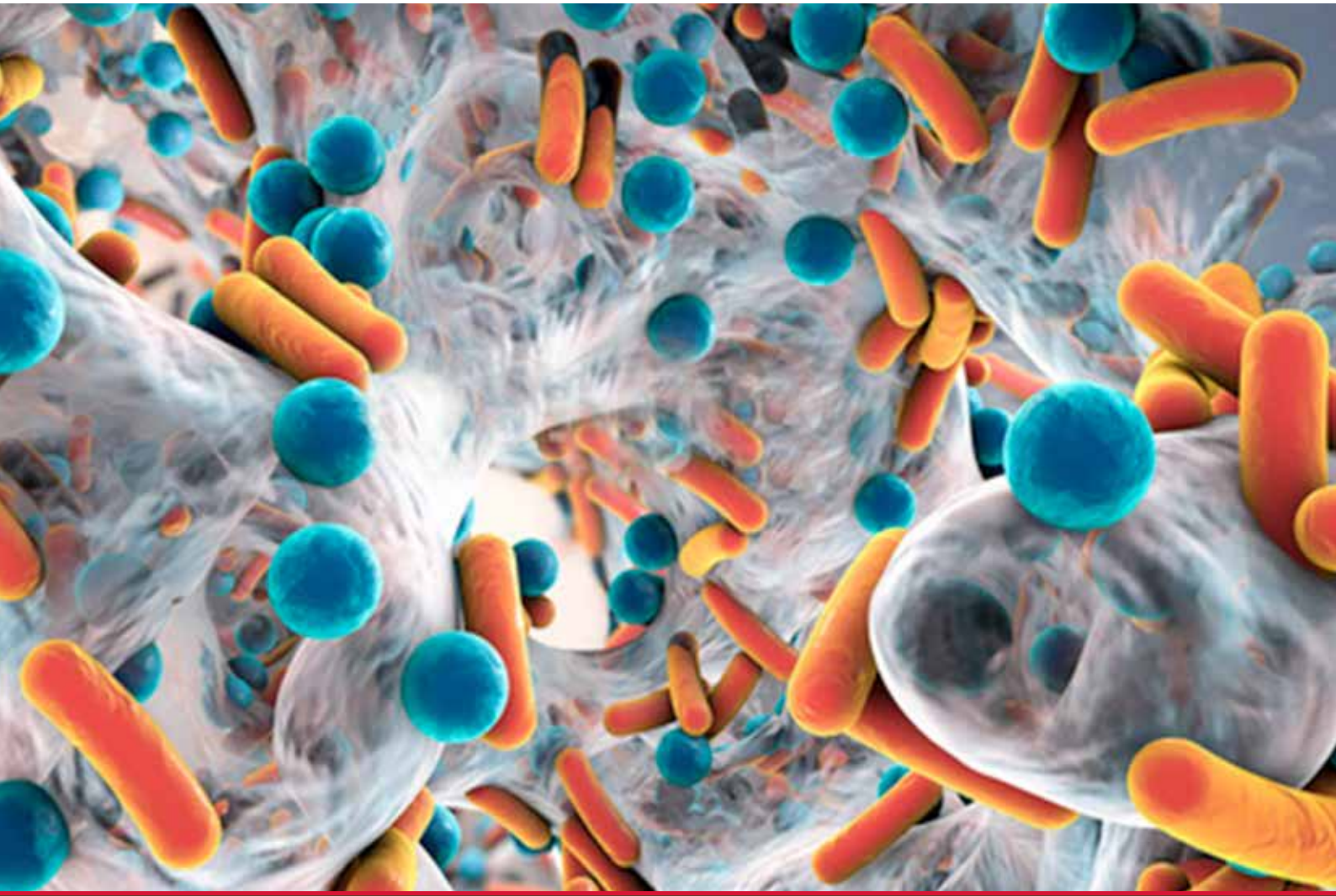
bio|vis' DIAGNOSTIK

The Intestinal Microbiome



Modern Stool Diagnostics
Make Molecular Human Gut-Microbiota Profiles Possible

The Gut Microbiome

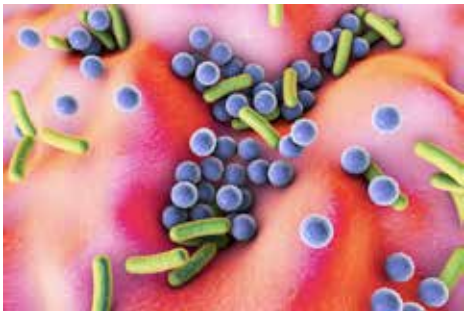
Microbiome is the name for all microorganisms populating the human body. Bacteria generally have a bad reputation – frequently they are simply regarded as pathogens. This is, however, only justified in case of pathogenic bacteria. But humans also live in symbiotic balance with bacteria – we need them to stay healthy.

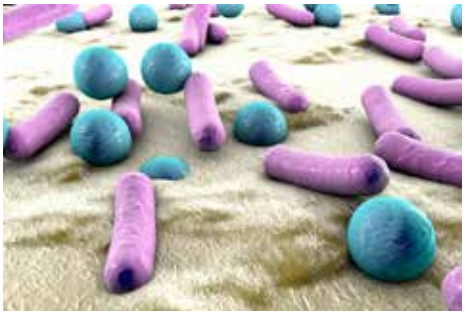
Digestive tract, parts of the respiratory and the urogenital tract are populated by a whole diversity of bacteria species. These protect us against pathogens.

The main colonisation organ of bacteria is the human colon. Here one finds a **community of microbes** consisting of more than one thousand species. This "intestinal microbiome" – recently defined as metabolic organ – outnumbers genetic information by 150 times and thus is the largest bacteria cluster of the human body (!). **Intestinal bacteria** influence a variety of complex interactions on metabolic as well as immune-regulatory levels: they control essential metabolic processes by providing – among others – energy carriers or release immune modulating substances.

Commensal gut bacteria are not only able to utilize consumed food and to cleave indigestible substances, they also synthetize vital vitamins and anti-microbial substances, which control the growth of pathogenic bacteria. In addition they positively influence intestinal epithelia and mucosa as well as the immune system (2).

Various bacteria clusters on the intestinal surface 3-D-Illustration,

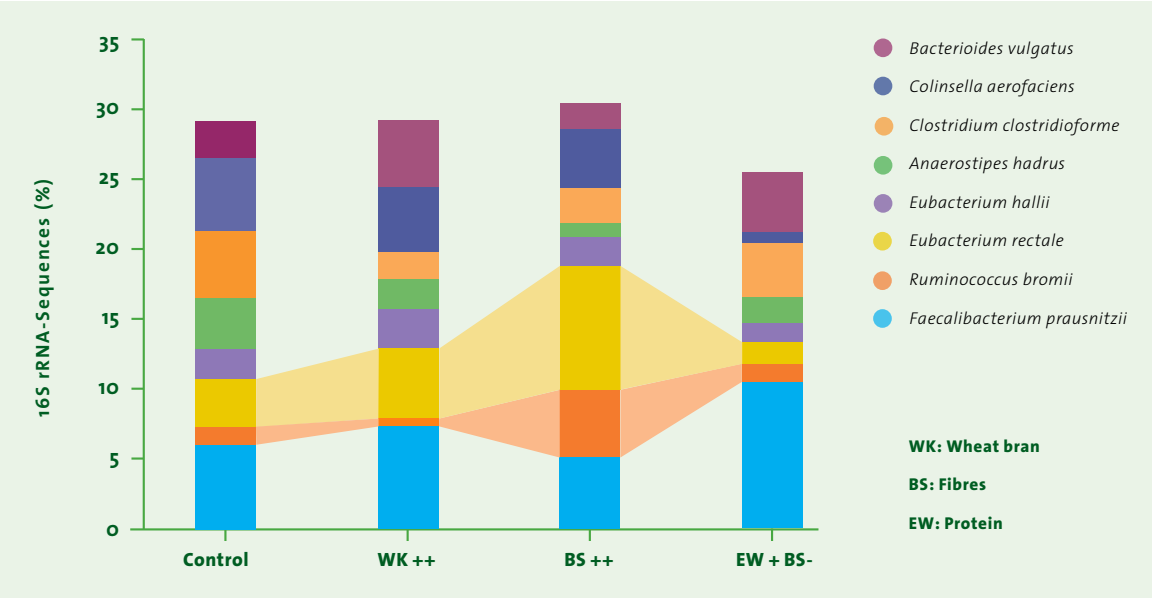




Further important functions are also ascribed to them:

- Stimulation of the **immune system**: stabilization of the mucosa immune system (MIS), expulsion of pathogens by developing ß-defensin and sIgA
- **Vitamin supply:** synthesis of B1, B2, B6, B12 and K vitamins in the intestines
- They support **digestion**: decomposition of indigestive carbohydrates or fibres
- Production of **short-chain fatty acids** like acetic acid (acetate) and butyric acid (butyrate), which also affect the intestinal milieu
- Short-chain fatty acids act as **energy source** for gut mucosa cells
- Support of the **intestinal peristalsis** with the aid of short-chain fatty acids
- Control of **inflammations**: especially butyrate has anti-inflammatory and mucosa protecting effects.
- **Detoxification** of foreign matters.

The composition of healthy microbiomes may indeed vary. They are influenced by the initial bacterial population after birth, genetic factors and very important by diets. How diets are able to influence bacteria strains and species in regard to prevalence is shown in Picture 1. Mainly fibres lead to proliferation of the firmicute group with prominent members like *Eubacterium rectale, Eubacterium hallii, Ruminococcus bromii* or various *Roseburia* types.



Picture 1.
The influence of diets on the intestinal microbiota
Diagram modified according to Flint *et al.*

The small intestine population differs considerably from that of the colon: the bacteria density of the small intestine is significantly lower than that of the colon –here bacteria counts of 10^{11} to 10^{12} bacteria/g stool can be reached. About half of the excreted stool of healthy adults consists of bacteria material (3).

The functions of the intestinal microbiome are, however, only carried out properly if suitable bacteria types in optimal organisation colonize the intestinal mucosa. If there are shifts within this balance the development of endogenic infections is promoted and serious systemic diseases may occur. Fluctuations within the intestinal microbiota may therefore occur in direct relation with **clinical symptoms**.

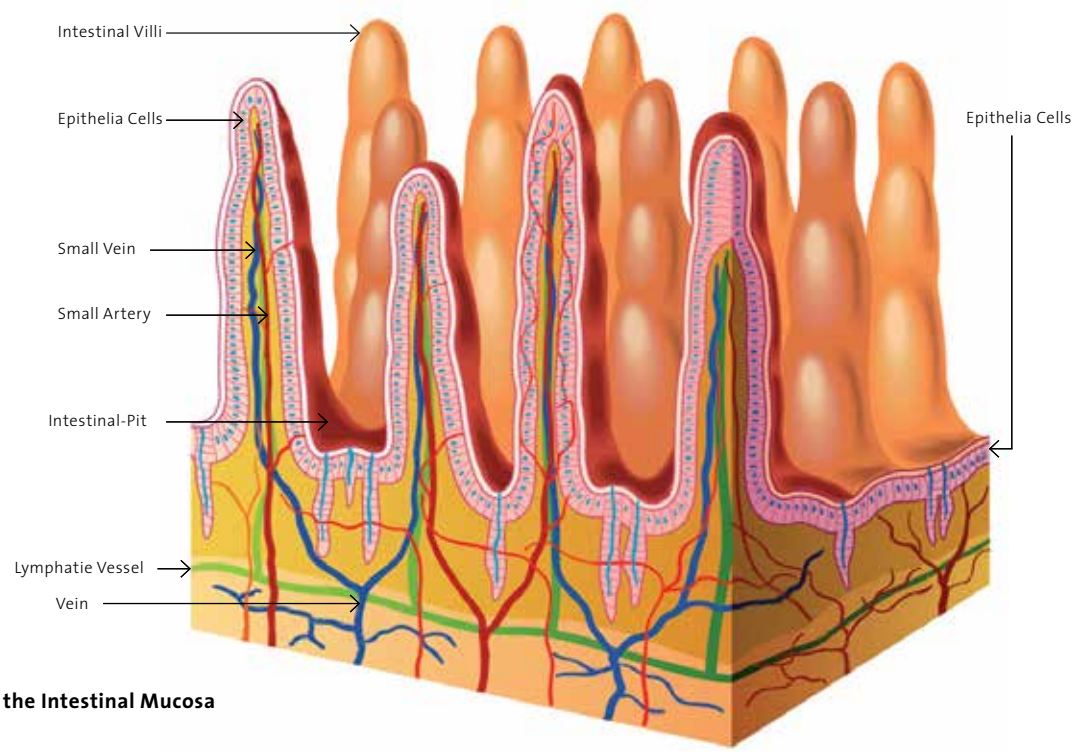
The Microbial Gut Community

Commensals

Domestic bacteria in the human colon ferment carbohydrates and proteins consumed with food to short fatty acid chains (lactic acid, acetic acid, butyric acid etc.) and gases (hydrogen, carbon dioxide). **Butyrate**, the salt of butyric acid, is the most important energy source for colonocytes – furthermore it has a strong anti-inflammatory effect. Especially butyrate developing firmicutes are regarded as important supplier of these short-chain fatty acids in particular *Faecalibacterium prausnitzi*i. It represents a total of 5-15 % of the human intestinal bacteria and is thus one of the most common intestinal inhabitants. As downright potent butyrate developer it plays a central role for the energy supply of intestinal cells. Aside from butyrate development *F. prausnitzii* also excels with its anti-inflammatory properties by inactivating the transcription factor NF- kB as well as IL-8 Production (4).

Often an increase of acute phase proteins in stool – e.g. α-1-antitrypsin or calprotectin – indicates inflammatory irritations of the intestinal mucosa. The molecular stool diagnostics of ***biovis‘*** provide conclusions about bacterial indicators. Often the absence of *F. prausnitzii* correlates with the degree of inflammation.

If the colon is healthy the epithelia cells are covered by a protective mucosa layer. If this **mucin layer** is damaged or only insufficient amounts of mucin are developed, pathogens, pollutants or allergens might come into direct contact with the mucosa. This will lead to inflammations. Therefore the maintenance of intact mucosa barriers is a protection against inflammations. In this case the bacterium *Akkermansia muciniphila* – a representative of the *verrucomicrobia* – is involved as it promotes mucosa production by goblet cells. The decomposition of mucosa promotes new production and at the same time provides substrate in form of oligosaccharides and short-chain fatty acid for butyrate development. This is an important correlation, which can be evaluated with the aid of microbiome diagnostics. Knowledge grows with every new analysis (2, 5)



Picture 2: **Structure of the Intestinal Mucosa**
3-D-Illustration,

Pathogens

Bacterial metabolism does not work exclusively for the benefit of humans. Via generating **hydrogen sulphide (H2S)** also sulphate reducing bacteria are involved in the development of gut diseases. H_2S is a toxic metabolic product, which damages the intestinal epithelia and thus promotes the occurrence of cellular atypia. The species *Bilophila wadsworthii, Desulfomonas pigra* and *Desulfovibrio piger* are considered to be especially potent H_2S developers.

The genus of obligatory anaerobic clostridia includes pathogenic as well as valuable bacteria, which have immune modulating effects and contribute to an increase of IL10. Especially clostridia of cluster I contain toxin developing types, which are frequently found in case of autistic spectrum disorders and are often the cause of intestinal as well as extra-intestinal complaints associated with autism.

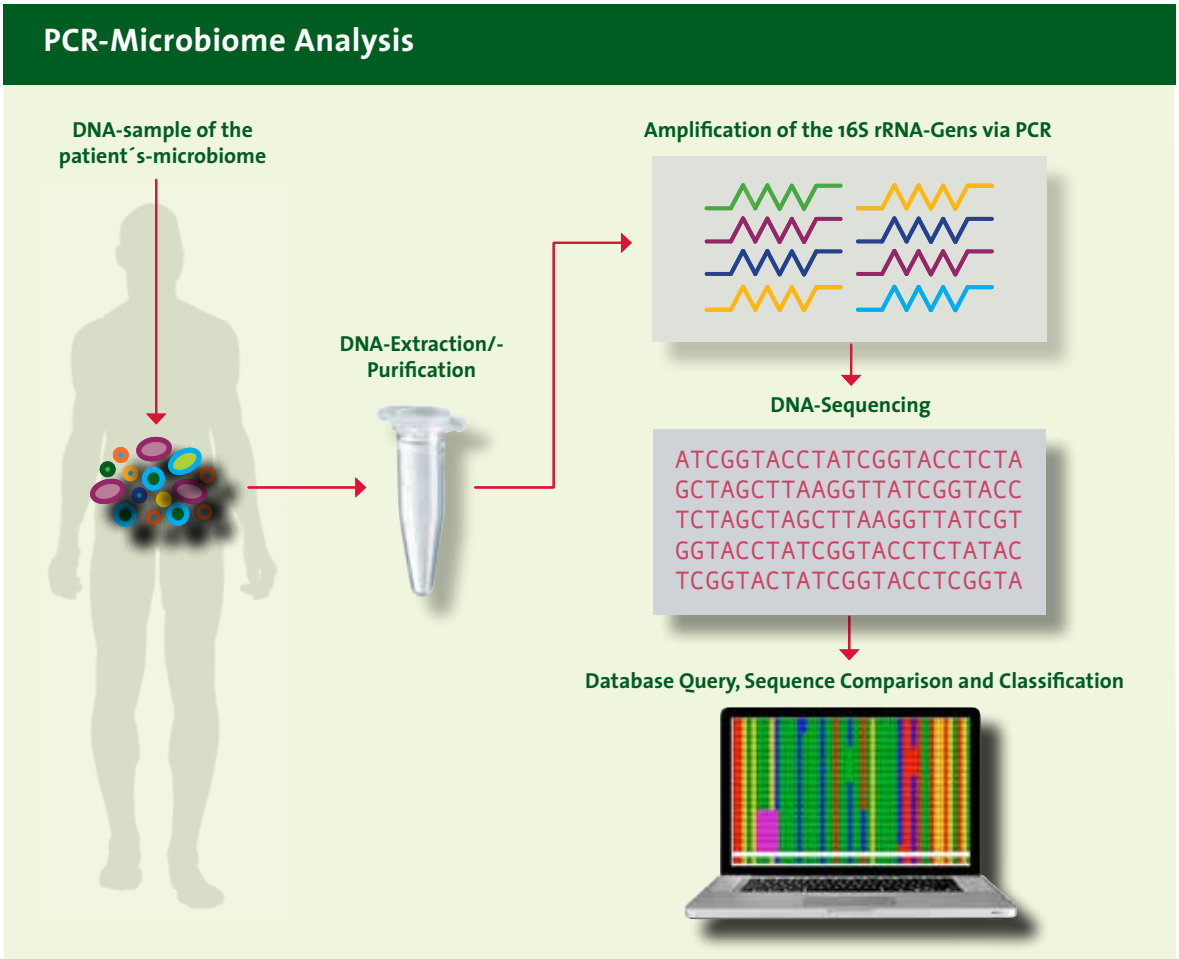
Furthermore often potentially **pathogenic species** like *Haemophilus* and *Fusobacteria* - both associated with the mucosa of the respiratory tract – can be found in the intestines. Recent results of basic research show the participation of these pathogenic species in case of chronic inflammatory bowel diseases (CIBD), colorectal carcinoma and appendicitis. Coherences like these and future findings can easily be integrated in molecular-genetic stool diagnostics. (6, 7)

Enteral Microbiome Analysis

Well-known gut bacteria like *E.coli, enterococcus, bifido bacterium* as well as various lactobacilli species can be cultivated reliably and cover an important part of the intestinal microbiota. A whole variety of anaerobes – microorganisms, which can only grow in oxygen-free living environments are either very difficult to cultivate or cultivation is not possible at all. Therefore large shares of these bacteria (e.g. *Faecalibacterium prausnitzii, Akkermansia muciniphila*) can only hardly or not at all be detected in the scope of conventional analyses. These bacteria, however, represent the largest group of the gut microbiota and have essential metabolic abilities. With hitherto existing diagnostic methods it has already been possible to come to manifold conclusions. Important information about butyrate, mucin or H_2S development and the responsible bacteria, however, remained unanswered so far.

As characteristics and individual functions of the bacteria are coded in their genomes, an extensive analysis of the intestinal microbiome is only possible with the aid of additional genetic analyses. The new ***biovis‘*** – diagnostics make use of this: The traditional stool test is complemented by more sophisticated modern molecular-genetic procedures providing for the examination of numerous aerobic and anaerobic bacteria as well as all metabolically relevant groups.

For distinct identification of bacteria genomes ***biovis‘*** uses the microbiome analysis. It is a molecular-genetic method based on sequencing of isolated bacteria DNA from clinical samples. During the process the signals, which exclusively occur in bacteria, are recorded. With the aid of individual 16S rRNA sequences of bacteria one can determine, which and exactly how many bacteria genoms are present in one sample. Thus the bacterial biodiversity is analysed. Science calls this technique 165-rRNA sequencing. Picture 2 shows a typical test procedure.



Picture 3:

Microbiome Analysis – Test Procedure

The bacteria DNA is isolated from the patient's stool sample and reproduced with the aid of PCR. The gene fragments, which are available in high counts now, are subsequently sequenced. The abundance of resulting data is evaluated with special computer programmes. Gene sequences are compared to reference genomes to allocate the bacteria correctly.

Picture acc. to Keller et al.

Microbiome analyses data provide for sequence comparisons in regard to intestinal flora composition of healthy people and the sequenced gene section can be assigned to certain bacteria species (8). This is done with the aid of **reference genomes**, which are provided by databases of the "Human Microbiome Project" – an initiative which was founded in 2008 to identify and document human microbes on molecular levels.

The microbiome analysis covers 250 parameters. Taking all verifiable species and generic groups into consideration one can come to conclusions about bacteria diversity. High bacteria diversity offers protection against endogenic infections in optimal cases, but it is often reduced as consequence of antibiotic therapies or in the scope of various disease patterns. In this case opportunistic bacteria like pathogens, fungi and viruses can easily proliferate (3, 10)

Classification – Enterotype

In the scope of the intestinal microbiome analysis also the individual enterotypes are determined – three main groups to which the human gut bacteria can be assigned. Enterotypes are defined by prevailing species *Bacteroides, Prevotella and Ruminococcus*, which cleave food components differently. This in turn has consequences for vitamin and mineral resorption. The enterotypes develop stable, clearly distinguishable bacteria clusters with typical metabolic properties. Enterotype 1 is mainly characterized by high *Bacteroides* counts, enterotype 2 by strong *Prevotella* population and enterotype 3 shows distinct *Ruminococcus* flora (10).

Bacteria Quantification

To complement the established cultivation methods and to determine individual parameters a molecular biological procedure based on qPCR (quantitative PCR) is applied. This method provides for precise quantification of individual bacteria on the bases of specific probes. With the aid of this technology it is possible to answer selected questions based on determined data and to recommend individual therapeutic measures.

Evaluation of Diagnostic Findings

To analyse the microbiome composition based on bacteria phyla, *Actinobacteria, Bacteroidetes, Firmicutes, Akkermansia muciniphila* and rarely detectable *Fusobacteria* are considered. On these highest taxonomic levels typical patterns can be recognized – for example increased *Firmicutes/Bacteroidetes* ratio or dominant *Proteobacteria* typical for various clinical patterns. The further report is structured based on contents and represents important species and their metabolic active genera – the most frequent are listed below in Table 1.

Bacteria Strains	Bacteria Species	Frequency
Bacteroidetes Metabolize soluble fibres, carbohydrates	*Bacteroides vulgatus*	++++
	Alistipes sp.	++
	Parabacteroides sp.	+
	Prevotella sp.	++
Firmicutes Metabolization of insoluble fibres	*Faecalibacterium prausnitzii**	++ to ++++
	*Eubacterium rectale**	++ to +++
	*Eubacterium hallii**	+ to +++
	Rominococcus bromii	+ to ++
	Clostridium clostridioforme	+++
	Anaerostipes hadrus	++
	Lachnospiraceae sp.	++
	*Roseburia sp.**	+ to +++
Actinobacteria	*Bifidobacterium sp.*	++ to +++
	Collinsella aerofaciens	+ to +++
Proteobacteria	*Escherischia coli*	+
Verrucomicrobia	*Akkermansia muciniphila***	+

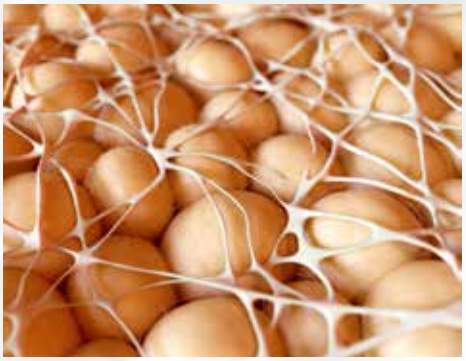
Table 1: **The Most Important Bacteria of the Intestinal Microbiome**

* = Butyric Acid Developer / ** = Mucin Developer

Correlations between Dysbiosis and Clinical Symptoms

Presently the intestinal microbiome is subject of intensive **research** – with very interesting results: The organisation of the human, gut populating bacteria flora helps to detect significant correlations in regard to physical health. Therefore it is also possible to treat the patient by influencing the intestinal microbiome with dietetic factors or prebiotic agents to reach a balanced ratio of strains and species to each other or balance selected deficits of important bacteria species. In the following please find a selection of **health impairments** caused by **shifts of the enteral microflora.**

1. Adiposity



Fat Cells, 3D-Illustration

Patients suffering from overweight and adiposity often have shifted ratios of *firmicutes* and *bacteroidetes* strains. Healthy people mostly show ***firmicutes/bacteroidetes* ratios** of 1:1 to 1:3, while 35% of overweight people show significantly shifted ratios in favour of *firmicutes* 3:1 to 25:1 (in extreme cases even up to 200 : 1). *Firmicute* predominance promotes metabolization of fibres as well as energy exploitation. Obesity is therefore also the long-term result of "additional supply" by excessively pronounced *firmicute* flora (19).

Adiposity is often also characterized by very low *Faecali prausnitzii* counts – a *firmicute* which belongs to the three most frequent bacteria of the intestinal tract. *F. prausnitzii* develops **butyrate**, which supports the gut mucosa and at the same time protects it against inflammations as the salt of butyric acid inhibits the activation of the transcription factor NFKB and blocks the release of the chemokine interleukin 8. In case of obese patients partly significantly increased hsCRP and interleukin-6 levels indicate inflammatory processes, which almost always come along with low *F. prausnitzii* counts. If the *F. prausnitzii* levels of these patients can be increased, the intestinal mucosa will be protected and local inflammatory reactions will decrease (4).

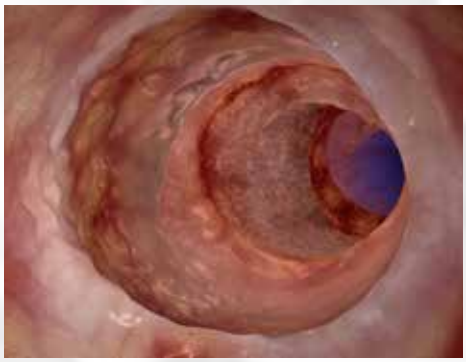
Also *Akkermansia muciniphila* counts are often low in case of obese patients. The bacterium is able to degrade **mucus** – a mucosa layer covering the intestinal epithelia cells. This does not lead to a reduction of the mucin layer – in fact the goblet cells are animated to develop more mucus which protects the mucosa and shields it against chemical, mechanical or inflammatory irritations. If high-fat diets lead to detectable *Akkermansia* reduction, taking oligosaccharides (e.g. prebiotic agents) lead to partly considerable increases of the bacteria count. In the scope of animal experiments this phenomena led to weight reduction, mucin layer development, mucosa barrier stabilization and positive influence on fasting blood sugar values and insulin resistance. Data presently available indicate that similar favourable effects can also be achieved for humans under the influence of *A. muciniphila* (11)

2. Metabolic Syndrome

Also for patients suffering from metabolic syndromes we frequently find changes of the intestinal microbiome – these are mainly low *Akkermansia muciniphila* counts. If the *A. muciniphila* counts can be increased insulin resistance and fasting blood sugar values will be positively influenced (12).

3. Intestinal Inflammation

The **irritable colon syndrome** frequently is a diagnosis of exclusion in case of diffuse, long lasting and recurring intestinal complaints. For quite some time there has been evidence that probiotic therapies are able to largely ease the symptoms. Recent studies revealed that *F. prausnitzii* counts are reduced by approximately 30 % in patients suffering from irritable colon. Even lower bacteria counts are found in case of people with Crohn's disease. As *F. prausnitzii* is the most important producer of anti-inflammatory butyrate, reduced bacteria counts are very negative: the inhibiting effect of *F. prausnitzii* on NF-$_K$B and interleukin-8 as well as mucosa stabilizing, anti-inflammatory and protecting effects of butyric acid are not available any longer. (4, 6)



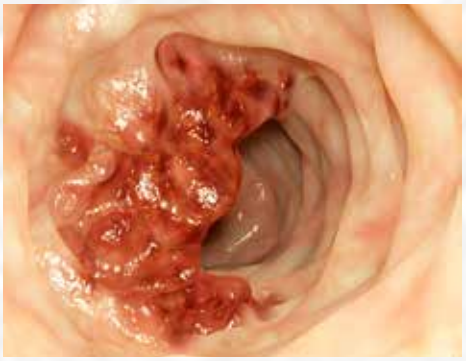
Crohn´s Disease, 3D-Illustration

If children with initial Crohn's disease diagnosis were concerned, *campylobacter* species could be isolated in up to 70 % of the cases. For this reason causal influence is being discussed constantly. If *campylobacter* species can be determined in patient samples, probiotic agents may be given, as these strongly counteract pathogenic bacteria (13).

The **leaky gut syndrome** is an another clinical pattern closely related to the intestinal microbiome. Stool analyses show low presence of mucin metabolizing *A. muciniphila* in the intestinal tract of persons suffering from permeability de-

fects of the tight junctions (14)

3. Intestinal Tumours and Intestinal Cancer



Intestina Cancer, 3D-Illustration

Aside from other known factors also hydrogen sulphide promotes the development of cell atypia and thus the formation of colorectal carcinoma by irritating the mucosa. Sulphate reducing bacteria (SRB) like *Desulfomonas piger* and *Desulfovibrio piger* as well as H2S developing *clostridia* are responsible for H2S development. If SRB counts are increased one should try to counteract further H2S proliferation by diet changes and milieu altering pre- or probiotic therapies (e.g. resistant starch). Intestinal tumour diseases also come along **with significant microbiome shifts**: Often *F. prausnitzii* counts are reduced below the limit of detection. As a consequence the anti-inflammatory butyrate is missing.

4. Arthritis

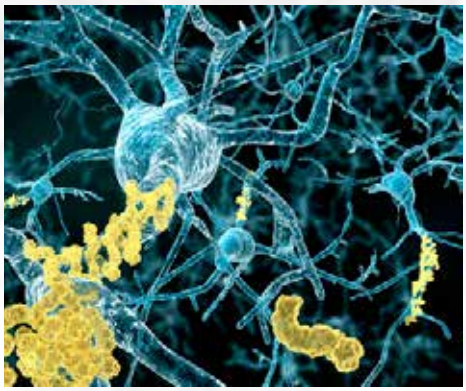
Arthritis, Deformed Hands

If people suffer from rheumatoid arthritis intestinal microbiome analyses may also **show bacterial imbalances**, which might correlate with disease development and progress. Possible consequences of bacterial imbalances can be recognized for example when looking at the intestinal bacterium *Prevotella copri*: If it populates the human intestines in physiological quantities both immune and digestive system profit. Patients with rheumatoid arthritis often have increased *Prevotella copr*i and *Prevotella sp.* counts. Scientists suspect that predominating counts of *P. copri* suppress growth and function of other intestinal bacteria (16).

5. Autism

During the genesis of autism **genetic factors** play a major role. In addition further factors co-determine the progress of development disorders. Children suffering from disorders of the autistic spectrum often complain about gastro-intestinal disorders. Studies showed that antibiotic therapies do not only ease gastro-intestinal complaints but also influence other symptoms associated with autism. According to some studies alterations of the intestinal microflora may influence brain development and behaviour (gut-brain-axis). This suggests that

impaired intestinal biodiversity may be connected to formation as well as progress of autism (17). In fact there are often increased bacteria counts of toxin developing *clostridia* in stool samples of children suffering from autism. Even *clostridia species* were found. They were exclusively detected in autistic children and not in the neurotypical control group. How exactly clostridia influence onset and progress of autism still remains unclear. If toxin developing clostridia are determined in the stool sample of patients (*clostridia* of cluster I), toxin development can be reduced by giving suitable probiotic agents (7).



Alsheimer´s Disease
Neurones withs Amyloid-Plaque,
3D-Illustration

6. Alzheimer`s Disease

Alterations of the intestinal microbiome are also found in Alzheimer patients. A recent study showed that almost 100 % of all tested Alzheimer patients (n=52) had low *F. prausnitzii* counts. In addition 87.5 % of the tested persons showed increased values of inflammation markers (calprotectin or antitrypsin). The hsCRP values of 91% of the patients indicated systemic inflammations in the body (20).

F. prausnitzii deficiency promotes intestinal mucosa inflammations. If it is possible to significantly increase the bacteria count of important species like *A. muciniphila* and *F. prausnitzii* anti-inflammatory and mucosa-protective effects will be achieved. Treatment with prebiotic and probiotic agents promote proliferation of certain bacteria species. This is also favourable for Alzheimer patients.

Diagnostics in Transition: The Future Has Begun!

With the aid of molecular-genetic stool diagnostics it is possible to detect alterations of the intestinal microbiome and to provide the basis for **prebiotic and probiotic therapies**. If the new diagnostics show individual changes of intestinal microbiomes, a differentiated therapy adapted to the patient's needs can be applied. There are various probiotic and prebiotics combinations available, which were successfully given to patients suffering from intestinal diseases (Crohn's disease, Colitis ulcerosa, leaky-gut-syndrome and others), adiposity and different types of diarrhoea associated with antibiotic therapies (18,19).

biovis' Diagnostik establishes individual and elaborate reports with respective therapy recommendations based on extensive molecular-genetic analyses of the intestinal microbiome and optionally supplemented by parameters like pancreas elastase, bile acids, calprotectin, α-1-antitrypsin and sIgA – the sample report (Picture 3) gives a first impression. The following table lists various therapy options, which can be adapted to laboratory results and therapy progress.

Picture 4:
First page of a ***biovis'*** sample report - The coloured bars show the laboratory results as well as scope of the values to be determined. Index and quotient describe patient values: if these are red this indicates lack of the respective parameter. Black is for values within normal range.

Probiotics	• Protection against pathogens by development of ß-defensin or SIgA • Reduction of clostridia toxins • Reduction of inflammatory gastro-intestinal diseases • Reduction of TNF-alpha development • Reduktion des α-1-Antitrypsin • Improves the mucosa barrier (leaky gut) • Promotes healthy intestinal microbiome during antibiotic therapies and intestinal infection	
Prebiotics	**Promote** ↑	**Inhibit** ↓
Starch	*R. bromii* *E. rectale / Roseburia sp.* *Bifido Bacteria*	Sulphate reducer
Inulin	*F. prausnitzii* *Bifidobacterium sp.* *Lactobacillus sp.*	*Bacteroides sp.* *Prevotella sp.* *Clostridium sp.* Sulphate reducer
Pectin	*Bacteroides*	
Fructose-oligosaccharides (FOS)	*F. prausnitzii* *A. muciniphila*	*Bacteroides sp.*
Galactose-oligosaccharides (GOS)	*Bifidobacterium sp.*	*Prevotella sp.*
Low-Carb diet		*R. bromii* *E. rectale / Roseburia sp.* *Bifidobacterium sp.* *Faecalibacterium prausnitzii*
High-fat diet		*A. muciniphila*
High-fat and protein diet	Sulphate reducer	
High-fat diet + FOS	*A. muciniphila* *F. prausnitzii*	
Supplement	**Glutamine** improves mucosa quality, regeneration and barrier function	

Table 2: Individual Therapy: Prebiotic and Probiotic Options

Genome Sequencing

Genome sequencing is the gold standard of basic research and covers more than 250 types and species - thus significantly more than all other methods. The sequence based microbiome analysis provides for easy handling and economic processing of patient samples. For precise analyses of the intestinal microbiome with molecular-genetic procedures biovis applies the newest insights of basic research

and thus up-to-date, innovative tools of stool diagnostics, which are of great medical relevance. Constant optimization with the aid of improved sample preparation and verification procedures as well as the application of additional relevant indicator bacteria with constantly new detection sequences of important intestinal bacteria will have its place in modern functional stool diagnostics.

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- **Do you still have questions?**
- **Are you interested in the new *biovis‘* diagnostic analyses?**
- **Would you like to have stool analyses done?**
- **Please call us or write us – we will be glad to help you!**

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