



universität
wien

MASTERARBEIT

Epigenetic Influences of Probiotics on the Expression of
Inflammation specific Genes and micro RNAs

Epigenetische Einflüsse von Probiotika auf die Expression von
Entzündungs-spezifischen Genen und micro RNAs

angestrebter akademischer Grad

Master of Science (MSc)

Verfasserin / Verfasser: Manuela Nestelberger

Studienrichtung (lt. Studien-
blatt):

A 066 838

066 Masterstudium

838 Ernährungswissenschaften

Betreuer:

Univ.-Doz. Dr. Alexander G. Haslberger

Wien, im Oktober 2010

Danksagung

Auf diesem Wege möchte ich mich natürlich vor allem bei dem Betreuer dieser Masterarbeit bedanken, Dr. Alexander Haslberger. Er hat mich im Laufe der Arbeit gefordert, mich fachlich und persönlich gestärkt und alle meine Launen geduldig ertragen.

Besonderer Dank gilt auch Frau Mag. Eva Aumüller, die mich mit sehr viel Geduld in dieses spannende Gebiet eingeführt hat und immer ein offenes Ohr und Zeit für meine Anliegen hatte, egal worum es ging.

Christine Mölzer hat alle Zellen für diese Arbeit bereitgestellt, nur durch sie konnten alle Zellversuche zügig durchgeführt werden. Sie wurde nie müde uns zu helfen – Vielen Dank Tine.

Herzliches Dankeschön auch an die gesamte Arbeitsgruppe Haslberger, die Menschen in dieser Abteilung machten einige Situationen überhaupt erst erträglich und halfen wo sie nur konnten. In dieser Gruppe kann man sich einfach nur wohl und geborgen fühlen.

Auch meinen Großeltern bin ich zu großem Dank verpflichtet, sie haben mich finanziell und moralisch auf meinem gesamten Lebensweg unterstützt.

Vielen Dank auch an Bernd, ohne den der Abschluss dieses Studiums wohl sehr viel länger gedauert hätte und der alle meine Launen zu 100% abbekommen hat. Er hat alles geduldig ertragen und mir geholfen wo er konnte.

Nicht zuletzt möchte ich natürlich meinen Eltern danken, die mich in jeder Hinsicht immer unterstützt haben. Allein durch sie konnte ich mich voll und ganz auf mein Studium konzentrieren, was wohl das Beste war, das sie mir auf meinen Lebensweg mitgeben konnten. Sie haben mir in all dieser Zeit geholfen, mich aufgebaut wenn ich am Boden war, mir zugehört wenn ich jemanden zum Reden brauchte und alle meine Stimmungsschwankungen mitgemacht, ohne sich zu beschweren. Sie sind die großzügigsten und warmherzigsten Menschen, die ich kenne. Ich verdanke euch alles!

„Leider läßt sich eine wahrhafte Dankbarkeit mit Worten nicht ausdrücken.“

(Johann Wolfgang von Goethe)

Content

Introduction	14
Gene expression in an eukaryotic cell.....	14
Transcription	14
Post-transcriptional modifications	15
5' Capping:	15
3' Polyadenylation:	15
Splicing:	16
microRNA	17
miRs used in this Thesis	18
RNA export.....	19
Translation.....	19
Folding	19
Protein transport.....	20
Signaling.....	20
Signaling pathways	21
Enzyme coupled Receptors	23
The way from the Receptor to the Nucleus	25
The Ras Protein.....	25
The fast Way to the Nucleus	26
Toll Like Receptors (TLR).....	30
Toll like Receptor 4	30
Toll like Receptor 2	31
Toll like Receptor 9	32
Interleukin 6 (IL6) Signaling.....	32
Tumor Necrosis Factor α (TNF α) Signaling.....	34

NF κ B signaling	35
PPAR γ signaling.....	37
Probiotics	42
LPS, used as a control	44
Objectives.....	45
Materials and Methods	46
Polymerase Chain Reaction (PCR) and Real time PCR	46
Cell Culture	49
Gene Expression.....	50
miR Expression	53
Statistical Analysis	55
Results.....	56
Gene Expression in IL1 β Prestimulated Cells	56
IL6 Expression after 3 hours	56
IL6 Expression after 24 hours	57
TNF α Expression after 3 hours	58
TNF α Expression after 24 hours	59
NF κ Bp65 Expression after 3 hours.....	60
NF κ Bp65 Expression after 24 hours.....	61
PPAR γ Expression after 3 hours	62
PPAR γ Expression after 24 hours	63
Gene Expression without Prestimulation.....	64
IL6 Expression after 3 hours	64
IL6 Expression after 24 hours	65
TNF α Expression after 3 hours	66
TNF α Expression after 24 hours	67

NFκBp65 Expression after 3 hours.....	68
NFκBp65 Expression after 24 hours.....	69
PPARγ Expression after 3 hours	70
PPARγ Expression after 24 hours	71
miR Level after Probiotic Treatment.....	72
Discussion.....	77
CACO2 cell model.....	77
IL6 Expression.....	77
TNFα Expression.....	79
NFκBp65 Expression.....	79
PPARγ Expression	80
miR 7i	81
miR 27b.....	81
miR 155.....	82
Summary	83
Zusammenfassung	85
Protocols	87
RNeasy Mini Kit (Qiagen).....	87
Phusion RT-PCR Kit Finnzymes	93
QuantiMir RT Kit - Small RNA Quantitation System (System Biosciences)...	95
Appendix	98
Lebenslauf.....	105
References.....	107

List of Figures

Figure 1 miRNA Function (Nelson, Baldwin et al. 2003)	17
Figure 2 Signaling ways (Alberts, Bray et al. 2005)	27
Figure 3 Signaling Conclusion (Alberts, Bray et al. 2005).....	28
Figure 4 An alternative way (Alberts, Bray et al. 2005)	29
Figure 5 IL6 Signaling	33
Figure 6 TNF α Signaling (Hong 2007)	35
Figure 7 Signaling through NFkB (Alberts, Bray et al. 2005)	36
Figure 8 Alternative Pathway (Alberts, Bray et al. 2005).....	37
Figure 9 PPAR γ Signaling.....	40
Figure 10 Active Repression (Bailey and Ghosh 2005)	41
Figure 11 Activated State (Bailey and Ghosh 2005)	41
Figure 12 Transrepression (Bailey and Ghosh 2005)	42
Figure 13 Principle of a PCR reaction (Torak et al.)	47
Figure 14 Real Time PCR Curve	53
Figure 15 IL6 Expression prestimulated 3 hours.....	56
Figure 16 IL6 Expression prestimulated 24 hours.....	57
Figure 17 TNF α Expression prestimulated 3 hours.....	58
Figure 18 TNF α Expression prestimulated 24 hours.....	59
Figure 19 NFkBp65 Expression prestimulated 3 hours.....	60
Figure 20 NFkBp65 Expression prestimulated 24 hours.....	61
Figure 21 PPAR γ Expression prestimulated 3 hours	62
Figure 22 PPAR γ Expression prestimulated 24 hours	63
Figure 23 IL6 Expression 3 hours	64
Figure 24 IL6 Expression 24 hours	65
Figure 25 TNF α Expression 3 hours	66
Figure 26 TNF α Expression 24 hours	67
Figure 27 NFkBp65 Expression 3 hours	68
Figure 28 NFkBp65 Expression 24 hours	69
Figure 29 PPAR γ Expression 3 hours.....	70
Figure 30 PPAR γ Expression 24 hours.....	71
Figure 31 miR Expression after Lactobacillus treatment.....	72

Figure 32 miR Expression after Streptococcus treatment.....	73
Figure 33 miR treatment after LPS treatment	74
Figure 34 IL6 Expression after Nano Silver Treatment	99
Figure 35 TNF α Expression after Nano Silver Treatment	99
Figure 36 Caspase3 Expression after Nano Silver Treatment	100
Figure 37 miR7i Expression after Nano Silver Treatment.....	101
Figure 38 miR27b Expression after Nano Silver Treatment.....	101
Figure 39 miR155 Expression after Nano Silver Treatment.....	102
Figure 40 Number of Cells after Nano Silver Treatment	102
Figure 41 Viability of Cells after Nano Silver Treatment.....	103

List of Tables

Table 1 List of Substances and their used Concentrations	49
Table 2 Temperature conditions of Real Time PCR	52
Table 3 Used Primers	52
Table 4 Real Time PCR Conditions for miRNAs	54
Table 5 Detected miRNAs	54

List of Abbreviations

5'UTR - five prime untranslated Region

3'UTR - three prime untranslated Region

15-d Ptg - 15-Deoxy-Delta Prostaglandin-J2

ATP – Adenosintriphosphat

BLP - bacterial Lipoprotein

cAMP - cyclic Adenosine-3, 5' Monophosphate

CBP - CREB-Binding Protein

CD14 – Cluster of Differentiation

cDNA – complementary DNA

CPSF - Cleavage and Polyadenylation Specificity Factor

CStF - Cleavage Stimulation Factor

DISC - Death Inducing Signaling Complex

dNTP – Deoxynucleotidtriphosphat

ECSIT - Evolutionary Conserved Signaling Intermediate in Toll Pathways

ELISA - Enzyme Linked Immuno Sorbent Assay

ERK - Extracellular Signal Regulated Kinase

GAPDH - Glycerinaldehyd-3-phosphate Dehydrogenase

GDP - Guanosindiphosphat

GPCR - G-Protein Coupled Receptor

GRB - Growth Factor Receptor Bound Protein

GTP – Guanosintriphosphat

HDAC - Histone Deacetylases

HSP – Heat Shock Protein

IKK - Inhibitor of Kappa Light Polypeptide

IL6 – Interleukin 6

IRAK – Interleukin Receptor Associated Kinase

IRF3 - Interferon Regulatory Factor-3

JAK - Janus-Kinases
JNK - c-Jun-kinase
LAM – Lipoarabinomannan
LBP - LPS-binding-Protein
LPS – Lipopolysaccharide
LTA - Lipotechoic Acid
M7G - 7-Methylguanosine
MAPK - Mitogen activated Protein Kinase
MAPKK - Mitogen activated Protein Kinase Kinase
MAPKKK - Mitogen activated Protein Kinase Kinase Kinase
MgCl₂ – Magnesiumchloride
miR – micro RNA
mRNA – messenger RNA
MyD88 - Myeloid Differentiaion Primary-Response Proetin-88
NCOR - Nuclear Receptor Corepressor
NFkB - Nuclear Factor kappa B
PBMC - Peripheral Blood Mononuclear cells
PCR – Polymerase Chain Reaction
PGN – Peptidoglycans
PI3K - Phosphoinositide-3-Kinase
PKA - Protein-Kinase-A
PPAR_γ - Peroxisome Proliferator Activated Receptor γ
RXR - Retinoid-X-Receptor
SMRT - Silencing Mediator For Retinoid and Thyroid Hormone Receptor
SOS - Son of Sevenless
SRC - Steroid Receptor Coactivator
STAT - Signal Transducers and Activators of Transcription
TAB – TAK Binding Protein

TAK - TGF-Beta-Activated Kinase

TGF - Transforming Growth Factor-Beta

TIR – Toll Interleukin-1-Receptor

TLR – Toll Like Receptor

TNF α – Tumor Necrosis Factor α

TRAF - Factor Associated Receptor Factor

tRNA – transfer RNA

TZD – Thiazolidinedione

UbC - Ubiquitin-Conjugating-Enzyme

Introduction

Gene expression in an eukaryotic cell

Transcription

A gene is encoded by a specific sequence in the DNA. This sequence must be transcribed into RNA and further processed to become the protein structure that it has to. The transcription from DNA into RNA copies is done by an enzyme called polymerase. This enzyme adds the complementary bases of the DNA sequence next to each other and in this way creates a complementary strand. This strand is complementary to the 3'- 5'DNA template strand, which is complementary to the 5'- 3'coding DNA strand. Thus the transcribed RNA strand is identical with the coding DNA strand, the only exception is that all thymines are replaced by uracils in the RNA sequence.

(Brueckner, Armache et al. 2009)

The result of this transcription process is the so called messenger RNA (mRNA). This mRNA is necessary to produce a protein via translation.

The sequence that results after transcription contains so called regulatory sequences which are necessary for direction and regulation of the synthesis of the protein. These non coding sequences are upstream the coding region (five prime untranslated region – 5'UTR) or downstream the coding region (three prime untranslated region – 3'UTR).

Only one of the two complementary DNA strands, the so called template strand, is transcribed. The other strand is called coding strand, because it has the same sequence like the new transcribed RNA. Contrary to the DNA replication, there are no Okazaki fragments needed in the transcription process because only the 3'- 5' sequence is used. (Brueckner, Armache et al. 2009)

Post-transcriptional modifications

The primary RNA transcript consists of coding regions, so called exons and non coding regions, so called introns. In the process of RNA maturation, the introns are cut out of the primary sequence. This process is called splicing. After cutting, the exons are tight together.

In the process of maturation the RNA undergoes three specific modifications :

- 5' capping
- 3' polyadenylation
- RNA splicing

All of these three modifications occur in the nucleus of the eukaryotic cell after transcription but before the RNA is translated. (Riccardo, Tortoriello et al. 2007)

5' Capping:

A phosphatase Enzyme removes the terminal 5' phosphate, so that a 7-methylguanosine (m^7G) can be added to the 5' end. The enzyme guanosyl transferase produces a diphosphat 5' end. This diphosphat end attacks the α phosphorus atom of the Guanosintriphosphat (GTP) complex and in this way adds a guanine residue in a 5'5' triphosphate link. An enzyme called S-adenosyl methionine methylates the guanine ring at the N-7 position. This cap is called the cap 0 structure, because it has only got the (m^7G) in position. For a cap 1 structure, the ribose of the next nucleotide has to be methylated too. When the methylation continues downstream, cap 2, 3 etc. These caps have the ability to protect the primary RNA transcript from ribonucleases. The methyl groups that are necessary to form the cap structure are added to the 2'OH groups of the ribose sugar. (Riccardo, Tortoriello et al. 2007)

3' Polyadenylation:

For a polyadenylation of the 3' end of the RNA tail a polyadenylation signal sequence (5' - AAUAAA - 3') is needed. This signal sequence is usually located

near the 3' end of the pre-mRNA and followed by another sequence which is usually (5' - CA - 3'). The second sequence is the signal site for cleavage, whereas further downstream the RNA tail a GU rich fragment is usually present in pre mRNAs. (Alberts, Bray et al. 2005)

Later on the RNA Polymerase2 enzyme transfers the Cleavage and Polyadenylation Specificity Factor (CPSF) and the Cleavage Stimulation Factor (CStF) to the RNA molecule. Together they form a complex that cleaves the RNA tail between the polyadenylation site and the GU rich sequence, at the cleavage site (5'-CA-3'). (Alberts, Johnson et al. 2002)

Another enzyme, called Poly(A)polymerase, adds up to 200 adenines to the new 3' end of the RNA tail. The needed substrate for this process is ATP, which serves as a precursor. Thus the 3' RNA end is protected from nuclease digestion by the Poly-A-tail. (Alberts, Johnson et al. 2002)

Splicing:

In the RNA splicing process, the introns, which are the non coding regions, are removed from the pre-mRNA. These introns do not code for any protein but are sometimes used to process small RNAs like non coding RNAs. After the process of splicing the RNA molecule consists only of exons. The process of splicing is catalyzed by the so called Spliceosome, which is a complex of specialized small nuclear RNAs and proteins. These small RNA molecules recognize splice sites in the pre-mRNA sequence, so that the process of splicing can start. (Alberts, Bray et al. 2005)

A lot of pre-mRNAs can be spliced in different ways and furthermore encode for different proteins, depending on the process of splicing. This ability to splice one pre-mRNA in different ways and produce different proteins is called alternative splicing. This process gives the facility to produce a range of different proteins from a limited number of DNA and belongs to the post-transcriptional modifications. (Alberts, Johnson et al. 2002)

microRNA

micro RNAs (miR) are small non coding RNAs of about 18-24 nucleotides length.

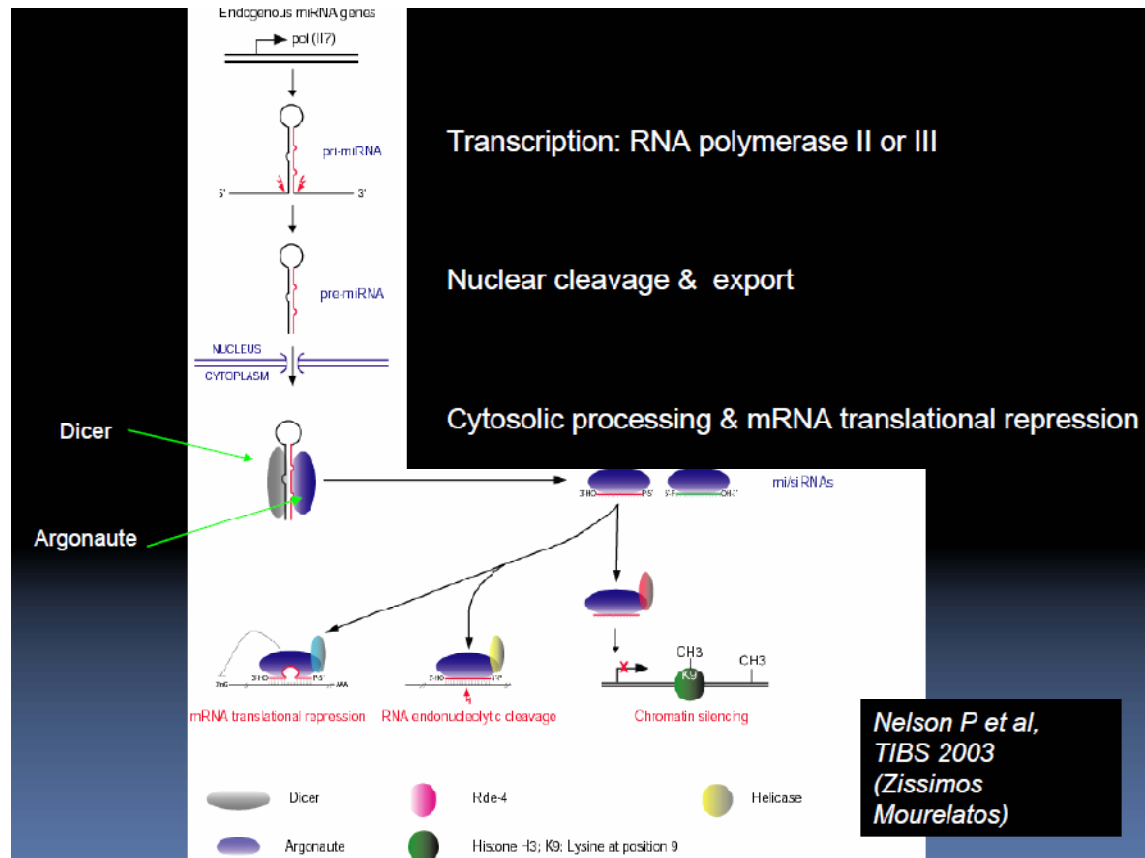


Figure 1 miRNA Function (Nelson, Baldwin et al. 2003)

miRs effect the gene expression after transcription. For this regulation they use evolutionary ancient mechanisms. They have the ability to “cleave” to their target-mRNA and in this way block the translation or they elicit target mRNA degradation. Furthermore the mRNA cannot be translated into the protein structure and degrades. This process is also a post-transcriptional modification, with the effect that the protein is not built and the cellular response is changed. Just like other RNAs, miR sequences are anchored in the DNA and get transcribed. This transcription includes stem-loop precursors and build up the precursor miR, including the miR itself. The first processing step inside the nucleus is done by the RNase Drosha. Afterwards the precursor miR gets exported into the cytoplasm, where the RNase Dicer is responsible for further

processing the structure. Furthermore the miR binds to a protein complex, called Argonaute-family, this newly synthesized structure is called RNA-induced silencing complex (RISC) and enables the miR to work. (System Biosciences)

miRs used in this Thesis

- miR7i

The importance of TLRs will be described in another chapter. MiR7i seems to have an influence on the expression of TLR 4, which is responsible for LPS signaling. The TLR 4 expression level is degraded in CACO2 cells, maybe because of the adaptation processes, which are necessary in the gut. Chen et al. already showed in 2007 that the miR7i directly regulates the expression of TLR4, in the way of blocking expression through mRNA translational repression.

(Chen, Splinter et al. 2007)

- miR27b

Jennewein et al. claimed 2009 that miR 27b destabilizes PPAR γ mRNA in macrophages after LPS treatment. PPAR γ is well known because of its anti-inflammatory effects. The activation of PPAR γ is very interesting and important for the field of cronic inflammatory diseases. (Jennewein, Knethen et al. 2010)

- miR155

Tili et al. reported in 2007 that miR155 plays an important role in innate immunity. They showed that miR155 targets some transcripts coding for proteins that are necessary in the LPS signaling pathway such as I κ B.

(Tili, Michaille et al. 2007)

O'Connell et al. also showed a miR155 responsiveness to LPS and an increased miR155 level in patients with acute myeloid leukemia. Furthermore, O'Connell et al. conclude that miR155 is a common target for a broad range of inflammatory mediators. (O'Connell, Taganov et al. 2006)

O'Connell et al. found that miR155 is involved in the first line of defense in macrophages, in response to different types of inflammatory mediators. This fact ensures an important role for miR155 in cancer development and innate immunity. (Connell, Rao et al. 2008)

RNA export

Most mature RNAs must be transported out of the nucleus to be further processed. These mature RNAs often use nuclear pores for the export into the cytosol. In this case, the RNA is an information carrier for the protein synthesis and it is called mRNA. (Anfinsen 1972)

Translation

For non-coding RNAs, the RNA construct is the finished product. Contrary, most of the RNA in the cell encodes for one or more proteins (mRNA). In the coding regions of the RNA sequence, each triplet of nucleotides corresponds to a binding site of the so called transfer RNA (tRNA). Later on this transfer RNA is translated into amino acids which are chained together by ribosomes. Thus the sequence of the tRNA becomes a protein. Where in the cell the protein synthesis takes place, depends on where the protein is needed. It can for example be formed in the cytoplasm or the endoplasmatic reticulum. (Alberts, Johnson et al. 2002)

Folding

After Translation proteins are unfolded polypeptides, so called random coils, when translated from a mRNA to a linear chain of amino acids. For the right function of a protein, it has to be folded specifically. Amino acids interact with each other to reach the specific 3 dimensional structure. Thus it seems clear that the specific 3D structure of the protein depends on the amino acid sequence.

A mistake in folding can lead to inactive proteins with different properties such as toxic prions or cause neurodegenerative and other diseases. (Anfinsen 1972)

The process of folding can already start during translation, in this case the N-terminus of the protein, which is translated first, starts to fold while the C-terminus is still in the translation phase. Chaperons, which are protein structures that appear during folding, but do not occur in the product, assist in the folding procedure of other proteins. These chaperons are commonly known as Heat Shock Proteins (HSP) whose expression is elevated when the temperature in the cell raises or other environmental stress situations disturb the cell. (Alberts, Johnson et al. 2002)

Protein transport

In some cases the produced proteins are not targeted for the places where they were produced. They have to be transported to their target location. For this transport system a wide range of signaling sequences are necessary, to lead the protein into the right direction.

A variety of proteins which are produced for extracellular mechanisms is exported out of the cell. Such proteins are for example: digestive enzymes, hormones or extracellular matrix proteins. In eukaryotes, most of the transports are done through translocation to the endoplasmatic reticulum and further to the Golgi apparatus, from where the export is completed. (Anfinsen 1972)

Signaling

Cell signaling is necessary for coordination of the functions in the cell and the communication that governs basic cellular activities. This ability to respond to environmental influences provides the cell's basis for development, tissue repair, immunity and homeostasis. Cancer, autoimmune diseases and diabetes

can occur because of errors in this complex signaling system. (LinkWitzany 2010)

The way of information transport is a complex signaling network. Changes in this network can influence transmission and flow of information. (Brueckner, Armache et al. 2009)

Cells can communicate with each other using the following ways:

- Juxtacrine signaling – means transport over direct contact
 - Information is transmitted through oligosaccharides, lipids or protein components
- Paracrine signaling – means transport over short distances
 - Information is transmitted over short scales because the information carrier degrade very early on their way. One example are growth factors.
- Endocrine signaling – means transport over large distances
 - Information is transmitted for example via hormones in the blood over large scales. (Brueckner, Armache et al. 2009)

Signaling pathways

Signaling pathways in the cell are possibilities for the cell to respond to environmental influences. The first step in this system is a protein complex called receptor. These receptors are often located at the cell surface and are able to stimulate specific interactions of protein structures which are activated or deactivated by mechanisms like phosphorylation, ubiquitination, etc. Depending on the ligand that binds to the receptor, the specific receptor can initiate a range of pathways. A cell-surface-receptor is necessary because these ligands are mostly hydrophilic and thus cannot pass the cell membrane. In some cases receptors are also located in the cytosol or at the nucleus, the so called nuclear-receptors. One of them is Peroxisome Proliferator Activated Receptor γ (PPAR γ) which will be discussed later in this work. Which ligand is activated and further binds to its receptor depends on the environmental exposure. There

are specific ligands and receptors for inflammatory and anti-inflammatory processes, called cytokines that will be the main theme in this ongoing work.

Taken together there are three classes of cell-surface-receptors:

- Ion-channel receptor: opens and closes after binding of a signal molecule
- G-protein-coupled receptor: binds to an extracellular signal molecule and first transmits the signal to a GTP-binding protein which is bound to the receptor. Furthermore, this activated G-protein leaves its receptor and activates an enzyme or a ion-channel in the plasma membrane. The so called G-protein is a complex of three subunits, which can dissociate from each other in response to a signal.
- Enzyme-coupled receptor: if a signal molecule binds to this sort of receptor, the enzyme inside the cell is activated. This activation can be limited to its own activity or also take other enzymes into account. (Alberts, Bray et al. 2005)

These three types of receptors differ in their intracellular signals. In the case of ion-channel receptors, the intracellular response to the binding of a signal molecule is the flowing of ions through the membrane. This flow produces electrical energy. G-protein coupled receptors activate a class of membrane bound proteins, so called G-proteins, which further can move in the plasma membrane and initiate a cascade of other effects. When the an enzyme-coupled receptor or a coupled enzyme is activated, additional signals are initiated, including small molecules which are released in the cytosol of the eukaryotic cell. (Alberts, Bray et al. 2005)

The number of receptor-types is even larger than the number of extracellular signals. This is because one extracellular signal molecule is able to bind to more than one receptor. In some cases, extracellular signals bind to different receptor-types in different tissues. This enables tissues to respond in different ways to the same signal molecule. (Alberts, Bray et al. 2005)

The discussed extracellular receptors can also bind to substances like heroin, nicotin, tranquilizers and chilipepper. These substances either act like natural ligands and initiate a physiological signal transduction or they bind onto different sites of the receptor and thereby block or over-stimulate the receptor activity. A lot of pharmaceuticals also affect these receptors to initiate the response of choice. (Alberts, Bray et al. 2005)

Enzyme coupled Receptors

Enzyme coupled receptors are transmembrane proteins and their ligand binding domain is directed outside the plasma membrane. This type of receptors responds to signals which control cellular differentiation, growth, proliferation and survival. Mistakes in these mechanisms can lead to the development of cancer. In this work mediators and signal transducers that aim at least one of these processes will be discussed.

Most of the signal molecules act as local mediators and initiate their effect even in low concentrations. The response to these mediators typically takes a few hours because a complex intracellular signaling cascade is needed. Finally the gene expression is changed.

The main types of these enzyme coupled receptors are those which have a cytoplasmatic domain, working as tyrosine-proteinkinase. This kinase is able to phosphorylate tyrosin at the sidechains of specific proteins inside the cell. These receptors are called receptor-tyrosine-kinases. (Alberts, Bray et al. 2005)

Scientists suggest that the enzyme coupled receptor protein consists of only one transmembrane segment which passes through the membrane in an α -helix structure. The kinase function is often activated by dimerisation. Thus, one receptor is able to phosphorylate the other. In the case of receptor-tyrosin-kinases, the phosphorylation takes place on a tyrosin-rest. After phosphorylation a complex signal transduction cascade begins.

The phosphorylated tyrosines serve as binding sites for 10-20 intracellular signaling proteins, which can be activated by this binding situation. As long as this complex exists, it sends signals over a wide range of possible ways with completely different aims at the same time. Furthermore a lot of biochemical changes appear in these pathways which are necessary to initiate such complex procedures like cell proliferation. To stop the process of receptor activation, protein-tyrosine-phosphatases are necessary. These enzymes cut the phosphates off again and in this way stop the activation. In some cases the activated receptors are digested in lysozymes after endocytosis.

Different receptor-tyrosine-kinases have different intracellular signaling proteins for different outcomes. Nevertheless, some seem to be more important than others and are used more often. One example is the phospholipase which is necessary for the activation of the inositolphospholipid-signaling way. Receptor-tyrosine-kinases are also able to activate an important enzyme in signal-transduction, called phosphatidylinositol-3-kinase, which phosphorylates inositolphospholipids in the plasmamembrane.

The main signaling way of receptor-tyrosine-kinases is a different one than that described above. Uncontrolled mutations that activate this signaling cascade lead to the development of a lot of cancers through stimulation of cell division. (Alberts, Bray et al. 2005)

The way from the Receptor to the Nucleus

Some of the signaling proteins are just used as adaptor molecules in the signaling process. This means that they help to build up a signaling aggregate through binding the receptor to other proteins, which are activated and transport the signal further on. An important role in this complex is hosted by the Ras protein. This protein is located tightly to the plasma membrane by a lipid structure. Nearly all receptor-tyrosine-kinases activate the Ras protein.

The Ras Protein

The Ras protein belongs to the family of GTP-binding proteins, but they only form monomers in contrast to the trimer-G-Proteins. Ras has a similar structure to the α subunit of a G-Protein and functions as a molecular switch. Ras is activated by binding of GTP and changes its confirmation status. Through hydrolysis of GTP to Guanosindiphosphat (GDP), Ras turns itself off again.

When Ras is activated it initiates a phosphorylation cascade that is defined by phosphorylation of proteinkinases. These kinases phosphorylate each other in the ongoing cascade. This way of phosphorylation and signal transduction is called MAP-Kinase-Cascade because the last kinase in the pathway is called Mitogen Activated Protein Kinase (MAP-Kinase). Within the signal transduction pathway, the MAP-Kinase is phosphorylated and thereby activated by an enzyme called MAP-Kinase-Kinase. This enzyme itself is activated by a MAP-Kinase-Kinase-Kinase, which is activated by Ras. At the end of the pathway MAP-Kinase phosphorylates serines and threonines on specific gene regulatory proteins and thereby changes their ability to regulate the expression of genes. Depending on which other signals the cell receives and which other genes are active in the cell, this signaling cascade can influence the gene expression, stimulate the cell proliferation, initiate cell survival or induce cell differentiation.

When Ras is deactivated by intracellular injections of antibodies, the cell loses the ability to respond to several extracellular signals. If, on the other hand, the

Ras activity is turned on over a period, the cell behaves as if it is treated with proliferation stimulating mitogens.

In 30% of all human cancer types, the gene coding for Ras is mutated. In other types of cancer, often genes involved in the same pathway are affected. The wild type of these genes, which is necessary for a “normal” function of a cell, is often called proto-oncogenes, because they can easily be turned into oncogenes by mutation. This is the start of the uncontrolled growth and division of cancer cells. (Alberts, Bray et al. 2005)

The fast Way to the Nucleus

Some hormones and a lot of local mediators, so called cytokines, bind to receptors that can activate gene regulatory proteins, which are near the membrane in a latent state. Once activated, these regulator proteins enter the nucleus and stimulate the transcription of specific genes. Interferons are one popular example for this pathway. They are cytokines which make cells produce the proteins they need for the defense against viral infections.

Cytokine-receptors do not have any enzyme activity like receptor-tyrosine-kinases have. Instead, cytokine-receptors are bound to tyrosine-kinases called Janus-Kinases (JAKs) which are activated in the case of cytokine binding to the receptor. When the JAKs are activated, they phosphorylate and activate cytoplasmic gene regulatory proteins, so called signal transducer and activators of transcription (STATs). These STATs move to the nucleus and stimulate the transcription of specific genes.

Different cytokine receptors stimulate different responses, by activating different STATs. The cytokine signaling gets deactivated by protein phosphatases, these phosphatases remove the phosphate groups from the activated signaling proteins.

Another class of fast receptors are the receptor-serine/threonine-kinases. They directly phosphorylate and activate cytoplasmic gene regulatory proteins after

stimulation by an extracellular signaling molecule. The hormones and local mediators that stimulate this kind of receptors belong to the Tumor Growth Factor (TGF)- β -superfamily of extracellular proteins, which have an important role in the development of oncogenic transformation. (Alberts, Bray et al. 2005)

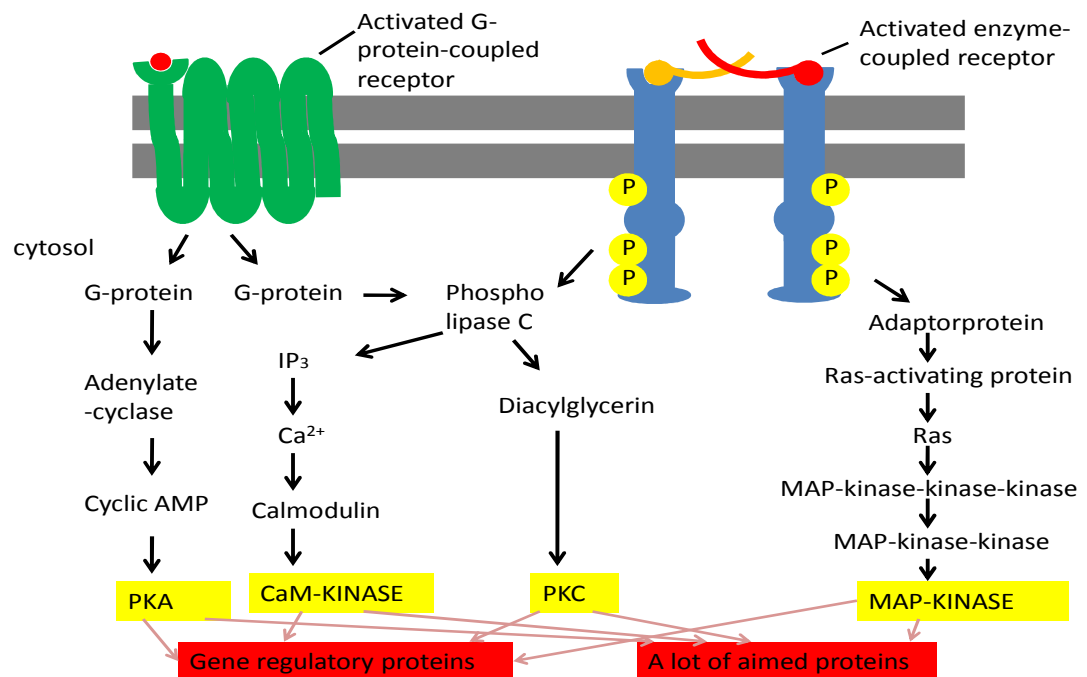


Figure 2 Signaling ways (Alberts, Bray et al. 2005)

Signaling ways are often tight to each other. The picture shows the signaling ways of G-protein coupled receptors and enzyme coupled receptors, both acting at least in one possible pathway over phospholipase C. The involved protein kinases phosphorylate a lot of proteins, including those proteins that belong to other pathways. The red arrows show the tight network of regulatory links. (Alberts, Bray et al. 2005)

Protein kinases are present in every signaling pathway. Through their overlapping phosphorylation they link different pathways to each other and build up a complex network. The single components of this network interact with each other, so that in fact nearly all control systems in the cell communicate with each other. Scientists believe that nearly 2% of all human genes encode for

protein kinases and that in a single eukaryotic cell hundreds of different protein kinases can be present.

When an extracellular signal stimulates the cell, a lot of further mechanisms are possible, for example differentiation, growth, settlement, division and so on. Intracellular signaling proteins are often responsible for the response of a cell to the different extracellular information. These “conclusion-proteins” have different potential phosphorylation sites and each one of them can be phosphorylated by a different protein kinase. In this way different signals are summarized and produce one common response in the ongoing pathway.

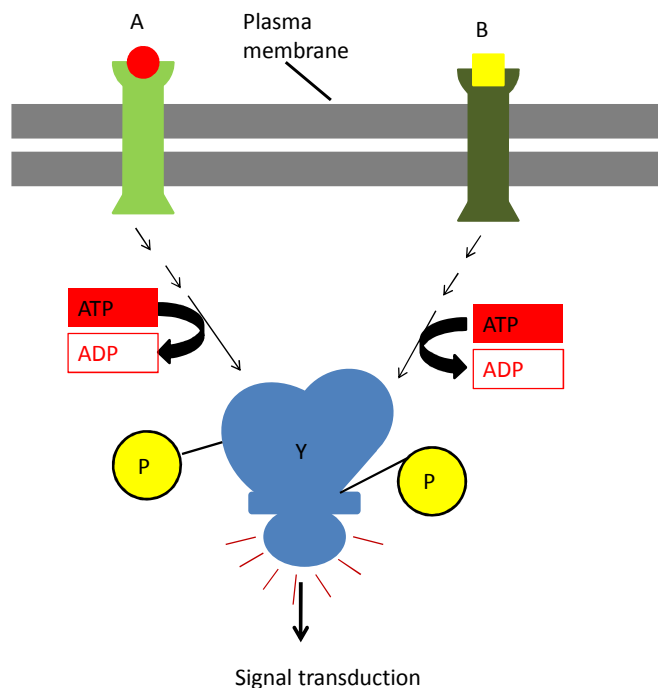


Figure 3 Signaling Conclusion (Alberts, Bray et al. 2005)

Signals A and B are able to activate different protein phosphorylating cascades, everyone of them leads to a phosphorylation of protein Y. But the phosphorylation happens on different sites of the protein. Protein Y is active when both sites are phosphorylated, furthermore the only possibility to activate protein Y is when both signals (A+B) appear at the same time. (Alberts, Bray et al. 2005)

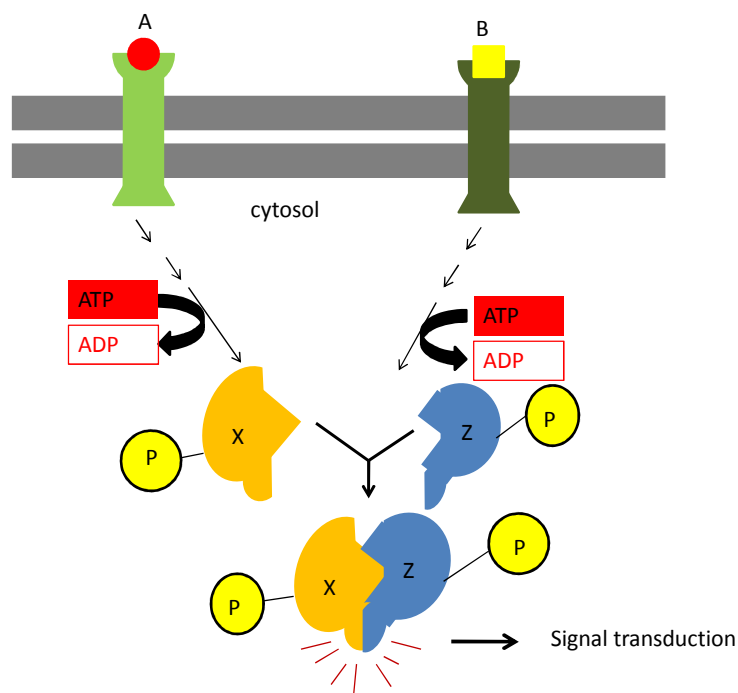


Figure 4 An alternative way (Alberts, Bray et al. 2005)

An alternative way for the signals A and B is the phosphorylation of the proteins X and Z. After phosphorylation of these proteins they can bind to each other and form the active heterodimere proteincomplex XZ. (Alberts, Bray et al. 2005)

Toll Like Receptors (TLR)

To initiate a signaling pathway, an extracellular signal has to bind to a receptor,. One important and popular family of receptors in the inflammatory network are TLRs. TLRs are transmembrane proteins which have the ability to recognize invading microbes and initiate a signaling machinery inside the cell, producing immune and inflammatory responses to destroy the “enemies”. At the moment, eleven members of the TLR family are identified, TLR1-TLR11. The structure of the TLRs shows an extracellular domain, a transmembrane region and a cytoplasmic tail. This cytoplasmic tail is called the TIR (Toll-IL-1R[Interleukin-1-Receptor]) homology domain. For an activation of a TLR, the cognate ligand must bind to the receptor. Different TLRs have different ligands for activation, ranging from bacterial cell wall components, viral double-stranded RNA to small molecule anti-viral or immunomodulatory compounds. After activation of TLRs, there are two possible pathways that can be initiated:

1. The core pathway: most TLRs act through this pathway, it leads to an activation of Nuclear Factor kappa B (NFκB) and the MAPKs p38 and JNK (c-Jun-kinase).
2. The second pathway: is activated by TLR3 and TLR4 and activates NFκB and Interferon Regulatory Factor-3 (IRF3), this enables the induction of an additional set of genes, like antiviral genes.

There is a difference between TLRs located at the cell surface and those TLRs that are located on the endosome. Membrane bound TLRs are: TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10. Intracellular TLRs are: TLR3, TLR7, TLR8 and TLR9. The newly discovered TLRs 11, 12 and 13 seem to belong to the group of intracellular TLRs. (Beutler 2004)

Toll like Receptor 4

This receptor is activated by Lipopolysaccharides (LPS), which are components of the cell surface of gram-negative bacteria, and Lipotechoic Acid (LTA). Both of these substances first bind to the Cluster of Differentiation 14 (CD14)

receptor, which transports the signal to the TLR4. Whereas LTA binds directly to the CD14 receptor, LPS must be transported by a LPS-binding-protein (LBP). The activation of TLR4 leads to a dimerization of the receptor, which further builds up a complex with the protein MD2, afterwards this complex is transported to the cell surface. The activation of TLR4 includes numerous Myeloid Differentiation Primary-Response Protein-88 (MyD88) adaptor family members. (Nishiya and DeFranco 2004)

Over a complex signaling process, a lot of proteins are activated through phosphorylation and complexing mechanisms. This activation-pathway includes the activation of: Interleukin-1-Receptor Associated Kinase-1 (IRAK1), IRAK4 Serine/Threonine Kinases, Tumor Necrosis Factor Associated Receptor Factor-6 (TRAF-6), TGF-Beta-Activated Kinase-1 (TAK1), TAK1-Binding-Protein (TAB1), TAK2-Binding Protein (TAB2), Ubiquitin-Conjugating-Enzyme 13 (Ubc13), Ubiquitin-Conjugating-Enzyme E2-Variant-1 (UEV1A), Evolutionary Conserved Signaling Intermediate in Toll Pathways (ECSIT). (Beutler 2004)

TAK1 also phosphorylates the Inhibitor of Kappa Light Polypeptide Gene Enhancer in B-Cells Kinase (IKK) complex, this complex consists of IKK α , IKK β and IKK γ . Furthermore this IKK complex phosphorylates I κ B, leading to an ubiquitylation and degradation of I κ B. NF κ B is not inhibited by I κ B anymore and able to translocate to the nucleus and induce the expression of its target genes. (Yamamoto and Akira 2004)

Toll like Receptor 2

The TLR2 is activated by bacterial Lipoarabinomannan (LAM), bacterial Lipoprotein (BLP) and Peptidoglycans (PGN). It is also responsible for the recognition of yeast cell wall particles. cPGN and LAM interact with the TLR2 over CD14, initiating a similar downstream signaling like the already discussed signaling after TLR4 stimulation. After stimulation by BLP, the TLR2 cascade leads to an activation of apoptosis as well as to an activation of NF κ B. When the yeast cell wall particle Zymosan binds to CD14 and is transported to TLR2, the

production of Tumor Necrosis Factor (TNF) through the NF κ B pathway is increased by a signaling mechanism. (Hallman, Ramet et al. 2001)

Toll like Receptor 9

This receptor recognizes CpG islands in the bacterial DNA. The mechanism is not really clear at the moment. Possible reactions are:

- TLR9 is activated by extracellular bacterial CpG islands, leading to the endocytosis of these particles.
- The other theory is that the bacteria are phagocytized and the TLR9, which has separately formed in the phagosome, is activated by the CpG islands. Afterwards TLR9 initiates a signaling pathway which stimulates NF κ B. (Hallman, Ramet et al. 2001)

Taken together, TLRs are responsible for the recognition of a range of extracellular signals, leading to an activation of immune- or inflammation specific genes. The TLR system is not fully understood at the moment and it seems as if not all TLRs are already identified. Animal models show new interesting TLRs, which have to be verified and also shown in the human model. Anyway the importance of TLRs is undisputed. (Cook, Pisetsky et al. 2002)

Interleukin 6 (IL6) Signaling

IL6 is a cytokine that is able to initiate several pathways responsible for inflammation, hematopoiesis and oncogenesis by regulation of cell growth, gene activation, proliferation, survival and differentiation. When IL6 binds to its receptor, the receptor dimerizes. The Janus Kinase (JAK kinase) is activated, followed by activation of Ras-mediated signaling. The activated JAK phosphorylates and in this way activates STAT, STAT3 in particular. Furthermore STAT3 forms a dimer and translocates into the nucleus, where it promotes the expression of genes containing the STAT3 response elements. The Ras-mediated components of the pathway (named SHC, Growth Factor

Receptor Bound Protein 2 (GRB2) and Son of Sevenless 1 (SOS)) activate MAP-Kinases downstream followed by activation of transcription factors. These factors are involved in a range of different signaling pathways and regulate a lot of complex promoters and enhancers that are activated in the IL6 and other signaling pathways. (Heinrich, Behrmann et al. 1998)

In its signaling pathway IL6 also activates Phosphoinositide-3-Kinase (PI3K), which functions in a cascade of PI3K/Akt/Nuclear Factor κ -light-chain-enhancer of activated B-cells and promotes the most effective anti-apoptotic characteristics of IL6 against the Transforming Growth Factor-Beta (TGF β). (Heinrich, Behrmann et al. 1998)

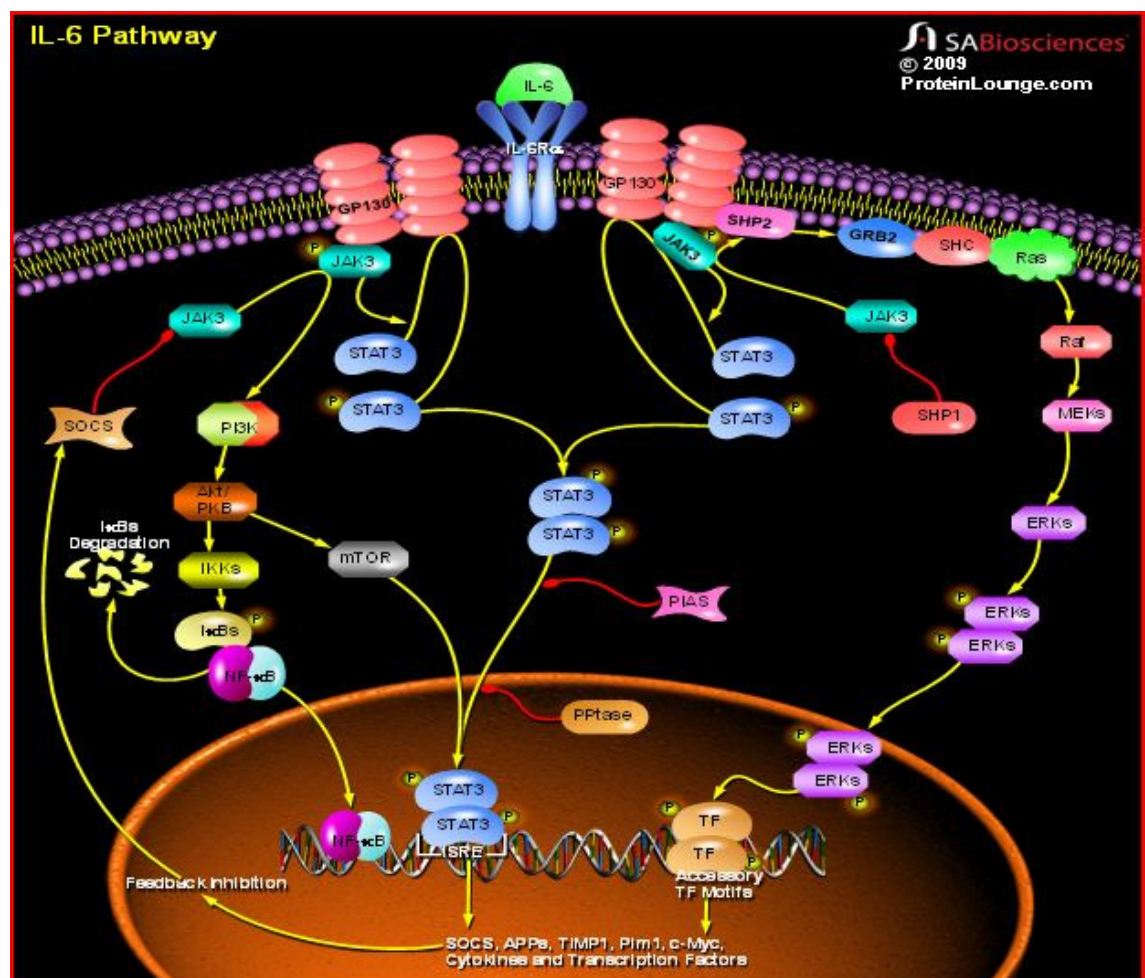


Figure 5 IL6 Signaling

Internet:http://www.sabiosciences.com/pathway.php?sn=IL-6_Pathway (accessed 6. March 2010)

Tumor Necrosis Factor α (TNF α) Signaling

Active TNF α binds to the TNF receptor, initiating the transduction of growth regulatory signals into the cell. It furthermore starts programmed cell death and apoptosis in transformed cells, followed by DNA fragmentation and cytolysis. The so called Death Domain is a protein-protein complex which is necessary to transport the apoptotic signals. TNF α is also able to mediate a survival pathway via NF κ B, for this pathway a phosphorylation of I κ B is necessary, because this enables NF κ B to dissociate from the inhibitory complex and act as a transcription factor in the nucleus. Cells in which the NF κ B signaling pathway is blocked more often undergo apoptosis in response to TNF than normal cells because the survival pathway, including NF κ B signaling is locked. It seems as if the availability of NF κ B influences the ability of TNF to act as an apoptosis inducer and anti tumor agent. (Hong 2007)

In the signal transduction of this pathway a lot of enzymes are involved again. Again they can activate each other through phosphorylation or build up protein complexes for a better signaling system.

In the case of apoptosis-induction, the TNF α pathway includes the proteins p55-TNFR (the TNF receptor), the Death Domain Proteins TRADD, FADD and RIP (these three proteins form the Death Inducing Signaling Complex [DISC]) and Caspase 8, which induces cell death.

In the protective pathway, additional proteins are needed: IKK, I κ B and NF κ B the mechanism of these proteins, their interactions and their inhibition have already been described above. (Hong 2007)

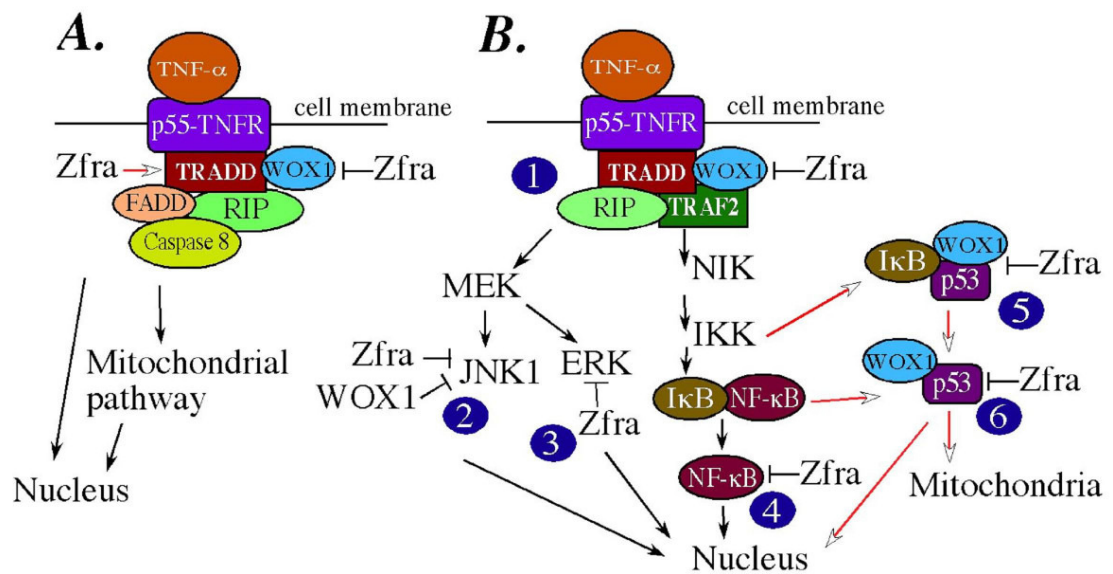


Figure 6 TNF α Signaling (Hong 2007)

NF κ B signaling

Nuclear Factor kappa B proteins are also called Rel proteins, they include p52/p100, p50/p105, c-Rel, RelA/p65 and RelB. These proteins can function as transcription factors when they are in their dimeric form. As transcription factors, they control gene expression of a broad range of genes, regulating inflammation, innate and adaptive immunity, stress responses, B-cell development and lymphoid organogenesis. Normally Rel proteins are inhibited by an I κ B complex, which binds to the proteins and thereby stops their function. The IKK complex is activated by LPS, proinflammatory cytokines, growth factors or antigen receptors. This activated IKK complex is able to dissociate I κ B from the Rel protein by phosphorylation. Phosphorylating I κ B leads to ubiquitylation and degradation and furthermore free Rel proteins. Furthermore, the NF κ B/Rel proteins can translocate to the nucleus and either alone or in combination with other transcription factor families start the transcription of its target genes. (Hayden and Ghosh 2008)

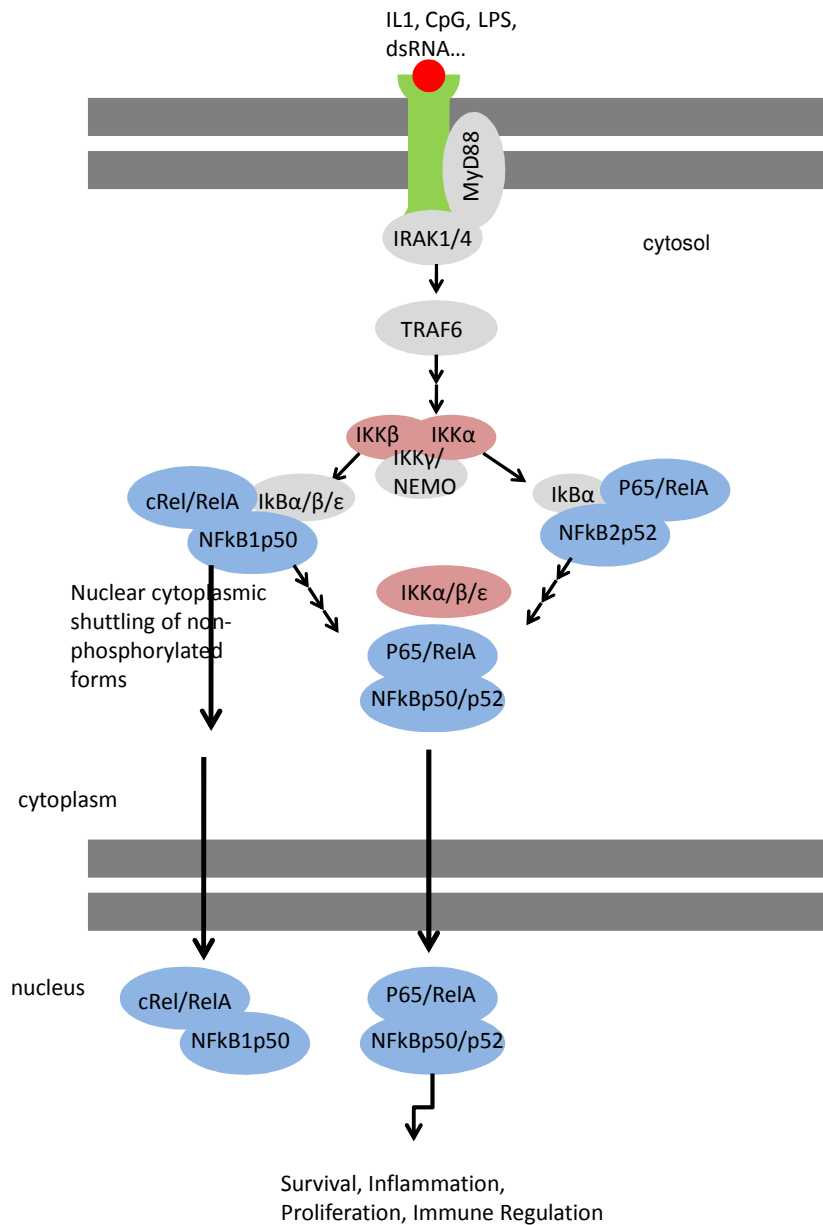


Figure 7 Signaling through NFκB (Alberts, Bray et al. 2005)

The alternative pathway signals through activation of the NFκBp100/RelB complex. This complex is inactive under normal conditions but a set of receptors is able to activate it. Furthermore a kinase, called NIK, is activated

and in turn activates the IKK α complex which is responsible for phosphorylation of the NFkBp100 complex. This phosphorylation leads to an ubiquitination and further proteasomal processing to NFkBp52. The newly formed NFkBp52/RelB complex is transcriptionally active, is able to translocate into the nucleus and induce target gene expression. (Hayden and Ghosh 2008)

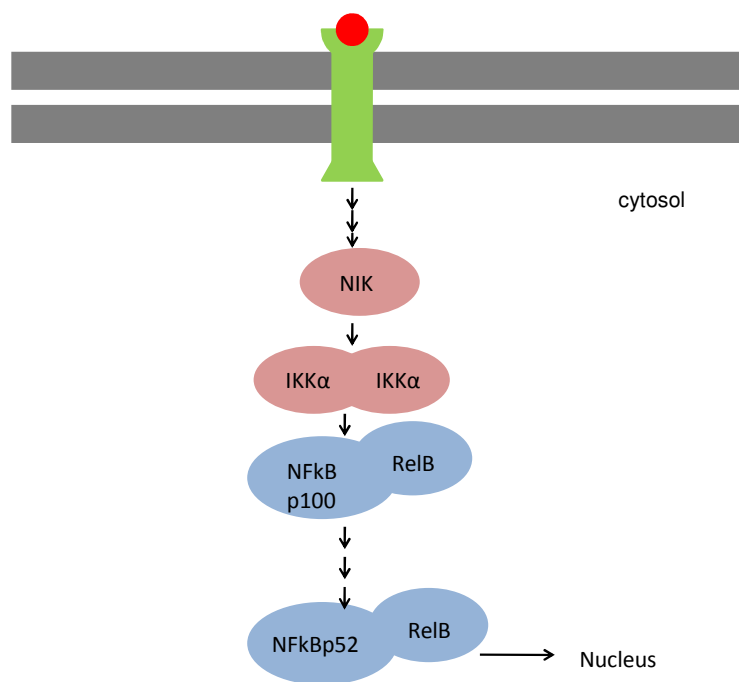


Figure 8 Alternative Pathway (Alberts, Bray et al. 2005)

PPAR γ signaling

The Peroxisome Proliferator-Activated Receptor (PPAR) belongs to the superfamily of hormone receptors. It regulates transcription over a ligand dependent manner. The regulation of transcription is managed by binding to the Retinoid-X-Receptor (RXR). The product is a heterodimeric transcription factor

complex. This complex binds to the promoter regions of its target genes and promotes transcription. The heterodimeric transcription factor complex targets genes that are involved in the catabolism of fatty acids. When the heterodimeric complex of PPAR is bound to the RXR, it can either be active or inactive. For the inactivation, it must be bound to the Nuclear Receptor Corepressor (NCOR) or to the Silencing Mediator for Retinoid and Thyroid Hormone Receptor (SMRT). When a ligand appears, the corepressor dissociates and an activation of co-activators, such as Steroid Receptor Coactivator1 (SRC1), CREB-Binding Protein (CBP/p300), the Tuberous Sclerosis Gene-2 product, the PPAR binding protein, PPAR-Gamma Coactivator 1 and 2 (P-GammaC1/2) and Ara70. The activated form of the PPAR:RXR complex binds to the PPRE region in the 5' region of the target genes and starts with the transcriptional regulation. When PPARs are activated, they bind to the DNA and regulate the transcription of their target genes. (Boitier, Gautier et al. 2003)

The necessary ligands for PPAR are either synthetic, such as hypolipidaemic drugs, anti-inflammatory or insulin-sensitizing drugs. There are also known endogenous PPAR ligands, like fatty acids or their derivatives. PPAR is included in a range of processes inside the cell, these processes can either be part of the normal regulatory system or disease-like. It is involved in lipid- and energy metabolism, inflammation, embryo implantation, diabetes and cancer. Different tissues show different balances of its isoforms ($\alpha, \beta, \delta, \gamma$). Every isoform shows a responsibility for the lipid metabolism, although every single isoform has been shown to have its own specific functions. (Park, Vogelstein et al. 2001)

PPAR α : It is highly expressed in tissues that show a high rate of mitochondrial fatty acid oxidation. Examples are liver, heart, muscle, kidney and cells of the arterial wall (for example smooth-muscle cells, endothelial cells and monocyte-derived macrophages). Furthermore PPAR α is activated by fibrates, fatty acids, eicosanoids, 15-Deoxy-Delta Prostaglandin-J2 (15-d Ptg) and oxidized fatty acids. (Martin 2009)

PPAR γ : It is especially expressed in adipose tissue but can be found in nearly every tissue because it is activated by fatty acids and their derivatives. It plays an

important role in insulin sensitivity, adipogenesis and placental function. PPAR γ has already been shown to have a function in transcription activation, neoplastic processes and colorectal cancer. Thiazolidinedione (TZD) is a class of antidiabetic drugs which act as PPAR γ ligands. (Martin 2009)

PPAR β : It is found in nearly every tissue and is only weakly activated by fatty acids, Ptg α s and leukotrienes. This receptor has no known physiological ligand.

The activity of PPAR γ and PPAR α can be regulated by phosphorylation of specific protein complexes. In the activation system, adaptor molecules, like SH2 containing protein (SHC) and the Growth Factor Receptor-Bound Protein-2 –Son of Sevenless (GRB-2-SOS) are recruited by specific growth factor receptors. Furthermore, the Ras protein is phosphorylated, which in turn activates MAPKs of the Extracellular Signal Regulated Kinase (ERK) type. This type of kinase occurs when Tumor-Growth-Factor (TGF)-Beta Activated Kinase-1 (TAK1) and MAPK/ERK (MEK) are activated. The activity of PPAR α and PPAR γ is inhibited by these kinases. On the other hand PPAR α is activated by G-Protein Coupled Receptor (GPCR) induced phosphorylation of Protein-Kinase-A (PKA) by cyclic Adenosine-3, 5' Monophosphate (cAMP) or p38 MAPK. The differential regulation of the activation of PPAR gives the cell the possibility to control the target gene expression of PPAR in a very rapid manner and to give a fast response to extracellular signals. (Kelly 2001)

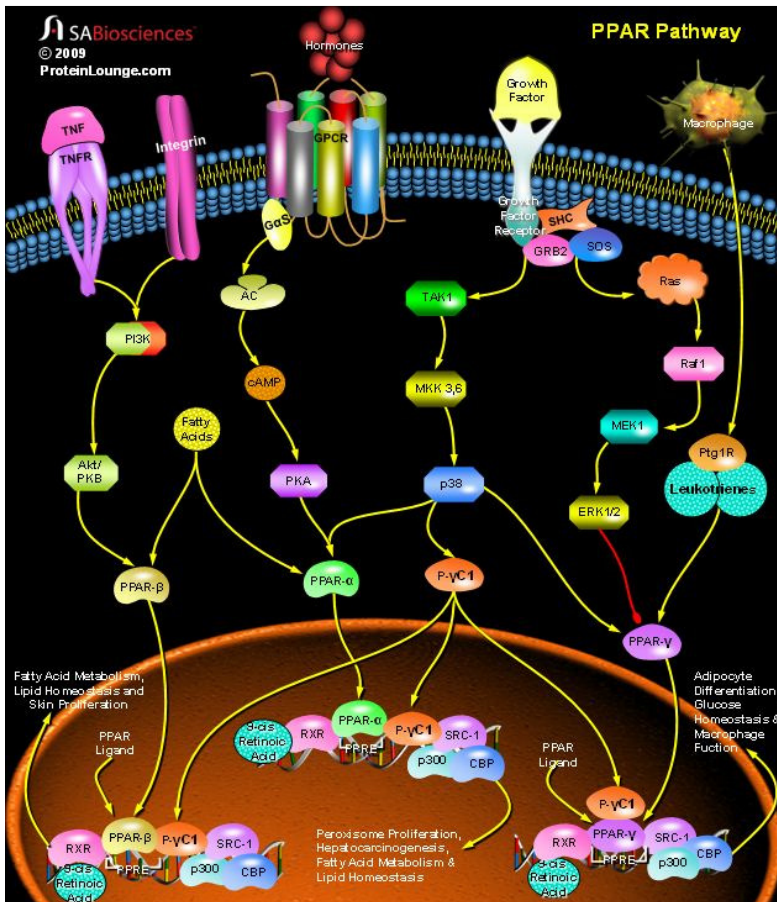


Figure 9 PPAR γ Signaling

Internet:

http://www.google.at/imgres?imgurl=http://208.52.138.117/iapp/maps/ppar.gif&imgrefurl=http://208.52.138.117/iapp/ppar.html&usg=__Q07pHLe_UKweuPIPhI2ivPUeEfA=&h=335&w=290&sz=31&hl=de&start=57&zoom=1&um=1&itbs=1&tbnid=PTzCvtZnwZjpfM:&tbnh=119&tbnw=103&prev=/images%3Fq%3Dpparg%2Bsignaling%26start%3D42%26um%3D1%26hl%3Dde%26sa%3DN%26ndsp%3D21%26tbs%3Disch:1 (accessed 22.September.2010)

Mutations in the structure of PPAR γ can change its functions and cause an insulin resistance syndrome, hypertension and dyslipidemia. PPAR γ inhibits the inflammatory response of monocytes and macrophages by prevention of an activation of NF κ B. When the cell is not stimulated, NF κ B is bound to a corepressor complex, existing of NCoR, Histone Deacetylases 3 (HDAC3), TBL1 and TBLR1. In this state NF κ B is actively repressed. Agents like LPS activate the NF κ B pathway and the corepressor complex is degraded by an ubiquitination-dependent mechanism. Furthermore, instead of the corepressor

complex a coactivator complex binds to NF κ B. This coactivator complex further initiates the transcription by activation of the transcription factor NF κ B. When PPAR γ ligands are present, they lead to a sumoylation of PPAR γ , which is afterwards able to prevent the exchange of corepressor and coactivator complex and lead to a transrepression of the NF κ B dependent promoters. This transrepression maintains the actively repressed state and prevents cytokine expression through the transcription factor NF κ B. (Bailey and Ghosh 2005)

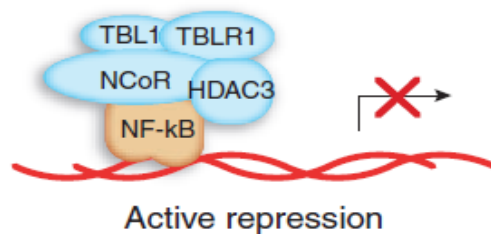


Figure 10 Active Repression (Bailey and Ghosh 2005)

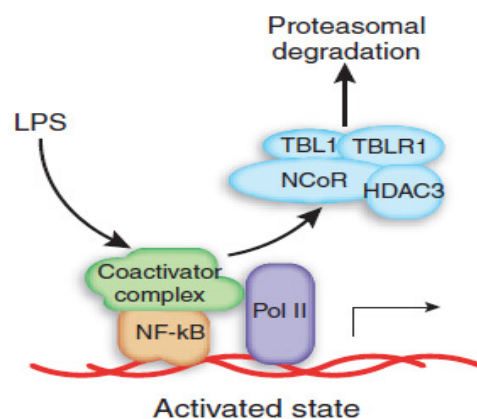


Figure 11 Activated State (Bailey and Ghosh 2005)

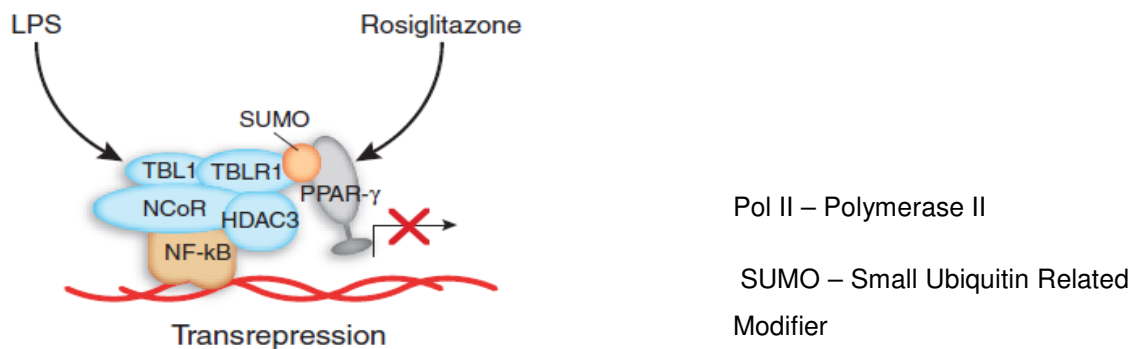


Figure 12 Transrepression (Bailey and Ghosh 2005)

Probiotics

The World Health Organization (WHO) defines probiotics as “Probiotics are essentially helpful, live organisms, usually bacteria, that are used to change or re-establish the intestinal or gut flora and improve our health. They are usually given as part of our food, but can be introduced into our bodies in other ways.” Internet:<http://www.paho.org/English/CFNI/NyamnewsNov1-205.pdf> (accessed 28.9.2010)

It has been shown several times that the microflora of the gut, containing a diverse population of nonpathogenic bacteria, has an influence on the health status and the innate immune system of all mammals. The complex cascades that are necessary for the signal transduction after pathogen microorganisms have attached to the cell surfaces have already been described. All these cascade-mechanisms are necessary for the development of an inflammatory process or even a disease. The estimated number of total microorganisms in the human gut, with around 10^{14} , is even higher than the number of mammalian cells, with around 10^{13} . Looking at the extreme range of bacteria in the gut and their important role for the development and shaping of the immune system, it is not surprising that the microorganisms communicate in some respect with each other and with the host. The way of communication, the interaction between probiotics and host and the cellular and molecular mechanisms of how bacteria

stimulate and influence the immune system, are not already clear. (Clarke and Sperandio 2005)

Bacterial moieties can bind to receptors expressed on the apical or basolateral side of the surface of epithelial cells. The binding activates a signaling cascade inside the cell, of course depending on the receptor and the substance or bacterium that binds. This signaling cascade might trigger the expression of proinflammatory genes. In the human gut, cells are tolerant against their usual microflora, to prevent an ongoing inflammatory response. This tolerance mechanism can be attributed to CD4-protein and T-cell (in the lamina propria) activities. (Kelly, Campbell et al. 2003)

- *Lactobacillus acidophilus* NCFM

Lactobacilli are the largest genus of the lactic acid bacteria group. They predominantly appear in the gut, vaginal and oral regions of mammals. *Lactobacilli* also play an important role in cheese and yoghurt production. By creating lactic acid, they decrease the pH-value in food. *Lactobacillus* is a short gram-positive rod. *Lactobacillus acidophilus* NCFM was first isolated from human excrements. It is commercially available in the USA since the 1970s. This specific strain produces Lactacin B, a bacteriocin, which shows antimicrobial activity against other *Lactobacilli* and *Enterococcus faecalis*. If *Lactobacillus acidophilus* colonizes in the gut, it is able to influence the host's microbial population in favor of his/her health. (Sanders and Klaenhammer 2001)

- *Streptococcus thermophilus*

This species is a lactic acid bacterium too and commercially used in the milk-, cheese- and dairy products production. It is thermophilic, gram-positive and occurs as coccus in pairs or chains. It is able to produce ATP in the presence of oxygen by aerobic respiration and without oxygen through fermentation (a facultative anaerobe bacterium). *Streptococcus thermophilus* does not have the

ability to move or form spores. It is closely related to other pathogenic Streptococci but is not pathogenic itself. (Bolotin, Quinquis et al. 2004)

- *Bifidobacterium lactis*

Belongs to *Bifidobacterium longum*, which colonize the gastrointestinal tract (GIT) and the vagina. Bifidobacteria are gram-positive, anaerobe, branched, rod-shaped, not able to move and not able to form spores. Bifidobacteria ferment sugars into lactic acid and lower the pH-value in the intestine. *Bifidobacterium longum* is the most often recognized species in the human gut and was found to be the first inhabitant in new born infants, especially in breast fed infants. It has beneficial effects on the host's health. Infants fed a formula diet seem to have a different microflora and are more at risk of diarrhea and allergies than breast fed infants. (Reinert 2002)

LPS, used as a control

Lipopolysaccharides are constructs of lipids and polysaccharides, building up large molecules. Gram-negative bacteria produce them on their outer cell membrane. LPS is necessary to protect the bacterium from outer influences, such as chemicals. LPS initiates strong immune responses in animals and human beings. The seriousness of its endotoxin abilities depends on the type of LPS. LPS binds to the CD14/TLR4/MD2 receptor complex, initiating the secretion of several cytokines through activation of a range of transcription factors in human cells. (Stewart, Schluter et al. 2005)

Objectives

The growing number of people suffering from chronic inflammatory diseases is increasing dramatically. The positive influences of probiotics on inflammatory processes have already been discussed in different publications. (Zhang, Li et al. 2005) The aim of this work was to observe the influences of food components, *Lactobacillus acidophilus* NCFM, *Streptococcus thermophilus* ST21, *Bifidobacterium lactis* 420 and LPS in particular, on the expression of inflammatory genes. The mRNA expression of signaling mediators, such as IL6 and TNF α was measured as well as the expression of the transcription factor NF κ B. To investigate anti-inflammatory activities, the expression of PPAR γ was tested too. These genes are important marker of inflammatory mechanisms in human cells. The cell system CACO2 was chosen because it serves as a model for mature enterocytes. The influence of probiotics and LPS on the expression of inflammatory genes was analyzed on both, inflammatory pre- and not-prestimulated cells. The outcomes may contribute to the understanding of how probiotics and LPS can influence inflammatory processes in the human gut and if manipulation with designed probiotics could alter inflammatory processes.

To further enhance this understanding, the expression of miRs after treatment with the already mentioned probiotics and LPS was analyzed. Again the CACO2 cell system was chosen as model for mature enterocytes. These outcomes may give some more information of how bacteria influence the gene expression in human cells.

Materials and Methods

Polymerase Chain Reaction (PCR) and Real time PCR

Both types follow the same principle of DNA amplification. Thus it is possible to detect even low amounts of DNA in a PCR. In the real time PCR the monitoring of the PCR products is done during the run (in real time), in the case of end point PCR this monitoring can soonest been done after the PCR run. All real time PCR analysis were done with the Applied Biosystems (ABI) StepOnePlus Instrument with 96 wells.

Things needed for a PCR reaction:

- Deoxynucleotidetriphosphats (dNTPs): dNTPs of the four DNA bases Adenine, Guanine, Cytosine and Thymine are needed for building up new DNA strands.
- DNA-Polymerase: This enzyme is needed to build up the new DNA strands out of the dNTPs.
- Magnesium-chloride: The DNA-Polymerase needs this substance as a cofactor. For a successful PCR Magnesium-chloride must be added.
- Primers: These oligonucleotides are about 15-40 base pairs long and serve as starting point for the DNA-Polymerase. Primers are necessary because the DNA-polymerase is not able to do the replication on its own, needs starting oligonucleotides which can be elongated during the PCR reaction. It is also necessary to add a forward and a reverse primer to amplify the double stranded DNA. The oligonucleotides furthermore bind to their complementary sequence on the DNA strand.
- Fluorescent reporter: This reporter has the ability to bind to the DNA and gives the possibility to detect and quantify it. This is only possible in real time PCR, not in the end-point system.

Different steps in the PCR reaction setup:

The setup consists of 30-40 cycles of denaturation, annealing and elongation

1. The first step is the denaturation step, at 90-99°C, this step is needed to denature the DNA.
2. In the annealing-step, the primers bind to their complementary sequence on the target DNA. The temperature of this step depends on the primer-structure and varies for every primer. Usually it is between 50°C and 70°C.
3. The Elongation step is necessary for the polymerase to build up the desired DNA strand, starting at the annealed primer. The temperature of this step is usually 72°C.

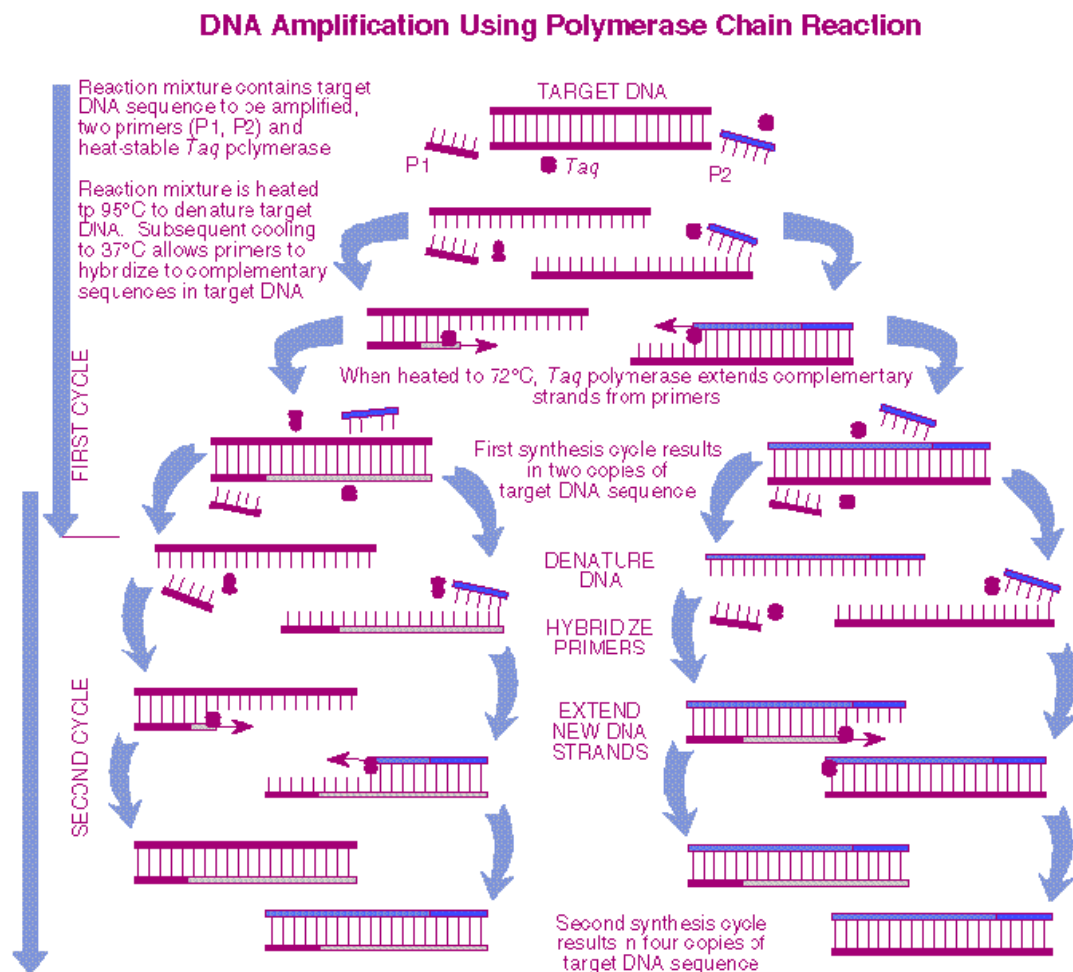


Figure 13 Principle of a PCR reaction (Torak et al.)

There are three principle methods for real time PCR detection:

1. Hydrolysis probe technique
2. Hybridization probe technique
3. DNA-binding agents

Ad 1.:

In addition to the two primers, a third oligonucleotide is added, a so called probe. This probe must fit to the sequence between the forward and the reverse primer. At the 3' and the 5' end of the probe there is a fluorescent dye (fluorochrome), this dye is also called reporter, and a quencher bound. As long as the probe is intact, the quencher absorbs the fluorescence signal of the reporter. But during elongation the probe is destroyed. Thus the fluorescent dye and the quencher are separated and the signal can be detected.

Ad 2.:

For this system two oligonucleotides, which bind directly next to each other on the Sequence, are needed as reporters. One of these oligonucleotides is labeled with a donor fluorochrome, the other one with an acceptor fluorochrome. When both fluorochromes anneal to the DNA, the emitted light of the donor excites the acceptor, whose emitted fluorescence can be detected during annealing. The signal increases after every PCR cycle, because there are more DNA copies after every cycle, which the probes can anneal to.

Ad 3.:

A fluorescent agent binds to the double stranded DNA sequence after the elongation step. In this way the signal increases with every PCR cycle, because after every cycle more DNA is available to bind to. One of the most popular dyes for this type of real time PCR is Sybr Green, which was also used for the experiments in this work.

Cell Culture

In this experiment, CACO2 cells were used. CACO2 cells are immortalized cells of a human colon carcinoma. These cells act as a model for the colon epithel. The cells were grown in a medium containing in 50ml Fetal Bovine Serum (FBS), 5ml P/S/G, 1 ml Ne-Pyr in 450ml medium. They were passaged every 3-4 days with acutase because trypsin was mentioned to be cell toxic.

For the following experiments, CACO2 cells were grown onto 24 well plates for the gene expression analysis and on 6-well plates for the miR analysis because for the miR analysis more cell material was necessary. In the 6 well plates, 3×10^6 cells were seed, in the 24 well plates 2×10^5 . They were kept in an incubator at 37°C and 5% CO₂. The experiments for gene expression were on the one hand done with prestimulation of IL1 β , on the other hand without prestimulation. The prestimulation was necessary to initiate an inflammatory status before the substances (probiotics and LPS) were added to see the effect on the inflammatory gene expression. In the case of prestimulation, cells were incubated with IL1 β for 24 hours before the treatment with food components started. The added concentration of IL1 β was 10ng/ml. Used food components were probiotics and LPS in the following concentrations:

Substance	Concentration
LPS	10 μ g/ml
Lactobacillus acidophilus NCFM	5×10^8 CFU/ml
Streptococcus thermophilus ST21	5×10^8 CFU/ml
Bifidobacterium lactis Bt420	1×10^9 CFU/ml

Table 1 List of Substances and their used Concentrations

All concentrations were chosen because of experiences of the lab or because of well established literature. The probiotics were achieved from Danisco (Madison, USA) in lyophilysed state. They were solved in ddH₂O and heat

inactivated by warming them for 30 minutes on the heat block at 96°C. Afterwards they were immediately cooled down to 4°C.

The cells were harvested, after incubation with the above described substances, after 3, 24 and 48 hours.

The harvesting procedure included:

1. Remove liquid media
2. Wash twice with ice-cold PBS from genXpress (Wiener Neudorf, Austria)
3. Harvesting cells with a policeman cell scraper and stored in PBS for further DNA analysis, in RLT buffer from Qiagen (Hilden, Germany) (first buffer of the RNA isolation kit) for further RNA analysis or in trizol from Invitrogen (Paisley, Scotland) for miR analysis.
4. Freezing cells down on -20°C before further processing

Gene Expression

In a gene expression assay, the content of mRNA of a specific gene is quantified. The mRNA is the product of DNA transcription and the content of mRNA is directly proportional to the gene activity. For the detection of mRNA, total RNA must be extracted from the cells and afterwards it is converted into cDNA. This is necessary for the stability of the sample because RNA tends to degrade very fast by RNases, which ubiquitous. The conversion into cDNA is called reverse transcription.

For the extraction of RNA the RNeasy Mini Kit from Qiagen (Hilden, Germany) was used, the extraction was done by the instruction applied.

The reverse transcription was done with the Phusion RT-PCR Kit from Finnzymes (Espoo, Finland). With the kit, three different primer are delivered for the reverse transcription of mRNA:

- Random-hexamer-primers: This is a mixture of oligonucleotides consisting of six randomly chosen base pairs, enabling the primers to bind to nearly every mRNA.
- Oligo(dT) primer: These primers consist of only Thymine and bind to the polyA-tail of the mRNA.
- Sequence specific primers: With this type of primers only single genes or parts of the target genes are converted into cDNA.

In this work the Oligo(dT) primer was chosen for the reverse transcription. 8 µl of mRNA template were taken as template for starting. All other steps and the PCR conditions were done following the instruction applied.

For the real time PCRs, the following reaction set up was chosen:

Per aliquot 10µl of total reaction volume was prepared, this volume consisted of:

- 5 µl MasterMix: SensiMix SYBR Kit from genXpress (Wiener Neudorf, Austria) was used
- 1 µl Primer: the correct concentration was prepared before adding the primer, from Biomers
- 3 µl nuclease free water
- 1 µl cDNA

The Magnesiumchloride (MgCl_2) concentration for all assays was 3mM. The SensiMix already contains the Sybr Green dye for the real-time analysis.

The temperature conditions were as follows:

40 cycles	Step	Annealing Temperature	Time
	Hot Start for the Activation of the Polymerase	95 °C	10 minutes
	Denaturation	95 °C	30 seconds
	Annealing	57 °C-60 °C	40 seconds
	Elongation	72 °C	30 seconds
		Melt Curve	

Table 2 Temperature conditions of Real Time PCR

Used primer pairs:

Primer	Sequence 5'-3'	Annealing temperature	Concentration
IL6	Forward:GGTACATCCTCGACGGCATCT Reverse:GTGCCTCTTTGCTGCTTTCAC	59 °C	10pmol/μl
TNFα	Forward:AAGAGGGAGAGAAGCAACTACAGA Reverse:GGTGGAGCCGTGGGTCAG	60 °C	2,5pmol/μl
NFκBp65	Forward:AGAAAGAGGACATTGAGGTG Reverse:CCCCCAGGTCTTCATCATCA	59 °C	5pmol/μl
PPARγ	Forward:AAGTTCAATGCACTGGAATTAGATGA Reverse:TGTAGCAGGTTGTCTTGAATGTCTTC	57 °C	10pmol/μl
GAPDH	Forward:CGACCACTTTGTCAAGCTCA Reverse:AGGGGAGATTCAGTGTGGTG	51 °C	5pmol/μl

Table 3 Used Primers

Typically real time PCR curves look like this:

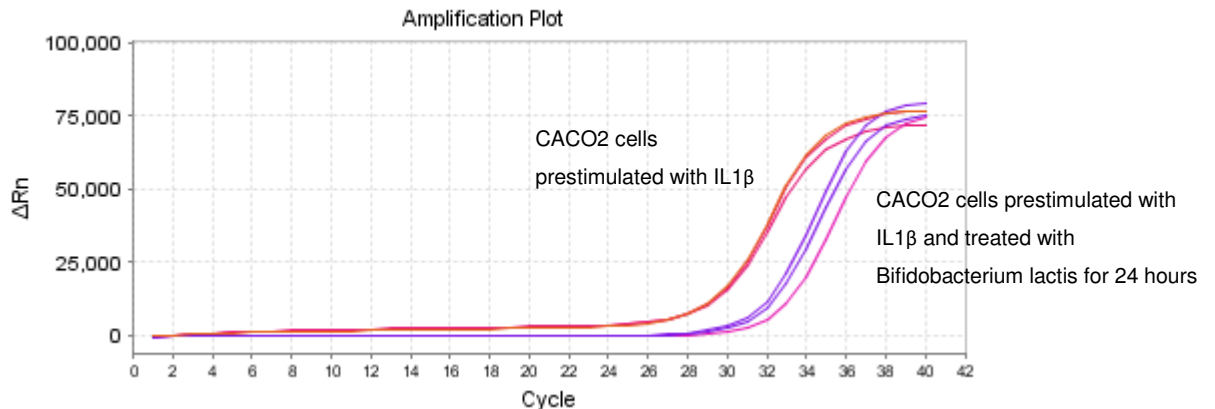


Figure 14 Real Time PCR Curve

This real time PCR run shows the expression of IL6 in CACO2 cells prestimulated with IL1 β . The shown results were detected from cells treated with Bifidobacterium lactis and cells without treatment after prestimulation.

miR Expression

The used extraction kit for miRs was mirVana miR Isolation Kit from Life Technologies (Carlsbad, California). Just like mRNA, extracted miR is not very stable, so it was immediately transcribed into cDNA. The used reverse transcription kit was the QuantiMir RT Kit – Small RNA Quantitation System from System Biosciences (ordered at BioCat – Heidelberg, Germany). Everything was done according to the manual applied. The primers for the detection of miR 7i, 27b and 155 were also from System Biosciences (ordered at BioCat – Heidelberg, Germany). Typically the primers for miR detection consist of the full sequence of the miR. In this kit a universal reverse primer for all miRs was included. The miR were quantified using real time PCR. The SensiMix SYBR Kit from genXpress (Wiener Neudorf, Austria) was used. (The MgCl₂ concentration of the SensiMix fit with the needed concentration of the polymerase for the reaction.)

The temperature conditions were as follows:

Step		Temperature	Time
		50 °C	2 minutes
hot start for the activation of the polymerase		95 °C	10 minutes
40 cycles	denaturation	95 °C	15 seconds
	annealing	60-64 °C	1 minute
	Melt curve		

Table 4 Real Time PCR Conditions for miRNAs

Specific primers needed their specific conditions:

Primer		Annealing Temperature	Concentration
miR7i		62 °C	10µM
miR27b		62 °C	10µM
miR155		62 °C	10µM
Control RNAs	miR16 (human/mouse)	60 °C	10µM
	U6 (human snRNA)	64 °C	10µM

Table 5 Detected miRNAs

The principle of the QuantiMir kit for reverse transcription of miRs is the following:

- The extracted miR becomes polyA tailed
- An oligo-dT adaptor anneals to the prepared polyA tail and furthermore
- Conversion to cDNA can start using
- Reverse transcriptase to create “first strand cDNAs”

This workflow ensures the conversion of all miRs in the sample to cDNAs.

Statistical Analysis

The real time data were analyzed with the StepOne software belonging to the StepOne real time PCR Life Technologies (Carlsbad, California). Statistical analysis were performed using OriginPro 8G from OriginLab (Northampton, USA) for calculation of two tailed test for variances and a two tailed (student's) t-test. Gene expression data were normalized to the expression of the housekeeping gene Glycerinaldehyde-3-phosphate Dehydrogenase (GAPDH) which is necessary for glycolysis. There is evidence that it is expressed constantly to all conditions in human cells. This fact gives the possibility to calculate a change in specific gene expressions, when the GAPDH expression stays the same. In the case of miR detection, the housekeeping RNA was U6, which is a snRNA, a small RNA. The principle for expression of this small RNA is the same for human cells like it is for GAPDH.

The figures and Box-Plots have also been made by OriginPro 8G. Results were considered significant at $p < 0.05$.

Results

Gene Expression in IL1 β Prestimulated Cells

The results of prestimulation with IL1 β for 24 hours, followed by a treatment with *Lactobacillus acidophilus* NCFM, *Streptococcus thermophilus* ST21, *Bifidobacterium lactis* Bt420 or LPS are declared for every figure in the following chapter.

Control means the ratio of gene expression in prestimulated cells compared to gene expression of not prestimulated cells.

(*shows significant alterations)

IL6 Expression after 3 hours

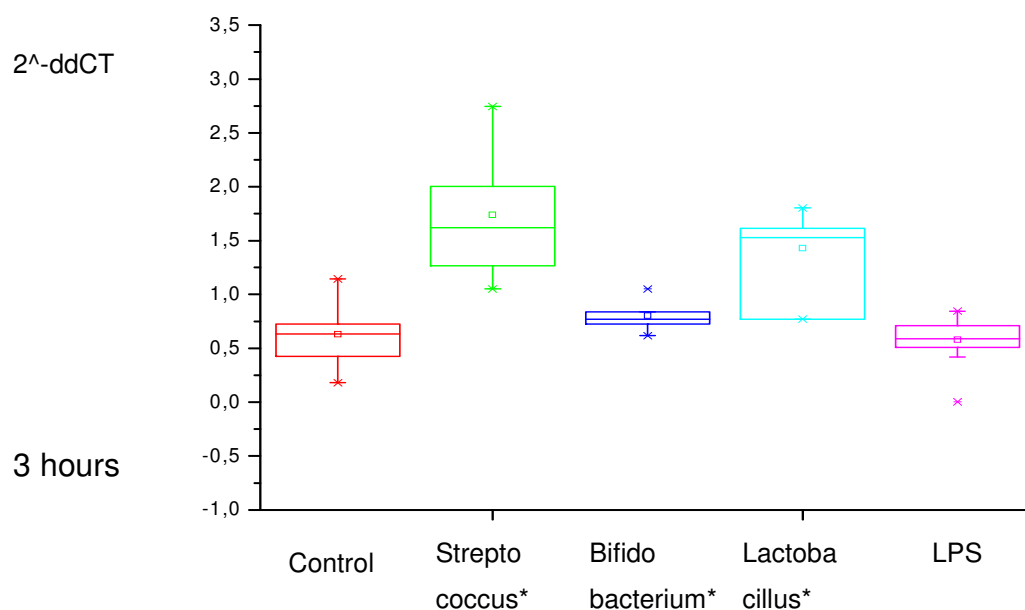


Figure 15 IL6 Expression prestimulated 3 hours

The expression of IL6 after *Streptococcus*, *Bifidobacterium* and *Lactobacillus* treatment was statistically significant higher than the expression of the control.

Streptococcus treatment depicted a 1.7-fold increase $\pm$ 0.5 SD, Bifidobacterium treatment a decrease to the 0.8-fold $\pm$ 0.2 SD, Lactobacillus a 1.4-fold increase $\pm$ 0.4SD and LPS a decrease to the 0.6-fold $\pm$ 0.2SD in IL6 expression.

IL6 Expression after 24 hours

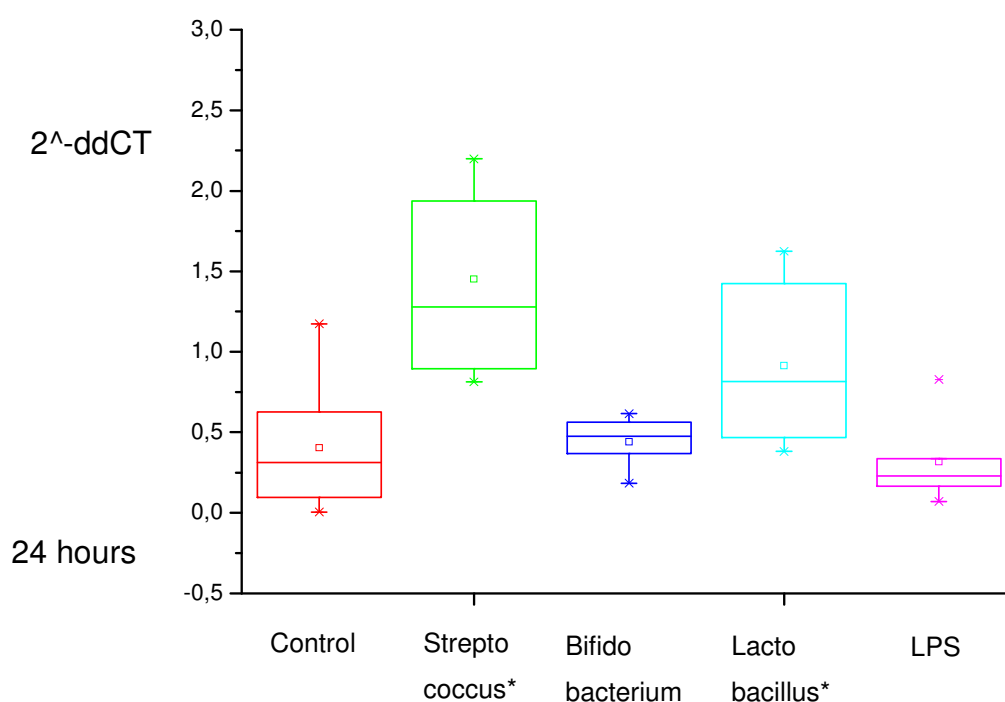


Figure 16 IL6 Expression prestimulated 24 hours

The expression after Streptococcus and Lactobacillus treatment was significantly higher than the control's.

The expression level of IL6 after 24 hours showed an increase to the 1.5-fold $\pm$ 0.6 SD after Streptococcus treatment, a decrease to 0.4-fold $\pm$ 0.2 SD after Bifidobacterium treatment, a decrease to the 0.9-fold $\pm$ 0.5 SD after Lactobacillus treatment and a decrease to the 0.3-fold $\pm$ 0.3 SD after LPS treatment.

TNF α Expression after 3 hours

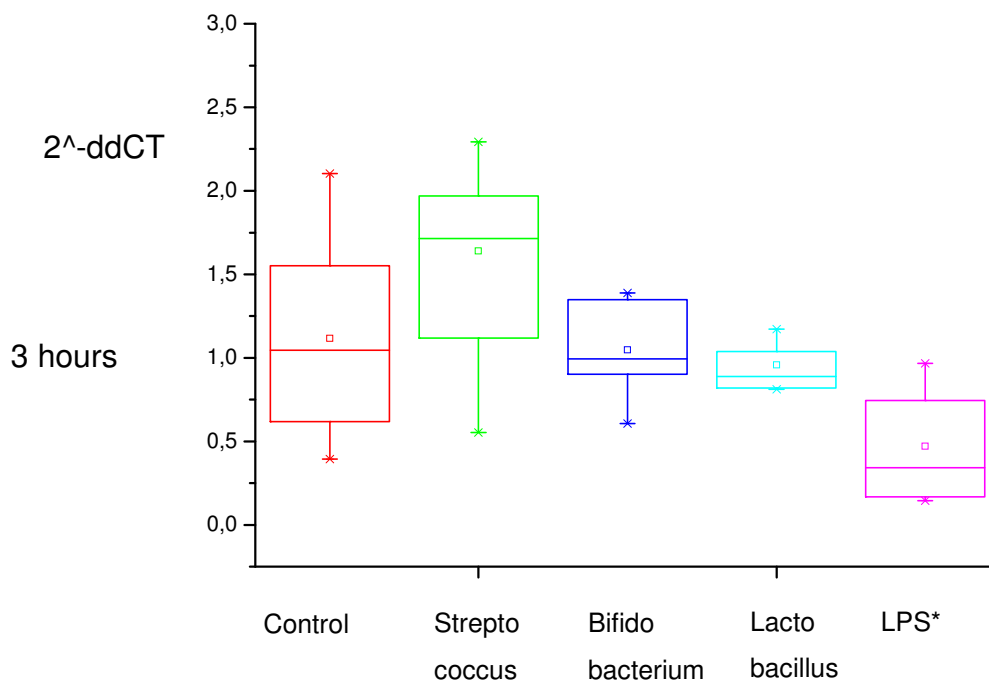


Figure 17 TNF α Expression prestimulated 3 hours

The Expression of TNF α after LPS treatment was significantly decreased, compared to the control.

After Streptococcus treatment the expression of TNF α was increased to the 1.6-fold $\pm$ 0.6 SD, to the 1.05-fold $\pm$ 0.3 SD after Bifidobacterium treatment, equal after Lactobacillus treatment 1 $\pm$ 0.1 SD and decreased after LPS treatment to the 0.5-fold $\pm$ 0.3 SD.

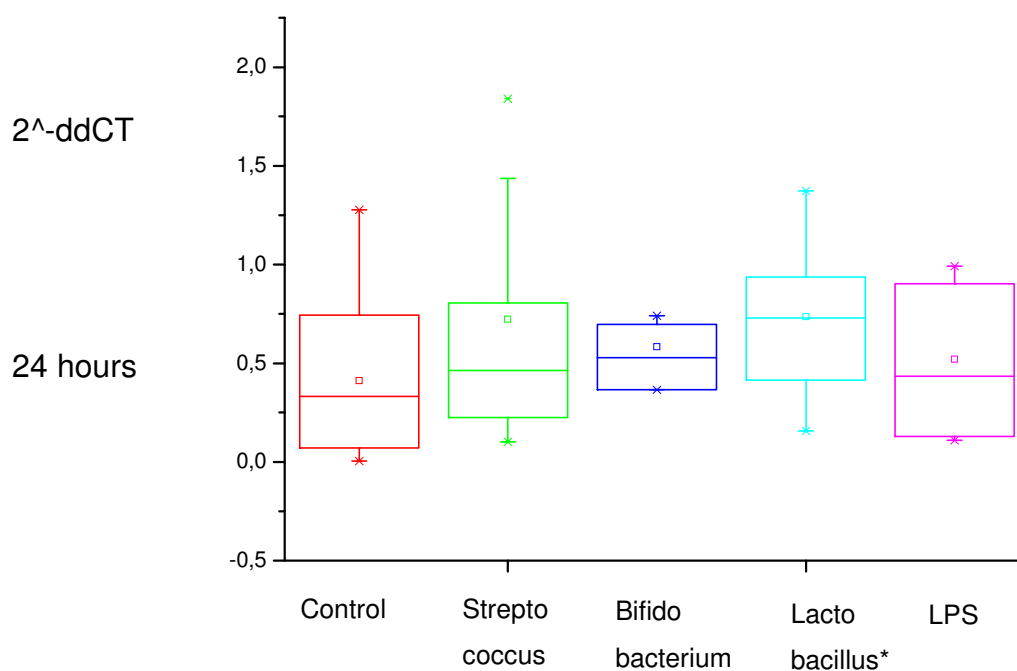
TNF α Expression after 24 hours

Figure 18 TNF α Expression prestimulated 24 hours

The Expression level after Lactobacillus treatment differed significantly from the control.

Treatment with Streptococcus showed a decrease in the expression of TNF α to the 0.7- fold $\pm$ 0.6 SD, a decrease to the 0.6-fold $\pm$ 0.2 SD after Bifidobacterium treatment, a decrease to the 0.7-fold $\pm$ 0.4 SD after Lactobacillus treatment and a decrease to the 0.5-fold $\pm$ 0.4 SD after LPS treatment.

NFκBp65 Expression after 3 hours

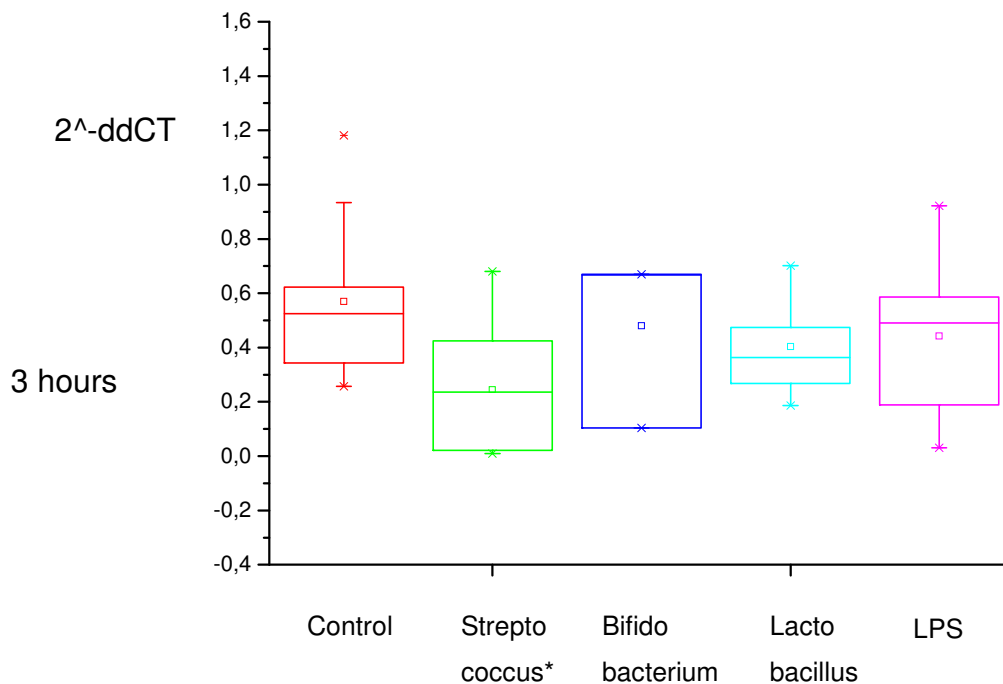


Figure 19 NFκBp65 Expression prestimulated 3 hours

Expression after Streptococcus treatment differed significantly from the control-expression.

The decreases in the expression of NFκB were as follows: after Streptococcus treatment 0.3-fold $\pm$ 0.2 SD, 0.5-fold $\pm$ 0.3 SD after Bifidobacterium treatment, 0.4-fold $\pm$ 0.2 SD after Lactobacillus treatment and 0.4-fold $\pm$ 0.3 SD after LPS treatment.

NFκBp65 Expression after 24 hours

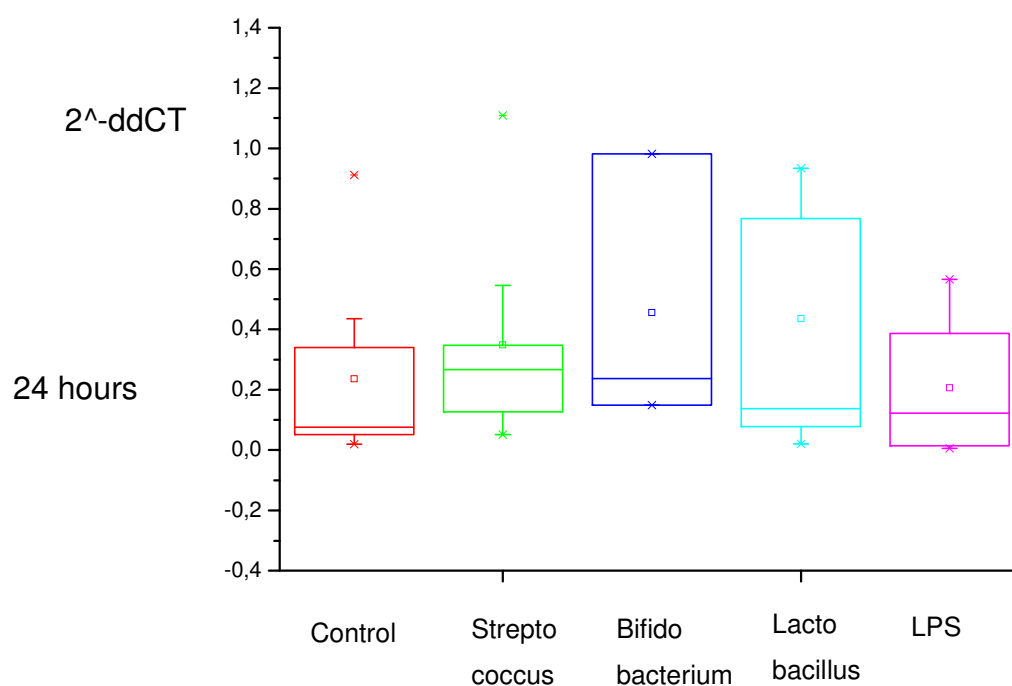


Figure 20 NFκBp65 Expression prestimulated 24 hours

No significant alterations were found.

The changes in the expression level after 24 hours showed a decrease to the 0.4-fold $\pm$ 0.3 after Streptococcus treatment, a decrease to the 0.5-fold $\pm$ 0.5 SD after Bifidobacterium treatment, a decrease to the 0.4-fold $\pm$ 0.4 SD after Lactobacillus treatment and a decrease to the 0.2-fold $\pm$ 0.2 SD after LPS treatment.

PPAR γ Expression after 3 hours

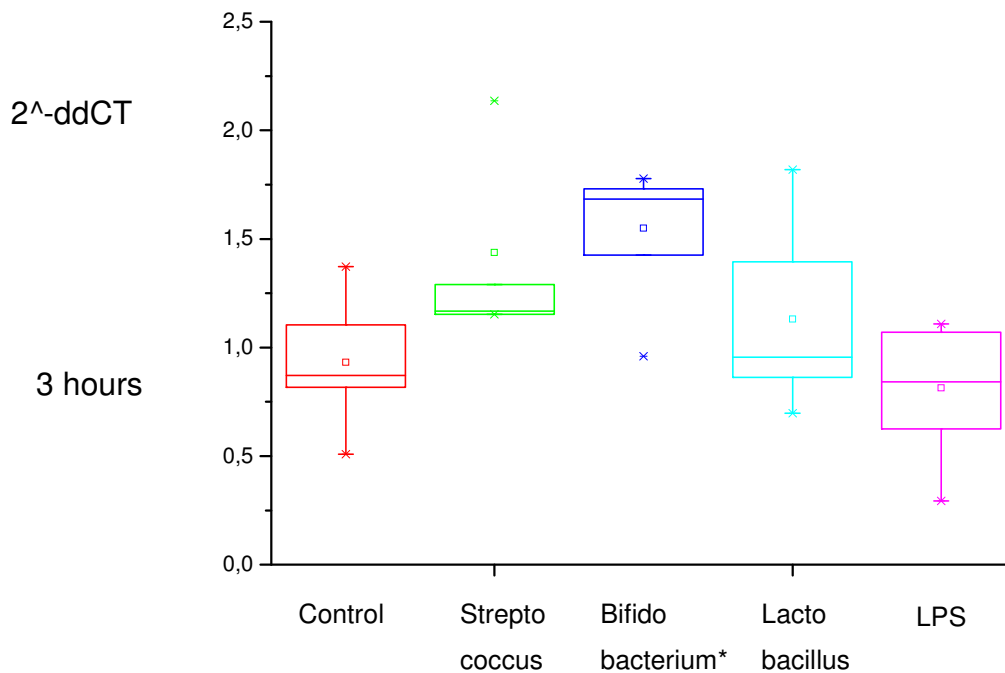


Figure 21 PPAR γ Expression prestimulated 3 hours

Bifidobacterium treatment increased the expression significantly compared to the control after 3 hours.

Treatment with Streptococcus increased the expression of PPAR γ to the 1.4-fold $\pm$ 0.5 SD, treatment with Bifidobacterium increased the expression to the 1.6 $\pm$ 0.3 SD, Lactobacillus treatment increased the expression to the 1.1-fold $\pm$ 0.4 SD and treatment with LPS decreased the expression to the 0.8-fold $\pm$ 0.3 SD.

PPAR γ Expression after 24 hours

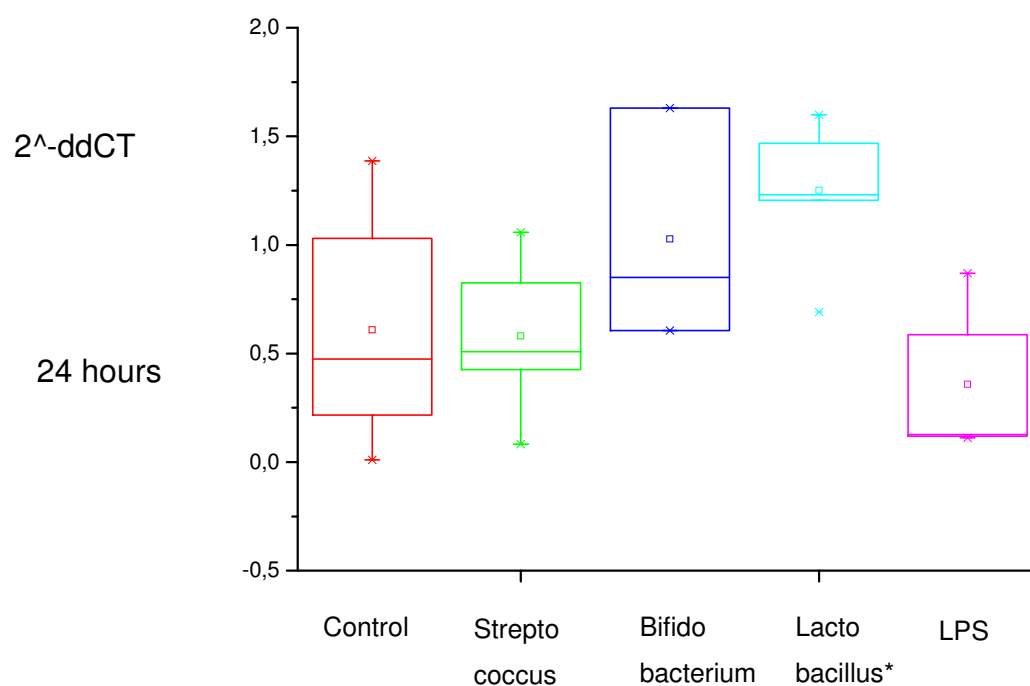


Figure 22 PPAR γ Expression prestimulated 24 hours

The Expression level after Lactobacillus treatment was significantly increased compared to the control-expression.

The expression after Streptococcus treatment showed a decrease in the expression level of PPAR γ to the 0.6-fold $\pm$ 0.4 SD, Bifidobacterium treatment showed an equal expression level 1 $\pm$ 0.5 SD, Lactobacillus treatment showed an increase to the 1.3-fold $\pm$ 0.3 SD and LPS treatment showed a 0.4-fold $\pm$ 0.3 SD decrease.

Gene Expression without Prestimulation

The same experiments, except *Bifidobacterium lactis*, have been done with CACO2 cells without prestimulation with IL1 β . The results were the following (The control is at 1):

IL6 Expression after 3 hours

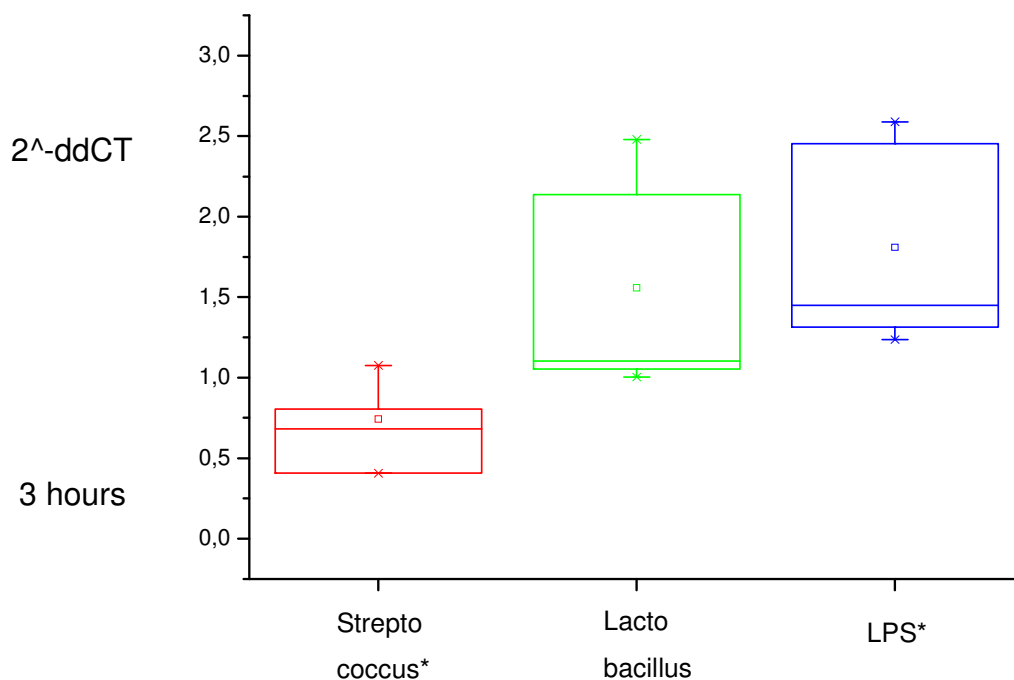


Figure 23 IL6 Expression 3 hours

The expression after LPS treatment was significantly higher than the expression of the control.

Streptococcus treatment decreased the expression to the 0.7-fold $\pm$ 0.3 SD, Lactobacillus treatment increased the expression to the 1.6-fold $\pm$ 0.6 SD and LPS increased the expression to the 1.8-fold $\pm$ 0.7 SD.

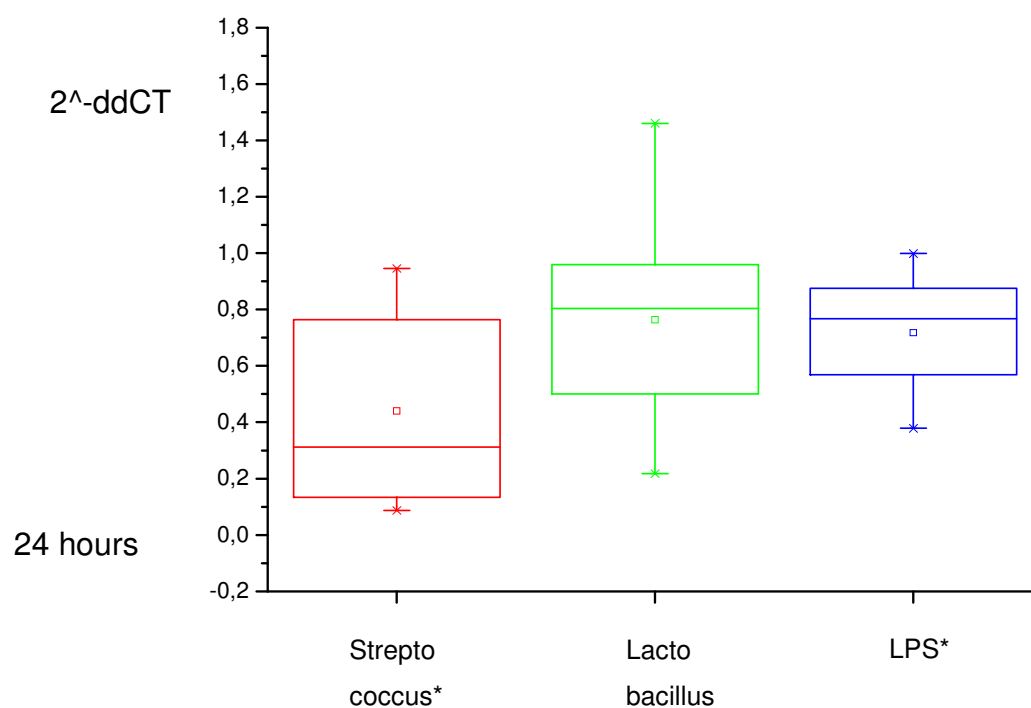
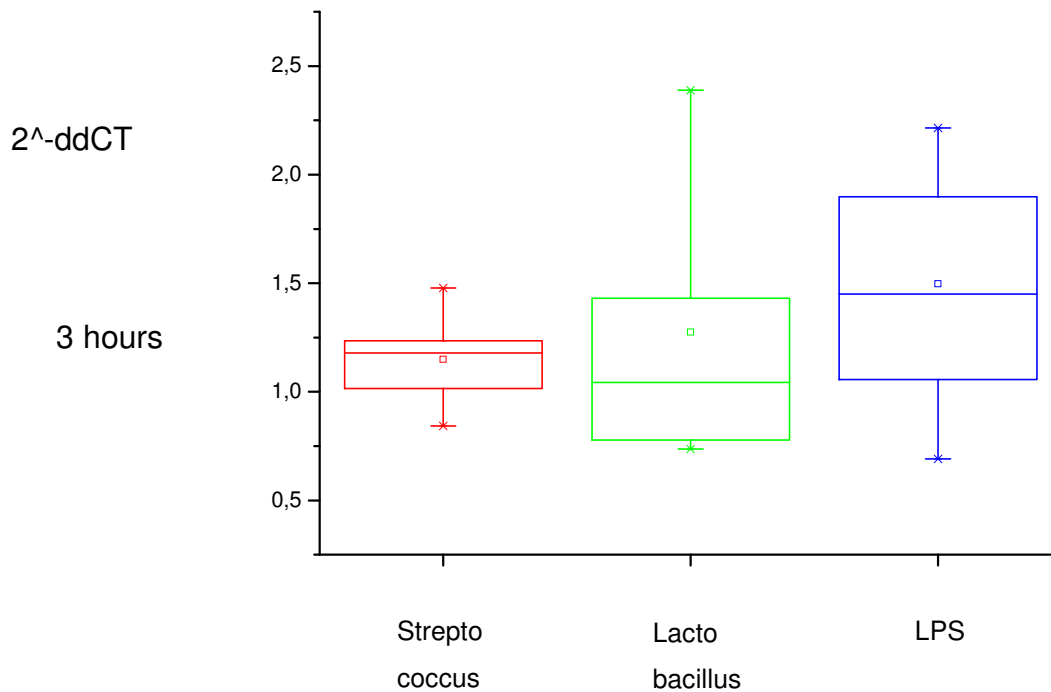
IL6 Expression after 24 hours

Figure 24 IL6 Expression 24 hours

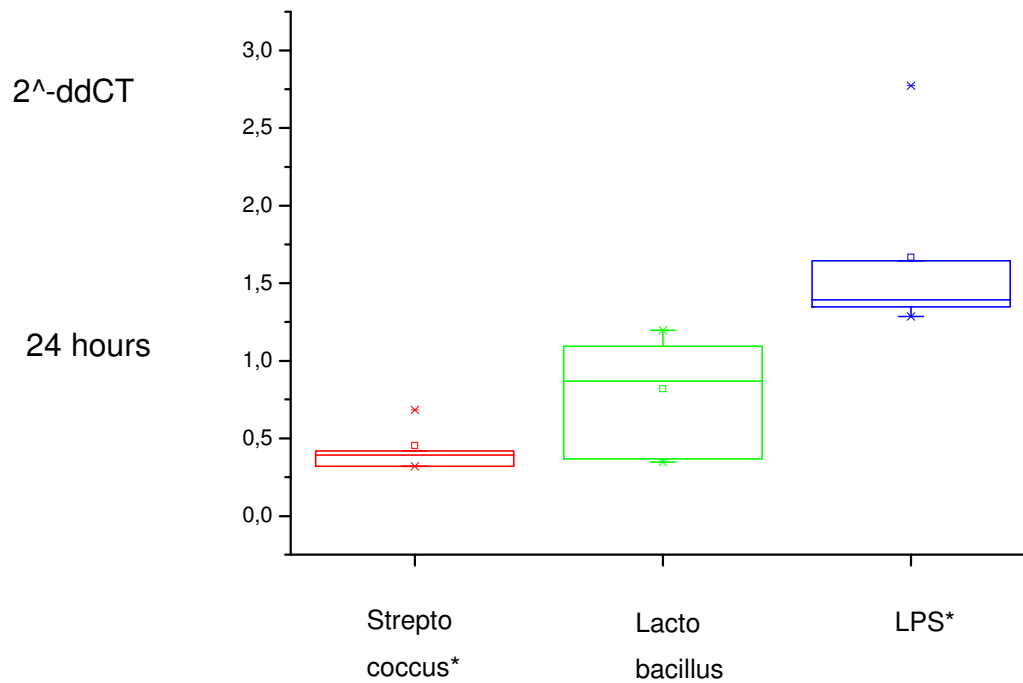
Alterations after Streptococcus and LPS treatment were statistically significant.

Streptococcus treatment decreased the expression to the 0.4-fold $\pm$ 0.3 SD, Lactobacillus treatment decreased the expression to the 0.8-fold $\pm$ 0.4 SD and LPS treatment decreased the expression to the 0.7-fold $\pm$ 0.2 SD.

TNF α Expression after 3 hours**Figure 25 TNF α Expression 3 hours**

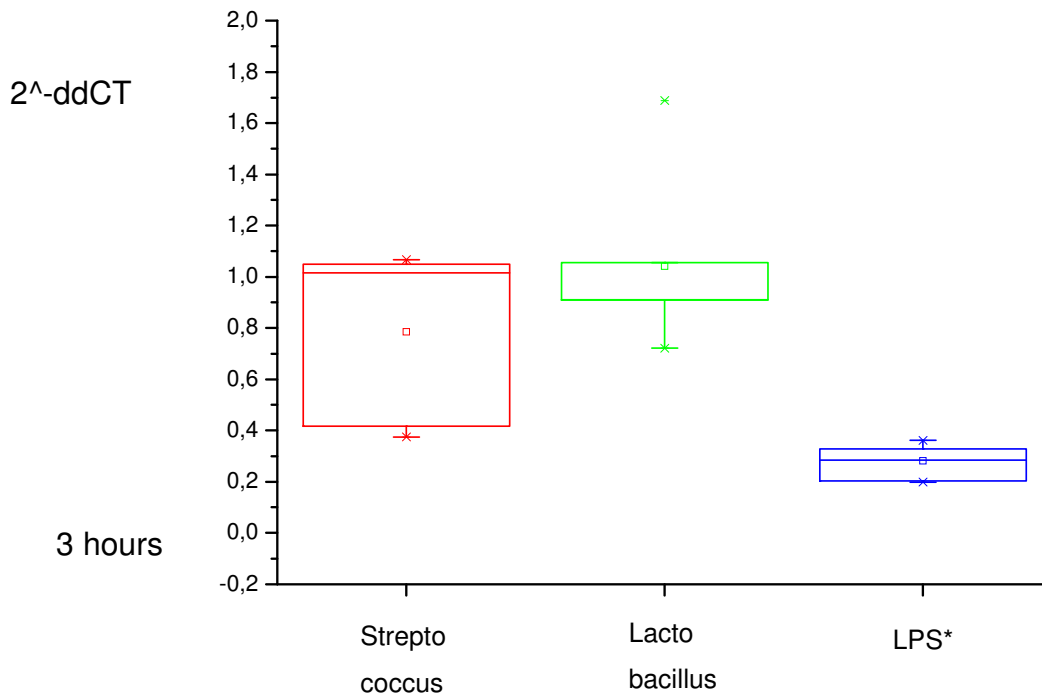
No significant alterations.

Streptococcus treatment changed the expression to the 1.1-fold $\pm$ 0.2 SD, Lactobacillus treatment changed the expression to the 1.3-fold $\pm$ 0.7 SD and LPS treatment changed the expression to the 1.5-fold $\pm$ 0.6 SD.

TNF α Expression after 24 hours**Figure 26 TNF α Expression 24 hours**

Streptococcus and LPS treatment changed the expression significant.

After treatment with Streptococcus the expression was decreased to the 0.5-fold $\pm$ 0.2 SD, treatment with Lactobacillus decreased the expression to the 0.8-fold $\pm$ 0.4 SD and LPS treatment decreased the expression to the 1.7-fold $\pm$ 0.6 SD.

NFκBp65 Expression after 3 hours**Figure 27 NFκBp65 Expression 3 hours**

LPS treatment altered the expression significantly.

The expression of NFκBp65 was decreased by Streptococcus treatment to the 0.8-fold $\pm$ 0.4 SD, after Lactobacillus treatment it was equal 1 $\pm$ 0.3 SD and LPS treatment decreased the expression to the 0.3-fold $\pm$ 0.07 SD.

NFκBp65 Expression after 24 hours

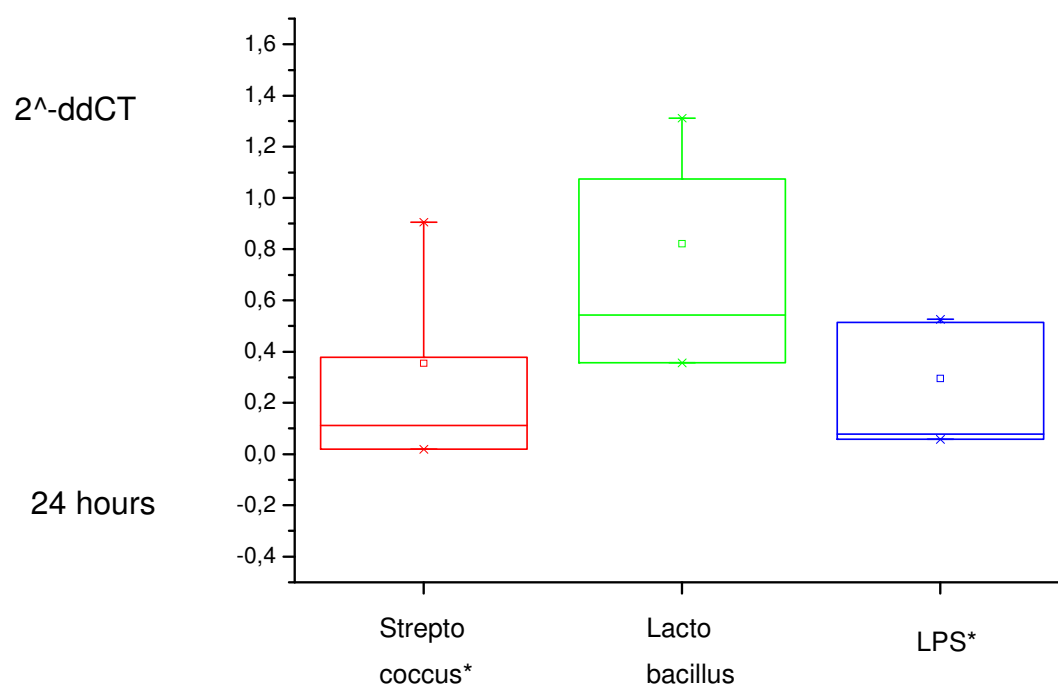
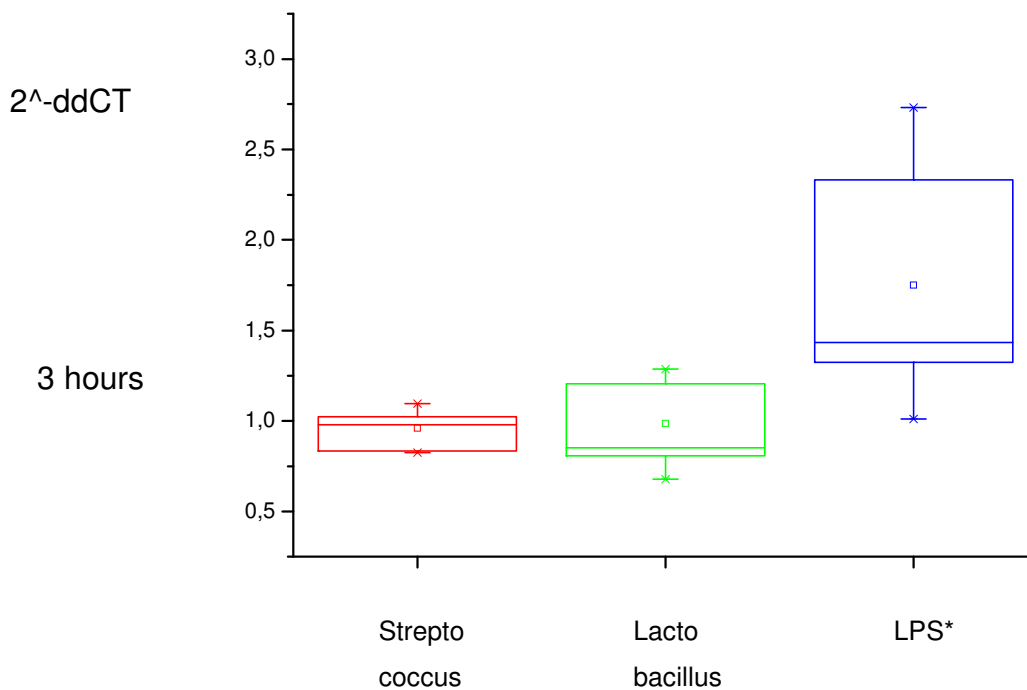


Figure 28 NFκBp65 Expression 24 hours

Streptococcus and LPS changed the expression significantly.

Streptococcus treatment showed a decrease in the expression to the 0.4-fold $\pm$ 0.4 SD, Lactobacillus showed a decrease to the 0.8-fold $\pm$ 0.4 SD and LPS showed a decrease to the 0.3-fold $\pm$ 0.3 SD.

PPAR γ Expression after 3 hours**Figure 29 PPAR γ Expression 3 hours**

The expression level after LPS treatment was significantly different than the expression level of the control.

Streptococcus treatment did not alter the expression 1 ± 0.1 SD, Lactobacillus treatment also did not show a changed expression level 1 ± 0.2 SD and LPS changed the expression to the 1.8-fold ± 0.7 SD.

PPAR γ Expression after 24 hours

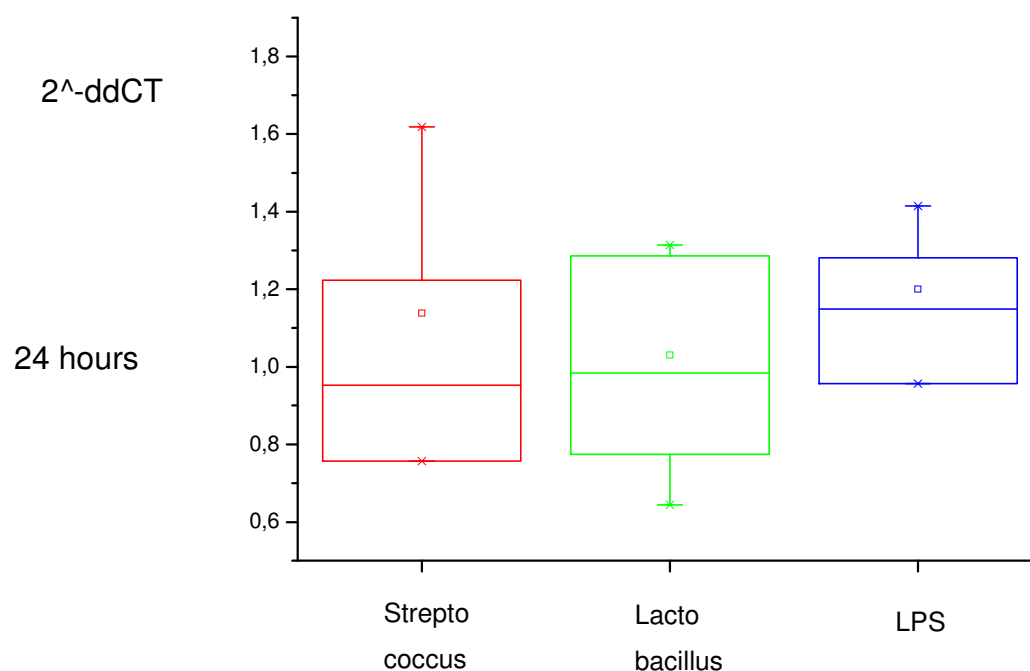


Figure 30 PPAR γ Expression 24 hours

No significant alterations.

Streptococcus treatment increased the expression to the 1.1-fold $\pm$ 0.4 SD, Lactobacillus treatment did not alter the expression 1 ± 0.3 SD and LPS increased the expression to the 1.2-fold $\pm$ 0.2 SD.

miR Level after Probiotic Treatment

Cells for miR detection have not been prestimulated with IL1 β . The control was set to 1. After treatment with *Lactobacillus acidophilus*, *Streptococcus thermophilus* or LPS for 24 hours, the miR levels were as follows:

Lactobacillus acidophilus:

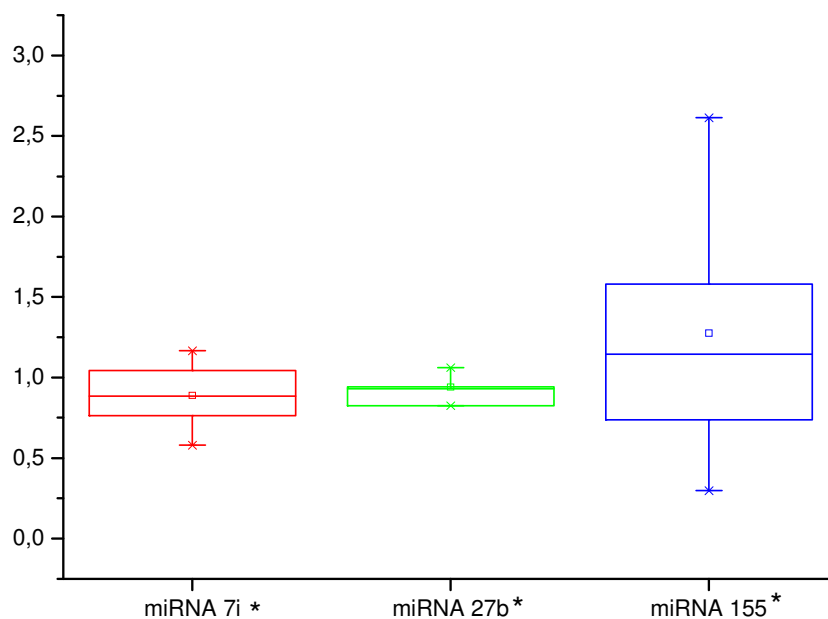


Figure 31 miR Expression after Lactobacillus treatment

The levels of all three miRs differed significantly from the control. All tested miRs were significantly decreased after 24 hours.

The treatment with *Lactobacillus* decreased the expression of miR7i to the 0.9-fold $\pm$ 0.2 SD, the miR27b expression was decreased to the 0.9-fold $\pm$ 0.09 SD and the miR155 expression was increased to 1.3-fold $\pm$ 0.9 SD.

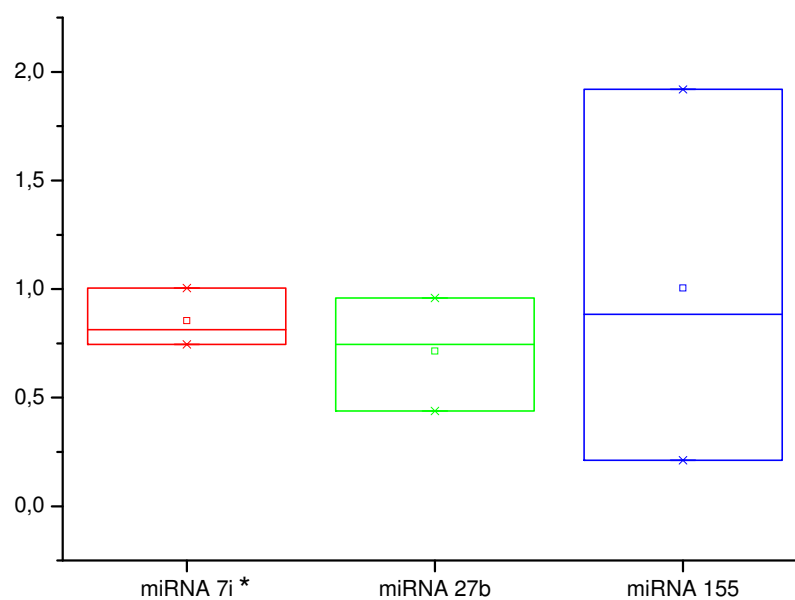
Streptococcus thermophilus:

Figure 32 miR Expression after Streptococcus treatment

The expression of miR7i was significantly lower after Streptococcus treatment than in the control.

After Streptococcus treatment, miR7i expression was decreased to the 0.9-fold $\pm$ 0.1 SD, miR27b expression was decreased to the 0.7-fold $\pm$ 0.3 and the miR155 expression was equal 1 $\pm$ 0.9.

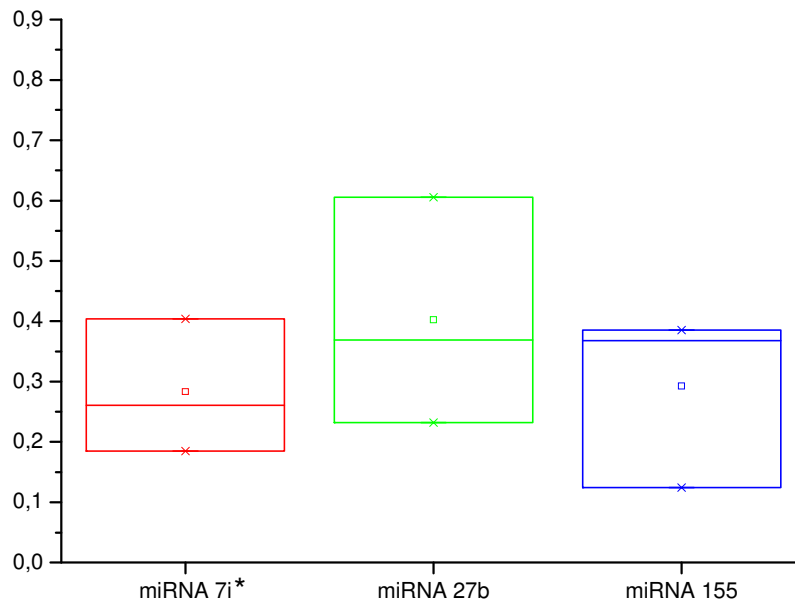
LPS:

Figure 33 miR treatment after LPS treatment

The treatment with LPS resulted in a decrease in all miRs. Only miR7i was statistically significant decreased.

The LPS treatment changed the miR expression as follows: miR7i was decreased to the 0.3-fold $\pm$ 0.1 SD, miR27b was decreased to the 0.4-fold $\pm$ 0.2 SD and miR155 was decreased to the 0.3-fold $\pm$ 0.1 SD

Abstract

Non invasive therapies for chronic inflammatory diseases are becoming of greater interest. Since several publications reported a positive influence of probiotics on inflammatory processes investigations in this direction are forced forward. The complex and specific signaling cascades, stimulated by probiotics show that the kind of bacteria has a high impact on the anti-inflammatory potential.

The aim of this study was to investigate if the expression of inflammatory mediators, Interleukin 6 (IL6) and Tumor Necrosis Factor α (TNF α) in particular, inflammatory transcription factors, NF κ B, and anti-inflammatory genes, such as PPAR γ , are altered after the exposure to probiotics and LPS. (LPS was used as a control) To investigate the direct influences on the gene expression, probiotic treatment was done directly onto the cells. Influences on already existing inflammatory processes have been observed by a prestimulation of cells with Interleukin 1 β (IL1 β), 24 hours before probiotical treatment. The expression of micro RNAs (miRs) which are presumably involved in the expression of the discussed genes was watched too, in particular miR7i, miR27b and mir155. The Colon Carcinoma cell system CACO2 was chosen for this study.

For the detection of gene expressions, as well as miR expression, quantitative real time PCR was used. *Lactobacillus acidophilus* NCFM, *Bifidobacterium lactis* 420 and *Streptococcus thermophilus* ST21, first lyophilized, were dissolved in water and heat inactivated. LPS was from *E. coli*.

The results showed a trend for probiotics to enhance the expression of inflammatory mediators, IL6 and TNF α in particular, but no increase in the expression of the transcription factor NF κ B. Different probiotics showed different effects on the expression levels, contributing to the suggestion that they initiate different signaling pathways in the host's enterocytes. Especially *Streptococcus* treatment often showed dramatically different effects on the gene expression than other probiotics. LPS showed expected different results than the probiotics but not expected decreases in the expression level of most genes after

prestimulation with IL1 β . PPAR γ expression was slightly increased after most treatments.

miR expression levels also changed dramatically after treatment with Lactobacillus, Streptococcus and LPS. A decrease in the miRs 7i and 27b could be observed after every probiotical treatment. Contrary miR 155 expression increased after Lactobacillus and Streptococcus treatment but not after LPS treatment.

Taken together the results of this study confirm with findings of other studies, claiming that probiotics can influence the inflammatory status. Further analysis must be done for a clear understanding of the initiated signaling pathways and gene expressions.

Draft – publication in progress.

Discussion

To investigate the influences of probiotics on inflammatory processes, in vitro studies are a popular method. They show clear results and can be easily controlled. Effects in vivo can be contemplated but no conclusions can be made for sure.

CACO2 cell model

All experiments were done with CACO2 cells which were harvested at 80-90% confluency. This experimental design allows a comparison with previous studies because the CACO2 cell model is a popular one. Of course CACO2 cells serve just as a model which makes it difficult to transfer the results to an in vivo system.

IL6 Expression

In IL1 β prestimulated CACO2 cells the expression of IL6 was significantly higher after treatment with *Lactobacillus acidophilus*, *Streptococcus thermophilus* and *Bifidobacterium lactis*, compared to untreated cells. The control was calculated as the difference in the expression of IL6 between prestimulated CACO2 cells without treatment and not prestimulated CACO2 cells without treatment. This experimental design made it possible to see the differences of the specific treatment. Contrary in the not prestimulated CACO2 cells the treatment with *Lactobacillus* did not alter the expression of IL6 significantly. Only the treatment with LPS after 3 and 24 hours and *Streptococcus* after 24 hours showed significantly increased IL6 mRNA levels. These findings have already been reported by Reilly et al. in 2007. They also treated prestimulated and not prestimulated CACO2 cells with *Lactobacillus paracasei* and *Lactobacillus plantarum*, to investigate the protective effect of

these probiotics. This group could not see an effect of the probiotics without prestimulation too but the treatment after prestimulation also showed an extreme increase in IL6 expression. They used living and heat inactivated Lactobacilli and found similar results. The group also showed that the effect was not initiated by any product secreted from the probiotics. In their publication, Reilly et al. stated that the effect of the heat inactivated probiotics may be caused by the heat shock proteins. When they knocked out hsp70 and hsp27, which are expressed at higher levels after heat inactivation, the potentiating effect of living probiotics on IL6 expression was gone. Furthermore, they claimed that the potentiating effect of IL6 expression has an anti-inflammatory background. Because it was shown that IL6 has anti-inflammatory effects on the intestinal mucosa. (Reilly, Poylin et al. 2007)

The IL6 expression without prestimulation was increased after 3 hours and decreased after 24 hours. Miettinen et al. already showed in 1996 that the treatment of Peripheral Blood Mononuclear cells (PBMC) with different lactic acid bacteria or LPS leads to an increased amount of TNF α and IL6, detected by Enzyme Linked Immuno Sorbent Assay (ELISA). These results suggest that the mRNA level is lowered after 24 hours because the translation of most of the mRNA into proteins has already taken place. (Miettinen, Vuopio-Varkila et al. 1996)

The expression of IL6 mRNA seems to take longer than the expression of TNF α mRNA. Thus in this work the peak of the expression level of IL6 might be not visible because it may be between 3 hours and 24 hours of treatment. Agarwal et al. showed that the increases in the expression of TNF α , IL1 β and IL8 appear soon after treatment with LPS but the IL6 expression increase takes longer. It seems that this cytokine differs from the others and this would contribute to the thesis that IL6 acts as an anti-inflammatory cytokine. (Agarwal, Piesco et al. 1995)

TNF α Expression

In this work, the mRNA level was analyzed after 3 and 24 hours. In the case of TNF α , the mRNA level was increased after 3 hours and decreased after 24 hours.

Miettinen et al. showed that after 24 hours treatment with different lactic acid bacteria, the protein level of TNF α increased dramatically. Like in the case of IL6, this could be the effect of an increased translation process. The authors concluded their findings in a stimulation of a nonspecific immune response by lactic acid bacteria. (Miettinen, Vuopio-Varkila et al. 1996)

Agarwal et al. studied the effect of different types of LPS onto the gene expression of different cytokines in monocytes. What they found was that E.coli LPS, which was used in this work too, increased the amount of TNF α and IL6 mRNA the least compared to LPS from other microbes. The time course of the expression levels showed that TNF α mRNA was rapidly expressed after LPS treatment and decreased fast after 3 hours.

It might be that the peak of TNF α expression could not be depicted in this study because it has already past after 3 hours. (Agarwal, Piesco et al. 1995)

NF κ Bp65 Expression

Rousseaux et al showed in 2007 that Lactobacillus acidophilus NCFM is able to induce, over direct contact with epithelial cells, an NF κ B directed expression of opioid and cannabinoid receptors. These results suggest a direct influence of Lactobacillus acidophilus NCFM onto the NF κ B signaling cascades. (Rousseaux, Thuru et al. 2007)

Su et al. claimed 1999 that they found a time kinetic in NF κ B expression. They stimulated CACO2 cells with IL1 β and recognized a rapid increase in IL8 mRNA. An immunoblot of I κ B (inhibitory protein of NF κ B which prevents the transcription factor to translocate into the nucleus and initiate the transcription of

inflammatory genes) showed that the protein was rapidly decreased within 10 minutes after stimulation with IL1 β . After 80 minutes a reappearance of I κ B could be recognized. After 180 minutes there was still less I κ B but more NF κ B followed by an increased expression of TNF α and IL6. After 24 hours, I κ B was back at the level it was before the treatment, followed by a decreased TNF α level. (Su, Wen et al. 1999)

In this work, the expression of NF κ B in prestimulated CACO2 cells was highest in the control after 3 hours, according to Su's findings that I κ B is decreased after 180 minutes. The treatment with probiotics and LPS seemed to decrease the inflammatory cascade because the expression of NF κ Bp65 was less than in the control with only *Streptococcus thermophilus* being statistically significant. After 24 hours, the NF κ B expression was decreased by all treatments, consistent with the claim that after 24 hours the protein level of I κ B is high enough again to inhibit the NF κ B function. Also consistent with the fact that within all treatments the IL6 and the TNF α expression was lower after 24 hours than after 3 hours in prestimulated cells.

PPAR γ Expression

Bailey et al. claimed that PPAR ligands are able to inhibit the expression of genes which are responsible for inflammatory activities in the cell. As already discussed, PPAR ligands play a very important role in inhibiting the transcription factor NF κ B. (Bailey and Ghosh 2005)

In this work, the expression of NF κ B in not prestimulated CACO2 cells was lowest after 3 hours treatment with LPS, consistent with Su's findings but also consistent with the PPAR γ expression, which was highest after 3 hours after LPS treatment.

In prestimulated CACO2 cells, the expression of PPAR γ was significantly increased after 3 hours treatment with *Bifidobacterium* and after 24 hours treatment with *Lactobacillus*.

Even though a real trend for an anti-inflammatory effect of PPAR γ after all treatments could not be reported in CACO2 cells, maybe due to the fact that PPAR γ appears and functions more often in adipocytes, skeletal muscle cells, osteoclasts, osteoblasts and several immunetype cells.(Martin 2009)

miR 7i

Chen et al. showed that *Clostridium parvum* decreases, over a MyD88/NF κ B dependent pathway, the expression of miR7i and in this way increases the expression of TLR4. (Chen, Splinter et al. 2007)

The findings of this work show that miR 7i was statistically significant decreased by all treatments, whereas the treatment with LPS showed the lowest levels of miR7i. Consistently, also NF κ B expression was lowest after LPS treatment. This may be due to an increased expression of TLR4, which is responsible for the LPS response in the cell.

miR 27b

Jennewein et al. showed a decrease in PPAR γ expression after LPS treatment, due to the repression through miR27b, which seems to regulate the PPAR γ mRNA translation, in monocytes. (Jennewein, Knethen et al. 2010)

In this work, the expression of PPAR γ increased after LPS treatment, but the miR27b level decreased. It is of course possible that these opposite results can be attributed to the different cell model. Another possible reason is that harvesting was too late for Jennewein's findings (24 hours) and the expression levels already reversed again. As already mentioned, PPAR γ does not have its target tissue in CACO2 cells.

miR 155

O'Connell et al. found an upregulation of miR155 after LPS stimulation in hematopoietic stem cells after LPS treatment. (Connell, Rao et al. 2008)

Furthermore, they found that miR155 was the only one out of 200 miRNAs they tested that was induced by all agents added, as there were different TLR ligands, as well as by LPS. Experiments were done in human monocytes. O'Connell et al. claim that the miR 155 pathway uses MyD88 and TNF α for signaling. The signaling cascade induced by TNF α is fast leading to a fast miR155 expression. (O'Connell, Taganov et al. 2006)

In this work, an increase of miR155 expression after LPS treatment could not be found. This may be either contributed to the different cell system or to the duration of the treatment, because O'Connell et al. measured the expression after 6 hours so that the peak of the expression level maybe was already gone.

Summary

In this work the changed expression of Interleukin 6 (IL6), Tumor Necrosis Factor α (TNF α), Nuclear Factor kappa B, subunit p65 (NFkBp65) and Peroxisome Proliferative activated Receptor γ (PPAR γ) was analyzed after treatment with the probiotics *Lactobacillus acidophilus* NCFM, *Bifidobacterium lactis* 420, *Streptococcus thermophilus* ST21 and Lipopolysaccharides (LPS). All of these components were added to either not prestimulated cells or to Interleukin 1 β (IL1 β) prestimulated cells. This ensured that on the one hand the effect of the probiotics and LPS on the cell system and on the other hand their effect on inflammatory processes, induced by IL1 β , in this specific cell system could be observed. The expression level of the micro RNAs miR7i, miR27b and miR155 was also measured after treatment with LPS, *Lactobacillus acidophilus* and *Streptococcus thermophilus*. The used cell system was the Colon-Carcinoma cell line CACO2.

Epigenetics is characterized by a change in gene expression without a change in the DNA sequence. Different food components are able to modify the expression level of specific genes. The signaling pathways that characterize the time kinetics of the cellular response to specific components are very complex systems that interact with each other. Micro RNAs (miRs) are important non-coding RNAs which regulate gene expression.

The results showed that the treatment with *Streptococcus thermophilus* often induced different expression levels than the other probiotics, more similar to LPS. LPS treatment itself showed significant decreases in the expression of distinct genes that were not expected, especially after prestimulation with IL1 β . Without prestimulation the only significant increases in gene expression could be seen after *Streptococcus* and LPS treatment. Within the expression detection of micro RNAs there was a clear trend that showed that miR7i was statistically significant decreased after every treatment. After the treatment with *Lactobacillus acidophilus*, every miR was decreased significantly. Taken

together no significant upregulation of micro RNA expression could be observed after 24 hours of treatment, although miR155 expression was increased after *Streptococcus* and *Lactobacillus* treatment.

To conclude the findings, probiotics and LPS have the ability to alter the expression of inflammatory genes and micro RNAs in CACO2 cells. Whereas micro RNA expression presumably influenced inflammatory gene expression. Especially effects on already existing inflammatory processes could be shown. Further investigations are necessary to claim the impact of specific probiotics and LPS on the human immune system.

Zusammenfassung

In dieser Arbeit wurde die veränderte Genexpression von Interleukin 6 (IL6), Tumor Nekrosis Faktor α (TNF α), Nuklear Faktor kappa B, Untereinheit p65 (NF κ Bp65) und Peroxisom Proliferativer aktivierter Rezeptor γ (PPAR γ) nach der Behandlung mit den Probiotika *Lactobacillus acidophilus* NCFM, *Bifidobacterium lactis* 420, *Streptococcus thermophilus* ST21 und mit Lipopolysacchariden (LPS) untersucht. All diese Stoffe wurden einerseits zu nicht vorstimulierten Zellen, andererseits zu Interleukin 1 β (IL1 β) vorstimulierten Zellen zugesetzt. Dieser Aufbau stellte sicher, dass auf der einen Seite die Auswirkungen der Probiotika und LPS auf die Zellen klar wurden und auf der anderen Seite ihr Einfluss auf entzündliche Vorgänge, die durch IL1 β hervorgerufen wurden. Die Expression von miR7i, miR27b und miR155 wurde ebenfalls nach der Behandlung mit LPS, *Lactobacillus acidophilus* und *Streptococcus thermophilus* bestimmt. Es wurde das Colon Karzinom Zellsystem CACO2 verwendet.

Epigenetik wird definiert als eine Veränderung in der Genexpression ohne Veränderungen in der DNA Sequenz. Verschiedene Nahrungsmittelinhaltsstoffe können die Genexpression verändern. Die Signaltransduktionswege, die die Zeitkinetik der zellulären Antwort auf spezifische Inhaltsstoffe bestimmen, sind komplex und interagieren auf verschiedenen Weisen miteinander. micro RNAs (miRs) sind wichtige nicht-kodierende RNAs, die die Genexpression regulieren.

Die Ergebnisse zeigten, dass die Behandlung mit *Streptococcus thermophilus* oft zu Expressionslevels führt, die sich von den anderen Probiotika unterscheiden. LPS zeigte, vor allem in IL1 β vorstimulierten Zellen, ein unerwartetes Herabsinken der Expression von Genen. In nicht vorstimulierten Zellen, konnten nur nach LPS Behandlung signifikante Steigerungen in der Genexpression festgestellt werden. Die Expressionen der miRNAs zeigten einen klaren Trend, so konnte die miR7i durch alle Behandlungen statistisch signifikant gesenkt werden. Nach Behandlung mit *Lactobacillus acidophilus* war die Expression aller miRs signifikant gesunken. Es konnte nach Auswertung aller Ergebnisse keine Erhöhung der micro RNA Expression nach 24 Stunden

Behandlung festgestellt werden. Obwohl die miR155 Expression nach Streptococcus und Lactobacillus Behandlung erhöht war.

Im Rahmen dieser Arbeit zeigten Probiotika und LPS die Fähigkeit in CACO2 Zellen die Expression von entzündungs-spezifischen Genen zu verändern. Wobei die micro RNA Expression wahrscheinlich die Expression entzündungsspezifischer Gene beeinflusst. Die Veränderungen konnten insbesondere bei bereits vorhandenen entzündlichen Prozessen gezeigt werden.

Weitere Studien sind notwendig um den Effekt von Probiotika und LPS auf das Immunsystem von Menschen klar nachweisen zu können.

Protocols

RNeasy Mini Kit (Qiagen)

Determining the correct amount of starting material

It is essential to use the correct amount of starting material in order to obtain optimal RNA yield and purity. The minimum amount is generally 100 cells, while the maximum amount depends on:

- The RNA content of the cell type
- The RNA binding capacity of the RNeasy spin column (100 µg RNA)
- The volume of Buffer RLT required for efficient lysis (the maximum volume of Buffer RLT that can be used limits the maximum amount of starting material to 1×10^7 cells)

RNA content can vary greatly between cell types. The following examples illustrate how to determine the maximum amount of starting material:

- COS cells have high RNA content (approximately 35 µg RNA per 10^6 cells). Do not use more than 3×10^6 cells, otherwise the RNA binding capacity of the RNeasy spin column will be exceeded.
- HeLa cells have average RNA content (approximately 15 µg RNA per 10^6 cells). Do not use more than 7×10^6 cells, otherwise the RNA binding capacity of the RNeasy spin column will be exceeded.
- NIH/3T3 cells have low RNA content (approximately 10 µg RNA per 10^6 cells). The maximum amount of starting material (1×10^7 cells) can be used.

If processing a cell type not listed in Table 2 (page 19) and if there is no information about its RNA content, we recommend starting with no more than $3\text{--}4 \times 10^6$ cells. Depending on RNA yield and purity, it may be possible to increase the cell number in subsequent preparations.

Do not overload the RNeasy spin column, as this will significantly reduce RNA yield and purity.

Counting cells is the most accurate way to quantitate the amount of starting material. As a guide, the number of HeLa cells obtained in various culture vessels after confluent growth is given in Table 4.

Table 4. Growth Area and Number of HeLa Cells in Various Culture Vessels

Cell-culture vessel	Growth area (cm ²)*	Number of cells [†]
Multiwell-plates		
■ 96-well	0.32–0.6	4–5 × 10 ⁴
■ 48-well	1	1 × 10 ⁵
■ 24-well	2	2.5 × 10 ⁵
■ 12-well	4	5 × 10 ⁵
■ 6-well	9.5	1 × 10 ⁶
Dishes		
■ 35 mm	8	1 × 10 ⁶
■ 60 mm	21	2.5 × 10 ⁶
■ 100 mm	56	7 × 10 ⁶
■ 145–150 mm	145	2 × 10 ⁷
Flasks		
■ 40–50 ml	25	3 × 10 ⁶
■ 250–300 ml	75	1 × 10 ⁷
■ 650–750 ml	162–175	2 × 10 ⁷

* Per well, if multiwell plates are used; varies slightly depending on the supplier.

[†] Cell numbers are given for HeLa cells (approximate length = 15 µm), assuming confluent growth. Cell numbers will vary for different kinds of animal cells, which vary in length from 10 to 30 µm.

Important points before starting

- If using the RNeasy Kit for the first time, read “Important Notes” (page 18).
- If working with RNA for the first time, read Appendix A (page 63).
- Cell pellets can be stored at –70°C for later use or used directly in the procedure. Determine the number of cells before freezing. Frozen cell pellets should be thawed slightly so that they can be dislodged by flicking the tube in step 2. Homogenized cell lysates from step 3 can be stored at –70°C for several months. Frozen lysates should be incubated at 37°C in a water bath until completely thawed and salts are dissolved. Avoid prolonged incubation, which may compromise RNA integrity. If any insoluble material is visible, centrifuge for 5 min at 3000–5000 × g. Transfer supernatant to a new RNase-free glass or polypropylene tube, and continue with step 4.

- Buffer RLT may form a precipitate upon storage. If necessary, redissolve by warming, and then place at room temperature (15–25°C).
- Buffer RLT and Buffer RW1 contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. See page 8 for safety information.
- Perform all steps of the procedure at room temperature. During the procedure, work quickly.
- Perform all centrifugation steps at 20–25°C in a standard microcentrifuge. Ensure that the centrifuge does not cool below 20°C.

Things to do before starting

- If purifying RNA from cell lines rich in RNases, we recommend adding β -mercaptoethanol (β -ME) to Buffer RLT before use. Add 10 μ l β -ME per 1 ml Buffer RLT. Dispense in a fume hood and wear appropriate protective clothing. Buffer RLT containing β -ME can be stored at room temperature for up to 1 month.
- Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%) as indicated on the bottle to obtain a working solution.
- If performing optional on-column DNase digestion, prepare DNase I stock solution as described in Appendix D (page 69).

Procedure

1. Harvest cells according to step 1a or 1b.
 - 1a. Cells grown in suspension (do not use more than 1×10^7 cells):
 Determine the number of cells. Pellet the appropriate number of cells by centrifuging for 5 min at $300 \times g$ in a centrifuge tube (not supplied). Carefully remove all supernatant by aspiration, and proceed to step 2.
Note: Incomplete removal of cell-culture medium will inhibit lysis and dilute the lysate, affecting the conditions for binding of RNA to the RNeasy membrane. Both effects may reduce RNA yield.
 - 1b. Cells grown in a monolayer (do not use more than 1×10^7 cells):
 Cells can be either lysed directly in the cell-culture vessel (up to 10 cm diameter) or trypsinized and collected as a cell pellet prior to lysis. Cells grown in cell-culture flasks should always be trypsinized.
 To lyse cells directly:
 Determine the number of cells. Completely aspirate the cell-culture medium, and proceed immediately to step 2.

Note: Incomplete removal of cell-culture medium will inhibit lysis and dilute the lysate, affecting the conditions for binding of RNA to the RNeasy membrane. Both effects may reduce RNA yield.

To trypsinize and collect cells:

Determine the number of cells. Aspirate the medium, and wash the cells with PBS. Aspirate the PBS, and add 0.1–0.25% trypsin in PBS. After the cells detach from the dish or flask, add medium (containing serum to inactivate the trypsin), transfer the cells to an RNase-free glass or polypropylene centrifuge tube (not supplied), and centrifuge at 300 x g for 5 min. Completely aspirate the supernatant, and proceed to step 2.

Note: Incomplete removal of cell-culture medium will inhibit lysis and dilute the lysate, affecting the conditions for binding of RNA to the RNeasy membrane. Both effects may reduce RNA yield.

2. Disrupt the cells by adding Buffer RLT.

For pelleted cells, loosen the cell pellet thoroughly by flicking the tube. Add the appropriate volume of Buffer RLT (see Table 5). Vortex or pipet to mix, and proceed to step 3.

Note: Incomplete loosening of the cell pellet may lead to inefficient lysis and reduced RNA yields.

Table 5. Volumes of Buffer RLT for Lysing Pelleted Cells

Number of pelleted cells	Volume of Buffer RLT (µl)
<5 x 10 ⁶	350
5 x 10 ⁶ – 1 x 10 ⁷	600

For direct lysis of cells grown in a monolayer, add the appropriate volume of Buffer RLT (see Table 6) to the cell-culture dish. Collect the lysate with a rubber policeman. Pipet the lysate into a microcentrifuge tube (not supplied). Vortex or pipet to mix, and ensure that no cell clumps are visible before proceeding to step 3.

Table 6. Volumes of Buffer RLT for Direct Cell Lysis

Dish diameter (cm)	Volume of Buffer RLT (µl)*
<6	350
6–10	600

* Regardless of the cell number, use the buffer volumes indicated to completely cover the surface of the dish.

3. Homogenize the lysate according to step 3a, 3b, or 3c.

See “Disrupting and homogenizing starting material”, pages 20–23, for more details on homogenization. If processing $\leq 1 \times 10^6$ cells, homogenize by vortexing for 1 min. After homogenization, proceed to step 4.

Note: Incomplete homogenization leads to significantly reduced RNA yields and can cause clogging of the RNeasy spin column. Homogenization with a rotor–stator or QIAshredder homogenizer generally results in higher RNA yields than with a syringe and needle.

3a. Pipet the lysate directly into a QIAshredder spin column placed in a 2 ml collection tube, and centrifuge for 2 min at full speed. Proceed to step 4.

3b. Homogenize the lysate for 30 s using a rotor–stator homogenizer. Proceed to step 4.

3c. Pass the lysate at least 5 times through a blunt 20-gauge needle (0.9 mm diameter) fitted to an RNase-free syringe. Proceed to step 4.

4. Add 1 volume of 70% ethanol to the homogenized lysate, and mix well by pipetting. Do not centrifuge.

Note: The volume of lysate may be less than 350 μ l or 600 μ l due to loss during homogenization.

Note: When purifying RNA from certain cell lines, precipitates may be visible after addition of ethanol. This does not affect the procedure.

5. Transfer up to 700 μ l of the sample, including any precipitate that may have formed, to an RNeasy spin column placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Discard the flow-through.*

Reuse the collection tube in step 6.

If the sample volume exceeds 700 μ l, centrifuge successive aliquots in the same RNeasy spin column. Discard the flow-through after each centrifugation.*

Optional: If performing optional on-column DNase digestion (see “Eliminating genomic DNA contamination”, page 23), follow steps D1–D4 (page 69) after performing this step.

6. Add 700 μ l Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to wash the spin column membrane. Discard the flow-through.*

Reuse the collection tube in step 7.

* Flow-through contains Buffer RLT or Buffer RW1 and is therefore not compatible with bleach. See page 8 for safety information.

Note: After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Be sure to empty the collection tube completely.

Skip this step if performing optional on-column DNase digestion (page 69).

7. **Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to wash the spin column membrane. Discard the flow-through.**

Reuse the collection tube in step 8.

Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use (see "Things to do before starting").

8. **Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to wash the spin column membrane.**

The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions.

Note: After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.

9. **Optional: Place the RNeasy spin column in a new 2 ml collection tube (supplied), and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge at full speed for 1 min.**

Perform this step to eliminate any possible carryover of Buffer RPE, or if residual flow-through remains on the outside of the RNeasy spin column after step 8.

10. **Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30–50 µl RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to elute the RNA.**

11. **If the expected RNA yield is >30 µg, repeat step 10 using another 30–50 µl RNase-free water, or using the eluate from step 10 (if high RNA concentration is required). Reuse the collection tube from step 10.**

If using the eluate from step 10, the RNA yield will be 15–30% less than that obtained using a second volume of RNase-free water, but the final RNA concentration will be higher.

Phusion RT-PCR Kit Finnzymes

Protocol for cDNA synthesis

Guidelines for reverse transcription

- Use gloves and RNase-free plasticware to prevent RNase contamination.
- Prepare premixes to avoid pipetting very small volumes.
- Pipet all components on ice.
- Reaction volume in cDNA synthesis is 20 μ l.
- Use up to 1 μ g of RNA template. The minimum amount depends on both the template and the primers used.
- Recommended primer amounts in a 20 μ l reaction:
 - 100 ng oligo(dT) primers (can be increased up to 1 μ g) or
 - 50 ng random primers (may require optimization) or
 - 5 pmol (2–10 pmol) gene-specific primers.

Note: When determining the amount of RNA template, the expression level of the target RNA molecule should be considered, as it affects the subsequent PCR step. The volume of the cDNA reaction mixture used as a source for template in PCR should not exceed 10 % of the final PCR reaction volume. A high RNA concentration in the PCR may also inhibit the reaction.

Protocol

It is recommended that control reactions be performed in parallel with all experiments. Control RNA and primers are provided with the kit. The setup for controls is given in chapter 6.

1. Thaw template RNA, 10x RT buffer, dNTPs and primers. Mix the individual solutions to assure homogeneity and centrifuge briefly before pipetting.

2. Combine the following components in reaction tubes

Template RNA x μ l (up to 1 μ g)

10 mM dNTP mix 1 μ l

Oligo(dT) primer* 1 μ l

RNase-free H₂O Add to 10 μ l

3. Incubate at 65°C for 5 minutes to predenature the RNA.

4. Place the reaction tubes on ice and add to each tube

10x RT buffer 2 μ l

RT enzyme mix 2 μ l

RNase-free H₂O 6 μ l

5. Program a thermal cycler as outlined in Table 1.

6. Place the tubes in the cycler and start the program.

Table 1. Cycler protocol for cDNA synthesis.

Step Temperature Time

Primer extension 25°C 10 min

cDNA synthesis 40°C 30 min

Reaction termination 85°C 5 min

Cooling of the sample 4°C Hold

Primer extension

The incubation for 10 minutes at 25°C extends oligo(dT) and random primers before the actual cDNA synthesis. Without the incubation at 25°C, the primers may dissociate from the template when the temperature is increased. When using gene-specific primers, this extension step is not necessary.

cDNA synthesis

Incubation at 40 °C will work for most templates, but the incubation temperature can be optimized between 37–48 °C, if necessary. Increasing the temperature can be helpful if the template has strong secondary structures. A higher temperature can also improve specificity if genespecific primers are used. Incubation above 48 °C is not recommended.

In most cases, incubation for 30 minutes is sufficient. If the target is located near the 5' end of a long transcript and oligo(dT) priming is used, or if the target is rare, cDNA synthesis time can be extended up to 60 min.

Reaction termination

The termination step at 85 °C inactivates the M-MuLV RT. This prevents the reverse transcriptase from inhibiting the subsequent PCR reaction.

Note: A separate RNase H treatment is not required. The cDNA can be directly used as a template in the subsequent PCR or stored at -20 °C, if not used immediately.

(April 2010)

QuantiMir RT Kit - Small RNA Quantitation System (System Biosciences)

II. Protocol

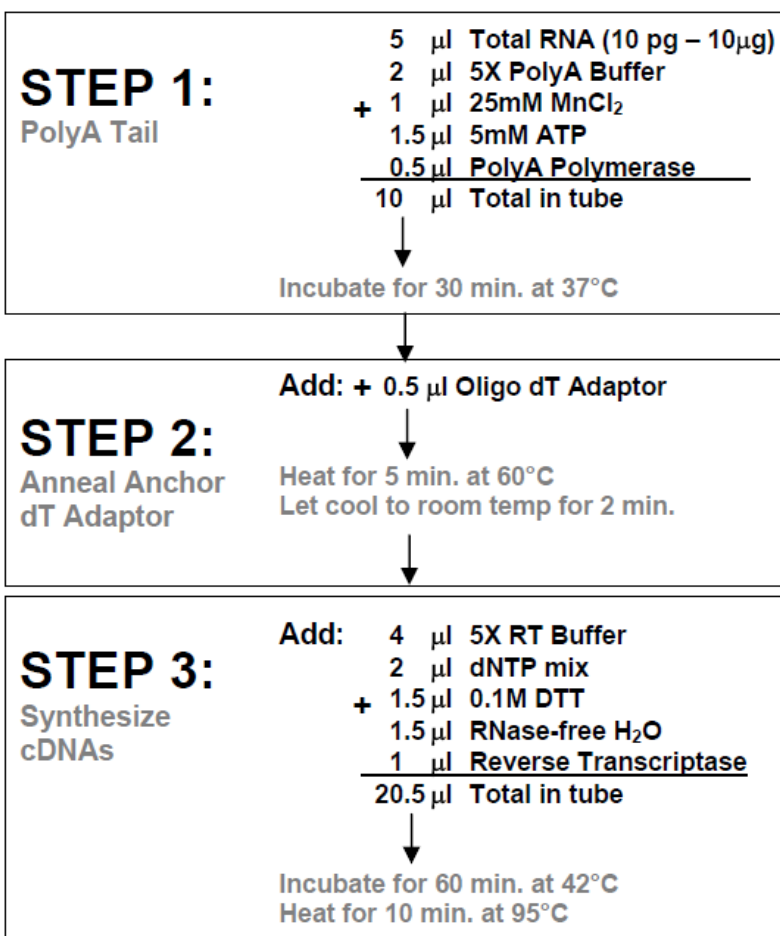
A. QuantiMir™ RT Reaction Setup



It is important to start with total RNA that includes the small RNA fraction.

Start:

In a thin-walled PCR tube or PCR-compatible plate well, combine:



*The QuantiMir™ cDNAs can be stored at -20°C.

Done!

How much cDNA template per qPCR reaction?

Starting RNA	QuantiMir cDNA per qPCR reaction
10pg -100 ng	0.5ul to 1ul
100ng – 1 µg	Dilute 1: 100
> 1 µg	Dilute 1: 1000

B. Primer Design Considerations for QuantiMir™ cDNA Detection and Quantitation

The user needs only to design the forward “sense” orientation primer to perform the end-point or qPCR reactions. MicroRNAs typically range in size from 19 – 24 nt. We recommend using the exact sequence of the miRNA or siRNA being studied when designing the forward primer. If the miRNA under study is known and documented, using the miRBase database can be an easy starting point:

(<http://microrna.sanger.ac.uk/sequences/search.shtml>).

An example of the known and documented miRNA, Human miR-16, is shown below.

Hsa-miR-16

Mature sequence MIMAT0000069	
Accession	MIMAT0000069
ID	hsa-miR-16
Sequence	14 - uagcagcacguaaaauuggcg - 35
	Get sequence
Evidence	experimental; cloned [1,5,7], Northern [1,6]

Simple: Directly use sequence of mature miRNA as forward primer in oligo design.

The mature miRNA sequence 5' – **uagcagcacguaaaauuggcg** – 3' can be simply converted to a DNA sequence and used directly as the forward primer for end-point and qPCR analysis.

Forward primer for hsa-miR-16 (included in kit):

5' – **TAGCAGCACGTAAATATTGGCG** – 3'

T_m = 58.9°C, 45% GC and length = 22 bases.

If the user is developing a new assay for a novel miRNA, follow the guidelines of the example above. Design the primer to have a T_m of at least 50°C and to have a length of at least 18 bases. If the primer being designed for the miRNA to be studied has a T_m below 50°C, lower the annealing temperature in your cycling conditions for end-point and qPCR instrument settings. An example of successfully using this approach is demonstrated in **Figure 5**.

D. Real-time qPCR Reactions using QuantiMir™ cDNAs

For end-point PCR reactions, please refer to the **Section C** above, “End-point PCR Reactions using QuantiMir™ cDNAs”.

1. qPCR Reaction Setup

To determine the expression profile for your miRNA under study, mix the following per well:

For Single well determination:

	15	μl	2X SYBR Green qPCR Mastermix buffer
	1	μl	User-designed Forward Primer (10 μM)
+	0.5	μl	Universal Reverse Primer (10 μM)
	1	μl	Diluted QuantiMir™ cDNA (see p.# 7)
	12.5	μl	RNase-free water
	30	μl	Total / well

The Mastermix contents can be scaled up or down depending upon on your experimental needs. Once reagents are loaded into the wells, cover the plate with the optical adhesive cover and spin briefly in a centrifuge to bring contents to bottom of wells. Place plate in the correct orientation (well A1, upper left) into the Real-time qPCR instrument and perform analysis run.

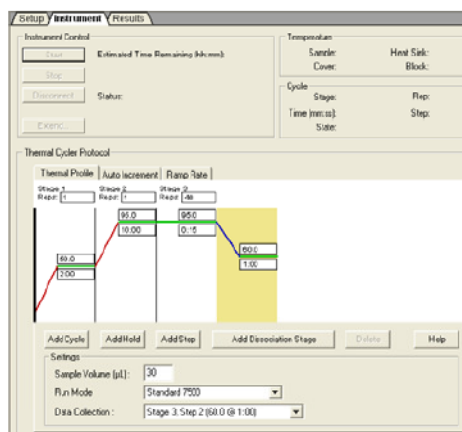
2. Real-time qPCR Instrument Parameters

Follow the guidelines as detailed for your specific Real-time instrumentation. The following parameters tested by SBI were performed on an Applied Biosystems 7300 Real-time PCR System but can also apply to an ABI 7500 or an ABI 7900 96-well system. The details of the thermocycling conditions used in testing at SBI are shown below. A screenshot from SBI's instrument set up is shown on the right. Default conditions are used throughout except for those cases where the Forward Primer's T_m is below 55°C, then the annealing temperature in Step 2 of Stage 3 is lowered from 60°C to 50°C.

qPCR cycling and data accumulation conditions:

1. 50°C 2 min.
2. 95°C 10 min.
3. 95°C 15 sec.
4. 60°C 1 min.

(40 cycles of steps 3 and 4), data read at 60°C 15 sec. Step (gold rectangle)



An additional recommendation is to include a melt analysis after the qPCR run to assess the T_m of the PCR amplicon to verify the specificity of the amplification reaction. Refer to the User Manual for your specific instrument to conduct the melt analysis and the data analyses of the amplification plots and Cycle Threshold (Ct) calculations. In general, Cycle thresholds should be set within the exponential phase of the amplification plots with software automatic baseline settings.

Appendix

Experiments with CACO2 cells have also been done with Nano Silver. Nano Silver is becoming of interest in nutrition because it is already used in packaging material in the food industry. It has become an important substance because of its antibacterial characteristics. Another cause for the interest in this “new” substance is that Nano Silver is sold as food additive. It is also often a component of deodorants, room sprays, water cleaners, laundry detergents and wall paint. Although it is already widely used, there is not much known about its biological effects. (Miura and Shinohara 2009)

The mechanism that makes Nano Silver toxic for bacteria depends on the ability of the ionic silver to bind to the negatively charged bacterial cell wall. Furthermore, Reactive Oxygen Species (ROS) are produced, followed by a deactivation of cellular enzymes. Possible mechanisms for Nano Silver to increase the cytotoxic effect are: on the one hand attachment to cell membranes but also changes of the membrane permeability and intracellular accumulation of ROS on the other hand. (Miura and Shinohara 2009)

Nano Silver is already discussed to have a pro-inflammatory impact on human cells, so that the objectives were the following: Proofing the inflammatory, cytotoxic and apoptotic effect of Nano Silver on CACO2 cells. This was done by stimulation of CACO2 cells with Nano Silver for 24 hours. Afterwards the mRNA level of the genes TNF α and IL6 as inflammatory genes and Caspase3 as apoptotic gene were measured. Additionally, a Trypanblue test was done to investigate the viability and cell survival after Nano Silver stimulation. Different concentrations were tested to proof the toxic potential of Nano Silver.

CACO2 cells were grown in the already described medium. No IL1 β was added, the concentrations of Nano Silver ranged from 0,01 μ g/ml to 50 μ g/ml. The used substance was Nano Silver: NM – 300 Silver, the size was <20nm, the concentration of the stock solution was 10⁵ ppm, the particles were citrate coated so that they should not form aggregates.

The results showed a pro-inflammatory effect of Nano Silver. Especially at the concentration of 50 $\mu\text{g/ml}$, IL6 and TNF α were dramatically increased. (The control is 0).

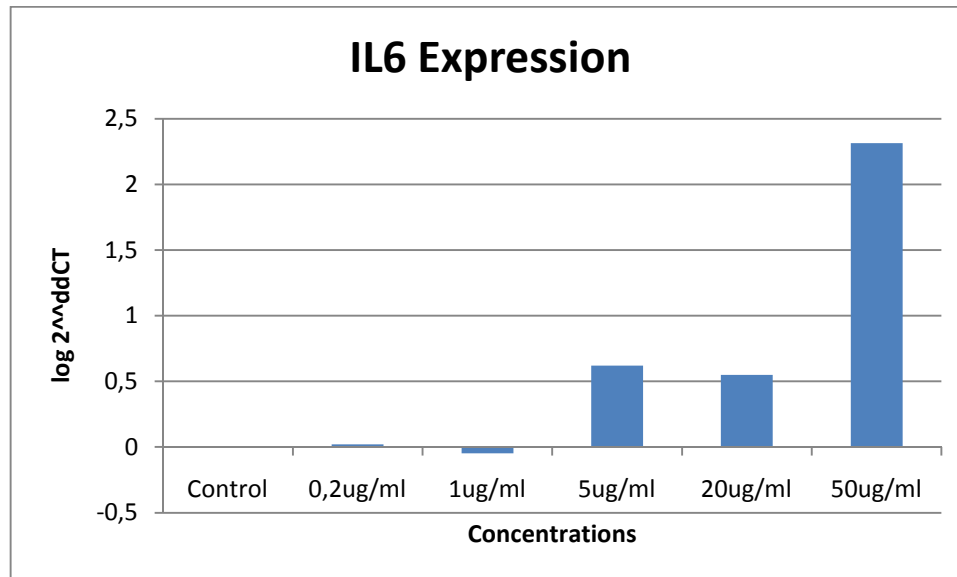


Figure 34 IL6 Expression after Nano Silver Treatment

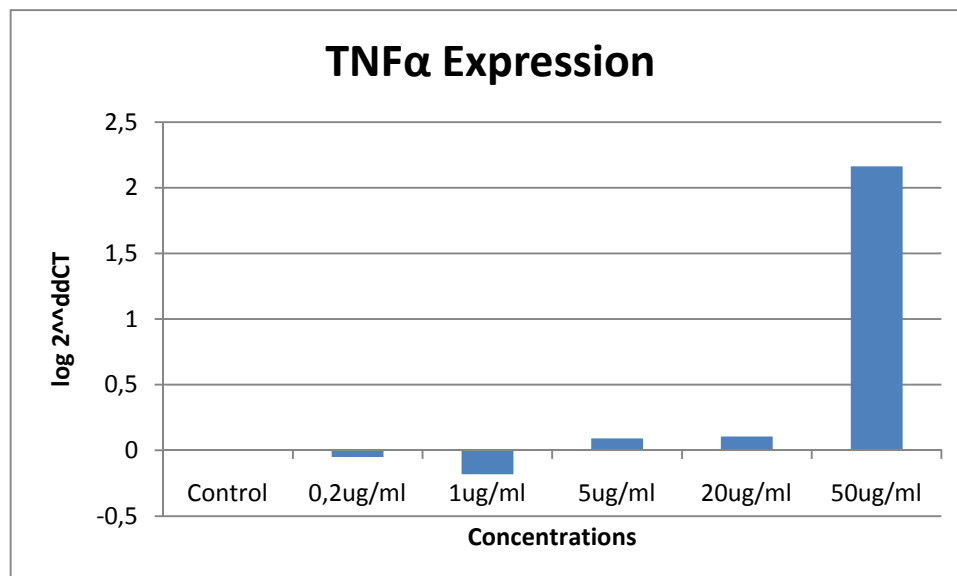


Figure 35 TNF α Expression after Nano Silver Treatment

The expression of the apoptotic gene Caspase 3 showed opposite expression levels:

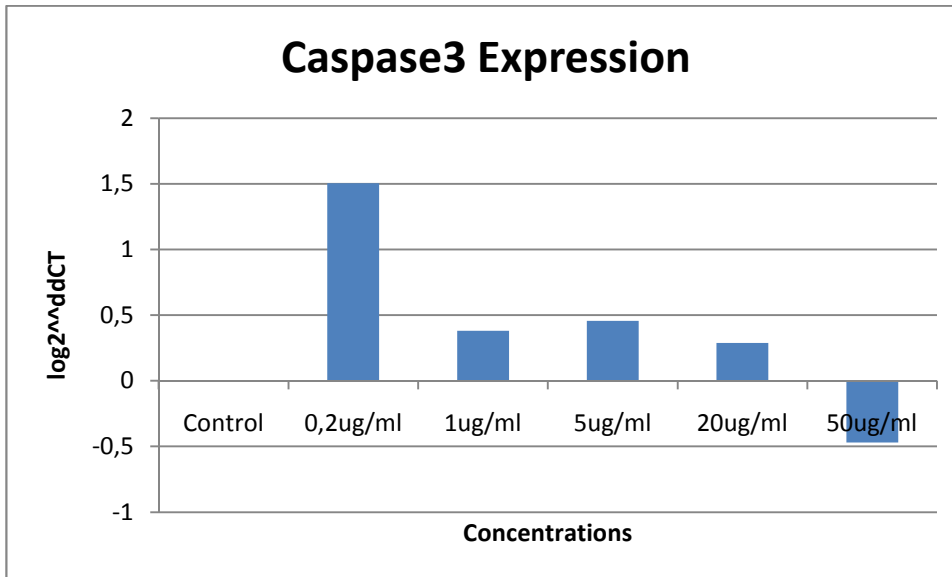


Figure 36 Caspase3 Expression after Nano Silver Treatment

The expression of inflammation specific micro RNAs also changed dramatically after Nano Silver stimulation:

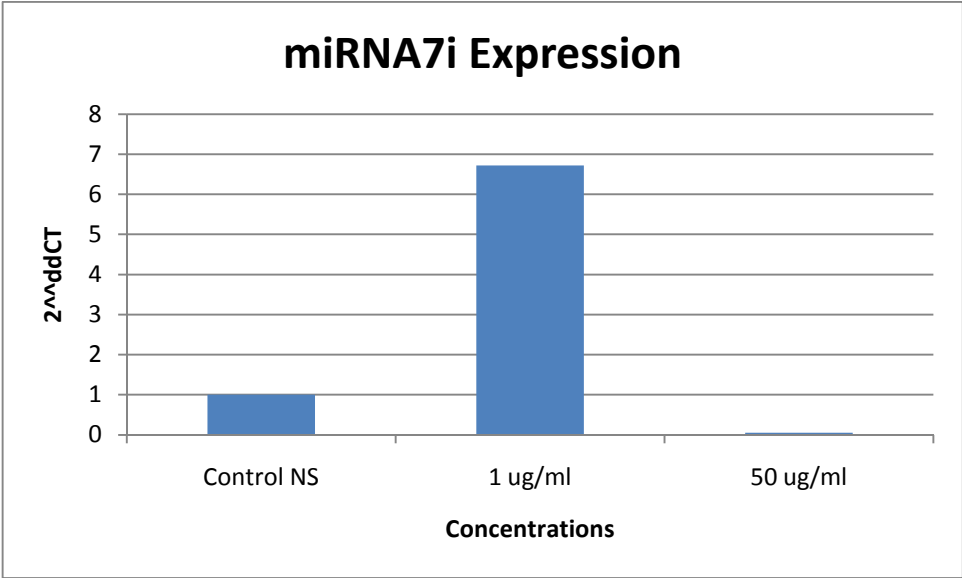


Figure 37 miR7i Expression after Nano Silver Treatment

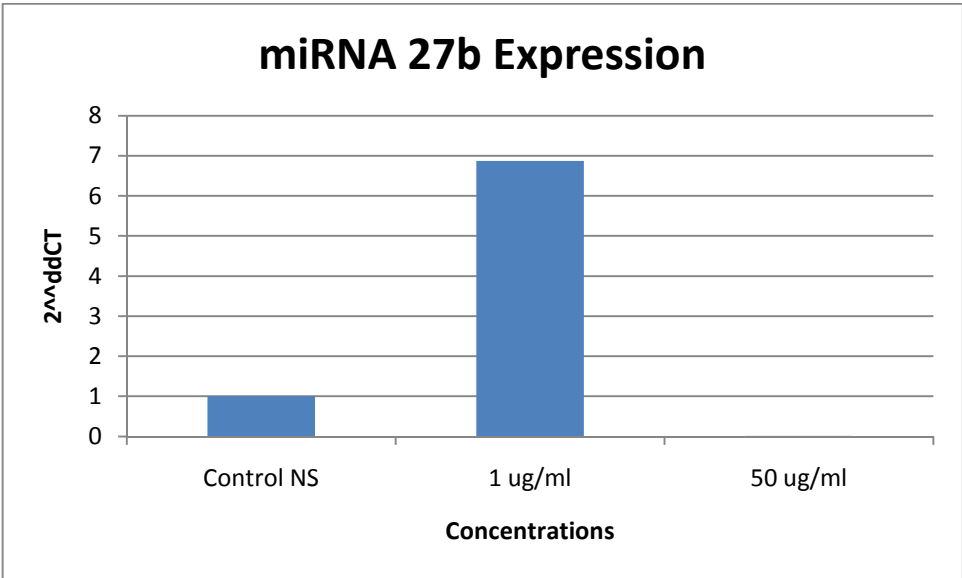


Figure 38 miR27b Expression after Nano Silver Treatment

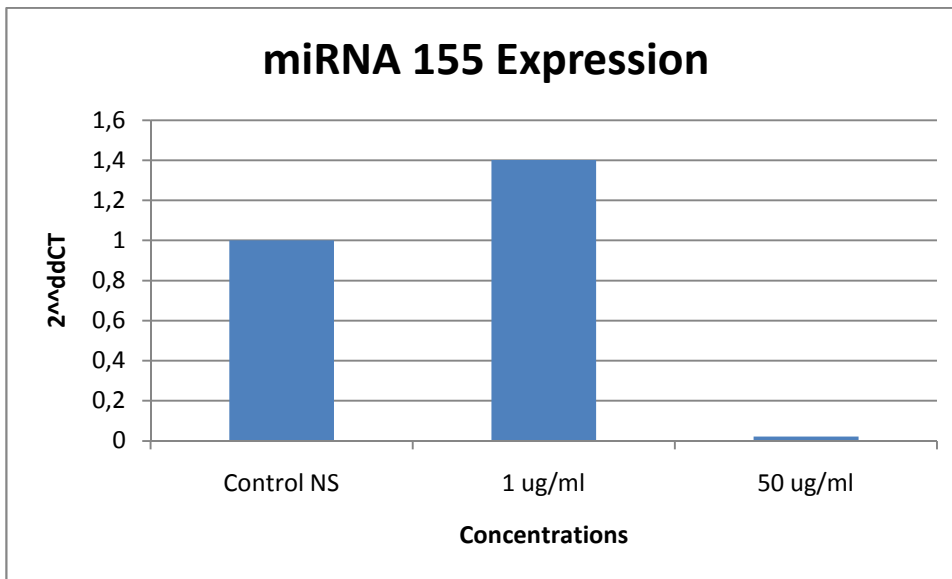


Figure 39 miR155 Expression after Nano Silver Treatment

Furthermore the Trypanblue test showed following results:

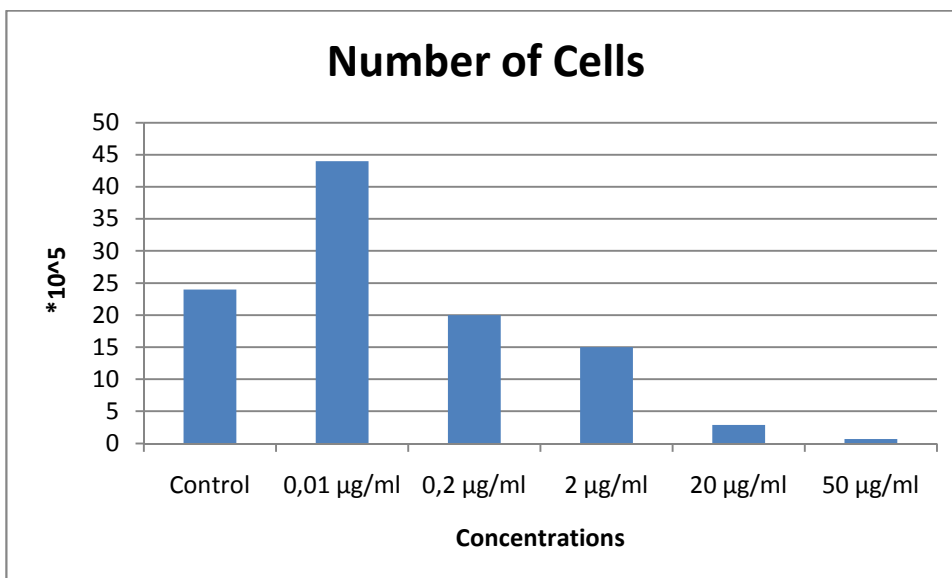


Figure 40 Number of Cells after Nano Silver Treatment

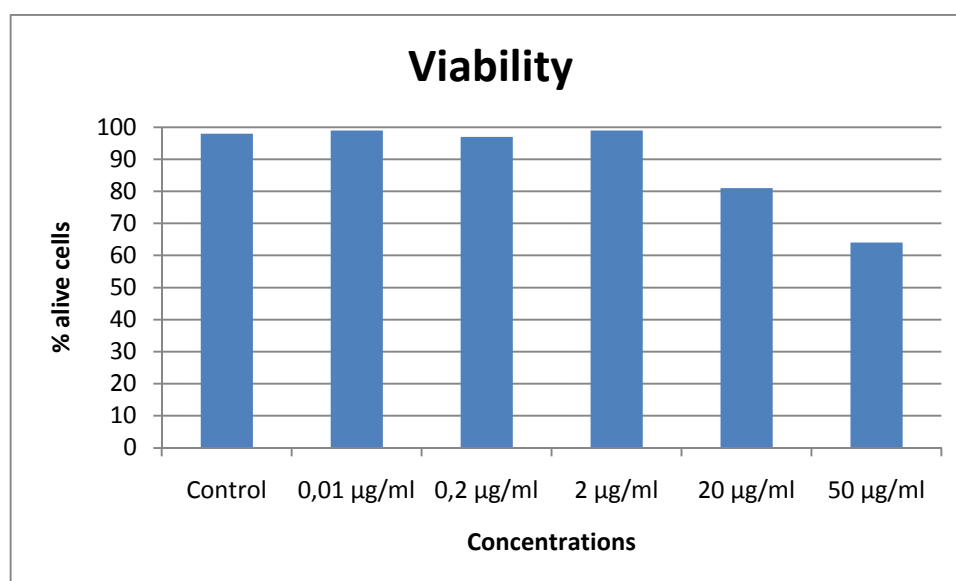


Figure 41 Viability of Cells after Nano Silver Treatment

These findings support the thesis that Nano Silver has inflammatory effects in higher concentrations. Shin et al. reported an anti-inflammatory effect of Nano Silver in PBMCs. The TNF α level decreased with higher concentrations of Nano Silver. The maximum concentration used was 20 ppm. The different findings of Shin and this experiment surely occur because of different cell systems and different concentrations (in this work the most toxic effect was at 50 μ g/ml), but the main difference between the experiments is the size of the particles. Shin et al. used Nano Silver of < 5 nm; in this work Nano Silver of < 20 nm was used. Shin et al. already mentioned in 2007 that the results of Nano Silver stimulation depend on the size of the particles. (Shin, Ye et al. 2007)

Miura et al. showed in 2009 that the viability of Hela cells (Henrietta Lacks – cervix carcinoma cells) starts to decrease at a concentration of 60 μ g/ml. They showed a clear cytotoxic effect of Nano Silver in high concentrations. Miura et al. also found that Nano Silver stimulation increases the expression of the “stress genes” ho-1 and mt-2A. (Miura and Shinohara 2009)

The experiments of this work must be repeated to be able to show significant alterations in Nano Silver treated cell. What already can be seen now is a trend

that shows an increasing Nano Silver concentration is followed by an increased concentration of mRNA of inflammatory genes. The viability and the number of cells decrease with higher concentrations. At a concentration of 50 µg/ml the viability nearly decreased to one half of the control. This concentration seems to be cytotoxic. The decreased expression of Caspase 3, miR7i, miR27b and miR155 at the concentration of 50 µg/ml are maybe the result of a decreased cell number, miRs could not be detected in samples with a low cell number in other experiments too. The results would point to an extreme apoptotic cell population. The cells that are left produce extremely elevated mRNA levels of inflammatory marker to either survive the inflammatory stress induced by Nano Silver stimulation or to initiate apoptosis too.

Biological effects of Nano Silver are still not clear and have not already been tested enough to see a clear impact. It is very important to find out more about cytotoxicity, pro-inflammatory or anti-inflammatory effects of Nano Silver. Another point for discussion is that the concentration of Nano Silver in food additives is 50 mg/l, the same concentration that was found to be cytotoxic in this work. Nano Silver is an agent that is already widely used but it is not fully understood at the moment.

Lebenslauf

Name: Manuela Nestelberger
Akadem. Grad: Bakk. rer. nat.
Geb.: 26.10.1985 in Tulln
Adresse: Oberbierbaum 70
 3454 Reidling
Email: nesis@gmx.at

Bisherige Ausbildung:

1992 – 1996	Volksschule	Zwentendorf
1996 – 2000	Musikhauptschule	Tulln
2000 – 2004	AHS – Oberstufe,	St. Pölten
	naturwissenschaftlicher Schwerpunkt	
09.2004 – 06.2005	Diplom-Studium	
	Ernährungswissenschaften	Universität Wien
09.2005 – 06.2006	Studium Humanmedizin	Meduni Wien
09.2005 – 12.2008	Bakkalaureatsstudium	Universität Wien
	Ernährungswissenschaften	
Seit Dezember 2008	Masterstudium	Universität Wien
	Ernährungswissenschaften	
	Schwerpunkt Food Quality	
	and Food Safety	

Arbeitserfahrung:

08.2008	NÖ Landesregierung, Abteilung Umwelthygiene (1-tägiges Praktikum im Trinkwasserlabor Frenzl Tulln)
07.-09. 2009	Agrana Bioethanol GmbH (Abteilung Qualitätssicherung und Qualitätskontrolle)
06. – 07. 2010	geringfügige Beschäftigung Arbeitsgruppe Haslberger Universität Wien

Fremdsprachen:

Englisch

Italienisch

References

- Agarwal, S., N. P. Piesco, et al. (1995). "Differential Expression of IL-1b, TNFa, IL6, and IL8 in Human Monocytes in Response to Lipopolysaccharides from Different Microbes." *Journal of Dental Research* **74**: 1057-1065.
- Alberts, Bray, et al., Eds. (2005). *Lehrbuch der Molekularen Zellbiologie*, Wiley-VCH.
- Alberts, Johnson, et al., Eds. (2002). *The shape and structure of proteins*.
- Anfinsen, C. B. (1972). "The formation and stabilization of protein structure." *Biochem J* **128**(4): 737-49.
- Bailey, S. T. and S. Ghosh (2005). "PPAR'ting ways with inflammation." *Nat Immunol* **6**(10): 966-7.
- Beutler, B. (2004). "Inferences, questions and possibilities in Toll-like receptor signalling. ." *Nature* **430**: 257-63.
- Boitier, Gautier, et al. (2003). "Advances in understanding the regulation of apoptosis and mitosis by peroxisome-proliferator activated receptors in pre-clinical models: relevance for human health and disease." *Comp Hepatol*. **2**.
- Bolotin, Quinquis, et al. (2004). "Complete sequence and comparative genome analysis of the dairy bacterium *Streptococcus thermophilus*." *Nature Biotechnology* **22**: 1554 – 1558.
- Brueckner, F., K. J. Armache, et al. (2009). "Structure-function studies of the RNA polymerase II elongation complex." *Acta Crystallogr D Biol Crystallogr* **65**(Pt 2): 112-20.
- Chen, X.-M., P. L. Splinter, et al. (2007). "A Cellular Micro-RNA, let-7i, Regulates Toll-like Receptor 4 Expression and Contributes to Cholangiocyte Immune Responses against *Cryptosporidium parvum* Infection." *The Journal of biological Chemistry* **282**: 28929-28938.
- Clarke, M. B. and V. Sperandio (2005). "Events at the Host-Microbial Interface

of the Gastrointestinal Tract III. Cell-to-cell signaling among microbial flora, host, and pathogens: there is a whole lot of talking going on." *Am J Physiol Gastrointest Liver Physiol* **288**: G1105–G1109.

Connell, R. M. O., D. S. Rao, et al. (2008). "Sustained expression of microRNA-155 in hematopoietic stem cells causes a myeloproliferative disorder." *The Journal of Experimental Medicine* **205**: 585-594.

Cook, D., D. Pisetsky, et al. (2002). "Toll-like receptors in the pathogenesis of human disease. ." *Nat. Immunol.* **5**: 975-9.

Hallman, M., M. Ramet, et al. (2001). "Toll-like receptors as sensors of pathogens. ." **50**: 315-21.

Hayden and Ghosh (2008). "Shared principles in NF-kappaB signaling." *Cell* **132**: 344–62.

Heinrich, P. C., I. Behrmann, et al. (1998). "Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway." *Biochem J* **334 (Pt 2)**: 297-314.

Hong, e. a. (2007). *BMC Molecular Biology* **8**.

Jennewein, C., A. v. Knethen, et al. (2010). "MicroRNA-27b Contributes to Lipopolysaccharide-mediated Peroxisome Proliferator-activated Receptor (PPAR) mRNA Destabilization." *The Journal of biological Chemistry* **285**: 11846-11853.

Kelly (2001). "The pleiotropic nature of the vascular PPAR gene regulatory pathway." *Circ Res.* **89**: 935-7.

Kelly, Campbell, et al. (2003). "Commensal anaerobic gut bacteria attenuate inflammation by regulating nuclear-cytoplasmic shuttling of PPAR-g and RelA." *Nature Immunology*.

LinkWitzany, Ed. (2010). *LinkBiocommunication and natural genome editing*, Springer

Martin, H. (2009). "Role of PPAR-gamma in inflammation. Prospects for

- therapeutic intervention by food components." *Mutation Research* **669**: 1–7.
- Miettinen, M., J. Vuopio-Varkila, et al. (1996). "Production of Human Tumor Necrosis Factor Alpha, Interleukin-6, and Interleukin-10 Is Induced by Lactic Acid Bacteria." *INFECTION AND IMMUNITY* **64**: 5403-5405.
- Miura, N. and Y. Shinohara (2009). "Cytotoxic effect and apoptosis induction by silver nanoparticles in HeLa cells." *Biochemical and Biophysical Research Communications* **390**: 733–737.
- Nelson, Baldwin, et al. (2003). "RAKE and LNA-ISH reveal microRNA expression and localization in archival human brain."
- Nishiya, T. and A. DeFranco (2004). "Ligand-dependent Toll-like receptor 4 (TLR4)-oligomerization is directly linked with TLR4-signaling. ." *J. Endotoxin Res.* **10**: 257-60.
- O'Connell, R. M., K. D. Taganov, et al. (2006). "MicroRNA-155 is induced during the macrophage inflammatory response." *PNAS* **104**: 1604-1609.
- Park, Vogelstein, et al. (2001). "Genetic disruption of PPARdelta decreases the tumorigenicity of human colon cancer cells." *Proc Natl Acad Sci U S A* **98**: 2598-603.
- Reilly, N., V. Poylin, et al. (2007). "Probiotics potentiate IL-6 production in IL-1-treated Caco-2 cells through a heat shock-dependent mechanism." *The American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*.
- Reinert (2002). "Friendly tenants in the human gut: The genome of *B. longum*." *Genome News Network*.
- Riccardo, S., G. Tortoriello, et al. (2007). "The coding/non-coding overlapping architecture of the gene encoding the *Drosophila* pseudouridine synthase." *BMC Mol Biol* **8**: 15.
- Rousseaux, C., X. Thuru, et al. (2007). "Lactobacillus acidophilus

modulates intestinal pain and induces opioid and cannabinoid receptors." *Nature Medicine* **13**: 35-37.

Sanders and Klaenhammer (2001). "The Scientific Basis of *Lactobacillus acidophilus* NCFM Functionally as a Probiotic." *Journal of Dairy Sciences* **84**: 319-331.

Shin, S.-H., M.-K. Ye, et al. (2007). "The effects of nano-silver on the proliferation and cytokine expression by peripheral blood mononuclear cells." *International Immunopharmacology* **7**: 1813–1818.

Stewart, I., P. J. Schluter, et al. (2005). "Cyanobacterial lipopolysaccharides and human health – a review." *BioMed Central* **5**.

Su, C. G., X. Wen, et al. (1999). "A novel therapy for colitis utilizing PPAR-gamma ligands to inhibit the epithelial inflammatory response." *J Clin Invest* **104**(4): 383-9.

Tili, E., J.-J. Michaille, et al. (2007). "Modulation of miR-155 and miR-125b Levels following Lipopolysaccharide/TNF- Stimulation and Their Possible Roles in Regulating the Response to Endotoxin Shock." *The Journal of Immunology* **179**: 5082-5089.

Yamamoto, M. and S. Akira (2004). "TIR domain--containing adaptors regulate TLR-mediated signaling pathways. ." *Nippon. Rinsho*. **62**: 2197-203.

Zhang, L., N. Li, et al. (2005). "Alive and dead *Lactobacillus rhamnosus* GG decrease tumor necrosis factor-alpha-induced interleukin-8 production in Caco-2 cells." *J Nutr* **135**(7): 1752-6.

Ich habe mich bemüht, sämtliche Inhaber der Bildrechte ausfindig zu machen und ihre Zustimmung zur Verwendung der Bilder in dieser Arbeit eingeholt. Sollte dennoch eine Urheberrechtsverletzung bekannt werden, ersuche ich um Meldung bei mir.