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Diversität der intestinalen Mikrobiota am Beispiel der Darmflora einer Anorexia Patientin

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1. Introduction

Gut flora

The total of all microorganisms residing in our gastro-intestinal tract is called the gut flora. In the last years many studies have shown the significance of this flora for humans. There are 10-100 times more bacteria residing in our intestine than the total number of cells in our body. 10^{14} bacteria out of more than 1000 species, and thereof at least 160 species per individual, are colonizing our intestine [1]. Out of the 70 known phyla, only 7 are found in human intestine until now and therefrom *Firmicutes* and *Bacteroides* constitute 90% [2]. In addition there are eukaryotes, archaea and viruses.

The composition of bacterial species differs from individual to individual and is dependent on many different factors. The most important are ethnicity, geography, genetics and later on age and diet. Furthermore in present times there are also factors like antibacterial treatment, hygiene and lifestyle which can influence and disturb the natural balance of gut microflora.

Humans are born with a sterile intestine. The first essential influence on the constitution of our intestinal microbiota is the way of delivery (vaginal versus caesarian) [3][4], followed by the infant's nutrition, meaning over all whether the mother is breastfeeding or not [4]. Probably the most important part for the development towards an individual microbiota is done in the first years of living and this is the time where it is the most influenceable.

Bacteria in our intestine have to deal with concurrence between different species, intestinal peristaltic and the human immune system. They adapt to these factors in a permanent dynamic. This is how a balance is created between those bacteria which establish themselves and constitute our gut microbiota. They develop adhesion systems to avoid being purged out by intestinal peristaltic [5]. Between the human immune system and the bacterial antigens a permanent exchange is happening. It has been shown that *Bacteroides* living in intestine make a DNA-transfer with their environment. In this way they make themselves a unique line ideally adapted to each host [6].

The symbiosis allowing the survival of gut bacteria has an important benefit for humans:

The major beneficial functions of intestinal bacteria are the metabolism of otherwise indigestible polysaccharides such as cellulose and some starches [7], the production of a high part of short chain fatty acids which are very important for the intestinal mucosa [8], the production of vitamin K and certain amino acids [9] and a protective effect on the intestinal mucosa [10].

The intestinal microbiota with its direct influence on intestine, digestions, energy balance and immune system has also an impact on our whole body. Early studies on the intestinal microbtiota have shown correlation between certain bacterial species and overweight: The ratio *Bacteroides/Firmicutes* is reduced in overweight individuals [11][12] and bacteria such as *Lactobacillus* are found to be increased [13]. Furthermore there was a report suggesting a link between a certain composition of intestinal microbiota and inflammatory bowel disease [14], irritable bowel syndrome and colorectal carcinoma [15].

The intestinal bacteria can also influence many extra intestinal organs. For example a link is hypothesized between intestinal microbiota and diabetes [16], allergies [17] and lymphomas [18]. Even on behavior, psyche, memory, learning, depression, cognition and pain the intestinal flora seems to have an impact [19][20].

How can this influence on health by gut bacteria be explained?

As mentioned above, there is a permanent exchange between gut flora and immune system. The consequence is finally a toleration of symbiotic bacteria on one side and a regulation – in some cases a deregulation- of the immune system on the other side. And this is probably where the most important interaction between bacteria and other body systems is made. The detailed mechanisms of these interactions are not yet completely understood but there are already many approaches:

Intestinal bacteria are foreign organisms to our body and possess antigens which are recognized from our dendritic cells. Conditioning of the dendritic cells in our intestinal mucosa occurs by intestinal bacteria [21]. Then dendritic cells present the antigens to T-cells in spleen and lymph nodes, which stimulate differentiation of leukocytes in the bone marrow. Pathogen-associated microbial pattern (PAMPs) and differentiation of the immune system is stimulated [22].

To avoid their elimination by local immune reaction, intestinal bacteria develop

mechanisms like inhibition of the immune regulating transcription factor Nf-kB [23], tolerance against endotoxins [24] or production of metabolites that take influence on the immune system. Short-chain fatty acids are produced by bacteria such as *Roseburia*, *Eubacterium*, *Bacteroides,* und *Faecalibacterium* and have an anti-inflammatory effect by inducing differentiation of regulatory T-cells (Treg-cells), which play an important role in autoimmune diseases by suppressing immune system to enable self-tolerance [25]. In intestine and CNS, Treg-cells prevent inflammation through IL-10 [26]. Polysaccharide A and niacin produced by certain intestinal bacteria also trigger Treg-cells [26][27]. Intestinal bacteria also play a role in the metabolism of arachidonic acid into leukotrienes and prostaglandins. These two products are signaling molecules of inflammation processes in our body. *Bacteroides thetaiotaomicron* for example is a bacterium of the intestinal flora which increases the level of prostaglandin in mice [28]. In general most of these processes are observed in mice. This does not always allow inference on human beings. Nevertheless these exemplary listed mechanisms give us a view into the effect of intestinal microbiota on our immune system and thereby connected diseases.

The functional association between gut and CNS is well known: With the N. vagus digestion is controlled and gut sensations are carried to the CNS. Through ghrelin, neuropeptide Y, peptide YY and cholecystokinin the intestine gives information about hunger and satiety to the CNS [29]. Interestingly messenger molecules can be influenced by the intestinal microbiota [32].

Enterochromaffin cells of the intestinal mucosa synthesize a large part of serotonin available in our body [30]. Serotonin does not only regulate digestion but is also responsible for multiple processes in other systems such as mood, behavior and psyche [31]. Production of serotonin and dopamine can be directly stimulated or made by bacteria [22]. Furthermore neuropeptides can be neutralized by antibodies. And this process can be influenced by bacteria with certain antigens, which increase the production of these antibodies by molecular mimicry [33].

Antibodies against neuropeptides stimulating hunger also seem to play a role in anorexia nervosa [33]-[36].

Anorexia nervosa

Anorexia nervosa will be described in detail in this chapter because the presented research project was made on a stool sample collected from an anorexic patient.
Anorexia nervosa is an eating disorder. Affected patients induce a loss of weight by very restrictive food intake and active measures such as use of laxatives, vomiting or extreme physical activity. Their body weight is at least 15% lower than normal weight. This disease is getting more important and has overall lifetime prevalence of 0,6% and the prevalence in women is 0,9% [37]. Comorbidity of this eating disorder is high because of underweight [38]. Mortality is around 10% and so the highest under psychological diseases [39].
In 1996 already it was hypothesized that certain neuropeptides are jointly responsible for the genesis of anorexia nervosa [40]. Later a correlation between antibodies against α-MSH and anorexia nervosa has been reported [35], respectively especially to the psychological characters of this disease like social insecurity, interpersonal distrust, impulse regulation and asceticism [36]. The fact that intestinal microbtiota can influence the production of antibodies by molecular mimicry, has finally proved in a study by Fetissov et al. [33]. It has to be mentioned that probably in all humans, neuropeptides with influence on food intake and emotions are physiologically regulated by antibodies [34]. But Ghrelin, an appetite stimulating hormone, is interestingly found in a higher level in free form in plasma of anorexic patients, because there are less antibodies against this hormone than in healthy subjects [41]. A reduced feeling of hunger in anorexia patients could be explained through a resistance against Ghrelin developed by this phenomena.
Supposedly the intestinal microbiota is influenced by the very restrictive food intake. A study showed that *Methanobrevibacter smithii* was found increased in the intestine of anorexia patients compared to healthy subjects [13]. *M. smithii* is able to metabolize H2 and CO2 to methane, which improves the bacterial metabolism and therefore the energy balance [42].
Apart from these discoveries there is not much known about special characteristics of anorexia nervosa patients' intestinal microflora. The above described effect of antibodies against neuropeptides has indeed been assigned to certain bacterial

populations [33], but we don't know yet if these are reduced or increased in anorexia nervosa patients.

However several metagenomic studies on the microbiome of undernourished children have been published [43]–[46]. Correlations between certain bacterial groups and children's weight have been found in all of these studies: Transplantation of intestinal microbiota of a undernourished child into germ-free mice resulted in a loss of weight for these mice [45]. On the other hand the microbiome of undernourished children could be changed by the intake of Ready-to-Use Therapeutic Food (RUTF) [45]. This is a proof that body weight is influenced by gut flora on one side and that the gut flora is dependent on diet on the other side. Protective bacteria for the intestinal mucosa which do either produce short-chain fatty acids such as *Butyrivibrio*, or do have anti-inflammatory effects such as *Eubacterium* and *Faecalibacterium*, were missing in undernourished children in India [43]. *Bacteroides*, which induce a loss of weight in overweight individuals, were increased in undernourished children [44] and were reduced after intake of RUTF in an other study [45], which let us suppose a very direct adaption of microbiome to diet. *Lactobacilli* and *Bifidobacteria* levels were increased after intake of RUTF [45]. These bacteria seem to have a positive effect concerning inflammation and pathogenic bacteria in human intestine. Pathogenic bacteria as well as bacteria linked to inflammatory bowel disease or irritable bowel syndrome in other studies, have been found in undernourished children's intestine [45][44]. Once the microbiome is altered due to undernourishment it is influencing energy balance and health negatively. In consequence there is a hope to find an other therapeutic approach apart food intake for malnutrition by using certain bacterial groups.

In general, considering these links between gut flora and organism found until now, one can suggest that a part of our modern civilization diseases can be explained by the altered constitution of our intestinal microbiota. A disturbed balance is resulting in a reduction of diversity, because different bacterial lines can not live next to each other anymore. But it is especially the large diversity which is important for human beings. Pathomechanisms, through which intestinal bacteria influence health and behavior, as well as resulting therapeutic approaches are currently intensively researched.

Metagenomics versus Culture and the field of unknown bacteria

A big progress has been made in the last twenty years in the field of metagenomics. With this new technique the step of culturing can be skipped and samples can be examined directly on the contained genes. In a relatively short time a very large view on the complete genes of all in this sample living microorganisms can be obtained – and depending on the technique either about metabolic functions of the microsystem, either about the contained species. Cultivation seemed to be unnecessary for several years. But in metagenomics also there are gaps. First, bacteria contained in a very low concentration are hardly captured by metagenomics [47]. Secondly, one study of the laboratory in which the present research has been made, showed that from the same 16 samples metagenomics and gram-straining obtained discrepant results and that with metagenomics an important part of gram-negative bacteria could not be captured [48]. Next to broad metagnomic studies, cultivation seems therefore to stay an essential element in research in the aim to obtain an as complete image as possible of the examined sample, in our case of the gut flora.

With metagenomics,microbiology gained new insights in the world of bacteria: Less than 1% of all existing bacteria could have been cultivated so far [49] and through metagenomic studies a large number of bacteria has been identified, which have not been isolated by culture until now. There are whole phyla of which no representative species could be cultivated [50]. These bacteria are classified as „uncultivable“ and thus seem to be accessible by metagenomics only. On one hand this points out the unique role of metagenomics. On the other hand those „uncultivable“ bacteria give a new challenge to microbiology. Many research projects work to solve this problem by developing new culture conditions for those bacteria and have success in this effort [51][52]. The description „uncultivable“ is therefore not definitive – it is rather visualizing the large unknown field of bacteria.

Finally, unknown bacteria also bring a challenge to metagenomics. It concerns unclassified gene segments, which cannot be assigned to known genes. This can be caused by a too short segment and by still existing technical inaccuracy and limits of metagenomics. But it can also be possible that these unclassified gene segments do not

have a correspondent known segment in the database, can therefore not be classified and must belong to yet unknown bacteria. Whether by culture or metagenomics: the biggest part of bacteria is still unknown.

This unexplored field is also present in gut microbiota, although it is relatively small compared to environment. In human gut flora, 70-90% of the bacteria have not been cultivated yet [55] and out of all genes from respective samples more than half cannot be assigned. The latter has been deduced of „An integrated catalog of reference genes in the human gut microbiome“ [56], in which genes of already published and of new studies have been integrated. This makes a pool of more than 1200 samples from three continents. The collected examined genes were compared to 3,449 reference-genes of bacteria and archaea. Here 21,3% could be assigned only on phylum level and 44,4% to a genus or species. In the „Genomes Online Database“ metagenomic and genomic results are collected and recapitulated worldwide [57]. All following listed numbers and percentages are taken from the internet page on January 12th 2016:
From all examined ecological systems, samples isolated from humans represent 23%. 37,4% of these microorganims isolated from human are obtained from the digestive system, and it has to be considered that further 37,1% remain unclassified. This shows that by far most microorganism in humans have been isolated from the intestine. Moreover from no class of organisms as many microorganisms have been isolated as from human digestive system. 5,8% of all genomes in the collection GOLD were obtained in the Human Microbiome Project. This means that a considerable part of unknown bacterias can be isolated from human stool samples.
This is how within two years 31 new species have been discovered in 4 examined human stool samples by cultivation [47].
With its large diversity of bacterial species, the gut flora can provide new insights into the yet relatively unexplored field of bacteria. To obtain a large view on bacterial species living in human intestine, the following project has been initiated by a research group of Prof. Raoult in the Research Unit in Infectious and Tropical Emergent Diseases, Marseille, France:

Culturomics project and aim of this thesis

The project is concentrating on two aspects: the inter-individual and the individual diversity of the gut flora. To explore both aspects, stool samples have been collected and examined firstly from different continents [47], from humans with and without antibiotic treatment [58], from overweight [47] and normal weight persons, in this work from an anorexia patient, and even from a gorilla [59] to get a big variation of influencing factors on the constitution of gut flora. Secondly many different culture conditions have been applied on each sample with the aim to isolate the most possible bacteria from each sample. In parallel, DNA extracted from the samples has been amplified on the 16S rRNA-sequence and sequenced to identify on metagenomic level the species contained in the stool sample. This approach is made to complement metagenomis and culturing with each other, to minimize respective gaps and to obtain the most possibly complete view on the microflora from one single individual.

The stool sample I examined as a part of this project was obtained from a 21-year old french anorexia nervosa patient. Which bacteria have been identified in this stool sample by metagenomics or culturing, in which culturing conditions they were isolated, the classification of the identified bacterial species, as well as a comparison of the two methods have been published in „Culturomics identified 11 new bacterial species from a single anorexie nervosa stool sample". This publication is following in chapter 2.1. Further articles describe the bacteria which have been newly discovered in this work. One of these articles is included in this dissertation (chapter 2.2.). Four more can be found in NCBI and the six left are in progress.

2. Material and methods / results.

2.1. Publication I:

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Culturomics identified 11 new bacterial species from a single anorexia nervosa stool sample

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ABSTRACT

The rebirth of the bacterial culture has been highlighted successively by environmental microbiologists, the design of axenic culture for intracellular bacteria in clinical microbiology, and more recently by the human gut microbiota studies. Indeed, microbial culturomics (large scale of culture conditions with identification of colonies by MALDI-TOF or 16S rRNA) allowed to culture 31 new bacterial species from only 4 stools samples studied.

We performed microbial culturomics in comparison with pyrosequencing 16SrRNA targeting the V6 region, on an anorexia nervosa stool sample because this clinical condition has never been explored before by culture while its composition has been observed atypical by metagenomic.

We tested 88 culture conditions generating 12,700 different colonies identifying

133 bacterial species with 19 bacterial species never isolated from the human gut before, including 11 new bacterial species which genome has been sequenced. These 11 new bacterial species isolated from a single stool allow to extend more significantly the repertoire in comparison to the bacterial species validated by the rest of the world during the 2 last years. Pyrosequencing indicated a dramatic discrepancy with the culturomics results, with only 23 OTUs assigned to the species level overlapping (17% of the culturomics results). Most of the sequences assigned to bacteria only detected by pyrosequencing belonged to *Ruminococcaceae*, *Lachnospiraceae* and *Erysipelotrichaceae* constituted by strictly anaerobic species, indicating the future route for culturomics.

This study revealed new bacterial species participating significantly to the extension of the gut microbiota repertoire, first step before to connect the bacterial composition with geographic or clinical status.

INTRODUCTION

Microbial culture has been neglected in clinical microbiology since few years compared to molecular tools [60]. This was particularly relevant for the gut microbiota study which is currently only studied by metagenomic and 16S rRNA pyrosequencing, abandoning progressively new culture conditions design [61]. This has been generalized in microbiology excepted for the fastidious and intracellular bacteria with the development of axenic culture [62][63]. Nevertheless, environmental microbiologists have continued to develop empirical strategies using respectively diffusion chamber and isolation chip allowing to enlarge dramatically the repertoire of cultured microorganisms [64]–[66]. Indeed, thanks to the design of new culture conditions and the improvement of identification methods, the number of bacterial species increased from 1,791 in 1980 to more than 12,000 in 2013 [60], [67]. We have recently used a large scale of culture conditions allowing to promote the growth of fastidious species or conversely to inhibit the growth of dominant bacterial populations [47]. The identification strategy was based on MALDI-TOF followed by 16S rRNA amplification and sequencing for the misidentified strains because of previously unknown spectra or of new bacterial species (microbial culturomics) [47]. The first culturomics study,

testing 32,500 colonies by MALDI-TOF, allowed to identify 340 bacterial species including more of half first described from human gut, and 31 new bacterial species which genome has been sequenced and a majority of these has been already described [68]–[79]. In addition this culture approach has allowed in 2012, 3 world records in Microbiology, the largest human bacterial genome (*Microvirga massiliensis*, 9.3 Mb), the largest human virus (Senegalvirus, 372 Kb) and the largest human archaeal genome (*Methanomassiliicoccus luminyensis*, 2.6 Mb) [80], [47].

In order to optimize our chances to recover unknown species, we have previously selected atypical human stool samples because of geographic provenance (Senegalese or French individuals), or because the patients were treated by large antibiotics regimen which changing the microbiota [81], [82]. In the aim to describe new microorganisms from human, we propose to continue to apply in different samples microbial culturomics, a time-consuming technique because it requires to test at least 10,000 colonies in each stool samples studied [47][81].

Herein, we propose to study for the first time an anorexia nervosa stool sample by culturomics because the gut microbiota composition in these patients, previously explored by molecular techniques and appeared atypical [13]. The new bacteria cultured, will not necessarily associated to the status of the individuals studied. Nevertheless, we propose a pioneer study allowing, foremost, to describe new bacterial species. A possible link with clinical condition or geographic provenance could be secondarily studied when the repertoire will be more comprehensively completed.

MATERIALS AND METHODS

Patient stool collection

The analyzed stool sample was obtained from a 21-year-old French Caucasian female who had suffered from a severe restrictive form of anorexia nervosa since the age of 12. Despite continuous nutritional and psychiatric support, the natural history of her disease was characterized by an absence of clinical remission (BMI fluctuating between 10 and 15 kg/m^2) and a succession of acute episodes inducing life-threatening malnutrition and the need for hospitalization in critical care units. At the time of sample collection, she was hospitalized in the Nutrition unit of our hospital due to recent

aggravation of her medical condition. Her weight was 27.7 kg, and her height was 1.63 m (BMI: 10.4 kg/m^2). The stool sample was collected on her first day of hospitalization, before the introduction of tube feeding. The dietary habits of the patient were surveyed and were mainly based on vegetables and fruits and milk product.

We collected 97 g of stool from the patient, which we aliquoted into 1 g samples and stored at -80°C immediately after collection because a low temperature has been reported as the best condition for longtime conservation [83]. The patient's written consent and the agreement of the local ethics committee of the Institut Fédératif de Recherche 48 (IFR48) were obtained (agreement number 09-022, Marseille, France).

Microbial culturomics:

Culture:

Each gram of stool was diluted in 9 ml of DPBS and inoculated into different culture media under variable conditions in a dilution series ranging from 1/10 to $1/10^{10}$. To isolate bacteria, referring to a preliminary study of the human intestinal microbiota, we inoculated stool samples into 88 preselected culture condition treatments, which produced a high diversity of isolated bacteria (Table S1). These culture conditions are based on multiple different physicochemical conditions, atmospheres, the use of *E. coli* phage, and passive and active filtration, including preincubation in blood culture bottles and utilizing rumen fluid and fresh sterile stools, with the aim of selecting for minority bacterial populations. Each set of treatment conditions was observed at least on day 1 and after one week, 2 weeks and 1 month of incubation to isolate colonies. Blood culture bottles were monitored until two months after inoculation. In addition, we applied 12 supplementary culture condition treatments. Among them, we developed empirically new culture media based on randomly chosen products belonging to the dietary habits of the patient: banana, camembert and yogurt.

Identification by mass spectrometry (MALDI-TOF):

Each of the 12,700 samples was covered with 2 mL of matrix solution (saturated α-cyano acid-4-hydroxycinnamic in 50% acetonitrile and 2.5% trifluoroacetic acid). This analysis was performed using a MICROFLEX spectrometer (Bruker Daltonics) according to the manufacturer's recommendations. For each spectrum, a maximum of 100 peaks were used, and these peaks were compared with

the computer databases at the Bruker base and the base-specific laboratory at La Timone hospital. We previously updated our database with the spectra of the new bacterial species cultured during our previous study. An isolate was labeled as correctly identified at the species level when at least one spectrum presented a score ≥ 1.9, and a spectrum had a score of ≥ 1.7 [84]. Every non-identified colony was verified three times. When a strain remained unrecognized, the 16S RNA gene was sequenced as previously described. All of the spectra of the species identified based on 16S rRNA sequencing have been added to the database. The software MALDI BIOTYPER 3 was a helpful tool for the classification of the non-identified isolates based on comparison of their spectra. Only one strain per group of strains with similar spectra was sequenced, while the other strains were verified via MALDI-TOF after adding the spectrum to the database.

DNA extracted from fungi isolated from plates was amplified with the primers ITS1/ITS4R and identified by direct sequencing (as described below).

16S rRNA amplification and sequencing of the unidentified bacteria:

For nucleotide sequence analyses, bacterial DNA was extracted using the MagNA Pure LC DNA isolation kit III (Roche) and a MagNA Pure LC instrument. The 16S rRNA gene was amplified via PCR using the universal primer pair fd1 and rp2 and an annealing temperature of 52°C. The obtained PCR products were purified using the NucleoFast 96 PCR kit (Macherey-Nagel). Sequencing reactions were carried out with the Big Dye Terminator Sequencing Kit, version 1.1 (Perkin-Elmer) using the primers 536F, 536R, 800F, 800R, 1050F, and 1050R. The products of the sequencing reactions were purified and were analyzed using an ABI PRISM 3130x Genetic Analyzer (Applied Biosystems). The obtained sequences were compared with the sequences available in the GenBank database using BLAST. A threshold similarity value of >98.7% was chosen for identification at the species level [85]. Below this value, a new species was suspected, and the isolate was characterized in detail using phenotypic analyses and electron microscopy. All sequences of the new species have been deposited in GenBank database with the accession numbers: JX041639 and from JX101683 to JX101692.

Genome sequencing

The 11 new bacterial genera and species were grown on 5 to 10 blood agar plates in Petri dishes. The biomass was resuspended in 750 µl of TE buffer. Each sample was divided into 7 replicates of 100 µl each. The samples were lysed using a mechanical treatment on the FastPrep-24 Sample Preparation System device (M.P. Biomedicals, USA) followed by a lysozyme treatment at 20 mg/ml and an incubation for 30 min at 37°C. The preparations were then extracted on an EZ1 advanced XL device (Qiagen, Courtaboeuf, France) with kit and electronic card "bacteria". Each sample was eluted into 7 aliquots of 50 µL Tris-HCL (10 mM) and concentrated on a Qiamp column (Qiagen Courtaboeuf, France) in 100 µL of AE buffer. The DNA concentration was measured using a QuAnt-iT picogreen kit (Invitrogen) on a Tecan Genios fluorometer. A paired-end strategy was chosen for the high-throughput pyrosequencing on the 454-Titanium instrument. The 5 PicoTierPlate PTPs were loaded in 4 regions. An aliquot of 5 µg of DNA was fragmented in the range of 3-4 Kb on the Hydroshear device (GeneMachines, USA). The libraries were constructed according to the manufacturer's instructions for the 454-Titanium paired-end protocol. Each library was clonally amplified with 1 cpb in 4 emPCR reactions with the GS Titanium SV emPCR kit (Lib-L) v2. The yield of the titration was distributed in a range of 12 to 22%. A total of 790,000 beads per project and per region was loaded on the GS Titanium PicoTiterPlate PTP 70x75 kit and sequenced with the GS Titanium Sequencing XLR70 kit. The runs were performed overnight and then analyzed in cluster. The de novo assembly of the genome sequences was performed using the Newbler 2.5.3 program.

Pyrosequencing

Fecal DNA was extracted from the samples using the NucleoSpin® Tissue Mini Kit (Macherey Nagel, Hoerdt, France) with a previously described protocol [86]. A 577 nt region of the 16S rRNA gene was amplified via PCR with the primers *917F* (5'-GAATTGACGGGGRCCC) and *1391R* (5'-GACGGGCGGTGWGTRCA). These primers were selected because they can amplify the hypervariable V6 region (950 to 1,080 bp) and because they produce an amplicon length equivalent to the average length of the reads produced by GS FLX Titanium. High-throughput sequencing was realized via unidirectional sequencing. The forward and reverse primers were designated

ShotA_917F (CCATCTCATCCCTGCGTGTCTCCGACTCAGGAATTGACGGGGRCCC) and
ShotB_1391R (CCTATCCCCTGTGTGCCTTGGCAGTCTCAGGACGGGCGGTGWGTRCA). The amplicon library was generated based on a simple PCR assay using 1 μL of DNA template, extracted as described above, and a pair of special fusion primers composed of two parts with two approaches. PCR amplifications were performed in a volume of 20 μL over 30 cycles using Taq Phusion (FinnZymes, Thermo Scientific). The applied PCR reagents and thermocycling parameters were those suggested in the protocol (the annealing temperature was optimal at 58 °C, and the elongation time was 30 sec, with a final elongation time of 10 min). Amplicon lengths were visualized using the BioAnalyzer DNA 7500 labchip at 544 bp. The obtained products were purified as recommended using Ampure beads (Agencourt) and quantified via measurement in a fluorometer according to the 454_Roche *Amplicon Library Preparation Method Manual*. The unidirectional library was amplified with 1.5 cpb using the GS Titanium LV emPCR Kit (Lib-L). This project was loaded onto one-half of a GS Titanium PicoTiterPlate PTP Kit 70x75 and sequenced with the GS Titanium Sequencing Kit XLR70.

The raw 16S pyrosequencing data were trimmed based on the SOP procedure [87] and using the mothur software package, version 1.2.5 [88]. The reads were trimmed using a minimum average quality score of 35 in a windows size of 50 nucleotides. Only the reads with a minimum length of 180 pb were kept. The data was dereplicated and aligned using the SILVA reference bacteria alignment. After pre-clustering [89] the trimmed reads were also checked for chimera using an implementation of the Uchime program in the mothur package. A distance matrix was build and Operational Taxonomic Units were defined using a dissimilarity cut-off of 0.03. The representative sequence of each OTUs was assigned at the genus level using the RDP classifier and the RDP training set 9 **(http://www.mothur.org/)**. A database was created using criteria selected from the Hierarchy Browser of the RDP 16S rRNA database release 10 (http://rdp.cme.msu.edu/). A “Type” database was built with sequences labeled “Type strains”, “Isolates” and “length >1200 bp” with “good quality”. The database was formatted using Taxcollector [90]. Species-level

identification was defined with a minimum sequence identity of 98.7% [91] with the unique best Blast hit from the "Type" database. Graphic model of pyrosequencing and culture results (Fig. 2) has been assembled using Cytoscape software [92].

RESULTS

Culturomics

Bacterial species cultured

We analyzed 12,700 colonies using mass spectrometry (MALDI-TOF). Using the aforementioned strategy, we identified 133 different bacterial species from 4 phyla, including 11 previously undescribed bacterial species (Table 1, 2, Fig. 1, 2 and S1). These species included 79 *Firmicutes* species (59.4%), 25 *Actinobacteria* species (18.8%), 18 *Bacteroidetes* species (13.5%) and 11 *Proteobacteria* species (8.3%). We cultured 57 different bacterial genera, including 19 different species of *Clostridium*, which was the most represented genus (14%), 16 species of *Bacillus* (12%) and 9 species of *Bacteroides* (7%).

Identification strategy

Among the colonies tested by MALDI-TOF only 206 colonies could not be directly identified with this technique (Fig. S2). Considering that each non-identified colony was verified by three passages through MALDI-TOF, approximately 5% of the spots were not identified. A MALDI Biotyper allowed us to classify these colonies into 57 different clusters. The majority of the strains that could not be identified emerged during the 10 first weeks. After identification via 16S rRNA amplification and sequencing and the addition of the spectra to the MALDI-TOF database, the number of non-identified species was reduced to an average of 5 strains per week (Fig. S2).

Among the 57 sequenced strains, we identified 31 different types of bacteria, of which 11 were new species, and 20 were previously known species that were not identified via MALDI-TOF. Among these 20 species, 8 previously had no spectra available in the spectrometry database used for this study, and 12 species had an insufficient number of spectra in the database to allow identification. The discrepancy between the number of sequenced strains and the number of different bacteria can be explained by 3 contaminants (*Staphylococcus spp.*) that were tested erroneously and 23

strains that were tested to confirm the first identification or because of insufficient clustering that did not cluster 2 specimens of the same species into one group. All of the spectra for these species have been added to the spectrometry database to facilitate the rapid identification of colonies.

Bacterial species from the human gut:

Of the 133 species of bacteria cultured, 114 (85.7%) have already been described in the human gut. The previous culturomics studies have allowed to identify 349 different bacteria. From this sample, we identified by culturomics, 36 supplementary bacterial species including 11 new species, eight species that have been previously described but not isolated from the human gut, including 3 *Actinobacteria* (*Corynebacterium ureicelerivorans, Microbacterium aurum, Kytococcus sedentarius)*, 4 *Firmicutes* (*Bacillus marislfavi*, *Lysinibacillus fusiformis*, *Facklamia tabacinasalis*, *Bacillus polyfermenticus*) and 1 *Bacteroidetes* (*Chryseobacterium hominis*) and 17 species previously known from human gut. Among these 36 bacteria first isolated by culturomics, 20 bacterial species grew preferentially or strictly in anaerobic conditions. In addition, 25 species of the previously known bacterial species (22%) have only once been described from the human gut by previous culturomics studies [81], [47]. Moreover, 4 bacterial species, *Peptoniphilus grossensis* (GenBank JF837491)*, Peptoniphilus timonensis* (GenBank JN657222)*, Bacillus massiliosenegalensis* (GenBank JF824800)*,* and *Actinomyces grossensis* (GenBank JF837492), that were first isolated in our previous study were also isolated from the stool sample studied here (Table 1) [79].

New bacterial species:

The new bacterial species included nine new species from three phyla and two new genera. Among these isolates, we cultured fivr *Firmicutes* species "*Candidatus* Holdemania massiliensis" (JX101683)*, "Candidatus* Dorea massiliensis" (JX101687)*, "Candidatus* Clostridium anorexicus" (JX101685)*, "Candidatus* Clostridium anorexicamassiliense" (JX101686)*, "Candidatus* Bacillus marseilloanorexicus" (JX101689), two *Actinobacteria* species *"Candidatus* Streptomyces massiliensis" (JX101691) and "Candidatus *Blastococcus massiliensis*" (JX101684) and 2 *Bacteroidetes* species *"Candidatus* Bacteroides timonensis" (JX041639) and

"*Candidatus* Alistipes marseilloanorexicus" (JX101692) for the first time. The 2 new genera are represented by "*Candidatus* Stoquefichus massiliensis" (JX101690) and "*Candidatus* Soleaferrea massiliensis" (JX101688), of the *Firmicutes* phylum. "*Candidatus* Soleaferrea massiliensis" was so named because it resembles a horseshoe (Fig. S1). The names of the new species have been selected preferentially in the reference of 'Marseille', our laboratory's city or 'Timone' for the hospital where our laboratory is localized, as previously employed [81], [47]. In the cases where the species name has been already used, we added "anorexica" with "massiliensis" in reference of the source of the sample, although no link exists between the clinical status and these new bacterial species. All of these species were deposited in the Collection de Souches de l'Unité des Rickettsies (CSUR).

Culture conditions for new species

Eight out of the 11 new bacterial species and genera require anaerobic conditions for growth. Among the 8 anaerobic bacteria, one was cultured after a long incubation (1 month) in 5% sheep blood agar; one was cultured at 28°C; and 5 were isolated after preincubation in a blood culture bottle with or without sheep blood and sheep rumen fluid to mimic the natural environment, as proposed by environmental microbiologists [65] (Table 2 and S1).

Genome sequencing

Each new species will be described using the new concept of microbiogenomics, adding the MALDI-TOF spectra and genome sequences to the classical description. Ten out of the 11 new bacterial species and genera isolated from stool sample have already been sequenced (Table 1, Table S1), generating a total of 41.2 Mb unique sequence. The genomes sizes of the new bacteria ranged from 2.75 Mb to 7.13 Mb (Table 1B). Based on our previous results, altogether, the present work yielded approximately at least 3,000 previously unknown genes.

Culture conditions:

Among the 133 bacterial isolates (Table 1), 130 (97.7%) were identified using 70 different types of basic culture conditions (Table S3), selected because they had allowed to identify the 340 different bacterial species in our seminal study [47]. The culture condition that produced the best yield was preincubation in an anaerobic blood culture

bottle with thioglycolate, from which 49 species (37%) were isolated. Anaerobic incubation directly on 5% sheep blood agar led to 8 additional species being identified (*Bacteroides caccae, B. fragilis, B. nordii, Butyricimonas virosa, Corynebacterium amycolatum, Parabacteroides goldsteinii, Staphylococcus saprophyticus, Streptococcus salivarius*), and passive filtration in Leptospira broth resulted in 6 additional species (*Bacillus marisflavi, B. pumilus, B. flexus, Brevibacillus borstelensis, Enterococcus avium, Paenibacillus barengoltzii*). Three species (*Enterobacter cloacae, Bacillus clausii and Staphylococcus cohnii*) were identified using newly designed culture conditions.

Pyrosequencing

The pyrosequencing analysis generated 83,951 reads. The abundance of reads classified at the phylum level was 64,216 reads from *Firmicutes* (76.49%), 8,439 reads from *Actinobacteria* (10.05%), 4,983 from *Bacteroidetes* (5.94%), 2,434 from *Proteobacteria* (2.9%), 25 for *Cyanobacteria/Chloroplasts* (0.03%), 17 from *Synergistetes* (0.02%), and 2 from *Verrucomicrobia*. Finally, 3,834 reads (4.57%) were not assigned at the phylum level and were designated as unclassified.

Diversity was represented by 1,273 different OTUs. Of these OTUs, 1,026 were assigned to the *Firmicutes* phylum, 86 to the *Actinobacteri*a phylum, 58 to the *Bacteroidetes* phylum, 32 to the *Proteobacteria* phylum, and 66 different OTUs remained unclassified. Among the OTUs assigned to the *Firmicutes*, 22% belonged to the *Clostridium* genus and 16 % to the *Oscillibacter* genus, while 37 % remained unclassified at the genus level. Among the OTUs assigned to the *Actinobacteria* phylum, most belonged to the *Bifidobacteria* genus (62%).

Comparison between microbial culturomics and pyrosequencing results:

A dramatic difference was observed between the groups of microbial species identified by culturomics and by pyrosequencing. At the species level, only 23 of the isolates were common to the two approaches (17% of the culturomics group) (Fig. 2). The 10 most abundant OTUs assigned to the species level corresponded to 13,979 reads (16.7%). Among these species, 5 bacteria, representing 9% of the total of reads (*Bifidobacterium animalis, Clostridium ramosum, Turicibacter sanguinis, Streptococcus salivarius, Bacteroides uniformis*), were concomitantly cultured. In

addition, without taking into account the species detected by both techniques, more of the half of the sequences (45,317 reads) were assigned to *Ruminococcaceae*, *Erysipelotrichaceae* or *Lachnospiraceae* families respectively, that are constituted by strictly anaerobic species.

DISCUSSION

Here, we carried out the first study combining microbial culturomics and pyrosequencing in the gut of an anorexia nervosa patient and we have been lucky enough to isolate for the first time 11 completely new species. As mass spectrometry is used in routine bacteriological analyses, including in two previous culturomics study [81], [47] completed using 16S rRNA amplification and sequencing [85] for unidentified colonies, we are confident in the results of this study [84]. Genome sequencing has been performed as previously described [68]–[79]. In parallel, we conducted pyrosequencing of 16S rRNA amplicons targeting the hypervariable V6 region, previously described as a reference method for analyses of the human gut [93], [82], [47]. The large-scale nature of this work involving complementary techniques explains why we analyzed only a single stool sample. Nevertheless, the uniqueness of these results (19 bacterial species first described from human gut including 11 new bacterial species and genome sequences) allows us to draw useful information about the gut microbiota repertoire [47].

The 11 new bacterial species isolated in this study demonstrate that each stool sample studied by culturomics in a particular condition (here anorexia nervosa) can lead to a significant increase in the number of new bacterial species isolated from the gut microbiota (Fig. 1 and 2). These 11 new species cultured from a single stool sample demonstrates the potential of this technique [94] when compared to only 8 species from human gut microbiota described in International Journal of Systematic and Evolutionary Microbiology during the 2 last years! As a matter of fact, from the 5 stools published using culturomics so far, we have identified 42 new bacterial species comparable to these validated in IJSEM by the rest of the world from human gut since 5 years (Fig. 1) [81], [47]. As this are pioneering studies, only the extension of this strategy will allow to determine if these new species are linked to the clinical and

epidemiological patients status studied (rural healthy African individuals, obese and anorexic French patients, patients treated with wide-spectrum antibiotics) [81], [47]. Four new bacterial species discovered in our first culturomics study have been cultured in this sample (Table 1). When a higher number of stool samples will be studied by culturomics, specific PCR will be designed for each 42 new bacterial species in the aim to associate these with different clinical status. In addition, if a link between new bacterial species and clinical status was highlighted, molecular tools will be easily used to study the evolution of gut microbiota composition after treatment.

The pyrosequencing results highlighted, as previously reported, a low overlapping with culturomics results with only 17 % of the species detected by the 2 techniques. In addition, most of the OTU detected only by pyrosequencing were assigned mainly to *Ruminococcaceae*, *Lachnospiraceae* and *Erysipelotrichaceae* families which are constituted by stringent anaerobic species. The best future route for culturomics will be to improve the anaerobic culture conditions. As previously reported, we could propose, in the future, to collect fecal samples directly in containers with a gas generation system and to transport immediately at 4°C before processing in an adapted anaerobic chamber in the aim to reduce the redox potential [95] and the bacterial viability reduction caused by freezing [83], or use roll-tubes initially designed for the methanogenic archaeal species culture [96]. Finally, we could use supplementary culture conditions with various antibiotics with different critical concentration in the aim to select minority bacterial populations [97].

In conclusion, this approach using microbial culturomics and culture-independent techniques has been yet incredibly fertile to describe new microbes from human gut microbiota. In the future, pyrosequencing results will help to design specific new culture conditions for the more represented bacterial families. Once the repertoire will be comprehensively described, supplementary studies with more samples will connect the gut microbiota composition with the clinical or geographical status.

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Table 1. Bacterial species identified via culture. The 19 bacterial species described from the human gut for the first time in this work are presented in bold.

Phylum	Species	Phylum	Species
Actinobacteria	*Actinomyces grossensis*	*Firmicutes*	*Clostridium baratii*
	Adlercreutzia equolifaciens		*Clostridium bifermentans*
	Aeromicrobium massiliense		*Clostridium butyricum*
	Arthrobacter castelli		*Clostridium citroniae*
	Blastococcus massiliensis		*Clostridium glycolycum*
	Bifidobacterium animalis		*Clostridium hathewayi*
	Cellulosimicrobium cellulans		*Clostridium inocuum*
	Collinsella aerofaciens		*Clostridium orbiscindens*
	Corynebacterium afermentans		*Clostridium oroticum*
	Corynebacterium amycolatum		*Clostridium paraputrificum*
	Corynebacterium mucifaciens		*Clostridium perfringens*
	Corynebacterium tuberculostearicum		*Clostridium ramosum*
	Corynebacterium ureicelerivorans		*Clostridium scindens*
	Dermabacter hominis		*Clostridium sordellii*
	Eggerthella lenta		*Clostridium sporogogenes*
	Gordonibacter pamelaeae		*Clostridium symbiosum*
	Kytococcus schroeteri		*Clostridium tertium*
	Kytococcus sedentarius		*Coprobacillus cateniformis*
	Microbacterium aurum		***Dorea massiliensis***
	Micrococcus luteus		*Enterococcus avium*
	Micrococcus lylae		*Enterococcus casseliflavus*
	Propionibacterium acnes		*Enterococcus faecalis*
	Rothia aeria		*Enterococcus faecium*
	Rothia dentocariosa		*Eubacterium limosum*
	Streptomyces massiliensis		*Eubacterium tenue*
Bacteroidetes	***Alistipes marseilloanorexicus***		***Facklamia tabacinasalis***
	Alistipes onderdonkii		*Finegoldia magna*
	Bacteroides cellulosilyticus		***Holdemania massiliensis***
	Bacteroides caccae		*Lactobacillus rhamnosus*
	Bacteroides fragilis		*Lactobacillus paracasei*
	Bacteroides nordii		*Lactococcus lactis*
	Bacteroides ovatus		***Lysinibacillus fusiformis***
	Bacteroides thetaiotaomicron		*Negativococcus succinicivorans*
	Bacteroides timonensis		*Paenibacillus barcinonensis*
	Bacteroides uniformis		*Paenibacillus barengoltzii*
	Bacteroides vulgatus		*Peptinophilus grossensis*
	Barnesiella intestinihominis		*Peptinophilus timonensis*
	Butyricimonas virosa		***Soleaferrea massiliensis***

	Chryseobacterium hominis		*Staphylococcus aureus*
	Parabacteroides goldsteinii		*Staphylococcus capitis*
	Parabacteroides distasonis		***Staphylococcus caprae***
	Parabacteroides merdae		*Staphylococcus cohnii*
	Porphyromonas somerea		*Staphylococcus epidermidis*
Firmicutes	*Bacillus badius*		*Staphylococcus haemolyticus*
	Bacillus cereus		*Staphylococcus hominis*
	Bacillus circulans		*Staphylococcus pasteuri*
	Bacillus clausii		*Staphylococcus saprophyticus*
	Bacillus flexus		*Staphylococcus warneri*
	Bacillus licheniformis		***Stoquefichus massiliensis***
	Bacillus marisflavi		*Streptococcus gordonii*
	Bacillus marseilleoanorexicus		*Streptococcus mitis*
	Bacillus megaterium		*Streptococcus oralis*
	Bacillus polyfermenticus		*Streptococcus parasanguinis*
	Bacillus pumilus		*Streptococcus pneumoniae*
	Bacillus senegalensis		*Streptococcus salivarius*
	Bacillus simplex		*Turicibacter sanguinis*
	Bacillus sonorensis	*Proteobacteria*	*Bilophila wadsworthia*
	Bacillus subtilus		*Citrobacter freundii*
	Bacillus vallismortis		*Enterobacter cloacae*
	Blautia coccoides		*Escherichia coli*
	Blautia hydrogenotrophica		*Escherichia hermannii*
	Brevibacillus borstelensis		*Klebsiella pneumoniae*
	Brevibacillus agri		*Moraxella osloensis*
	Catabacter hongkongensis		*Neisseria flavescens*
	Clostridium anorexicamassiliense		*Neisseria perflava*
	Clostridium anorexicus		*Pantoea agglomerans*
			Proteus mirabilis

Table 2. Main characteristics of the new bacterial species and respective genome sequence.

Phylum	Bacterial species	Culture condition	16S number	Bioproject number	Genome size
Firmicutes	*Soleaferrea massiliensis*	Inoculation in blood culture bottle, 5% sheep blood agar, anaerobe, 37°C	JX101688	PRJEB375	In progress
	Stoquefichus massiliensis	5% sheep blood agar, anaerobe, 28°C	JX101690	PRJEB377	3,46Mb
	Dorea massiliensis	Inoculation in blood culture bottle + rumen fluid, 5% sheep blood agar, anaerobe, 37°C	JX101687	PRJEB374	3,6Mb
	Holdemania massiliensis	Inoculation in blood culture bottle + Thioglycolate, 5% sheep blood agar, anaerobe, 37°C	JX101683	PRJEB187	3,8 Mb
	Clostridium anorexicus	Inoculation blood culture bottle + sheep blood, 5% sheep blood agar, anaerobe, 37°C	JX101685	PRJEB372	3,52Mb
	Clostridium anorexicamassiliense	Inoculation blood culture bottle + sheep blood, 5% sheep blood agar, anaerobe, 37°C	JX101686	PRJEB373	4,42Mb
	Bacillus marseilloanorexicus	Inoculation in blood culture bottle 1 month + rumen fluid, 5% sheep blood agar, aerobe, 37°C	JX101689	PRJEB376	4,62Mb
Bacteroidetes	*Bacteroides timonensis*	5% sheep blood agar 1 month, anaerobe, 37°C,	JX041639	PRJEB186	7,13Mb
	Alistipes marseilloanorexicus	Inoculation in blood culture bottle + Thioglycolate, 5% sheep blood agar, anaerobe 37°C	JX101692	PRJEB379	2,75Mb
Actinobacteria	*Streptomyces massiliensis*	Filtration 0,45µm, Brain Heart Infusion agar, aerobe, 37°C	JX101691	PRJEB378	In progress
	Blastococcus massiliensis	Brucella agar, aerobe 37°C	JX101684	PRJEB371	3.87 Mb

Table 3. OTUs assigned to the species level identified via pyrosequencing.

Phylum	Species	Phylum	Species
Actinobacteria	*Actinomyces odontolyticus*	*Firmicutes*	*Clostridium saccharoperbutylacetonicum*
	Asaccharobacter celatus		*Clostridium scindens*
	Bifidobacterium animalis		*Clostridium spiroforme*
	Collinsella aerofaciens		*Clostridium vincentii*
	Eggerthella lenta		*Enterococcus saccharolyticus*
	Propionibacterium acnes		*Eubacterium callanderi*
	Rothia mucilaginosa		*Eubacterium tenue*
Bacteroidetes	*Alistipes putredinis*		*Lactobacillus rogosae*
	Alistipes shahii		*Lactococcus raffinolactis*
	Bacteroides caccae		*Lactonifactor longoviformis*
	Bacteroides fragilis		*Parvimonas micra*
	Bacteroides ovatus		*Pediococcus damnosus*
	Bacteroides thetaiotaomicron		*Phascolarctobacterium faecium*
	Bacteroides uniformis		*Pseudoflavonifractor capillosus*
	Parabacteroides distasonis		*Roseburia faecis*
Firmicutes	*Brevibacterium massiliense*		*Roseburia hominis*
	Clostridium aldenense		*Roseburia intestinalis*
	Clostridium asparagiforme		*Ruminococcus flavefaciens*
	Clostridium baratii		*Ruminococcus torques*
	Clostridium bartlettii		*Staphylococcus aureus*
	Clostridium celatum		*Streptococcus mutans*
	Clostridium chauvoei		*Streptococcus salivarius*
	Clostridium glycolicum		*Turicibacter sanguinis*
	Clostridium glycyrrhizinilyticum		*Veillonella rogosae*
	Clostridium leptum	*Proteobacteria*	*Enterobacter cloacae*
	Clostridium orbiscindens		*Pelomonas saccharophila*
	Clostridium oroticum		*Serratia ficaria*
	Clostridium perfringens	*Verrucomicrobia*	*Akkermansia muciniphila*
	Clostridium ramosum		

Table 4. Bacterial species and OTUs assigned to the species level identified via both pyrosequencing and culture

Phylum	Species
Actinobacteria	*Bifidobacterium animalis*
	Collinsella aerofaciens
	Eggerthella lenta
	Propionibacterium acnes
Bacteroidetes	*Bacteroides caccae*
	Bacteroides fragilis
	Bacteroides ovatus
	Bacteroides thetaiotaomicron
	Bacteroides uniformis
	Parabacteroides distasonis
	Parabacteroides merdae
Firmicutes	*Clostridium baratii*
	Clostridium glycolicum
	Clostridium orbiscindens
	Clostridium oroticum
	Clostridium perfringens
	Clostridium ramosum
	Clostridium scindens
	Eubacterium tenue
	Staphylococcus aureus
	Streptococcus salivarius
	Turicibacter sanguinis
Proteobacteria	*Enterobacter cloacae*

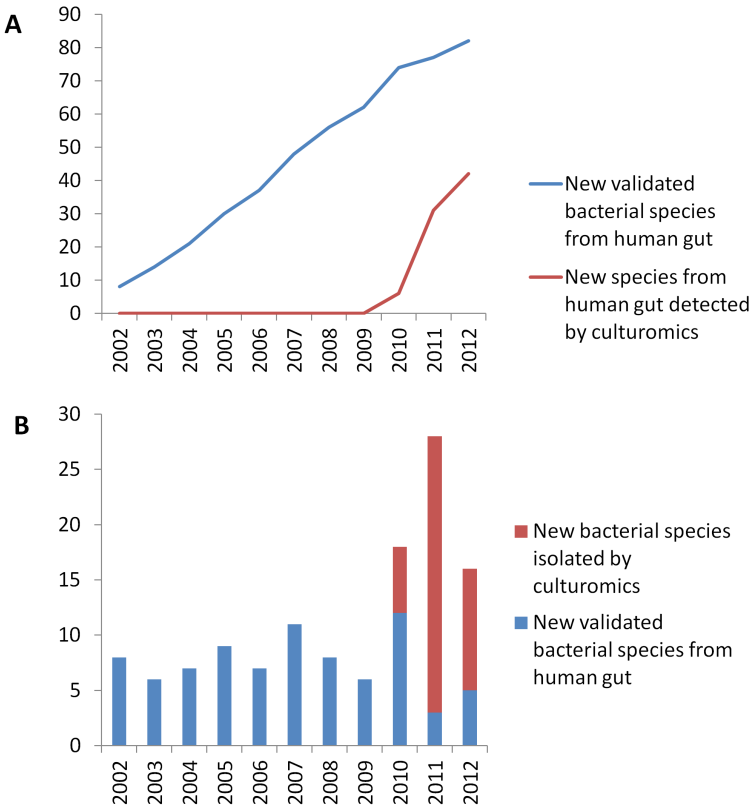


Fig. 1 Number of bacterial species found in the human gut validated in the literature and isolated via culturomics between 2000 and 2012 (A) and the proportion of bacterial species validated or isolated by culturomics each year (B).

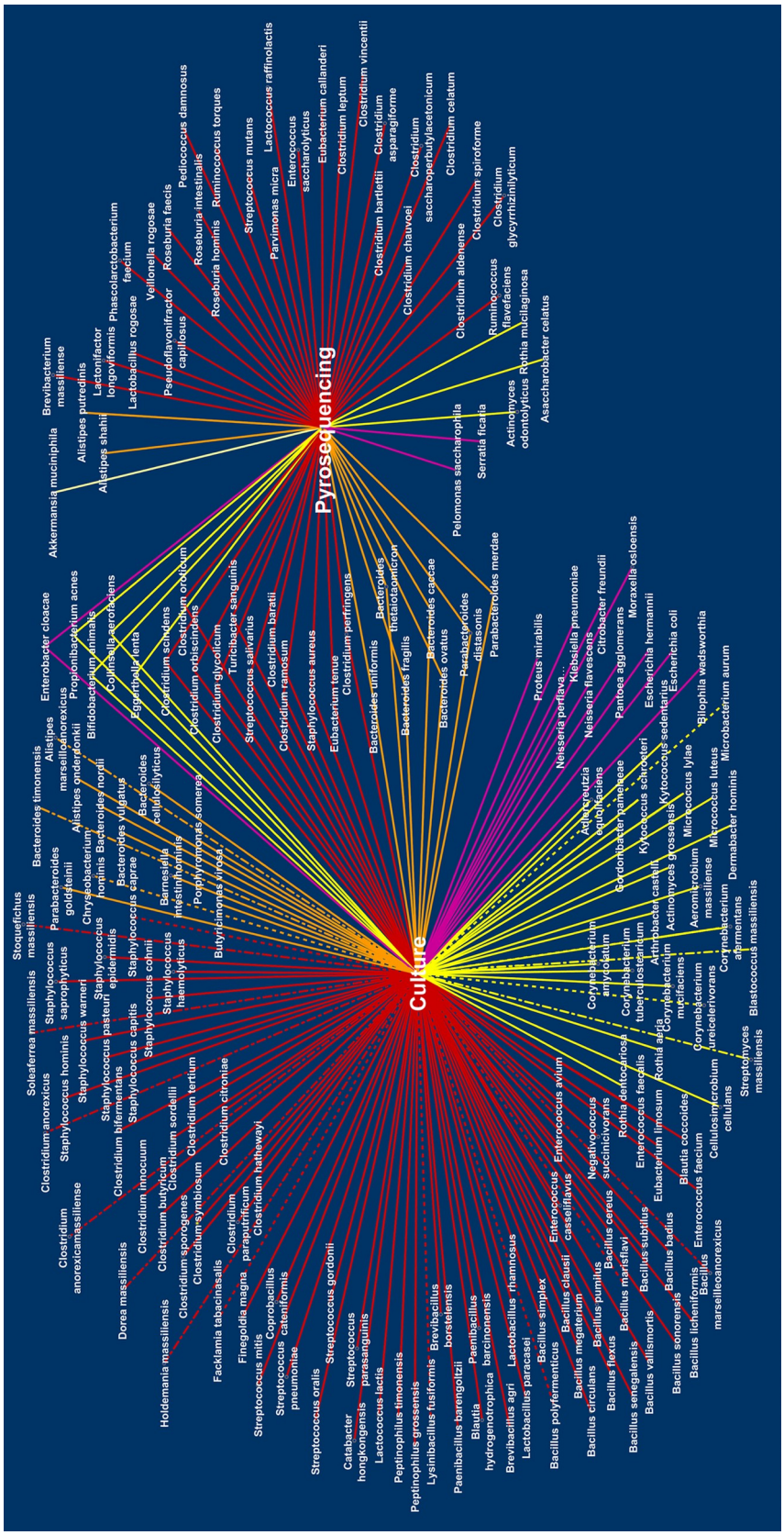


Fig. 2 Comparison of the pyrosequencing and culture results. Broken lines containing points and dashes represent new bacterial species, while a simple dotted line represents a species isolated for the first time from the human gut. Different colors represent each phylum: red, *Firmicutes*; orange, *Bacteroidetes*; yellow, *Actinobacteria*; pink, *Proteobacteria*; and light yellow, *Verrucomicrobia*.

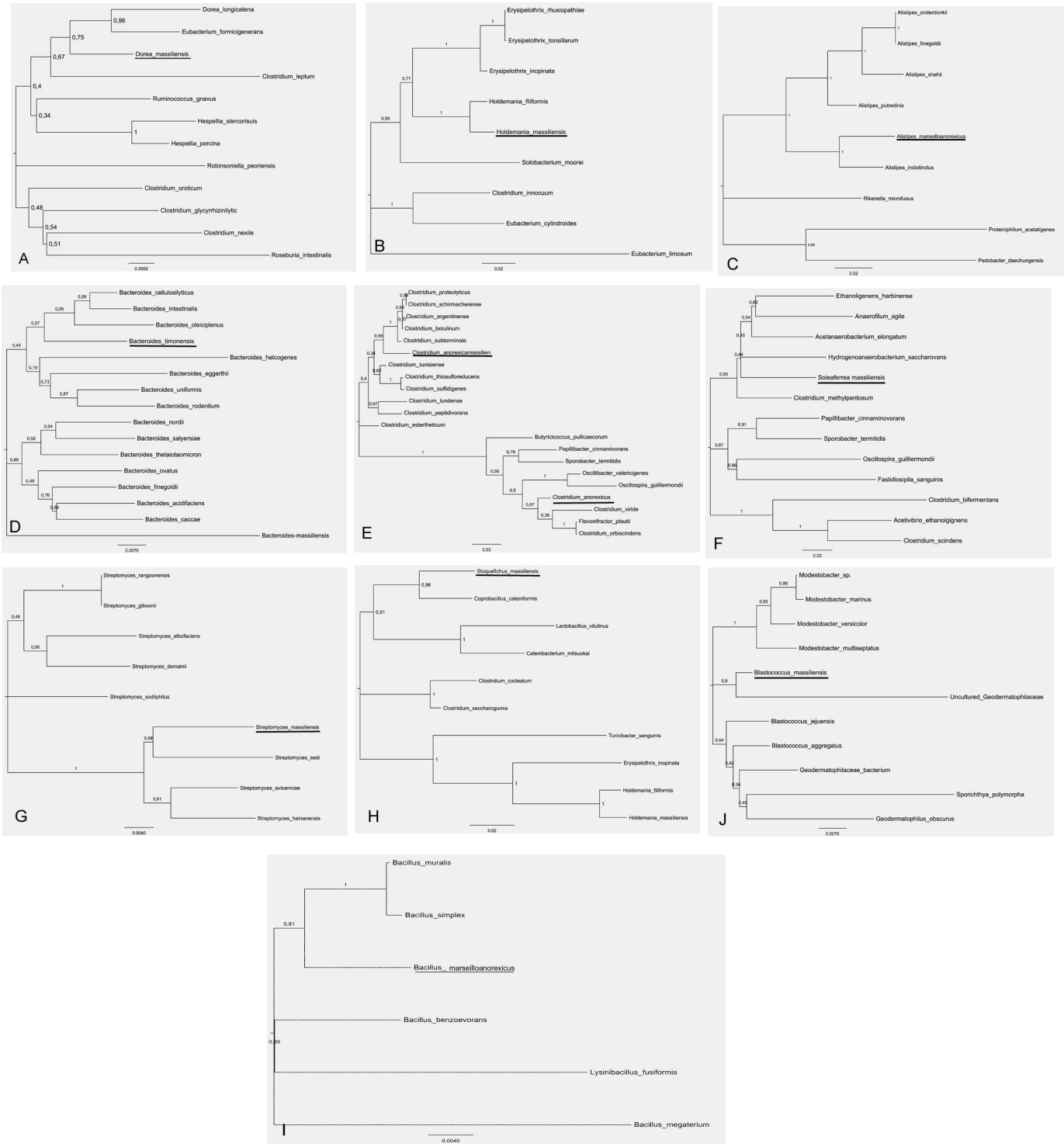
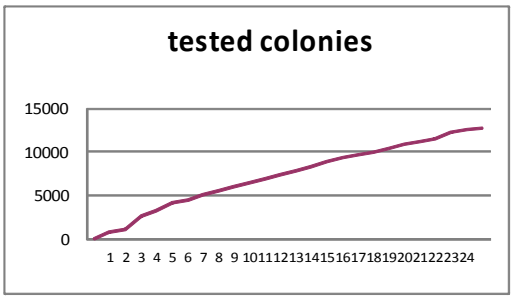
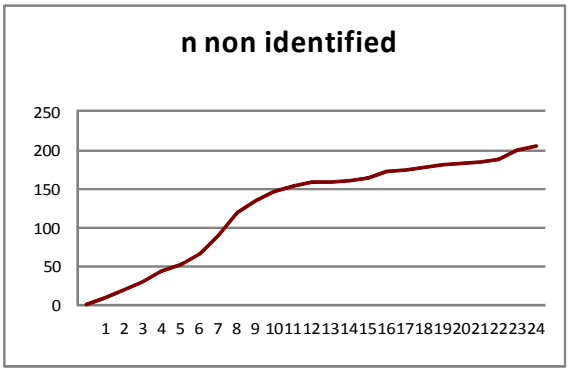


Fig. S1. Phylogenetic trees of the new species cultured: A: *Dorea massiliensis* **B:** *Holdemania massiliensis* **C:** *Alistipes marseilloanorexicus* **D:** *Bacteroides timonensis* **E:** *Clostridium anorexicamassiliense* and *Clostridium anorexicus* **F:** *Soleaferrea massiliensis* **G:** *Streptomyces massiliensis* **H:** *Stoquefichus massiliensis* **I:** *Bacillus marseilloanorexicus* **J:** *Blastococcus massiliensis*



A



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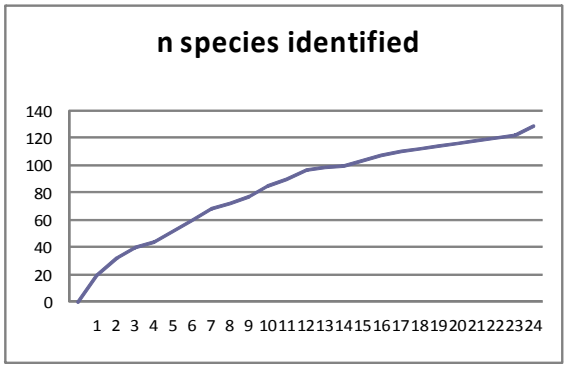

C

Fig. S2. Evolving progress of culturomics. Visual representations of the number of colonies tested each week (A); the number of non-identified species isolated each week (B); and of the number of supplementary species identified each week (C). No plateau was observed in the number of supplementary species identified.

Table S1: Description of the 88 culture conditions used.

Basic culture conditions

COS, anaerobic 37°C	BHI + filtered stool, aerobic 37°C	Schaedler, anaerobic 37°C
COS, anaerobic 28° C	BHI + sheep blood, aerobic 37°C	Sabouraud, anaerobic 37°C
COS, 5µm anaerobic 37°C	BHI + Colimycine + Vancomycine, aerobic 37°C	MRS, anaerobic 37°C
COS, µaerobic 37°C	BHI + Vancomycine, aerobic 37°C	Schaedler KV, anaerobic 37°C
COS, aerobic 25°C	Mueller Hinton + Vancomycine, aerobic 37°C	BHI + sheep blood, anaerobic 37°C
COS, aerobic 37°C	BHI, filtration 0,45µm, aerobic 37°C	BHI, filtration 5µm, anaerobic 37°C
Orange, aerobic 37°C	BHI, filtration 5µm, aerobic 37°C	Leptospira, passive filtration, µaerobic 37°C
Marin, aerobic 28°C	BHI + 15g/l NaCl, aerobic 37°C	BSKH, passive filtration, aerobic 37°C
MOD4, aerobic 37°C	BHI + 3g/l NaCl, aerobic 37°C	Mueller Hinton, aerobic 37°C
BCP, aerobic 37°C	BHI + 1g/l NaCl, aerobic 37°C	HTM, 2,5% CO2 37°C
M17, aerobic 37°C	Bordatella, 5%CO2 37°C	Shaedler KV, aerobic 37°C
Brucella, aerobic 37°C	R2A, aerobic 37°C	TSA, aerobic 37°C
EMB, aerobic 37°C	PVX, 5%CO2 37°C	Wilkins, aerobic 37°C
CaCO3, aerobic 37°C	ISP, aerobic 37°C	BCYE, 5%CO2 37°C

BCYE, Inoculation blood culture, anaerobic 37°C, D7	COS, Inoculation blood culture + sheep rumen, anaerobic 37°C, D2
BHI, Inoculation blood culture, anaerobic 37°C, D2	COS, Inoculation blood culture + sheep rumen, anaerobic 37°C, D10
BHI, Inoculation blood culture, anaerobic 37°C, 2weeks	COS, Inoculation blood culture + sheep rumen, anaerobic 37°C, 1m
Schaedler KV, Inoculation blood culture, anaerobic 37°C	COS, Inoculation blood culture + sheep rumen, anaerobic 37°C, D7
COS, Inoculation blood culture, anaerobic 37°C, D2	COS, Inoculation blood culture + sheep rumen + sheep blood, anaerobic 37°C, 1m
COS, Inoculation blood culture, anaerobic 37°C, 2weeks	COS, Inoculation blood culture + sheep rumen + sheep blood, anaerobic 37°C, D3
COS, Inoculation blood culture, anaerobic 37°C, 1month	COS, Inoculation blood culture + sheep blood, anaerobic 37°C, D2
COS, Inoculation blood culture + Thioglycolate, anaerobic 37°C, D2	COS, Inoculation blood culture + sheep blood, anaerobic 37°C, D20
COS, Inoculation blood culture + Thioglycolate, anaerobic 37°C, D5	COS, Inoculation blood culture + sheep rumen + filtered stool, anaerobic 37°C, D2
COS, Inoculation blood culture + Thioglycolate, anaerobic 37°C, D10	COS, Inoculation blood culture after thermic shock 60°C 20min, anaerobic 37°C
COS, Inoculation blood culture + Thioglycolate, anaerobic 37°C, 1m	COS, Inoculation blood culture + sheep rumen, aerobic 37°C
	COS, Inoculation blood culture after thermic shock 60°C 20min, aerobic 37°C

Supplementary culture conditions

PVX, aerobic 45°C	Orange ph6, aerobic 37°C	Yoghurt medium, 5% CO2 37°C
PVX, anaerobic 45°C	Orange ph6, anaerobic 37°C	Yoghurt medium, anaerobic 37°C
PCA, aerobic 37°C	Chapman, aerobic 37°C	Cheese medium, anaerobic 37°C
	Brewer's yeast medium, 5% CO2 37°C	Brewer's yeast medium, anaerobic 37°C

COS, Inoculation blood culture bottle + banana, anaerobic 37°C	COS, Inoculation blood culture bottle + yoghurt, anarobic 37°C
TSA, Inoculation blood culture bottle + banana, anaerobic 37°C	COS, Inoculation blood culture + cow rumen, anaerobic 37°C
COS, Inoculation blood culture bottle + cheese, anarobic 37°C	COS, Inoculation blood culture after filtration 0,8µm, aerobic 37°C
TSA, Inoculation blood culture bottle + cheese, anarobic 37°C	COS, Inoculation blood culture after filtration 0,8µm, anaerobic 37°C
COS, Inoculation blood culture bottle + brewer's yeast, anarobic 37°C	COS, Inoculation blood culture, aerobic 37°C
	COS, Inoculation blood culture + sheep blood, aerobic 37°C

2.2 Publication II:

(To be adjusted to this dissertation, the numbering of tables and figures have been changed in the following article by addition of "2" in front of the initial number.)

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Non-contiguous finished genome sequence and description of *Bacillus massilioanorexius sp. nov.*

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Abstract

Bacillus massilioanorexius strain AP8^T sp. nov. is the type strain of *B. massilioanorexius* sp. nov., a new species within the genus *Bacillus*. This strain, whose genome is described here, was isolated from the fecal flora of a 21-year-old Caucasian French female suffering from a severe form of anorexia nervosa since the age of 12 years. *B. massilioanorexius* is a Gram-positive aerobic bacillus. Here we describe the features of this organism, together with the complete genome sequence and annotation. The 4,616,135 bp long genome (one chromosome but no plasmid) contains 4,432 protein-coding and 87 RNA genes, including 8 rRNA genes.

Introduction

Bacillus massilioanorexius strain AP8^T (= CSUR P201 = DSM 26092) is the type strain of *B. massilioanorexius* sp. nov. This bacterium is a Gram-positive, non-spore-forming, aerobic and motile bacillus that was isolated from the stool of a 21-year-old Caucasian French female suffering from a severe form of anorexia nervosa since the age of 12 years and is part of a "culturomics" study aiming at cultivating all species within human feces individually

[47][81][98]. This bacterium was one of the 11 new bacterial species isolated from this single stool sample [98].

The current classification of *Bacteria* and *Archaea* remains a subject of debate and currently relies on a combination of phenotypic and genotypic characteristics [99]. Genomic data has not yet been routinely incorporated into descriptions. However, as more than 6,000 bacterial genomes have been sequenced including 982 type strains [100][101] and another 15,000 genomic projects are ongoing including 2,120 type strains [100][59], we recently proposed to integrate genomic information in the description of new bacterial species [68]-[79], [102]-[111].

The genus *Bacillus* (Cohn 1872) was created in 1872 [112]. It consists mainly of Gram-positive, motile, spore-forming bacteria classified within 251 species and 3 subspecies with validly published names [113]. Members of the genus *Bacillus* are ubiquitous bacteria isolated from various environments including soil, fresh and sea water and food. In humans, *Bacillus* species may be opportunists in immunocompromised patients or pathogenic, such as *B. anthracis* [84] and *B. cereus*. However, in addition to these two species, various *Bacillus* species may be involved in a variety of aspecific human infections, including cutaneous, ocular, central nervous system or bone infections, pneumonia, endocarditis and bacteremia [115].

Here we present a summary classification and a set of features for *B. massilioanorexius* sp. nov. strain AP8^T (= CSUR P201 = DSM 26092), together with the description of the complete genomic sequence and its annotation. These characteristics support the circumscription of the species *B. massilioanorexius*.

Classification and information

A stool sample was collected from a 21-year-old Caucasian French female suffering from a severe restrictive form of anorexia nervosa since the age of 12 years. She was hospitalized in the nutrition unit of our hospital for recent aggravation of her medical condition. At the time of hospitalization, her weight and height was 27.7 kg, and 1.63 m (BMI: 10.4 kg/m2) respectively. The patient gave an informed and signed consent. This study and the assent procedure were approved by the Ethics Committee of the Institut Fédératif de Recherche IFR48, Faculty of Medicine, Marseille, France (agreement 09-022). The fecal specimen was preserved at -80°C after collection. Strain AP8^T (Table 21) was isolated in March 2012 by aerobic cultivation on Columbia agar (BioMerieux, Marcy l'Etoile, France) after one month of preincubation of the stool sample with addition of 5ml of sheep rumen in blood bottle culture. This strain exhibited a 97% nucleotide sequence similarity with *B. sim-plex* [116], t

the phylogenetically closest validated *Bacillus* species (Figure 21). This value was lower than the 98.7% 16S rRNA gene sequence threshold recommended by Stackebrandt and Ebers to delineate a new species without carrying out DNA-DNA hybridization [85].

Different growth temperatures (25, 30, 37, 45°C) were tested. Growth was observed between 25 and 45°C, with optimal growth at 37°C after 24 hours of incubation. Colonies were 3 mm in diameter and 0.5 mm in thickness and gray in color with coarse appearance on blood-enriched Columbia agar. Growth of the strain was tested under anaerobic and microaerophilic conditions using GENbag anaer and GENbag microaer systems, respectively (BioMerieux), and under aerobic conditions, with or without 5% CO2. Growth was obtained in all the above mentioned conditions except in anaerobic conditions, where weak growth was observed.

Gram staining showed Gram-positive rods. The motility test was positive. Cells grown on agar are Gram-positive rods (Figure 22), have a mean diameter of 0.77 µm and a mean length of 2.27 µm in electron microscopy (Figure 23).

Strain AP8^T exhibited catalase and oxidase activity. Substrates oxidation and assimilation were examined with an API 50CH strip (BioMerieux) at the optimal growth temperature. Positive reactions were observed for D-glucose, D-fructose, D-saccharose, ribose, mannose, mannitol and D-trehalose and weak reactions were observed for L-rhamnose, esculine, salicine, D-cellobiose and gentiobiose. Using an API 20E strip (BioMerieux, Marcy l'Etoile), positive reactions were observed for tryptophane deaminase, acetoin and gelatinase production. Negative reactions were found for urease and indole production.

B. massilioanorexius is susceptible to amoxicillin, rifampicin, ciprofloxacin, gentamicin, doxycycline and vancomycin but resistant to trimethoprim/sulfamethoxazole and metronidazole. When compared with representative species from the genus *Bacillus*, *B. massilioanorexius* strain AP8^T exhibited the phenotypic differences detailed in Table 22.

Matrix-assisted laser-desorption/ionization time-of-flight (MALDI-TOF) MS protein analysis was carried out as previously described [84] using a Microflex spectrometer (Brüker Daltonics, Leipzig, Germany). Twelve individual colonies were deposited on a MTP 384 MALDI-TOF target plate (Brüker). The twelve AP8^T spectra were imported into the MALDI BioTyper software (version 2.0, Brüker) and analyzed by standard pattern matching (with default parameter settings) against the main spectra of 3,769 bacteria, including 129 spectra from 98 validly named *Bacillus* species, used as reference data in the BioTyper database. A score enabled the presumptive identification and discrimination of the tested species from those in a database: a score > 2 with a validated species enabled the identification at the species level; and a score < 1.7 did not enable any identification. For strain AP8^T, no significant

score was obtained, suggesting that our isolate was not a member of any known species (Figures 24 and 25).

Genome sequencing information

Genome project history

The organism was selected for sequencing on the basis of its phylogenetic position and 16S rRNA similarity to other members of the *Bacillus* genus, and is part of a "culturomics" study of the human digestive flora aiming at isolating all bacterial species within human feces. It was the twentyseventh genome of a *Bacillus* species and the first genome of *Bacillus massilioanorexius* sp. nov. A summary of the project information is shown in Table 23. The Genbank accession number is CAPG00000000 and consists of 120 contigs. Table 23 shows the project information and its association with MIGS version 2.0 compliance [117].

Growth conditions and DNA isolation

Strain AP8^{T} was grown aerobically in Columbia broth (BioMerieux, Marcy l'Etoile, France). Extraction of chromosomal DNA was performed by using 50 mL of 48-72 h culture of *B. massilioanorexius*, centrifuged at 4°C and 2000 × g for 20 min. Resuspension of cell pellets was done in 1 mL Tris/EDTA/NaCl [10mM Tris/HCl (pH7.0), 10 mM EDTA (pH8.0), and 300 mM NaCl] and recentrifugation was done under the same conditions. The pellets were resuspended in 200µL TE/lysozyme [25 mM Tris/HCl (pH8.0), 10 mM EDTA (pH8.0), 10 mM NaCl, and 10 mg lysozyme/mL]. The sample was incubated at 37°C for 30 min and then 30 µL of 30% (w/v) sodium N- lauroyl-sarcosine (Sarcosyl) was added to it, incubated for 20 min at 65°C, followed by incubation for 5 min at 4°C. Purification of DNA with phenol/chloroform/isoamylalcohol (25:24:1) was followed by precipitation with ethanol. DNA concentration was 64.3 ng/µl as determined by Genios Tecan fluorometer, using the Quant-it Picogreen kit (Invitrogen).

Genome sequencing and assembly

A 3kb paired-end sequencing strategy (Roche, Meylan, France) was used. Five µg of DNA were mechanically fragmented on the Covaris device (KBioScience-LGC Genomics, Middlesex, UK) through miniTUBE-Red with an enrichment size at 3-4kb. The DNA fragmentation was visualized through the Agilent 2100 BioAnalyzer on a DNA labchip 7500 with an optimal size of 2.95 kb. The library was constructed according to the 454 GS FLX Titanium paired-end protocol. Circularization and nebulization were performed which generated a pattern of 553 bp optimal size. PCR amplification was performed for 17 cycles followed by double size selection. The single stranded paired-end library was quantified using Quant-it Ribogreen kit (Invitrogen) with Genios Tecan fluorometer that yielded concentration

of 556 pg/µL. The library concentration equivalence was calculated as 1.82E+09 molecules/µL. The library was stored at -20°C until further use.

The shotgun library was clonally amplified with 5cpb in 4 emPCR reactions and the 3kb paired end library was amplified with lower cpb in 4 emPCR reactions at 1cpb and 2 emPCR at 0.5 cpb with the GS Titanium SV emPCR Kit (Lib-L) v2 (Roche). The yield of the shotgun emPCR reactions was 16.9 and 5.62% respectively for the two kinds of paired-end emPCR reactions according to the quality expected (range of 5 to 20%) from the Roche procedure. Two libraries were loaded on the GS Titanium PicoTiterPlates (PTP Kit 70x75, Roche) and pyrosequenced with the GS Titanium Sequencing Kit XLR70 and the GS FLX Titanium sequencer (Roche). The run was performed overnight and analyzed on the cluster through the gsRunBrowser and Newbler assembler (Roche). A total of 410,883 passed filter wells were obtained and generated 144.49 Mb with a length average of 344 bp. The passed filter sequences were assembled Using Newbler with 90% identity and 40 bp as overlap. The final assembly identified 20 scaffolds and 120 contigs and generated a genome size of 4.61Mb which corresponds to a coverage of 31.34 × genome equivalent.

Genome annotation

Open Reading Frames (ORFs) were predicted using Prodigal [118] with default parameters but the predicted ORFs were excluded if they were spanning a sequencing gap region. The predicted bacterial protein sequences were searched against the GenBank database [119] and the Clusters of Orthologous Groups (COG) databases using BLASTP. The tRNAScanSE tool [120] was used to find tRNA genes, whereas ribosomal RNAs were found by using RNAmmer [121] and BLASTn against the GenBank database. Lipoprotein signal peptides and the number of transmembrane helices were predicted using SignalP [122] and TMHMM [123] respectively. ORFans were identified if their BLASTP *E*-value was lower than $1e^{-03}$ for alignment length greater than 80 amino acids. If alignment lengths were smaller than 80 amino acids, we used an *E*-value of $1e^{-05}$. Such parameter thresholds have already been used in previous works to define ORFans. Ortholog sets composed of one gene from each of six genomes (*B. massilioanorexius* strain $AP8^T$, *B. timonensis* strain DSM 25372 (GenBank accession number CAET00000000), *B. amyloliquefaciens* strain FZB42 (GenBank accession number NC_009725), *B. massiliosenegalensis* strain JC6T (GenBank accession number CAHJ00000000), *B. mycoides* strain DSM 2048 (GenBank accession number CM000742) and *B. thuringiensis* strain BMB171 (GenBank accession number CP001903),) were identified using the Proteinortho software (version 1.4) [124] using a 30% protein identity and $1e^{-05}$ *E*-value. The average percentage of nucleotide sequence identity between corresponding

orthologous sets were determined using the Needleman-Wunsch algorithm global alignment technique. Artemis [125] was used for data management and DNA Plotter [126] was used for visualization of genomic features. Mauve alignment tool was used for multiple genomic sequence alignment and visualization [127].

Genome properties

The genome of *B. massiliensis* strain AP8^T is 4,616,135 bp long (1 chromosome, but no plasmid) with a 34.10% G + C content (Figure 26 and Table 24). Of the 4,519 predicted genes, 4,432 were protein-coding genes, and 87 were RNAs. Eight rRNA genes (one 16S rRNA, one 23S rRNA and six 5S rRNA) and 79 predicted tRNA genes were identified in the genome. A total of 3,290 genes (72.80%) were assigned a putative function. Three hundred fifty-four genes were identified as ORFans (7.98%). The remaining genes were annotated as hypothetical proteins. The properties and the statistics of the genome are summarized in Table 24 and Table 5. The distribution of genes into COGs functional categories is presented in Table 25.

Comparison with other *Bacillus* species genomes

Here, we compared the genome of *B. massilioanorexius* strain AP8^T, *B. timonensis* strain DSM 25372, *B. amyloliquefaciens* strain FZB42, *B. massiliosenegalensis* strain JC6T, *B. mycoides* strain DSM 2048 and *B. thuringiensis* strain BMB171. The draft genome of *B. massilioanorexius* is larger in size than that of *B. amyloliquefaciens* (4.6 vs 3.9 Mb, respectively), similar in size than that of *B. timonensis* (4.6 Mb) and smaller in size than those of *B. massiliosenegalensis*, *B. mycoides* and *B. thuringiensis* (4.9, 5.5 and 5.6 Mb, respectively). The G+C content of *B. massilioanorexius* is lower than those of *B. massiliosenegalensis*, *B. timonensis*, *B. amyloliquefaciens*, *B. mycoides* and *B. thuringiensis* (34.10, 37.60, 37.30, 46.48, 35.21 and 35.18%, respectively). The gene content of *B. massilioanorexius* is larger than that of *B. amyloliquefaciens* (4,519 and 3,814, respectively) and fewer than those of *B. massiliosenegalensis*, *B. timonensis*, *B. mycoides* and *B. thuringiensis* (4,997, 4,684, 5,747 and 5,495, respectively). The ratio of genes per MB of *B. massilioanorexius* is greater than that of *B. amyloliquefaciens* (982 and 978, respectively), comparable to that of *B. thuringiensis* (982) and smaller to those of *B. massiliosenegalensis*, *B. timonensis* and *B. mycoides* (1,019, 1,018 and 1,044, respectively). However, the distribution of genes into COG categories was not entirely similar in all the three compared genomes (Figure 27). The nucleotide sequence identity ranged from 66.09 to 83.69% among *Bacillus* species, and from 66.09 to 70.10% between *B. massilioanorexius* and other *Bacillus* species, thus confirming its new species status. Table 26 summarizes the numbers of

orthologous genes and the average percentage of nucleotide sequence identity between the different genomes studied.

Conclusion

On the basis of phenotypic, phylogenetic and genomic analyses, we formally propose the creation of *Bacillus massilioanorexius* sp. nov. that contains the strain AP8^T. The strain has been found in France.

Description of *Bacillus massilioanorexius* sp. nov.

Bacillus massilioanorexius (ma.si.li.o.a.no.rex'i.us. L. masc. adj. massilioanorexius, combination of Massilia, the Latin name of Marseille, France, where the type strain was isolated, and anorexia, the disease presented by the patient from whom the strain was cultivated).

Colonies were 3 mm in diameter and 0.5 mm in thickness, gray in color with a coarse appearance on blood-enriched Columbia agar. Cells are rod-shaped with a mean diameter of 0.77 μm. Optimal growth occurs aerobically, weak growth was observed under anaerobic conditions. Growth occurs between 25 and 45°C, with optimal growth observed at 37°C. Cells stain Gram-positive, are non-endospore forming and are motile. Cells are Gram-positive, catalase-positive, oxidase-positive. D-glucose, D-fructose, D-saccharose, D-trehalose, ribose, mannitol, mannose were used as carbon source. Positive reactions were observed for tryptophane deaminase, acetoin and gelatinase production. Weak reactions were obtained for L-rhamnose, esculine, salicine, D-cellobiose and gentiobiose. Cells are susceptible to amoxicillin, rifampicin, ciprofloxacin, gentamicin, doxycycline and vancomycin but resistant to trimethoprim/sulfamethoxazole and metronidazole.

The G+C content of the genome is 34.10%. The 16S rRNA and genome sequences are deposited in GenBank under accession numbers JX101689 and CAPG00000000, respectively. The type strain AP8^T (= CSUR P201 = DSM 26092) was isolated from the fecal flora of a female suffering from anorexia nervosa in Marseille, France.

Acknowledgements

The authors thank the Xegen Company (www.xegen.fr) for automating the genomic annotation process. This study was funded by the Mediterranée-Infection Foundation.

Table 21. Classification and general features of *Bacillus massilioanorexius* strain AP8^T

MIGS ID	Property	Term	Evidence codea
		Domain *Bacteria*	TAS [36]
		Phylum *Firmicutes*	TAS [37-39]
		Class *Bacilli*	TAS [40,41]
		Domain *Bacteria*	TAS [36]
	Current classification	Order *Bacillales*	TAS [42,43]
		Family *Bacillaceae*	TAS [42,44]
		Genus *Bacillus*	TAS [29,42,45]
		Species *Bacillus massilioanorexius*	IDA
		Type strain AP8^T	IDA
	Gram stain	Positive	IDA
	Cell shape	Bacilli	IDA
	Motility	Motile	IDA
	Sporulation	Nonsporulating	IDA
	Temperature range	Mesophile	IDA
	Optimum temperature	37°C	IDA
MIGS-6.3	Salinity	Unknown	IDA
MIGS-22	Oxygen requirement	Aerobic	IDA
	Carbon source	Unknown	NAS
	Energy source	Unknown	NAS
MIGS-6	Habitat	Human gut	IDA
MIGS-15	Biotic relationship	Free living	IDA
	Pathogenicity	Unknown	Pathogenicity
	Biosafety level	2	Biosafety level
MIGS-14	Isolation	Human feces	MIGS-14
MIGS-4	Geographic location	France	IDA
MIGS-5	Sample collection time	August 2011	IDA
	Latitude	43.296482	IDA
MIGS-4.1	Longitude	5.36978	IDA
MIGS-4.3	Depth	Surface	IDA
MIGS-4.4	Altitude	0 m above sea level	IDA

Evidence codes - IDA: Inferred from Direct Assay; TAS: Traceable Author Statement (i.e., a direct report exists in the literature); NAS: Non-traceable Author Statement (i.e., not directly observed for the living, isolated sample, but based on a generally accepted property for the species, or anecdotal evidence). These evidence codes are from the Gene Ontology project [128]. If the evidence is IDA, then the property was directly observed for a live isolate by one of the authors or an expert mentioned in the acknowledgements.

Table 22. Differential characteristics of *Bacillus massilioanorexius* strain AP8^{T}, *B. timonensis* strain DSM 25372, *B. amyloliquefaciens* strain FZB42, *B. massiliosenegalensis* strain JC6T, *B. mycoides* strain DSM 2048 and *B. thuringiensis* strain BMB171

Properties	***B. massilio-anorexius***	***B. timonensis***	***B. amyloli-quefaciens***	***B. massilio-senegalensis***	***B. mycoides***	***B. thuringi-ensis***
Cell diameter (μm)	0.77	0.66	0.8	0.65	1.1	1.0
Oxygen requirement	aerobic	aerobic	aerobic	aerobic	facultative anaerobic	facultative anaerobic
Pigment production	+	–	–	–	–	–
Gram stain	+	–	+	+	+	+
Salt requirement	–	+	+	+	+	
Motility	+	+	+	+		–
Endospore formation	–	+	+	+	+	+
Production of						
Acid phosphatase	na	na	+	w	+	+
Catalase	+	–	+	+	+	+
Oxidase	+	+	+	–	–	+
Nitrate reductase	na	na	+	+	v	+
Urease	–	na	–	–	v	+
β-galactosidase	na	+	v	na	+	–
N-acetyl-glucosamine	na	+	+	+	+	+
Acid from						
L-Arabinose	–	+	+	–	+	na
Ribose	+	–	+	–	+	+
Mannose	+	–	+	–	+	+
Mannitol	+	–	+	–	+	+
Sucrose	–	–	+	–	+	v
D-glucose	+	–	+	+	+	+
D-fructose	+	–	+	–	+	+
D-maltose	–	–	+	+	+	+
D-lactose	–	+	+	–	+	+
Hydrolysis of						
Gelatin	+	–	+	–	+	+
Starch	na	na	+	na	+	+
G+C content (mol %)	34.10	37.30	46.48	37.6	35.21	35.18
Habitat	human gut	human gut	Soil	human gut	soil	soil

na = data not available; w = weak, v = variable reaction

Table 23. Project information

MIGS ID	Property	Term
MIGS-31	Finishing quality	High-quality draft
MIGS-28	Libraries used	One 454 paired end 3-kb library
MIGS-29	Sequencing platforms	454 GS FLX Titanium
MIGS-31.2	Fold coverage	31.34 ×
MIGS-30	Assemblers	Newbler version 2.5.3
MIGS-32	Gene calling method	Prodigal
	Genbank ID	CAPG00000000
	Genbank Date of Release	November 28, 2012
	Gold ID	Gi20708
MIGS-13	Project relevance	Study of the human gut microbiome

Table 24. Nucleotide content and gene count levels of the genome

Attribute	Value	% of total[a]
Genome size (bp)	4,616,135	
DNA coding region (bp)	3,750,534	81.24
DNA G+C content (bp)	1,574,102	34.10
Number of replicons	1	
Extrachromosomal elements	0	
Total genes	4,519	100
RNA genes	87	1.92
rRNA operons	1	
Protein-coding genes	4,432	98.07
Genes with function prediction	3,524	77.98
Genes assigned to COGs	3,290	72.80
Protein coding genes assigned Pfam domains	3,807	84.24
Genes with peptide signals	270	5.97
Genes with transmembrane helices	1,241	27.46
CRISPR repeats	2	

[a] The total is based on either the size of the genome in base pairs or the total number of protein coding genes in the annotated genome

Table 25. Number of genes associated with the 25 general COG functional categories

Code	Value	%age [a]	Description
J	171	3.86	Translation
A	0	0	RNA processing and modification
K	335	7.56	Transcription
L	200	4.51	Replication, recombination and repair
B	1	0.02	Chromatin structure and dynamics
D	37	0.83	Cell cycle control, mitosis and meiosis
Y	0	0	Nuclear structure
V	76	1.71	Defense mechanisms
T	212	4.78	Signal transduction mechanisms
M	147	3.32	Cell wall/membrane biogenesis
N	70	1.58	Cell motility
Z	0	0	Cytoskeleton
W	0	0	Extracellular structures
U	48	1.08	Intracellular trafficking and secretion
O	121	2.73	Posttranslational modification, protein turnover, chaperones
C	245	5.53	Energy production and conversion
G	221	4.99	Carbohydrate transport and metabolism
E	405	9.14	Amino acid transport and metabolism
F	98	2.21	Nucleotide transport and metabolism
H	135	3.05	Coenzyme transport and metabolism
I	136	3.07	Lipid transport and metabolism
P	258	5.82	Inorganic ion transport and metabolism
Q	81	1.83	Secondary metabolites biosynthesis, transport and catabolism
R	527	11.89	General function prediction only
S	349	7.87	Function unknown
-	1,142	25.77	Not in COGs

[a] The total is based on the total number of protein coding genes in the annotated genome.

Table 26. Orthologous gene comparison and average nucleotide identity of *Bacillus* species *B. massilioanorexius*[1] with *B. massiliosenegalensis*[2]; *B. timonensis*[3], *B. thuringiensis*[4]; *B. mycoides*[5]; *B. amyloliquefaciens*[6†].

	B. massilio-anorexius	***B. massilios-enegalensis***	***B. timonensis***	***B. thuringiensis***	***B. mycoides***	***B. amyloliquefaciens***
B. massilioanorexius	**4,432**	1,897	1,864	1,887	1,794	1,709
B.massiliosenegalensis	70.10	**4,895**	1,965	1,863	1,765	1,755
B. timonensis	69.84	70.33	**4,610**	1,864	1,762	1,742
B. thuringiensis	69.35	68.88	69.31	**6,243**	2,210	1,832
B. mycoides	69.41	69.11	69.41	83.69	**5,885**	1,719
B. amyloliquefaciens	66.09	67.02	67.12	66.35	66.57	**3,823**

†Upper right, numbers of orthologous genes; lower left, mean nucleotide identities of orthologous genes. Bold numbers indicate the numbers of genes or each genome.
[1]Genbank accession number CAPG00000000, [2]CAHJ00000000, [3]CAET00000000, [4]CP001903, [5]CM000742, [6]NC_009725

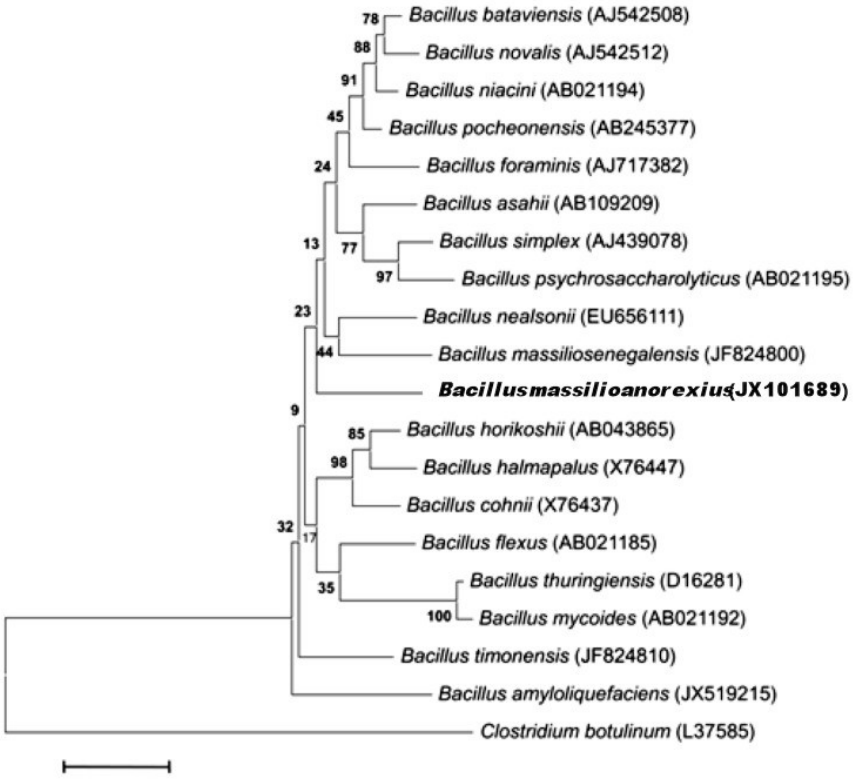

Figure 21. Phylogenetic tree highlighting the position of *Bacillus massilioanorexius* strain AP8^T relative to a selection of type strains of validly published species of *Bacillus* genus. GenBank accession numbers are indicated in parentheses. Sequences were aligned using CLUSTALW, and phylogenetic inferences obtained using the maximum-likelihood method within MEGA program. Numbers at the nodes are percentages of bootstrap values obtained by repeating the analysis 500 times to generate a majority consensus tree. *Clostridium botulinum* was used as outgroup. The scale bar represents a 2% nucleotide sequence divergence.

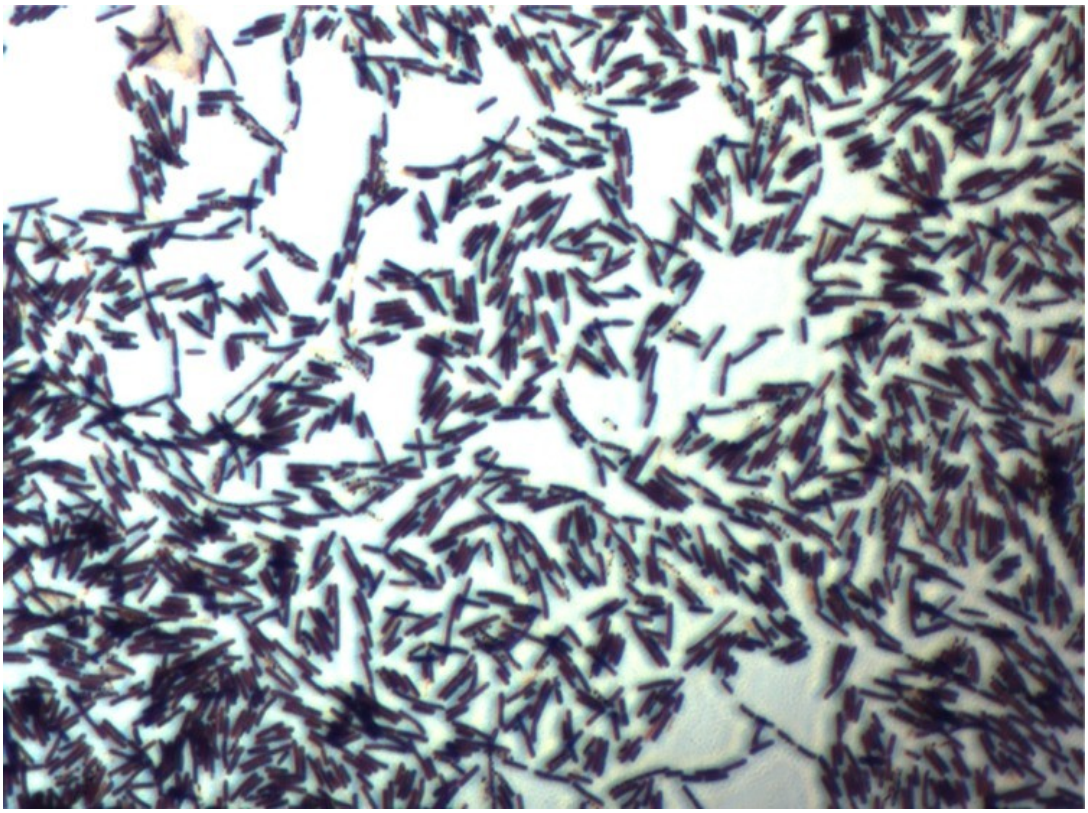

Figure 22. Gram staining of *B. massilioanorexius* strain AP8^{T}

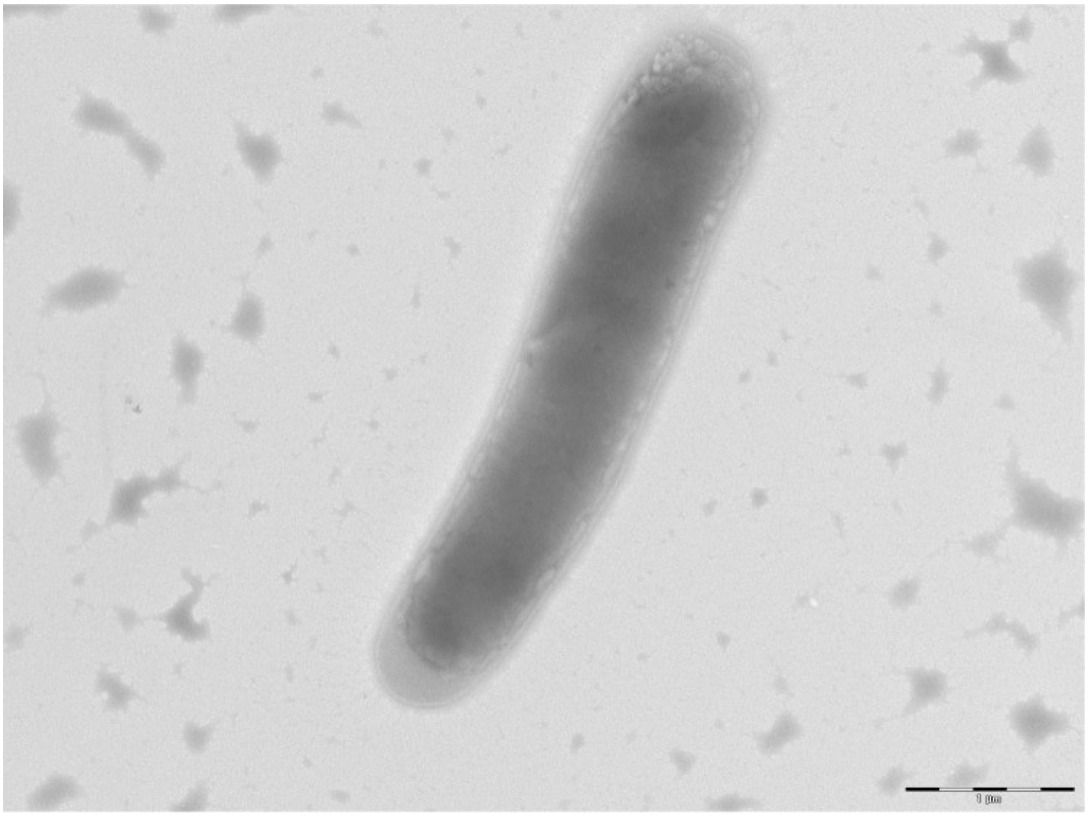

Figure 23. Transmission electron microscopy of *B. massilioanorexius* strain AP8^{T}, using a Morgani 268D (Philips) at an operating voltage of 60kV. The scale bar represents 1µm.

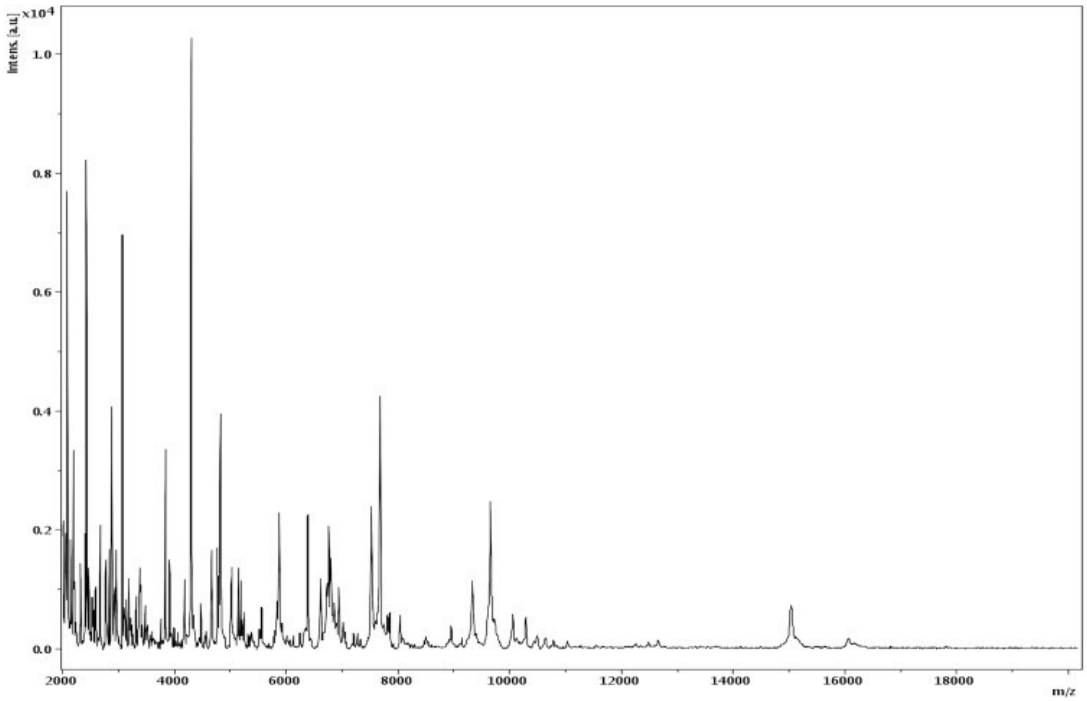

Figure 24. Reference mass spectrum from *B. massilioanorexius* strain AP8^T. Spectra from 12 individual colonies were compared and a reference spectrum was generated.

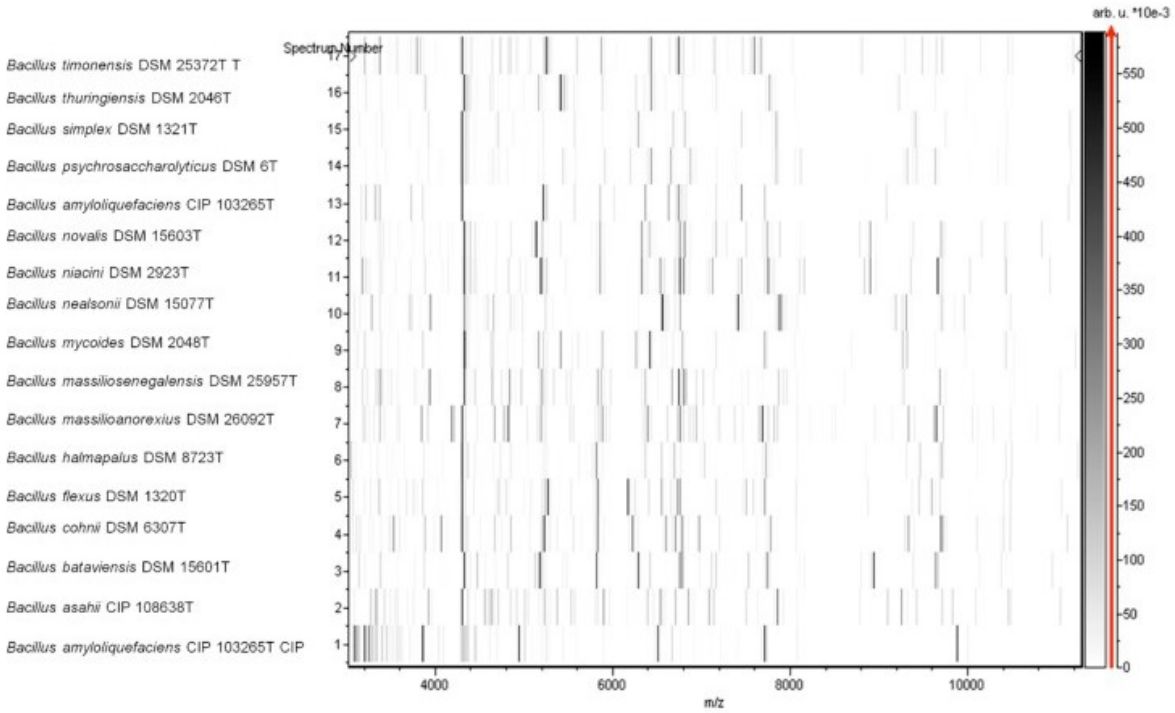

Figure 25. Gel view comparing *B. massilioanorexius* sp. nov strain AP8^T and other *Bacillus* species. The gel view displays the raw spectra of loaded spectrum files arranged in a pseudo-gel like look. The x-axis records the m/z value. The left y-axis displays the running spectrum number originating from subsequent spectra loading. The peak intensity is expressed by a Gray scale scheme code. The color bar and the right y-axis indicate the relation between the color a peak is displayed with and the peak intensity in arbitrary units. Displayed species are indicated on the left.

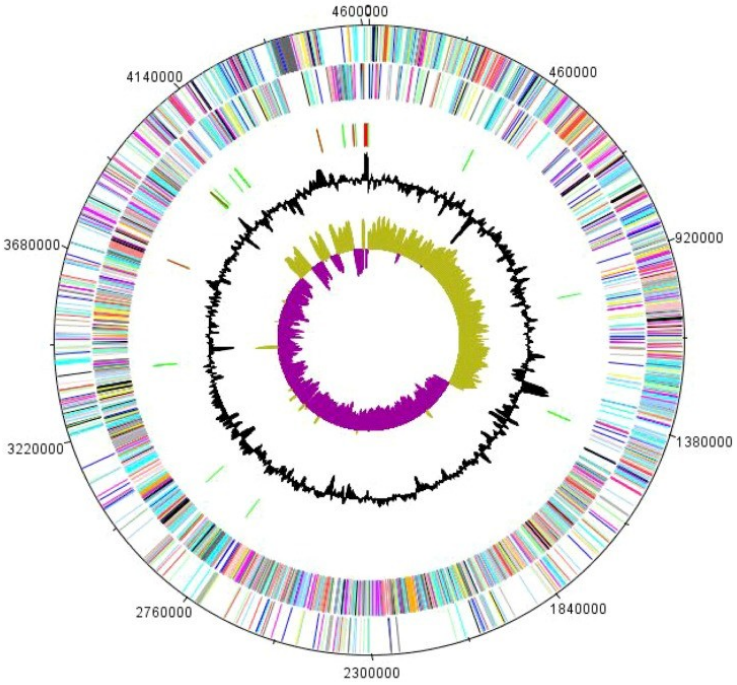

Figure 26. Graphical circular map of the chromosome. From the outside in, the outer two circles show open reading frames oriented in the forward and reverse directions (colored by COG categories), respectively. The third circle shows the rRNA gene operon (red) and tRNA genes (green). The fourth circle shows the G+C% content plot. The inner-most circle shows GC skew, purple and olive indicating negative and positive values, respectively.

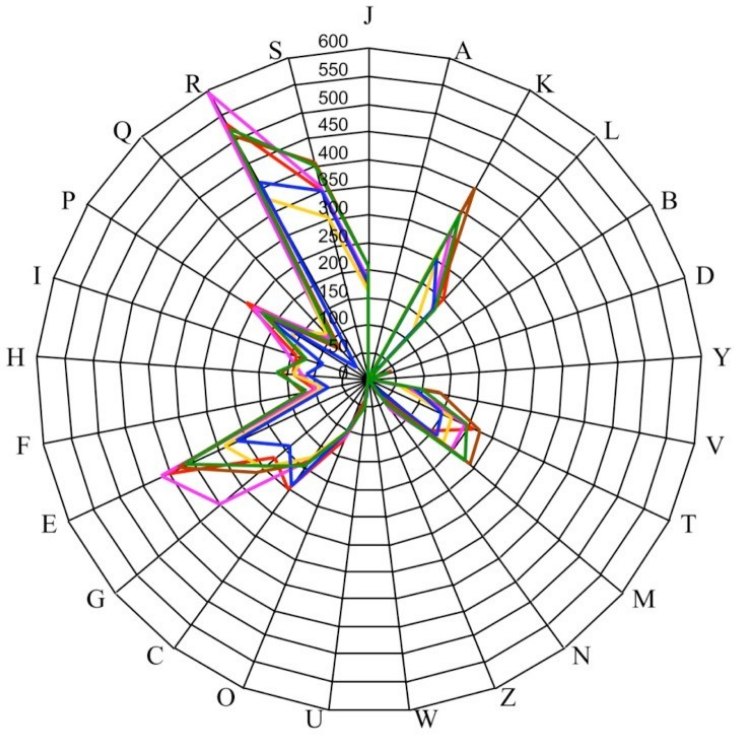

Figure 27. Distribution of functional classes of predicted genes in *B. massilioanorexius* (red), *B. massiliosenegalensis* (blue), *B. timonensis* (pink), *B. amyloliquefaciens* (yellow), *B. mycoides* (brown) and *B. thuringiensis* (green) chromosomes according to the clusters of orthologous groups of proteins.

2.3. Description of further bacteria isolated for the first time in this work

In the following, the 10 further newly discovered bacteria will be described. In contrast to the previous article on *Bacillus massilioanorexius* only results generated by my own work will be stated. The most important phenotypical characters and growth conditions will be described in detail. Except oxidase and catalase activity it will be passed on listing biochemical characterization. Phylogenetic trees of all newly discovered bacterial species can be found in figure S1 in chapter 2.1.1 and are not displayed here.

2.3.1 Alistipes ihumii

(The name has been changed from „*Alistipes marseilloanorexicus*“ used in „Culturomics identified 11 new bacterial species from a single anorexia nervosa stool sample“ to current and definitive name „*Alistipes ihumii*“)

Alistipes ihumii AP11^T belongs to the phylum of *Bacteroidetes* and has been isolated from Columbia-Agar in anaerobic atmosphere after preincubation in anaerobic blood culture with addition of Thioglycolate. Colonies of *A. ihumii* AP11^T appeared transparent on Columbia-Agar, with a diameter of 0,2 mm, grew very close to each other and showed ß-hemolysis. Optimal growth occurred in anaerobic conditions, only weakly in microaerophilic and not in aerobic conditions or with 5% CO_2. Colonies grew between 25-45°C, with optimal growth at 37°C. Cells are non-motile rods with a mean diameter of 0.72 µm. They are gram-negative and have a negative catalase and positive oxidase activity.

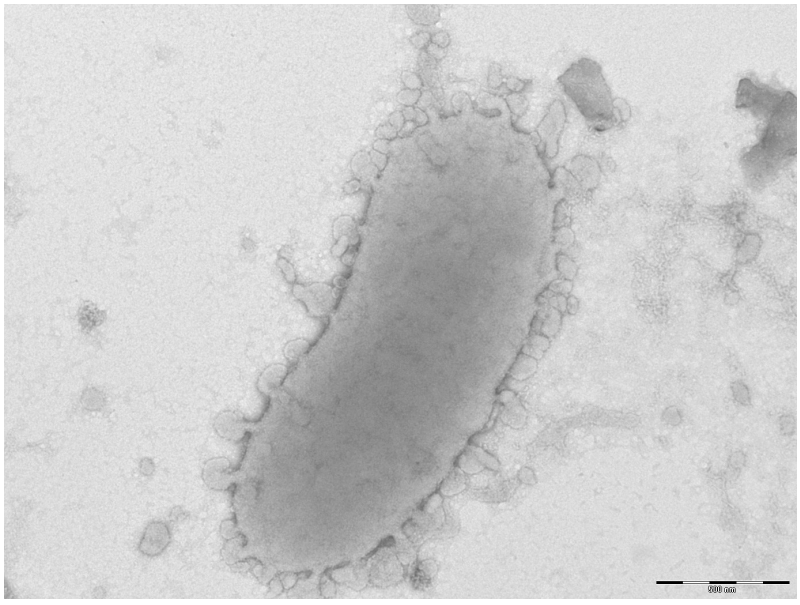

Fig. 31. Electron microscopy of *Alistipes ihumii* AP11^T. Scale bar: 500 nm.

2.3.2 Bacteroides timonensis

Bacteroides timonensis AP1^T belongs to the phylum of *Bacteroidetes.* The sample has been incubated for 1 month on Columbia-Agar in anaerobic atmosphere. Colonies of *B. timonensis* AP1^T appeared transparent on Columbia-Agar with a creamy solid consistence and a diameter of 0,33 mm. They grew on each other, creating smears with thickness upon 0,5 mm. Optimal growth occurred in anaerobic and microaerophilic conditions, only weakly under 5% CO_2 and not in aerobic conditions. Colonies grew between 25-37°C, with optimal growth at 37°C. Cells are non-motile rods with a mean diameter of 0.88 µm. They are gram-negative and have a positive catalase and negative oxidase activity. *B. timonensis* AP1^T is susceptible to doxycycline, amoxicillin + clavulanic acid, metronidazole, imipenem, weakly to vancomycin and is resistant to penicillin G.

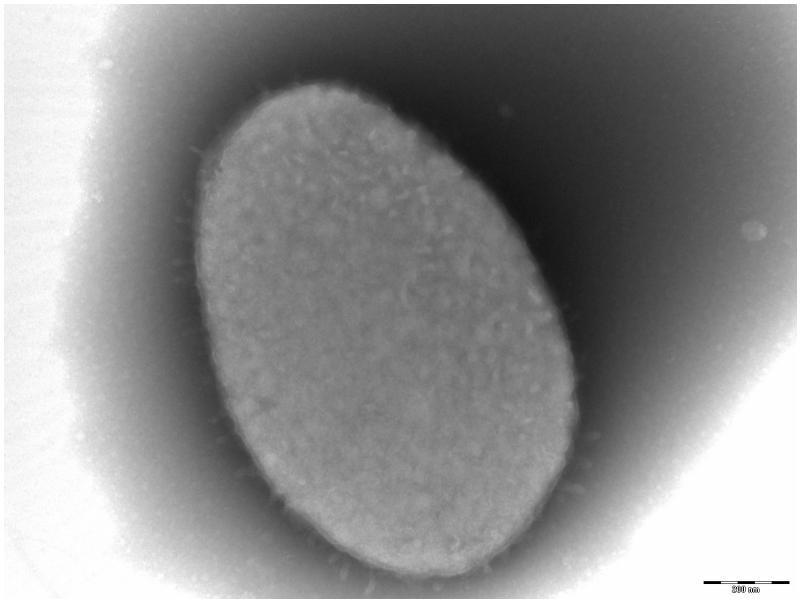

Fig. 32. Electron microscopy of *Bacteroides timonensis* AP1^T. Scale bar: 200 nm.

2.3.3 Blastococcus massiliensis

Blastococcus massiliensis AP4^T belongs to the phylum of *Actinobacteria* and has been isolated on Brucella-Agar in aerobic atmosphere. Colonies of *B. massiliensis* AP4^T appeared opaque whitely on Columbia-Agar, had a creamy consistence and a diameter of 0,5 mm. Growth occurred in aerobic, microaerophilic conditions and under 5% CO2, very weakly after 2 days in anaerobic conditions. Colonies grew between 25-37°C, after 2 days very weakly at 45°C, with optimal growth at 37°C. Cells are non-motile elongate cocci with a mean diameter of 0.4 µm. They are gram-positive and have a positive catalase and negative oxidase activity. *B. massiliensis* AP4^T is susceptible to vancomycin, imipenem, amoxicillin + clavulanic acid,

doxycycline, penicillin and resistant to metronidazole.

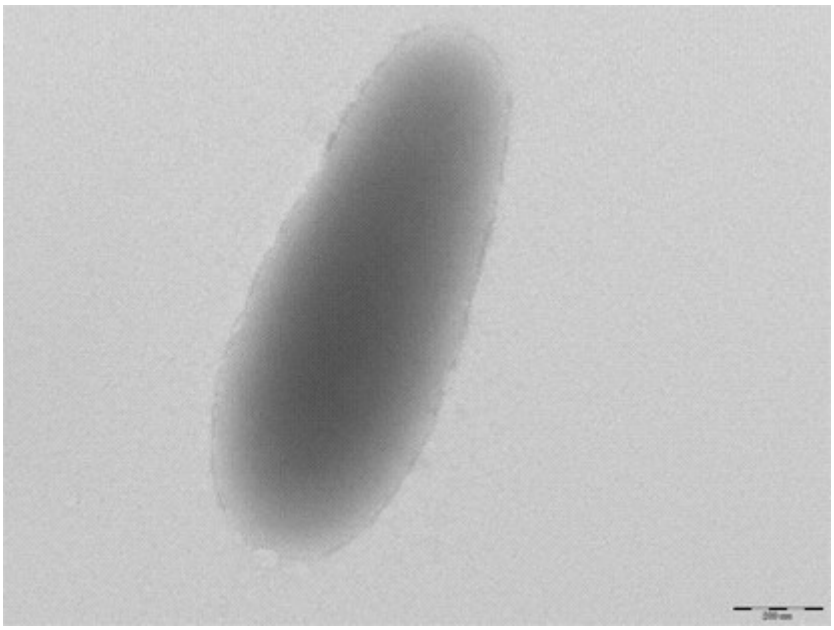

Abb. 33. Electron microscopy of *Blastococcus massiliensis* AP4^{T}. Scale bar: 300nm.

2.3.4 Clostridium ihumii

(The name has been changed from „*Clostridium anorexicus*“ used in „Culturomics identified 11 new bacterial species from a single anorexia nervosa stool sample“ to current and definitive name „*Clostridium ihumii*“)

Fig. 34. Electron microscopy of *Clostridium ihumii* AP6^{T}. Scale bar: 900nm.

Clostridium ihumii AP6^{T} belongs to the phylum of *Firmicutes*. The sample has been incubated on Columbia-Agar in anaerobic atmosphere after preincubation in anaerobic blood culture

with addition of sheep blood. Colonies of *C. ihumii* AP6^T appeared whitely on Columbia-Agar, with a diameter of 0,5 mm and grew in large distance to each other. Growth only occurred in anaerobic conditions. Colonies grew between 25-45°C, with an optimal growth at 37°C. Cells are non-motile rods with a mean diameter of 0.69 µm. They are gram-positive and have a negative catalase and a positive oxidase activity.

2.3.5 Clostridium anorexicamassiliense

Clostridium anorexicamassiliense AP5^T belongs to the phylum of *Firmicutes*. The sample has been incubated on Columbia-Agar in anaerobic atmosphere after preincubation in anaerobic blood culture with addition of sheep blood. Colonies of *C. anorexicamassiliense* AP5^T had a irregular appearance on Columbia-Agar, were blue-green to gray, opaque with a displeasing smell and had a diameter of 5 mm. Growth only occurred in anaerobic condition. Colonies grew between 25-45°C, with optimal growth at 37°C. Cells are non-motile rods with very variegate diameter. They are gram-positive and have a positive catalase and negative oxidase activity.

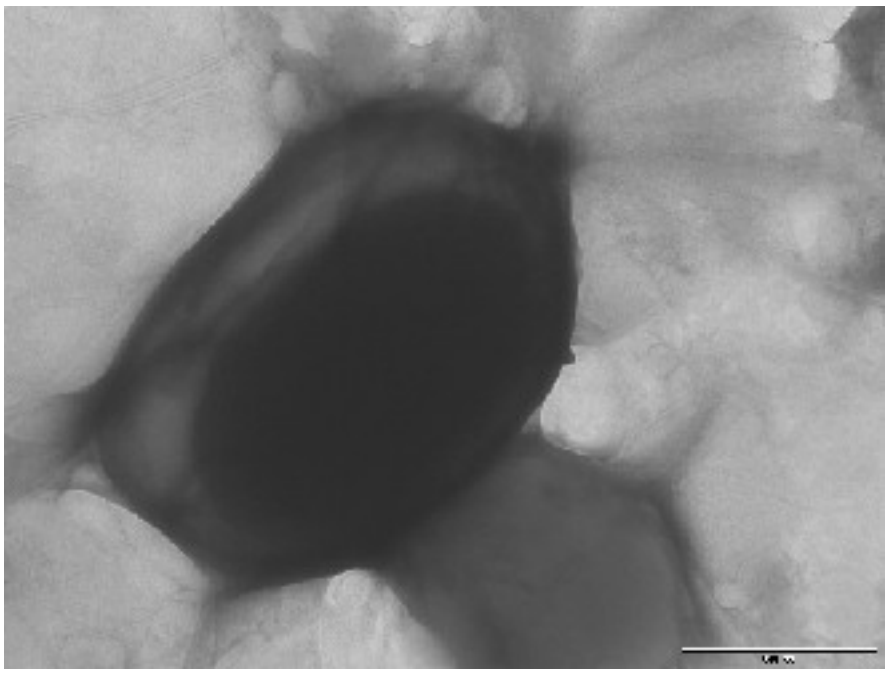

Fig. 35. Electron microscopy of *Clostridium anorexicamassiliense* AP5^T. Scale bar: 500nm.

2.3.6 Dorea massiliensis

Dorea massiliensis AP3^T belongs to the phylum of *Firmicutes*. The sample has been incubated on Columbia-Agar in anaerobic atmosphere after preincubation in anaerobic blood culture with addition of rumen of a sheep. Colonies of *D. massiliensis* AP3^T appeared light brown on

Columbia-Agar and were very small and plane. Growth occurred in anaerobic, microaerophilic, aerobic condition with and without 5% CO_2. Colonies grew between 25-45°C, with optimal growth at 37°C. Cells are non-motile gram-positive rods.

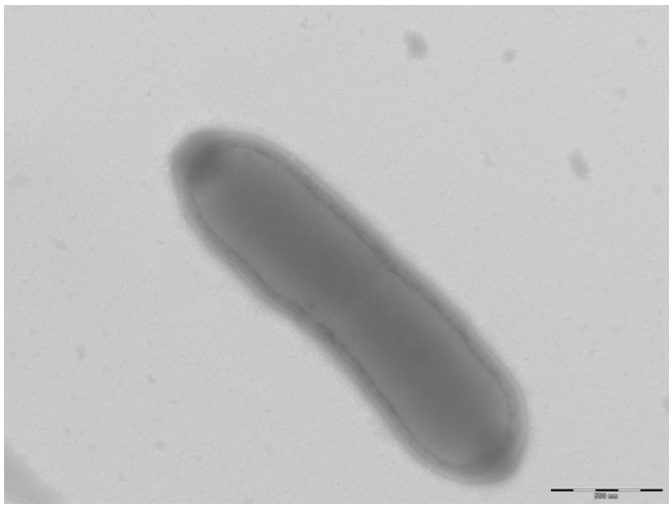

Abb. 36. Electron microscopy of *Dorea massiliensis* AP3^T. Sale bar: 500nm.

2.3.7 Holdemania massiliensis

Holdemania massiliensis AP2^T belongs to the phylum of *Bacteroidetes*. The sample has been incubated on Columbia-Agar in anaerobic atmosphere after preincubation in anaerobic blood culture with addition of Thioglycolate. Colonies of *H. massiliensis* AP2^T appeared light gray on Columbia-Agar, had a creamy consistence, a diameter of 0,2 mm and showed ß-hemolysis. Growth only occurred in anaerobic atmosphere. Colonies grew after 72 hours between 25-30°C, with optimal growth after 24 hours at 37°C. Cells are non-motile rods with a mean diameter of 0.57 µm. They are gram-positive and have a negative catalase and positive oxidase activity.

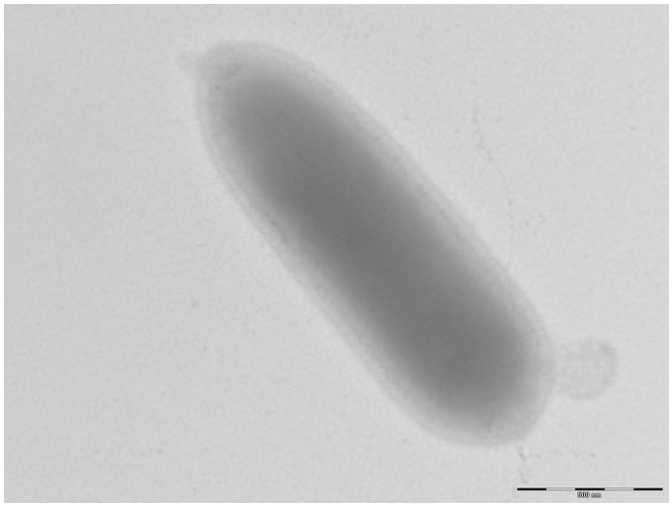

Abb. 37. Electron microscopy of *Holdemania massiliensis* AP2^T. Scale bar: 900nm.

2.3.8 Streptomyces massiliensis

Streptomyces massiliensis AP7^T belongs to the phylum of *Actinobacteria.* The sample had been diluted and filtrated through 0,45 µm and incubated on BHI-Agar in aerobic condition. Colonies of *S. massiliensis* AP7^T appeared greenly gray on Columbia-Agar, had a diameter of 0,4 mm and were very dry and adhesive. Optimal growth occurred in aerobic conditions, weakly under 5% CO_2 and not in anaerobic or microaerophilic atmospheres. Colonies grew between 30-45°C, with optimal growth at 37°C-45°C. Cells are non-motile linear rods with a mean diameter of 0.54µm. They are gram-positive and have a positive catalase and oxidase activity.

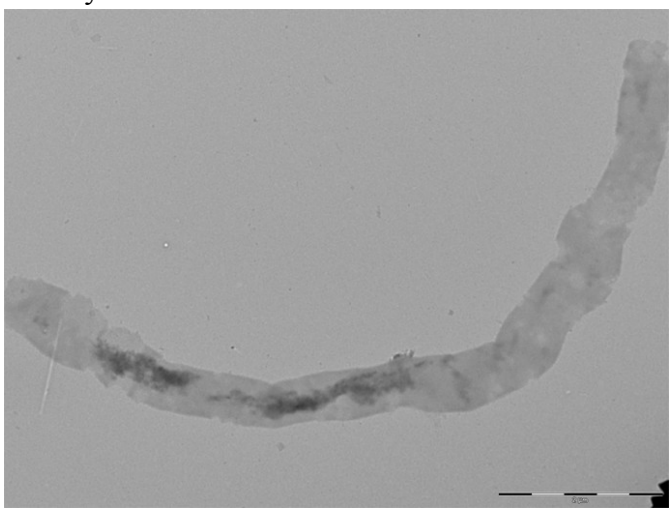

Abb. 38.1. Electron microscopy of *Streptomyces massiliensis* AP7^T. Scale bar: 2µm.

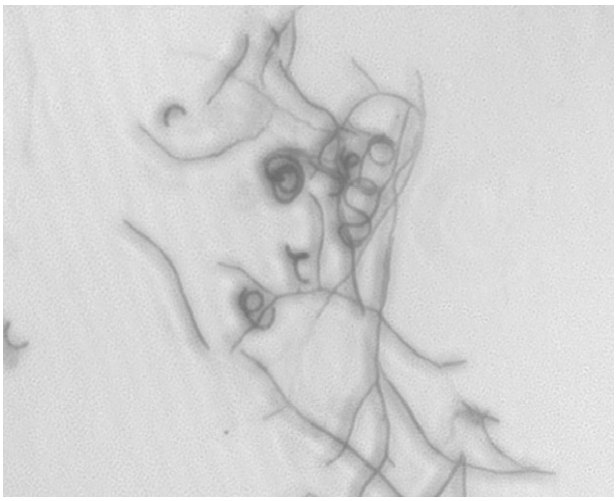

Abb. 38.2. Gram-straining of *Streptomyces massiliensis* AP7^T

2.3.9 Soleaferrea massiliensis

Soleaferrea massiliensis AP10^T belongs to the phylum of *Firmicutes* and to the family of *Ruminococcaceae*. It is a new genus. The sample has been incubated on Columbia-Agar in anaerobic atmosphere after preincubation in anaerobic blood culture with addition of Thioglycolate. Colonies of *Soleaferrea massiliensis* AP10^T appeared gray on Columbia-Agar, had a rough surface, a diameter of upon 3 mm and a thickness of 0,5mm. Optimal growth occurred in anaerobic but also in aerobic condition with or without CO_2. Colonies grew between 35-45°C, with optimal growth at 37°C. Cells are non-motile curved rods with a mean diameter of 0.64µm. They are gram-positive and have a negative catalase and positive oxidase activity.

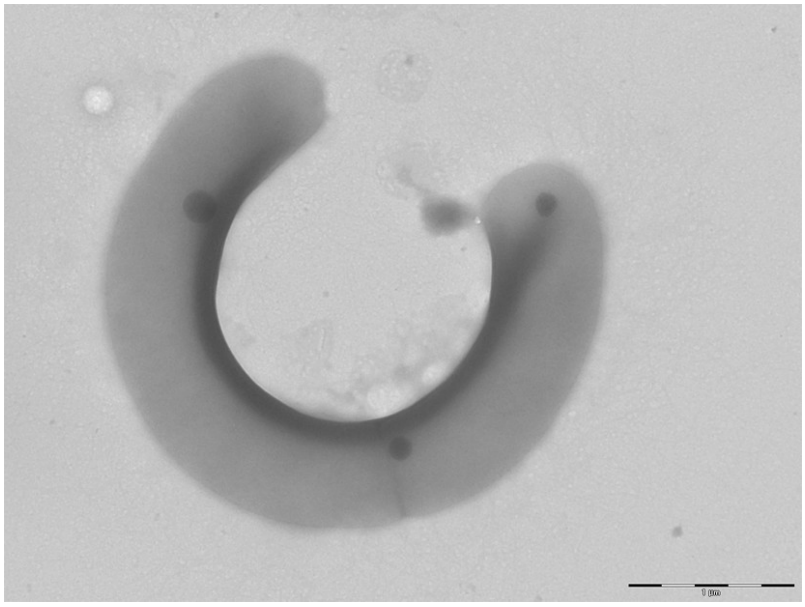

Abb. 39.1. Electron microscopy of *Soleaferrea massiliensis* AP10^T. Scale bar: 1µm.

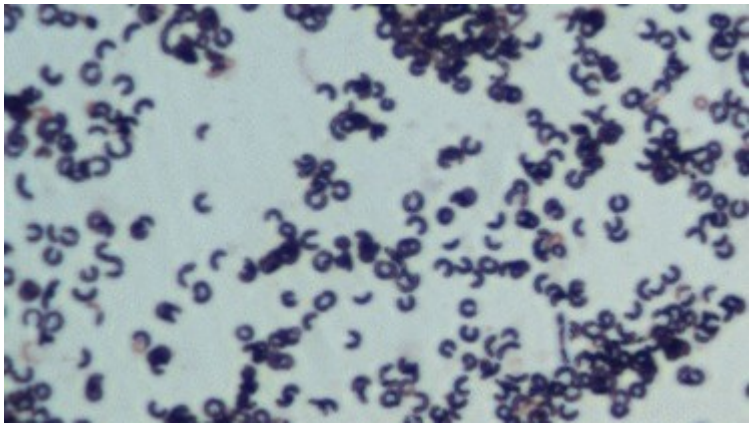

Abb. 39.2. Gram-straining of *Soleaferrea massiliensis* AP10^T

2.3.10 Stoquefichus massiliensis

Stoquefichus massiliensis AP9^T belongs to the phylum of *Firmicutes* and to the family of *Erysipelotrichaceae*. The new genus has been isolated after anaerobic incubation at 28°C on Columbia-Agar.

Colonies of *Stoquefichus massiliensis* AP9^T appeared silver on Columbia-Agar, with a diameter of 0,5 mm and grew close to each other thus forming either a carpet of small colonies, or 1 mm thick drops of irregular colonies. Optimal growth occurred in anaerobic condition, only weakly in microaerophilic and not in aerobic conditions with or without 5% CO_2. Colonies grew between 25-45°C, with optimal growth at 37°C. Cells are non-motile rods with a mean diameter of 0.62 µm. They are gram-positive and have a positive catalase and oxidase activity.

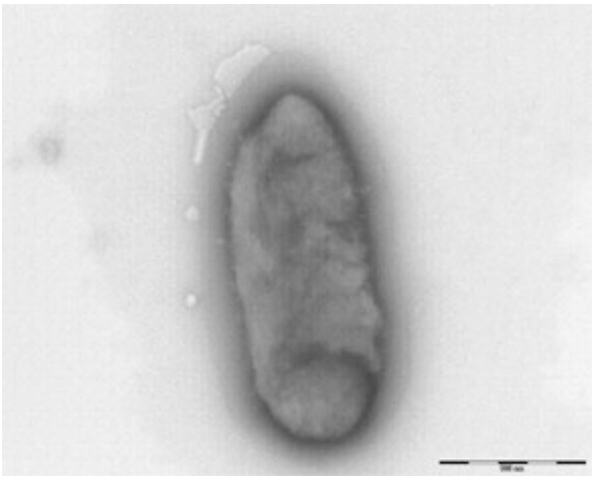

Abb. 30. Electron microscopy of *Stoquefichus massiliensis* AP9^T. Scale bar: 500nm.

3. Discussion

In this study, the intestinal microbiota of an anorexia nervosa patient has been examined for the first time.

The identification of the cultivated bacteria was worked out either by mass spectrometry or by sequencing of 16S rRNA. Mass spectrometry is a validated, time effective method for identification of bacteria in clinical laboratories of microbiology [129][130] and the gene sequence 16S rRNA is the standard for sequencing bacterial genes [84]. Therefore the results of this study have been supported with validated methods.

Anorexia nervosa

As this study examines a single sample of one person, no conclusions can be made on correlations between bacterial species and development of disease. The patient regrettably died due to her illness. A longitudinal study which could have observed transformation and adaption of intestinal flora to an eventual change of diet or treatment becomes thereby impossible. But the obtained results of this study on the intestinal microflora of an anorexia patient can be compared to other data as presented in introduction.

Firstly there are the studies on gut flora of undernourished children. The restrictive diet of an anorexia patient differs naturally from classical undernourishment: the food of our patient was in fact quantitatively poor but she had payed attention to ingest all necessary nutrients and vitamins. Nevertheless in both forms in total a shortage of nutrients is dominating. This shortage affects the intestinal mucosa and the gut flora probably adapts to the negative energy balance. The resulted data of the sample of the anorexia patient features analogies to the gut flora of undernourished children. This concerns over all a group of bacteria which do either have anti-inflammatory or inflammatory effects: *Bilophila wadsworthia* (correlating with inflammatory bowel diseases) and *Clostridium innocuum* (in relation with opportunistic infections) can be found as well in undernourished children [45] as well as in our patient. Anti-inflammatory bacteria such as *Eubacterium*, *Phascolarctobacterium*, *Roseburia* and *Faecalibacterium* were in contrast only missing in undernourrished children [43]. In addition to the pathogen species *Haemophilus parainfluenzae* found in both forms, *Porphyromonas somerae* has been detected in the sample of the Anorexie nervosa patient. Interestingly this bacterial group has been only found in stool samples of patients with M. Crohn until now [131].

It could be examined in future studies whether restrictive diet of anorexia nervosa also results in increased intestinal inflammatory activity as it seems to be the case in undernourishment due to poverty.

Secondly there is the approach to explain the development of anorexia nervosa by immunological processes, as described in introduction. This approach postulates a stimulation of antibodies by molecular mimicry with intestinal bacteria.
The first neuropeptide which seems to play a role in this context is α-MSH. Bacteria with influence on this molecule are *Bifidobacterium longum*, *Bacteroides, Bacillus cereus* and *Escherichia coli* [34]. All these bacteria have been isolated in this study. Apart from that there is *Enterobacteria phage* and the fungal species *Yarrowia lipolytica, Candida albicans, Cryptococcus neoformans* and *Aspergillus fumigatus* [34]. On viruses and fungi this study can not make a statement.
Antibodies against Ghrelin are expected to be reduced in anorexia patients [41] and can also be stimulated by certain bacteria. The bacteria known to influence this stimualation, *Enterococcus faecalis* and *Cl. Perfringens* [34], have been isolated from the sample of our patient. Again an other study has to be made for the virus *Lactobacillus casei bacteriophage* and for the eukaryotes *Mycobacteriophage, Saccharomyces cerevisiae, Yarrowia lipolytica, Candida albicans* and *Cryptococcus neoformans* [34].
Finally only one bacterial species has been found in this study which stimulates the production of antibodies against the „satiety hormone“ Leptin: *Lactococcus lactis* [34]. Apart from *Lactobacillus bacteriophage* and the fungi (*Candida albicans, Yarrowia lipolytica* and *Aspergillus fumigatus)* there are also bacteria missing in our study for the stimulation of antibodies against Leptin: *H. pylori* and *Campylobacter* [33].
Lactococcus lactis has been identified with 13 reads in metagenomics. *L. lactis* is thereby one of the bacteria of this sample only appearing in a small concentration. Also in culturing *L. lactis* has been isolated comparatively rarely. It could be supposed that in the patient of our study only few bacteria stimulate the production of antibodies against Leptin through molecular mimicry and that in consequence free Leptin is increased in plasma and gives a feeling of satiety to the patient. But this is contradicting results of previous studies where Leptin is found to be reduced in anorexia patients [132]. For the moment it is impossible to state effects of bacteria on the regulation of food intake, or to directly conclude the concentration of neuropeptides from the number of bacteria which have an influence on these respective neuropeptides. It still has to be researched how in detail the mechanism works to

change the balance of respective hormones in anorexia patients compared to healthy persons through stimulation of antibodies by intestinal bacteria. For this aim more microbiota of anorexia patients could be examined in future and be compared to microbiota of healthy subjects concerning the bacteria with influence on neuropeptides listed above.
In this study it is certain that the microflora of the anorexia patient possesses bacteria which can make molecular mimicry with all of the neuropeptides known until now to have an effect on hunger and food intake.
If the influence of the microbiome - and antibodies against neuropeptides regulating food intake- on the development of anorexia nervosa will be confirmed, it would have an important impact. On one hand it would change the image of a disease which has been classified to be purely psychological until now. This would influence the interaction with these patients and of course the therapy. To heal anorexia nervosa by modifying the intestinal microbiota seems to be scientifically unrealistic up to date. But there might be a possibility to bring hormones regulating food intake back into a natural balance and by this process influence positively the organic component of the disease.

All bacteria which have an effect on neuropeptides have been cultivated. But two of them have not been identified by metagenomics. These are *Bacillus cereus* and *Enterococcus faecalis*. In future metagenomic studies on microbiota of anorexia nervosa patients this should be considered and these bacteria should be researched separately because they might play an important role in this disease.
This leads to the next point: the relevance of culturing.

Culturomics and newly discovered species

This study affirms the benefit of cultivation to complete metagenomic results.
109 species have been isolated by culture which have not been identified by metagenomics. This constitutes with 65% a large part of all identified bacteria in this sample. Only 23 species have been detected by both methods. This accounts only 17% of all cultivated bacteria. In this study cultivation contributed more than metagenomics to explore the bacterial diversity. If one sample is examined by only one of these techniques, with the current existing possibilities a complete view on the bacterial diversity will not be obtained.
Why did 109 cultivated bacteria could not be detected by metagenomics?
Apparently some genes could not be assigned although their respective bacteria have been described and their reference genes added to the database before. This could be caused by too short reads or other technical limits of metagenomics [55]. The metagenomic study has been

performed in 2010/11. Since this a lot of advancements have been made in metagenomics [133] and it can be assumed that metagenomics today would detect more bacteria in the same sample. The number of not detected gene sequences should therefore continue falling in future.

One part of those bacteria which were not detected by metagenomics probably occurred in a too small concentration. This challenge will remain [134]. Here, with selective growth inhibition, culturing can detect some species which can not be identified by metagenomics. The question is if these bacteria occurring in a low concentration play an important role in the microbiome. But if this is the case, cultivation stays indispensable as long as metagenomics has not developed better techniques.

The time effort to complete each examination of micobiota by a culturing of the sample is regrettably too high to be made in parallel in each study. To discover correlations between certain bacterial populations and diseases or other characteristics, studies have to be mainly supported by metagenomics. Nevertheless metagenomic results should currently be regularly controlled and completed by culturing.

The newly identified 9 bacterial species and 2 bacterial genera which have been isolated from this sample continue giving value to cultivation. Their genome sequences have been published and are now accessible to metagenomic studies.

Which significance does cultivation have in the discovery of new bacterial species? From genes gained without cultivation, encoded enzymes and other proteins can be deduced. The genome of one bacteria contains per definition all information. However natural products of bacteria can only be gained after cultivating the bacterium itself [53]. These products are for instance the base of antibiotics. Medicine needs new antibiotics, because more and more bacteria are getting resistant to the common products [54]. This aspect demonstrates the opportunities included in cultivation of new species. Also the „behavior“ of bacteria and the signal transmission in vivo can only be described by cultivation [53]. A bacterium which is only registered by metagenomics and described by its genome, is not completely explored and it is at this point that cultivation becomes indispensable.

Compared to the estimated number of yet unknown bacteria, the count 11 is vanishing small. But only the bacteria newly discovered in the last 5 years in the Culturomics-project counted together constitute 75% of all newly described bacteria in this time [135]. This way, the study has reached its aim to explore the bacterial diversity in general and especially in human gut flora, and contributes to the expansion of its repertoire.

Moreover 8 known bacteria have been added to the Human Gut Repertoire which were

isolated from human gut for the first time in this study.
A part of the newly described bacteria from other stool samples of this project were also isolated in this study. Therefore it can be suspected that these newly discovered bacteria are part of the Human Gut Repertoire which also occurs in other individuals. For the study of anorexia it will be interesting whether the newly discovered bacterial species in this sample will be found increased in other samples of persons with eating disorders or undernourishment.
In the sense of the project it can be continued to explore samples from persons with different influencing factors on the intestinal flora, such as geography, special dietary habits like the patient in this study, and intake of medicaments, especially antibiotics. On the strength of past experience there is an important potential to isolate further intestinal bacteria and new species.

Challenges

How can we reach cultivation of bacteria which have only been detected by metagenomics so far? Most of these species in our study (28 out of 36, 77,8%) are anaerobic. The strict anaerobic conditions can be in fact improved. Anaerobic cultures in this study have indeed been incubated in anaerobic jars with respective atmosphere generators, but inoculation on agar was carried out in normal atmosphere containing oxygen. Also the first steps meaning transport, partition into aliquots and the freezing of the sample have been conducted without any special adherence to anaerobic atmosphere. In future one part of the sample could be treated anaerobically as soon as obtained to avoid destruction of strictly anaerobic bacteria. Also further steps of dilution and inoculation could be made in optimized, anaerobic conditions.
Aerobic or facultative anaerobic bacteria of this sample, which could not be cultivated are following: *Enterococcus saccharolyticus, Brevibacterium massiliense, Streptococcus mutans, Rothia mucilaginosa, Serratia ficalia, Pelomonas saccharophila, Lactococcus raffinolactis,* and *Lactobacillus rogosae.*
The first four species do not have any special characteristics which could explain why they were not detected by culture. These bacteria have been probably destroyed while preparation, did not grow on the used culture medium or their colonies have been missed while isolating. But then it is noticeable that *Serratia ficalia, Lactococcus raffinolactis* and *Pelomonas saccharophila* have been isolated only out of food or water until now and need special culture conditions which have not been applied in this study [136][137]. Particularly they all grow at temperatures not exceeding 30°C. By broadening the diversity of culture conditions, the gap

in culturing could be continued to be reduced. Finally *Lactobacillus rogosae* seems to be a bacterial species which does not exist in any collection of microorganisms [138] and which is therefore either not cultivable or not valid. *L. rogosae* should be examined more in detail to develop adapted conditions for its cultivation. In further studies of the Culturomics project, more cultures could be incubated with temperatures between 25°- 30°C and more special culture media such as R2A-Agar could be used [137].

All listed uncultured bacteria were detected in metagenomics with only few reads (at the most 7, mostly with only one read). This means that they occur in a small concentration. This increases the risk in culture to be dominated by other species and not to be isolated by the searcher. This can also explain why they have not been found in cultivation.

But many other bacteria with only one read in metagenomics were isolated nevertheless and partly even in simple culture conditions. One aim of this research work was to suppress some of the bacterial populations to enable others to grow. For this special culture media, addition of antibiotics, filtration and other conditions have been applied. Within these culture conditions there was no one which was especially advantageous. It is rather the multitude of different conditions which makes the large diversity of cultivated bacteria and characterizes the Culturomics project and its study presented here.

Finally there is the unknown part of microorganisms which becomes visible in metagenomic studies but cannot be identified and characterized. Almost 5% of gene sequences obtained in this study could not be classified. Beside technical limits this could present an indication of unknown bacteria which have to be discovered in future.

The presented research work, as a part of the Culturomics project, contributed to the exploration of the inter-individual as well as the individual diversity of intestinal microbiota. The present study provides a high number of identified species, including 11 newly described bacteria. Exploring the diversity of gut flora also contributes to make the large field of unknown bacteria more accessible.

4. Summary

10^{14} bacteria live in the human intestine. The composition of the gut flora differs from individual to individual and is dependent on multiple factors. Bacteria can play important roles in processes concerning our whole body. As the development of disease can also be explained by this approach, the intestinal microbiota is currently intensively explored.

The presented study is part of a project exploring the diversity of the human gut flora. In this context a stool sample of a 21 year old anorexia nervosa patient has been examined on the contained bacteria. For this aim 88 different culture conditions have been applied to enable growth of the most possible different species. Multiple established and new developed culture media have been inoculated and atmosphere, temperature and time of incubation has been varied. In addition active and passive filtration, phages and blood culture bottles with or without addition of nutriments have been applied.
In parallel the same sample has been examined by colleagues using pyrosequencing.

133 species have been isolated, belonging to the phyla *Firmicutes, Actinobacteria, Bacteroidetes* and *Proteobacteria*. 19 bacteria have been isolated from human intestine for the first time. Out of these 19, 11 species have been newly discovered and described in this study. These are 2 new genera *Stoquefichus massiliensis* and *Soleaferrea massiliensis* and 9 new species *Dorea massiliensis, Clostridium ihumii, Clostridium anorexicamassiliense, Holdemania massiliensis, Bacillus marseilloanorexius, Alistipes ihumii, Bacteroides timonensis, Streptomyces massiliensis* and *Blastococcus massiliensis.*
The new bacteria have been described by me and their genomes sequenced by colleagues. In consequence more genes should be assigned successfully in future metagenomic studies. Between the results of culturing and metagenomics there is only a small overlap. Only 17% of the cultivated bacteria have been also identified by metagenomics. On the other hand metagenomics detected 36 species which could not be cultivated in this study.

The intestinal microbiota of the anorexia nervosa patient possesses bacteria which can stimulate the production of certain antibodies by molecular mimicry with antigens. These antibodies influence the balance of hormones which regulate food intake and could play a role in the development of anorexia nervosa. Parallels to the intestinal flora of undernourished children concern overall bacteria with influence on inflammation. Eventually some of the newly discovered bacteria also have a relation with anorexia nervosa.
As this study is made on a single sample of one patient, we need more studies on the

constitution of the intestinal flora of anorexia nervosa patients for concluding correlations between microflora and disease. This work provides first data.
The study also compares culturing and metagenomics. The results show that with current methods a complete view on the intestinal flora can only be obtained by combining both methods.
The present research contributes to an extended description of human intestinal microflora and led to the discovery of 11 new bacteria.

Deutsche Zusammenfassung

Im menschlichen Darm leben 10^{14} Bakterien. Die Zusammensetzung der Darmflora ist von Mensch zu Mensch unterschiedlich und von verschiedenen Faktoren abhängig. Bei Vorgängen in unserem gesamten Körper spielen die Bakterien eine zum Teil wichtige Rolle. Da damit auch die Entstehung vieler Krankheiten erklärt werden kann, wird die intestinale Mikrobiota zur Zeit intensiv erforscht.

Die hier vorgestellte Studie ist Teil eines Projektes zur Erforschung der Diversität der menschlichen Darmflora. In diesem Sinne wurde die Stuhlprobe einer 21-jährigen Anorexia Patientin auf die darin enthaltenen Bakterien untersucht. Dazu wurden 88 verschiedene Kulturkonditionen angewandt um möglichst vielen Arten das Wachstum zu ermöglichen. Verschiedene bekannte und neu entwickelte Nährböden wurden beimpft und Atmosphäre, Temperatur und Inkubationszeit variiert. Außerdem wurde aktive und passive Filtration angewandt, Phagen benutzt, in Blutkulturflaschen mit oder ohne Beimengung von Verdauungssäften oder sterilem Stuhl präinkubiert und schließlich neu entwickelte Nährböden mit Banane, Camembert oder Joghurt in Anlehnung an die Ernährungsgewohnheiten der Patientin angewandt. Die Kolonien wurden per MALDI-TOF identifiziert oder die DNA der Bakterien extrahiert, das 16s rRNA-Gen amplifiziert, sequenziert und mit BLAST mit der Datenbank abgeglichen. Bei einer Similarität <98.7 % zu bekannten Spezien wurde eine neue Spezies vermutet und die Bakterien weiter biochemisch, physikalisch und phänotypisch analysiert.
Parallel dazu wurde die selbe Probe von Kollegen per Pyrosequencing untersucht.

Es wurden 133 Spezies isoliert, die den Phyla *Firmicutes, Actinobacteria, Bacteroidetes* und *Proteobacteria* angehören. 19 der Bakterien wurden zum ersten Mal aus dem menschlichen Darm isoliert. Von diesen wiederum sind 11 Bakterien von mir neu entdeckt und beschrieben worden. Es handelt sich um die 2 neuen Gattungen *Stoquefichus massiliensis* und *Soleaferrea massiliensis* und die 9 neuen Spezies *Dorea massiliensis, Clostridium ihumii, Clostridium anorexicamassiliense, Holdemania massiliensis, Bacillus marseilloanorexius, Alistipes ihumii, Bacteroides timonensis, Streptomyces massiliensis* und *Blastococcus massiliensis*. 8 dieser neuen Bakterien sind anaerob, 7 wurden nach Präinkubation in Blutkulturflaschen entdeckt, 2 davon bei Zugabe von Verdauungssäften.
Die neuen Bakterien wurden beschrieben und deren Genome von Kollegen sequenziert. Damit können in Zukunft mehr Gene in metagenomischen Studien erfolgreich zugeordnet

werden.
Mit den Ergebnissen der metagenomischen Untersuchung zeigt sich eine nur geringe Überlappung. Nur 17% der kultivierten Bakterien wurden auch in der Metagenomik identifiziert, die ihrerseits 36 Spezies erfasste, die nicht kultiviert werden konnten.

Die intestinale Mikrobiota der Anorexia Patientin enthält Bakterien, die durch Molekulares Mimikry mit Antigenen die Produktion von bestimmten Antikörpern stimulieren. Diese Antikörper beeinflussen das Gleichgewicht von Hormonen, die die Nahrungsaufnahme regulieren und in der Krankheitsentstehung von Anorexia nervosa eine Rolle spielen könnten. Parallelen zur Darmflora von unterernährten Kindern zeigen sich vor allem bei bestimmten Bakterienarten mit Einfluss auf Entzündung. Eventuell stehen auch manche der neu entdeckten Bakterien im Zusammenhang mit Anorexia nervosa.
Da es sich um eine einzelne Probe einer einzigen Patientin handelt, braucht man weitere Studien über die Zusammensetzung der Darmflora von Anorexia Patienten, um Aussagen über einen Zusammenhang zwischen Mikrobiota und Krankheit machen zu können. Diese Arbeit liefert hierfür erste Daten.
Die Studie vergleicht außerdem Kultivierung mit Metagenomik. Die Ergebnisse zeigen, dass man beim aktuellen Stand der Wissenschaft nur dann ein vollständiges Bild der Darmflora erhalten kann, wenn beide Methoden kombiniert werden.
Die vorliegende Forschungsarbeit trägt zu einer erweiterten Beschreibung der menschlichen Darmflora bei und führte zur Entdeckung von 11 bisher unbekannten Bakterien.

Referenzen

[1] J. Qin, R. Li, J. Raes, M. Arumugam, K. S. Burgdorf, C. Manichanh, T. Nielsen, N. Pons, F. Levenez, T. Yamada, D. R. Mende, J. Li, J. Xu, S. Li, D. Li, J. Cao, B. Wang, H. Liang, H. Zheng, Y. Xie, J. Tap, P. Lepage, M. Bertalan, J.-M. Batto, T. Hansen, D. Le Paslier, A. Linneberg, H. B. Nielsen, E. Pelletier, P. Renault, T. Sicheritz-Ponten, K. Turner, H. Zhu, C. Yu, S. Li, M. Jian, Y. Zhou, Y. Li, X. Zhang, S. Li, N. Qin, H. Yang, J. Wang, S. Brunak, J. Doré, F. Guarner, K. Kristiansen, O. Pedersen, J. Parkhill, J. Weissenbach, P. Bork, S. D. Ehrlich, and J. Wang, "A human gut microbial gene catalogue established by metagenomic sequencing.," *Nature*, vol. 464, no. 7285, pp. 59–65, Mar. 2010.

[2] P. J. Turnbaugh, R. E. Ley, M. Hamady, C. Fraser-liggett, R. Knight, and J. I. Gordon, "The human microbiome project: exploring the microbial part of ourselves in a changing world," *Nature*, vol. 449, no. 7164, pp. 804–810, 2007.

[3] J. Penders, K. Gerhold, C. Thijs, K. Zimmermann, U. Wahn, S. Lau, and E. Hamelmann, "New insights into the hygiene hypothesis in allergic diseases: mediation of sibling and birth mode effects by the gut microbiota.," *Gut Microbes*, vol. 5, no. 2, pp. 239–44.

[4] J. Penders, C. Thijs, C. Vink, F. F. Stelma, B. Snijders, I. Kummeling, P. A. van den Brandt, and E. E. Stobberingh, "Factors influencing the composition of the intestinal microbiota in early infancy.," *Pediatrics*, vol. 118, no. 2, pp. 511–21, Aug. 2006.

[5] M. K. Gibson, M. W. Pesesky, and G. Dantas, "The Yin and Yang of Bacterial Resilience in the Human Gut Microbiota.," *J. Mol. Biol.*, Jun. 2014.

[6] M. J. Coyne, N. L. Zitomersky, A. M. McGuire, A. M. Earl, and L. E. Comstock, "Evidence of Extensive DNA Transfer between Bacteroidales Species within the Human Gut.," *MBio*, vol. 5, no. 3, pp. e01305–14, Jan. 2014.

[7] J. Xu, M. K. Bjursell, J. Himrod, S. Deng, L. K. Carmichael, H. C. Chiang, L. V Hooper, and J. I. Gordon, "A genomic view of the human-Bacteroides thetaiotaomicron symbiosis.," *Science*, vol. 299, no. 5615, pp. 2074–6, Mar. 2003.

[8] S. Shoaie and J. Nielsen, "Elucidating the interactions between the human gut microbiota and its host through metabolic modeling.," *Front. Genet.*, vol. 5, no. April, p. 86, Jan. 2014.

[9] E. M. M. Quigley, "Gut Bacteria in Health and Disease," vol. 9, no. 9, pp. 560–569, 2013.

[10] F. Sommer and F. Bäckhed, "The gut microbiota--masters of host development and physiology.," *Nat. Rev. Microbiol.*, vol. 11, no. 4, pp. 227–38, Apr. 2013.

[11] S. Sanderson, W. Boardman, C. Ciofi, and R. Gibson, "Human gut microbes associated with obesity," vol. 444.

[12] P. J. Turnbaugh, R. E. Ley, M. a Mahowald, V. Magrini, E. R. Mardis, and J. I. Gordon, "An obesity-associated gut microbiome with increased capacity for energy harvest.," *Nature*, vol. 444, no. 7122, pp. 1027–31, Dec. 2006.

[13] F. Armougom, M. Henry, B. Vialettes, D. Raccah, and D. Raoult, "Monitoring bacterial community of human gut microbiota reveals an increase in Lactobacillus in obese patients and Methanogens in anorexic patients.," *PLoS One*, vol. 4, no. 9, p. e7125, Jan. 2009.

[14] D. A. Peterson, D. N. Frank, N. R. Pace, and J. I. Gordon, "Metagenomic Approaches for Defining the Pathogenesis of Inflammatory Bowel Diseases," vol. 3, no. 6, pp. 417–427, 2010.

[15] P. D. Scanlan, F. Shanahan, Y. Clune, J. K. Collins, G. C. O'Sullivan, M. O'Riordan, E. Holmes, Y. Wang, and J. R. Marchesi, "Culture-independent analysis of the gut

microbiota in colorectal cancer and polyposis.," *Environ. Microbiol.*, vol. 10, no. 3, pp. 789–98, Mar. 2008.
[16] I. Sekirov, S. L. Russell, L. C. M. Antunes, and B. B. Finlay, "Gut Microbiota in Health and Disease," pp. 859–904, 2010.
[17] S. Hazebrouck, L. Przybylski-Nicaise, S. Ah-Leung, K. Adel-Patient, G. Corthier, J.-M. Wal, and S. Rabot, "Allergic sensitization to bovine beta-lactoglobulin: comparison between germ-free and conventional BALB/c mice.," *Int. Arch. Allergy Immunol.*, vol. 148, no. 1, pp. 65–72, Jan. 2009.
[18] M. L. Yamamoto and R. H. Schiestl, "Lymphoma caused by intestinal microbiota.," *Int. J. Environ. Res. Public Health*, vol. 11, no. 9, pp. 9038–49, Sep. 2014.
[19] J. F. Cryan and T. G. Dinan, "Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour.," *Nat. Rev. Neurosci.*, vol. 13, no. 10, pp. 701–12, Oct. 2012.
[20] J. Dore, M. Simren, L. Buttle, and F. Guarner, "Hot topics in gut microbiota," *United Eur. Gastroenterol. J.*, vol. 1, no. 5, pp. 311–318, Oct. 2013.
[21] L. H. Zeuthen, L. N. Fink, and H. Frokiaer, "Epithelial cells prime the immune response to an array of gut-derived commensals towards a tolerogenic phenotype through distinct actions of thymic stromal lymphopoietin and transforming growth factor-beta.," *Immunology*, vol. 123, no. 2, pp. 197–208, Feb. 2008.
[22] N. L. Baganz and R. D. Blakely, "A dialogue between the immune system and brain, spoken in the language of serotonin.," *ACS Chem. Neurosci.*, vol. 4, no. 1, pp. 48–63, Jan. 2013.
[23] J. Lee, J.-H. Mo, K. Katakura, I. Alkalay, A. N. Rucker, Y.-T. Liu, H.-K. Lee, C. Shen, G. Cojocaru, S. Shenouda, M. Kagnoff, L. Eckmann, Y. Ben-Neriah, and E. Raz, "Maintenance of colonic homeostasis by distinctive apical TLR9 signalling in intestinal epithelial cells.," *Nat. Cell Biol.*, vol. 8, no. 12, pp. 1327–36, Dec. 2006.
[24] M. Lotz, D. Gütle, S. Walther, S. Ménard, C. Bogdan, and M. W. Hornef, "Postnatal acquisition of endotoxin tolerance in intestinal epithelial cells.," *J. Exp. Med.*, vol. 203, no. 4, pp. 973–84, Apr. 2006.
[25] B. L. Kelsall and F. Leon, "Involvement of intestinal dendritic cells in oral tolerance, immunity to pathogens, and inflammatory bowel disease.," *Immunol. Rev.*, vol. 206, pp. 132–48, Aug. 2005.
[26] J. L. Round and S. K. Mazmanian, "Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 107, no. 27, pp. 12204–9, Jul. 2010.
[27] N. Singh, A. Gurav, S. Sivaprakasam, E. Brady, R. Padia, H. Shi, M. Thangaraju, P. D. Prasad, S. Manicassamy, D. H. Munn, J. R. Lee, S. Offermanns, and V. Ganapathy, "Activation of Gpr109a, receptor for niacin and the commensal metabolite butyrate, suppresses colonic inflammation and carcinogenesis.," *Immunity*, vol. 40, no. 1, pp. 128–39, Jan. 2014.
[28] C. M. Rath, T. Alexandrov, S. K. Higginbottom, J. Song, M. E. Milla, M. A. Fischbach, J. L. Sonnenburg, and P. C. Dorrestein, "Molecular analysis of model gut microbiotas by imaging mass spectrometry and nanodesorption electrospray ionization reveals dietary metabolite transformations.," *Anal. Chem.*, vol. 84, no. 21, pp. 9259–67, Dec. 2012.
[29] B. Perry and Y. Wang, "Appetite regulation and weight control : the role of gut hormones," vol. 2, no. 1, pp. e26–7, 2012.
[30] M. D. Gershon and J. Tack, "The serotonin signaling system: from basic understanding to drug development for functional GI disorders.," *Gastroenterology*, vol. 132, no. 1, pp. 397–414, Jan. 2007.

[31] M. Berger, J. A. Gray, and B. L. Roth, “The expanded biology of serotonin.,” *Annu. Rev. Med.*, vol. 60, pp. 355–66, Jan. 2009.
[32] S. M. O’Mahony, G. Clarke, Y. E. Borre, T. G. Dinan, and J. F. Cryan, “Serotonin, Tryptophan Metabolism and the Brain-Gut- Microbiome Axis,” *Behav. Brain Res.*, Jul. 2014.
[33] S. O. Fetissov, M. Hamze Sinno, M. Coëffier, C. Bole-Feysot, P. Ducrotté, T. Hökfelt, and P. Déchelotte, “Autoantibodies against appetite-regulating peptide hormones and neuropeptides: putative modulation by gut microflora.,” *Nutrition*, vol. 24, no. 4, pp. 348–59, Apr. 2008.
[34] S. O. Fetissov and P. Déchelotte, “The putative role of neuropeptide autoantibodies in anorexia nervosa.,” *Curr. Opin. Clin. Nutr. Metab. Care*, vol. 11, no. 4, pp. 428–34, Jul. 2008.
[35] S. O. Fetissov, J. Hallman, L. Oreland, B. Af Klinteberg, E. Grenbäck, A.-L. Hulting, and T. Hökfelt, “Autoantibodies against alpha -MSH, ACTH, and LHRH in anorexia and bulimia nervosa patients.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 99, no. 26, pp. 17155–60, Dec. 2002.
[36] S. O. Fetissov, J. Harro, M. Jaanisk, A. Järv, I. Podar, J. Allik, I. Nilsson, P. Sakthivel, A. K. Lefvert, and T. Hökfelt, “Autoantibodies against neuropeptides are associated with psychological traits in eating disorders.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 102, no. 41, pp. 14865–70, Oct. 2005.
[37] J. I. Hudson, E. Hiripi, H. G. Pope, and R. C. Kessler, “The prevalence and correlates of eating disorders in the National Comorbidity Survey Replication.,” *Biol. Psychiatry*, vol. 61, no. 3, pp. 348–58, Feb. 2007.
[38] L. Erdur, B. Kallenbach-Dermutz, V. Lehmann, F. Zimmermann-Viehoff, W. Köpp, C. Weber, and H.-C. Deter, “Somatic comorbidity in anorexia nervosa: First results of a 21-year follow-up study on female inpatients.,” *Biopsychosoc. Med.*, vol. 6, no. 1, p. 4, Jan. 2012.
[39] J. Arcelus, A. J. Mitchell, J. Wales, and S. Nielsen, “Mortality rates in patients with anorexia nervosa and other eating disorders. A meta-analysis of 36 studies.,” *Arch. Gen. Psychiatry*, vol. 68, no. 7, pp. 724–31, Jul. 2011.
[40] W. H. Kaye, “Neuropeptide abnormalities in anorexia nervosa.,” *Psychiatry Res.*, vol. 62, no. 1, pp. 65–74, Apr. 1996.
[41] M. Terashi, A. Asakawa, T. Harada, M. Ushikai, Q. Coquerel, M. H. Sinno, P. Déchelotte, A. Inui, and S. O. Fetissov, “Ghrelin reactive autoantibodies in restrictive anorexia nervosa.,” *Nutrition*, vol. 27, no. 4, pp. 407–13, Apr. 2011.
[42] U. Deppenmeier, “The unique biochemistry of methanogenesis.,” *Prog. Nucleic Acid Res. Mol. Biol.*, vol. 71, pp. 223–83, 2002.
[43] T. S. Ghosh, S. Sen Gupta, T. Bhattacharya, D. Yadav, A. Barik, A. Chowdhury, B. Das, S. S. Mande, and G. B. Nair, “Gut microbiomes of Indian children of varying nutritional status.,” *PLoS One*, vol. 9, no. 4, p. e95547, Jan. 2014.
[44] S. Sen Gupta, M. H. Mohammed, T. S. Ghosh, S. Kanungo, G. B. Nair, and S. S. Mande, “Metagenome of the gut of a malnourished child.,” *Gut Pathog.*, vol. 3, no. 1, p. 7, Jan. 2011.
[45] M. I. Smith, T. Yatsunenko, M. J. Manary, I. Trehan, J. Cheng, A. L. Kau, S. S. Rich, P. Concannon, C. Josyf, J. Liu, E. Houpt, J. V Li, E. Holmes, and J. Nicholson, “Gut microbiomes of Malawian twin pairs discordant for kwashiorkor,” vol. 339, no. 6119, pp. 548–554, 2013.
[46] S. Subramanian, S. Huq, T. Yatsunenko, R. Haque, M. Mahfuz, M. a Alam, A. Benezra, J. DeStefano, M. F. Meier, B. D. Muegge, M. J. Barratt, L. G. VanArendonk, Q. Zhang, M. a Province, W. a Petri, T. Ahmed, and J. I. Gordon, “Persistent gut microbiota

immaturity in malnourished Bangladeshi children.," *Nature*, vol. 509, no. 7505, pp. 417–21, Jun. 2014.
[47] J.-C. Lagier, F. Armougom, M. Million, P. Hugon, I. Pagnier, C. Robert, F. Bittar, G. Fournous, G. Gimenez, M. Maraninchi, J.-F. Trape, E. V Koonin, B. La Scola, and D. Raoult, "Microbial culturomics: paradigm shift in the human gut microbiome study.," *Clin. Microbiol. Infect.*, vol. 18, no. 12, pp. 1185–93, Dec. 2012.
[48] P. Hugon, J.-C. Lagier, C. Robert, C. Lepolard, L. Papazian, D. Musso, B. Vialettes, and D. Raoult, "Molecular studies neglect apparently gram-negative populations in the human gut microbiota.," *J. Clin. Microbiol.*, vol. 51, no. 10, pp. 3286–93, Oct. 2013.
[49] S. Kumar, K. K. Krishnani, B. Bhushan, and M. P. Brahmane, "Metagenomics: Retrospect and Prospects in High Throughput Age.," *Biotechnol. Res. Int.*, vol. 2015, p. 121735, Jan. 2015.
[50] M. S. Rappé and S. J. Giovannoni, "The uncultured microbial majority.," *Annu. Rev. Microbiol.*, vol. 57, pp. 369–94, Jan. 2003.
[51] S. S. Epstein, "The phenomenon of microbial uncultivability," *Curr. Opin. Microbiol.*, vol. 16, no. 5, pp. 636–642, 2013.
[52] J. Song, H.-M. Oh, and J.-C. Cho, "Improved culturability of SAR11 strains in dilution-to-extinction culturing from the East Sea, West Pacific Ocean.," *FEMS Microbiol. Lett.*, vol. 295, no. 2, pp. 141–7, Jun. 2009.
[53] E. J. Stewart, "Growing unculturable bacteria.," *J. Bacteriol.*, vol. 194, no. 16, pp. 4151–60, Aug. 2012.
[54] "WHO | Antimicrobial resistance," *WHO*, 2016.
[55] W.-L. Wang, S.-Y. Xu, Z.-G. Ren, L. Tao, J.-W. Jiang, and S.-S. Zheng, "Application of metagenomics in the human gut microbiome.," *World J. Gastroenterol.*, vol. 21, no. 3, pp. 803–14, Jan. 2015.
[56] J. Li, H. Jia, X. Cai, H. Zhong, Q. Feng, S. Sunagawa, M. Arumugam, J. R. Kultima, E. Prifti, T. Nielsen, A. S. Juncker, C. Manichanh, B. Chen, W. Zhang, F. Levenez, J. Wang, X. Xu, L. Xiao, S. Liang, D. Zhang, Z. Zhang, W. Chen, H. Zhao, J. Y. Al-Aama, S. Edris, H. Yang, J. Wang, T. Hansen, H. B. Nielsen, S. Brunak, K. Kristiansen, F. Guarner, O. Pedersen, J. Doré, S. D. Ehrlich, P. Bork, and J. Wang, "An integrated catalog of reference genes in the human gut microbiome.," *Nat. Biotechnol.*, vol. 32, no. 8, pp. 834–41, Aug. 2014.
[57] "JGI GOLD | Statistics." [Online]. Available: https://gold.jgi.doe.gov/statistics. [Accessed: 12-Jan-2016].
[58] G. Dubourg, J. C. Lagier, C. Robert, F. Armougom, P. Hugon, S. Metidji, N. Dione, N. P. M. Dangui, A. Pfleiderer, J. Abrahao, D. Musso, L. Papazian, P. Brouqui, F. Bibi, M. Yasir, B. Vialettes, and D. Raoult, "Culturomics and pyrosequencing evidence of the reduction in gut microbiota diversity in patients with broad-spectrum antibiotics.," *Int. J. Antimicrob. Agents*, vol. 44, no. 2, pp. 117–24, Aug. 2014.
[59] F. Bittar, M. B. Keita, J.-C. Lagier, M. Peeters, E. Delaporte, and D. Raoult, "Gorilla gorilla gorilla gut: a potential reservoir of pathogenic bacteria as revealed using culturomics and molecular tools," *Sci. Rep.*, vol. 4, p. 7174, Jan. 2014.
[60] J. M. Janda and S. L. Abbott, "16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls.," *J. Clin. Microbiol.*, vol. 45, no. 9, pp. 2761–4, Sep. 2007.
[61] J.-C. Lagier, M. Million, P. Hugon, F. Armougom, and D. Raoult, "Human gut microbiota: repertoire and variations.," *Front. Cell. Infect. Microbiol.*, vol. 2, no. November, p. 136, Jan. 2012.
[62] S. Singh, C. Eldin, M. Kowalczewska, and D. Raoult, "Axenic culture of fastidious and intracellular bacteria.," *Trends Microbiol.*, vol. 21, no. 2, pp. 92–9, Feb. 2013.

[63] A. Omsland, D. C. Cockrell, D. Howe, E. R. Fischer, K. Virtaneva, D. E. Sturdevant, S. F. Porcella, and R. A. Heinzen, “Host cell-free growth of the Q fever bacterium Coxiella burnetii.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 106, no. 11, pp. 4430–4, Mar. 2009.
[64] A. Bollmann, K. Lewis, and S. S. Epstein, “Incubation of environmental samples in a diffusion chamber increases the diversity of recovered isolates.,” *Appl. Environ. Microbiol.*, vol. 73, no. 20, pp. 6386–90, Oct. 2007.
[65] T. Kaeberlein, K. Lewis, and S. S. Epstein, “Isolating ‘uncultivable’ microorganisms in pure culture in a simulated natural environment.,” *Science*, vol. 296, no. 5570, pp. 1127–9, May 2002.
[66] D. Nichols, N. Cahoon, E. M. Trakhtenberg, L. Pham, A. Mehta, A. Belanger, T. Kanigan, K. Lewis, and S. S. Epstein, “Use of ichip for high-throughput in situ cultivation of ‘uncultivable’ microbial species.,” *Appl. Environ. Microbiol.*, vol. 76, no. 8, pp. 2445–50, Apr. 2010.
[67] “LPSN - List of Prokaryotic names with Standing in Nomenclature.” [Online]. Available: http://www.bacterio.net/. [Accessed: 20-Jan-2016].
[68] S. Kokcha, A. K. Mishra, J.-C. Lagier, M. Million, Q. Leroy, D. Raoult, and P.-E. Fournier, “Non contiguous-finished genome sequence and description of Bacillus timonensis sp. nov.,” *Stand. Genomic Sci.*, vol. 6, no. 3, pp. 346–55, Jul. 2012.
[69] S. Kokcha, D. Ramasamy, J.-C. Lagier, C. Robert, D. Raoult, and P.-E. Fournier, “Non-contiguous finished genome sequence and description of Brevibacterium senegalense sp. nov.,” *Stand. Genomic Sci.*, vol. 7, no. 2, pp. 233–45, Dec. 2012.
[70] D. Ramasamy, S. Kokcha, J.-C. Lagier, T.-T. Nguyen, D. Raoult, and P.-E. Fournier, “Genome sequence and description of Aeromicrobium massiliense sp. nov.,” *Stand. Genomic Sci.*, vol. 7, no. 2, pp. 246–57, Dec. 2012.
[71] V. Roux, K. El Karkouri, J.-C. Lagier, C. Robert, and D. Raoult, “Non-contiguous finished genome sequence and description of Kurthia massiliensis sp. nov.,” *Stand. Genomic Sci.*, vol. 7, no. 2, pp. 221–32, Dec. 2012.
[72] J.-C. Lagier, F. Armougom, A. K. Mishra, T.-T. Nguyen, D. Raoult, and P.-E. Fournier, “Non-contiguous finished genome sequence and description of Alistipes timonensis sp. nov.,” *Stand. Genomic Sci.*, vol. 6, no. 3, pp. 315–24, Jul. 2012.
[73] J.-C. Lagier, G. Gimenez, C. Robert, D. Raoult, and P.-E. Fournier, “Non-contiguous finished genome sequence and description of Herbaspirillum massiliense sp. nov.,” *Stand. Genomic Sci.*, vol. 7, no. 2, pp. 200–9, Dec. 2012.
[74] J.-C. Lagier, K. El Karkouri, T.-T. Nguyen, F. Armougom, D. Raoult, and P.-E. Fournier, “Non-contiguous finished genome sequence and description of Anaerococcus senegalensis sp. nov.,” *Stand. Genomic Sci.*, vol. 6, no. 1, pp. 116–25, Mar. 2012.
[75] J.-C. Lagier, D. Ramasamy, R. Rivet, D. Raoult, and P.-E. Fournier, “Non contiguous-finished genome sequence and description of Cellulomonas massiliensis sp. nov.,” *Stand. Genomic Sci.*, vol. 7, no. 2, pp. 258–70, Dec. 2012.
[76] A. K. Mishra, G. Gimenez, J.-C. Lagier, C. Robert, D. Raoult, and P.-E. Fournier, “Genome sequence and description of Alistipes senegalensis sp. nov.,” *Stand. Genomic Sci.*, vol. 6, no. 3, pp. 1–16, Jul. 2012.
[77] A. K. Mishra, J.-C. Lagier, R. Rivet, D. Raoult, and P.-E. Fournier, “Non-contiguous finished genome sequence and description of Paenibacillus senegalensis sp. nov.,” *Stand. Genomic Sci.*, vol. 7, no. 1, pp. 70–81, Oct. 2012.
[78] A. K. Mishra, J.-C. Lagier, C. Robert, D. Raoult, and P.-E. Fournier, “Non-contiguous finished genome sequence and description of Clostridium senegalense sp. nov.,” *Stand. Genomic Sci.*, vol. 6, no. 3, pp. 386–95, Jul. 2012.
[79] A. K. Mishra, J.-C. Lagier, C. Robert, D. Raoult, and P.-E. Fournier, “Non contiguous-

finished genome sequence and description of Peptoniphilus timonensis sp. nov.," *Stand. Genomic Sci.*, vol. 7, no. 1, pp. 1–11, Oct. 2012.
[80] A. Gorlas, C. Robert, G. Gimenez, M. Drancourt, and D. Raoult, "Complete genome sequence of Methanomassiliicoccus luminyensis, the largest genome of a human-associated Archaea species.," *J. Bacteriol.*, vol. 194, no. 17, p. 4745, Sep. 2012.
[81] G. Dubourg, J. C. Lagier, F. Armougom, C. Robert, I. Hamad, P. Brouqui, and D. Raoult, "The gut microbiota of a patient with resistant tuberculosis is more comprehensively studied by culturomics than by metagenomics.," *Eur. J. Clin. Microbiol. Infect. Dis.*, vol. 32, no. 5, pp. 637–45, May 2013.
[82] G. Dubourg, J.-C. Lagier, F. Armougom, C. Robert, G. Audoly, L. Papazian, and D. Raoult, "High-level colonisation of the human gut by Verrucomicrobia following broad-spectrum antibiotic treatment.," *Int. J. Antimicrob. Agents*, vol. 41, no. 2, pp. 149–55, Feb. 2013.
[83] M. Dan, J. Richardson, M. D. Miliotis, and H. J. Koornhof, "Comparison of preservation media and freezing conditions for storage of specimens of faeces.," *J. Med. Microbiol.*, vol. 28, no. 2, pp. 151–4, Feb. 1989.
[84] P. Seng, M. Drancourt, F. Gouriet, B. La Scola, P.-E. Fournier, J. M. Rolain, and D. Raoult, "Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry.," *Clin. Infect. Dis.*, vol. 49, no. 4, pp. 543–51, Aug. 2009.
[85] E. J. Stackebrandt E, "Taxonomic parameters revisited: tarnished gold standards.," *Microbiol To-day*, vol. 33, pp. 152–15, 2006.
[86] B. Dridi, M. Henry, A. El Khéchine, D. Raoult, and M. Drancourt, "High prevalence of Methanobrevibacter smithii and Methanosphaera stadtmanae detected in the human gut using an improved DNA detection protocol.," *PLoS One*, vol. 4, no. 9, p. e7063, Jan. 2009.
[87] P. D. Schloss, D. Gevers, and S. L. Westcott, "Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-based studies.," *PLoS One*, vol. 6, no. 12, p. e27310, Jan. 2011.
[88] P. D. Schloss, S. L. Westcott, T. Ryabin, J. R. Hall, M. Hartmann, E. B. Hollister, R. A. Lesniewski, B. B. Oakley, D. H. Parks, C. J. Robinson, J. W. Sahl, B. Stres, G. G. Thallinger, D. J. Van Horn, and C. F. Weber, "Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities.," *Appl. Environ. Microbiol.*, vol. 75, no. 23, pp. 7537–41, Dec. 2009.
[89] S. M. Huse, D. M. Welch, H. G. Morrison, and M. L. Sogin, "Ironing out the wrinkles in the rare biosphere through improved OTU clustering.," *Environ. Microbiol.*, vol. 12, no. 7, pp. 1889–98, Jul. 2010.
[90] A. Giongo, A. G. Davis-Richardson, D. B. Crabb, and E. W. Triplett, "TaxCollector: Modifying Current 16S rRNA Databases for the Rapid Classification at Six Taxonomic Levels," *Diversity*, vol. 2, no. 7, pp. 1015–1025, Jul. 2010.
[91] R. Schlaberg, K. E. Simmon, and M. A. Fisher, "A systematic approach for discovering novel, clinically relevant bacteria.," *Emerg. Infect. Dis.*, vol. 18, no. 3, pp. 422–30, Mar. 2012.
[92] P. Shannon, A. Markiel, O. Ozier, N. S. Baliga, J. T. Wang, D. Ramage, N. Amin, B. Schwikowski, and T. Ideker, "Cytoscape: a software environment for integrated models of biomolecular interaction networks.," *Genome Res.*, vol. 13, no. 11, pp. 2498–504, Nov. 2003.
[93] M. J. Claesson, Q. Wang, O. O'Sullivan, R. Greene-Diniz, J. R. Cole, R. P. Ross, and P. W. O'Toole, "Comparison of two next-generation sequencing technologies for

resolving highly complex microbiota composition using tandem variable 16S rRNA gene regions.," *Nucleic Acids Res.*, vol. 38, no. 22, p. e200, Dec. 2010.
[94] G. Dubourg, J. C. Lagier, F. Armougom, C. Robert, I. Hamad, P. Brouqui, and D. Raoult, "The proof of concept that culturomics can be superior to metagenomics to study atypical stool samples.," *Eur. J. Clin. Microbiol. Infect. Dis.*, vol. 32, no. 8, p. 1099, Aug. 2013.
[95] A. Arank, S. A. Syed, E. B. Kenney, and R. Freter, "Isolation of anaerobic bacteria from human gingiva and mouse cecum by means of a simplified glove box procedure.," *Appl. Microbiol.*, vol. 17, no. 4, pp. 568–76, Apr. 1969.
[96] C. Eller, M. R. Crabill, and M. P. Bryant, "Anaerobic roll tube media for nonselective enumeration and isolation of bacteria in human feces.," *Appl. Microbiol.*, vol. 22, no. 4, pp. 522–9, Oct. 1971.
[97] M. W. Wren, "Multiple selective media for the isolation of anaerobic bacteria from clinical specimens.," *J. Clin. Pathol.*, vol. 33, no. 1, pp. 61–5, Jan. 1980.
[98] A. Pfleiderer, J. C. Lagier, F. Armougom, C. Robert, B. Vialettes, and D. Raoult, "Culturomics identified 11 new bacterial species from a single anorexia nervosa stool sample," *Eur. J. Clin. Microbiol. Infect. Dis.*, vol. 32, pp. 1471–1481, 2013.
[99] B. J. Tindall, R. Rosselló-Móra, H.-J. Busse, W. Ludwig, and P. Kämpfer, "Notes on the characterization of prokaryote strains for taxonomic purposes.," *Int. J. Syst. Evol. Microbiol.*, vol. 60, no. Pt 1, pp. 249–66, Jan. 2010.
[100] "JGI GOLD | Home." [Online]. Available: https://gold.jgi.doe.gov/index. [Accessed: 19-Jan-2016].
[101] "MEP Home." [Online]. Available: http://www.microbial-earth.org/cgi-bin/index.cgi. [Accessed: 20-Jan-2016].
[102] P. Hugon, D. Ramasamy, J.-C. Lagier, R. Rivet, C. Couderc, D. Raoult, and P.-E. Fournier, "Non contiguous-finished genome sequence and description of Alistipes obesi sp. nov.," *Stand. Genomic Sci.*, vol. 7, no. 3, pp. 427–39, Jan. 2013.
[103] A. K. Mishra, P. Hugon, J.-C. Lagier, T.-T. Nguyen, C. Robert, C. Couderc, D. Raoult, and P.-E. Fournier, "Non contiguous-finished genome sequence and description of Peptoniphilus obesi sp. nov.," *Stand. Genomic Sci.*, vol. 7, no. 3, pp. 357–69, Jan. 2013.
[104] A. K. Mishra, P. Hugon, J.-C. Lagier, T.-T. Nguyen, C. Couderc, D. Raoult, and P.-E. Fournier, "Non contiguous-finished genome sequence and description of Enorma massiliensis gen. nov., sp. nov., a new member of the Family Coriobacteriaceae.," *Stand. Genomic Sci.*, vol. 8, no. 2, pp. 290–305, Jan. 2013.
[105] A. K. Mishra, P. Hugon, C. Robert, D. Raoult, and P.-E. Fournier, "Non contiguous-finished genome sequence and description of Peptoniphilus grossensis sp. nov.," *Stand. Genomic Sci.*, vol. 7, no. 2, pp. 320–30, Dec. 2012.
[106] A. K. Mishra, J.-C. Lagier, T.-T. Nguyen, D. Raoult, and P.-E. Fournier, "Non contiguous-finished genome sequence and description of Peptoniphilus senegalensis sp. nov.," *Stand. Genomic Sci.*, vol. 7, no. 3, pp. 370–81, Jan. 2013.
[107] A. K. Mishra, J.-C. Lagier, C. Robert, D. Raoult, and P.-E. Fournier, "Genome sequence and description of Timonella senegalensis gen. nov., sp. nov., a new member of the suborder Micrococcinae.," *Stand. Genomic Sci.*, vol. 8, no. 2, pp. 318–35, Jan. 2013.
[108] D. Ramasamy, J.-C. Lagier, A. Gorlas, D. Raoult, and P.-E. Fournier, "Non contiguous-finished genome sequence and description of Bacillus massiliosenegalensis sp. nov.," *Stand. Genomic Sci.*, vol. 8, no. 2, pp. 264–78, Jan. 2013.
[109] D. Ramasamy, J.-C. Lagier, T. T. Nguyen, D. Raoult, and P.-E. Fournier, "Non contiguous-finished genome sequence and description of Dielma fastidiosa gen. nov., sp. nov., a new member of the Family Erysipelotrichaceae.," *Stand. Genomic Sci.*, vol.

8, no. 2, pp. 336–51, Jan. 2013.
[110] J.-C. Lagier, K. Elkarkouri, R. Rivet, C. Couderc, D. Raoult, and P.-E. Fournier, “Non contiguous-finished genome sequence and description of Senegalemassilia anaerobia gen. nov., sp. nov.,” *Stand. Genomic Sci.*, vol. 7, no. 3, pp. 343–56, Jan. 2013.
[111] J.-C. Lagier, K. El Karkouri, A. K. Mishra, C. Robert, D. Raoult, and P.-E. Fournier, “Non contiguous-finished genome sequence and description of Enterobacter massiliensis sp. nov.,” *Stand. Genomic Sci.*, vol. 7, no. 3, pp. 399–412, Jan. 2013.
[112] F. Cohn, “Untersuchungen über Bakterien.,” *Beitrage zur Biol. der Pflanz. H.*, vol. 1, pp. 127–224., 1872.
[113] M. H. Mathews WC, Caperna J, Toerner JG, Barber RE, “Neutropenia is a risk factor for gram-negative bacillus bacteremia in human im-munodeficiency virus-infected patients: results of a nested case-control study.,” *Am J Epidemiol*, vol. 48, pp. 1175–1183.25., 1998.
[114] J. A. Jernigan, D. S. Stephens, D. A. Ashford, C. Omenaca, M. S. Topiel, M. Galbraith, M. Tapper, T. L. Fisk, S. Zaki, T. Popovic, R. F. Meyer, C. P. Quinn, S. A. Harper, S. K. Fridkin, J. J. Sejvar, C. W. Shepard, M. McConnell, J. Guarner, W. J. Shieh, J. M. Malecki, J. L. Gerberding, J. M. Hughes, and B. A. Perkins, “Bioterrorism-related inhalational anthrax: the first 10 cases reported in the United States.,” *Emerg. Infect. Dis.*, vol. 7, no. 6, pp. 933–44, Jan.
[115] E. J. Bottone, “Bacillus cereus, a volatile human pathogen.,” *Clin. Microbiol. Rev.*, vol. 23, no. 2, pp. 382–98, Apr. 2010.
[116] J. Purswani, C. Pozo, M. Rodríguez-Díaz, and J. González-López, “Selection and identification of bacterial strains with methyl-tert-butyl ether, ethyl-tert-butyl ether, and tert-amyl methyl ether degrading capacities.,” *Environ. Toxicol. Chem.*, vol. 27, no. 11, pp. 2296–303, Nov. 2008.
[117] D. Field, G. Garrity, T. Gray, N. Morrison, J. Selengut, P. Sterk, T. Tatusova, N. Thomson, M. J. Allen, S. V Angiuoli, M. Ashburner, N. Axelrod, S. Baldauf, S. Ballard, J. Boore, G. Cochrane, J. Cole, P. Dawyndt, P. De Vos, C. DePamphilis, R. Edwards, N. Faruque, R. Feldman, J. Gilbert, P. Gilna, F. O. Glöckner, P. Goldstein, R. Guralnick, D. Haft, D. Hancock, H. Hermjakob, C. Hertz-Fowler, P. Hugenholtz, I. Joint, L. Kagan, M. Kane, J. Kennedy, G. Kowalchuk, R. Kottmann, E. Kolker, S. Kravitz, N. Kyrpides, J. Leebens-Mack, S. E. Lewis, K. Li, A. L. Lister, P. Lord, N. Maltsev, V. Markowitz, J. Martiny, B. Methe, I. Mizrachi, R. Moxon, K. Nelson, J. Parkhill, L. Proctor, O. White, S.-A. Sansone, A. Spiers, R. Stevens, P. Swift, C. Taylor, Y. Tateno, A. Tett, S. Turner, D. Ussery, B. Vaughan, N. Ward, T. Whetzel, I. San Gil, G. Wilson, and A. Wipat, “The minimum information about a genome sequence (MIGS) specification.,” *Nat. Biotechnol.*, vol. 26, no. 5, pp. 541–7, May 2008.
[118] “Prodigal: Microbial Gene Prediction Software.” [Online]. Available: http://prodigal.ornl.gov/. [Accessed: 20-Jan-2016].
[119] “GenBank Home.” [Online]. Available: http://www.ncbi.nlm.nih.gov/genbank. [Accessed: 19-Jan-2016].
[120] T. M. Lowe and S. R. Eddy, “tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence.,” *Nucleic Acids Res.*, vol. 25, no. 5, pp. 955–64, Mar. 1997.
[121] K. Lagesen, P. Hallin, E. A. Rødland, H.-H. Staerfeldt, T. Rognes, and D. W. Ussery, “RNAmmer: consistent and rapid annotation of ribosomal RNA genes.,” *Nucleic Acids Res.*, vol. 35, no. 9, pp. 3100–8, Jan. 2007.
[122] J. D. Bendtsen, H. Nielsen, G. von Heijne, and S. Brunak, “Improved prediction of signal peptides: SignalP 3.0.,” *J. Mol. Biol.*, vol. 340, no. 4, pp. 783–95, Jul. 2004.
[123] A. Krogh, B. Larsson, G. von Heijne, and E. L. Sonnhammer, “Predicting

transmembrane protein topology with a hidden Markov model: application to complete genomes.," *J. Mol. Biol.*, vol. 305, no. 3, pp. 567–80, Jan. 2001.
[124] M. Lechner, S. Findeiss, L. Steiner, M. Marz, P. F. Stadler, and S. J. Prohaska, "Proteinortho: detection of (co-)orthologs in large-scale analysis.," *BMC Bioinformatics*, vol. 12, p. 124, Jan. 2011.
[125] K. Rutherford, J. Parkhill, J. Crook, T. Horsnell, P. Rice, M. A. Rajandream, and B. Barrell, "Artemis: sequence visualization and annotation.," *Bioinformatics*, vol. 16, no. 10, pp. 944–5, Oct. 2000.
[126] T. Carver, N. Thomson, A. Bleasby, M. Berriman, and J. Parkhill, "DNAPlotter: circular and linear interactive genome visualization.," *Bioinformatics*, vol. 25, no. 1, pp. 119–20, Jan. 2009.
[127] A. C. E. Darling, B. Mau, F. R. Blattner, and N. T. Perna, "Mauve: multiple alignment of conserved genomic sequence with rearrangements.," *Genome Res.*, vol. 14, no. 7, pp. 1394–403, Jul. 2004.
[128] M. Ashburner, C. A. Ball, J. A. Blake, D. Botstein, H. Butler, J. M. Cherry, A. P. Davis, K. Dolinski, S. S. Dwight, J. T. Eppig, M. A. Harris, D. P. Hill, L. Issel-Tarver, A. Kasarskis, S. Lewis, J. C. Matese, J. E. Richardson, M. Ringwald, G. M. Rubin, and G. Sherlock, "Gene ontology: tool for the unification of biology. The Gene Ontology Consortium.," *Nat. Genet.*, vol. 25, no. 1, pp. 25–9, May 2000.
[129] B. Schulthess, G. V. Bloemberg, A. Zbinden, F. Mouttet, R. Zbinden, E. C. Böttger, and M. Hombach, "Evaluation of the Bruker MALDI Biotyper for the identification of fastidious Gram-negative rods," *J. Clin. Microbiol.*, no. December, pp. JCM.03107–15, 2015.
[130] J. Mestas, T. Quias, and J. Dien Bard, "Direct Identification of Aerobic Bacteria by Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry Is Accurate and Robust.," *J. Clin. Lab. Anal.*, Dec. 2015.
[131] C. Manichanh, L. Rigottier-Gois, E. Bonnaud, K. Gloux, E. Pelletier, L. Frangeul, R. Nalin, C. Jarrin, P. Chardon, P. Marteau, J. Roca, and J. Dore, "Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach.," *Gut*, vol. 55, no. 2, pp. 205–11, Feb. 2006.
[132] N. Germain, B. Galusca, C. W. Le Roux, C. Bossu, M. a. Ghatei, F. Lang, S. R. Bloom, and B. Estour, "Constitutional thinness and lean anorexia nervosa display opposite concentrations of peptide YY, glucagon-like peptide 1, ghrelin, and leptin," *Am. J. Clin. Nutr.*, vol. 85, no. 4, pp. 967–971, 2007.
[133] A. Escobar-Zepeda, A. Vera-Ponce de León, and A. Sanchez-Flores, "The Road to Metagenomics: From Microbiology to DNA Sequencing Technologies and Bioinformatics," *Front. Genet.*, vol. 6, p. 348, Dec. 2015.
[134] P. Hugon, J.-C. Dufour, P. Colson, P.-E. Fournier, K. Sallah, and D. Raoult, "A comprehensive repertoire of prokaryotic species identified in human beings.," *Lancet. Infect. Dis.*, vol. 15, no. 10, pp. 1211–9, Oct. 2015.
[135] J.-C. Lagier, P. Hugon, S. Khelaifia, P.-E. Fournier, B. La Scola, and D. Raoult, "The rebirth of culture in microbiology through the example of culturomics to study human gut microbiota.," *Clin. Microbiol. Rev.*, vol. 28, no. 1, pp. 237–64, Jan. 2015.
[136] P. A. D. Grimont, F. Grimont, and M. P. Starr, "Serratia ficaria sp. nov., a bacterial species associated with Smyrna figs and the fig waspBlastophaga psenes," *Curr. Microbiol.*, vol. 2, no. 5, pp. 277–282, Sep. 1979.
[137] M. Gomila, B. Bowien, E. Falsen, E. R. B. Moore, and J. Lalucat, "Description of Pelomonas aquatica sp. nov. and Pelomonas puraquae sp. nov., isolated from industrial and haemodialysis water.," *Int. J. Syst. Evol. Microbiol.*, vol. 57, no. Pt 11, pp. 2629–35, Nov. 2007.

[138] G. E. Felis, S. Torriani, and F. Dellaglio, “The status of the species Lactobacillus rogosae Holdeman and Moore 1974. Request for an Opinion,” *Int. J. Syst. Evol. Microbiol.*, vol. 54, no. 5, pp. 1903–1904, 2004.

Erklärung zum Eigenanteil

„Culturomics identified 11 new bacterial species from a single anorexia nervosa stool sample“

Die Studie wurde von Prof. D.Raoult konzipiert und geleitet. J.C. Lagier betreute die Arbeit. Die Stuhlprobe und Informationen über die Patientin erhielten wir von B.Vialettes. Die Vorbereitung der Probe, die gesamte Kultivierung sowie Identifizierung der Kolonien per MALDI-TOF oder 16S rRNA-Sequenzierung führte A.Pfleiderer durch. F.Armougoum und C.Robert untersuchten die Probe metagenomisch.

Die Datenrecherche stammt von A.Pfleiderer und J.C.Lagier. Einleitung, Resultate und Diskussion wurden von A.Pfleiderer geschrieben und von J.C.Lagier korrigiert. Bei Material und Methoden wurde der Teil über Metagenomik von F.Armougoum, C.Robert und J.C.Lagier verfasst, der Rest von A.Pfleiderer. Tabelle 1 und die ersten 3 Kolumnen von Tabelle 2, sowie Abbildungen1 und 2, Abbildungen S1 und S2 und Tabelle S1 stammen von A.Pfleiderer. Die letzten 3 Kolumnen von Tabelle 2, sowie Abbildung 3 sind von F.Armougoum und C.Robert.

„Non-contiguous finished genome sequence and description of *Bacillus massilioanorexius sp.nov.*“

Die Studie, in der die Bakterien entdeckt wurden, wurde von Prof. D.Raoult geleitet. J.C. Lagier betreute die Arbeit. Isolierung der neuen Bakterien, 16S rRNA-Sequenzierung und Einordnen als neue Spezies wurde von A.Pfleiderer durchgeführt. Weiterhin untersuchte und beschrieb A.Pfleiderer die biochemischen, phänotypischen und physikalischen Eigenschaften der Bakterien. Das Genom sequenzierten und beschrieben C.Robert und A.K. Mishra. A.K. Mishra verglich das Genom mit verwandten Spezies. Tabelle 21 und 22, sowie Abbildungen 21, 22, 23 und 24 stammen von J.C.Lagier und A.Pfleiderer, Tabellen 23, 24, 25 und 26, sowie Abbildungen 25, 26 und 27 von C.Robert und A.K.Mishra. Einleitung und Zusammenfassung wurden von J.C.Lagier verfasst.

Weitere Veröffentlichungen

- Pfleiderer, A., Lagier, J. C., Armougom, F., Robert, C., Vialettes, B., Raoult, D., „Non-contiguous finished genome sequence and description of Holdemania massiliensis sp. nov.“, Standards in genomic sciences, vol. 9, no.2, p.395-409, 2013.
- Pfleiderer, A., Mishra, A. K., Lagier, J.C., Robert, C, Caputo, A., Raoult, D., Fournier, P.E., „Non-contiguous finished genome sequence and description of Alistipes ihumii sp. nov.“, Standards in genomic sciences, vol. 9, no.3, p.1221-35, 2014.
- Ramasamy, D., Lagier, J.C., Rossi-Tamisier, M., Pfleiderer, A., Michelle, C., Couderc, C., Raoult, D., Fournier, P.E., „Genome sequence and description of Bacteroides timonensis sp. nov.“, Standards in genomic sciences, vol. 9, no.3, p.1181-97, 2014.

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