102–104] and other animals [19]. Some genera of bacteria, such as *Pseudobutyrivibrio*, *Ruminococcus*, *Coprococcus*, and *Clostridium*, seem to be part of the GIT core microbiome in bovines, as these genera were detected in all GIT samples of the Nelore, as well in the ruminal core microbiome described for other bovines [105, 106]. An over-enrichment of OTUs belonging to the phyla Bacteroidetes, followed by Firmicutes, was detected in the Nelore rumen, but the abundance of these phyla was different in other GIT segments; namely, in the small and large intestine.

When compared with the microbial community in other GIT segments, that in the small intestine was dominated by members of the phylum Firmicutes, with greater abundance of OTUs from the families Clostridiaceae and Prevotellaceae. Given the known role of these bacteria in carbohydrate metabolism and the close proximity of the lumen components to the host tissue, it seems these bacteria may further contribute to nutrient acquisition within the small intestine, as has been proposed recently for humans [107]. The microbial community in the large intestine of the Nelore is also enriched for OTUs from Firmicutes, and the presence of OTUs from the Ruminoccocaceae family in this segment indicates that these communities may also contribute to the further downstream feed fermentation of forage that bypasses rumen degradation.

The microbiome of the Nelore GIT has also been investigated using molecular techniques such as PCR-DGGE and real-time PCR. These analyses demonstrated the patterns of archaeal, bacterial, and fungal diversity through the entire GIT by comparing the microbial populations in the lumen with populations of mucosa-associated microorganisms [100]. A clear segregation of the microbiota (including bacteria, archaea, and fungi) was observed among mucosa and digesta samples, suggesting that microbes in close association with the host tissue can be exposed to different selection driving forces. Therefore, these populations should be considered in order to gain a better understanding of the microbial ecology in the GIT of ruminants. The study complemented findings from a pyrosequencing study [100], in which 26 digesta and 30 mucosa samples from all GIT segments (forestomach, small and large intestine) were processed separately. This approach confirmed the existence of a high intra-individual variation, even within the same GIT segment, in microbial populations that cannot be assessed only by sampling rumen contents or feces. While inter-microbiota variation is important for assessing differences in GIT segments between individuals, intra-microbiota variation is equally important, given that health assessments for individuals are primarily conducted using fecal and/or ruminal microbiota analysis.

In addition to the GIT microbiome studies, the Nelore vaginal microbiota has been characterized by next-generation sequencing [101]. Twenty animals, divided into four groups – non-pregnant heifers, pregnant heifers, non-pregnant cows, and pregnant cows – were sampled for their vulva-associated microbial communities, which were sequenced using Miseq (Illumina, San Diego, CA, United States). Regarding the bacterial community, the main phyla found were Firmicutes, Bacteroidetes, and Proteobacteria, which are commonly described in the GIT of animals. Members of the phylum Euryarchaeota, mainly the genus *Methanobrevibacter*, dominated the archaeal community and the dominating member of the eukaryotic community was a fungus from the genus *Mycospharella* (phylum Ascomycota). A comparison of OTU abundance and alpha-diversity calculations showed no significant differences among the four groups of animals studied, indicating that hormonal maturity does not affect the microbial diversity in the vulva. The vaginal microbiota appeared to be affected by the animals' GIT communities, probably because of the anatomical characteristics of the animals, and most differences in the microbiota could be explained by individual variation rather than by other factors investigated [101].

From an evolutionary perspective, the microbial interactions in the GIT of vertebrates are conserved and result from the processes of microbial diversification; these interactions are probably shaped by the eating habits and the GIT anatomy of the animals [20]. Understanding the structure of the microbial community and the factors that affect microbial assemblage in the GIT and other bovine parts may be useful for developing new livestock management technologies, particularly for nutrition and sustainability systems. Based on microbiome studies involving humans, the relevance of this type of approach for nutrition and management systems in Nelore cattle can be anticipated, aiming for higher animal efficiency and maximization of the genetic potential of this important tropical breed. More studies associating the microbiome with the genome of the animal [108] and management practices can have positive impacts on animal productivity and may help to mitigate some environmental problems, such as enteric methane emission and the expansion of grazing areas in Brazil.

The Buffalo Microbiome

The domesticated water buffalo (*Bubalus bubalis*) has been used for centuries in animal husbandry for meat and milk production in several regions of the world, especially in Asia, Southern Europe, and North Africa [109]. In more recent times, water buffaloes have also become relevant in South America, with buffalo milk and meat being widely commercialized in many cities in the Amazon region. The global buffalo population is estimated to be at least 195 million animals [110]. In 2014, the Brazilian buffalo herd was approximately 1.31 million head, with over 60% of the animals raised in the North Region of the country [85]. Typically, four different buffalo breeds (Jafarabadi, Mediterranean, swamp buffalo, and Murrah) are raised in Brazil. Because these animals are often more resistant to diseases and parasites than cattle and show great adaptability to different environments and geographical locations, buffaloes are considered by farmers and animal scientists as a suitable livestock choice for tropical regions [111].

As do other ruminants, buffaloes rely on complex communities of mutualistic microorganisms to convert plant cell biomass to microbial proteins, volatile fatty acids, gases (CH_4), and ammonia. The composition of the microbial community of the buffalo may vary according to various factors such as diet changes, breed, age, geographical and environmental factors, and host genetics and physiology [112, 113].

Lin et al. [114] studied the ruminal microbial community in 12 buffaloes of the Murrah breed and 12 buffaloes of the Nili-Ravi breed, separated into groups fed high-concentrate and low-concentrate diets, with 6 animals of each breed per diet group. As has been reported for other ruminants, the majority (53.4%) of the bacterial community at the phylum level corresponded to Bacteroidetes, and *Prevotella* was the dominant genus, with an average relative abundance of 35.9% of the total bacteria. Cellulolytic bacteria (*Fibrobacter, Ruminococcus,* and *Ruminobacter*)

represented less than 10% of the total bacteria found in the rumens of the Murrah and Nili-Ravi breeds and no significant differences in relative abundance were observed between the animals fed high- or low-concentrate diets. On average, 6.3% of the total bacterial sequences obtained from both buffalo breeds and both diets were assigned to *Paludibacter*, a gram-negative, strictly anaerobic, propionate-producing bacterium. Methanogens of the *Methanobrevibacter gottschalkii* clade represented the majority of the archaea found in the ruminal microbial community of the Murrah (65.7%) and Nili-Ravi (66.9%) buffaloes [114].

In an earlier study, Franzolin et al. [111] investigated the diversity of ruminal methanogens in 13 Mediterranean water buffaloes (*Bubalus bubalis*) fed three different diets. Five males were maintained in a feedlot for 21 days consuming a diet containing 45% corn silage and 55% concentrate, while six females grazed on pasture of *Brachiaria brizantha* for at least 12 months. Two males were fed a diet of 80% sugar cane and 20% concentrate for 120 days. Analysis of the 16S rRNA gene libraries for the ruminal methanogens revealed that most of the 467 clones had high sequence identity with species of the genus *Methanobrevibacter* [111]. Nineteen species-level OTUs were identified among the total number of clones, eight OTUs were unique to a specific diet, and only four OTUs were shared by all diets [111]. These observations distinguished the methanogenic community of the water buffaloes from that of other buffalo breeds and even from that of other herbivores.

Recent studies have also emphasized the effects of diet on the composition and diversity of microbial communities in water buffaloes. In a study carried out in Pirassununga, São Paulo, Brazil, Franzolin and Wright demonstrated that grazing buffaloes of the Mediterranean breed had higher populations of ciliate protozoa than those in animals maintained in a feedlot on a concentrated diet [115]. In another study, metatranscriptomic analysis of rumen fiber-adherent and fiber-free active bacteria, followed by functional annotation using the Kyoto Encyclopedia of Genes and Genomes orthology database, revealed that diet treatments (different forage-to-concentrate ratios) led to significant differences in the proportions of enzymes involved in metabolic pathways for volatile fatty acid (VFA; propionate and butyrate) production in the microbiome of the Indian water buffalo [116].

Further studies based on deep sequencing and metagenomic analysis are needed to provide further insights into the diversity and function of the buffalo microbiome under various husbandry conditions. These insights may contribute to developing strategies that could help to improve the overall productivity of buffalo herds and the quality of the animal products (milk and meat).

Impact of Diet on Rumen Microbial Diversity and Function

The rumen is a functionally stable ecosystem, although it is widely recognized that changes in microbial community composition occur according to the diet (substrates available for fermentation) and that the ecosystem is influenced by the host genetics,

geographical location, and environmental factors [117]. Several ecological properties that define the ruminal ecosystem and other gut microbiomes (e.g., resistance, redundancy, and resilience) have been described [12, 45]. The ruminal ecosystem shows functional redundancy because of the overlapping physiological activities carried out by ruminal microorganisms, which can utilize a range of catabolic pathways to metabolize complex polymers, macromolecules, monomers, and soluble substrates present in the food ingested by ruminants [118]. Even though there is great diversity of dietary substrates susceptible to microbial attack, the typical stoichiometry of fermentation products tends to be maintained in adult ruminants [119]. However, variations in the molar ratios of organic acids may occur even if animals are consuming feeds with a similar chemical composition. Some studies highlight the individuality of the host as a factor influencing the microbial community structure and biochemical parameters of the rumen [120], but diet and host species appear to play a major role in determining community composition [117].

When Holstein heifers received orchardgrass (*Dactylis glomerata*) as pasture or hay, the total concentration of ruminal VFAs was higher in animals fed hay (182.2 mmol/l) than in animals kept on pasture (132.7 mmol/l) [121]. Heifers fed hay showed higher proportions of acetate and valerate, while the molar ratio of butyrate, isobutyrate, and isovalerate +2-methylbutyrate was higher in animals kept on pasture. Interestingly, analysis of the bacterial community composition through 454 pyrosequencing showed that heifers receiving orchardgrass pasture also had an increased abundance of sequences from the genus *Butyrivibrio* compared with the animals fed hay. These observations allowed the authors to demonstrate that the diet-dependent shifts (pasture vs. hay) in bacterial composition were correlated with a higher proportion of butyrate in the ruminal VFA of the heifers kept on pasture [121]. In recent years, attempts have been made to describe the core microbiome (taxa shared by all animals under study) of bovines and to evaluate the stability of the microbial community and the variations resulting from changes in diet [105, 122, 123].

Petri et al. [122] evaluated populations of ruminal bacteria during the transition from forage to concentrate diet and during and after acidosis induction in eight Angus heifers. The composition of the ruminal microbiota was analyzed in 36 DNA samples by pyrosequencing of the 16S rRNA V1–V3 hypervariable region. Bacteria were classified into 44 genera, which varied significantly according to diet composition or rumen fraction (liquid or solid). Some genera were associated with bacteria commonly studied in the ruminal ecosystem, including *Fibrobacter*, *Prevotella*, *Rumminococcus*, *Selenomonas*, *Streptococcus*, and *Succinivibrio*. When heifers were subjected to induced acidosis, the population of Proteobacteria increased by up to 20.1% of the population after 12 h of induced acidosis, while the phylum Firmicutes decreased by 10% 4 h post-acidotic challenge [122].

The abundance of Bacteroidetes and Firmicutes in the rumen of dairy cows was also reported by de Menezes et al. [102] after they analyzed the microbial community by terminal restriction fragment length polymorphism and pyrosequencing and compared the effect of grazing and a total mixed ration (TMR) on bacterial community composition in solid and liquid fractions of the rumen. Among the 14 phyla

identified, members of the phyla Firmicutes and Bacteroidetes represented up to 80% of the total sequences obtained in the rumen samples. Sequences associated with the phylum Firmicutes were more abundant in the liquid fraction of the animals receiving a TMR, while bacteria of the phyla Fibrobacteres and Spirochaetes were more abundant in the solid fraction [102]. Fibrobacteres represents a small phylum constituted only by *Fibrobacter* species actively involved in ruminal cellulose degradation [124]. Additionally, de Menezes et al. showed that members of the Prevotellaceae family represented more than 20% of the total sequences obtained from bacteria associated with rumen liquid and solid fractions in the grazing cows [102]. Members of the Lachnospiraceae family, which specialize in pectin degradation, were prevalent (12–21% of sequences) in all the samples (liquid and solid fraction) analyzed in the grazing animals.

The relationships between specific microbial groups and the relationships of these groups with the physiological and metabolic parameters of the host have also been reported in rumen microbiome studies. Jami et al. [9] showed that the ratio of Firmicutes/Bacteroidetes was strongly correlated (Pearson $R = 0.72$, $P = 2 \times 10^{-3}$) with the yield (kg/day) of milk fat in dairy cows, suggesting an effect similar to that observed in mice, where a lower abundance of Bacteroidetes in the gut microbiota was correlated with an increase in fat in both the blood and the tissues of the host [125]. Differences in ruminal microbiota have also been associated with high and low emissions of enteric methane in ruminants. Kittelmann et al. [126] have demonstrated that differences in methane emissions (g CH_4/kg of dry matter intake) are related to factors that, in animals with low methane emission, select microbial communities producing smaller quantities of hydrogen (H_2), resulting in the reduced availability of substrates for hydrogenotrophic metanogenic archaea. Animals classified as low methane producers showed a higher proportion of propionate in the rumen and a greater abundance of organisms phylogenetically related to *Quinella ovalis*, a bacterial species involved in the production of lactic and succinic acids, fermentation products that are generally associated with lower hydrogen production [126].

An interesting aspect related to the bacterial species described as major catalysts of cellulose degradation in the rumen (*Fibrobacter*, *Ruminococcus*) is the fact that these bacteria have been found to show less abundance than expected in some microbiome studies of cattle fed forage [102, 127, 128]. Some studies have recognized that members of the less abundant microbial communities or "rare" taxa may also play a role in animal nutrition [129]. Although bioinformatic tools such as the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) software package [130] have been developed to predict metagenome functional content from marker genes (e.g., 16S rRNA), it is urgent that we continue our efforts to characterize the diversity and function of the gut microbiome of livestock animals raised under various management conditions and in different geographical regions or fed diets with different compositions. Also, the culturing of ecologically relevant microbes and the study of their genomes, physiology, and responses to changes in the environment (e.g., substrate availability, pH, etc.) could expand our knowledge of the interactions between microbial populations in the animal gut and their role in the nutrition and health of the host.

Closing Remarks

The advances in next-generation sequencing technologies in recent years have enabled researchers to analyze complex microbial communities from different ecosystems in great depth. Research of the gut microbiome has benefited from the approaches used for the Human Microbiome Project and several research groups are now applying similar strategies to investigate the gut microbial communities associated with livestock and production animals. In Brazil, research groups devoted to the study of the animal gut microbiome are still scarce, but there is an increasing awareness of the need for more research in this field, especially for livestock animals, such as cattle, goats, swine, and poultry. Such studies are currently limited by the lack of investment in microbiome research and the high costs of next-generation sequencing in Brazil compared with these factors in developed countries. To change this scenario, decision-making leaders and the general population must be convinced of the key role played by microbes in sustaining the livestock animals that provide protein and essential nutrients for humans. In addition, multidisciplinary work must be developed, involving microbiologists, animal scientists, molecular biologists, nutritionists, statisticians, geneticists, and bioinformaticians from Brazil and abroad to characterize and explore the Brazilian biodiversity, including the microbiomes of production animals and their impact on feed efficiency and the quality of animal products. The information derived from these studies could have a major impact in reducing the environmental costs (e.g., emission of greenhouse gases) associated with ruminant production at both local and global levels and could improve our understanding of the relationships between animal feed, the gut microbiome, and animal genetics and production traits, leading to improvements in the health and efficiency of livestock animals in the tropics.

Acknowledgments The authors gratefully acknowledge financial support from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; Brasília, Brazil), the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES; Brasília, Brazil), the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG; Belo Horizonte, Brazil), and the INCT Ciência Animal.

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Human Microbiome in Brazil

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Abstract The human body is inhabited by complex microbial communities, which positively impact different aspects of our health and are also associated with the development of diseases. Microbiomes from different organs and body sites vary in composition, structure, and gene repertoire, revealing strong niche specialization. Various lifestyle aspects, including diet, health care, hygiene habits and living conditions might influence the human-associated microbiome, highlighting the importance of comparative studies of populations from different countries. Specific cultural traits from Brazil, together with genetic, climatic and socio-economic conditions suggest that microbial communities associated with Brazilian population might contain particular features. This chapter reviews the progress of human microbiome research in Brazil in the past 10 years, focusing on broad-range analyses by DNA sequencing technologies. Microbial communities from skin, gut and oral cavity from Brazilian subjects, as well as microbiome from built environment and human-associated objects are discussed. Issues of specific interest for the country are approached, including endemic as well as prevalent diseases and conditions, and future challenges are identified.

Introduction

For years, our perception of the interaction of microorganisms with the human body has focused mainly on their role as causal agents of diseases. In the nineteenth century, the work of Louis Pasteur (1822–1895) revolutionized medicine by formally demonstrating the "germ theory of disease" thus providing evidence that some diseases are caused by microorganisms [1]. A series of experimental steps proposed by German physician Robert Koch (1843–1910), referred as Koch's postulates, aimed to link a disease to a specific microorganism [2].

The concept of "bad microbe" has now shifted, and we are aware of a much more complex picture. Our body is host to a variety of organisms including Bacteria,

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V. Pylro, L. Roesch (eds.), *The Brazilian Microbiome*,
DOI 10.1007/978-3-319-59997-7_5

Fungi, Archaea and viruses, collectively known as the human microbiome [3]. Human-associated microbial communities interact with each other and with the host, and impact multiple aspects of health [4]. The number of microbial cells inhabiting the human body had been estimated to outnumber human cells tenfold, and the total number of genes in the microbial genomes exceeds human genes by two orders of magnitude [5–8]. More recently, the numbers of human and bacterial cells in the body have been revised, suggesting that a 1:1 ratio would be more accurate [9]. Nevertheless, the importance of microorganisms in our body is evident, expanding our view of "self": we can be considered superorganisms, constituted by the host and its associated microbiome [10]. The collective of genomes from microorganisms and host comprises the hologenome, which functions and evolves as a unit [11].

Research groups from several countries and large consortia, such as The Human Microbiome Project by the US National Institutes of Health [8, 12, 13], have used "omic" approaches to unravel the microbiome associated with the human body and its influences on health and disease. It has been shown that phyla Firmicutes, Actinobacteria, Bacteroidetes and Proteobacteria dominate human-associated bacterial microbiome [8]. Various organs and body sites are inhabited by distinct microbial communities, revealing strong niche specialization [8, 14, 15]. Even organs that were previously thought to be inhospitable environments, such as the stomach, are now known to harbor specific populations [16, 17]. Temporal stability and dynamics of microbiome from different body sites in healthy subjects have been addressed [15, 18–22], as well as inter-individual variation [23, 24]. The vast majority of human microbiome studies have been focusing on Bacteria: knowledge regarding fungal, archaea, and viral microbiome are still limited (reviewed in [25–27]).

Microbial communities associated with our bodies can be beneficial to health. Comparative genomic functional analyses revealed that microbial genomes provide us essential traits that are absent from the human genome [6]. Gut microorganisms carry out fermentation and catalysis of complex polysaccharides, as well as synthesis of essential amino acids and vitamins [28]. Additionally, indigenous microbiome can protect the host against invaders [29]. Microbial communities also play a role in host immunity, as they shape and modulate the host immune system [30, 31]. This is a two-way interaction, as microbiome composition is shaped by immune system [32], and is related to host genetic variation [33].

The human microbiome has also been implicated in the development of a variety of diseases and conditions, including obesity [34], inflammatory bowel disease [35], diabetes [36, 37], neurodevelopmental disorders [38], and cancer [39]. These discoveries have resulted in a paradigm shift: a disease could be a consequence of an imbalance of the microbiome, referred to as dysbiosis. Thus, rather than specific pathogenic microbes as perceived by Pasteur and Koch, we should consider disease as a result of changes in the complex interactions between microbial communities and the host [40].

Our microbiome could be changing as a consequence of modern lifestyle: diet [41], use of antibiotics [42, 43] and Cesarean section (C-section) delivery [44] can lead to dysbiosis, which might be related to the increased prevalence of allergies,

autoimmune and inflammatory disorders [45, 46]. Gut and skin bacterial microbiome studies comparing Venezuelan Amazon Amerindians and subjects from the United States revealed differences in composition and functional gene repertoires, suggesting that Western lifestyle is altering the microbiome [47–49]. Variations in microbial communities shaped by cultural traits emphasize the need for studies in different countries, approaching a variety of cultures and habits, as well as ethnic backgrounds and climate variations.

In Brazil, this field of inquiry has been expanding over the past decade and the number of studies has been increasing, although it is still in the early stage. Human-associated microorganisms have been investigated using culture-dependent techniques and molecular approaches targeting specific microbial taxa; however, there are fewer broad-range analyses using sequencing technologies. Overall, the composition and structure of microbial communities were approached using Sanger sequencing and next generation sequencing (NGS). Gene content metagenomics, metatranscriptomics and metaproteomics have not yet been extensively explored. As is the general tendency, information regarding non-bacterial microbiome in Brazil is limited.

Specific cultural traits and habits from Brazil, including diet, hygiene, heath care, housing and the organization of the family structure, combined with genetic, environmental and climatic aspects, suggest that the human-associated microbiome in the country might contain particular characteristics. Geographic and socioeconomic differences within Brazil might also contribute to increase variations in microbial communities. Moreover, issues of local interest, such as endemic diseases [50], could benefit from knowledge derived from microbiome studies. This chapter approaches the research on human microbiome from Brazil, focusing on broad-range analyses by DNA sequencing technologies. Studies of the microbiome from skin, gut, oral cavity, built environment and human-associated objects are discussed, as well as their relevance and potential implications for Brazilian health care.

Skin Microbiome

The skin is considered to be the largest human organ, and serves as a barrier as well as an interface with the external environment [51]. Microbial communities inhabiting the skin can modulate immune response [52] and protect the host against pathogens [53]. There are also data supporting the association between microbiome and several skin diseases (reviewed in [54]). Analyses of healthy skin microbiome performed in the United States showed variation across body sites as well as individuals [8, 15, 23, 55, 56].

Skin microbiome was analyzed in Brazilian healthy subjects and patients with three skin conditions that have different characteristics: dandruff, leprosy and cutaneous leishmaniasis (Table 1). Dandruff is a very prevalent condition worldwide, affecting approximately half of adult population [60]. In contrast, leprosy and leishmaniasis are considered by the World Health Organization to be neglected tropical diseases: infectious diseases prevalent in tropical and subtropical conditions that affect low- and middle-income countries [61].

Table 1 Studies of human skin microbiome from Brazil using broad-range sequencing

References	Location[a]	Subjects	Biospecimen	Microbial group	Method
[57]	Belo Horizonte-MG	3 skin biopsies from leprous lesions from hospital's archives	Formalin-fixed paraffin embedded biopsies	Bacteria	V2-V4 16S rDNA Sanger sequencing and V3-V4 16S rDNA sequencing on Illumina MiSeq platform
[58]	Corte de Pedra-BA	10 adults with cutaneous leishmaniasis	Swabs from healthy and skin lesions	Bacteria	V4 16S rDNA sequencing on Illumina MiSeq platform
[59]	São Paulo-SP	24 adults (11 healthy and 13 with dandruff)	Skin swabs from scalp and forehead	Bacteria Fungi	V4 16S rDNA and ITS1 sequencing on Illumina MiSeq platform

[a]City where subjects were recruited

According to Brazilian Ministry of Health, in 2010 Brazil had nearly 30,000 cases of leprosy in treatment, and more than 34,000 new cases detected [50]. Silva et al. [57] analyzed bacterial microbiome in leprous lesion biopsies from Brazilian subjects, showing that communities were dominated by genera *Burkholderia, Pseudomonas and Bacillus*. In contrast, genera typically found to be abundant in healthy skin, such as *Propionibacterium, Corynebacterium and Staphylococcus* [54], were underrepresented in leprous lesions. Surprisingly, *Mycobacterium leprae,* the etiological agent of leprosy, was found in low proportions.

Cutaneous leishmaniasis is a zoonotic infection caused by protozoa from *Leishmania* genus and transmitted by infected sandflies, with approximately 25,000 notified cases in 2005 in Brazil according to Brazilian Health Ministry data [62]. The pathology causes ulcerative skin lesions that are subject to secondary bacterial infections [63]. Salgado et al. [58] used an NGS approach to analyze bacterial microbiome from cutaneous leishmaniasis lesions, in comparison with contralateral healthy skin sites. The subjects were recruited from a leishmaniasis endemic area. Data showed that bacterial communities clustered according to healthy status. Diversity was decreased in lesions, which were dominated by *Streptococcus, Staphylococcus, Fusobacterium* and other strict or facultative anaerobic bacteria. In addition, bacterial genera not previously associated with skin microbiome were detected in this study.

Leprosy and leishimaniasis have been less explored in the international scientific literature compared with diseases that have greater impact across the economic spectrum of countries worldwide. It would be beneficial for Brazil to take a deeper interest in these diseases, which could have a positive influence even beyond its borders.

Dandruff is a chronic inflammatory condition that has been commonly associated with *Malassezia* organisms, and anti-fungal shampoos are normally used to control the symptoms [64]. However, the role *Malassezia* plays has not been elucidated, and the etiology of the condition remains unclear. In addition to the medical concern, dandruff is also a matter of economic interest in Brazil. According to the Brazilian Association for the Industry of Cosmetic, Toiletry and Fragrance, the sector had net sales of US$ 6 billion in the first half of 2014 [65]. Brazil holds the third place in the consumer global market for this category of products, and the second for hair supplies [65], which include anti-dandruff products. Our research group used high-throughput 16S rDNA and ITS1 (Internal Transcribed Spacer 1) sequencing to characterize cutaneous bacterial and fungal microbiomes from healthy and dandruff subjects, including scalp and forehead samples (lesional and non-lesional skin sites) [59]. Findings revealed that bacterial and fungal communities differed according to health status, and diversity was higher in samples from dandruff compared with healthy subjects. The microbial shift extended also to non-lesional sites from dandruff subjects, suggesting that the condition may be related to a systemic process, and not restricted to the skin site showing the symptoms. Highly prevalent uncharacterized *Malassezia* organisms were detected; however, *Malassezia* microbiota analyzed at species level did not reveal association with health condition. An NGS-based study performed in China showed that scalp microbiome is influenced by host factors, such as demographics and physiological conditions of the skin, and consistent with our findings, did not show association between *Malassezia* at species level and health status [66].

Gut Microbiome

The majority of the microorganisms in the human body is concentrated in the gastrointestinal tract [67]. A study performed by The NIH Human Microbiome Project Consortium with 300 subjects in the United States showed that bacterial gut microbiome was highly diverse [8]. More recently, Belgian and Dutch cohorts totaling almost 4 thousand subjects were analyzed, identifying a core fecal microbiome and a set of covariates, such as medication usage [68].

In Brazil, studies of gut microbiome have addressed the association with obesity, as well as microbiome development in early life in relation to delivery mode and socio-economic status (Table 2).

Obesity is an important health issue in Brazil. A 2014 Brazilian Heath Ministry survey of health plan beneficiaries in all 26 state capitals and the Federal District estimated that more than half of the adult population surveyed was overweight (Body Mass Index-BMI $\geq$ 25 kg/m^2), and 16.8% were obese (BMI $\geq$ 30 kg/m^2) [73]. The association between obesity and gut microbiome has been demonstrated in animal models [74] and in human subjects from the United States [75, 76]. In Brazil, de Souza et al. [69] analyzed the composition of gut bacterial microbiome from 33 overweight and obese adults using NGS strategy in order to evaluate the effect of oral supplementation with L-glutamine. Subjects who received glutamine showed a decrease in Firmicutes, similar to the weight loss effect verified in study from the United States [75].

Mueller et al. [70] investigated the influence of maternal weight on intestinal bacterial microbiome of babies delivered vaginally and those delivered by C-section. Fecal samples from 74 Brazilian neonates were analyzed employing an NGS approach. Gut microbiome from vaginally delivered neonates differed according to maternal weight, with babies born to overweight or obese mothers showed distinct microbial community structure as compared with babies born to normal weight mothers, especially the difference in relative abundance of *Bacteroides*. In contrast, maternal weight did not seem to affect the microbiome from Cesarean delivered babies. Maternal weight was also a factor in predicted metagenomic functional profiling, which revealed differences in vaginally delivered neonates: babies born to overweight or obese mothers had higher gene content related to carbohydrate metabolism, whereas fatty acid metabolism pathways are overrepresented in babies born to normal weight mothers. The acquisition and development of microbiome in early life have been previously investigated in the United States [77, 78], and the influence of delivery mode on microbiome has been addressed in studies with Venezuelan [79], Swedish [80, 81], Singaporean [82], and Puerto Rican subjects [83]. Additionally, the association between C-section delivery and childhood obesity has been reported in various countries, including Brazil (reviewed in [84]). The study with Brazilian subjects confirmed that C-section delivery alters the babies' microbiome as reported in other countries, and showed for the first time that the microbiome association with maternal weight is related to transmission during delivery [70].

Alterations of the microbial communities in relation to delivery mode is a very important matter in Brazil, where the percentage of C-section deliveries is very high

Table 2 Studies of human gut microbiome from Brazil using broad-range sequencing

References	Location[a]	Subjects	Biospecimen	Microbial group	Method
[69]	Sumaré-SP	33 overweight and obese adults	Fecal samples	Bacteria	V3 16S rDNA sequencing on Illumina MiSeq platform
[70]	Porto Alegre-RS	74 neonates (18 born vaginally and 56 by C-section)	Fecal samples	Bacteria	V4 16S rDNA sequencing on Illumina MiSeq platform
[71]	São Paulo-SP	10 neonates from low socioeconomic status (2, 7 and 30 days after birth)	Fecal samples	Bacteria	16S rDNA cloning and Sanger sequencing
[72]	São Paulo-SP	10 babies from low socioeconomic status (3, 6, and 12 months of age)	Fecal samples	Bacteria	16S rDNA cloning and Sanger sequencing

[a]City where subjects were recruited

and has been increasing progressively. Data from Brazilian Ministry of Health shows that C-sections have increased from 37.8% in 1994 to 57.1% of deliveries in 2014 [85, 86]. World Health Organization recommends 10–15% of C-section deliveries, and estimates that in 2008 over 6 million unnecessary C-sections worldwide resulted in over 2 billion dollars in expenditures [87]. Studies addressing the influence of delivery mode on the microbiome in Brazil might contribute to the establishment of public health policies and help to decrease the high rate of unnecessary C-sections in the country.

The establishment and development of gut bacterial microbiome in children from low-socioeconomic status were also investigated in Brazil. Brandt et al. [71] used Sanger sequencing strategy to analyze fecal samples from ten neonates obtained on days 2, 7, and 30 after birth. Overall, *Escherichia* and *Clostridium* were dominating genera, and *Staphylococcus* was identified at a low rate. Most sequences from the day 2 to day 7 groups were assigned to *Escherichia*, and a higher bacterial diversity was found in the day 30 group. A baby who received antibiotics presented lower proportions of *Escherichia* and anaerobes, and an increase of *Klebsiella*. A more recent study performed by the same research group used similar methodology to analyze the fecal microbiome from children of low socioeconomic status, comparing samples obtained at 3, 6, and 12 months of age. Once again, *Escherichia* was found to be a dominating genus, followed by *Streptococcus*. An increase of diversity was observed at 12 months of life. Breastfeeding seems to influence the microbiome: babies who were exclusively breastfed until 5 months of age showed distinct bacterial profile compared with babies who had mixed feeding [72]. The authors suggested that the microbial colonization patterns might be related to environmental conditions to which the infants are exposed: families that participated in the studies had low income and lived in poor conditions, with inadequate sewer systems. Such conditions reflect the situation of a substantial portion of the Brazilian population. When considering sanitation, for example, data from the Brazilian Institute of Geography and Statistics showed that in 2008 approximately half of municipalities in the country did not have sewerage services [88]. Moreover, the socioeconomic status probably influences diet, health care and hygiene practices, which possibly impact gut microbiome in Brazilian population. Microbial shifts associated with diarrhea in young children from low-income countries in Africa and Asia have been reported [89], and it is a relevant matter of public health that concerns Brazil.

Oral Cavity Microbiome

The oral cavity maintains direct contact with the external environment, as well as with the interior of the body, which has implications for the microbiome and consequently for health [90]. Different microenvironments within the oral cavity harbor distinct microbial communities [8]. The role of Bacteria in dental caries and periodontal diseases has been known for a long time, but more recently the association of oral microbiome with a variety of other pathologies including non-oral diseases, has been shown (reviewed in [91]).

In Brazil, data obtained in 2010 by the Brazilian Health Ministry showed that among people 35–44 years old, a mere 0.9% were free of caries, 17.8% did not present periodontal diseases, and 31% did not need dental prostheses. In the age group from 65 to 74 years, the situation was even more drastic, as the percentages dropped to 0.2%, 1.8% and 7.3%, respectively [92]. The oral cavity microbiome has been the subject of the majority of the human microbiome studies in Brazil. Microbial communities from various oral sites were investigated in healthy subjects, tobacco and alcohol users, subjects with implants, and patients with oral and non-oral diseases (Table 3).

Bacterial microbiome from root canal was analyzed in patients with endodontic infections using Sanger sequencing [93–95]. Overall, Firmicutes was the most abundant phylum. Additionally, bacterial communities differed comparing samples from symptomatic and asymptomatic infections [94].

Two studies from the same research group used NGS to assess the bacterial microbiome from root canal in extracted teeth with apical periodontitis lesions [96, 97]. Interestingly, the studies diverged in phyla dominance: Siqueira et al. [96] reported that Proteobacteria was the most abundant phylum, and Santos et al. [97] found Firmicutes to be dominant in their samples. Moreover, bacterial communities clustered according to the type of infection (acute or chronic), and samples from acute infection presented higher diversity [97].

Gomes et al. [98] analyzed bacterial microbiome in combined endodontic-periodontal lesions in Brazilian subjects through NGS. Root canal microbiome was compared before and after chemomechanical preparation for disinfection, and periodontal samples were also obtained (before and after root canal treatment). Root canal microbiome was compared before and after chemomechanical preparation for disinfection, and periodontal samples were also obtained (before and after root canal treatment). Firmicutes was the dominating phylum in both root canal and periodontal sites. Root canal communities shifted after treatment, and surprisingly higher numbers of phyla and genera were detected; although culture-based analyses revealed a decrease of colony forming units. The authors suggested that it might be due to exposure of infected dentinal tubules caused by the treatment. In contrast, periodontal communities did not vary significantly, and were similar to those from root canal, suggesting a possible infection pathway between pulp and periodontium.

The increases in the prevalence and severity of periodontitis have been associated with diabetes [109]. Data from 2013 estimated that diabetes affected 6.2% of Brazilian population over the age of 18 years, and approximately 20% of the population between 65 and 74 years of age [110]. The effect of diabetes and periodontitis on subgingival bacteria has been investigated mostly by culture-based methods or molecular approaches targeting specific taxa (reviewed in [109]). A study performed in Brazil used Sanger sequencing to characterize bacterial microbiome from subgingival periodontal pockets in patients with chronic periodontitis and uncontrolled type-2 diabetes, in comparison with periodontitis patients with no diabetes [99]. Firmicutes was the most abundant phylum in both groups. Nevertheless, findings indicated significant differences in the percentages of clones comparing diabetic and

Table 3 Studies of human oral cavity microbiome from Brazil using broad-range sequencing

References	Location[a]	Subjects[b]	Biospecimen	Microbial group	Method
[93]	Piracicaba- SP	7 teenagers and adults with pulpal necrosis and periapical lesions	Root canal samples	Bacteria	16S rDNA cloning and Sanger sequencing
[94]	Rio de Janeiro-RJ	4 adults with asymptomatic or symptomatic teeth	Samples from root canal and abscess exudates	Bacteria	16S rDNA cloning and Sanger sequencing
[95]	São Paulo-SP	12 adults with endodontic infection and periradicular lesions	Root canal samples	Bacteria	16S rDNA cloning and Sanger sequencing
[96]	Rio de Janeiro-RJ	10 subjects with apical periodontitis lesions	Apical root canal samples from extracted teeth	Bacteria	16S rDNA 454 pyrosequencing
[97]	Rio de Janeiro-RJ	10 adults with apical periodontitis lesions	Apical root canal samples from extracted teeth	Bacteria	16S rDNA 454 pyrosequencing
[98]	Piracicaba-SP	15 adults with endodontic- periodontal diseases	Samples from root canal and periodontal plaque	Bacteria	V3-V4 16S rDNA sequencing on MiSeq Illumina platform
[99]	Guarulhos-SP and Piracicaba-SP	23 adults with periodontitis (12 with type-2 diabetes and 11 non-diabetics)	Subgingival biofilms samples from periodontal pockets	Bacteria	16S rDNA cloning and Sanger sequencing
[100]	São José dos Campos-SP	20 adults (10 healthy and 10 with aphthous ulcers) (2 sample pools)	Swabs of aphtous ulcers and healthy oral mucosa	Bacteria	16S rDNA cloning and Sanger sequencing
[101]	São José dos Campos-SP	20 adult denture wearers (10 healthy and 10 with stomatitis) (2 sample pools)	Swabs of palatal tissue and fitting surface of the denture	Bacteria Fungi	16S rDNA and ITS2 cloning and Sanger sequencing

[102]	São Paulo-SP	22 subjects (7 alcohol and tobacco users, 6 tobacco only, 9 non-users)	Oral biofilm swabs (tongue, mouth floor and buccal mucosa)	Bacteria	V1 16S rDNA sequencing on Ion Torrent PGM platform
[103]	Location not specified	10 teenagers and adults with deep occlusal caries	Samples from occlusal caries in permanent molars	Bacteria	V4 16S rDNA sequencing on MiSeq Illumina platform
[104]	Guarulhos-SP	20 adults with dental implants (10 healthy and 10 with peri-implantitis)	Samples from periodontal pockets, implants, and subgingival biofilm	Bacteria	16S rDNA cloning and Sanger sequencing
[105]	Ribeirão Preto-SP	20 healthy adults with dental implants (10 titanium and 10 zirconia)	Biofilm of implants, prostheses, teeth, peri-implant and periodontal sulcus	Bacteria	16S rDNA 454 pyrosequencing
[106]	Guarulhos-SP	20 adults (10 healthy and 10 with periodontitis)	Subgingival biofilm samples	Archaea	16S rDNA cloning and Sanger sequencing
[107]	Guarulhos-SP	50 adults (25 with healthy implants and 25 with peri-implantitis)	Subgingival biofilm samples from implants and teeth	Archaea	16S rDNA cloning and Sanger sequencing
[108]	Pará and São Paulo states (cities not specified)	12 adults with myocardial infarction and periodontal diseases	Atheromatous plaque samples	Bacteria	16S rDNA cloning and Sanger sequencing

[a]City where subjects were recruited

[b]Only subjects from whom samples were analyzed by broad-range sequencing were considered

nondiabetic subjects, at phylum, genus and species level. The results are in coherence with a broad-range pyrosequencing study done in China showing that bacterial subgingival microbiome in periodontitis patients clustered according to diabetes status, and specific taxa were also associated with diabetic and non-diabetic samples [111].

Aphtous ulcers and denture stomatitis have been investigated in two studies by the same research group from Brazil [100, 101]. First, oral mucosa swabs from healthy subjects and patients with recurrent aphthous ulcers were analyzed through Sanger sequencing, and findings indicated that bacterial communities differed significantly between groups [100]. Subsequently, Campos et al. [101] investigated both bacterial and fungal microbiome from oral biofilm in denture wearers with generalized denture stomatitis, in comparison with healthy denture wearers. This study is of particular interest in that it includes fungal microbiome, whereas most studies concentrate on Bacteria. Bacterial-fungal mixed biofilms are clinically relevant, as they might be associated with increased antibiotic or antifungal resistance [112, 113]. Findings showed that both bacterial and fungal communities differed between groups. Many bacterial taxa were detected exclusively in patients with denture stomatitis, others only in healthy subjects. Regarding Fungi, only *C. albicans* was found in denture stomatitis samples, whereas healthy denture wearers showed higher diversity [101].

The effects of alcohol and tobacco consumption on oral microbiome were also evaluated. A Brazilian National Health Survey from 2013 showed that 15% of the total population were tobacco users, and 24% of the population over 18 years of age consumed alcohol regularly [110]. The combination of these drugs increases the potential health damage, and the use of alcohol and tobacco is likely interrelated [114]. Thomas et al. [102] sampled biofilm from tongue, floor of the mouth, and the buccal mucosa from Brazilian subjects, and characterized bacterial microbiome using NGS. Beta diversity analysis showed that bacterial communities clustered preferentially according to alcohol/tobacco usage. Moreover, samples from smokers and drinkers showed higher intra-group similarity and lower richness (alfa diversity) as compared with control group.

Dentinal caries is a prevalent health issue in Brazil, affecting nearly all age groups, and is related to dental loss [92]. Bacterial microbiome associated with advanced dentinal caries in Brazilian subjects was studied through NGS approach [103]. In half of the samples the dominating genus was *Lactobacillus,* however in the other half this genus was detected in low proportions. *Lactobacillus* sp. was found to be abundant in caries-associated oral microbiome in studies from the United Kingdom [115], Australia [116] and China [117]. The authors of the Brazilian study suggested that the difference in *Lactobacillus* abundance might be related to the transition to pulp tissue infection, although they stated that this aspect should be further investigated.

Oral microbiome associated with oral implants was also studied in Brazil. da Silva et al. [104] accessed bacterial microbiome from subgingival biofilm samples from implants with or without peri-implantitis using Sanger sequencing, and showed that composition of the communities differed between groups. More recently, Nascimento et al. [105] performed a longitudinal study to characterize bacterial microbiome associated

with titanium and zirconia dental implants at baseline–implant loading, 3 months, and 6 months after the intervention. NGS was used to analyze samples from subgingival and supragingival biofilms, as well as biofilm from surface and internal parts of the implants. Results showed that pathogenic species were present in the implant-related sites and persisted over time. Community composition varied according to the implant material (titanium and zirconia), which might be related to differences in roughness and susceptibility to bacterial adhesion [105].

In contrast with most Brazilian oral microbiome studies, which approached bacterial communities, two reports from the same research group characterized Archaea oral microbiome from subgingival biofilm in periodontitis and healthy subjects [106], and in patients with peri-implantitis and subjects with healthy implants [107], both by Sanger sequencing. Most of the clones corresponded to *Methanobrevibacter oralis*, which was previously reported in other countries in different oral environments (reviewed in [27]). Nevertheless, differences between groups were detected, suggesting that a potential association of Archaea with periodontitis and peri-implantitis should be further investigated.

Bacteria found in other body sites might originate from oral cavity. For example, cardiovascular diseases have been associated with periodontal diseases, and studies from several countries have investigated their relation to oral microbiome (reviewed in [118]). Myocardial infarction and stroke, major causes of death worldwide, are associated with atherosclerosis [119]. In Brazil, cardiovascular diseases were responsible for more than 30% of deaths in 2011, according to data from Brazilian Ministry of Health [120]. Calandrini et al. [108] used Sanger sequencing to analyze bacterial microbiome from atherosclerotic plaques in Brazilian patients indicated for aorta endarterectomy due to myocardial infarction. Subjects also presented different periodontal diseases. The majority of the sequences were assigned to phylum Proteobacteria, followed by Firmicutes. Bacteria previously found in oral cavity were detected, including pathogenic species. A Swedish study also surveyed bacterial microbiome from atherosclerotic plaques, as well as oral and gut microbiome from atherosclerosis patients and healthy controls [121]. As corroborated by the Brazilian report, Proteobacteria and Firmicutes were found to be the most abundant phyla. Furthermore, results from Sweden showed that bacterial communities clustered according to body site, and the relative abundance of some taxa in plaques correlated with their abundance in oral cavity. Some bacteria were shared between plaques and oral or gut within the same subject, suggesting possible bacterial migration to the plaques.

Microbiome of Built Environments and Human-associated Objects

We now spend much of our time indoors, and microbiome from built environments might influence human microbiome and impact our health [122]. Studies performed in the United States using NGS approaches revealed that bacterial communities from indoor surfaces, air and dust are influenced by architectural design,

ventilation, and external air sources, as well as human occupancy and usage of the spaces [123–125].

It has also been shown that the human microbiome, especially from skin, might shape the microbiome from built environments [126–128]. Using climate chambers, it was demonstrated that airborne bacterial cloud differed between individuals, and each person contributes to the environmental microbiome with specific microorganisms [129]. It has also been suggested that microbial fingerprints on surfaces and objects could be suited to forensic applications [130, 131]. Furthermore, US studies have demonstrated that people sharing a home have similar bacterial microbiome [132, 133]. Such effect goes beyond human relationships, as skin bacterial communities of dog-owners were found to be similar to their dogs [132].

In Brazil, three studies from the same research group accessed bacterial communities on currency notes [134], surfaces in a large public hospital [135], and surfaces from a research institute [136] through NGS (Table 4). Overall, Proteobacteria, Firmicutes and Actinobacteria were the most abundant phyla, and potentially pathogenic genera were detected, raising the awareness of the importance of personal hygiene and efficient methods for cleaning and disinfection.

Bacterial microbiome from residential environments was analyzed across a gradient of urbanization, in a study with the participation of our research group [137]. Four locations at the same latitude in the Amazon Basin were selected: an Amerindian isolated jungle village, a rural community, an urban town, and an urban city (the first three in Peru and the last in Brazil). Results showed that the composition of bacterial communities differed according to the degree of urbanization. The microbiome allowed the classification of functional spaces in houses, and kitchen and bathroom could be more efficiently differentiated by bacteria communities from walls in urban than in rural houses. A progressive separation between indoor and outdoor environments was observed as urbanization increases. Thus human bacteria were enriched in the town and city houses. In contrast, environmental bacteria were higher in the jungle and rural village houses [137].

Concluding Remarks

Studies of human-associated microbiome from Brazilian subjects using broad-range sequencing approached in this chapter showed dominance of bacterial phyla Firmicutes, Proteobacteria, Bacteroidetes and Actinobacteria, in coherence with reports from other countries [8], although the proportions differed among body sites. Results suggest that human microbiome from Brazil is associated with various diseases, as well as specific habits, lifestyles, and living conditions. Issues of particular interest to the country were addressed, and findings should contribute to guide public health policies in Brazil.

Many studies reported high amounts of uncharacterized organisms, suggesting unexplored biodiversity associated with our body. Future studies should analyze larger subject cohorts in order to access interpersonal microbiome variation, as well as to explore other body habitats and non-bacterial microbiomes. Further expansion

Table 4 Studies of built environment and human-associated objects microbiome from Brazil using broad-range sequencing

References	Location of sampling	Number of samples	Environmental sites or objects	Microbial group	Method
[134]	Metropolitan area of São Paulo-SP	5 pools of samples combined according to note denomination	Banknotes collected from street markets	Bacteria	V4 16S rDNA sequencing on Illumina MiSeq platform
[135]	São Paulo-SP	4 pools of samples combined according to surface type	Surfaces from functional objects and restrooms from a public hospital	Bacteria	V4 16S rDNA sequencing on MiSeq platform
[136]	São Paulo-SP	2 pools of samples combined according to surface type	Surfaces from functional objects and restrooms from a research institute	Bacteria	V4 16S rDNA sequencing on Illumina MiSeq platform
[137]	Manaus-AM (also includes three locations in Peru)	Total of 270 (78 from Manaus)	Surfaces of functional house spaces	Bacteria	V3-V4 16S rDNA sequencing on Illumina HiSeq platform

of studies to include more geographic regions would allow to access genetic, socio-economic, climatic and cultural variations, thus providing a more accurate panorama of human microbiome from Brazil.

Whole-genome shotgun metagenomic sequencing, metatranscriptomic and metaproteomic approaches would contribute to unravel the functional role of microbial communities, and consequently their impact on health as well as various diseases and conditions. Exploring microbial-host interactions, such as immune response, would also help to elucidate the influence of microbiome in various aspects of our life, contributing to establish more efficient therapeutic approaches and public health strategies in Brazil. Moreover, investigating potentially beneficial microbial organisms could provide possibilities to improve our health.

The establishment of biobanks properly regulated to host human biological samples, as well as culture collections specifically for clinical isolates would promote advances in the field of human microbiome in Brazil. Sequence databases of human-associated microorganisms and standardization of protocols are also needed.

Studies of human microbiome have ethical and legal implications. There are potential issues of privacy, as microbial inter-personal variation could be sufficiently high to allow the identification of individuals. Brazil needs to advance the development of laws and regulations to assure the rights of subjects, while encouraging research to progress. Finally, due to the direct involvement of volunteers, human microbiome research field can offer the possibility to bring science education to the general public.

Acknowledgements The author thanks Daniel Littwin for language revision.

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Bioprospecting Studies: Transforming the Natural Genetic Heritage into Biotechnological Richness

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Abstract The Brazilian microbiota has great potential richness for industrial use, given its mega-diversity. Despite the advances in international research that have provided access to such microbiota, via several approaches (metagenomics; second-generation DNA sequencing, in situ cultivation, and as a consequence high-throughput screening, etc.) a glimpse into the research output in Brazil demonstrates that such immense potential has been poorly explored. Even though the Brazilian scientific community has reached a degree of international excellence in research recognition, there is still strong centralization of knowledge and of biotechnology enterprises in the Southeast Region of the country, which greatly limits access to our multitude of biomes and ecosystems. Another problem is the lack of communication between the knowledge generation centers and practical efforts in the field, resulting in very little national intelligence reaching the consumer market. Consequently, the internal biotechnology market prioritizes imports, even though there are available domestic resources to generate competitiveness at a global level. Academic and industry integration initiatives through innovative agencies have demonstrated a path to bridge the gap between the "ownership" and the "usage capacity" of the country's rich microbial diversity.

Introduction

Brazil has a land mass of 8.5 million square kilometers, which makes it the largest country in South America. The country consists of 26 States and a Federal District, and contains five geographic regions. The great extent of the land reflects a variety

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V. Pylro, L. Roesch (eds.), *The Brazilian Microbiome*,
DOI 10.1007/978-3-319-59997-7_6

of biomes that have enormous biodiversity. Thus, Brazil is a country that harbors mega-diversity, containing around 20% of the described species of the planet [1].

The diversity of the Brazilian biomes—Cerrado, Caatinga, Atlantic and Amazon forests, Pantanal, and Pampas [2]—allows the diversification of a variety of living forms. The considerable literature on the biodiversity of the country's plants and animals has outlined fundamental parameters that are followed for the management of conservation areas [3]. In contrast, studies considering microbial biodiversity are fewer and are still insufficient to understand the microbial biology and functional diversity of a particular environment [4]. Therefore, the great diversity of Brazilian microbial ecosystems represents a reservoir to be explored, and it may contain genes for new enzymes and products for biotechnological use.

The biomolecules produced by microorganisms may act as bioindicators of soil quality, and may be used for bioremediation. Also, biomolecules such as biopolymers have various uses, including their use as bioemulsifiers, while exopolysaccharides [5, 6] are used in medicine as drug carriers to deliver therapeutic molecules such as drugs and genes, and in chemotherapeutics as nanodrugs, and even for tissue engineering [7–9]. Brazil leads in studies of agricultural plant-bacterial interactions and in the selection of more efficient bacterial strains to fix nitrogen [10–12], mostly under harsh conditions. As a result, biological nitrogen fixation has been largely adopted in the country, promoting the replacement of conventional fertilization by inoculants, providing social, environmental, and economic benefits for the producer as well as for the consumer. For soybean the advantages of symbiotic nitrogen fixation are already well known, resulting in an annual revenue of US$7 billion for the country [13]. Soybean (*Glycine max*) and sugarcane (*Saccharum* spp.) are intensively cultivated throughout Brazil, thanks to the favorable soil and topological conditions, allowing the production of biofuels—biodiesel and bioethanol, respectively—both of which, with the great recent advances in production, use enzymes for their synthesis.

Biodiesel, when compared with fuels derived from oil, is less toxic and more easily degraded because it has fewer components, and it is considered a sustainable source of energy [14]. Oil/fat of animal or plant origin and a short-chain alcohol are used for its production, generating biodiesel and glycerol as by-products. The production of enzymes that use agroindustry by-products, with the aim of the further utilization of these enzymes for biofuel production is an alternative method to be considered, once the reduction of tailings and the economic feasibility of such procedures are considered [15].

In 2014, Raizen Energia S/A (Piracicaba, Brazil) and GranBio (São Miguel dos Campos, Brazil) started producing ethanol from sugarcane bagasse (second-generation ethanol) in Brazil, with the intention of producing it on a commercial scale. The enzymes involved were to be supplied by a Danish company (Novozymes, Bagsvard city, Denmark) that intends to build a plant in Brazil to better serve the needs of all three companies. While this might be considered as a breakthrough for biofuel production, it repeats a common scenario in Brazil: the export of primary commodities and the import of finished products, a situation that can be considered unfavorable to the economy of any country.

As well as its very large territory that is favorable for agriculture, Brazil has around 8000 km of marine coast. Microbial marine communities can be found from the surface of the water to deeper areas, such as the abyssal zone [16, 17], excluding the microorganisms associated with other marine species [18]. Important biotechnological products, such as cellulolytic enzymes from fungi in symbiosis with cnidarians, can be obtained from the marine environment [16]. Mangroves, which inhabit transition zones between the terrestrial and marine environments, seem to have unique features, and as a consequence they show autochthonous bacterial species adaptation, representing an important biotechnological resource [15].

Thus, although Brazil has great biodiversity and availability of fertile land for biomass production, as well as a number of different environments to be explored, at present only primary commodities are commercialized. So biotechnological development is compromised and the country faces serious challenges, because less than 1% of its scientific productivity reaches the consumer market [19]. One of the obstacles to be overcome is access to the country's immense biodiversity. However, despite its importance, knowledge of Brazilian microbial diversity is still sparse.

This chapter considers barriers to the search for new bioactive compounds for biotechnological and economic development in Brazil, as well as the search for promising methods for large-scale production of these compounds. For these purposes, we carried out a review of the legal procedures and an assessment of the potential rules governing microbiological research. In addition, we present an overview of microbiological research in the country, with special focus on the main biomes already mentioned above. A picture of some of the most commercialized products of microbial origin is drawn, stressing national participation in this scenario, contextualizing and emphasizing the main perspectives under development.

We also reflect on what needs to be improved so that national biotechnological efforts become competitive; academic and industrial research needs to be integrated with either public or private initiatives, leading to better use of the resources. The country still faces many problems that hinder advances in biotechnology, including a low level of investment in research and development, dependence on public financial support, poor intellectual property protection, the lack of public/ private sector arrangements, and other structural deficiencies. The distribution of scientific production in Brazil is still unequal, with biotech companies being concentrated in the Southeast Region, where the strongest academic centers can also be found; this unequal distribution is the main obstacle to potential access to biotechnology in the nation as a whole.

Use of Microorganisms and Legal Information on Biological Resources

The microbiota represents an immense reservoir of enzymes and bioactive metabolites with great potential for industrial utilization. Microbial resources, particularly enzymes, have been used by humans for at least 2000 years to fulfill human needs,

and in the past 50 years they have been used in many industrial processes (for domestic needs and in the production of food for humans and animals; for chemistry compounds from technical to high-quality (or A.C.S.) grades and in the pharmaceutical industry; in biofuel production; and in water treatment); this has increased their presence in the market in an exponential fashion. These enzymes are produced by submerged or solid-state fermentation, by either bacteria or fungi isolated from the environment or by recombinant bacteria [19–22].

Because of the importance of such resources for the production of wealth, the biochemical and functional characterization of the microbiota has become essential to protect world biodiversity. Projects on a global scale such as "The Earth Microbiome Project" (http://www.earthmicrobiome.org), "International Census of Marine Microbes" (http://icomm.mbl.edu/index.html), and "Human Microbiome Project" (http://hmpdacc.org) all have some focus on such protection. Considering the strong impact of biotechnology on industry, the Economic Development and Cooperation Organization (EDCO) proposed the creation of the Global Biological Resource Centre Network (GBRCN), which consists of the biological collections of each country, and aims to promote access to high-quality biological material (http://www.gbrcn.org/).

Within Brazil, efforts are being made to evaluate the possibilities of the sustainable exploration of different ecosystems, with organizations such as the "Biota-FAPESP Project" and the "Brazilian Microbiome Project", which are standardizing the analytical profiles of Brazilian microbial communities using data from different DNA sequencing platforms [23].

In 2007, the "Rede Brasileira de Recursos Biológicos" (CRB-Br; the "Brazilian Network of Biological Resources") was established, created by Law Decree 6041, to maintain the diversity of macro- and microbiotas in Brazil. The CRB-Br covers all the major biotechnological sectors and consists of the Ministry of Science, Technology, and Innovation; the National Council of Metrology, Standardization, and Industrial Quality; the Technology Institute of Paraná; the Oswaldo Cruz Foundation (FIOCRUZ); the Brazilian Agricultural Research Corporation (EMBRAPA); the University of Campinas; the Rio de Janeiro Cell Bank; the Reference Centre on Environmental Information; the National Institute for National Property; and the Brazilian Society of Microbiology [24].

Once the guidelines for accessing biotechnological potential are established, one of the main questions to be considered is "how is this access going to be carried out?"; this is a subject that will be considered in the following section.

How to Access the Diversity of the Microbiota?

Despite all the potential represented by the microbiota, it is not always possible to access these microorganisms; this is because of the complexity required to faithfully represent the set of physicochemical factors that is necessary for their development [25]. So, it is estimated that only around 1% of these microorganisms can actually

be cultivated using traditional growth techniques and culture media plating on Petri dishes [21, 26–28], and because of these difficulties much of the microbiota's genetic diversity is not available to be retrieved.

In this context, with the development of research in the past few decades, some techniques have been developed to assess this vast diversity (microbial and molecular). The main techniques used are the metagenomic approach, second-generation large-scale DNA sequencing, in situ cultivation, and high-throughput screening. In the following sections, brief descriptions of each technique are presented, along with discussions on how research groups are currently using these techniques in institutions and universities.

Almost Two Decades of Metagenomics

Metagenomics is a technique that allows us to access and to study collective genomes, without the need for previous cultivation of donor organisms [29]. This technique makes it possible for scientists to better investigate the vast potential of different microbial sources, such as, for example, soils; microbial consortia; mangrove sediments; river, lake, and marine water samples; insect-associated microbiota; and the bovine rumen, etc.

The development of metagenomics has taken place in the context of advanced molecular biology technology and functional assays [29]. Studies that involve metagenomics include a DNA extraction phase; a second phase of cloning the DNA fragments using vectors that can be cosmids, fosmids, plasmids, or even vectors that can harbor large DNA inserts such as artificial bacterial or yeast chromosomes; a third phase with the growth of transformed clones harboring the cloned DNA fragments, using the host; for example, *Escherichia coli* competent cells. The obtained clones are then collected and kept as metagenomics libraries, which can be used for screening new natural products.

In recent years, function-driven searches for several genes and/or proteins of biotechnological importance have been made in these metagenomics libraries, with the aim being to conduct initial screening for the identification of a desired activity or expected expression, while sequence-driven searches have been used to identify DNA conserved target sequences [30].

Using function-driven metagenomic searches it was possible to identify a novel member of the GH16 family derived from sugarcane soil [31] and it was also possible to identify clones that showed excellent (>70%) hexadecane biodegradation in a metagenomics library from a Brazilian oil reservoir sample [32].

Still considering findings using a function-driven search of metagenomic samples, the group from the Plant and Microorganisms Biochemistry Laboratory (LBMP) of São Paulo State University at the Jaboticabal campus in Brazil has identified 30 clones with lipolytic activity in a metagenomic library derived from a microbial consortium able to degrade diesel oil.

Clones that showed great potential for tributyrin hydrolysis on Petri dishes assays were selected and, after DNA sequencing and annotation, three open reading frames

(ORFs), identified as coding for esterase/lipases, were cloned on an expression vector, with the aim being functional characterization and further biotechnological applications. The results showed three proteins, named ORF2, Est16, and EST3, which were expressed and purified as soluble and stable proteins. Functional characterization of Est16 [33] and EST3 [34] indicated that these esterases seemed to have strong potential for assays involving organic solvents and biofuel production, respectively. Sequence analysis and molecular modeling of ORF2 have shown that this protein is a new and undescribed member of the lipolytic bacterial family V [35], which, according to Arpigny and Jaeger [36], has members of mesophilic origin that are adapted to cold and heat.

In Brazil various other lipolytic enzymes of different origins are being investigated using metagenomics: in mangrove sediments [37] and fat-contaminated soil [38], in microorganisms such as *Lactobacillus plantarum* [39] and *Rhizomucor pusillus* [40], and also in plant material [41, 42].

Using the sequence-driven approach, the LBMP group has also been able to find two antibiotic gene groups (PKS I and II) in environmentally derived eucalyptus samples [43]. Also in Brazil, using the same approach, it was possible to identify, by metagenomic analysis, epoxide hydrolases and haloalkane dehalogenases originating from mangrove soil samples [44], laccases originating from sugarcane [45], and genes coding for proteins involved in biomass degradation, such as hydrolases and dehydrogenases. It was also possible to identify genes associated with bacterial efflux pumps or ABC-type transport systems from the metagenomic analysis of composting animal material samples [46]. In addition to these examples, the LBMP metagenomic libraries were mined, using both sequence- and function-driven mining approaches, to search for several genes of industrial interest, including genes for catalases, amylases, peptidases, cellulases, and laccases; genes for other antibiotic pathways; and genes for xyloseisomerases and phosphatases.

Various other metagenomic projects have been or are still being developed in Brazil by other research teams, such as those from EMBRAPA, FIOCRUZ, “Universidade de São Paulo” (USP, São Paulo State University) and the “Centro de Energia Nuclear na Agricultura” (CENA/USP, Center for Nuclear Energy in Agriculture) among others [23].

Next-Generation DNA Sequencing (NGS)

Next-generation DNA sequencing (NGS) techniques, and progress in data analysis methods and platforms, have allowed the exploration of microbial diversity in microbiota that are still not cultivable (including non-abundant microbiota) and the search for genes with high technological value; the new techniques even allow the detection of differences within a set of genomes (like the human genome). These new DNA sequencing techniques could cause a revolution in genetics, because the high-throughput systems used can generate thousands or even millions of nucleotide sequences in one single run, allowing us to answer questions at an unprecedented speed [47].

Various technologies are used for NGS, such as the commercial platforms Illumina Miseq and HiSeq2000 (Illumina, San Diego, CA, USA), Ion PGM and Ion Proton (Life Technologies, Carlsbad, CA, USA), and Pacbio (Pacific Biosciences, Menlo Park, CA, USA). These DNA sequencers differ based on their adopted chemistry or amplification method, the resultant Mb amount per run, run duration, amplified fragment length, and Mb cost. These platforms are currently used for quite different purposes; one example being for studying the microbial ecology of fermented or unfermented food [48, 49]. By using NGS it has become possible to identify new microbial species and to correlate them with particular food and production steps, knowledge that is very important for promoting quality control and food production security [48].

Against this background, the Brazilian Microbiome Project (BMP), which aims to build a Brazilian metagenomic database, was developed [23]. The BMP intends to link their information to systems of functional genetic diversity, and compare these systems with other microbiome projects throughout the world. The BMP also intends to describe microorganisms that can be used for the production of new products, with the purpose of improving the use of Brazilian biodiversity that favors biotechnology.

High-Throughput Screening (HTS)

High-throughput screening (HTS) is a system that is able to identify chemical probes on libraries containing a large number of compounds, using sophisticated assays and detection platforms [50]. As this technology can be used to evaluate a great number of chemical substances, it can be adopted for key targets of biomedical research, which could lead to the screening of new drugs [51]. HTS is reported to be the most productive technique for use on different targets [51].

Drug screening is just one of the many uses or strategic options that can be implemented for the control of diseases that, to date, are not amenable to cure. In a recently published paper [52], a research group reported on developing and improving a drug-screening assay for human African trypanosomiasis (HAT) and identifying potential candidates for the development of new drugs against HAT. After screening a library of 4000 putative kinase inhibitors, they found 13 scaffolds that indicated activity against *Trypanosoma brucei*. Their SYBR Green-based HTS is an effective way of detecting *T. brucei* when compared with resazurin (standard assay), as it is faster and more sensitive and reliable.

Cultivating the Uncultivable: Ichip

The isolation chip (Ichip) is a new method for the in situ cultivation of environmental microbial communities, the aim being to access the great hitherto inaccessible microbial diversity [53]. With this method the Ichip, which consists of

hundreds of micro-chambers, is used to allow the separation of each single cell of a cellular mixture into individual micro-chambers, after the environmental sample has been distributed over the specimen slide. The assembled Ichip is returned to the environment for incubation, because the development of colonies is based on diffusion by natural sources; after the incubation period, the material is recovered for further analysis, such as, for example, microscopy, DNA sequencing, etc. [53].

As recently reported, a new antibiotic, named teixobactin, was isolated from a soil sample using the Ichip as a tool [21]; besides allowing the discovery of this natural product, the Ichip also enabled the isolation of the relevant microorganism, tentatively named *Eleftheria terrae*, which, up to that time, had not been cultivable. With a high-throughput method, the Ichip promoted the recovery of 50% of the inoculated microorganisms in the micro-chambers; in other words, its use has made possible the in situ cultivation of observed colonies [53].

Teixobactin has shown high activity against gram-positive pathogens and drug-resistant strains, and interestingly, this antibiotic action was found to be related to the main biosynthetic pathway of *Staphylococcus aureus*, indicating that, in view of its interaction with peptidoglycan precursors, this antibiotic might be a new inhibitor of peptidoglycan synthesis. The efficient ligation of teixobactin to bacterial wall teichoic acid, a precursor of undecaprenyl-PP-GlnNAc (lipid III), liberates autolysis, producing cell wall lysis, consequently resulting in the pathogen´s death [21]. Even if other new drugs in the class are not developed, teixobactin is the first member of a new antibiotic class that targets lipid III [21, 54].

Brazil Compared with Other Countries

The choice of a technique or a set of techniques is a key point to be taken into account when an experimental design is to be considered for a scientific project. In this phase, it is essential to raise the questions to be answered by the project´s aims and/or to state the study's hypotheses, while bearing in mind the infrastructure available for the correct development of the experiments, the necessary equipment and reagents, the time required for the experiments to be carried out, and the data analysis.

We analyzed how Brazilian procedures compare with those of other countries in terms of the different techniques and methods used for investigating biomolecules. Does Brazil implement the currently available scientific and technological methods in its laboratories and research groups? If the answer is yes, which of the techniques listed above (metagenomic approach, NGS, HTS, and Ichip), are currently more accepted by the Brazilian scientific community?

To answer these two questions, a search was done of published scientific papers, using the databank from the Web of Science (http://wokinfo.com/), which is based on one of the most important world sources of information, the Thomson Reuters BiologyBrowser. A search for published scientific papers and not for other types of

information was performed because this is a standard scientific procedure worldwide, and with such a search it is possible to obtain information on the methods used and results, as well as the names of the research groups who carried out the experiments and the names of the relevant institutions and countries.

For this particular analysis two search mechanisms were used: the first one looked for works that had used the above-mentioned techniques and so the keywords "metagenome", "next-generation sequencing", "high-throughput screening", or "Ichip" were inserted in the search field. For the second analysis, as well as using the topic search field, we searched for the location at which the experiments were carried out, so for this option a second search field was added, in which data from countries such as Brazil, the United States, India, China, and Japan were individually analyzed. The data were collected in mid-August 2015 and Excel software generated the graphics.

The data clearly showed that, of the four above-mentioned techniques, HTS was the most commonly used worldwide by the scientific community, with 42,667 published articles in the period 2010–2015, almost double the number of articles using NGS (24,412). The number articles using the metagenomic approach was significantly lower (1883), followed by those using the Ichip (14 articles).

Alhough the HTS technique was the one most commonly used by the laboratories involved with this type of research in scientific institutions and/or private companies abroad, within Brazil this technique was listed as the second option, after NGS, in terms of the number of published articles, at 152 and 181, respectively.

It was interesting to observe that, in Brazil, although the NGS technique was used in the greatest number of published studies, metagenomics was, by far, the most prominent technique in comparison with other countries. The number of articles from Brazilian groups using the metagenomic approach corresponded to 2% of all the published articles worldwide (Fig. 1a), followed by NGS with 1% of all the published articles (Fig. 1b), and then HTS (Fig. 1c) and Ichip (Fig. 1d). These findings emphasize that, to date, and according to the data obtained in this analysis, there have been no publications by Brazilian research groups or institutions using the Ichip technique, in contrast with results in the United States, with five articles involving this option.

With scientific and technological advances, new methodologies are being proposed for the search of biomolecules, such as, for example, ultrahigh-throughput screening using drop-based microfluidics [55]. According to our analysis, within the past 5 years three articles in which this particular technique was used were published by Brazilian research groups in partnership with United States institutions, while none has been published in Brazil alone.

Thus, our data analysis has revealed that, although Brazilian research groups are implementing different bioprospecting methods for biomolecules, this is being done at a much lower speed than that in developed countries such as Japan and the United States. When we compare Brazil with India, whose human development index (HDI) for 2014 was lower than the Brazilian HDI (http://hdr.undp.org/en/countries), at 0.586 and 0.744, respectively, it can be seen that Brazil has less significant

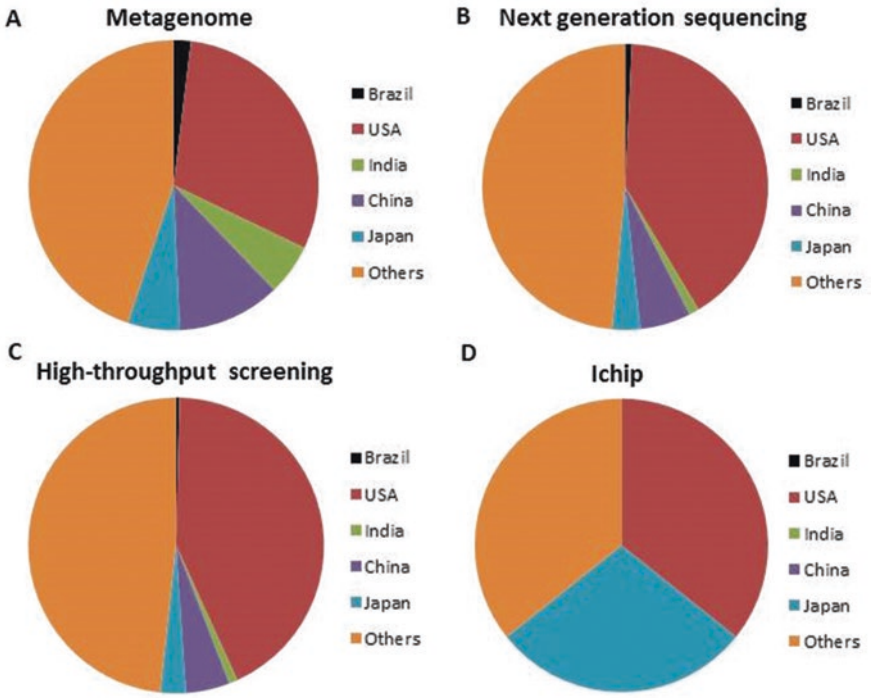

Fig. 1 Comparative analysis of bioprospecting in Brazil and other countries. The search for scientific articles that adopted metagenomic (**a**), next-generation sequencing (**b**), high-throughput screening (**c**), or Ichip (**d**) techniques was carried out using the Web of Science databank. The data were collected in August 2015 and the graphics were generated using Excel software

participation in bioprospecting than India (Fig. 1a, b, and c). This situation prompts us to think about the reasons that could be slowing the development of Brazilian science, particularly in regard to bioprospecting assays, which can lead to a better understanding of our own biodiversity.

Research Frontiers: Where the Microbial Metabolic Wealth is Mostly Explored

Brazil has the world's sixth largest microbial culture collection, holding 109,626 microbial isolates shared by 75 collections throughout the country (World Data Center for Microorganisms, 2015; accessed on August 23, 2015).

Surprisingly, this great biochemical and genetic microbial diversity has rarely been explored, especially when one takes into account the diversity of biomes in Brazil; opposed to this diversity is the extremely unequal centralization of the human and financial resources that are commonly destined for scientific research.

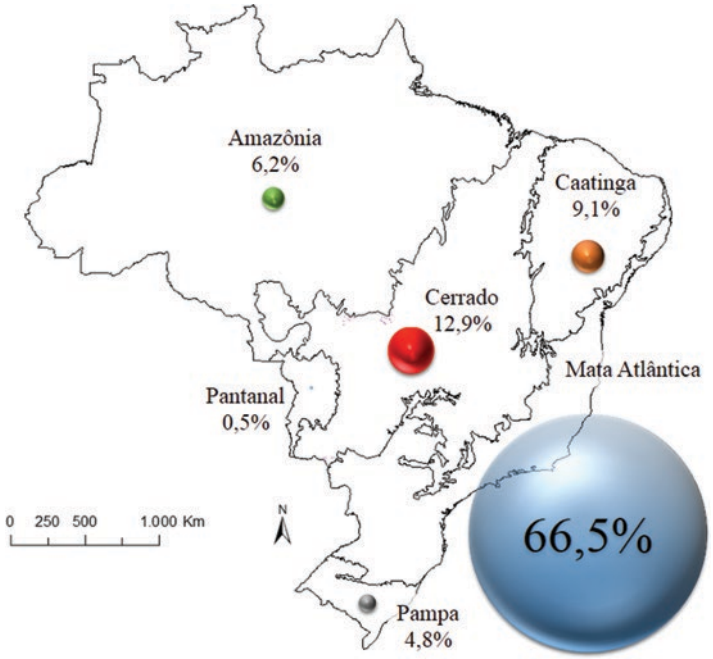

Fig. 2 Impact of studies involving microorganisms or their products isolated in Brazilian territory in articles published within the past 5 years in Brazil. The articles were searched for using the Web of Science tool [56] and the following keywords: "Microorganism", "Biomolecules", "Enzymes", and "Brazil". The data were collected in August 2015

Using the Web of Science tool (accessed between August 17 and 23, 2015), we carried out a bibliographic review of articles involving microorganisms or their products isolated in Brazilian territory published in the past 5 years in indexed periodicals. Most of the articles related to prospective studies of microbial communities and their metabolites (66.5%), with results from research developed at São Paulo State University and most referred to the Atlantic forest biome (Fig. 2). The second most commonly studied biome was the Cerrado (12.9%), followed by the Caatinga (9.1%). Despite their importance, the other biomes were mostly not studied, and biodiversity research and microbial prospecting for biotechnological purposes in these biomes were almost completely absent.

Economic Importance of Microorganisms: Global and National Impacts

Enzymes and antibiotics, mainly those of microbial origin, are among the biotechnological resources of high economic importance [19–22, 57].

The world market for enzymes is constantly expanding. Around 50% of this world market is destined for technical applications (bioenergy, textiles, etc.), while 37% of the market is used for food production and 12% is directly used for nourishment [58].

Of note, antibiotics and other bioactive entities originating from microbes raise billions of dollars in the world economy, because there is a constant need for new molecules for the control of human and animal diseases [19, 21, 43, 57].

According to available data for the year 2012 in the Observatory of Economic Complexity data bank (available at http://atlas.media.mit.edu/en/), world export transactions involving enzymes reached US$4.7 billion during this period. The countries that had the highest export transactions were Denmark (US$1.08 billion), the United States (US$487 million), and Finland (US$383 million), while Brazil (US$45.5 million) was ranked in the 16th position and the 5th position in the Americas. For antibiotics, the most relevant export transactions involved China (US$3.19 billion), Switzerland (US$1.92 billion), and the United States (US$1.7 billion), with Brazil in the 22nd position, with exports of US$69.68 million. More details for the world percentage distributions are shown in Fig. 3.

According to BCC Research (Fig. 4a), the best growth projections for enzymes related to biofuels are for the cellulase class, with a prospective doubling of the

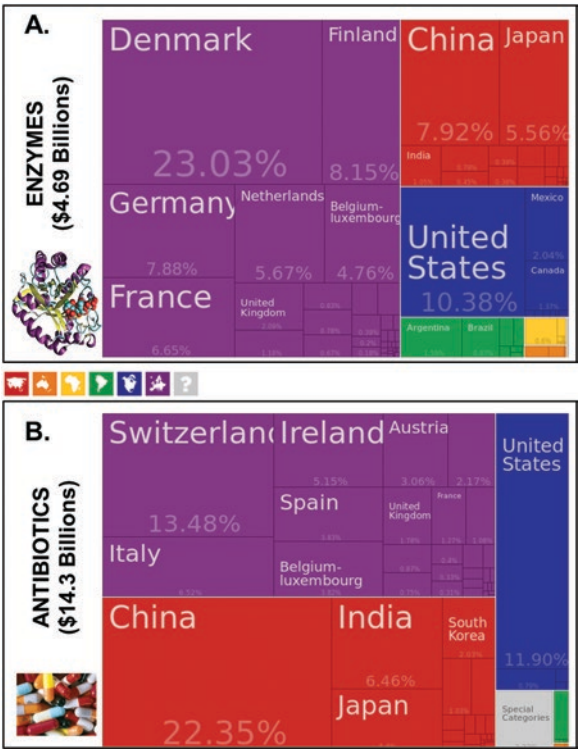

Fig. 3 World enzyme (**a**) and antibiotic (**b**) markets in 2012. Source: Observatory of Economic Complexity, accessed in August 2015

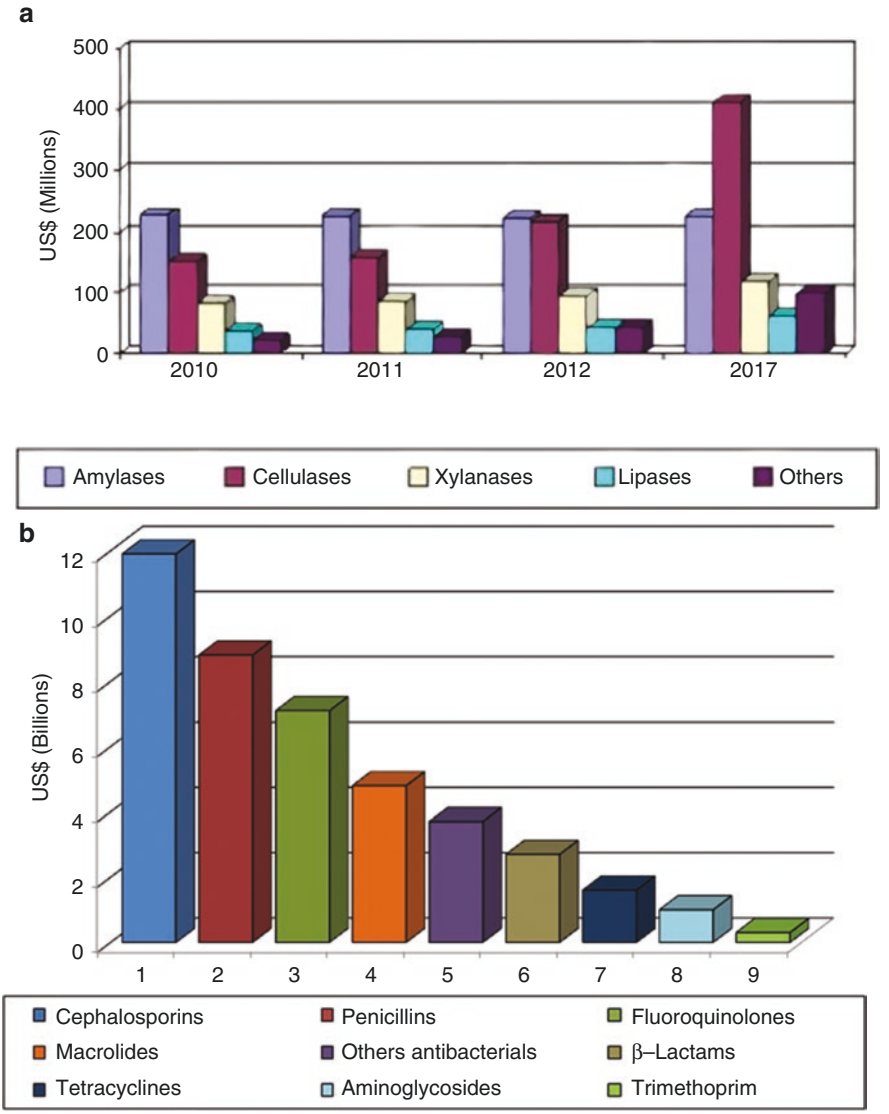

Fig. 4 Economic importance of microorganisms. (**a**) Projections of global enzyme markets for biofuels. Source: BCC Research (2013) [20]; (**b**) main commercial molecules worldwide in 2009 (adapted from Hamad, 2010 [57], with permission)

revenue by the year 2017, reaching a value of US$400 million on the world´s enzyme market. The main use of cellulases is related to the expansion of new technologies for the production of second-generation biofuels based on industrial residues, such as, for example, second-generation ethanol [59–62].

Figure 4b illustrates the worldwide revenues for commercial pharmaceuticals in the year 2009, with emphasis on the polypeptide class and cephalosporins, whose market share was US$11.9 billion [57].

Brazil is the world's second greatest ethanol producer, with vast experience in production scaling; thus, the country has an important reference role for the development of this biofuel. Annual Brazilian ethanol production has already reached 28 billion liters [63]. Of the by-products of ethanol production in the country, sugarcane bagasse, with an annual amount of around 160 million tons, should be emphasized. This lignocellulosic biomass (at present used for the cogeneration of power in production plants) has great potential to increase the production of biofuels, via second-generation ethanol, and its use could raise overall ethanol production by 40% compared with that obtained by first generation process [64]. However, there is still a need for better coordination among the various biotechnology research centers in Brazil so that the country can also be a leading second-generation ethanol producer [59, 65].

In addition to its strategic internal market for enzymes, Brazil has great potential for the production of enzymes that are used in different industrial sectors, especially considering the use of cheap raw material obtained from the country's own industrial waste. According to a recent study [58], the national capacity to produce concentrated amylases, cellulases, and lipases could reach annual levels of 31, 32, and 310 million tons, respectively; with respect to xylanases the value might hit 2.9 million tons.

Based on a rough idea of how these values are actually expressed, within the past 15 years Brazil has imported 14,401 tons of amylases and exported only 3517 tons (data obtained from the web server AliceWeb of the Brazilian Ministry of Development, Industry, and Foreign Trade, available at http://aliceweb.desenvolvimento.gov.br/; Fig. 5). Together, these values are equivalent to 0.05% of the estimated amylase production during a 1-year period, based exclusively on our own net resources (31 million tons) [58].

In 2012, the total revenues generated by the export of amylases and cellulases in Brazil were, respectively, US$7.5 and US$1.6 million, while the expenditures for these items were, respectively, US$12.3 and US$1.2 million . The world transactions for the same period for each of these enzymes were above US$200 million (Fig. 4).

Brazil's share of the total world market for enzymes is still narrow, despite its great biodiversity, as can be seen by the national data in comparison with those of the United States (Fig. 6).

Brazil occupied a strategic position in the portfolio of United States imports (Fig. 7), mainly during the period from 2011 to 2014 (fifth position). According to the Intelligence Base for US Imports and Exports (Zepol web server, available at http://www.datamyne.com/zepol-archived-trade-reports/), Brazil was the world's second largest enzyme importer. In June 2015, a total of US$6.7 million was expended, with the export revenue being around US$65 million for the same period.

The main destinations for enzymes exported from Brazil were Venezuela, Argentina, Japan, and Denmark (Fig. 8).

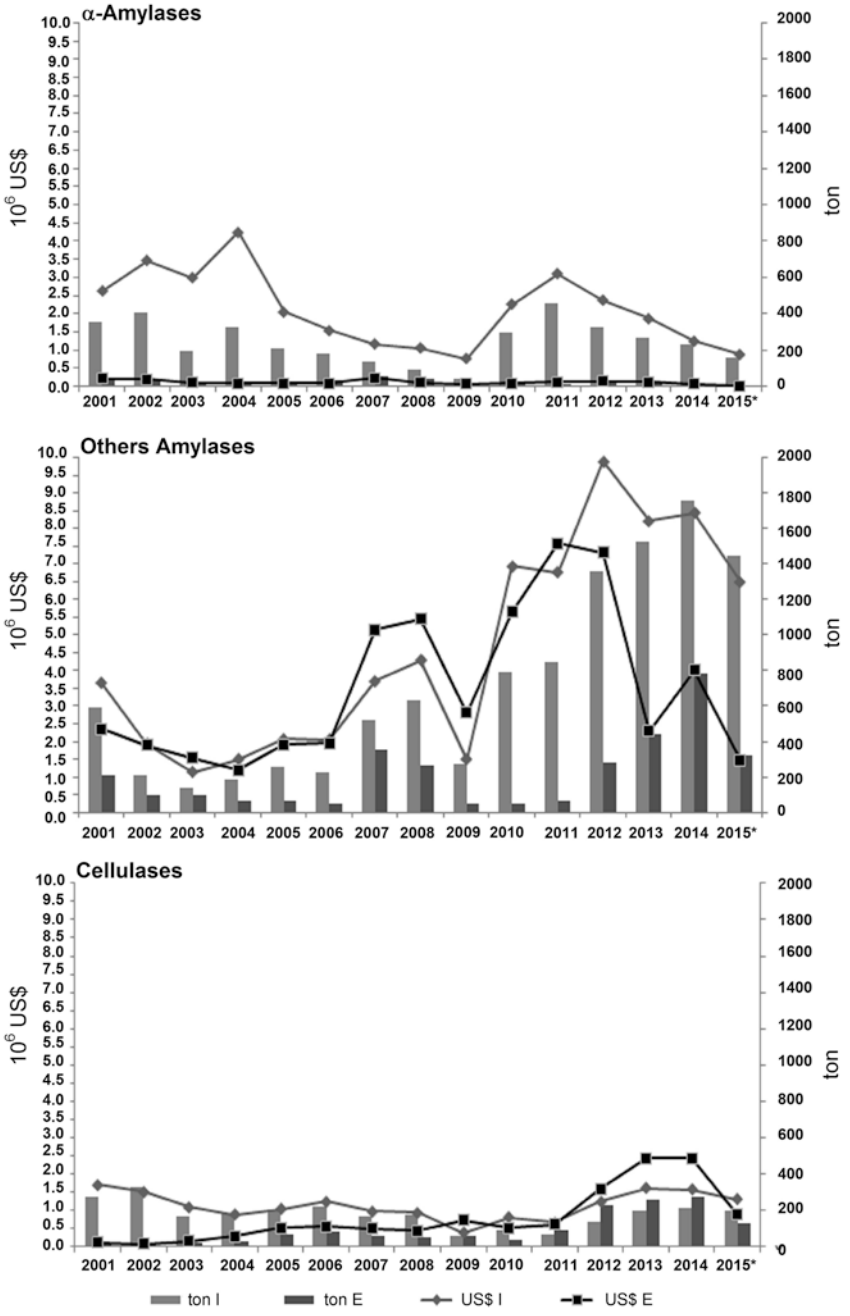

Fig. 5 Data for Brazilian enzyme import (*tonI*; US$ I) and export (*tonE*; US$ E) markets. The data were recovered from the webserver AliceWeb, using the terms "NCM 35079011" (α-amylases from *Aspergillus oryzae*); "NCM 35079019" (other amylases), and "NCM 35079041" (cellulases), in August 2015. The graphics were generated by Excel software

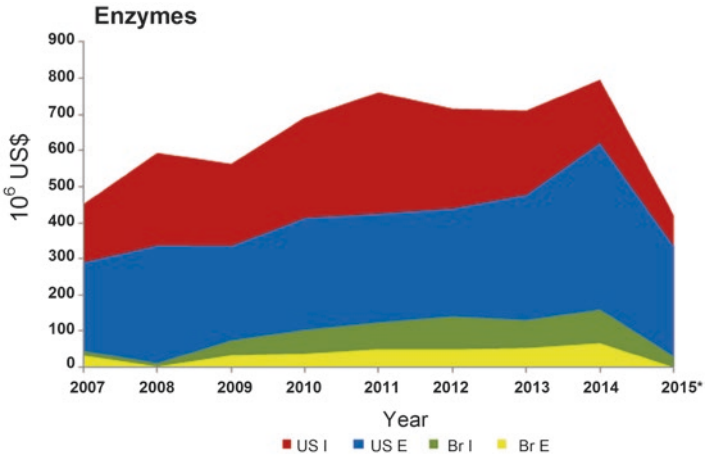

Fig. 6 Import/export data for enzymes in the United States (*USI*, *USE*) and Brazil (*BrI*, *BrE*). The data were recovered by the web servers AliceWeb (Brazil) and Zepol (United States). The data were obtained in August 2015 and the graphics were generated by Excel

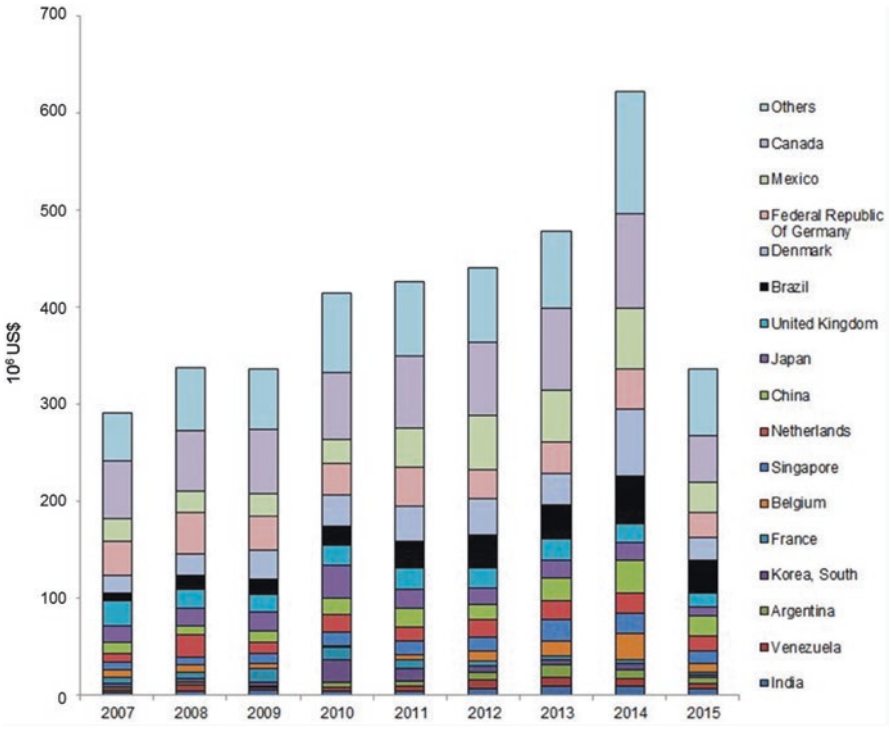

Fig. 7 Main destinations of enzymes exported from the United States. The data were recovered by the web server Zepol, using the keyword "NCM 3507" (Enzymes; Prepared Enzymes). The data were obtained in August 2015 and Excel generated the graphics

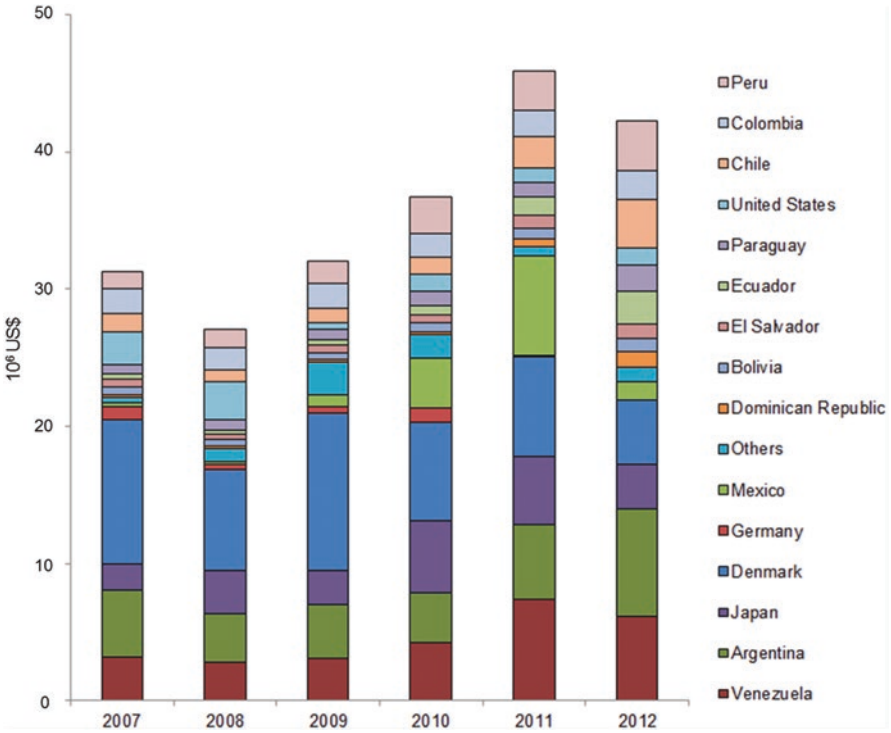

Fig. 8 Main destinations of enzymes exported from Brazil. The data were recovered by the web server Observatory of Economic Complexity, using the keyword "NCM 3507" (Enzymes; Prepared Enzymes). The data were obtained in August 2015 and Excel generated the graphics

Although Brazilian biotechnology has achieved several successful breakthrough initiatives for the development of plant cultivars, such initiatives have not taken place in the pharmaceutical industry, which is developing slowly because of the importation of the active principles of drugs [19]. Based on data from the Observatory of Economic Complexity, in 2012 Brazil exported less than 0.5% of the total world demand for commercial antibiotics, as opposed to China, which exported 22.3% of the total world demand (Fig. 3).

Despite its low export flow of antibiotics, Brazil has a strong internal market for these agents, both for human medical treatment and for agricultural use. The import and export volumes of chloramphenicol are low, in contrast to the import volumes of penicillin and tetracycline, both of which are high (Fig. 9).

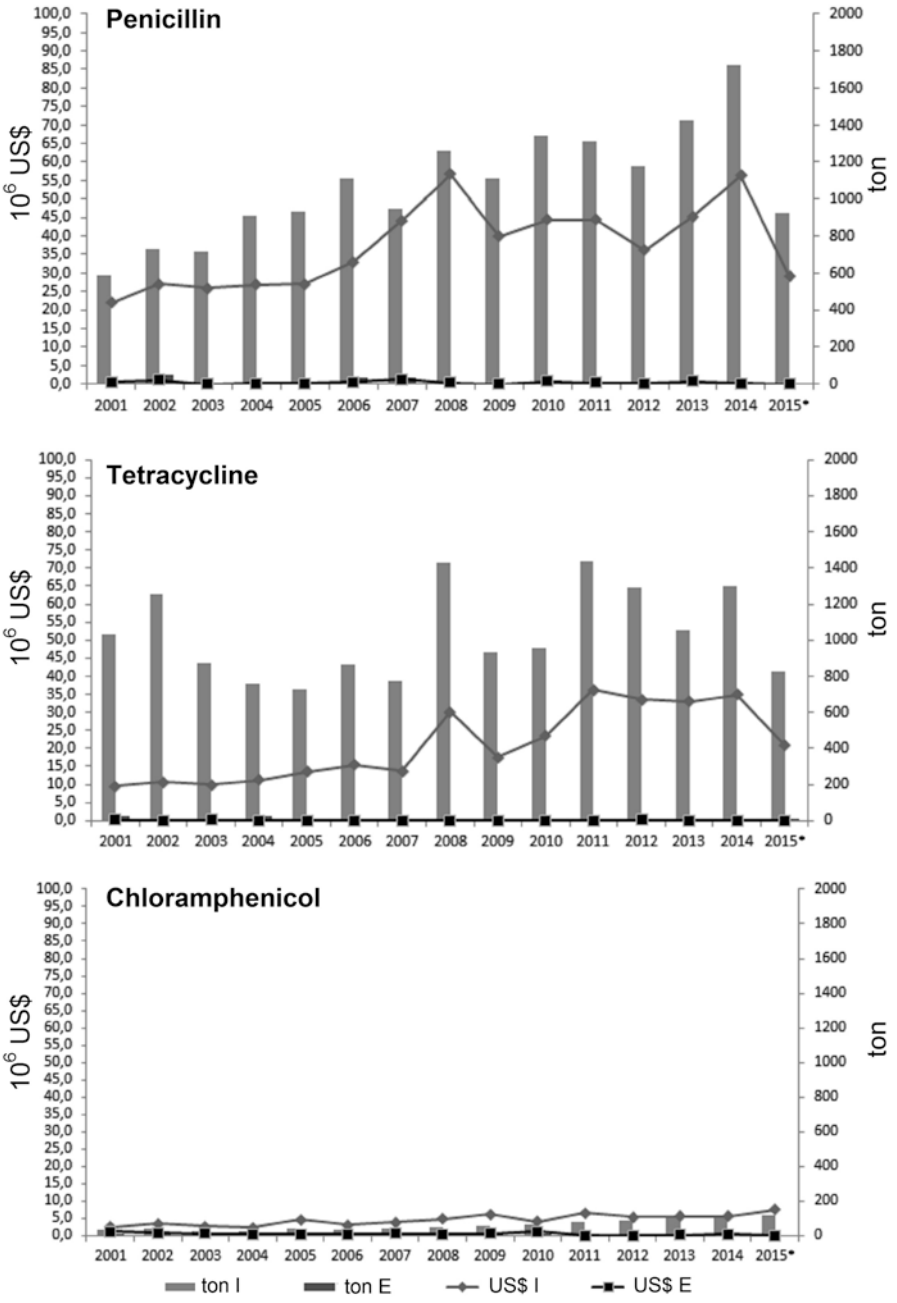

Fig. 9 Antibiotic import data (*ton I*, *US$ I*) and export data (*tonE US$ E*) for the Brazilian market. The data were recovered by the web server AliceWeb, using the key words "NCM 294110" (penicillin), "NCM 294130" (tetracycline), and "NCM 294140" (chloramphenicol). The data were obtained in August 2015 and Excel generated the graphics

The Gap Between the University and Industry: Challenges for Biotechnological Advances

Over several years of efforts and dedication, Brazilian science has gained the recognition and respect of the international scientific community [59, 66]. In fact, born in the birthplace of a developing nation, Brazilian academia seems to have been permeated by an increasing desire to find itself in the world context.

One of the first great Brazilian endeavors that have raised the country's scientific research status in international competition has been the *Xylella fastidiosa* DNA sequencing project [67]. The maturity acquired by the group in this project has generated involvement in other research approaches, such as that of cancer genome sequencing and the sugarcane expressed sequence tag project [68]. The Allelix (an anagram of *Xylella*) company is one example of this successful scientific research [66].

However, in the search for recognition, the national research goals seem to have become an entity turned to the academic world itself, centering efforts on university science and innovation, where there is still an unwise aspect regarding biotechnological potential. Academic research has progressed with its own expertise, hoping that this aspect would attract the eyes of industry [19, 69].

Despite sharing common interests, the academic and private biotechnological sectors are mostly not integrated, a situation that jeopardizes our ability to extend the Brazilian biotechnological network to reach world visibility [19]. There are 237 private biotechnology companies in Brazil, with almost 80% being in the Southeast Region; 63% of the companies have been active for only 15 years and most companies can be considered as micro or small companies (85%). Also, most companies are involved with human (39.75%) and animal (14.3%) health; while only 9.7% are related to agriculture, with 14.8% using environmental and bioenergy biotechnological solutions. Around 25% of the companies export some products, while 85% import mainly equipment and reagents [70].

The Brazilian academic sector is considered to be significant and well structured, as mentioned previously, although it is greatly centralized in certain regions of the country [59, 66]. About 1000 PhDs in science are awarded each year in Brazil, but only a small proportion of this human resource finds employment in the private sector. Consequently, the university-generated knowledge is underutilized, with less than 1% of this knowledge reaching the commercial market. This situation is rather different in other countries, with the United States accounting for 70% of the global biotechnology market [19]; this explains the results shown in Figs. 6, 7, and 8.

Biotechnology companies in Brazil do not attract Brazilian partners, as there is a lack of incentives, such as poor venture capital conditions and poor patent policies, which are considered unsuitable for large investors. Government involvement is also not appropriate, often being seen as only promises [19].

The absence of dialogue between the academic and industrial sectors is a fact. We need a bridge to unite university interests (frequently based on the number of publications) and those of the community, such as knowledge transference to industry, with rare interfaces aiming to measure this process [19, 58].

Some initiatives to improve knowledge transfer have emerged inside the universities themselves, through the "Agência Unesp de Inovação" (AUIN, São Paulo State University the Innovation Agency) and through "Agência USP de Inovação" (INOVA, University of São Paulo the Innovation Agency), both of which are specific entities that support researchers in the development and recognition of their patents. Together with these initiatives are projects to evaluate the possibility of sustainable exploration of the country's different ecosystems, such as the Biota-FAPESP Project and the Brazilian Microbiome Project [23], discussed at the beginning of this chapter.

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Bioinformatics for Microbiome Research: Concepts, Strategies, and Advances

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Abstract Advances in next-generation sequencing technologies allow comparative analyses of the diversity and abundance of whole microbial communities, and of important ecosystem functional genes, at far greater depths than ever before. However, the current major challenge for the use of this immense amount of genetic information is undoubtedly how to convert the information into rational biological conclusions. As an attempt to solve this issue, we now rely on a set of complex computational/statistical analyses, the use of which, however, could be a drawback for most researchers in the biological sciences. In this chapter, we outline the main approaches applied for microbiome studies based on high-throughput sequencing technologies and we introduce the most commonly used strategies for data handling, sequence clustering, taxonomic and functional assignment, and microbial community comparisons. We also draw readers' attention to recent advances in the microbiome research field, illustrating the Brazilian case.

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V. Pylro, L. Roesch (eds.), *The Brazilian Microbiome*,
DOI 10.1007/978-3-319-59997-7_7

Introduction

Current scientific and technological advances have revolutionized the way that we usually studied microbiological resources [1]. Since the introduction of next-generation sequencing (NGS) about 15 years ago, scientists have generated an unprecedented amount of genomic information, which has been cataloged in multiple biological databases [2–6]. However, these improvements in DNA sequencing methodologies arrived before we had the ability to comprehensively analyze the huge amount of data that was generated, and this makes bioinformatics one of the main bottlenecks in microbiome studies.

Studies that gather genomic information from single microbial populations, or even single-cell genomic studies, are useful for separating closely related strains, finding small genomic changes by comparative genomics, and disentangling the "microbial dark matter" as well (see more in [7]). These kinds of studies rely heavily on genomic annotation, which reveals information regarding a microbe's complete metabolic potential, indicating what makes this organism different from others. Therefore, precise annotation of the genome and standardization of the nomenclature of each identified gene (the term "high-quality annotation" is used in the literature) is of fundamental importance. Comparisons between genomes may provide evidence of the biological processes involved in differentiation and genomic evolution, as well as revealing important aspects of the genotype and phenotype relationship.

Besides the strategies used for analyzing single populations or cells, there are also other approaches focused on profiling entire microbial communities. With the possibility of obtaining millions (or billions) of microbial sequences from complex samples (e.g., environmental and host-associated samples), these approaches are now widely used by researchers. The computational analysis of these big datasets is now allowing us to reveal the microbial taxonomic structure in each sample—through data analyses of microbial phylogenetic marker genes, e.g., rRNA 16S (metataxonomics)—and their potential functional traits, by shotgun metagenomic (DNA) and/or metatranscriptomic (RNA) analyses. In fact, the generation of data for the target sequencing of phylogenetic markers, metagenomics, and metatranscriptomics is now reasonably well established and several DNA sequencing platforms based on different technologies are currently available [8]. However, considerable computational effort is required for the processing of NGS sequencing data and this sudden reliance on computing has been problematic for most researchers in the biological sciences. Without programming skills or expertise in computer science, researchers who rely on computational approaches are troubled by issues such as software installation and efficient software combinations, the determination of parameters, and the manipulation of large data files. Thus, to enable the systematic processing of large volumes of sequence data, including the structured storage of sampled data and metadata and the standardization of data analyses, there are fundamental requirements both for computers with a scalable structure and for well-trained bioinformaticians.

In this chapter, we intend to broaden readers' views of the main bioinformatics strategies for studying microbes using high-throughput sequencing technologies. This outline includes the most commonly used approaches for data handling, sequence clustering, taxonomic and functional assignment, and microbial community comparison. Finally, we highlight recent advances in the microbiome research field, with emphasis on the advances in Brazil.

Strategies

Taxonomic Profile Based on 16S Amplicon Data: 'Who is There?'

Picking Operational Taxonomic Units

Taxonomic identification is an important step in microbial community analyses. The robustness of these analyses depends on a series of initial processing steps, including raw data filtering, chimera identification, and the removal of spurious non-biological sequences [9]. An important concept used in microbial community analysis is the grouping of sequences into operational taxonomic units (OTUs). This concept was applied for the first time in botanical research by Sokal [10], but with the advances in molecular methods, this concept began to be used by microbiologists [11]. Multiple DNA sequences are clustered into an individual OTU by an arbitrary level of sequence identity (for example, 97% identity roughly representing genus and 95% identity representing family) [12]. The great advantage of grouping sequences into OTUs is the reduction of computational needs, once the number of sequences is reduced by picking a representative sequence from a pool of sequences in an OTU. Although this concept is widely applied and accepted by the scientific community, its application is questionable, because the similarity cutoffs applied to partial 16S gene sequences have no biological meaning and different biological entities present different identity levels. However, the lack of a better approach to deal with this issue justify its current use.

The strategy of picking OTUs has been applied since the beginning of microbial community analysis and may be used with three different options for OTU picking: closed reference-based (BLAST [13], UCLUST [14], USEARCH [14]), open-reference-based (UCLUST, USEARCH), and de novo (CD-HIT [15], Mothur [16], prefix/suffix, trie, UCLUST, USEARCH) [17] (Fig. 1). The closed-reference strategy is based on comparative identity between amplicon sequences and a reference database (e.g., Greengenes [18]) (Fig. 1a). The open-reference strategy is also based on alignment against a reference database; however, sequences that do not cluster with the reference are subsequently clustered by the de novo approach (Fig. 1b). The de novo approach is used for clustering amplicon sequences by pairwise comparison, without the need for a reference database (Fig. 1c). These algorithms are implemented in different softwares and they have been evaluated by numerous benchmarking studies [19–21]. The softwares most widely used to cluster biological

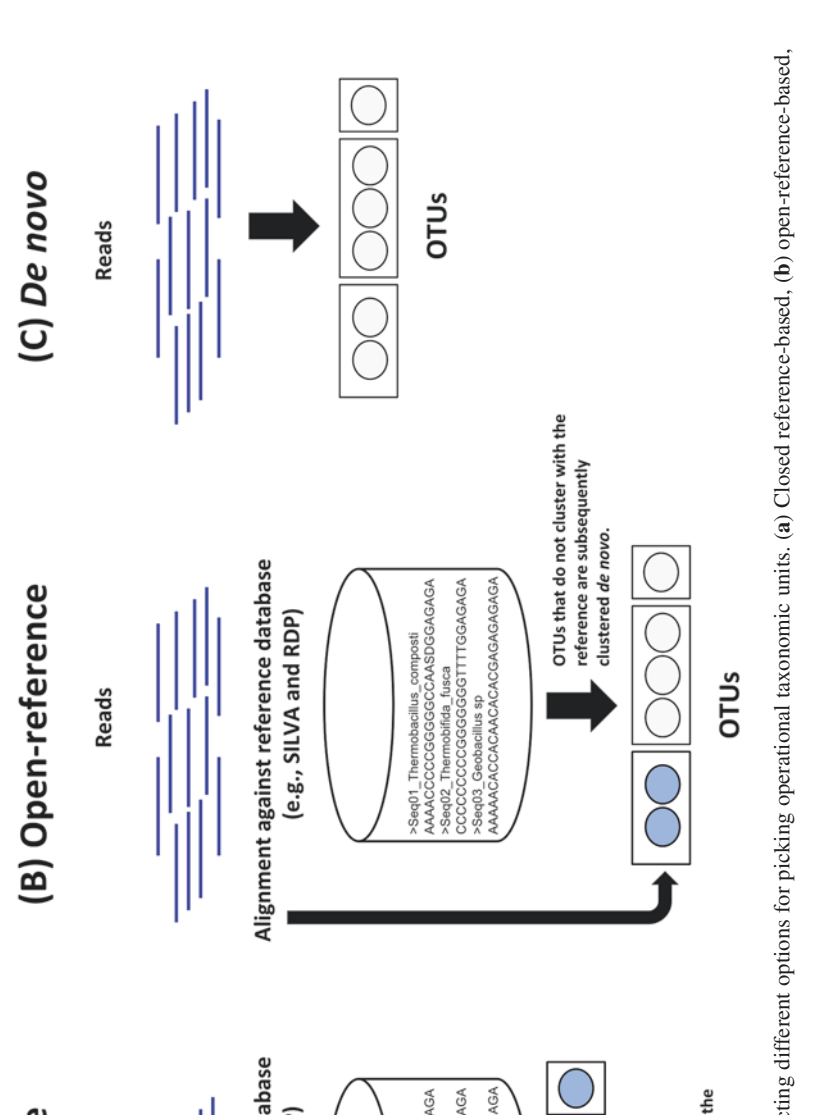

Fig. 1 Flowchart of data analysis depicting different options for picking operational taxonomic units. (**a**) Closed reference-based, (**b**) open-reference-based, and (**c**) de novo

sequences are UCLUST [14] (which is applied as a default method in the QIIME pipeline for all OTU picking approaches), Mothur [16] (picking OTUs by a de novo approach, based only on genetic distance methods), and UPARSE [22] (which uses USEARCH to pick OTUs by a de novo approach). However, none of these softwares or algorithms is free of bias, so the researcher must evaluate which algorithm or software is best for their dataset. For example, the QIIME pipeline keeps a large fraction of chimeric OTUs, inflating microbial diversity estimates [22]. On the other hand, UPARSE [22] might discard true OTUs because of its highly stringent default filtering parameters, thus making a false-negative type of error [23]. Genetic-distance methods implemented in Mothur, such as the average neighbor algorithm, seem to be the most robust approach [24], but these methods require great computational power, which might prevent the analysis of very large datasets in ordinary desktop computers. A common problem of open-reference strategies is the creation of unstable OTUs, where the cluster that a sequence is assigned to is affected by the number of sequences in the dataset [25]. Close-reference approaches generate stable OTUs; however, a considerable disadvantage of such approaches is the unavailability of complete public datasets if the approach excludes any OTUs that are not defined in a pre-existing reference dataset. The choice of the best algorithm to use depends on the biological and ecological question and the throughput of data.

Assigning Taxonomy

Several methods have been developed aiming to predict microbial taxonomy based on partial sequences of the 16S rRNA gene. The most widely used is the naïve Bayesian classifier implemented in the Ribosomal Database Project (RDP) [26]. With this method, sequences of 400 bp in length can be classified at genus level, and the method also uses bootstrap confidence scores to support the taxonomic assignment [26]. Other methods available are implemented in QIIME [27] and Mothur [16]. QIIME default classification uses only similarity among sequences to infer taxonomy [28]. Mothur uses *k*-mer counting and the Wang naïve Bayesian classifier, similarly to the RDP method [16].

Few studies have been conducted to compare the performance of the taxonomic prediction algorithms used in microbial diversity studies. Bokulich and colleagues [29] have demonstrated that the RDP classifier and Mothur provide the same results for taxonomy prediction, although the RDP classifier has the advantage of discovering novel taxa [30]. The RDP [31], Greengenes [18], and SILVA [32] are the main databases used for taxonomy assignment. The RDP database covers 27 phyla (RDP Release 11), including those that are uncultivable (e.g., Bathyarchaeota archaea). Greengenes had its last update in 2013, with the implementation of the tax2-tree tool to transfer taxonomy to a phylogenetic tree [33], but this database does not contain any new recently described phyla [34]. SILVA is the most complete database, covering all phyla in its last update (Release 128).

Measuring Alpha and Beta Diversity

Several tools are available to measure the alpha and beta diversity of an ecological community. These include statistical packages (e.g., Vegan [35]) that are implemented in general pipelines, such as QIIME and Mothur. Alpha diversity is the local diversity of a single sample and beta diversity is the diversity among different samples [36]. Specific methods are available for determining each type of diversity (alpha or beta). Alpha diversity indexes, such as the Shannon diversity index [37] and the Simpson diversity index [38], measure the species richness and evenness of the community structure. On the other hand, beta diversity indexes are applied for direct comparisons of the abundance profile or presence/absence of OTUs using distance metrics, either by counting methods (e.g., Bray-Curtis [39]) or by phylogenetic reconstruction methods (e.g., UniFrac [40]). The advantage of using phylogenetic approaches for comparisons of microbial communities is the possibility of using low sequence coverage. However, the use of methods based on absolute counting needs high sequence coverage to improve accuracy [36].

Functional Profile Based on Metagenomic and Metatranscriptomic Data: 'What can/do they do?'

Gene Prediction and Functional Gene Annotation

Finding the encoding genes in metagenomic DNA sequences is the first step in predicting protein function. This is a big challenge in bioinformatics, because the prediction needs to be performed on short fragmented reads (incomplete genes). Many softwares, such as Ophelia [41], FragGeneScan [42], MetaGeneMark [43], and Glimmer-MG [44] have been developed to annotate short metagenomic reads. For example, Ophelia [41] uses fragment length-specific models for gene prediction, while FragGeneScan [42] also combines sequencing error information and codon usage in a probabilistic model. This information improves accuracy in the prediction of coding sequences.

Methods based only on homology, such as BLASTx [13] and DIAMOND [45], do not use ab initio gene prediction. Homology-based methods allow searching for similar sequences in protein databases [46] e.g., non-redundant database (nr/National Center for Biology Information [NCBI]). The similarity search is slower than the direct comparison of ab initio predicted sequences, because the sequences must be translated into the six reading frames. Currently, DIAMOND is an alternative for annotating metagenomic reads, because of its speed in annotating millions of sequences in a short time [45].

Assigning Taxonomy

Assigning the taxonomy of short metagenomic reads may be done in two ways: (i) by approaches based on comparative analysis with all genome regions, including conserved housekeeping genes and highly variable genes; or (ii) by approaches based only on similarity to conserved housekeeping genes. The first option is used in most softwares, including homology-based methods such as MEGAN [47]. MEGAN uses an output BLAST score (best hits) search for taxonomic prediction from the lowest common ancestor. In this case, all metagenomic reads are aligned against a protein database of all microbial genomes deposited in the NCBI, for example. The limitation of this algorithm is the low speed of the BLAST search, which uses millions of reads. However, other softwares have been developed to align metagenomic reads against databases; for instance, DIAMOND [45], Kraken [48], and Centrifuge [49]. There are also taxonomic prediction methods based on comparisons of each read against a clade-specific gene marker catalog, such as that in MetaPhlAn [50].

Genome Assembly from Metagenomic Data

Currently, metagenomes are analyzed by two main approaches: gene-centric and genome-centric. Gene-centric approaches are based on unassembled individual genomes and individual genes are predicted from short fragmented reads [51, 52]. On the other hand, genome-centric approaches consider individual microbial populations reconstructed by total metagenome assembly [53, 54].

Strategies based on gene-centric analysis are limited by the length of short metagenomic reads. Although specific software exists for gene prediction based on short sequences, assembling short reads into contiguous sequences (contigs) is more powerful. Currently, softwares such as MetaVelvet [55] and metaSPAdes [56] subdivide short reads in graphs per *k*-mer lengths (*De Brujin* graphs). There are several methods for assembling short reads; however, here we focus only on De Brujin methods, because they are the most commonly used metagenomic assemblers. MetaVelvet divides the graph into sub-graphs and each sub-graph represents an individual genome [55].

Post-assembly analysis may enable improved gene prediction and functional annotation. Because contigs are longer than the usual short reads, they can be used for the reconstruction of near, partial, or complete microbial genomes of uncultivable bacteria [53]. This approach is known as “binning”. The main idea of binning is the clustering of assembled contigs into individual populations according to the compositional content of sequences, such as guanine-cytosine (GC) content, tetranucleotide frequency, and sequence coverage [57]. Some softwares available for binning and reconstructing individual microbial genomes from metagenomic data are MaxBin [57], GroopM [58], and MetaBAT [59].

3. Advances and Trends: Establishment of Microbiome Study Consortiums and Their Impact on Bioinformatics. The Brazilian Case

Overcoming the challenges in any field frequently requires groups working together [60]. Assembling heterogeneous groups, with multiple fields of expertise, seems to be, so far, the best strategy to tackle complicated problems such as microbiome studies. By definition, the microbiome is:

> *the entire habitat, including the microorganisms (bacteria, archaea, lower and higher eurkaryotes, and viruses), their genomes (i.e., genes), and the surrounding environmental conditions. This definition is based on that of "biome," the biotic and abiotic factors of given environments* [61].

Thus, the term "microbiome" refers not only to the microorganisms, but to all the interactions in which they are involved. In this view, given the interdisciplinary nature of the field, microbiome studies must rely on consortiums to gather information and human resources for dealing with the current avalanche of microbiome data.

In the past ten years, microbiome surveys targeting different subjects have been developed, shedding light on and expanding our knowledge of the microbial world, as well as fostering the development of novel bioinformatics tools and workflows to address a plethora of demands in microbiome studies. For instance, some human-related microbiome projects [62], including the Human Microbiome Project [63] and the MetaHIT consortium [64], as well as others related to soil, such as TerraGenome [65], or more general ones such as the Earth Microbiome Project [66], have not only increased our knowledge of the factors driving the assembly and function of microbial communities in various samples, but have also emphasized the importance of the microbiome field for scientific and technological advances, as well as suggesting standards [67] to be applied by the scientific community.

In a mega-diverse country like Brazil, with specific local features, an initiative already exists—The Brazilian Microbiome Project (BMP; http://www.brmicrobiome.org/) [68]. This Project forms the basis of a strong national program, and it is ready to be prompted worldwide [69]. In summary, this initiative aims to assemble a consortium/database devoted to the collating of information regarding microbiome studies undergone in Brazil, evaluating the methods being applied to these studies, defining and standardizing methods for data analyses, designing a structured database comprising sequences and metadata, and developing user-friendly tools to help in the distribution of data-analysis capacity. This initiative represents a solution for the inequality of research opportunities in Brazil, not only providing access to analysis guidelines and partnerships, but also helping to instruct researchers in bioinformatics, a field that is well known for overcoming some of the complications in the deciphering of genomic big data. The BMP has achieved several of its primary objectives, being able to define standards for data analysis for microbial profiling used and recognized in Brazil and abroad [70], create methods for making

data from different sequencing technologies compatible [71], develop user-friendly data analysis tools [72], and generate proficiency in the analysis of microbial community data.

Now, the next step in Brazil is the creation of a National Institute of Science and Technology for Microbiome studies, the INCT-Microbiome (http://inct-microbiome.org). The INCT-Microbiome will extend BMP endeavors, uniting microbiome investigators to support the development of the unprecedented knowledge base of Brazil's microbial resources. The INCT is focused on increasing information about the microbial community in Brazil through democratizing access to technology (sequencing generation and data analysis), highlighting the importance of microbiome studies, advising on the creation of new projects, and driving resources for new research. The INCT-Microbiome is structured to optimize the exploration of microbial communities associated with specific plant and animal hosts, many unique to Brazil, as well as microbial communities associated with terrestrial and aquatic environments, including those with links to human health. To achieve these goals, the INCT is based on a structured organization model comprising specific committees from various research domains, with other committees focused on training in bioinformatics and the transfer of knowledge and technology. This organization will be fundamental for translating microbiome research into socioeconomic benefits [73].

Acknowledgments Both Leandro Nascimento Lemos and Victor Satler Pylro received fellowships from FAPESP (São Paulo Research Foundation) (Processes 2016/18215-1 and 2014/50320-4 and Processes 2016/02219-8 and 2014/50320-4, respectively). All authors are supported by the Brazilian Microbiome Project (http://www.brmicrobiome.org/).

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