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DIPLOMARBEIT

ITS2 based analysis of the human gastrointestinal fungal microbiota of geriatrics, oncology patients and healthy volunteers

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Verfasserin: Natalie Alexandrow

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DANKE!

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3 LIST OF ABREVIATIONS

DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
rRNA	ribosomal RNA
rDNA	ribosomal DNA
PCR	Polymerase Chain Reaction
CFU	Colony forming units
min	minutes
sec	seconds
mM	millimoles
µM	micromoles
pM	picomoles
vol	Volume
V	Volt
°C	degree Celsius
SCFAs	Short chain fatty acids
qPCR	quantitative real time polymerase chain reaction
ITS	internal transcribed spacer region
IGS	ingtergenic spacer region
ETS	external transcribed spacer region
BTS	Biotherapeutic agent
dNTPs	Desoxynucleotidetriphosphates
PCA	principal component analysis
LAB	lactic acid bacteria

4 OBJECTIVES

Bacteria, archeae, yeasts and filamentous fungi are commensals of the adult gastrointestinal tract. Up to the present in the majority of cases the gastrointestinal bacterial diversity was investigated and the fungi community was nearly ignored (Rajilic-Stojanovic M, Smidt H et al. 2007; Zoetendal EG, Rajilic-Stojanovic M et al. 2008) .
This study seeks to contribute to enlighten the role of fungi in the human gastrointestinal tract.

It was suggested that the normal human intestine harbours a rich and diverse fungal community (Bernhardt H and Knoke M 1997), but results of the latest research indicated that the gastrointestinal fungal diversity of healthy individuals is low and stable compared with that of immunosuppressed individuals (Scanlan PD and Marchesi JR 2008). Furthermore composition of fungal gastrointestinal community is affected by antibiotics, antifungal agents, and diet (Chen Y, Chen Z et al. 2010).

On the basis of cultivation studies, the total number of fungal microorganisms is calculated to be 10^0-10^2 (0,0000000001-0,00001%, respectively) colony-forming units (CFUs/ml) in the oral cavity and increases to up 10^6 CFUs/ml in the faeces (Simon GL and Gorbach SL 1984; Bernhardt H and Knoke M 1997). However the problem of cultivation studies is that many fungal species are not cultivable (Ott SJ, Kühbacher T et al. 2008).
Scupham et al. were the first ones who analysed the mammalian fungal microbiota in the murine gut with culture-independent techniques (Scupham AJ, Presley LL et al. 2006).
So in recent years researchers began using molecular, culture-independent techniques for analyzing the human gastrointestinal fungal microbiota (Ott SJ, Kühbacher T et al. 2008; Scanlan PD and Marchesi JR 2008; Chen Y, Chen Z et al. 2010).
Especially the 18S rDNA is a widely used target for studies analyzing fungal diversity.

The 18S rDNA is an ideal target for molecular analysis, because it is ubiquitous and has a specific structure consisting of evolutionary conserved and variable regions (Muyzer G and K 1998).

The purpose of this diploma thesis was on the one hand to investigate the gastrointestinal fungal microbiota of young, healthy omnivores compared with geriatrics and on the other hand the changes of fungal microbiota in oncology patients in course of their therapy compared with healthy controls.

For this thesis a quantitative real time polymerase chain reaction (qPCR) approach, PCR-DGGE (denaturating gradient gel electrophoresis), clone library and sequencing analysis were used.

5 INTRODUTION

5.1 The Internal transcribed spacer regions

In clinical microbiology applications DNA and RNA have been used for the classification and identification of eukaryotic pathogens, such as fungi for many years (Versalovic J, Swanson DS et al. 1996).

The organization of fungi into taxonomic groups are genetically based on sequence homology within the fungal rDNA genes (18S, 5.8S, 28S) and the differences of the internal transcribed spacer regions (ITS1, ITS2)(Iwen PC, Hinrichs SH et al. 2002).

All eukaryotic cells have these ITS regions in their rDNA gene complex. The ITS1 region is located between the 18S and 5.8S rDNA genes, between the 5.8S and 28S rDNA genes the ITS2 region is found (Powell KA, Renwick a et al. 1994).

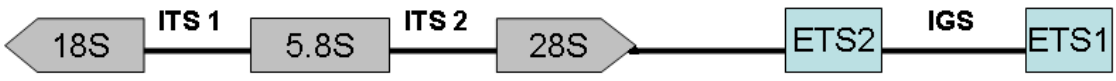


Figure 1 Location of the ETS regions of the IGS in relation to the rRNA genes and the ITS regions.

From 5’ to 3’ orientation the overall rDNA gene complex includes the ingtergenic spacer region (IGS). The IGS contains the external transcribed spacer (ETS) 1 region which is also called nontranscribed spacer on the 5’ end and the ETS2 region on the 3’ end. Further the 18S rDNA gene, the ITS 1 region, the 5.8S rDNA gene, the ITS 2 region and the 28S rDNA gene follow (Lott TJ, Kuykendall RJ et al. 1993).

ETS1 and ETS2 regions contain conserved sequences among fungal cells and although their biological function is not fully understood, both seem to be necessary for early rRNA processing during transcription (Lalev AI and RN 1999).

Otherwise the ITS1 and ITS2 regions show remarkable sequence diversity in major groups of eukaryotic microorganisms and even within species of the same organism group (Joseph N, Krauskopf E et al. 1999).

5.2 The fungal cell wall

The fungal cell wall is a dynamic organelle that plays a decisive role in several processes. On the one side it must protect the cell mechanically to withstand changes in osmotic pressure imposed by the environment, but on the other side the cell wall must keep enough plasticity to allow cell growth, cell division and the formation of multitudinous cell types during the life cycle of the fungus. Furthermore the cell wall is responsible for interaction of the fungi with its surroundings.

If the cell wall structure is disordered it has a severe effect on the growth and morphology of the fungal cell, often ending in lysis and death.

Glycoproteins and polysaccharides, primarily glucan and chitin, are the main components of the fungal cell wall. In addition the cell wall has also minor components which vary among fungal species.

The cell wall glycoproteins are modified with both N- and O-linked carbohydrates and, in many cases, also contain a glycosylphosphatidylinositol (GPI) anchor.

The cell wall components glycoprotein, glucan and chitin are cross-linked together and so they form the structural basis of the cell wall.

The composition of the cell wall is able to change and may vary within a single fungal isolate depending on the environmental conditions and stage of growth (Bowman SM and Free SJ 2006; Latge 2010).

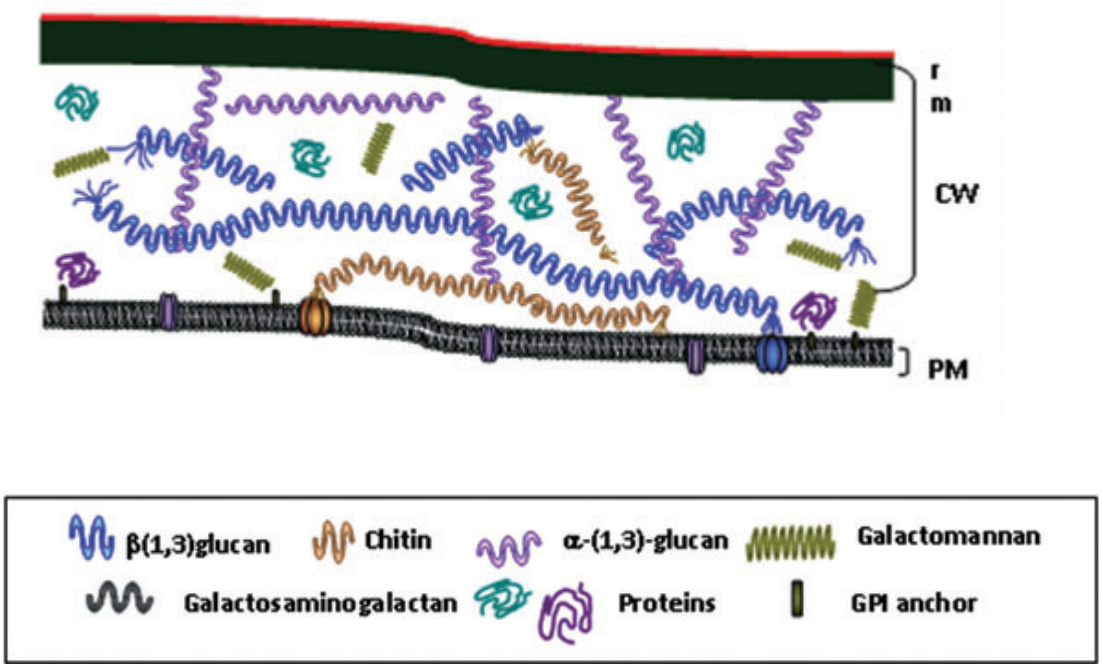


Figure 2 The fungal cell wall (Latge 2010).

5.2.1 Chitin

A relatively minor component of the fungal cell wall is Chitin, a linear homopolymer of ß-1,4-linked N-acetylglucosamine. Only 1-2% of the yeast cell wall consists of chitin (Klis 1994; Klis FM, Mol P et al. 2002), whereas in cell walls of filamentous fungi, e.g. Aspergillus, chitin accounts 10-20% (De Nobel JG, Van den Ende H et al. 2000).

When the synthesis of chitin is interrupted, the wall becomes disordered and the fungal cell becomes fragile and osmotically unstable (Specht CA, Liu Y et al. 1996).

5.2.2 Glucan

The major structural polysaccharide, namely 50-60%, of the fungal cell wall is glucan (Kapteyn JC, Van den Ende H et al. 1999). Generally 65% up to 90% of the cell wall glucan is ß-1,3-linked, but also other glucans, such as ß-1,6- or mixed ß-1,3- and ß-1,4-

glucan have been found in some fungal cell walls (Bernard M and JP 2001; Klis FM, De Groot P et al. 2001). The main structural component is ß-1,3-glucan to which other cell wall components are covalently attached. Therefore the synthesis of ß-1,3-glucan is important for proper cell wall formation and the normal development of fungi (Bowman SM and Free SJ 2006).

5.2.3 Glycoprotein

Within the structural matrix of chitin and glucan all fungal cell walls have a protein component, which account for 30-50% of yeasts cell walls (*S.cerevisiae, C.albicans*) whereas the cell wall of filamentous fungi consists approximately for 20-30% of proteins (Fleet 1991; Brown JA and BJ 1992).

Most cell wall proteins are integrated into the wall via covalent linkages between the sugars present at the N- and O-linked sites and/or in the GPI anchor with those in the polymers of chitin or glucan. Cell wall proteins play an important role in maintaining cell shape, mediating adhesion for cell migration and fusion, protecting the cell against foreign substances, mediating the absorption of molecules, transmitting intercellular signals from external stimuli, and synthesizing and remodelling cell wall components.

5.3 Metabolism

Fungal cells utilize a diverse range of nutrients and have equally diverse nutrient acquisition strategies. Fungi are non-motile, saprophytic (and sometimes parasitic) and chemo-organotrophic organisms. Fungi exhibit dynamic interactions with their environment that may be exemplified by certain morphological changes depending on nutrient availability.

Fungi, but few yeast species, extracellularly decompose polymeric compounds by secreted enzymes before utilize the monomers as carbon and energy sources.

The degradation of polymeric compounds by fungi is executed by various enzyme systems; some examples:

- starch —(amylases, glucoamylase)→ glucose
- inulin —(inulinase)→ fructose
- lipids —(lipases)→ fatty acids
- proteins —(proteinases)→ amino acids

5.4 Fungi in health and disease

Human intestinal microecology plays an constitutive role in metabolic activities, immunity, and trophic function (Guarner F and JR 2003; Blaut M and T 2007). Eckburg et al described the human intestinal microbiota actually as a novel 'organ' integral to human health and disease (Eckburg PB, Bik EM et al. 2005).
One explanation for the increasing number of immunosuppressed individuals and subsequent opportunistic infections is the therapeutic application of immunosuppressive drugs and the use of broadspectrum antibiotics in clinical settings (Kothavade RJ, Kura MM et al. 2010).
One of the major causes for the increasing number of patients with fungal infections is AIDS (Nissapatorn V, Lee C et al. 2003; Singh A, Bairy I et al. 2003).
C. albicans is a commensal of humans, but also the most frequently cause of nosocomial infections by yeasts. In fact infection caused by non-albicans Candida species, yeast other than Candida and mould fungi are increasing (Weinberger M, Sacks T et al. 1997; Baran J Jr, Muckatira B et al. 2001). For instance, *C. parapsilosis* occurred as a frequent cause of intravascular device-associated candidemia especially in children but its reservoir remains undefined (Welbel SF, McNeil MM et al. 1996; Levy I, Rubin G et al. 1998). *C. glabrata* was also noted to be a major nosocomial pathogen (Vazquez JA 1998).

It is suspected that the reservoir of *C. albicans* is the gastrointestinal tract and translocation of intestinal fungi may cause systemic infections in immunocompromised hosts (Ott SJ, Kühbacher T et al. 2008). Moreover also exogenous sources can be responsible for nosocomial infections by yeast (Voss A, Pfaller MA et al. 1995).

In an animal model with BALB/c mice it was shown that gastrointestinal Candida colonization stimulates increased serum IgG and IgE specific to intragastrically administered ovalbumin. So the researchers suggested, that gastrointestinal Candida colonization promotes sensitisation against food antigen (Yamaguchi N, Sugita R et al. 2006).

5.4.1 Fungi and cancer

The improvement of the antimicrobial agents against formerly severe bacterial infections might be the cause of increasing mycoses leading to morbidity and mortality in cancer patients (Lin SJ, Schranz J et al. 2001; McNeil MM, Nash SL et al. 2001).
Patients with acute myeloid leukaemia have the highest incidence of infections due to filamentous fungi. The infections are predominantly caused by *Aspergillus sp*. and less frequently by Zygomycetes (Pagano L, Caira M et al. 2006). The gastrointestinal tract of the patient is often the origin of Candida infections, but the reservoir for infections caused by *Aspergillus sp.* is environmental (Hammond SP, Marty FM et al. 2010).
Candida species infiltrate, in addtition to the oral mucosa, frequently the gastric mucosal layers and subsequent causes infections in immunocompromised patients (Stone HH, Kolb LD et al. 1974).
C. tropicalis, colonizing the human gastrointestinal tract seems to be more invasive than *C. albicans* in oncology patients (Wingard JR, Merz WG et al. 1979; Walsh TJ and Merz WG 1986).
Invasive candidiasis in patients with acute leukaemia or bone marrow transplantation is increasingly caused by *C. tropicalis* (Wingard JR, Merz WG et al. 1979; Sandford GR, Merz WG et al. 1980). However, it remains unclear which mechanisms permit *C.*

tropicalis to colonize the intestine and make dissemination possible (Fromtling RA, Abruzzo GK et al. 1987).

About 30% of cancer patients suffer from invasive fungal infections, but clinical diagnoses of those are often established to late (Chamilos G, Luna M et al. 2006).

It was reported that introduction of antifungal prophylaxes with fluconazole resulted in a significant reduction of invasive Candida infections. However after introduction these patients had a conspicuous risk of becoming colonized with *Candida sp.* (particularly *C. albicans, C. krusei* and *C. glabrata*), which are resistant to fluconazole (Marr KA, Seidel K et al. 2000).

5.4.2 Fungi and elderly

Elderly are more frequent affected by fungal infections which are also associated with higher morbidity and mortality. Besides diagnosis is more difficult because elderly often show different symptoms than non-elderly adults (Gavazzi G and KH 2002; Htwe TH, Mushtaq A et al. 2007).

C. glabrata infections in elderly are more common than in younger adults (Diekema DJ, Messer SA et al. 2002; Malani A, Hmoud J et al. 2005). Besides this yeast is uncommonly found in neonates and young children (Pappas PG, Rex JH et al. 2003).

In elderly also nosocomial infections are more common (Ohlsen K and J 2005). Reasons for that are an increased hospitalization rate and also the increased risk of infection per day of hospitalization (Emori TG, Banerjee SN et al. 1991). Especially Candida infections are a major problem in hospitals (Zilberberg MD, Shorr AF et al. 2008). However the transmission of *C. glabrata* to the patients is not clear, because this yeast was only found on environmental surfaces (Vasquez J, Dembry LM et al. 1998), but not on hands of health care workers (Hedderwick SA, Lyons MJ et al. 2000).

The increasing number of *C. glabrata* infections appears to have several causes, such as increased use of fluconazole and geographical differences (Marr KA, Seidel K et al. 2000; Malani PN, Bradley SF et al. 2001).

Already in the recent years studies reported that denture use is a risk factor for oral cavity yeast colonization in elderly (Berdicevsky I, Ben-Aryeh H et al. 1980; Cardash HS,

Helft M et al. 1989). Malani et al. reported in an actual study that denture wearers are even three times more likely to harbour *C. glabrata* than those who wear no dentures. So they suggested that the use of dentures is the strongest risk factor for oral yeast colonization in elderly. Though oral colonization will not preferentially lead to dissemination and bloodstream infection (Malani A, Psarros G et al. 2010).

5.4.2.1 Intestinal infections in elderly

In elderly physiological changes in the gastrointestinal tract such as decreased acid secretion by gastric mucosa take place. This facilitates the passage for microbes, like yeasts, and so they can dispread in new niches. The consequence is a shift in the composition of the gastrointestinal flora upon age (Bartosch S, Woodmansey EJ et al. 2005).

So an overgrowth of for example *Candida sp.* in the gut might be the consequence of partial lack of digestive enzymes and because of that plenty of nutrients are available for yeasts (Hof 2010).

5.5 Interaction Candida albicans – epithelial cells

The interaction with epithelial cells by *Candida sp.* occurs during mucosal colonization and initiation of disease (Zhu W and Filler SG 2009).

The first step of *C. albicans*-epithelial cell interaction is adherence, followed by invasion and finally induction of epithelial cell damage. In consequence the epithelial cells secret pro-inflammatory cytokines and produce factors that inhibit the growth of the yeast (Dongari-Bagtzoglou A, Kashleva H et al. 2004; Feng Z, Jiang B et al. 2005; Villar CC, Kashleva H et al. 2005).

For *C. albicans* the adherence step is essential for colonization and induction of mucosal disease. Therefore *C. albicans* possesses various surface structures which mediate adherence to epithelial cells. These so called adhesins exhibit differential expression on yeast versus hyphae (Zhu W and Filler SG 2009).

The invasive form of *Candida sp.* seems to be hyphae, because most of intracellular organisms are hyphae. The yeast form is normally located between or on the surface of epithelial cells (Scherwitz 1982; Ray TL and Payne CD 1988). Per in vitro studies two mechanisms responsible for invading epithelial cells were identified. The first one is that *C. albicans* activates epithelial cell endocytosis, which is induced by invasin-like proteins. These proteins are expressed on the surface of hyphae. The second mechanism of invasion is infiltration of a hyphae into or between epithelial cells (Phan QT, Myers CL et al. 2007; Dalle F, Wachtler B et al. 2010).

The way how *C. albicans* induces epithelial cell damage is to date not fully understood. For instance some researcher suggest that phopholipases secreted by *C. albicans* induce epithelial damage (Leidich SD, Ibrahim AS et al. 1998; Theiss S, Ishdorj G et al. 2006). However also other mechanism were discussed (Lermann U and Morschhauser J 2008; Naglik JR, Moyes D et al. 2008).

5.6 Interaction fungi – bacteria

Host-associated fungi are often found within communities comprised of various bacterial and fungal species. The diverse mechanisms of interaction between fungi and bacteria are illustrated in Figure 3 (Wargo MJ and Hogan DA 2006).

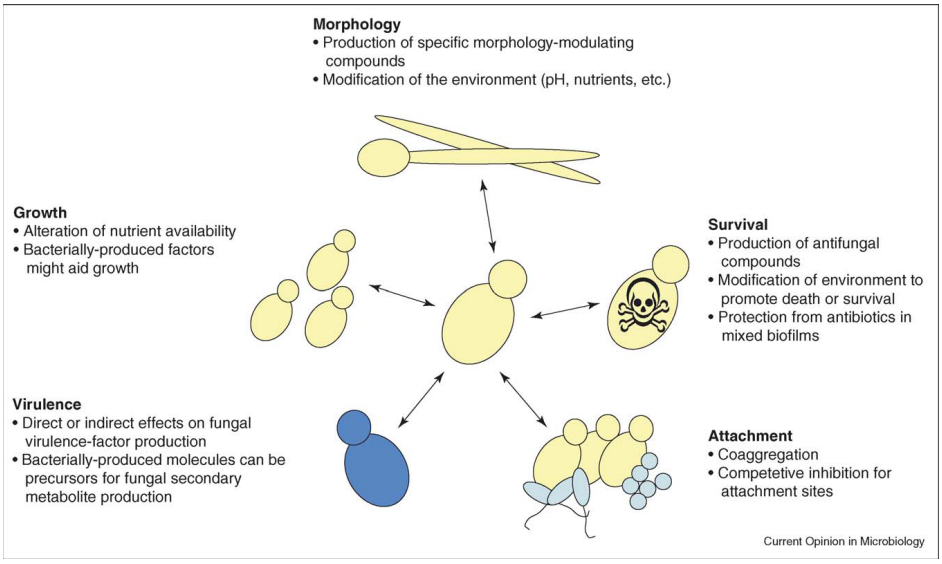


Figure 3 Different ways that bacteria can affect fungi. How fungal growth, survival and virulence are effected by bacteria. (Wargo MJ and Hogan DA 2006).

5.6.1 Candida sp. - bacteria interactions

Candida sp. and bacteria can form mixed microbial biofilms and infections caused by this communities are difficult to treat with antibiotics and antifungals. *C. albicans* and *Staphylococcus aureus* and their co-existence were identified in patients with denture stomatitis; in 39 of 50 patients with denture stomatitis a co-colonization of *C. albicans* and *S. aureus* was detected (Baena-Monroy T, Moreno-Maldonade V et al. 2005).

In vitro *Pseudomonas aeruginose* can inhibit the growth of *C. albicans* and is actually able to kill the yeast (Kerr 1994; Hogan DA and R 2002). This observation was strengthened by Gupta et al., who detected that in many cases *Candida sp.* co-infected burn wounds with bacteria. Moreover they found out when *P. aeruginose* was involved fungal colonization was inhibited (Gupta N, Haque A et al. 2005).

Some studies also report the detection of mixed-species biofilms on catheters (Marrie TJ and JW 1984; Crump JA and PJ 2000; Ramage G, Saville SP et al. 2005).

Biofilms of mixed-species are probably more resistant because of a more complex matrix composition. Besides antibiotic resistance profiles might change in infections caused by co-species communities (Wargo MJ and Hogan DA 2006).

Furthermore surface polysaccharides might also play an important role in the colonization of bacterial biofilms by *C. albicans* and the other way around (El-Azizi MA, Starks SE et al. 2004). Interestingly El-Azizi et al. also reported that glycocalyx-producing bacteria better adhere to *C. albicans*, but the yeast could not attach as easily to existing biofilms of glycocalyx-producing bacteria.

Bacteria also interact with other fungi than Candida, such as *Malassezia sp*.(Curvale-Fauchet N, Botterei F et al. 2004), *Cryptococcus neoformans* (Frases S, Chaskes S et al. 2006) and *Aspergillus sp.* (Ritz N, Ammann RA et al. 2005), but concerning these interactions only few data are available at the moment.

5.7 Probiotics – control of fungal infections

The most common and widely used probiotic organism is *Lactobacilli*. Probiotic organisms are effective against fungal overgrowth and infection in the host. They have

also an effect on fungal morphogenesis. The transition of *C. albicans* from yeast to hyphae can be inhibit by culture supernatant from lactic acid bacteria (LAB) (Noverr MC and Huffnagle GB 2004). Many LAB species produce butyric acid, a member of short chain fatty acids (SCFAs) and it is suggested that butyric acid is one of the most important factors controlling *C. albicans* infection (Wargo MJ and Hogan DA 2006).

5.8 Yeast as probiotics - Saccharomyces boulardii

Fuller defined the term probiotic as a 'live non-pathogenic microbial feed or food supplement which beneficially affects the host by improving its intestinal microbial balance' (Fuller 1991). This definition has extended to human health as 'non-pathogenic microorganisms that, when ingested, exert a positive influence on the health or the physiology of the host' (Marteau RP, deVrese M et al. 2001).
Another important term is 'Biotherapeutic agents' (BTAs). They are defined as 'living microorganisms used either to prevent or to treat human diseases by interacting with the natural microecology of the host' (Elmer GW, Surawicz CM et al. 1996; Elmer GW and McFarland LV 2001).
The group of BTAs consists of bacterial species such as various strains of *lactobacilli* and *bifidobacteria* and a strain of yeast, *Saccharomyces boulardii (Elmer GW and McFarland LV 2001)*.
After some discussion (McFarland 1996) *S. boulardii* is regarded to be identical to a particular strain of *S. cerevisiae* (Hennequin C, Thierry A et al. 2001).
S. boulardii is a non-pathogenic yeast which is isolated from lychee fruit in Indochina and not a part of the normal gastrointestinal microbiota. *S. boulardii* is a potential probiotic agent, because e.g. it survives transit through the gastrointestinal tract, its temperature optimum is 37 °C and it inhibits the growth of a number of microbial pathogens (Czerucka D, Piche T et al. 2007).
Therefore different types of diarrheal diseases such as antibiotic-associated diarrhea, traveler's diarrhea, acute gastroenteritis in adults and children, diarrhea in tube-fed patients, and chronic diarrhea in HIV-infected patients are often treated with *S.*

boulardii (Elmer GW, Surawicz CM et al. 1996; Marchand J and Vandenplas Y 2000; Elmer GW and McFarland LV 2001).

In genereal the treatment with *S. boulardii* is safe and well-tolerated. Only few reports show that this yeast is also cause of fungemia in patients with severe general or intestinal disease who had an indwelling catheter (Hennequin C, Kauffmann-Lacroix C et al. 2000; Munoz P, Bouza E et al. 2005).

5.8.1 Saccharomyces boulardii – Clostridium difficile

Antibiotic-associated colitis in humans and animals is often associated with *Clostridium difficile* (Bartlett JG, Chang TW et al. 1978). *C. difficile* colonizes the intestine and releases two potent protein exotoxins (toxin A and toxin B) after antibiotic intake. These two toxins are responsible for diarrhea and colitis caused by this bacterium (Lyerly DM, Kriven HC et al. 1988; Kelly CP 1994).

S. boulardii possess a 54-kDA protease, which has enzymatic activity against *C. difficile* toxin B.

Moreover, because of the protease the ability of toxins A and B to bind to human colonic brush-border membran is decreased and also the effects of both toxins on colonic epithelial cells and native human colonic mucosa in vitro are inhibited. This fact may be relevant to the mechanism by which *S. boulardii* exerts its beneficial effects in human *C.difficile* colitis (Castagliuolo I, Riegler MF et al. 1999).

5.8.2 Anti – inflammatory effect of Saccharomyces boulardii

Another probiotic effect of *S. bouldardii* is that it is able to suppress proinflammatory cytokine response in intraepithelial lymphocytes (IELs) and so anti-inflammatory cytokine response is increased. (Fidan I, Kalkanci A et al. 2008). In the yeast supernatant a small (<1kDa) heat stable, water-soluble anti-inflammatory molecule was found. It is assumed that this Saccharomyces anti-inflammatory factor (SAIF) plays a major role in the anti-inflammatory effect of the yeast (Sougioultzis S, Simeonidis S et al. 2006).

5.8.3 S. boulardii – short-chain fatty acids (SCFA)

Butyrate, a member of short chain fatty acids, is known to inhibit inflammatory response via inhibition of NF-κB (Sergain JP, Raingeard de la Bletiere D et al. 2000). Schneider et al. investigated the effect of *S. boulardii* on SCFAs in patients receiving enteral nutrition. These patients received *S. boulardii* and subsequent the faecal concentration of SCFAs was analyzed.

The total faecal SCFAs levels in the patients were significantly increased in comparison to controls. Therefore they assumed that *S. boulardii* induced increase of faecal SCFAs concentration, especially butyrate, may offer another explanation of the preventive effect of this yeast (Schneider SM, Girard-Pipau F et al. 2005).

6 METHODS AND MATERIALS

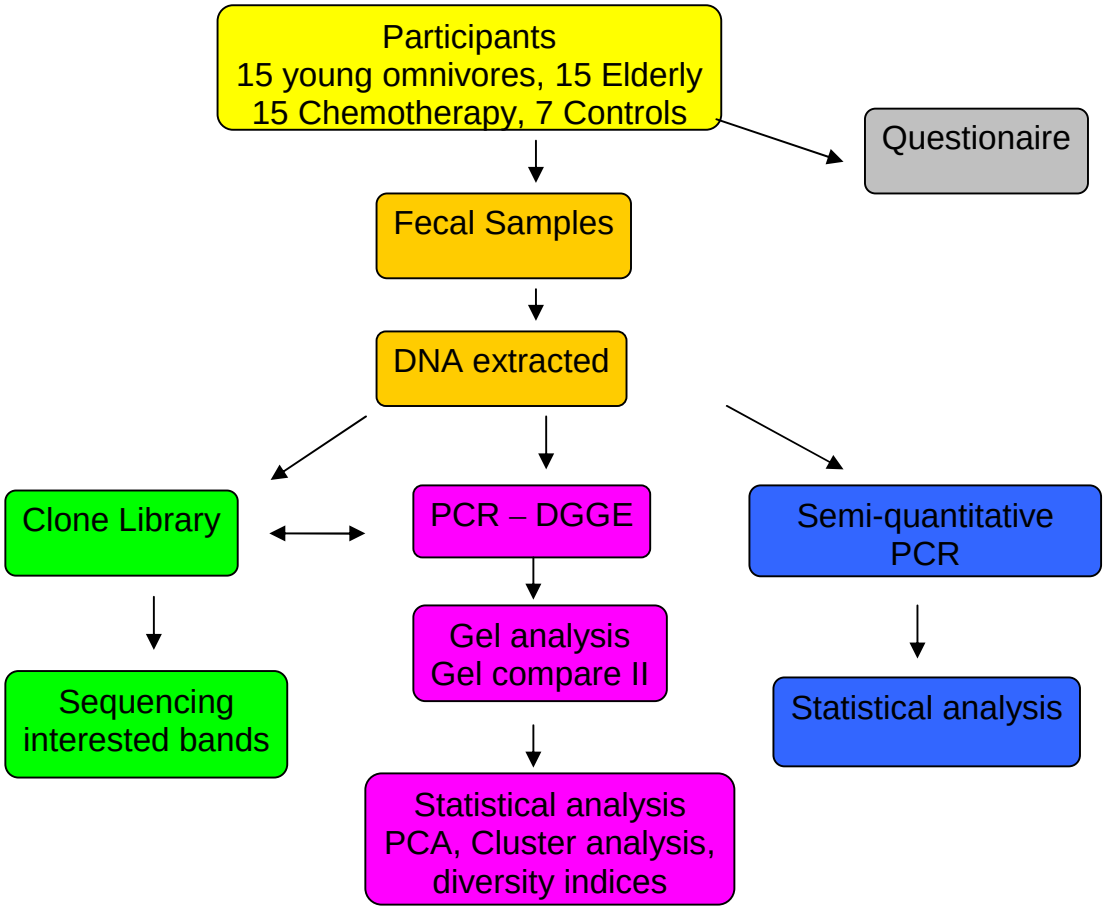


Figure 4 Study design of this thesis

6.1 Elderly and young participants

Faecal samples of 15 institutionalized elderly aged 86±8years, BMI 21,75±5,08, from a geriatric department in Vienna and 15 students aged 24±2,5 years, BMI 22,68±3,41, from Vienna were used for this study. The participants were interviewed following a questionnaire assessing: age; gender; body length and weight; individual health status, including chronic or acute diseases, blood lipid levels; and life-style aspects, such as physical activity and dietary habits.

Five percent of geriatric patients suffered from manifest diabetes mellitus type 2. Ten patients were bed-ridden and seven mobile. Causes of loss of mobility were Parkinson's disease, dementia and osteoporosis. Nursing staff reported the application of NSAIDs (non-steroidal anti-inflammatory drugs) on demand. Besides they received supplements with soluble fiber (Benefiber, Novartis).

The dietary habits of the 15 young healthy volunteers were typical for Central Europe.

Study populations were gender balanced, with 55% females in the group of elderly and 50% in the young group of omnivores.

Only non-pregnant subjects with no diagnosed gastrointestinal disease and no antibiotic or chemotherapeutic treatment three months prior to sampling were included in the study. All subjects agreed to participate in the study and gave their informed consent.

6.2 Oncology patients and healthy controls

Faecal samples of 15 subjects receiving chemotherapy with or without antimicrobial therapy (aged 58±12 years, BMI 26±5) from the Sozialmedizinisches Zentrum Ost in Vienna and seven healthy individuals were used for this study. At four time points faecal samples of each ambulant oncology patient were collected within two weeks before and after the onset of treatment. Two out of fifteen patients (ON004, ON013) had never received any chemotherapy before, while the others had a history of chemotherapy. One oncology patient additionally suffered from rheumatism while

another subject suffered from diabetes mellitus type II, hypertension and obesity. Anonymous medical records reported types of malignancies as well as chemotherapeutic and antimicrobial treatment. Six individuals suffered from a form of leukaemia, three patients suffered from a form of lymphoma (non-Hodgkin). Other malignancies were breast cancer, bladder cancer, ovarian arrhenoblastoma, multiple myeloma and bowel cancers. Among the chemotherapeutic regimens were bendamustin, bortezomib, cytarabin, dexamethosane, doxorubicin, etoposid, gemcitabine, idarubicin and melphalan. Leukaemia patients furthermore received G-CSF (neupogen) and/or radiated erythrocyte concentrate. In addition, patients ON004, ON005, ON007, ON009 and ON010 took antibiotics (Zwielehner J, Lassl C et al. 2010; under review).

Three stool samples of healthy individuals were collected during two weeks; they were composed of 56% females, whereas 47% of oncology patients were female.

All study participants were interviewed assessing: age; gender; body length; weight; health status (chronic and acute diseases); life-style aspects such as alcohol consumption as well as physical activity. Dietary habits were assessed using a food frequency questionnaire. Exclusion criteria for healthy controls were antimicrobial medication, chemotherapeutic treatment and pre- and probiotics at least three months before sample collection. All subjects gave written informed consent.

6.3 Sampling and DNA extraction from stools

After collection, stool samples were brought to the laboratory and immediately stored at -70°C. A 200mg aliquot of each sample was treated twice for 45s in a bead-beater (Mini-Beadbeater-8). Then DNA was extracted with the QIAamp® DNA Stool Mini Kit (QIAGEN) following the manufacturer's protocol and finally stored at -20°C until analysis.

6.4 Polymerase Chain Reaction (PCR)

The principle of PCR is that small regions of single or double stranded DNA are amplified by an enzymatic reaction and so it is possible to copy immeasurably small amounts of DNA for analysis with usual laboratory methods or for sequencing.

The PCR mixture is composed of:

- DNA template with sequence section which should be amplified
- Primers (forward and reverse) with a length of 15-20 nucleotides
- Thermostabile DNA polymerase (e.g. Taq polymerase from Thermus aquaticus)
- Desoxynucleotidetriphosphates (dNTPs)
- Buffer and $MgCl_2$ (polymerase cofactors)

The first step of PCR is initialization. In this step high temperature activates the DNA polymerase. After the initialization the PCR cycling (Figure 5), which consists of three recurring steps, follows:

1. Denaturation:

Heating the reaction mixture to approximately 95°C for melting the DNA template to achieve single-stranded DNA molecules – the DNA denatured

2. Annealing:

Annealing of the primers to single-stranded DNA at a lower temperature and the polymerase begins DNA synthesis.

3. Elongation:

The polymerase used defines the temperature of this step (e.g. Taq polymerase needs 72°C). A complementary DNA strand is synthesized by adding dNTPs. This reaction is exponential, which means that under optimum conditions (no limitations of substrates and reagents) the DNA target is doubled at each elongation step (Figure 6).

After cycling a final elongation is conducted. This single step assures that any single-stranded DNA is fully elongated.

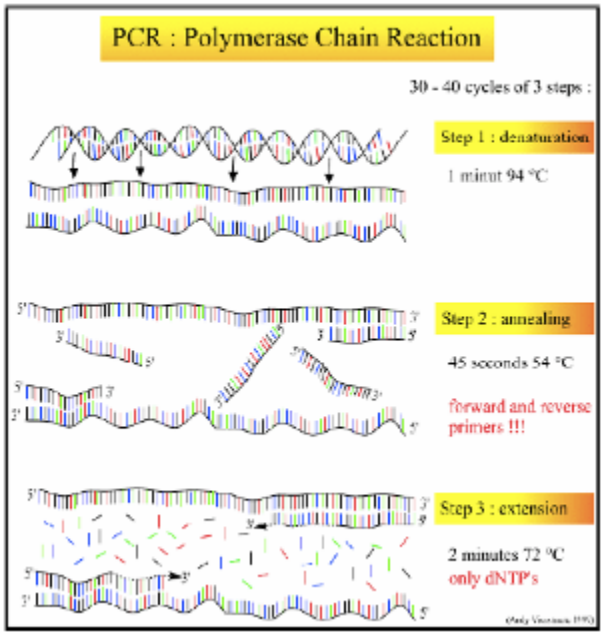


Figure 5
The PCR cycle.

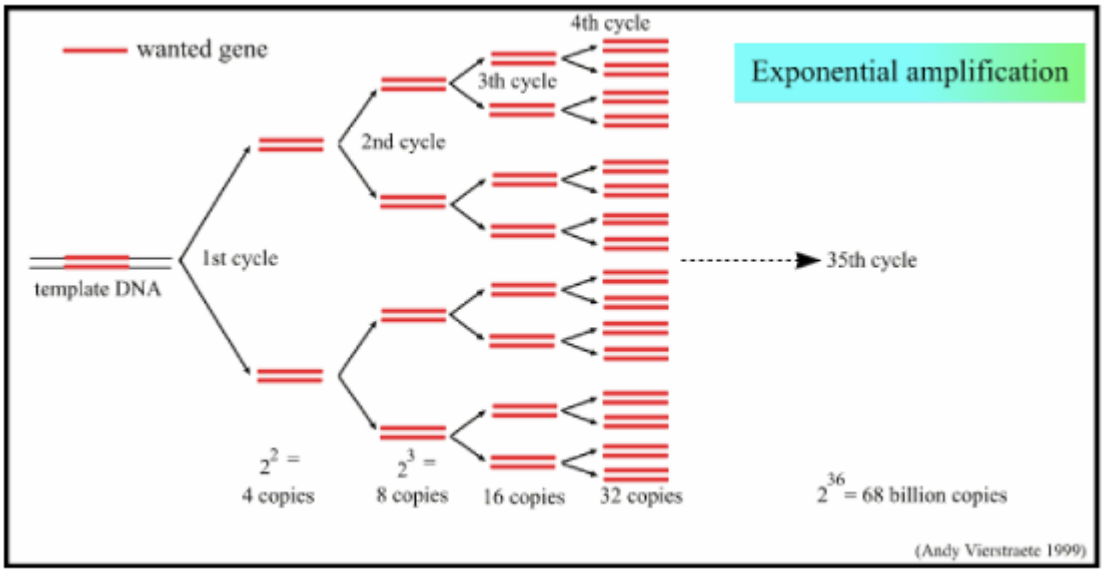


Figure 6 The exponential principle of PCR.

PCR with fungi specific primers, which ware listed in Table 1, targeted toward conserved sequences of the 5.8S and 28S ribosomal DNA (rDNA) and results in amplification of the species specific ITS 2 regions, which are variable in amplicon length (Figure 7).

Target	Primer	Sequences (5' – 3')	PCR product (bp)	References
ITS 2	ITS86 – F	gtgaatcatcgaatctttgaa	400	Turenne et al. 1999
	ITS4 – R	tcctccgcttattgatagc		White et al. 1990

Table 1 Primer used for amplification of the ITS2 region.

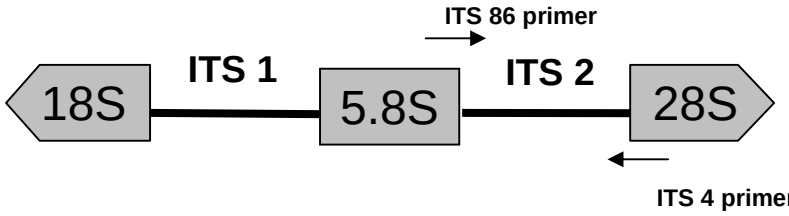


Figure 7 Schematic representation of the primer target areas used for amplification of the ITS2 region.

For amplification of faecal samples High Fidelity Enzyme PCR-mix (Fermentas) was used. Two PCRs were run to increase recovery of fungal rDNA. In the first PCR reaction mixture bovine serum albumin (Fermentas) was added to a final concentration of 400µg/ml. In the first PCR run 3µl DNA template were used. The second PCR reaction mix contains 3,5µl PCR-product of the first run and primer ITS86 with a GC-clamp for DGGE analysis. The other components of the reaction mixture are shown in Table 2. The lack of volume to 50µl was filled with nuclease free water.

Reagent	Quantity for 50**µl**	Final concentration
High Fidelity Enzyme Mix	0,25	1,25u/µl
10X High Fidelity PCR Buffer	5µl	1X
2mM dNTP mix	5µl	0,2mM of each
Primer ITS86 (50pmol/µl)	0,375µl	0,375mM
Primer ITS4 (50pmol/µl)	0,375µl	0,375mM
$MgCl_2$ (25mM)	6µl	3mM

Table 2 Composition of PCR mixture.

The PCR was performed at 95°C for 7 min, 30 respectively 25 cycles of 50 s at 95°C, 40 s at 55°C and 1 min at 72°C, followed by a final extension of 5 min at 72°C.
The fragment length of the PCR products was checked by gel electrophoresis on 2% agarose gel and visualized under UV-light after staining with GelRed.

Agarose gel electrophoresis makes it is possible to separate DNA and RNA fragments by lengths. So it is possible to estimate the size of the PCR product of the template. DNA is negatively charged because of its phosphate groups. Therefore nucleic acid molecules move to the anode in an electric field. The migration rate depends on the length of the DNA fragment, concentration of the agarose gel and the strength of the electric field.

6.5 Diversity measurement – PCR-DGGE

Denaturing gradient gel electrophoresis (DGGE) is a genetic fingerprinting technique and a form of electrophoresis which basis is the GC-content of the template. A similar method is TGGE (temperature gradient gel electrophoresis) where nucleic acids are separated in a temperature gradient.
The partially melted double-stranded DNA fragments are separated in polyacrylamide gels containing a linear gradient of DNA denaturants (urea and formamide). The leading thought of DGGE is that molecules have a different melting behaviour, because of different sequences and so they will stop migrating at different positions in the gel (Muyzer 1999).

Type strains (fungi and bacteria), knowing to be a part of the human gastrointestinal and two fungal clones, were used to create a DGGE standard lane (shown in Figure 8). The type strains were *C. albicans* ATCC 90028, *Clostridium leptum* and *Bifidobacterium longum* DSM 20219.

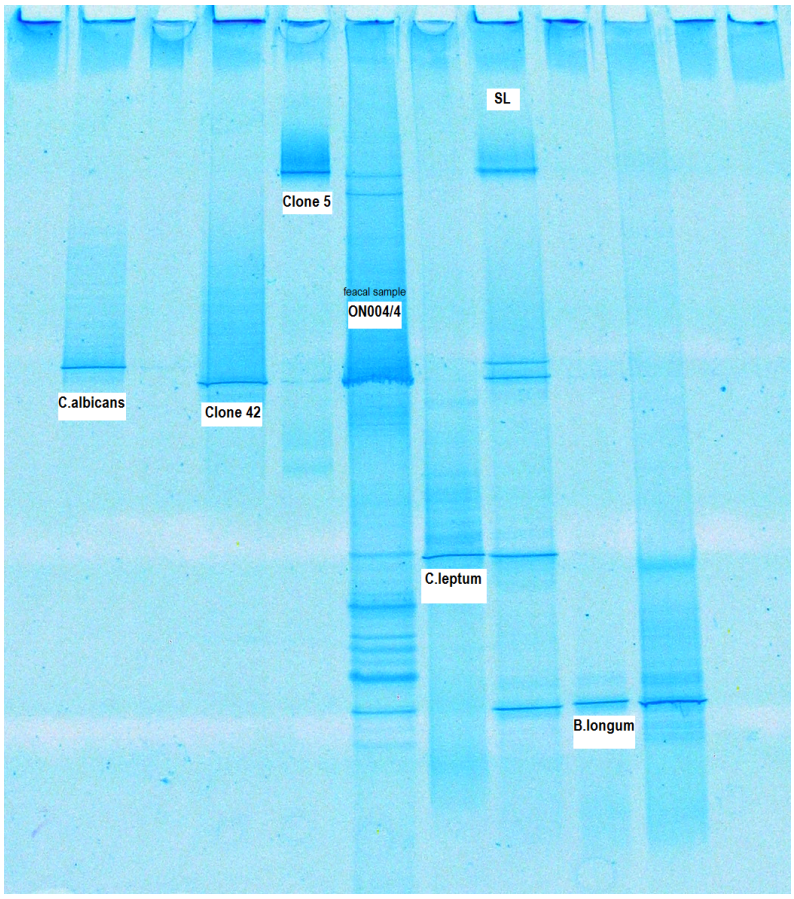


Figure 8 DGGE standard lane.

For DGGE analysis, PCR was performed in 2 x 50 µl reaction volume with primer ITS86 plus GC-clamp at the 5' end and ITS4. Fragment length was checked on a 2% agarose gel and visualized under UV light after Gelred staining and precipitated over night at –20°C.

PCR products were separated by polyacrylamid gels with a denaturing gradient of 18-50% using a gradientmixer (Hoefer SG 30) and a peristaltic pump. Electrophoresis was performed 6h at 150V and 60°C. The standard lane markers were loaded in triplicate on each gel to adjust gradient-variations within one gel.

6.6 Cloning

Cloning is the insertion of DNA in a vector and subsequent contribution in an adequate host cell.

Four steps are needed cloning a DNA fragment:

1. Fragmentation of plasmid with restriction enzymes – the plasmid is linearized.
2. Ligation: enzyme catalyzed linkage of two DNA or RNA segments (plasmid and DNA of interest); DNA and plasmid are incubated together with a ligase and so the ends get connected with each other.
3. Transformation: the ligation product is transformed in bacteria (E.coli) for multiplication and plated on selective agar in presence of X-gal.
4. Blue-white screening: it is the control if ligation was successful. White colonies imply that ligation was successful, blue colonies stand for the opposite.

To identify some members of fungi in the gastrointestinal tract and to check primer specificity a clone library was constructed. Therefore PCR-products amplified with ITS86 and ITS4 were inserted into a p-GEM Easy Vector (Promega) following the manufacturer's instructions. Several clones were picked and amplified with the primer pair T7/SP6 (Promega). Fragment length was checked on a 2% agarose gel; the PCR products were purified and finally sequenced by Confidence DNA-Analysen GmbH (Vienna). After nucleotide sequences were corrected for vector and primer with CodonCodeAligner (www.codoncode.com) the sequences were compared to previously published sequences in BLAST (www.ncbi.nlm.nih.gov/BLAST).

6.7 Semi-Quantitative measurement – SybreGreen qPCR

The Real-Time, also called quantitative real-time PCR (qPCR) is based on the principle of conventional polymerase chain reaction, but also allows the quantification of the obtained DNA template. Besides every phase of the reaction is continuous visualized on the computer monitor and compared with traditional end-point PCR it is not necessary to control the amplification fragment on agarose gel.
Fluorescent dyes (e.g. SybrGreen, ethidiumbromid) that intercalate with double stranded DNA fragment are often used in detection of products in qPCR. The increase of the DNA target correlates with the increase of fluorescence, which is measured at

the end of the elongation, from cycle to cycle. A disadvantage of this method is the low specificity. This can be offset by analyzing the DNA fragments in a melt curve after PCR amplification. In the melt curve the temperature is increased step by step until the PCR products melt. Here the DNA separates, the fluorescent dye is released and a decrease of fluorescence will be registered.

The SybrGreen assay was carried out in a Rotorgene 3000 (Corbett Life Sience). Each reaction was done in duplicate in a volume of 10 µl containing 5 µl SybrGreen SensiMix DNA Kit (Quantance), 1 µl of each primer and 2 µl DNA (20ng).
Amplification programs included an initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation step at 95 °C for 20 s, primer annealing at 56 °C for 25 s and extension at 72 °C for 30 s.
We used tenfold serial DNA dilutions of type strains *C. albicans* ATCC 90028 to construct standard curves for comparison of PCR reaction efficiencies among different experiments. DNA of *C.albicans* was quantified using the nanodrop method and DNA copies/µl were calculated through mean G+C content of the strain. All samples were measured in double approach and the average was used for calculation.

6.8 Statistical analysis

Statistical evaluation of differences between groups (chemotherapy and control, geriatrics and young omnivores) and changes within the chemotherapy group (all time points during chemotherapy, time points after chemotherapy or out of chemotherapy) was carried out using the OriginPro version 8 (OriginLab, Northampton, MA). For two group comparisons of independent ordinal and interval values the twosample - T-test and the non parametric Mann-Whitney-U-test were used. For the analysis of related data the paired-sample-T-test or the non paremetric Wilcoxon-signed-ranked-test were used. Pearson correlation was applied for comparison of fungal and bacterial numbers in the different groups of chemotherapy.

Food frequency data were analyzed based on Chi-square approximation. P values below 0.05 were considered statistically significant.
DGGE fingerprints were compared using GelComparII (www.applied-maths.com).

7 RESULTS

7.1 Dietary aspects

7.1.1 Geratrics and young omnivores

Analysis of the participants' dietary habits indicated similar consumption patterns of liquids, alcohol, fruits, grains and milk products in both groups. Omnivores stated significantly less frequent (Chi_2 Test; $p<0.04$) consumption of meat than elderly participants and regular consumption of whole grain products several times a week. The institutionalized elderly of this study did not consume any whole grain products at all but received supplements with soluble fiber (Benfiber®, Novartis).

7.1.2 Chemotherapy patients and controls

Dietary habits of the participants were evaluated using a food frequency questionnaire. All oncology patients and healthy controls were omnivores and showed similar eating habits concerning liquids, alcohol, fruits, vegetables, grains and milk products. Healthy controls stated more frequent consumption of fruits, whole grain products and alcohol several times a week compared to patients receiving chemotherapy (not significant).

7.2 qPCR quantification

7.2.1 Omnivores and geriatrics

Table 3 shows results from the real time PCR quantification in absolute numbers of fungi, including mean values and standard deviations for omnivores and geriatrics. Figure 9 and Figure 10 illustrate the comparison of the two groups. The absolute numbers are related to 2µl of faeces extract.

Geriatrics	copies/2ul
8g	2.773,5
10g	6.552,0
12g	60.381,5
13g	38.898,5
14g	1.582,7
15g	47.045,7
25g	44.792,1
26g	319.902,4
27g	222.152,5
29g	27.621,1
30g	4.308,9
31g	440,6
37g	294.326,7
44g	2.462,9
mean value	76660,1
standard deviation	113090,3

Omnivores	copies/2ul
3m	769,6
4m	38.167,4
11m	1.108,2
17m	49.047,2
18m	681,1
19m	755,7
33m	4.757,3
34m	17.720,6
35m	30.294,9
39m	1.810,5
40m	547,4
41m	34.116,5
42m	103.568,8
43m	150.624,1
mean value	30997,8
standard deviation	44935,2

Table 3 Results from qPCR in absolut numbers.

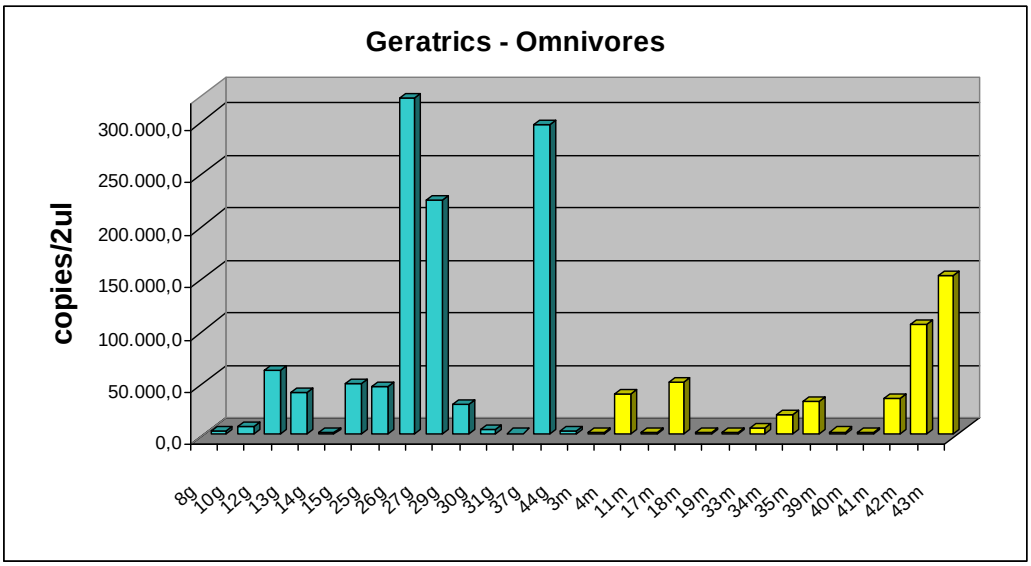


Figure 9 Fungi in faecal samples of geriatrics and young omnivores.

= geriatrics; = young omnivores

Statistical analyses show no significant difference in the amount of fungi between geriatrics and omnivores. But Figure 10 shows that the group of omnivores is more consistent because the geriatric group has three (27g, 29g, 44g) outliers with extreme high numbers of fungi.

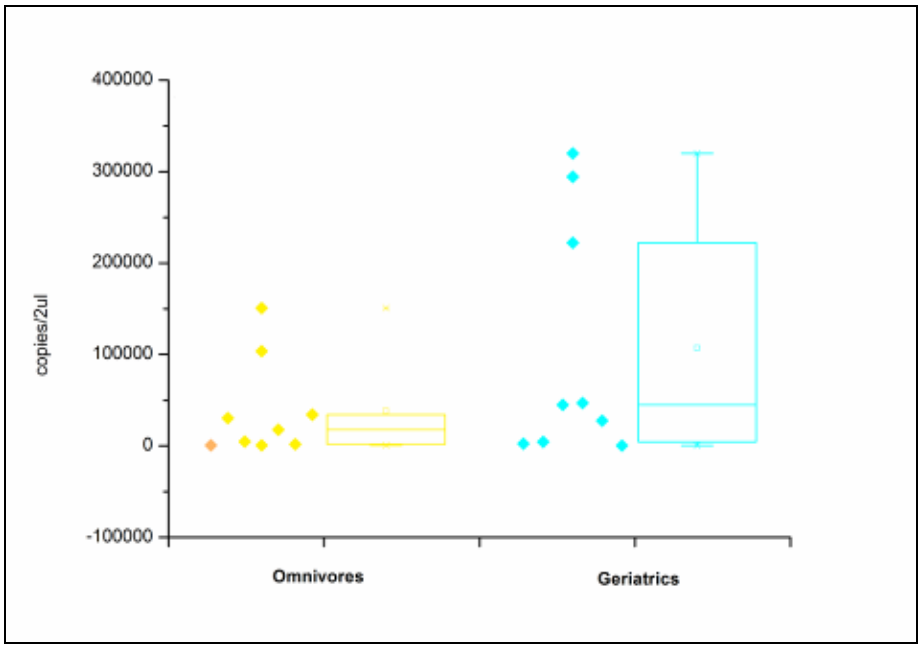


Figure 10 Boxplots: comparison number of fungi in young omnivores and geriatrics.

= young omnivores; = geriatrics

7.2.2 Oncology patients and healthy controls

Table 4 shows results from the real time PCR quantification in absolute numbers of fungi, related to 2µl of faeces extract, in oncology patients and Table 5 those from healthy controls.

Figure 11 and Figure 12 illustrate the two groups separate; Figure 14 shows all groups in one graphic. In comparison Table 6 and Figure 15 show the results of all bacteria analysis in the oncology group, part of a recent study done by Zwielehner et al. (Zwielehner J, Lassl C et al. 2010; under review).

	before Chemo		Chemo		after Chemo	recovery	
ON001	15.321,14	24.902,61	14.948,61	8.798,42	x	x	
ON002		87.962,35		149.203,93	699.758,13	293.603,57	
ON004		4.298,52		x	772.162,73	63.430,05	90.748,15
ON005	368,20	9.503,88		x	6.264,40	27.165,45	
ON006		14.688,44		x	181.082,57	140.049,91	3.527,66
ON007		21.756,32	205738,65	16702,15	30.269,97	x	
ON009		1.613,24		2972,29	1.332,66	148,80	
ON010		3.206,25	80524,29	2554,34	4.538,73	x	
ON011		723,31		x	980,99	326,99	2.672,71
ON012		x		x	2.674,41	2263,1	1876,76
ON013		x		3815,85	1.974,37	1.803,58	3461,91
ON014	398,63	598,66		x	175,78	x	
ON015		12.605,61		219,62	4.469,17	14.529,03	
ON016		398,88		208,74	6.485,06	942,47	
ON017		x		3098,57	966,92	8.646,53	112,5

Table 4 Absolute numbers of fungi, related to 2µl of faeces, in oncology patients. X stands for no data available.

	TP 1	TP 2	TP 3
1	1927,78	777,52	757,17
4	1395,51	4407,01	5688,07
16	51828,52	12928,05	7805,68
17	3976,75	45885,69	57865,95
18	46267,42	67116,37	24528,079
24	40557,02	3363,93	1789,27
25	1226,32	5977,96	14895,67

Table 5 Absolute numbers of fungi, related to 2µl of faeces extract, of the three time points (TP) in healthy individuals.

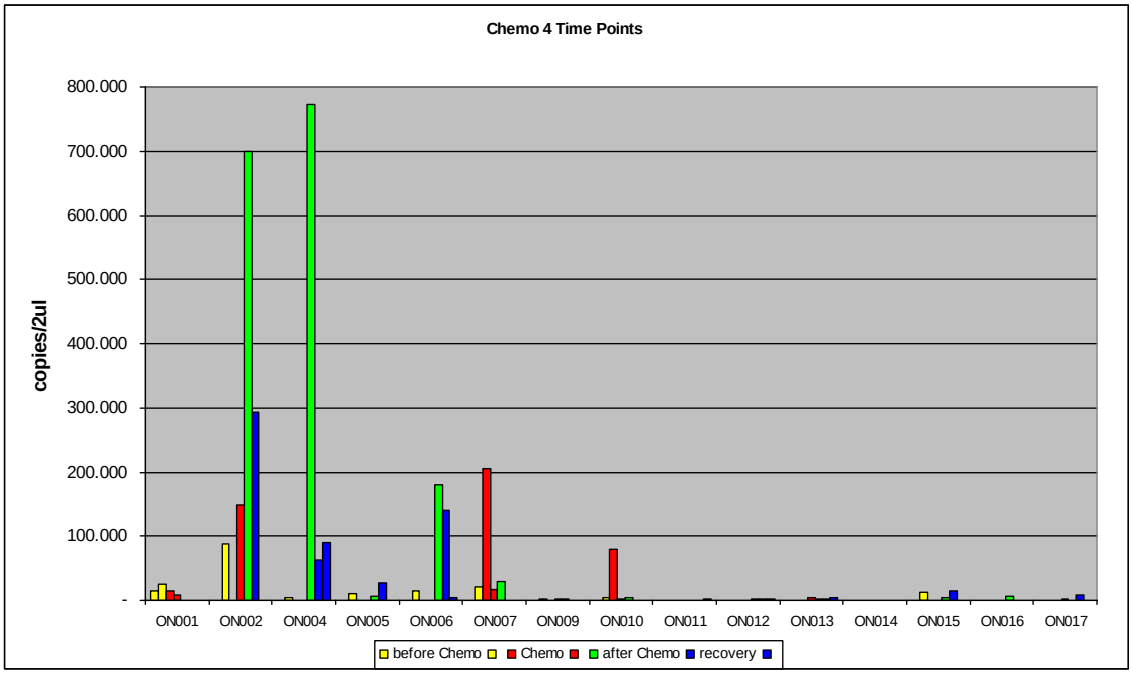


Figure 11 Absolute numbers of fungi in faecal samples in the course of chemotherapy.

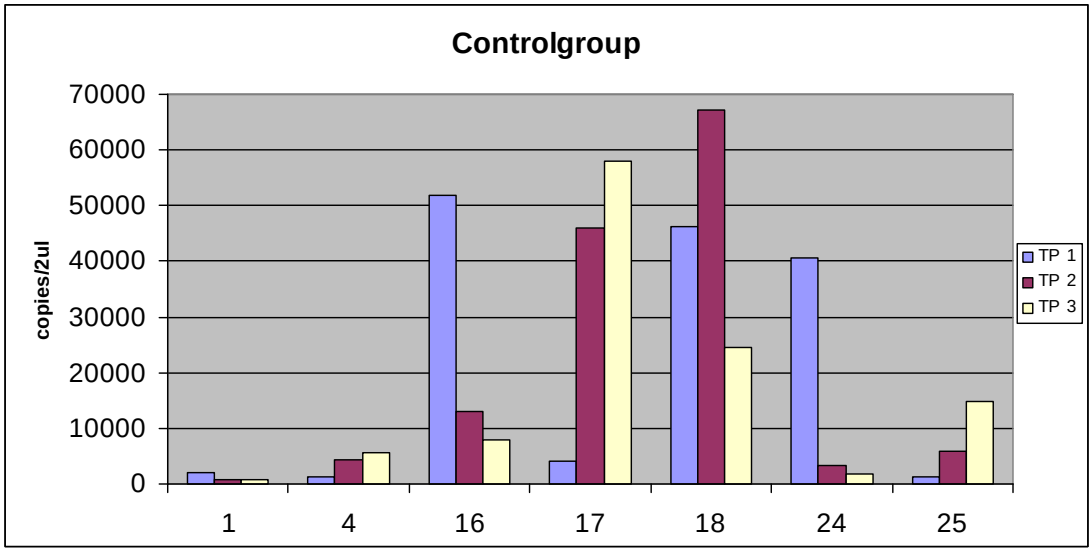


Figure 12 Absolute numbers of fungi in faecal samples of healthy individuals at three timepoints (TP).

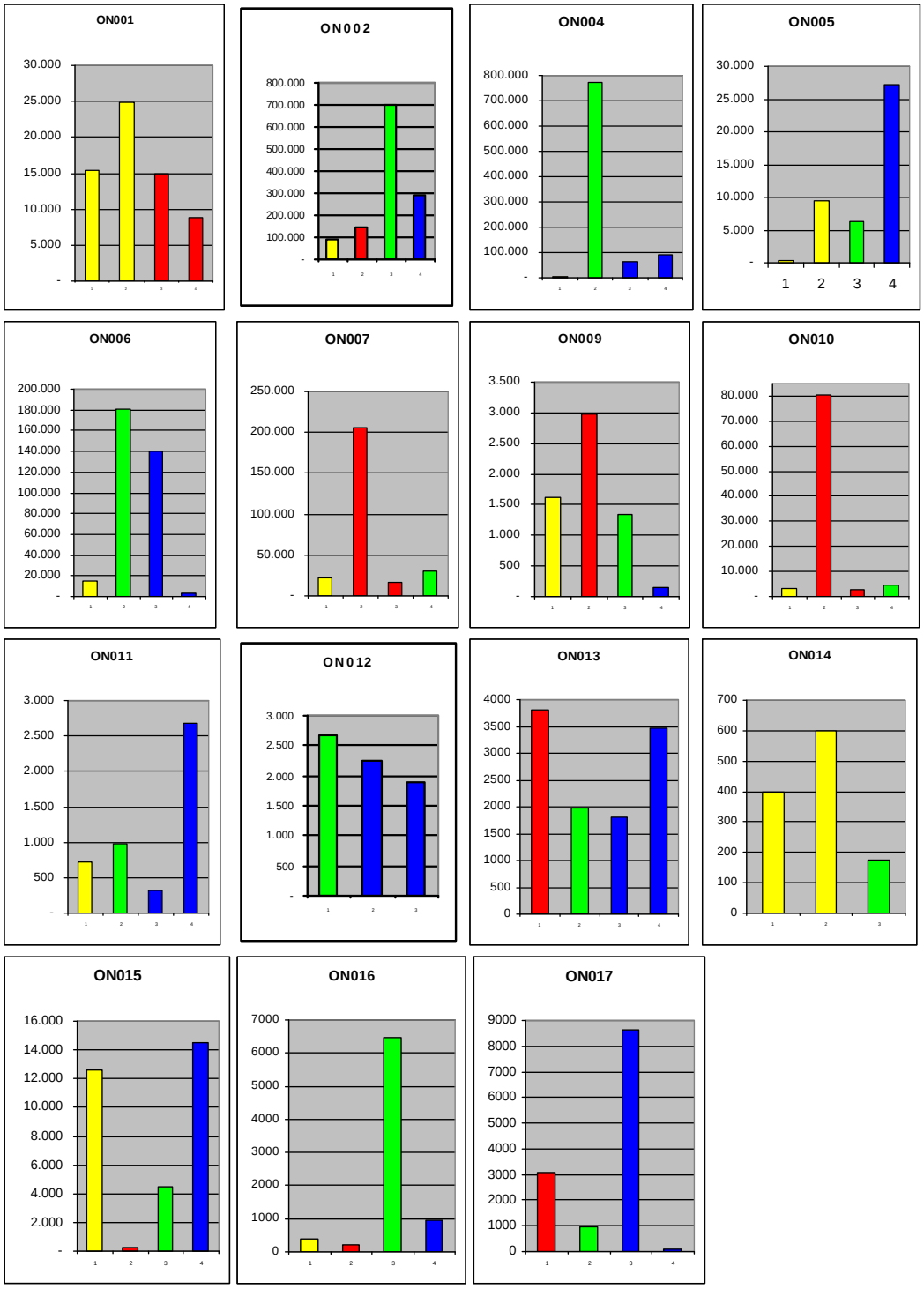


Figure 13 Absolute numbers of fungi in faeces related to 2µl in oncology patients, shown in detail.

= before chemotherapy; = chemotherapy;

= after chemotherapy; = recovery

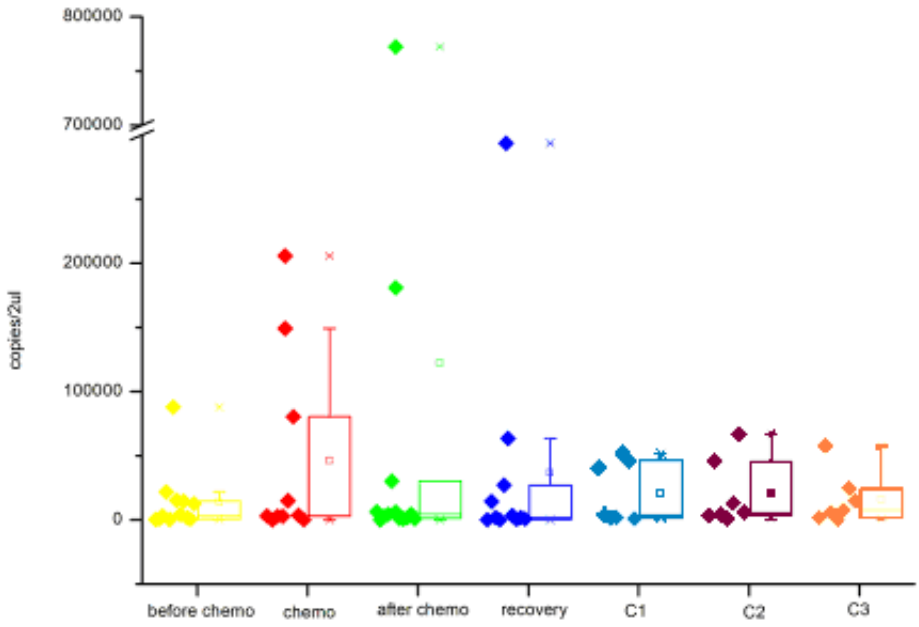


Figure 14 Comparison of chemotherapy group with control group (C).

Statistically there are no significant results concerning the numbers of fungi in both groups; neither in the course of chemotherapy nor in comparison to control group. There are also no significant changes in the number of fungi in the three time points of healthy persons.

Two outliers with high numbers of fungi are found in the oncology group (ON002, ON004). In most cases there is one higher data in course of the four time points of chemotherapy (detailed results are shown in Figure 13). However no trend, if these high numbers of fungi are in a certain time point of chemotherapy (before chemo, chemo, after chemo, recovery), is noticeable.

Furthermore also in the control group fluctuations among the healthy participants and within the three time points are observed.

Comparison of absolute numbers of bacteria and fungi during the course of chemotherapy results in no significant correlation.

	before Chemo		Chemo		after Chemo	recovery	
ON001	1798805,9	372479,3	1183463,8	4900917,4			
ON002		20212193,8		23903696,4	3129907,8	42134076,5	
ON004		10176085,8		X	2041417,9	9397403,8	17611326,1
ON005	58606024,9	77914671,2		X	47867203,6	45546066,2	
ON006		4254777,8		X	19663552,6	5817626,4	11491330,2
ON007		32882071,4	14010666,5	31475072,5	210972,4	X	
ON009		60928403,7		79557247,6	18692507,8	22422312,3	
ON010		13477917,7	18520677,8	52591368,1	21962667,1	X	
ON011		44187524,4		X	9505774,7	4652268,1	30489412,5
ON012		X		X	1101929,6	6715972,2	2775027,9
ON013		X		1417002,1	1743935,2	9419678,5	2220094,3
ON014	2812806,5	1898857,8		X	216491,6	X	
ON015		819799,6		515193,9	809754,5	1534925,2	
ON016		1378646,1		1343463,9	1152274,8	1229934,9	
ON017		X		1221647,5	661624,5	2197256,2	1698079,8

Table 6 Absolute numbers of bacteria, related to 2µl of faeces, in oncology patients. X stands for no data available.

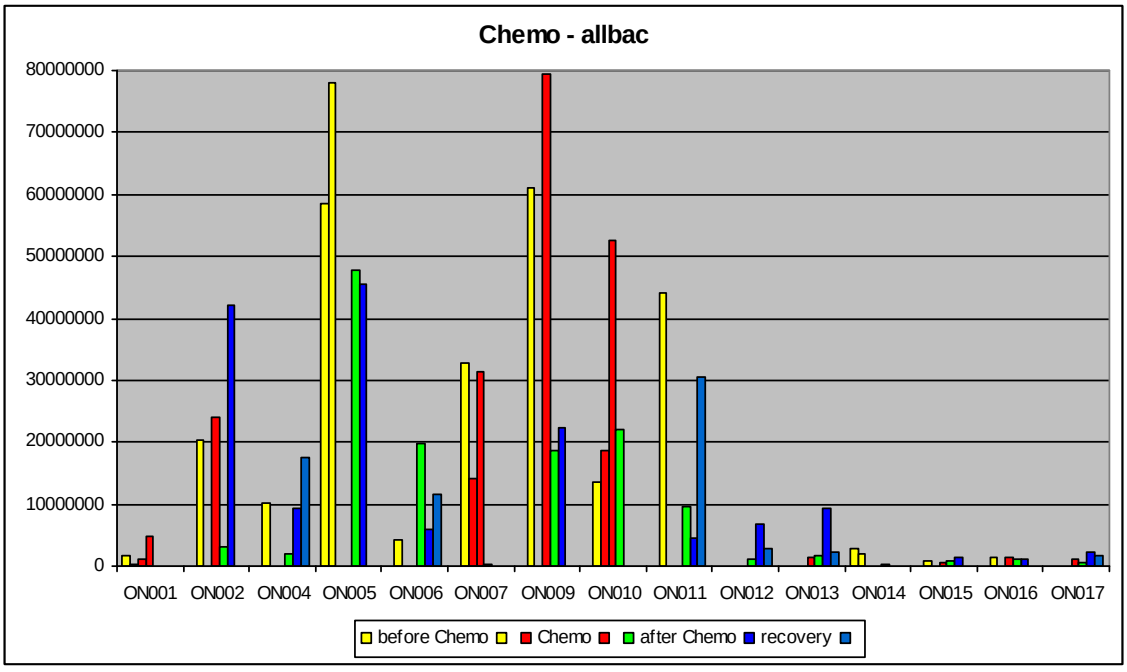
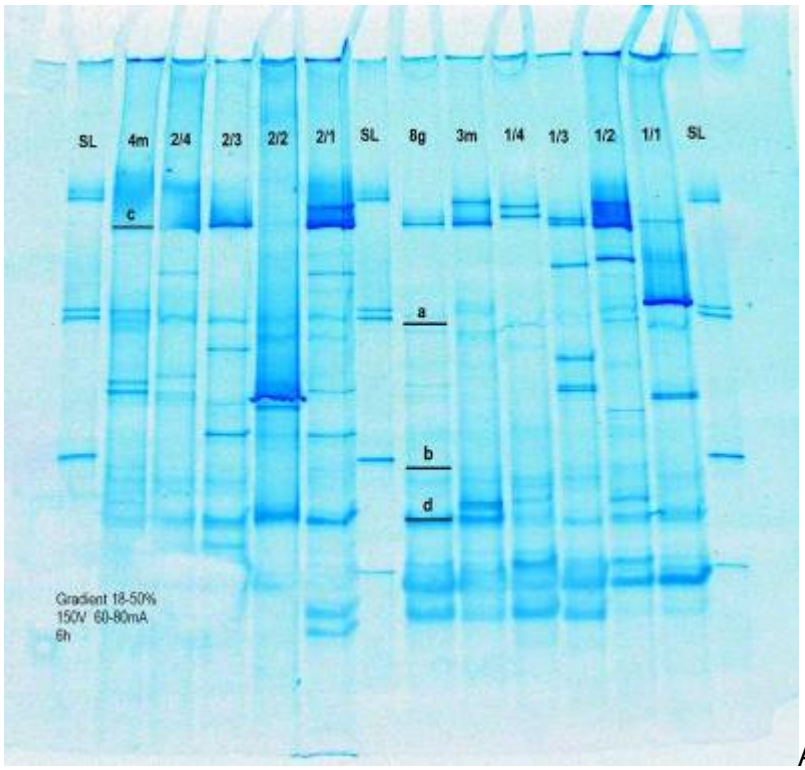
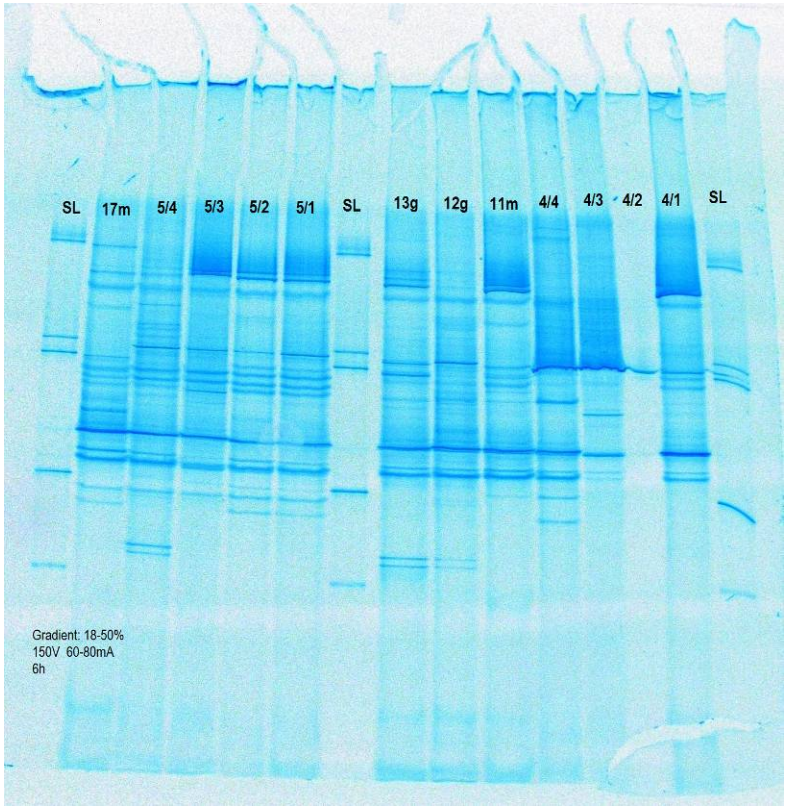


Figure 15 Absolute numbers (copies/2µl) of all bacteria in course of chemotherapy.

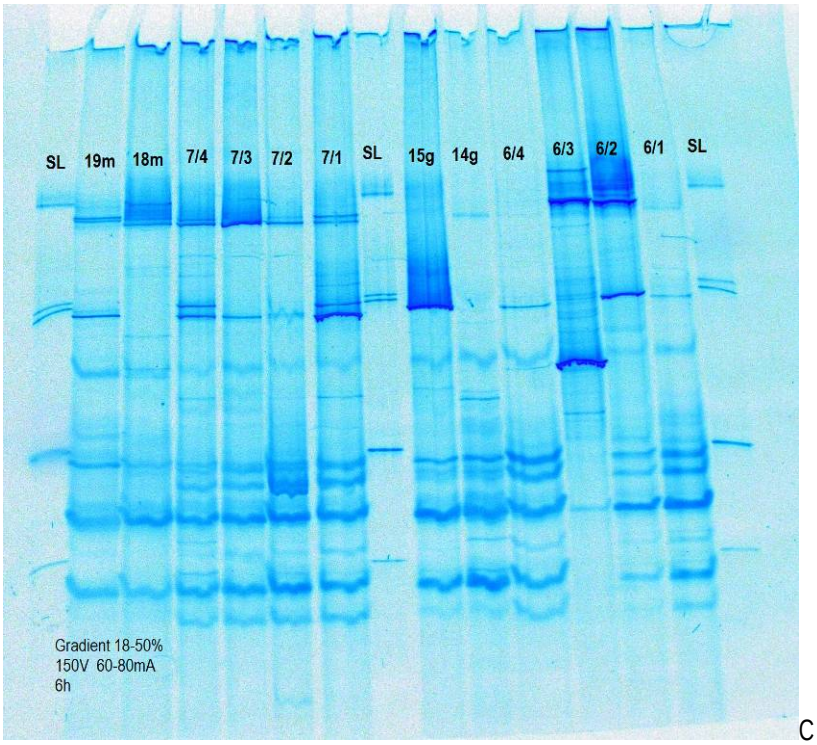
7.3 PCR-DGGE fingerprinting



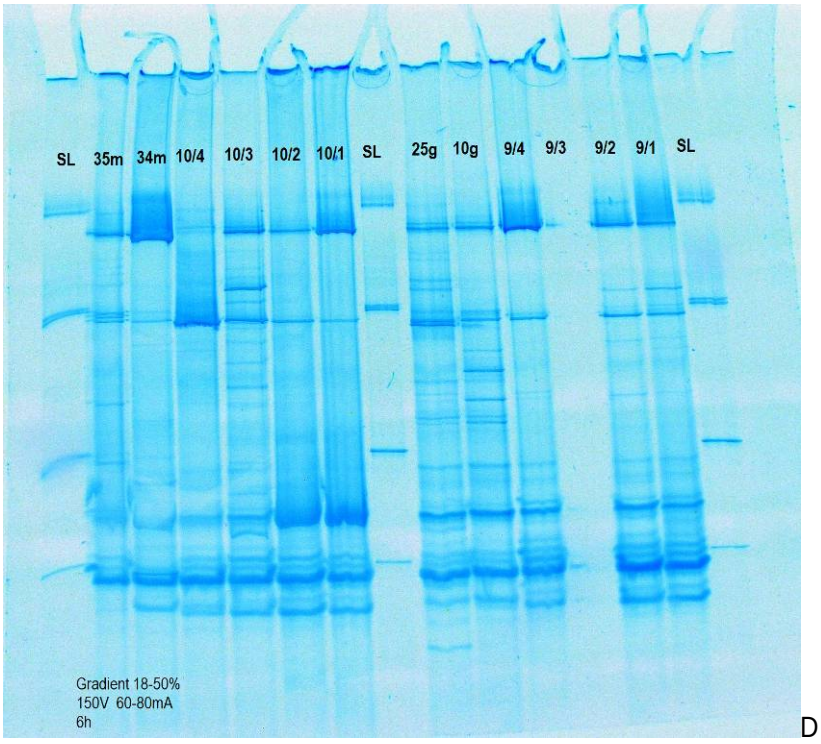
A



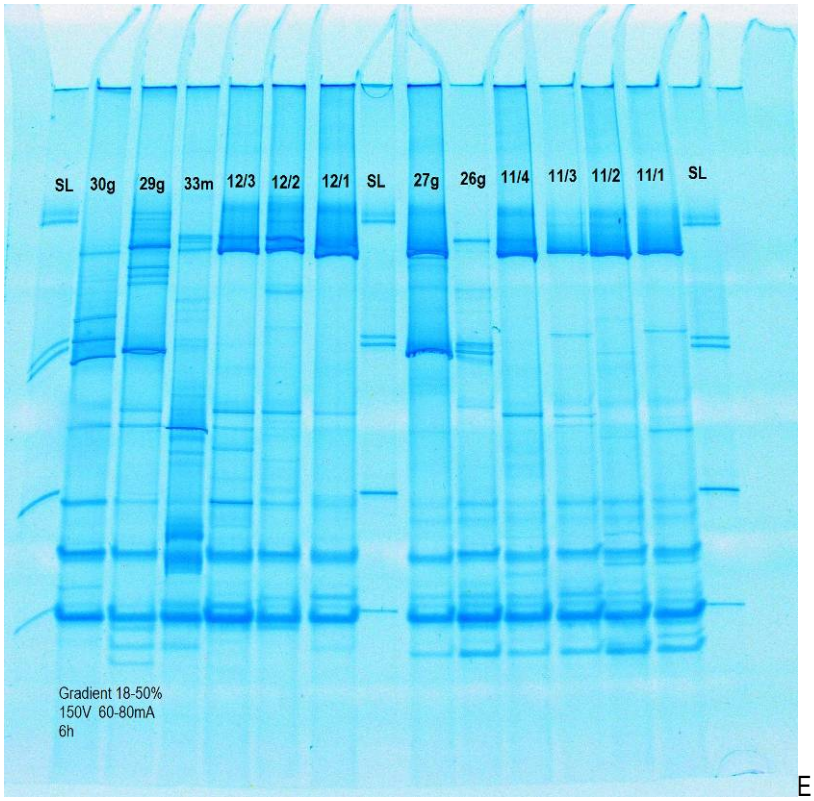
B


SL 19m 18m 7/4 7/3 7/2 7/1 SL 15g 14g 6/4 6/3 6/2 6/1 SL
Gradient 18-50%
150V 60-80mA
6h

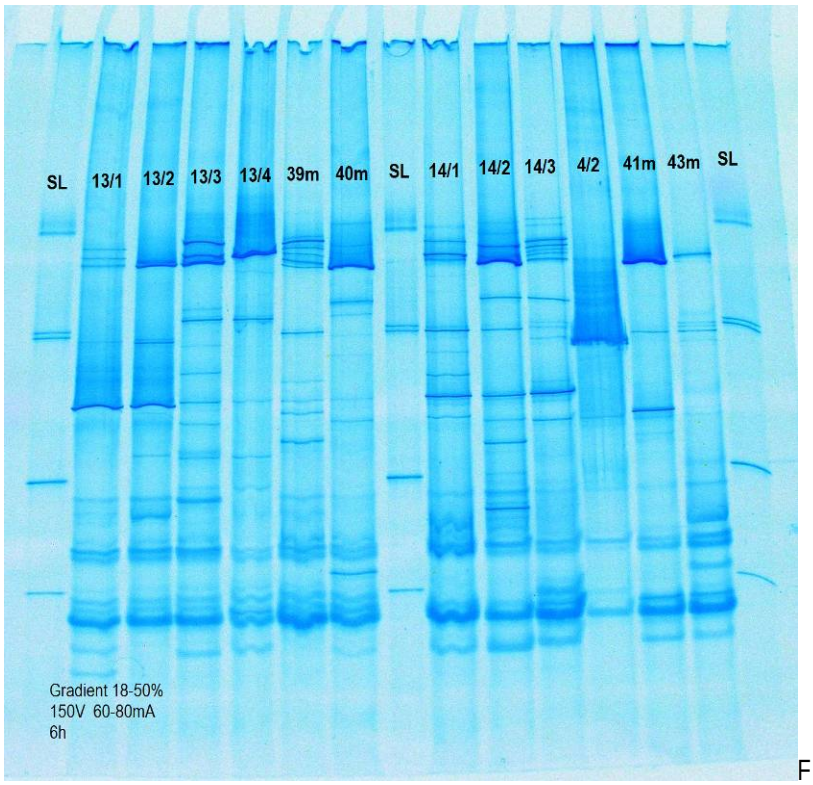
C


SL 35m 34m 10/4 10/3 10/2 10/1 SL 25g 10g 9/4 9/3 9/2 9/1 SL
Gradient 18-50%
150V 60-80mA
6h

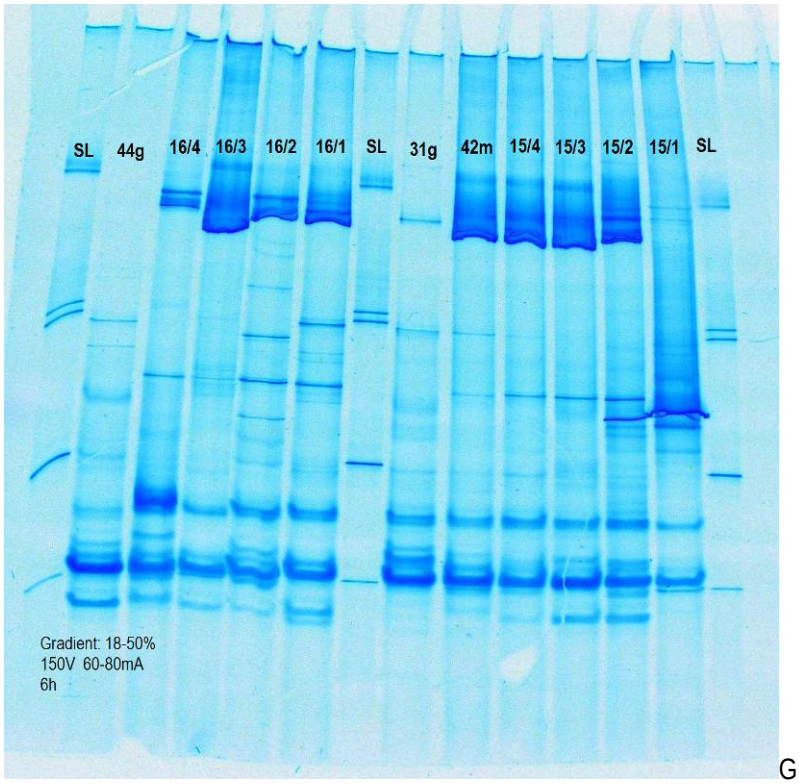
D


SL
30g
29g
33m
12/3
12/2
12/1
SL
27g
26g
11/4
11/3
11/2
11/1
SL
Gradient 18-50%
150V 60-80mA
6h

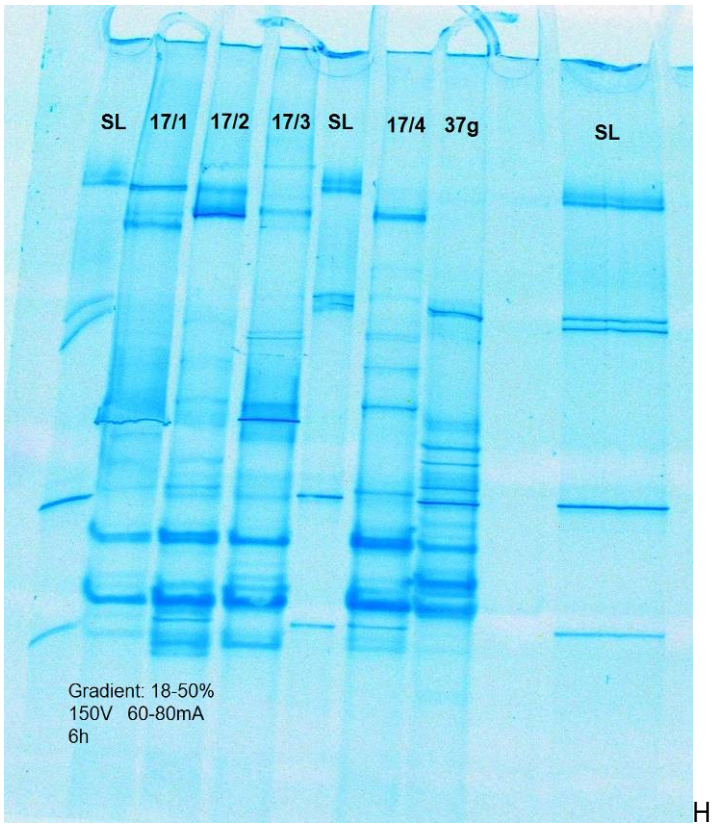
E


SL
13/1
13/2
13/3
13/4
39m
40m
SL
14/1
14/2
14/3
4/2
41m
43m
SL
Gradient 18-50%
150V 60-80mA
6h

F



G



H

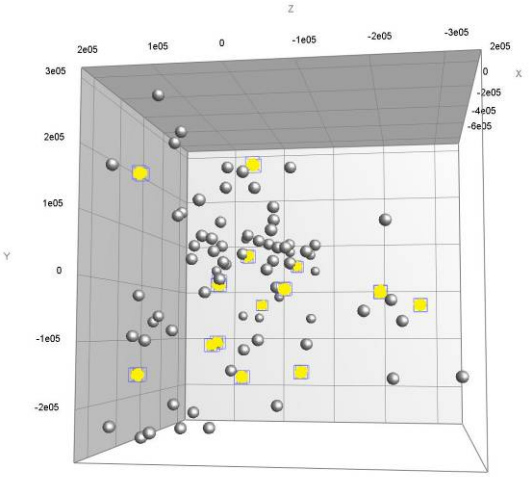
Figure 16 DGGE fingerprinting of fungi

Figure 16 shows the PCR-DGGE fingerprints of all samples, which have been analyzed with Gelcompar II.
The mean number of bands were 11,2±4,8 for omnivores and 11,7±5 bands for geriatrics. Furthermore 11,7±3,3 Bands in the group 'before chemotherapy', 10,4±4 during chemo, 9,1±4,8 after chemotherapy and 12,1±4,7 bands in the phase of recovery.
The combination of the bands a and b occur in all geriatric samples, except for participant 13g, whose fingerprinting does not consist of band b. Interestingly also some samples of oncology patients show this combination, just in the phase before chemotherapy and rarely after chemotherapy or in recovery. Only patient ON007 and ON013 have these two bands also during chemotherapy.
In all samples of young omnivores and also in some of the other groups, band c can be found. Band d appears in all analyzed samples except for those shown in gel B.
All in all the fingerprints of the various participants are very divers. During chemotherapy cycles it can be observed that several bands are found in all time points of one individual and some occur only at one time point.

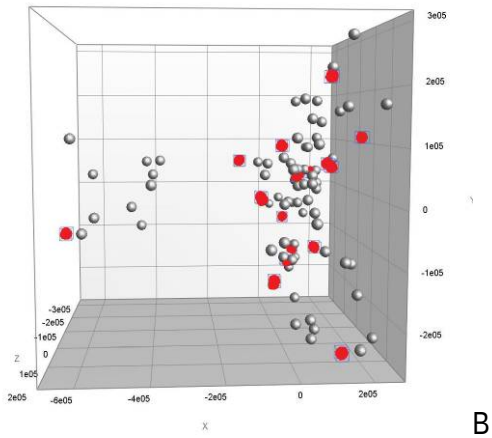
7.3.1 PCA-Analysis

The diverse dataset of fungi in different groups were analysed with principal component analysis (PCA) which extracts underlying components within the dataset separating samples according to their variance.
Figure 17 A, B, and C display PCA of fungi at different time points of chemotherapy. Graph A shows oncology patients before chemotherapy (yellow), graph B during chemotherapy (red) and graph C the phase of recovery (blue).
Figure 18 illustrates PCA analysis of fungi in all groups analyzed in this thesis, each group in a different colour.

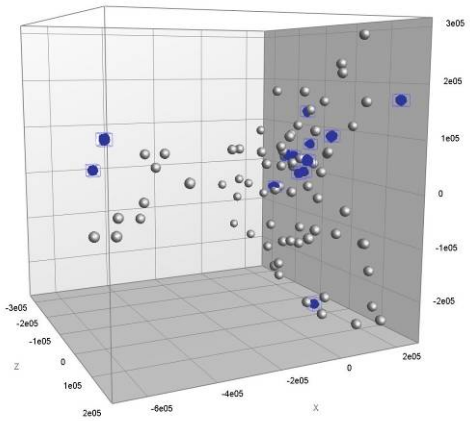
In oncology patients no clustering of the different groups is noticed. Figure 18 reveals a separation of the geriatric group (violet).



A



B

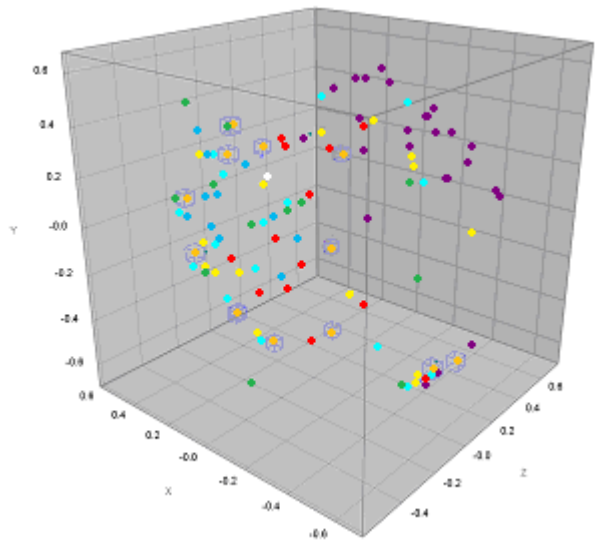


C

Figure 17
PCA of fungi DGGE fingerprints of the oncology group

= before chemo, = chemo
= recovery

Figure 18 PCA of all groups.



7.3.2 Cluster analysis

Cluster analysis of PCR-DGGE fingerprinting showed a tendency for clustering of elderly, but all the other groups could not be grouped (Figure 19).

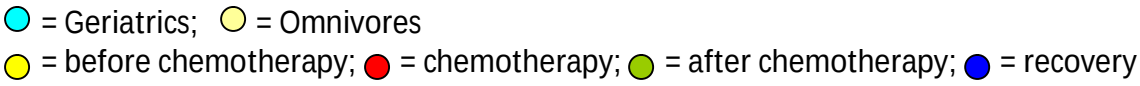
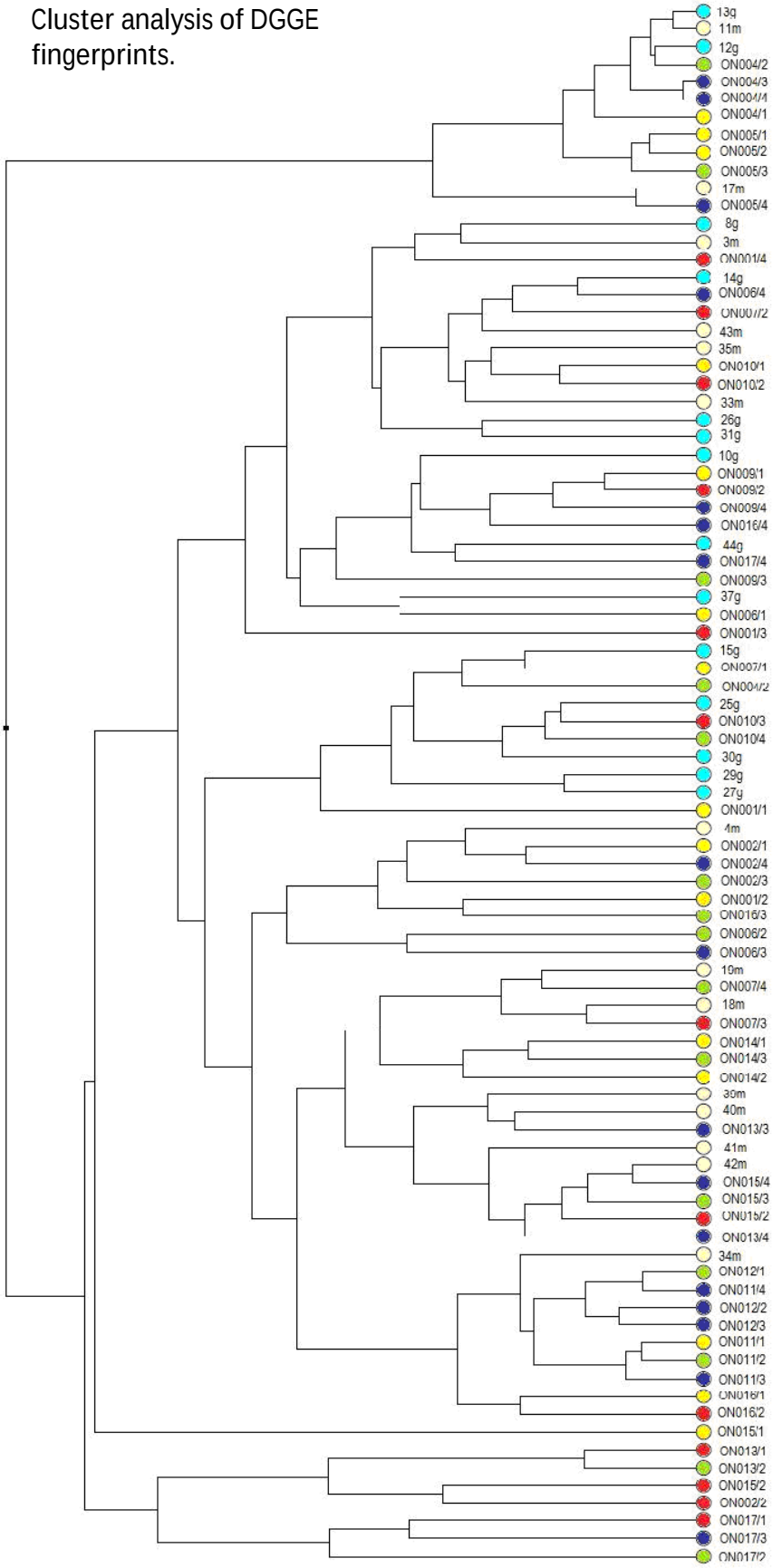


Figure 19
Cluster analysis of DGGE fingerprints.



7.4 Sequencing

Eight clones were sequenced and seven of them were identified as fungi. Clone ON14/2 17 was identified as Brassica oleracea.

Clones	Sequence name	Max. ident
ON006/2 5	Mucor fragilis	98%
ON006/2 4	Psathyrella calcarea voucher	97%
ON013/3 3	Candida tropicalis	97%
ON014/2 17	Sordaria alcina	100%
ON014/2 18	Brassica oleraceae	98%
39m 5	Saccharomyces cerevisiae	99%
39m 14	Penicillium digitatum	99%
29g 20	Candida glabrata	99%

Table 7 Identified clones

8 DISCUSSION

8.1 Study population

8.1.1 Oncology patients

Except for two patients, which had never received any cancer therapy before, all study participants had a longer history of chemotherapy. However they had different types of cancer and received different cancer treatment. Also the four samples of each participant were collected at distinct time points.

8.1.2 Geriatrics

The majority of institutionalized elderly were bed ridden and supplements with soluble fiber (Benfiber, Novartis) were administered to them. Perhaps the fungal microbiota was influenced by those two points. These circumstances and also the low number of study participants are maybe the explanation why no significant statistical differences in comparison to the group of young omnivores were detected.

8.2 METHODS

8.2.1 Faecal samples

Faecal samples were used in this study to examine the fungal microbiota in the intestine, because they are easily collected and need no ethical issue.
However you have to keep in mind that it is not clear to which degree the composition and function of the faecal microflora differ from mucosal microflora (Eckburg PB, Bik EM et al. 2005).
Moreover only few studies exist which analysis the fungal microbiota in the gastrointestinal tract using faecal samples. Therefore no statement about the representativeness of faecal samples concerning fungal microbiota in the intestine can be made.
Concerning the bacterial microbiota researchers suggest that faecal samples do represent the bacterial community only in the human colon and not in other parts of the gastrointestinal tract (Zoetendal EG, vonWright A et al. 2002; Zoetendal EG, Vaughan EE et al. 2006).
On the other side Van der Waaij et al. suggested that colonic bacteria are not in direct contact with the mucosa and found no significant difference between colonic biopsies and faeces (Van der Waaij LA, Harmsen HJ et al. 2005).

8.2.2 Culture - culture independent methods

In the past some studies analyzed the fungal microbiota of the human gastrointestinal tract with cultivation-dependant methods (Agirbasli H, Özcan SA et al. 2005), but these studies are encumbered by the fastidious nature of several fungal species (Ott SJ, Kühbacher T et al. 2008).
The first culture-independent analysis of fungal communities in the mammalian intestine was made by Scupham et al.. They analyzed the fungal population in the murine intestine. The results showed unexpected diversity and abundance of fungal species (Scupham AJ, Presley LL et al. 2006).

However, in recent years only a few studies were made analyzing the fungal microbiota in the human intestine with more sensitive and accurate molecular techniques like PCR-DGGE (Ott SJ, Kühbacher T et al. 2008; Scanlan PD and Marchesi JR 2008).
Scanlan et al. used culture-dependant and –independent methods for qualitative assessment of eukaryotic diversity in the human gut (Scanlan PD and Marchesi JR 2008). Chen et al. also compared those two methods in their work (Chen Y, Chen Z et al. 2010). Both groups highlighted the incongruence between cultivation and cultivation-independent analysis. Culture-dependent methods analyzing faeces detected mainly *Candida spp.,* whereas the results of culture-independent method were dominated by *Gleotinia/Paecilomyces* and *Galactomyces*.
Because only rare information about the fungal community in the human gastrointestinal tract on the basis of culture-independent methods is available, qPCR and PCR-DGGE with primers targeting the ITS2 region were used in this study.

8.2.3 PCR-DGGE

Nowadays PCR-DGGE is a well-established method in molecular biology for analyzing microbial communities and their diversity in different environments.
DGGE has also limitations like every method. However, apart from those, DGGE is a reliable, rapid and reproducible method to study the human microbiota.

8.2.4 qPCR

If it is accurate conducted, qPCR is a very sensitive, specific, efficient and rapid molecular method.
To assure great sensitivity of all PCR reactions stepwise dilutions of the standard were used to create a standard curve. Besides primer concentration and temperature profiles were optimized to get a good efficiency.

Maybe it would be better using more fungal strains for standards to imitate the human fungal microbiota in the gastrointestinal tract, because some species might be preferred for amplification.
In this study only *C. albicans* was used, because it is known to be a commensal in the human intestine and at the beginning of this study it was not clear which fungal species we would find in the faecal samples.
The comparison of several faecal samples is complicated, because they differ in their fiber, water and microorganism content. So we decided to standardize all samples by using 20ng of each as template.

8.2.5 Internal transcribed spacer region 2 – primer specificity

For this study the primer pair ITS86-ITS4 (White T, Bruns T et al. 1990; Turenne CY, Sanche SE et al. 1999), targeting ITS2 region was used. Turenne et al. tested the primers for their specificity and no amplification product was detected with template from human leukocytes, human whole blood, or human liver and also bacterial organisms *(Staphylococcus epidermidis, Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa,* and *Bacteroides fragilis*) were not targeted. Vancov and Keen also reported the fungal specificity of the primer set (Vancov T and B 2009). This good reputation concerning specificity was the reason why the primer set ITS86-ITS4 was used for analyses in this study.
Unfortunately one of the sequences from the clone library was identified as Brassica *oleracea*. This is the evidence that the primer set is not only fungal specific, but also amplifies plant rDNA. However, Bellemain et al. reported that all commonly used primers for amplifying fungi in environmental samples amplify plant ITS sequences (Bellemain E, Carlsen T et al. 2010).
Another point of discussion is which ITS region is more suitable for analysis of environmental samples. An in silico study demonstrated when using the ITS2 or the whole ITS region, ascomycetes will be targeted to a greater extend compared to basidiomycetes. Otherwise using primers amplifying ITS1 would tend to preferential amplification of ‘non-dikarya’ fungi. Therefore Bellemain et al. recommended the

parallel use of different primer pairs amplifying different parts of the ITS region (Bellemain E, Carlsen T et al. 2010).
For quantitative analysis of the fungal microbiota with qPCR the primer set ITS86-ITS4 was maybe not the best choice. The quantitative evaluation in this study was done in copies/2µl, but the fact that the rDNA copy number in fungi often vary was not considered (Maleszka R and GD 1990; Belkhiri A and GR 1996; Howlett BJ, Rolls BD et al. 1997). The copy number can be 10 to 100 times that of single-copy genes (Maleszka R and GD 1990; Maleszka R and GD 1993). The answer to this problem is for example primers which amplify the gene for actin. The gene for actin is highly conserved and a functionally essential element in eukaryotic cellular processes (Voigt K and J 2000). Hence the samples of this study should be also analyzed by primers amplifying the gene for actin and then results of both primer pairs should be compared.

8.3 RESULTS

8.3.1 Results Elderly

Zwielehner et al. investigated the bacterial microbiota of the geriatric patients and young omnivores in a previous study. They detected a significant less diversity in the PCR-DGGE the group of geriatrics and concluded that this observation depends either on lesser amount of bacterial DNA in these samples or on inadequate growing conditions in the gut of elderly (Zwielehner J, Liszt K et al. 2009).
The analysis of fungal microbiota in geriatric patients was conducted with the same samples mentioned above.

The core question of this study was if abundance and diversity of fungi also change with age.
We suggested that the number of fungi increases with age, because in elderly physiological changes in the gastrointestinal tract such as decreased acid secretion by gastric mucosa take place. This results in a shift of the composition of the gastrointestinal microbiota upon age (Bartosch S, Woodmansey EJ et al. 2005).

However this study shows no significant differences in absolute number of fungi. Unfortunately no comparable study with elderly was found in literature.
For instance Malani et al. investigated only oropharyngeal fungal colonization of elderly (Malani A, Psarros G et al. 2010) and other researches concentrate on already existing fungal infections in patients, but none of them analysed the 'basic gastrointestinal fungal microbiota' of elderly.

8.3.2 Results – chemotherapy

Zwielehner et al. investigated the bacterial microbiota and their alteration over the course of chemotherapy. They detected for example a recovery, in some patients even a 'rebound effect' of bacterial microbiota a few days after chemotherapeutic cycles (Zwielehner J, Lassl C et al. 2010).
In this study the same faecal samples were used for analysis. The aim was to figure out if the fungal microbiota of oncology patients changes during chemotherapy and if there is some correlation between the bacterial and the fungal microbiota.
Results show no significant change during chemotherapy cycles and also no significant difference compared to the control group.
We expected an increase in fungal number when the amount of bacterial decreased, but no correlation was detected.
Our findings do not go confirm with results from Nyhlen et al.. They reported a significant increase of yeast in patients with leukaemia, but they used culture-dependent techniques (Nyhlen A, Ljungberg B et al. 2002).
Other studies concentrated only on fungal infections and the risk of those in cancer patients (Marr KA, Seidel K et al. 2000; Hammond SP, Marty FM et al. 2010). However no comparable literature concerning gastrointestinal fungal microbiota in course of chemotherapy in patients who did not suffer from any mycoses was found.
Zwolinska-Wcislo et al. hypothesized that fungal overgrowth is the result of any bacterial imbalance (Zwolinska-Wcislo M, Brzozowski T et al. 2006). Zwielehner et at. reported such an bacterial imbalance of the gastrointestinal microbiota during

chemotherapy cycles (Zwielehner J, Lassl C et al. 2010; under review), but we could not verify fungal overgrowth in those patients compared with controls.
Maybe the cause of no significant results is the low number of participants, because some of the patients had very high amounts of fungi during their cancer therapy.

8.3.3 Results DGGE fingerprinting

Scanlan et al. reported that the fungal diversity in the human gastrointestinal tract is low, because their PCR – DGGE gels show only one to three bands per participant (Scanlan PD and Marchesi JR 2008). Therefore we expected that our DGGE fingerprints would also show only few bands per individual, but finally a greater fungal diversity was found. The various DGGE gels display approximately 10 bands per analyzed individual.
Furthermore two bands (band a and b in Figure 16) seem to be specific for geriatrics, because the combination of these bands are found in all participants except one in this group. Also some oncology patients show this combination, especially before chemotherapy. Most of the participants of the chemotherapy group have an older age; so it can be suggested that those two bands represent a fungal species which occurs specifically in elderly.
It seems that few fungal species constitute the basis of the human fungal microbiota in the gastrointestinal tract (e.g. band d in Figure 16) and that each individual has additionally its specific composition of fungal commensals.

8.3.4 Results clone-library – sequencing

The sequenced clones were identified with BLAST (www.ncbi.nlm.nih.gov/BLAST). However, a major problem in taxonomic classification of fungal rDNA sequences is the lack of substantial databases and comparative sequences from intestinal habitats. Furthermore also the inconsistencies in the systematic of fungal taxonomy is problematic (Cappa F and PS 2001; Gomes NC, Fagbola O et al. 2003; Hunt J, Boddy L et al. 2004).

Moreover Nilsson et al. suggested that about 20% of the fungal DNA sequences from sequence databases are identified to incorrect species (Nilsson R, Ryberg M et al. 2006).

8.3.4.1 Candida glabrata

One clone from the clone library of a geriatric person was identified as *Candida glabrata* with a maximal identity of 99%.

C. glabrata is in addition to *C. albicans* an important cause of mucosal and bloodstream infections (Pfaller 1996; Vasquez J, Dembry LM et al. 1998).

In the past *C. glabrata* was considered as a non-pathogenic commensal in healthy individuals (Stenderup A and GT 1962), but in recent years this yeast is increasingly prevalent in systemic infections (Hajjeh RA, Sofair AN et al. 2004). Besides the mortality rate of *C. glabrata* infections is the highest compared to infections with other non-albicans Candida species (Abi-Said D, Anaissie E et al. 1997; Krcmery 1999).

Old age is often associated with increased oral Candida colonization (Lockhart SR, Joly S et al. 1999) and also the carriage rate has been suggested to be a function of aging process (Kleinegger CL, Lockhart SR et al. 1996).

Although the participant didn't suffer from any known fungal infection the finding of Candida glabrata goes confirm with results from other studies (Lockhart SR, Joly S et al. 1999) (Kleinegger CL, Lockhart SR et al. 1996).

8.3.4.2 Mucor fragilis; Mucor Circinelloides; Mucor racemosus

One of the sequenced clones from an oncology patient was identified as *Mucor fragilis* to 98%, *Mucor circinelloides* and *Mucor racemosus* to 95%.

Mucor species are saprophytes and they are found in the whole world in soil and environmental samples (Farrow 1954; Goos 1963; Pandey A, Agarwal GP et al. 1989). Even in air or dust samples from home and hospital settings spores of *Mucor sp.* were found (Nobel WC and YM 1963; Bokhary H A and S 1995; Levy V, Rio B et al. 1996).

The following species of Mucor can cause disease in humans: *M. circinelloides, M. hiemalis, M. rouxianus, M. ramosissimus and M. racemosus*. However, specific information about the species *Mucor fragilis* couldn't be found in literature.
In general people who suffer from mucormycosis had a foregoing disease that cause immunosuppression (Ribes JA, Vanover-Sams CL et al. 2000) such as cancer and chemotherapy. The immune status of the patient plays an important role concerning the manifestation of infection with *Mucor sp.*. For instance in immunocompromised hosts exist the risk of developing invasive solid-organ diseases, whereas healthy host predominantly suffer from cutaneaous or nail infections (Medoff G and GS 1972; Levy V, Rio B et al. 1996).

8.3.4.3 Psathyrella calcareae

The second clone from an oncology patient was identified as *Psathyrella calcarea*, but also as an uncultured fungus with a maximal identity of 97%.
No specific information could be found about the species *Psathyrella calcarea*, but Scanlan et al. found another Psathyrella species, namely *Psathyrella candolleana,* in the human intestine (Scanlan PD and Marchesi JR 2008).
The genus Psathyrella belongs to the mushroom family Psathyrellaceae and consists of 400-600 species (Kirk PM, Cannon PF et al. 2001).

8.3.4.4 Sordaria sp.

Another clone of a patient receiving anti cancer therapy was identified to 100% as *S. alcina, S. lappae, S. fimicola* and *S. humana*.
However, the only useful information about Sordaria species found was that especially *S. fimicola* is commonly found in the faeces of herbivores, especially (Kirk PM, Cannon PF et al. 2008).

8.3.4.5 Candida tropicalis

The last sequenced clone from the clone library of a chemotherapy patient was identified as *C.tropicalis* to 97%.

Candida albicans is the most pathogenic yeast species causing candidiasis, but in recent years *C. tropcalis* infections dramatically increased worldwide, especially in immunocompromised patients (Kothavade RJ, Kura MM et al. 2010), who suffer for example from hepatitis B virus infection (Chen Y, Chen Z et al. 2010).

For instance in India *C. tropicalis* is the major cause of nosocomial infection (Kothari A and V 2009).

With this information in mind it is possible that *C.tropicalis* was an inhabitant of the gastrointestinal tract of this patient, although it was identified with only 97% maximal identity.

8.3.4.6 Saccharomyces cerevisiae

Saccharomyces cerevisiae was found in the faecal sample of a young omnivore and identified with 99% maximal identity.

The main field of application of the *S. cerevisiae* is the food industry.

S. cerevisiae is a commensal of mucosal surfaces and part of the normal microbiota in the gastrointestinal tract, the respiratory tract and the vagina (Salonen JH, Richardson MD et al. 2000).

However, the subtype *S. boulardii* is also known as the cause of different forms of invasive infection (Eddy JT, Stamatakis MK et al. 1997; Cassone M, Serra P et al. 2003).

S. cerevisiae was found in several other studies which investigated the fungal microbiota of healthy and immunocompromised individuals (Agirbasli H, Özcan SA et al. 2005; Ott SJ, Kühbacher T et al. 2008; Scanlan PD and Marchesi JR 2008; Chen Y, Chen Z et al. 2010).

8.3.4.7 Penicillium digitatum

P. digitatum is known as green-mold and it grows on the surface of citrus fruits and produces powdery olive-colored conidia.
This fungal species was found in the faecal sample of a young omnivore with 99% maximal identity, but was also identified as an uncultivatable soil fungus to 98%.
So maybe this fungus is no part of the human gastrointestinal fungal microbiota, but rather a nutritional contamination.

9 CONCLUSION

This diploma thesis verifies that fungi are part of the normal human gastrointestinal microbiota, because in the various participants, who all do not suffer from any known mycoses, fungi were detected.
However further investigations on this topic are necessary, because many questions are not answered; for example:
What is the 'basic' amount of fungi in the gastrointestinal tract and up to which number fungi are safe inhabitants?
How do diet and/or the environment affect the basic fungal composition?
Furthermore the set of samples used in this study should be analyzed with other primer sets to compare specificity. Maybe a primer set which target the ITS1 region for PCR-DGGE analysis and for qPCR a set targeting the actin gene.

10 SUMMARY

Several microbes like bacteria, yeasts and filamentous fungi constitute the human microbiota of the gastrointestinal tract. In the last years the main focus was concentrated on the investigation of bacterial diversity and the fungal microbiota was nearly ignored (Rajilic-Stojanovic M, Smidt H et al. 2007; Zoetendal EG, Rajilic-Stojanovic M et al. 2008) .

Therefore the role of fungi in the human gastrointestinal tract is poorly defined today. The aim of this diploma thesis was to investigate fungal abundances and diversity in the human gastrointestinal tract on the basis of faecal samples from 15 geriatrics and 15 young omnivores. Furthermore faecal samples of 15 oncology patients were used to analyze the fungal microbiota in course of chemotherapy and in comparison to healthy controls.

Fungal diversity was evaluated with PCR-DGGE fingerprinting and principal components analysis (PCA). Real time PCR (qPCR) was used for quantitative measurement of fungi in faecal samples. Moreover clone libraries were created to check primer specificity and also for sequencing. Sequences were identified with BLAST (www.ncbi.nlm.nih.gov/BLAST).

Statistical assessment shows no significant differences between the various groups, neither between geriatrics and young omnivores nor in oncology group compared with healthy controls, possibly because of small number of samples. Also in course of chemotherapy no significant changes of fungal microbiota could be detected. Furthermore no correlation was found between the fungal microbiota of patients receiving cancer therapy and the bacterial one, which was assessed in a recent study by Zwielehner et al. (Zwielehner J, Lassl C et al. 2010; under review).

PCR-DGGE fingerprinting illustrated a greater fungal diversity than expected. Each individual analyzed with DGGE show approximately 10 bands in the gel.

This result is controversial to those of Scanlan et al. who reported that fungal diversity in the gastrointestinal tract is low, because their PCR-DGGE fingerprints show only one to three bands per individual (Scanlan PD and Marchesi JR 2008).
Unfortunately identification of sequenced clones demonstrated that the primer set used is not absolutely fungal specific, because also some plant rDNA was targeted.

Altogether the methodological approach of this thesis is a good approach for further investigation of the human gastrointestinal fungal microbiota.

11 ZUSAMMENFASSUNG

Bakterien, Archeae, Hefen und Hyphomyceten sind Bewohner des Gastrointestinaltraktes eines gesunden, erwachsenen Menschen. Die bakterielle Diversität wurde in den letzten Jahren in vielen Studien untersucht und beschrieben. Pilze im Gastrointestinaltrakt des Menschen wurden allerdings bis zum heutigen Zeitpunkt nur sehr spärlich analysiert (Rajilic-Stojanovic M, Smidt H et al. 2007; Zoetendal EG, Rajilic-Stojanovic M et al. 2008). Aus diesem Grund ist die Rolle der Pilze im Darmtrakt noch nicht klar definiert.

Das Ziel der vorliegenden Diplomarbeit war es die Anzahl und die Diversität der Pilze im menschlichen Gastrointestinaltrakt anhand von Stuhlproben von 15 Geriatrikern und 15 jungen Mischköstlern zu bestimmen. Weiters wurde Fäzes von 15 Onkologie Patienten analysiert, um die Veränderungen der Pilz-Mikrobiota im Verlauf einer Chemotherapie zu untersuchen. Diese Ergebnisse wurden mit jenen einer Kontrollgruppe, bestehend aus gesunden Probanden, verglichen.
Die Diversität der Pilze wurde mittels PCR-DGGE fingerprinting und PCA (principal components analysis) evaluiert. Für die Quantifizierung der Pilze in den Stuhlproben wurde eine real time PCR (qPCR) durchgeführt. Außerdem wurden Klonbibliotheken angefertigt, um die Primerspezifität zu überprüfen und um einige Klone zu sequenzieren. Diese Sequenzen wurden anschließend mit BLAST (www.ncbi.nlm.nih.gov/BLAST) identifiziert.
Die statistische Auswertung zeigte keine signifikanten Unterschiede zwischen den verschiedenen Gruppen; weder zwischen Geriatrikern und jungen Mischköstlern, noch zwischen Onkologie Patienten und gesunden Freiwilligen. Auch im Verlauf der Chemotherapie konnten keine signifikanten Veränderungen beobachtet werden. Weiters wurde überprüft, ob es einen Zusammenhang zwischen der Pilz-Mikrobiota und der Bakterien-Mikrobiota, welche schon in einer vorherigen Studie von Zwielehner et al. (Zwielehner J, Lassl C et al. 2010; under review) analysiert wurde, der Onkologie Patienten gibt. Allerdings konnte keine Korrelation festgestellt werden.

Die Ergebnisse des PCR-DGGE fingerprintings machten eine größere Pilzdiversität, als erwartet, deutlich. Jede untersuchte Fäzesprobe der verschiedenen Probanden zeigte ungefähr 10 Banden im Gel. Dieses Ergebnis ist gegensätzlich zu dem von Scanlan et al., welche berichteten, dass die Pilzdiversität im Gastrointestinaltrakt niedrig ist, da ihre PCR-DGGE fingerprints nur ein bis drei Banden pro Individuum hatten (Scanlan PD and Marchesi JR 2008).
Unglücklicherweise zeigte die Identifikation der sequenzierten Klone, dass das gewählte Primerset nicht zu hundert Prozent Pilz spezifisch ist, da es auch pflanzliche rDNA detektierte.

Alles in allem stellt diese Diplomarbeit einen guten Beginn für weitere Untersuchungen der gastrointestinalen Pilz-Mikrobiota des Menschen dar.

12 PROTOCOLS

12.1 QIAamp® DNA Stool Handbook - Isolation of DNA from Stool for Human DNA Analysis

1. Weigh 180–220 mg stool in a 2 ml microcentrifuge tube (not provided) and place tube on ice.
This protocol is optimized for use with 180–220 mg stool but can also be used with smaller amounts. There is no need to reduce the amounts of buffers or InhibitEX matrix when using smaller amounts of stool.
If the sample is liquid, pipet 200 µl into the microcentrifuge tube. Cut the end of the pipet tip to make pipetting easier.
If the sample is frozen, use a scalpel or spatula to scrape bits of stool into a 2 ml microcentrifuge tube on ice.
Note: When using frozen stool samples, take care that the samples do not thaw until Buffer ASL is added in step 2 to lyse the sample; otherwise the DNA in the sample may degrade. After addition of Buffer ASL, all following steps can be performed at room temperature (15–25°C).
2. Add 1.6 ml Buffer ASL to each stool sample. Vortex continuously for 1 min or until the stool sample is thoroughly homogenized.
Note: It is important to vortex the samples thoroughly. This helps ensure maximum DNA concentration in the final eluate.
3. Centrifuge sample at full speed for 1 min to pellet stool particles.
4. Pipet 1.4 ml of the supernatant into a new 2 ml microcentrifuge tube (not provided) and discard the pellet.
Note: The 2 ml tubes used should be wide enough to accommodate an InhibitEX Tablet. Transferring small quantities of pelleted material will not affect the procedure.
5. Add 1 InhibitEX Tablet to each sample and vortex immediately and continuously for 1 min or until the tablet is completely suspended. Incubate suspension for 1 min at room temperature to allow inhibitors to adsorb to the InhibitEX matrix.
6. Centrifuge sample at full speed for 3 min to pellet stool particles an inhibitors bound to InhibitEX matrix.
Note: For most samples, 3 min centrifugation is sufficient. With some samples, however, centrifugation for 3 min may result in a pellet that is not sufficiently compact. Therefore it may be difficult to remove enough supernatant to transfer 600 µl supernatant after the next centrifugation step (step 9). In these cases, we recommend to centrifuge for 6 min.
Note: When processing more than 12 samples, for this step and step 7 we recommend processing batches of no more than 12 samples each. This is because the pellets formed after centrifugation will break up quickly if the supernatant is not removed immediately.
7. Immediately after the centrifuge stops, pipet all of the supernatant into a new 1.5 ml microcentrifuge tube (not provided) and discard the pellet. Centrifuge the sample at full speed for 3 min.

Transferring small quantities of pelleted material from step 6 will not affect the procedure.
8. Pipet 25 µl proteinase K into a new 2 ml microcentrifuge tube (not provided).
9. Pipet 600 µl supernatant from step 7 to the 2 ml microcentrifuge tube containing proteinase K.
10. Add 600 µl Buffer AL and vortex for 15 s.
Note: Do not add proteinase K directly to Buffer AL.
It is essential that the sample and Buffer AL are thoroughly mixed to form a homogeneous solution.
11. Incubate at 70°C for 10 min.
Centrifuge briefly to remove drops from the inside of the tube lid (optional).
12. Add 600 µl of ethanol (96–100%) to the lysate, and mix by vortexing.
Centrifuge briefly to remove drops from the inside of the tube lid (optional).
13. Label the lid of a new QIAamp spin column provided in a 2 ml collection tube. Carefully apply 600 µl lysate from step 12 to the QIAamp spin column without moistening the rim. Close the cap and centrifuge at full speed for 1 min. Place the QIAamp spin column in a new 2 ml collection tube, and discard the tube containing the filtrate.
Close each spin column in order to avoid aerosol formation during centrifugation.
If the lysate has not completely passed through the column after centrifugation, centrifuge again until the QIAamp spin column is empty.
14. Carefully open the QIAamp spin column, apply a second aliquot of 600 µl lysate and centrifuge at full speed for 1 min. Place the QIAamp spin column in a new 2 ml collection tube, and discard the tube containing the filtrate.
Close each spin column in order to avoid aerosol formation during centrifugation.
If the lysate has not completely passed through the column after centrifugation, centrifuge again until the QIAamp spin column is empty.
15. Repeat step 14 to load the third aliquot of the lysate onto the spin column.
16. Carefully open the QIAamp spin column and add 500 µl Buffer AW1. Close the cap and centrifuge at full speed for 1 min. Place the QIAamp spin column in a new 2 ml collection tube, and discard the collection tube containing the filtrate.
17. Carefully open the QIAamp spin column and add 500 µl Buffer AW2. Close the cap and centrifuge at full speed for 3 min. Discard the collection tube containing the filtrate.
Note: Residual Buffer AW2 in the eluate may cause problems in downstream applications. Some centrifuge rotors may vibrate upon deceleration, resulting in the flow-through, which contains Buffer AW2, contacting the QIAamp spin column. Removing the QIAamp spin column and collection tube from the rotor may also cause flow-through to come into contact with the QIAamp spin column.
18. Recommended: Place the QIAamp spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.
This step helps to eliminate the chance of possible Buffer AW2 carryover.
19. Transfer the QIAamp spin column into a new, labeled 1.5 ml microcentrifuge tube (not provided). Carefully open the QIAamp spin column and pipet 200 µl Buffer AE

directly onto the QIAamp membrane. Close the cap and incubate for 1 min at room temperature, then centrifuge at full speed for 1 min to elute DNA.

Note: When using eluates in PCR, for maximum PCR robustness we highly recommend adding BSA to a final concentration of 0.1 µg/µl to the PCR mixture. For maximum PCR specificity we recommend using QIAGEN HotStarTaq Plus DNA Polymerase (see ordering information on page 39). For best results in downstream PCR, use the minimum amount of eluate possible in PCR; the volume of eluate used as template should not exceed 10% of the final volume of the PCR mixture. Also, note that high amounts of template DNA may inhibit the PCR.

DNA yield is typically 15–60 µg but, depending on the individual stool sample and the way it was stored, may range from 5 to 100 µg. DNA concentration is typically 75–300 ng/µl.

For more information about elution and how to determine DNA yield, purity, and length, see the Appendix, page 36.

For long-term storage, we recommend keeping the eluate at –20°C.

12.2 Yeast Extraction (Candida spp. Cultures) with Lyticase

1. centrifuge (8000rpm) 1ml of the cell suspension 5 min.

2. discard 800µl of the supernatant

3. add 20µl lyticase solution and incubate at 37°C for 30min

4. centrifuge at full speed for 10min. Discard the supernatant

5. resuspend the pellet in 180µl Buffer ATL and 50µl Protease stock solution. Incubate at 55°C for 15min (do not use Proteinase K from the Kit, because it's isolated from fungi -> contamination!)

6. Continue with step 3a of the Tissue Protocol in the QIAamp DNA Mini Kit and QIAamp DNA Blood Mini Kit Handbook

è QIAamp® DNA Mini Kit - Tissue Protocol (QIAamp DNA Mini Kit only)

1. Cut up to 25 mg of tissue (up to 10 mg spleen) into small pieces, place in a 1.5 ml microcentrifuge tube, and add 180 µl of Buffer ATL.
It is important to cut the tissue into small pieces to decrease lysis time.
If DNA is prepared from spleen tissue, no more than 10 mg should be used.
The yield of DNA will depend on both the amount and the type of tissue processed.
1 mg of tissue will yield approximately 0.2–1.2 µg of DNA.
2 ml microcentrifuge tubes are better suited for lysis.

2. Add 20 µl Proteinase K, mix by vortexing, and incubate at 56°C until the tissue is completely lysed. Vortex occasionally during incubation to disperse the sample, or place in a shaking water bath or on a rocking platform.
Note: Proteinase K must be used. QIAGEN Protease has reduced activity in the presence of Buffer ATL.
Lysis time varies depending on the type of tissue processed. Lysis is usually complete in 1–3 h. Lysis overnight is possible and does not influence the preparation. In order to ensure efficient lysis, a shaking water bath or a rocking platform should be used. If not available, vortexing 2–3 times per hour during incubation is recommended.
3. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid. Continue with step 3a, or if RNA-free genomic DNA is required, continue with step 3b.
Transcriptionally active tissues, such as liver and kidney, contain high levels of RNA which will copurify with genomic DNA. RNA may inhibit some downstream enzymatic reactions, but will not inhibit PCR.
3a. Add 200 µl Buffer AL to the sample, mix by pulse-vortexing for 15 s, and incubate at 70°C for 10 min. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid.

It is essential that the sample and Buffer AL are mixed thoroughly to yield a homogeneous solution.
A white precipitate may form on addition of Buffer AL, which in most cases will dissolve during incubation at 70°C. The precipitate does not interfere with the QIAamp procedure, or with any subsequent application.
OR
3b. First add 4 µl RNase A (100 mg/ml), mix by pulse-vortexing for 15 s, and incubate for 2 min at room temperature. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid before adding 200 µl Buffer AL to the sample. Mix again by pulse-vortexing for 15 s, and incubate at 70°C for 10 min. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid.
It is essential that the sample and Buffer AL are mixed thoroughly to yield a homogeneous solution. A white precipitate may form on addition of Buffer AL. In most cases it will dissolve during incubation at 70°C. The precipitate does not interfere with the QIAamp procedure or with any subsequent application
4. Add 200 µl ethanol (96–100%) to the sample, and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid.
If samples are larger than 25 mg (10 mg spleen), increase the amount of ethanol proportionally; e.g., a 50 mg (360 µl) sample will require 400 µl ethanol.
It is essential that the sample, Buffer AL, and the ethanol are mixed thoroughly to yield a homogeneous solution.
A white precipitate may form on addition of ethanol. It is essential to apply all of the precipitate to the QIAamp spin column. This precipitate does not interfere with the QIAamp procedure or with any subsequent application.
Do not use alcohols other than ethanol because this may result in reduced yields.
5. Carefully apply the mixture from step 4 (including the precipitate) to the QIAamp spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.
Close each spin column to avoid aerosol formation during centrifugation.
It is essential to apply all of the precipitate to the QIAamp spin column.
Centrifugation is performed at 6000 x *g* (8000 rpm) in order to reduce noise.
Centrifugation at full speed will not affect the yield or purity of the DNA. If the solution has not completely passed through the membrane, centrifuge again at a higher speed until all the solution has passed through.
6. Carefully open the QIAamp spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap, and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate.
It is not necessary to increase the volume of Buffer AW1 if the original sample volume was larger than 25 mg (180 µl).
7. Carefully open the QIAamp spin column and add 500 µl Buffer AW2 without

wetting the rim. Close the cap and centrifuge at full speed (20,000 x *g*; 14,000 rpm) for 3 min. Continue directly with step 8, or to eliminate any chance of possible Buffer AW2 carryover, perform step 7a, and then continue with step 8.

Note: Residual Buffer AW2 in the eluate may cause problems in downstream applications. Some centrifuge rotors may vibrate upon deceleration, resulting in the flow-through, which contains Buffer AW2, contacting the QIAamp spin column. Removing the QIAamp spin column and collection tube from the rotor may also cause flow-through to come into contact with the QIAamp spin column. In these cases, the optional step 7a should be performed

7a. (Optional): Place the QIAamp spin column in a new 2 ml collection tube (not provided) and discard the collection tube containing the filtrate. Centrifuge at full speed for 1 min.

8. Place the QIAamp spin column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp spin column and add 200 **µ**l Buffer AE or distilled water. Incubate at room temperature for 1 min, and then centrifuge at 6000 x *g* (8000 rpm) for 1 min.

9. Repeat step 8.

A 5 min incubation of the QIAamp spin column loaded with Buffer AE or water, before centrifugation, generally increases DNA yield.

A third elution step with a further 200 µl Buffer AE will increase yields by up to 15%. Volumes of more than 200 µl should not be eluted into a 1.5 ml microcentrifuge tube because the spin column will come into contact with the eluate, leading to possible aerosol formation during centrifugation.

Elution with volumes of less than 200 µl increases the final DNA concentration in the eluate significantly, but slightly reduces the overall DNA yield (see Table 3, page 16). Eluting with 4 x 100 µl instead of 2 x 200 µl does not increase elution efficiency.

12.3 Cloning - pGEM®-T and pGEM®-T Easy Vector Systems (Promega) – Protocol for Ligations Using the pGEM®-T and pGEM®-T Easy Vectors and the 2X Rapid Ligation Buffer

Ligation Protocol

1. Briefly centrifuge the pGEM®-T or pGEM®-T Easy Vector and Control Insert DNA tubes to collect the contents at the bottom of the tubes.
2. Set up ligation reactions as described below.

7,5µl Buffer
+1µl Vector
+1µl ligase
+5,5µl PCR pur. or +3µl of control
15µl total volume à incubate at 4°C

Note: Use 0.5ml tubes known to have low DNA-binding capacity. Vortex the 2X Rapid Ligation Buffer vigorously before each use.

3. Mix the reactions by pipetting. Incubate the reactions for 1 hour at room temperature.

Alternatively, if the maximum number of transformants is required, incubate the reactions overnight at 4°C.

Notes:

1. Use only the T4 DNA Ligase supplied with this system to perform pGEM®-T and pGEM®-T Easy Vector ligations. Other commercial preparations of T4 DNA ligase may contain exonuclease activities that may remove the terminal deoxythymidines from the vector.
2. 2X Rapid Ligation Buffer contains ATP, which degrades during temperature fluctuations. Avoid multiple freeze-thaw cycles and exposure to frequent temperature changes by making single-use aliquots of the buffer.
3. Longer incubation times will increase the number of transformants. Generally, incubation overnight at 4°C will produce the maximum number of transformants.
4. An aliquot of the PCR reaction should be analyzed on an agarose gel before use in the ligation reaction to verify that the reaction produced the desired product. The PCR product to be ligated can be gel-purified or purified directly from the PCR amplification using the Wizard® SV Gel and PCR

Clean-Up System (Cat.# A9281). Clean-up of reactions prior to ligation is recommended to remove primer dimers or other undesired reaction products, and to improve ligation efficiency. Exposure of PCR products to shortwave ultraviolet light should be minimized in order to avoid the formation of pyrimidine dimers.

Transformations Using the pGEM®-T and pGEM®-T Easy Vector

1. Prepare two LB/ampicillin/IPTG/X-Gal plates for each ligation reaction, plus two plates for determining transformation efficiency. Equilibrate the plates to room temperature.
2. Centrifuge the tubes containing the ligation reactions to collect the contents at the bottom. Add 2µl of each ligation reaction to a sterile (17 × 100mm) polypropylene tube or a 1.5ml microcentrifuge tube on ice (see Note 1). Set up another tube on ice with 0.1ng uncut plasmid for determination of the transformation efficiency of the competent cells.
3. Remove tube(s) of frozen JM109 High Efficiency Competent Cells from storage and place in an ice bath until just thawed (about 5 minutes). Mix the cells by gently flicking the tube. Avoid excessive pipetting, as the competent cells are extremely fragile.
4. Carefully transfer 50µl of cells into each tube prepared in Step 2 (use 100µl of cells for determination of transformation efficiency).
5. Gently flick the tubes to mix and place them on ice for 20 minutes.
6. Heat-shock the cells for 45–50 seconds in a water bath at exactly 42°C (do not shake).
7. Immediately return the tubes to ice for 2 minutes.
8. Add 950µl room-temperature SOC medium to the tubes containing cells transformed with ligation reactions and 900µl to the tube containing cells transformed with uncut plasmid (LB broth may be substituted, but colony number may be lower).
9. Incubate for 1.5 hours at 37°C with shaking (~150rpm).
10. Plate 100µl of each transformation culture onto duplicate LB/ampicillin/IPTG/X-Gal plates. For the transformation control, a 1:10 dilution with SOC medium is recommended for plating. If a higher number of colonies is desired, the cells may be pelleted by centrifugation at 1,000 × *g* for 10 minutes, resuspended in 200µl of SOC medium, and 100µl plated on each of two plates.
11. Incubate the plates overnight (16–24 hours) at 37°C. If 100µl is plated, approximately 100 colonies per plate are routinely seen using competent cells that are 1 × 108cfu/µg DNA. Use of ultra-high- efficiency competent cells may result in a higher number of background colonies. Longer incubations or storage of plates at 4°C (after 37°C overnight incubation) may be used to
facilitate blue color development. White colonies generally contain inserts; however, inserts may also be present in blue colonies.

Notes:

1. We have found that use of larger (17 × 100mm) polypropylene tubes (e.g., Falcon™ Cat.# 2059) increases transformation efficiency. Tubes from some manufacturers bind DNA and should be avoided.
2. Colonies containing β-galactosidase activity may grow poorly relative to cells lacking this activity. After overnight growth, the blue colonies may be smaller than the white colonies, which are approximately one millimeter in diameter.
3. Blue color will become darker after the plate has been stored overnight at 4ºC.

Screening Transformants for Inserts
Successful cloning of an insert into the pGEM®-T or pGEM®-T Easy Vector interrupts the coding sequence of β-galactosidase; recombinant clones can be identified by color screening on indicator plates. However, the characteristics of the PCR products cloned into the vectors can significantly affect the ratio of blue:white colonies obtained. Usually clones containing PCR products produce
white colonies, but blue colonies can result from PCR fragments that are cloned in-frame with the *lac*Z gene. Such fragments are usually a multiple of 3 base pairs long (including the 3´-A overhangs) and do not contain in-frame stop codons. There have been reports of DNA fragments up to 2kb that have been cloned in-frame and have produced blue colonies. Even if your PCR product is not a multiple of 3 bases long, the amplification process can introduce mutations (deletions or point mutations) that may result in blue colonies. The Control Insert DNA supplied with the pGEM®-T and pGEM®-T Easy Systems is a 542bp fragment from pGEM®-*luc* Vector DNA (Cat.# E1541). This sequence has been mutated to contain multiple stop codons in all six reading frames, which ensures a low background of blue colonies for the control reaction. Results obtained with the Control Insert DNA may not be representative of those achieved with your PCR product.

<u>LB/ampicillin/IPTG/X-Gal plates</u>

1.) Prepare 4 LB/ampicillin/IPTG/X-Gal plates for each ligation reaction
+ 1 plate for determining transformation efficiency

LB Medium hard: Mix for 500ml LB-plating- media:

5g Tryptophan
2,5g Yeast Extract
2,5g NaCl
7,5g Agar
è Autoklav (1h)
At 50°C: +1ml Ampicilin (Final concentration: 200mg/L Medium)

Streak 20µl IPTG/X-Gal on each plate

LB Medium liquid 100ml:

1g Trypton
0,5g Yeast Extrakt
0,5g NaCl
Mit H20 dest. auf 100ml auffüllen, anrühren
Autoklav (1h)

PCR with Primern T7-Sp6:

1 Sample		
Gesamt V	Anteil Komponente	µL
25µL	$\frac{1}{2}$ MasterMix	12,5
	Primer T7	0,1875
	Primer Sp6	0,1875
	$\frac{1}{50}$ BSA	0,5
	Auffüllen mit NFW	
	Jeweiliges Template	1,5

PCR Programm (testing clone libraries):

		temperature	time	cycles
Initial denaturation	(denaturation &cell lysis)	95°C	10 min	1 x
Cycling	Denaturation	94°C	1 min	30 x
	Annealing	46°C	45 sec	
	Elongation	72°C	1 min	
Final Elongation		72°C	7 min	1 x

è View in Agarose; checking fragment length

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14 CURRICULUM VITAE

Personal Information

Name	Natalie Alexandrow
Date of birth	06.02.1986
Place of birth	Vienna/Austria
E-mail	nati_alex@yahoo.de

Education

Since 10/2004	Student of Nutritional Sciences at the Department of Life Sciences at University of Vienna
06/2004	Matura - general qualification for university entrance

Practical experiences

Diploma thesis since 07/2009	'ITS2 based analysis of the human gastrointestinal fungal microbiota of geriatrics, oncology patients and healthy volunteers'
April-June 2009	Internship AG Haslberger, Department of Nutritional Sciences; University of Vienna
February 2009	Internship, Lagerhaus, Weinlabor Kompetenzzentrum Guntramsdorf, Austria
August-September 2007	Internship, Jungbunzlauer Austria AG, Anwendungstechnik Ladenburg, Germany
July 2007	Internship, Staud's, Austria