Survival and uptake of feed-derived DNA in the mammalian intestinal tract

Lise Nordgård

A dissertation for the degree of Philosophiae Doctor

UNIVERSITY OF TROMSØ
Faculty of Medicine
Department of Pharmacy
GENØK – Centre for Biosafety
2009

ACKNOWLEDGEMENTS

The present work has been carried out at GenØk – Centre for Biosafety in the period 2003-2009. Financial support was received from the Research Council of Norway (140890/720 and 140870/130) and GenØk.

I wish to express my gratitude to supervisor Kaare M. Nielsen for creating a stimulating scientific environment. Thanks for your guidance, incredible enthusiasm, and patience with my work, for all fruitful discussions and for sharing chocolate in your office. My gratitude also goes to supervisor Terje Traavik for an incredible enthusiasm and optimism and important contributions to my work. Thanks for introducing me to the field of GeneEcology and for sending me all around the world.

Thanks to co-authors; Tore Midtvedt, Yoshimi Benno, Thuy Nguyen, Arpad Pusztai, Susan Bardocz, Kristin Fenton, Beate Hegge, Lorenzo Brusetti and Noura Raddadi for important contributions to my work. Thanks to the animal department, UiTø, for excellent guidance and assistance during my work with the rats. Thanks to Lissa Nordin and colleagues at the laboratory of Medical Microbial Ecology at Karolinska Institute, Stockholm for excellent guidance and assistance during my work with the gnotobiots. Thanks to Dr Idun Grønsberg, my closest co-worker and co-author, and to Elisabeth Olsen. Thanks to Ane, Klaus, Kristine and Montse for reading through parts of my thesis.

Travel grants have given me the opportunity to attend meetings, conferences and participate in exchange programs world-wide. I am grateful to Bengt E. Gustafsson Symposie fund for a grant that allowed me to participate in "The 15th International Symposium on Gnotobiology (ISG)" in Tokyo, Japan, from June 20 to 24, 2005, a FEMS Young Scientist Meeting Grant allowing me to participate at the FEMS Meeting 06-05: "5th Joint-INRA Symposium on Gut Microbiology – Research to Improve Health, Immune Response and Nutrition" in Aberdeen, UK, from June 21 to 23, 2006 and the Fredskorpset (FK) exchange program for funding my exchange period at National Institute for Scientific and Industrial Research Centre (NISIR), Lusaka, Zambia, from September 2006 to December 2007.

Thanks to people working in the “GenØk lab” and “Microbiology lab”, brightening up the daily life in the laboratories. Thanks to everybody at NISIR, Lusaka, Zambia for making my stay an unforgettable time. Thanks to Trine Glad, for a lot of fun, coffee breaks and productive discussions throughout the years. Thanks to office mates Julia, Ane and Tracy for sharing days and evenings in the office.

Thanks to everyone at GenØk, for creating a pleasant and inspiring working atmosphere.

Family and friends – thank you for being patient with me and supporting me. You all deserve an enormous Thank You!!

Lise

TABLE OF CONTENTS

LIST OF PAPERS

Paper I

Grønsberg IM, Nordgård L, Fenton K, Hegge B, Nielsen KM, Bardocz S, Pusztai A and Traavik T. The uptake and organ distribution of plasmid DNA added to the feed of actively growing or pregnant rats.

Submittet to J Agr Food Chem, June 2009.

Paper II

Nordgård L, Nguyen T, Midtvedt T, Benno Y, Traavik T and Nielsen KM. Lack of detectable uptake of DNA by bacterial gut isolates grown in vitro by *Acinetobacter baylyi* colonizing rodents *in situ* (2007) Environ Biosafety Res 6: 149-160.

Paper III

Nordgård L, Brusetti L, Raddadi N, Traavik T and Nielsen KM. An investigation of the potential of horizontal gene transfer of feed derived DNA in the gastrointestinal tract of rats.

Manuscript.

Paper IV

Grønsberg IM, Nordgård L, Nielsen KM, Østerud B and Traavik T. Human peripheral blood mononuclear cells (PBMCs) bind foreign DNA and protect it against DNase degradation in an *ex vivo* whole blood model.

Manuscript.

INTRODUCTION

DNA is found in all living organisms. This macromolecular compound exhibits relatively high chemical stability, can survive various environmental conditions and has been detected in organic remnants after thousand of years (Pääbo et al., 1988). However, degrading reactions like hydrolysis (enzymatic and non-enzymatic), oxidation and methylation of DNA occur at significant rates (Lindahl, 1993; Reuter and Aulrich, 2003). The matrix within which the DNA is contained influences its stability. For instance, DNA adsorbed to clay in soil has shown to be more stable than DNA in water solution (Thomas and Nielsen, 2005; Pontiroli et al., 2007). Variable stability has also been observed with DNA in food exposed to different processing conditions which has shown to influence the degradation rate and fragment size (Jonas et al., 2001; Bauer et al., 2004).

In the last decade, recombinant DNA technologies have permitted the introduction of foreign genes into unrelated species crossing species barriers at elevated frequencies (Pirondini and Marmiroli, 2008). Genetically modified (GM) organisms like GM microorganisms, GM plants and GM animals are examples of organisms that contain transgenic genes from different species including from different kingdoms of life (Thomson, 2001; Traavik et al., 2007). The purpose of producing new GM organisms can be to change the activity of a gene, to insert a new gene or to upregulate or turn off the activity of a gene. Biological risk assessment of GMOs has exposed knowledge gaps related to how DNA is degraded, or survive degradation in different environments such as in different compartments of the gastrointestinal tract of mammals (Thomson, 2001; Nielsen et al., 2005b; Traavik et al., 2007).

There are hypothesized side-effects of introducing recombinant DNA in mammalian systems, including the possibility of horizontal transfer of recombinant DNA from genetically modified plants by the intestinal microflora or by enterocytes in mammalian organisms. Potential risks may be the possible transfer of antibiotic resistance genes used as marker genes in a GMO to a pathogen, or unintended effects of integration into the host genome of GM-feed consumers (Kurth et al., 1998; Ho et al., 2000; Reuter and Aulrich, 2003; van den Eede et al., 2004; EFSA, 2009). However it is important to note that horizontal gene transfer (HGT)

is widespread in nature and in some cases occurs frequently and may not lead to any harm (Keese, 2008; Kelly et al., 2009).

The ways an organism can be exposed to foreign DNA molecules differ. It may be e.g. by inhalation, through infections or through food. Since such DNA may have deleterious genetic effect on the host, it implies that organisms have evolved defense systems against foreign DNA (Forsman et al., 2003). However, some foreign DNA is able to escape these degradation mechanisms and the DNA molecules can then be transmitted between species and possibly be taken up by the exposed organism (Doerfler and Schubbert, 1998; Kurth, 1998; Bushman, 2001; Tonheim et al., 2008). However, for uptake of foreign DNA in a mammalian system to occur, several conditions have to be met. Key requirements such as DNA survival in food and in the gastrointestinal tract have been identified.

The introduction will focus on knowledge of DNA passing through the gastrointestinal tract of mammals and the general mechanisms for uptake of DNA by bacteria or mammalian cells generated from research performed on different mammalian and bacterial species.

DNA stability / DNA degradation in mammalian systems

DNA in food /feed

All plant and animal food sources contain RNA, DNA, nucleosides and free nucleic bases. The diary intake and content of DNA varies depending on the diet and also on the effects of processing. Concentrations of RNA and DNA in foods depend mainly on cell density of food sources, and with our traditional diet we normally ingest relatively high amounts of DNA from animal muscle tissues whereas plant derived food contains lower concentrations (Gil, 2002). The average intake of dietary RNA and DNA in humans varies but is calculated to approximately 0.1-1 g / person / day (Doerfler and Schubbert, 1998). All DNA, including the DNA from GMOs, is composed of the same four nucleotides, meaning that the present use of recombinant techniques in the food chain does not introduce major changes in the chemical characteristics of the DNA (Jonas et al., 2001).

The conditions of food processing and storage as well as the food matrix and the processing will affect the extent of DNA degradation (Hupfer et al., 1998; Master et al., 1998; Guoli et al., 1999; Jonas et al., 2001). This may lead to partial or complete degradation of DNA molecules that may be present in the consumed product. This means that extensively processing of food may decrease the size of DNA making it undetectable by the detection methods used today (Pauli et al., 2000; Kharazmi et al., 2003).

Laboratory studies have demonstrated the persistence of DNA in food, for instance in canned food, whole seeds, cracked seeds and meal of canola, wet sugar, beet pulp, cereal grains (Bauer et al., 1999; Chiter et al., 2000; Einspanier et al., 2001; Bauer et al., 2003; Duggan et al., 2003). Large fragments of DNA may be present in various processed food product, such as biscuits, polenta, baking products (Hupfer et al., 1998; Lipp et al., 2001), cooked meat (Gouli et al., 1999), soymilk and tofu (Bauer et al., 2003).

Temperature and pH are important parameters in food processing. Studies have shown the the DNA double helix is unstable at a temperature between 60ºC and 90ºC, causing fragmentation of high-molecular weight DNA. At acidic pH (pH 2-3), DNA strands are damaged because purines are removed from the nucleic backbone due to cleavage of bonds between

deoxyribose residues and these bases. Also shear forces, chemical agents and enzymes may affect the DNA structure and cause depurination, deamination and strand breaks which may lead to further degradation and loss of biological activity. (Lindahl, 1993; Hupfer et al., 1998; Master et al., 1998; Kharazmi et al., 2003; Weiss et al., 2007).

DNA in food has not been considered to constitute a health risk, but it has been reported that dietary nucleotides have a modulatory effect on the immune system. The molecular mechanism by which dietary nucleotides modulate the immune system is not known (Gil, 2002). However, DNA in food can be an agent capable for transforming a wide range of bacteria, including phatogens (Nielsen et al., 2005). Concern about its possibility of transforming bacteria has gained increased focus after a wide range of foods containing GM appeared in the market. This has led to an increased focus on risk assessment of novel ingredients derived from genetically modified organisms in the food chain and the partial resistance of DNA to physical and chemical treatments and the potential for DNA persistence and possible horizontal spread (Sharma et al., 2006; Weiss et al., 2007). Many locally produced feedstuffs are not treated with high temperatures, and intact DNA will be present and potentially capable of being taken up by microbes in the food or human cells in the digestive tract.

Food constitutes suitable environment for numerous microorganisms as they usually provide readily available nutrients and ecological conditions for rapid growth. It has been demonstrated that food-associated bacteria, like *Bacillus subtilis*, which occur in foods as natural contaminants, can develop competence during growth in foods and then become transformed with free DNA in the food matrix (Bräutigam et al., 1997; Kharamazi et al., 2003). The effect of food components and processing parameters on DNA degradation in food was also monitored by using a detection system based on electrotransformation of *Esherichia coli*, which is an intestinal bacteria (Bauer et al., 1999; Bauer et al., 2004). In these studies *Esherichia coli* was shown to be transformable in different foodstuff. These observations suggest that food associated bacteria or contaminating intestinal bacteria may become transformed and may further spread the acquired DNA to bacteria of the digestive tract upon ingestion.

DNA in the gastrointestinal tract of mammals

The gastrointestinal tract is a complex ecosystem consisting of the GI epithelium, immune cells and the bacterial microbiota (McCracken and Lorenz, 2001). It is the main portal of entry for foreign macromolecules, and its epithelial lining forms the main site of contact with feed-derived DNA and proteins in mammals and many other organisms. Most free DNA molecules entering the digestive system undergo substantial degradation, and to be able to understand the dynamics of DNA, the stability of DNA in all regions of the digestive system must be taken into consideration. Free DNA may be broken down into small fragments by the mechanical processes of mastication, acid hydrolysis and gastrointestinal enzymatic activity involving nucleases, and DNases released from the pancreas, and by bacteria present in the intestine (Beever and Kemp, 2000; Wilcks et al., 2004; Mazza et al., 2005).

The oral cavity is the first site of contact between incoming bacteria, free DNA in food and the resident microflora. Salivary nucleases play an important role in DNA degradation but studies investigating the survival of plasmid DNA in human saliva have demonstrated that plasmid DNA can remain incompletely degraded for a significant time in samples from human saliva *in vitro*. Approximately 65 % of a 520 bp target was found to be amplifiable after 10 min of incubation in saliva (Mercer et al., 1999b; 2001; Duggan et al., 2000). Later, Duggan et al. (Duggan et al., 2003) demonstrated that plasmid DNA was extensively degraded after one min incubation *in vivo*, in the ovine oral cavity. Approximately 70 % of the plasmid DNA added to the mouth was lost within a minute and the physical integrity was also destroyed. However, some DNA survived in an incompletely degraded state and remained in a biological active state being able to transform electro competent *E. coli* cells up to 8 min of incubation in saliva (Duggan et al., 2003). A later study by Shedova et al. (Shedova et al., 2009), demonstrated that growing cells of *S. gordonii* incubated with saliva collected from cows were competent for DNA uptake. After passing the oral cavity, the food enters the stomach where gastric juice is produced. The median pH has been reported to be 1, 4 and this creates a hash environment for DNA. Under low pH conditions depurination of the nucleic acid backbone will take place followed by hydrolysis of adjacent 3`, 5-phosphodiester linkages resulting in shortening of DNA strand and this is a significant step in the degradation of DNA.

The next location is the small intestine which is the main site of digestion and absorption. Chyme with enzymes produced by epithelial cells, intestinal juice, pancreatic juice (secreted by the pancreas into the duodenum), and bile (produced by the liver and

transported via the gall bladder into the duodenum) aids the enzymatic degradation of food macromolecules.

The large intestine is primarily involved in water absorption. There are no digestive enzymes secreted by the mucosa of the large intestine so further breakdown of dietary constituents in this region is carried out only by the resident microbiota. DNA is less rapidly degraded here and could therefore be available for transformation of competent cells in the microbiota (Wilson et al., 2005). A study by Wilcks et al. (Wilcks et al., 2004) indicates that bacterial nucleases play only a minor role in DNA degradation since hardly any differences in DNA breakdown were observed in germfree and human flora associated (HFA) rats. In these *ex vivo* experiments, the major proportion was degraded in the upper part of the gastrointestinal system.

Studies examining the DNA persistence in the gastrointestinal tract of mammals

The fate of nucleic acids in the gastrointestinal tract of mammals, e.g ruminants and rats, was first investigated years ago when the catabolism of DNA to nitrogenous bases, free bases and secondary metabolites were determined. However, the limited sensitivity of the methods available could not eliminate the possibility that trace amounts of intact DNA fragments could survive passage through the mammalian gut system (Maturin and Curtiss, 1977; McAllans 1980; 1982). Later, studies by Rainer Schubbert, Walter Doerfler, and coworkers received increased attention after detecting bacteriophage-, plasmid- and plant DNA at different fragment sizes and frequencies in the gastrointestinal tract, circulating blood cells and organs like liver, spleen and kidneys of mice (Schubbert et al., 1994; 1997; 1998; Hohlweg and Doerfler, 2001; Palka-Santini et al., 2003).

Rodents: The research group of Walter Doerfler in Germany has performed investigations on the fate of feed-derived DNA of different sources consumed by rodents. Their studies demonstrated that feed-derived DNA was not completely degraded in the mouse intestinal tract (Schubbert et al., 1994; 1997; 1998; Hohlweg and Doerfler, 2001; Palka-Santini et al., 2003). In their work they demonstrated that 1-2 % of orally ingested bacteriophage DNA survived the enzymatic repertoire of the gastrointestinal tract and was detected in the feces. The main size of the DNA fragments detected ranged in size of a few hundred bp up to about 1700 bp in a few exceptions and were found in fecal samples 1-7 h after feeding (Schubbert et al., 1994; 1997; 1998). In another feeding experiment with mice fed soybeans, the plant specific rubisco gene survived in different parts of the gastrointestinal tract up to 121 h, indicating that plant-associated fed DNA is more stable in the gastrointestinal tract than "naked" DNA (Hohlweg and Doerfler, 2001). A further study by the same authors demonstrated that with higher fiber content in the diet, the transit time of food in the digestive tract was shortened and foreign DNA was cleared more rapidly (Palka-Santini et al., 2003). An increase in fat or cellulose content in the diet did not show a change in the digestion or extend DNA persistence time in the gastrointestinal tract, but the impact of stomach filling was shown to influence the degradation of DNA. In animals that had been starved prior to receiving plasmid DNA, the DNA was much more rapidly degraded (Palka-Santini et al., 2003).

Fish: In a feeding study with Atlantic salmon, Sanden et al. (Sanden et al., 2004) demonstrated that plant (180 bp)- and transgenic DNA fragments (120 bp and 195 bp) could be detected in different parts of the gastrointestinal tract of fish fed diets containing GM soybeans. Also Nielsen et al. (Nielsen et al., 2005a) were able to detect dietary DNA in samples from the gastrointestinal tract after adding extracted DNA from GM maize and soya to fish-feed. In another study with rainbow trout fed a mixed diet containing GM-soybeans (Chainark et al., 2008), chloroplast DNA fragments (257 bp) were detected at different levels in GI contents.

Poultry: Studies with poultry report detection of plant DNA fragments in the gastrointestinal tract while recombinant DNA only has been detected in different parts of the GIT (Einspanier et al., 2001; Chambers et al., 2002; Klotz et al., 2002; Tony et al., 2003; Nemeth et al., 2004; Aeshbacher et al., 2005; Deaville et al., 2005; Rossi et al., 2005). The main "bulk" of detected fragments in these studies are 100-500 bp long. However, Rossi et al. (Rossi et al., 2005) were able to detect an 1800 bp fragment of transgenic DNA in the crop and gizzard of birds fed Bt-corn. Also, DNA fragments from high copy number alleles/organelles are much more frequently detected than fragments from single copy genes.

Pig: High copy-number chloroplast-specific DNA fragments have been detected in gastrointestinal tract contents up to 72 h in pigs (Klotz et al., 2002; Reuter and Aulrich, 2003; Chowdhury et al., 2003 a; 2003 b; Nemeth et al., 2004; Sharma et al., 2006). The biggest fragment, a chloroplast specific 1028 bp fragment, was detected in the stomach, duodenal and cecal samples of both Bt-11 and non-GM corn fed pigs (Chowdhury et al., 2003 a). Most of the recombinant DNA was degraded in the gastrointestinal tract and only small fragments (110 bp) could be detected in all stomach, duodenal, ileal and cecal samples.

Sheep: Most studies with sheep have investigated the fate of foreign DNA through the GIT in *ex vivo* and *in vivo* models (Duggan et al., 2000; 2003; Alexander et al., 2004; 2006; Sharma et al., 2006). The results of *ex vivo* experiments demonstrated that both plasmid DNA and chromosomal DNA was incompletely degraded in gastrointestinal tract environments. A 350 bp target sequence of the plasmid DNA was amplifiable after 30 min incubation in rumen fluid and up to 2 h after incubation in fresh ovine saliva. The same target sequence for maize chromosomal DNA was, however, only amplified after up to 1 min after addition of rumen fluid and up 24 h in fresh ovine saliva (Duggan et al., 2000).

Later, the stability of free plant DNA was examined in ruminal, duodenal and fecal fluids (Alexander et al., 2004). Here, the results demonstrated a clear difference in the stability of free plant DNA depending on the digestive site and the pH of the fluid, when screening for fragments from 300 up to 1363 bp in size. Free transgenic DNA was the least stable in duodenal fluid at pH 7 where fragments less than 527 bp were detected for up to 2 min and fragments as large as 1363 bp were detected for 0, 5 min.

The results from a feeding experiment with sheep fed maize silage and maize grains demonstrated that a 1914 bp transgenic fragment was amplifiable in rumen samples 5 h after feeding maize grain. However, this sequence could not be amplified in silage-fed sheep (Duggan et al., 2003). After reducing the target sequence to 211 bp a more sensitive detection was received and the transgene was detected both in maize-silage and maize-grain fed sheep up to 3 h and 24 h. Neither in silage nor maize grain fed sheep could any plant DNA target sequences be detected in feces which may be explained by a slow rate of passage of digesta in ruminants. In addition to examinating the fate of a transgene in maize fed to sheep, this study also investigated the survival of free DNA in the oral cavity. The results showed that approximately 70 % of both plasmid DNA and maize chromosomal DNA was lost within the first minute but fragments of 1914 bp could be recovered up to 30 min after incubation of plasmid DNA and up to 5 min after incubation of maize chromosomal DNA.

In a study by Sharma et al. (Sharma et al., 2006) the fate of recombinant and endogenous plant DNA in the gastrointestinal tract was examinated (Sharma et al., 2006). Here, high-copy chloroplast-specific DNA fragments (520 bp) were detected in digesta samples. Low-copy plant DNA fragments (from 186 to 540 bp long) were also present in the gastrointestinal tract samples but at lower and more variable frequencies. The transgenic fragments (197-527 bp) were more common in intestinal digesta than in ruminal or abosomal content. In another study, the same group focused on quantifying the persistence of transgenic DNA in the rumen, at the proximal duodenum, and the feces of sheep fed diets containing Roundup Ready rapeseed meal (Alexander et al., 2006). Here a fragment of 1365 bp was quantifiable in rumen fluid and duodenal fluid for up to 13 h and a 108 bp fragment for up to 29 h, while no DNA was detected in feces.

Cattle: Some *in vitro* and *in situ* studies involving cattle fed GM-based diets, have been investigating the persistence of DNA from different plant formulations typical for use as animal feed, in rumen contents (Alexander et al., 2002; Sharma et al., 2004; Wiedemann et al., 2006). The different results demonstrated that plant DNA fragments (ranging from 179-

527 bp) from whole and cracked seeds, compared to more processed seeds as in pellets and flour, could be detected for the longest incubation time (up to 48 h) in ruminal fluid (Alexander et al., 2002; Sharma et al., 2004). This was also confirmed in an *in situ* study (Wiedemann et al., 2006). Here, plant DNA from whole plant corn could be detected for the longest incubation time. Further, quantification of both chloroplast- and transgenic specific DNA fragments showed a sharp decrease during the first 4 h of ruminal incubation (Wiedemann et al., 2006). In a study by Einspanier et al. (Einspanier et al., 2001) cows fed transgenic plant material were analyzed. Both chloroplast specific (199 bp) and transgenic DNA (189 bp) fragments were found in duodenal juice. In contrast, no signals were detected in feces. However, in one feeding study with cows and one with calves (Phipps et al., 2003; Chowdhury et al., 2004), fragments of chloroplast and transgenic DNA survived passage through the gastrointestinal tract. Phipps et al. (Phipps et al., 2003) detected fragments of chloroplast and transgenic DNA in the majority of the ruminal and duodenal samples, while only chloroplast DNA fragments were detectable in feces. The size of the chloroplast DNA fragments detected decreased from 1176 bp in the ruminal and duodenal digesta to 351 bp in fecal samples. In the study with calves, chloroplast- and transgenic DNA fragment (ranging from 110 – 1000 bp) were detected inconsistently in abomasal, jejunal and cecal contents from 5 to 18 h after feeding (Chowdhury et al., 2004).

Wild animals: So far only two studies have investigated the fate of genetically modified maize in the gastrointestinal tract of wild animals, one on fallow deer and another on wild boar (Guertler et al., 2008; Wiedemann et al., 2008). The first study detected chloroplast-specific (from 173 bp up to 896 bp) and maize-specific (329 bp) plant DNA in the contents from the gastrointestinal tract of fallow deer. Recombinant DNA fragments, ranging in size from 204 up to 1423 bp, were not detected in any samples (Guertler et al., 2008). In the study involving wild boar, both fragments of chloroplast-specific plant DNA (173 bp) and recombinant DNA fragments (from 211-727 bp long fragments) were detected in the contents from the gastrointestinal tract (Wiedemann et al., 2008).

Humans: When in comes to humans, only a few attempts have been made to study the stability of DNA in the gastrointestinal tract. Martin-Oruè et al. (Martin-Oruè et al., 2002) incubated GM-foods and DNA from genetically modified soya and maize in human intestinal simulations to investigate protection of the DNA by the food matrix. The results demonstrated that plant associated naturally fed DNA was more stable compared to naked

DNA in the gastrointestinal simulations. The incubation of plant material did not result in a significant decrease in recombinant DNA fragments when incubated in the intestinal simulations, while when naked DNA was incubated there was a relatively high decrease in the amount of recombinant DNA fragments. The results showed some differences between the two foods. Roundup Ready soya was shown to be much more sensitive to degradation compared to maize. The size of DNA extracted from soya and maize were quite different. The maize nucleic acid was of high molecular weight while the soya derived material had been extensively fragmented and had a molecular weight that ranged from 100 to 1000 bp. This is most likely to reflect the source of the material. The Roundup Ready soya was a blended product containing only small proportions of material while the maize seeds were obtained directly from Monsanto. Later, Netherwood et al. (Netherwood et al., 2004) evaluated the survival of soybeans in the gastrointestinal tract of human ileostomists. The meal fed to the ileostomists contained 3×10^{12} copies of the transgene and of these, a maximum of 3,7 % could be recovered in the digesta of the stoma from the individuals. To quantify the survival of transgene fragments in the feces, another experiment with individuals with an intact gastrointestinal tract was performed. Here, the transgene could not be detected. In summary, these results indicated that a small proportion of transgenes in soya survives passage through the human upper gastrointestinal tract but is completely degraded in the large intestine.

In summary, the studies on DNA in the gastrointestinal tract reflect that the majority of feed introduced DNA becomes reduced to a fragmented form and that the detection of it is dependent on the selection of the fragment size to be amplified. Besides, the results also reflect differences in the degradation process in the processing of food and in the gastrointestinal tract of feed-derived DNA in different animal models.

Studies examining the host uptake of DNA from the gastrointestinal tract

The huge resorptive surface of the gastrointestinal tract exposes all organisms to macromolecules from the foreign environment. As a consequence, the epithelial lining of the gastrointestinal tract is constantly exposed to foreign DNA, and the question of to what extent this DNA can be taken up and incorporated in the cells in the gastrointestinal tract or pass from the gastrointestinal tract into the circulation has gained increased attention.

A small proportion of ingested DNA (≤ 0,1 %) was detected in the blood stream of mice between 2-8 h after feeding and in spleen or liver cells up to 24 h after feeding (Schubbert et al., 1997). After feeding bacteriophage- or plasmid DNA to pregnant mice, foreign DNA was detected in the fetuses and of newborn animals (Schubbert et al., 1998). In a follow-up experiment with pregnant mice given a daily dose of plasmid DNA for 8 generations, the results did not provide any evidence for germ line transmission of DNA ingested (Hoelweg and Doerfler, 2001). The same group performed a feeding experiment with mice fed soybeans, and plant specific DNA fragments were detected in samples from the liver and spleen (Hoelweg and Doerfler, 2001). On the basis of the findings in these studies, the authors suggested that DNA fragments routinely exposed to epithelial cells of the GIT may be presented to the M-cells in the Peyer`s patches of the intestine wall which transfer the DNA molecules into the bloodstream where the DNA molecules may further be localized to various host cells and tissues.

The above findings also made it necessary to investigate the fate of foreign DNA in farm animals, since questions about the digestive fate of DNA and proteins have been raised with regard to human consumption of animal products (e.g. meat, milk and eggs) from farm animals fed transgenic crops. Farm animals ingest a considerable amount of foreign DNA via feed and the possibility of DNA transfer from GM crops into animal tissues and organs has gained increased attention. So far there are reports investigating the uptake of feed-derived DNA in farm animals by applying a variety of techniques: Southern Hybridization (SH), Polymerase Chain Reaction (PCR), Southern blot hybridization (SBH), *in situ* hybridization (ISH), fluorescent *in situ* hybridization (FISH), in rodents, fish, poultry, pigs, sheep, cattle, wild animals and humans. The majority of the studies is summarized in Table 1, and presented in more detail below.

Fish: In a feeding study with Atlantic salmon, Sanden et al. demonstrated that dietary DNA was taken up by intestinal tissue in Atlantic salmon (Sanden et al., 2007). Also Nielsen et al.

(Nielsen et al., 2005a) were able to detect dietary DNA in samples from the GIT, liver, kidney and blood, after adding extracted DNA from GM maize and soya to fish-feed. In another study with rainbow trout fed a mixed diet containing GM-soybeans (Chainark et al., 2008), chloroplast DNA fragments (257 bp) were detected at different levels in leucocytes and muscle, confirming the results about possible persistence and uptake of foreign DNA in fish from the studies with Atlantic salmon. In studies where foreign DNA was intravenously- and intramuscular injected in Atlantic salmon, foreign DNA was detected in different tissues and organs like liver, kidney, spleen and muscles (Nielsen et al., 2006; Tonheim et al., 2007).

Poultry: Studies investigating the fate of plant DNA in poultry show some variable results. One study by Jennings et al. was not able to detect any plant DNA in muscle from broilers fed transgenic corn (Jennings et al., 2003). Other studies report detection of plant DNA fragments in different organs and tissues such as liver, spleen, muscles and blood while transgenic DNA only has been detected in different parts of the gastrointestinal tract (Einspanier et al., 2001; Chambers et al., 2002; Klotz et al., 2002; Tony et al., 2003; Nemeth et al., 2004; Aeshbacher et al., 2005; Deaville et al., 2005; Rossi et al., 2005). The main "bulk" of detected fragments in organs and tissues in these studies are 100-500 bp long. Also, DNA fragments from high copy number alleles/organelles are much more frequently detected than fragments from single copy genes.

Pig: In contrast to the studies with rodents and poultry, plant derived DNA has been detected to a lesser extent in organs and tissues from pigs. Both Klotz et al. (Klotz et al., 2002) and Jennings et al. (Jennings et al., 2003) reported that plant DNA (both plant specific- and transgenic DNA fragments) could not be detected in samples from blood and different organs and tissues. DNA fragments have only been detected at different levels in gastrointestinal tract content except in a study by Sharma et al. (Sharma et al., 2006). Here, DNA fragments were detected by PCR and Southern blot hybridization in duodenal- and cecal tissues and in one kidney- and one liver sample.

Sheep: Most studies with sheep have investigated the fate of foreign DNA through the gastrointestinal tract in *ex vivo* and *in vivo* models (Duggan et al., 2000; 2003; Alexander et al., 2004; 2006; Sharma et al., 2006) except from one study by Sharma et al. (Sharma et al., 2006) which also examinated the fate of recombinant and endogenous plant DNA in GI tract tissues and organ tissues from sheep (Sharma et al., 2006). Here, high-copy chloroplast-

specific DNA fragments (520 bp) were detected not only in digesta samples, but also in the majority of intestinal tissues and in a few samples from tissues from liver and kidney of sheep. Low-copy plant DNA fragments (from 186 to 540 bp long) were also present in the same samples but at lower and more variable frequencies. The recombinant DNA fragments (197-527 bp) were not detected in blood or any tissues or from any visceral organs.

Cattle: Feeding studies involving cows and calves have been searching for both multi- and monocopy plant DNA and transgenic DNA fragments in the gastrointestinal tract, blood and visceral organs and tissues. In a study by Einspanier et al. cows fed transgenic plant material were analyzed. Both chloroplast specific (199 bp) and transgenic DNA (189 bp) fragments were found in duodenal juice. In contrast, no signals were detected in blood, feces and tissues of liver, spleen, kidney and muscle (Einspanier et al., 2001). Yonemochi et al. investigated samples from blood, liver and muscles of cows fed conventional and transgenic maize for transgenic DNA fragments (379 bp) (Yonemochi et al., 2003). These results confirmed the findings of Einspanier with no detection of transgenic DNA in samples from muscles and visceral organs. Moreover, in another study with cows fed a mixed diet containing transgenic soya and maize, a 118 bp fragment of the soya lectin gene and a 226 bp fragment of the maize invertase gene could not be detected in blood or urine, here the feces samples were positive for the same fragments (Poms et al., 2003). In this latter study extracted DNA from transgenic plants was injected intravenously and the results showed a fast elimination of marker DNA in the blood. The 118 bp fragment of soya lectin could be amplified only up to 2 min. Jennings et al. investigated the fate of chloroplast-specific and recombinant plant DNA in cattle and were not able to detect any plant DNA fragments by PCR in samples from any tissues of muscle, liver, spleen, or kidney from cattle (Jennings et al., 2004). In a study by Bertheau et al., chloroplast- and plant DNA fragments were variously detected in the blood leucocytes, liver, spleen, kidney, mesenteric lymph node and muscles of calves. The presence of plant DNA (endogenous and transgenic) in the blood of cows fed with silage of either conventional or GM maize was searched for, and the results showed that both mono- and multicopy sequences from maize DNA were less detectable than chloroplast DNA. The presence of transgenic DNA could not be demonstrated.

Milk: The only route that fragments of plant DNA can be detected in milk is by transfer from the gastrointestinal tract and into the blood, as long as the samples has not been contaminated during the sample collection. In studies with cows fed genetically modified maize, soy and

cottonseed, plant DNA fragments in milk were not detected (Poms et al., 2003; Castillo et al., 2004; Phipps et al., 2003; 2005; Jennings et al., 2004). Later, Agodi et al. (Agodi et al., 2006) were the first group to report that small GM DNA fragments can be detected in milk samples from the Italian marked. In a later study investigating milk from lactating goats fed GM maize, chloroplast-specific gene fragments were detected. In contrast, no chromosomally located maize gene or recombinant DNA fragments were detected (Rizzi et al., 2008).

Wild animals: There are two studies investigating the possible uptake of GM maize in wild animals, one on fallow deer and another on wild boar (Guertler et al., 2008; Wiedemann et al., 2008). The first study detected chloroplast-specific (from 173 bp up to 896 bp) and maize-specific (329 bp) plant DNA occasionally in samples from the spleen, kidney, lymphatic node, liver and muscle of fallow deer. Transgenic DNA fragments, ranging in size from 204 up to 1423 bp, were not detected in any samples (Guertler et al., 2008). The study involving wild boar, neither fragments of chloroplast-specific plant DNA (173 bp) nor transgenic fragments (from 211-727 bp long fragments) were detected in samples from liver, kidney, spleen, heart or lung (Wiedemann et al., 2008).

Humans: Only a few attempts have been made to study the transfer of DNA from ingested food across the intestinal barrier. A study by Forsman et al. (Forsman et al., 2003) demonstrated uptake of fragments up to $\geq$ 250 bp of high-copy rabbit endogenous retrotransposon DNA (RERV-H) and rabbit mitochondrial DNA into the peripheral blood of humans that had ingested a meal of 600 g rabbit meat (10^{14} copies of RERV-H) (Forsman et al., 2003). Up to 5 h after the experimental meal, a maximum of 200 RERV-H copies per ml blood could be detected, corresponding to approximately up to 10^6 RERV-H molecules in the circulation.

In summary, the differences in the detection of feed-derived DNA may be due to different plant formulations used as feed, species differences, in the distinct digestive systems, differences in developmental stage (juvenile vs. adult), difference in how the experiments were performed and also variations in method sensitivity and detection limits.

Table 1: Studies of food introduced- or injected DNA in mammalian systems

Animal species	DNA source	Exposure / (detection) [a)]	Exposure/results	Reference
Mouse	PY pDNA	Intact rec pDNA were inoculated subcutaneously into weanling mice Cleaved rec pDNA were inoculated subcutaneously into weanling mice	No antibody response to PY Nearly all injected animals developed PY infection	Israel et al., 1979
	Rec phage	Rec phage DNA were inoculated parenterally into weanling mice Cleaved rec phage DNA were inoculated parenterally into weanling mice	No PY infections PY infections were induced with regularity in the injected animals	Chan et al., 1979
	Bacteriophage	A single dose of 10-50 μg circular or linearized double-stranded bacteriophage DNA (M13mp18) was administered orally by pipette or in food pellets. The mice were killed at different time points after feeding before sampling of blood and GIT contents / (SBH, DBH, PCR)	Bacteriophage DNA fragments were detected in GIT samples and blood	Schubbert et al.,1994
	Bacteriophage	A single dose of 50 μg circular or linearized double-stranded bacteriophage DNA (M13mp18) was orally administered. The mice were killed at different time points up to 24 h after feeding before sampling of spleen-, liver- and blood cells / (PCR, SBH, FISH, Recloning of bacteriophage DNA sequences)	Bacteriophage DNA fragments were detected in the GIT, columnar epithelial cells in the cecum, leucocytes in Peyer`s patches of the cecum wall, cytotoxic T cells, B cells, macrophages from spleen	Schubbert et al., 1997
	Bacteriophage pDNA	A daily dose of 50 μg circular or linearized double-stranded bacteriophage DNA (M13mp18) or pDNA (pEGFP-C1) was orally administered by pipette to pregnant mice up to 14 days or from day 6-14 of gestation before sampling of organs and tissues, blood and fetus were sampled in the end of each experimental period / (PCR, FISH, SBH, Sequencing)	pDNA fragments were detected in GIT samples, liver, spleen and kidney and in cell nuclei in the intestinal wall, spleen and liver Bacteriophage or pDNA- fragments were detected in different tissues of fetuses and newborn animals from pregnant mice	Schubbert et al., 1998
	pDNA Soybean leaves	A single dose of pDNA (pEGFP-C1, pRSVGFP or pSVGFP) was injected into skeletal muscles and the animals were killed at different time sets after injection. Soybean leaves (Glycine max) or pDNA (pEGGT-C1) were fed to mice for different periods before sampling of organs, tissues and blood / (PCR, SBH, FISH, RT-PCR)	I.m injected pDNA showed **transcription** in the injected muscle. No transcription in gut, spleen or liver cells or any germline cells after orally administering the pDNA Plant specific DNA fragments were detected in GIT contents, liver and spleen feeding soy-bean leaves. No germline transmission of pDNA	Hoelweg and Doerfler, 2001

Table 1: continues

Animal species	DNA source	Exposure / (detection) [a)]	Exposure/results	Reference
	Bacteriophage pDNA	A single dose of 50 μg circular or linearized double-stranded pDNA (pEGFP-C1) (or in some cases bacteriophage DNA (M13mp18) and adenovirus type 2 (ad2) DNA) was orally administered. In addition diets with different fiber- and fat content were given in some experiments before sampling of liver, spleen, kidney, blood and content from stomach, small intestine, cecum and large intestine at various times after feeding (up to 5 days)/ (SH, PCR, FISH)	Bacteriophage DNA fragments were detected up to 2 h in contents from the stomach and small intestine, up to 6h in cecum and large intestine under different feeding regimes pDNA fragments were detected in the nuclei of cecal epithelial cells up to 18 h after feeding A higher fiber content of the diet decreased the transit time of food thorough the GIT, and thus DNA fragments were eliminated faster. Higher fat content of the diet had no demonstrable effect on the persistence and degradation of pDNA	Palka-Santini et al., 2003
Rats	Bacterial- and pDNA	Chormosomal- and plamsid DNA from *E.coli* λ1776 was mixed with intestine contents from conventional rats *ex vivo* up to 4 h / (Whatman No.3 filters, Beckman scintillation counter)	Bacterial- and pDNA was rapidly degraded when added to low dilutions of rat intestinal contents	Maturin and Curtiss, 1977
	pDNA Plant DNA	Different experiments were performed: 1) *ex vivo:* **a)** 2 ml intestinal contents from mono-associated rats with *E.coli* were mixed with extracted DNA from GM potato (Apriori) (40 μg/ml) and incubated at 37ºC up to 43 h **b)** 25 mg maize flour DNA was mixed with 100 mg content from the GIT from germfree and HFA up to 22 h at 37ºC 2) *in vivo*: **a)** pDNA (pMR2) was gavage fed in 3 monoassociated rats (*E.coli* strain MS15979) every day for 2 weeks **b)** Mono associated rats (*B. subtilis* 168) were gavage fed 1 ml (100 ug/ml) pDNA (pAW105) for 3 weeks 3) HFA rats were fed maize flour for three days / (PCR)	*Ex vivo* experiment demonstrated rapidly degradation of maize flour DNA and naked potato DNA in the upper part of the GIT *In vivo*, pDNA fragments were detected up to 5 h after feeding in all parts of the GIT. Maize DNA was only detectable in the upper part (stomach and duodenum). The presence of chloroplast DNA could be detected in all compartments of the GI tract	Wilcks et al., 2004

Table 1: continues

Animal species	DNA source	Exposure / (detection) [a)]	Exposure/results	Reference
	Soybeans	Mixed diets containing 30, 60, 90% GM soya (RRS: *cp4epsps*) or non-GM soya were given after weaning and up to week 13 before sampling of muscles / (PCR)	No plant- or transgenic DNA fragments were detected	Zhu et al., 2004
	Potato	A mixed diet with 30% genetically modified potato (Spunta lines G2 and G3) or the unmodified counterpart were given for 30 days before sampling of liver, kidney, spleen, heart, testes, lung, skin, blood, muscles, GI content from esophagus, stomach, duodenum, jejunum, ileum, cecum and rectum	Chloroplast DNA fragments were detected in GIT, excreta, liver, kidney, spleen and muscle and transgenic DNA fragments were detected in GIT and excreta	El Sayed al., 2006
Rabbit	Soybeans	A mixed diet (130 g/day) containing 20% GM soya (RRS: *cp4epsps*) was given for a period from 30 day-old up to 70 days of age before sampling of liver, muscles, kidney, heart and blood / (PCR	Chloroplast fragments were detected in blood, muscle, heart, liver and kidney at different levels. No recombinant DNA fragments were detected	Tudisco et al., 2006
Atlantic salmon	Soybeans	A mixed diet where part of the fishmeal protein was replaced with 17,2% DNA from GM soy beans (RRS: *cp4epsps*) or non GM soybeans was given for 6 weeks before sampling fish GI content from stomach, pyloric region, mid intestine and distal intestine and tissue from liver, muscle, brain and GIT / (PCR, sequencing, ISH)	Transgenic DNA fragments could be detected in the stomach, pyloric region, mid intestine and distal intestine.	Sanden et al., 2004
	Maize Soybeans	Amplified DNA from GM maize (Bt176) and GM soybeans (GTS40-3-2 leaf material – RRS) from certified reference material were mixed with a prepared feed before force-fed to fish. Sampling of blood, liver, kidney, GIT content was done up to 64 h AFF / (Real-Time PCR)	Dietary DNA fragments were detected in samples from the GIT, liver, kidney and blood	Nielsen et al., 2005a
	Maize Soybeans	1×10^{10} copies of different fragments of DNA extracted from maize (Bt176) and soybeans (RRS) from certified reference material were intravenously **injected** in tail vein before sampling of muscles, liver, gonads, kidney, blood up to 24 h after injection / (Real-Time PCR)	Target DNA fragments were detected in samples from blood, liver, muscle and gonad samples at different times after intravenously injection	Nielsen et al., 2006

Table 1: continues

Animal species	DNA source	Exposure / (detection) [a)]	Exposure/results	Reference
	Soybeans	*In vivo:* A mixed diet where part of the fishmeal protein was replaced with DNA from 30% GM full-fat soy beans (RRS) or non GM soybeans was given for 28 days before sampling of GI contents and tissues. *Ex vivo*: intestinal sleeves were incubated with different concentration of PCR-amplified test DNA from RRS genome / (PCR, Sequencing, ISH)	*In vivo*: target DNA fragments were detected in mid-intestinal tissue cells *Ex vivo*: target DNA fragments were detected in mid- and distal intestine cells	Sanden et al., 2007
	pDNA	A single dose of 100 µg pDNA (R70pRomiLuc) was **injected** intramuscularly before sampling of liver, kidney, spleen, heart, gills, muscle, anterior and posterior intestine and tissue containing the injection site (up to 525 days after injection) / (SBH, Real-Time PCR)	pDNA was detectable up to 350 days in liver, kidney, spleen, heart, gills, muscle, anterior intestine, posterior intestine, injection site after intramuscular injection	Tonheim et al., 2007
Trout	Soybeans	A mixed diet containing 31% GM soybeans and another w 30 % non GM soybeans was fed for 2 weeks before sampling of blood, GIT contents from the stomach, intestines and of tissues from muscle, kidney, spleen liver and brain / (Nested – PCR, ISH)	Chloroplast DNA fragments were detected in the GI tract contents, leucocytes and spleen. Transgenic DNA fragments were detected in GI contents, leucocytes head kidney and muscle	Chainark et al., 2008
Poultry	Maize	115 g of a mixed diet containing Bt-maize (*cry1Ab*) was given up to 12 weeks before sampling of blood, liver, spleen, kidney and muscle / (PCR)	Plant DNA fragments were detected in muscle, liver, spleen or kidney. No transgenic DNA fragments were detected	Einspanier et al., 2001
	pDNA Maize	100 g feed seeded with approximately 5 x 10^9 bacteria containing pDNA (*E coli* DH5α (pUK18)) was given for 3 days before sampling of digesta from the crop, stomach, duodenum, three sites along the intestine, cecum and rectum. In the maize feeding experiment chickens were fed a diet containing transgenic maize (CG00526-176) or conventional maize for 5 days before sampling of digesta / (PCR)	Plant DNA fragments were detected in samples from crop and stomach. Transgenic DNA fragments were detected in the crop	Chambers et al., 2002

Table 1: continues

Animal species	DNA source	Exposure / (detection) [a)]	Exposure/results	Reference
	Maize	A diet containing yield gard corn (Event MON810) or conventional corn was fed for 42 days before sampling of breast muscle tissue / (PCR, SBH)	No plant- or transgenic DNA fragments were detected in breast muscle tissue	Jennings et al., 2003
	na	Chicken hens were fed a standard breeding diet before sampling of embryos. Poultry samples (muscles, stomach and wings) were received form the local supermarket and / (PCR)	Chloroplast DNA fragments were detected in all field samples and plant DNA (*zein*) fragments were detected in chicken leg muscle and stomach. Non of these fragments were detected in chicken embryos	Klotz et al., 2002
	Maize	A mixed diet containing maize (Bt176) or its unmodified-GM counterpart was fed 35 days before sampling of blood, GI contents from crop, proventriculus, gizzard, duodenum, jejunum, ileum, cecum and rectum and tissues from muscles, liver, heart, spleen, kidney, bursa and thymus glands / (Real-time PCR)	Chloroplast DNA fragments were detected in the GIT, blood, skeletal muscles, liver, spleen and kidney. Maize specific – and transgenic DNA fragments were detected in GIT contents	Tony et al., 2003
	Maize	The muscle samples in this study were collected from a feeding experiment with a diet containing MON810 hybrid maize / (PCR, Sequencing)	Chloroplast DNA fragments were detected in 15% of the samples. No transgenic DNA fragments were detected	Nemeth et al., 2004
	Maize	A mixed diet containing GM maize (Bt176: *cry1Ab*) was given for up to 39 d before sampling of spleen, liver, heart, breast muscle, digesta samples from crop, gizzard, small intestine and cecum at different time periods / (PCR)	Corn-specific DNA fragment were detected in all poultry digesta samples as far as small intestine and in muscle, liver and spleen. Transgenic DNA could only be detected in digesta samples from the crop	Aeschbacher et al., 2005
	Maize Soybeans	Mixed diets containing transgenic maize and soya (The GM maize contained the *cry1Ab* gene and the GM soybean meal contained the *cp4epsps* event) or their isogenic counterpart were given from 0-6 weeks before sampling of blood, breast tissue, liver, gizzard, heart, spleen, kidney, bursa and digesta samples / (PCR)	Fragments of the multicopy rubisco gene were detected in WBC, serum, tissues of breast, bursa, spleen and GIT contents. Transgenic DNA fragments were detected in digesta samples	Deaville and Maddison, 2005

Table 1: continues

Animal species	DNA source	Exposure / (detection) [a)]	Exposure/results	Reference
	Maize	A mixed diet containing Bt maize (Dekalb; *cry1Ab*) was prepared and given for 42 days before sampling of blood and digesta samples / (PCR)	The high-copy maize specific zein gene was detected in all GIT samples (cecum, jejunum, gizzard and crop) and in blood samples. Transgenic DNA fragments were detected in samples from the crop and gizzard	Rossi et al., 2005
Pigs	Maize	A mixed diet containing 20-25% transgenic- (Bt176) or conventional maize was given for approximately 100 days before sampling of muscle, liver, spleen, lymph nodes and blood / (PCR)	Plant DNA fragments were detected in stomach and small intestinal contents	Klotz et al., 2002
	Maize	A mixed diet containing 60% GM maize (Bt11 event *Cry1Ab*) or conventional maize was given daily for 4 weeks before sampling of blood and GIT contents from stomach, duodenal, ileal, cecal and rectal / (PCR, Sequencing)	Plant- and transgenic DNA fragments were detected in the contents of the GIT at variable levels but not in blood	Chowdhury et al., 2003a
	Maize	A mixed diet containing 70% GM maize (Star LinkCBH351: event *cry9C*) or non-GM maize was given daily for 4 weeks before sampling of GIT contents (cecal, duodenal and rectal) / (PCR, Sequencing)	Plant- and transgenic DNA fragments were detected in cecal and rectal contents in the GIT	Chowdhury et al., 2003b
	Maize	A mixed diet containing 70 % GM maize (Bt maize NX6262) or the parental maize was given until a body weight (BW) of 80 kg (the average initial BW was measured to be 23.0 ± 3 kg) before sampling at different time sets after the last feeding of blood, liver spleen, kidney, lymphatic glands, ovary, muscles and GIT contents from stomach, duodenum, jejunum, ileum, cecum, colon and rectum / (PCR)	Plant DNA fragments were detected in samples from the GIT, blood and all tissue samples. Transgenic DNA fragments were detected up to 48 h up in GIT contents	Reuter and Aulrich, 2003
	Soybeans	A mixed diet containing 24, 19 or 14% GM soy (RRS) was given during grower (24-55 kg), early-finisher (55-87 kg) and late-finisher (87-111 kg) phases of growth before sampling of muscles / (PCR)	Plant- or transgenic DNA fragments were not detected in breast loin tissue	Jennings et al., 2003
	Maize	The muscle samples in this study were collected from a feeding experiment with a diet containing MON810 hybrid maize / (PCR, Sequencing)	Plant DNA fragments were detected in 53 % of the samples. No transgenic DNA fragments were detected	Nemeth et al., 2004

Table 1: continues

Animal species	DNA source	Exposure / (detection) [a)]	Exposure/results	Reference
	Maize	A mixed diet with containing 58% glutamas-dehydrogenase (*ghdA*) containing corn was fed for1 week before sampling of liver, rib muscle, blood and GI content / (PCR, Real-Time PCR)	Transgenic DNA fragments were detected in stomach- and ileal content	Beagle et al., 2005
	Maize	A mixed diet containing 50% GM maize (Bt maize: *cry1Ab*) or non-GM maize was given for approximately 35 days, from weight 9 kg until they reached 35 kg of average, before study termination and sampling of blood, liver, spleen, kidney and tight muscle / (PCR, SBH)	Maize specific- and transgenic DNA fragments were detected in blood, liver, spleen and kidney with different frequencies	Mazza et al., 2005
	Canola	A mixed diet containing transgenic canola (RR canola: *cp4epsps*) or non transgenic canola was prepared and given from weight 30 ± 3 kg until grower (60 ± 3 kg) and finisher (108 ± 5 kg) phases of growth before sampling of blood, kidney, spleen, liver, duodenum, cecum and cecum digesta / (PCR, SH)	Chloroplast DNA fragments were detected in cecal content, duodenal and cecal tissues. Low copy plant DNA fragments were detected in cecal content, liver, spleen, kidney at variable frequencies. Transgenic DNA fragments were detected in cecal content, duodenal and cecal tissues at variable frequencies except from one positive liver and one positive kidney sample	Sharma et al., 2006
Wild boar	Maize	Two feeding experiments were performed with a mixed diet containing GM-maize or non-GM maize, isogenic maize, isogenic maize kernels and rapeseed given for 35 days with animals of 45-55 kg in exp 1 and with animals of weight 35-40 kg in experiment 2. Samples from GI contents (stomach, jejunum, caecum, colon) and visceral organs (liver, kidney, spleen, heart and lung), muscle, lymph node and blood were taken in the end.	Chloroplast specific (rubisco)- and transgenic DNA fragments were detected in digestive samples	Wiedemann et al., 2008
Sheep	pDNA Maize	pDNA (pUC18) and maize chromosomal DNA from frozen maize leaves (Zea maize line CG00526-17) were added to ovine saliva, rumen fluid obtained via a cannula and silage effluent before incubation at 39ºC and termination at different time sets / (PCR)	pDNA and chromosomal maize DNA survived in a biologically functional state in ovine saliva for a considerable time and for shorter time in rumen fluid and silage effluent	Duggan et al., 2000

Table 1: continues

Animal species	DNA source	Exposure / (detection) [a)]	Exposure/results	Reference
	Maize	A mixed diet containing GM (*cry1Ab*) - or non-GM maize silage / maize grains (Zea maize line CG00526-17) was fed for 3 days before sampling of rumen and fecal content.	Plant- and transgenic maize DNA fragments were detected in rumen fluid of sheep fed both maize silage and maize grain.	Duggan et al., 2003
	Canola	Ruminal-, duodenal fluid and feces were sampled 4-5 h after feeding and filtrated before chromosomal DNA from RR canola (event GT73) was added. The samples were analyzed at different time sets / (PCR)	Transgenic DNA fragments were detected in digesta samples (ruminal fluid, duodenal fluid at different pH and in feces) at different time intervals	Alexander et al., 2004
	Rapeseed	A mixed diet containing 15% GM RR rapeseed (event GT73) or non-GM rapseed was given ruminally and duodenally cannulated sheep for three different feeding periods: 1) 14 d adaption to non-GM rapeseed feed 2) The GM-rapeseed were given to one group for 11 d 3) feeding of the non-GM was resumed for 11 d, and sampling of blood, ruminal fluid , duodenal fluid and feces was done at different time intervals during and after feeding / (PCR, Real-Time PCR)	Transgenic DNA fragments were detected in ruminal and duodenal fluids up 29 h after feeding	Alexander et al., 2006
	Canola	A mixed diet containing transgenic canola (RRcanola: *cp4epsps*) or non transgenic canola was prepared and given from initial body weight (BW) of 21.5 ± 1.0 kg to final BW of >45 kg before sampling of blood, esophagus, rumen, abomasums, small intestine, large intestine, kidney, spleen, liver, duodenum and cecum / (PCR, SH)	Chloroplast DNA fragments were detected in ruminal, abomasal, and large intestinal content and the low copy plant DNA fragments were detected in lower frequencies in ruminal, abomasal and intestinal samples. Transgenic DNA fragments were detected in digesta samples and in GI tract tissues at variable frequencies	Sharma et al., 2006
Cattle	Maize	Approximately 18,8 kg of a mixed diet containing Bt-maize (*cry1Ab*) or conventional maize silage was given up to 246 day before sampling of blood, liver, muscle, spleen and kidney / (PCR)	Plant DNA and transgenic maize DNA were detected in duodenal juice	Einspanier et al., 2001
	Canola	A mixed diet containing canola substrates (Parental and RR canola: *cp4epsps*) was prepared for batch culture fermentation and was incubated up to 48 h in rumen contents from rumen-fistulated steers / (PCR)	Plant DNA- and transgenic DNA fragments were detected in rumen fluid (*ex vivo*) in pellets containing plant debris	Alexander et al., 2002

Table 1: continues

Animal species	DNA source	Exposure / (detection) [a)]	Exposure/results	Reference
	Maize Soybeans	Genomic DNA was extracted from soybeans slugs for **i.v injection** before sampling of blood, urine and feces at different time sets up to 24 h after injection. In a feeding experiment a mixed diet containing 31 % maize silage and 53,5% soya slugs were given for 7 d before sampling of blood, urine and feces / (PCR)	Plant DNA fragments were detected in blood up to 2 min after i.v. injection of genomic DNA and in feces after feeding with soybeans	Poms et al., 2003
	Soybeans Maize	Mixed diets containing transgenic soy (*cp4epsps*) and maize (MON810) and their non-transgenic counterparts were given for a period of 4 weeks before sampling of ruminal fluid, duodenal digesta, feces and blood / (PCR, sequencing)	Plant DNA fragments were detected in ruminal-, duodenal digesta, feces and blood. Transgenic DNA fragments were detected in ruminal and duodenal digesta.	Phipps et al., 2003
	Maize	A mixed diet containing 35% Star Link (SL)- (event CBH351) or non-transgenic corn was given for 5 weeks before sampling of blood, liver and muscles / (PCR)	No transgenic DNA fragments were detected	Yonemochi et al., 2003
	Maize	A mixed diet containing 43% transgenic (Bt11: *cry1Ab*) or non transgenic maize was prepared and given for a period of 90 days before sampling of liver, spleen, mesenteric lymph nodes, muscles and GI contents from abomasums, jejunum and cecum / (PCR, Sequencing)	Plant DNA fragments were detected in the GIT, inconsistently in the blood, the visceral organs and the logissimus muscle. Transgenic DNA fragments were detected in GI contents	Chowdhury et al., 2004
	Cotton Maize	A diet containing transgenic cottonseed (RR-, Bollgard- and Bollgard II cotton) or transgenic maize (Yield Gard) and their non-transgenic counterparts was prepared for 2 different feeding studies over a period of 28 d before sampling of tissues from kidney, liver and spleen / (PCR, SBH)	No plant- or transgenic maize DNA fragments were detected in liver, kidney or spleen	Jennings et al., 2004
	Maize	Muscle samples for this study were collected from a feeding experiment with a diet containing MON810 hybrid maize / (PCR, Sequencing)	High copy endogenous plant DNA fragments were detected in 5% of the muscle samples	Nemeth et al., 2004
	Rapeseed	Mixed substrates containing rapeseeds (parental line and RR rapeseed:*cp4epsps*) were prepared and used for *in vitro* incubation up to 48 h using ruminal contents from a cannulated steer/ (PCR)	Plant- and transgenic DNA fragments were detected at different time points	Sharma et al., 2004

Table 1: continues

Animal species	DNA source	Exposure / (detection) [a)]	Exposure/results	Reference
	Maize	Mixed substrates containing whole plant isogenic-, whole plant transgenic-, ensiled isogenic- and ensiled transgenic corn (*Bt176*) were prepared for *in situ* experiments and incubated in the rumen of rumen-cannulated cows for a period of up to 48 h before sampling at different time sets / (PCR, Real-Time PCR)	Plant and recombinant maize DNA fragments were detected in ruminal samples	Wiedemann et al., 2006
	Maize	A mixed diet containing transgenic maize (*Bt176*) or conventional maize was given for a 9 weeks experimental period before sampling of blood, feces and rumen juice / (Real-Time PCR)	Chloroplast- and plant DNA fragments were detected at different frequencies in blood samples	Bertheau et al., 2009
Milk	Maize	Approximately 18,8 kg of a mixed diet containing Bt-maize (*cry1Ab*) or conventional maize silage was given up to 246 day before sampling of milk	Plant DNA fragments were detected	Einspanier et al., 2001
	Soybeans	A mixed diet containing 26,1% and 13,9% GM soybeans (*cp4epsps*) was given for a period up to 12 weeks and milk samples were collected at different time sets / (PCR)	Transgenic DNA fragments were not detected	Phipps et al., 2002
	Soybeans Maize	Mixed diets containing transgenic soy (*cp4epsps*) and maize (MON810) and their non-transgenic counterparts was given for a period of 4 weeks before sampling of milk / (PCR, Sequencing)	Multi-copy plant DNA fragments were detected. No transgenic DNA fragments were detected	Phipps et al., 2003
	Maize Soybeans	Genomic DNA was extracted from soybeans slugs for **i.v injection** before sampling of blood, urine and feces at different time sets up to 24 h after injection. In a feeding experiment a mixed diet containing 31% maize silage and 53,5% soya slugs were given for 7 d before sampling of blood, urine and feces / (PCR)	No maize – or soya DNA fragments were detected	Poms et al., 2003
	Maize	A mixed diet containing 35% Star-Link corn (event CBH351) or non-transgenic corn was given for 2 weeks in a pre-experimental period and further for 5 experimental weeks before sampling of blood, liver and muscles at the end / (PCR)	No transgenic DNA fragments were detected	Yonemochi et al., 2003

Table 1: continues

Animal species	DNA source	Exposure / (detection) [a)]	Exposure/results	Reference
	Cotton	Mixed diets containing transgenic cotton (Bollgard (*cry1Ac*)-, Bollgard II (*cry1Ac* and *cry2Ab*)- and RR (*cp4epsps*) cotton) was given for a period of week 1 to three for diet adaption and week 4 for sampling of milk / (PCR, SBH)	No cotton- or transgenic DNA fragments were detected	Castillo et al., 2004
	Cotton Maize	A diet containing transgenic cottonseed (RR-, Bollgard- and Bollgard II cotton) or transgenic maize (Yield Gard) and their non-transgenic counterpart was prepared for 2 different feeding studies over a period of 28 d before sampling of milk / (PCR, SBH)	No endogenous- or transgenic DNA fragments of cotton or maize were detected	Jennings et al., 2004
	Maize	Milk samples for this experiment were collected from 4 independent feeding studies with diets containing Mon 810 maize / (PCR, Sequencing)	High copy endogenous plant DNA fragments were detected in 86 % of the milk samples	Nemeth et al., 2004
	Maize	A mixed diet containing GM maize (Chardon LL) was used in a 12-week feeding study and collection of milk was done at different time sets / (PCR)	No endogenous- or transgenic DNA fragments were detected	Phipps et al., 2005
	Maize Soybeans	A number of milk samples from outlets in Catania, Sicily, Italy were tested for the presence transgenic sequences / (PCR, Gel Electrophoresis analysis)	Endogenous- and transgenic sequences of maize and soybeans were detected in a number of the samples	Agodi et al., 2006
	Maize	A mixed diet containing corn silage (GM crop (DK493RR/Bty)) was given and milk samples were collected from two different periods, each period was 28 d long / (PCR)	No plant- or transgenic DNA fragments were detected	Calsamiglia et al., 2007
	Maize	A mixed diet containing GM maize (E176) was prepared and given for a period before collection of milk samples / (PCR, Real Time PCR)	Chloroplast-specific gene fragments were detected. No transgenic DNA fragments were detected	Rizzi et al., 2008

Table 1: continues

Animal species	DNA source	Exposure / (detection) [a)]	Exposure/results	Reference
	Maize	A mixed diet containing GM maize (MON810: *Cry1Ab*) or non-GM maize was given for 6 months and milk samples were collected monthly / (Q-Real-Time PCR)	No detection of transgenic DNA fragments	Guertler et al., 2009
Fallow deer	Maize	In experiment 1 the animals were fed GM maize (Navares) and in experiment 2 the animals were fed GM maize and maize seeds (DKC3421, DKC3421-YG). The animals were adapted to the diet for 1 week and the experimental period was for 5 weeks before sampling of GI content and tissues from the rumen, abomasums, jejunum, cecum, colon, rectum and liver, kidney, spleen, lymph nodes and muscles / (PCR)	Plant specific DNA fragments were detected in the GIT contents and occasionally in the spleen, kidney, lymphatic node, liver, muscle. No transgenic DNA fragments were detected	Guertler et al., 2007
Humans	Maize Soybeans	Human digestion simulation was prepared and RRS and Bt-maize samples were incubated for up to 180 min / (QC-PCR)	Transgenic DNA fragments within soya and maize were detected in short time periods in ileal digesta and small intestinal simulation	Martin-Orùre et al., 2002
	Rabbit Meat	Volunteers were fed (3 x) 400 g meat cut from rabbits bought from a local butcher before sampling of blood before, during and after the meals (up to 432 h) / (PCR)	Both rabbit retrotransposon- and mitochondrial DNA fragments were detected in the blood	Forsman et al., 2003
	Soybeans	Volunteer human ileostomists were fed 190 g GM soya (*epsps*) burger and 264 g GM soya milk shake before sampling from their stoma bags every 30 min for 6 h. Subjects with intact GIT received the same meal before sampling of feces / (QC-PCR)	The transgene and native soya DNA fragments were detected in all ileostomist subjects. In the subjects with an intact GIT the transgene could not be detected in the feces	Netherwood et al., 2004

[a)] PY = polyomavirus, rec = recombinant, GIT= gastrointestinal tract, SBH = Southern Blot Hybridization, DBH = Dot Blot Hybridization, PCR = Polymerase Chain Reaction, FISH = Fluorescent *in situ* hybridization, pDNA = plasmid DNA, RT-PCR = Reverse transciptase Polymerase Chain Reaction, SH = Southern hybridization, Real-Time PCR = Real-Time Polymerase Chain Reaction, ISH = *in situ* hybridization, HFA = human flora associated, i.m = intra muscular, AFF = after force fed, WBC = white blood cells, QC-PCR = quantitative competitive PCR, na = not applicable, RR = Roundup Ready, Bt = a variant of maize, genetically altered to express the *Bacillus thuringiensis* toxin, EPSPS = 5-Enol-pyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. CP4, GM = genetically modified.

Suggested mechanisms of host DNA uptake from the gastrointestinal tract

As described previously, different studies have shown that ingested macromolecules like proteins and DNA are not necessarily fully degraded to nucleotides or amino acids in the gastrointestinal tract. In general, the intestinal barrier is permeable to digested nutrients and fluids but impermeable to macromolecules, particular antigens and most microorganisms (Kucharzik et al., 2000; O`Hara and Shanahan, 2006). Nevertheless, feed-ingested DNA has been detected in intestinal epithelia, in cells of the Peyer`s Patches (PPs) in the intestinal mucosa, in peripheral white blood cells and in spleen and liver cells, suggesting that the epithelial lining of the GI tract may be a portal of entry into the organism (Schubbert et al., 1994; 1997; 1998; Palka-Santini et al., 2003; Forsman et al., 2003). It has been estimated that approximately 0.1 %-1 % of dietary DNA is absorbed from the gastrointestinal tract. A precise measurement of this process is difficult because absorption takes place over several hours and DNA undergoes continuous transport, degradation and elimination (Nielsen et al. 2005).

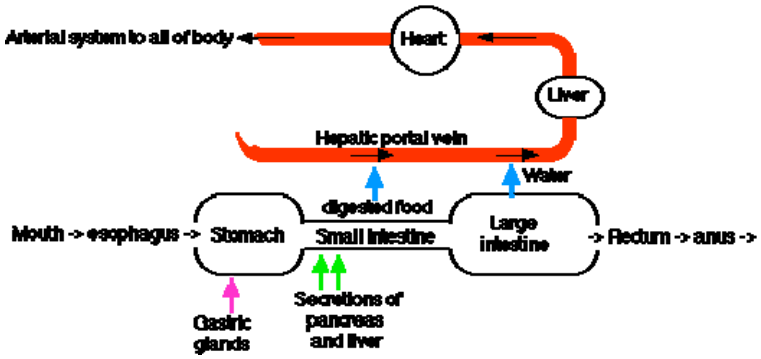


Figure 1: The veins draining the intestine. From, http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/G/GItract.gif reprinted with permission.

Role of the immune apparatus in the gastrointestinal tract

Because the mammalian gastrointestinal tract is both colonized by non-pathogenic bacteria and is also frequently exposed to many pathogenic organisms, it means that the host must discriminate between constituents of the external and the constituents of "self" (Ashkar et al., 2002; Harris et al., 2006; Kumagai et al., 2008). The epithelium of the gastrointestinal tract provides the first sensory line of defense and there are three main types of immunosensory cells here: 1) surface enterocytes, 2) M cells and 3) intestinal dendritic cells (O`Hara and Shanahan, 2006). The ability of these cells to discriminate between "self and non-self" is mediated in part by two major host pattern recognition receptor (PRR) systems: the family of Toll-like receptors (TLRs) and the nucleotide-binding oligomerization domain/capase recruitment domain isoforms (NOD/CARD).

The different TLRs respond to molecular motifs known as pathogen-associated molecular patterns (PAMPs), and are expressed by both epithelial and non-epithelial cells throughout the entire gastrointestinal tract and activate innate immune cells. PAMPs include lipopolysacahride (LPS), peptidoglycan, lipetechoid acid, bacterial flagellin and CpG DNA (Ashkar et al., 2002; Krieg et al., 2002; Harris et al., 2006; Tyrer et al., 2007).

CpG sites are regions of DNA were a cytosine nucleotide occurs next to a guanine nucleotide and is linked by a phosphodiester bound. These sequences (CpG motifs) are normally highly methylated in mammals, but bacterial and viral DNA possesses a lower methylation frequency. Thus, unmethylated CpG motifs represent a signature of non-host DNA. As such, CpG sequences in a plasmid DNA is often unmethylated due to its bacterial origin. By this signature, the vertebrate immune system and mammalian toll-like receptors (TLRs) are activated and this leads to the induction of cytokine release, macrophage activation, NK-cells stimulation, B-cell proliferation and immunoglobulin secretion etc. The CpG motifs of unmethylated nucleic acids are recognized by a well characterized receptor for foreign DNA, a TLR9. Not only naturally derived DNA from bacteria or viruses but also short (~20 bp) synthetic oligonucleotides (ODN) containing CG sequences are sensed by TLR9 (Hemmi et al., 2000; Harris et al., 2006; Heeg et al., 2008). This means that once inside lysosomal compartments, the CpG sequence (on e.g. the plasmid DNA or CpG ODN) could bind to TLR9 that initiates signal transduction which may result in inducing a cellular immune response. Recently a study by Roberts et al. (Roberts et al., 2009) indicates that another family of DNA binding proteins, HIN-200 proteins, also can act as PRR and mediate response to double-stranded DNA.

Endocytosis

The uptake and transport of DNA and other macromolecules is possible by using active vesicular transport and receptor mediated transport. The exact mechanisms for how these molecules are internalized are intriguing and not fully understood (Vellenga et al., 1985; Tsume et al., 1996; Kucharzik et al., 2000; Vlassov et al., 2007). There are of course factors affecting the transport of macromolecules across the GIT barrier like species, age, gastric function, mucosal immunity, intestinal proteolysis, membrane composition etc. (Udall and Walker, 1982). Undigested material is passed into the intercellular space by endocytosis-exocytosis (transcytosis), and further transported into the lymph, peritoneal cavity and/or blood. In the blood, DNase activity is one important factor regulating the concentration of foreign DNA in circulation, but foreign DNA is not necessarily present as free molecules and can be protected form nuclease degradation (Vlassov et al., 2007). This means that once the feed-ingested DNA crosses the intestinal barrier it may enter into circulation and reach other organs and tissues.

Endocytosis is a common name for the various mechanisms in mammalian cells used to internalize fluids, macromolecules and particles, involving the two main types: 1) phagocytosis and 2) pinocytosis (Hubbard, 1989; Belting et al., 2005) (Figure 2). In phagocytosis, the process of engulfment is triggered by pattern recognition receptors by which cells (principally macrophages) ingest large particles (> 0.3 μm), such as yeast and bacteria into phagosomes. This process proceeds through four steps: attachment, engulfment, fusion with lysosomes and degradation. Pinocytic vesicles containing small particles are constitutively formed and are subsequently fused with lysosomes to hydrolyze or break down the particles. Pinocytosis can further be divided in two main groups: 1) fluid phase endocytosis (macropinocytosis) and 2) a receptor mediated or clathrin mediated endocytosis.

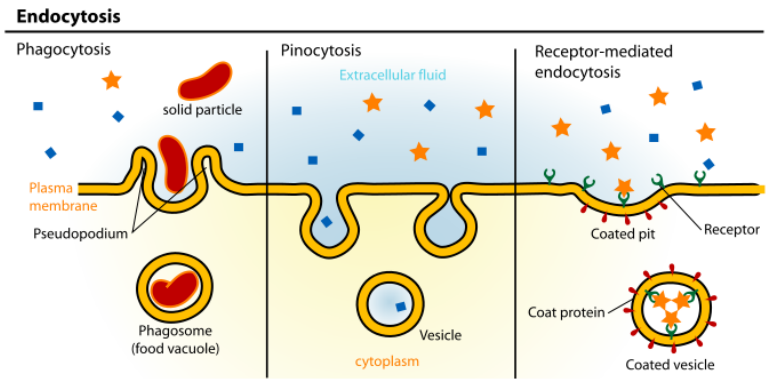


Figure 2: Three types of endocytosis. From http://cellbiology.med.unsw.edu.au/units/images/endocytosis_types.png, reprinted with permission.

Cell types active in DNA uptake

In studies of cell cultures and with macromolecular therapeutic agents injected systemically or locally in different tissues, it has been demonstrated that DNA can be taken up by different mammalian cell types. With molecular methods like PCR, Southern-blot hybridization, FISH, isotope and fluorescence labeling of plasmid DNA with subsequent analysis, DNA fragments have been detected in a variety of mammalian cell types, such as cytotoxic T-cells (Schubbert et al., 1997), B-cells (Schubbert et al., 1997; Coelho-Castelo et al., 2003), macrophages (Schubbert et al., 1997; Takakura et al., 1999; Ogawa et al., 2005) liver scavenger endothelial cells (Bijsterbosch et al., 1997; Hisazumi et al., 2004), hepatocytes (Budker et al., 2000), keratinocytes (Hengge et al., 1995) and myocytes (Bureau et al., 2004). Below, some examples of cell types that are known to take up DNA and their suggested mechanism are presented, keeping in mind that the mechanisms of DNA uptake by eukaryotic cells are still not completely understood. It is unknown how frequently the cells of an intact organism are exposed to, take up, and chromosomally integrate foreign DNA (Doerfler, 2001).

M cells

M cells (or microfold cells), which are specialized antigen sampling cells of the gut, are found in the follicle-associated epithelia (FAE) that overlie the mucosal lymphoid aggregates, known as Peyer`s patches. Here they constitute about 10-30 % of the epithelial cells in humans and mice (Tyrer et al., 2007). These cells are involved in transcytosis of bacteria, viruses and other macromolecules from the gut lumen to the mucosal immune cells, which contains dense populations of lymphocytes, macrophages and dendritic cells (Owen et al., 1999, Brayden et al., 2005; Tyrer et al., 2007). This is believed to happen by mechanisms such as endocytosis of clathrin-coated vesicle, or actin-dependent phagocytosis or engulfment by a fluid-phase pinocytosis or macropinocytosis (Kyd et al., 2008).

Enterocytes

Enterocytes are the predominant cells in the small intestinal mucosa, responsible for the final digestion and absorption of nutrients, electrolytes and water. These cells have been considered as a physical barrier limiting the uptake of macromolecules (Kaiserlian and Etchart, 1999). However, macromolecules have been shown to be transported through the intestinal enterocytes, form luminal to interstitial space and then further across the capillary wall to the

blood (Ziv and Bendayan, 2000). This transport was early suggested to happen by an active transport mechanism that Na^+ dependent (Bronk and Hastewell, 1987).

Dendritic cells

Dendritic cells (DCs) are immune cells that arise as immature cells in the bone marrow, and emerge from the bone marrow to migrate via the blood to peripheral tissues. They are found in small quantities in tissues that are in contact with the external environment, mainly the skin (Langerhans cells) and the inner lining of the nose, lungs, stomach and intestines. These cells become activated to mature DCs once they come into contact with a pathogen. Their main function is to process antigen material and present it on the surface of other cells of the immune system, thus functioning as antigen-presenting cells. These cells also play an important role in different immunological responses associated with the therapeutic use of CpG DNA (Yoshinga et al., 2002; Ogawa et al., 2005). The important roles of DCs are well known, and studies have shown that pDNA in mouse DCs can efficiently be taken up and rapidly degraded. The specific mechanism for cellular uptake of plasmid DNA in these cells remains poorly understood.

Cells in blood

The blood consists of several different cell types (erythrocytes, platelets, and the white blood cells) and circulating DNA appears as a result of nuclear cell death, erythrocyte and platelet maturation, and active secretion of nucleic acids into the extracellular space (Tamkovich et al., 2008). Purified DNA is “rapidly” broken when incubated in blood (Shaw et al., 1991; Tamkovich et al., 2008). However, a certain concentration of extracellular DNA is normally maintained in the blood, most likely associated with DNA binding proteins in the blood. This association makes DNA less vulnerable to breakdown and thus a candidate for uptake (Tamkovich et al., 2008). It has been described that B-lymphocytes can take up plasmid DNA and express the encoded protein (Coelho-Castelo et al., 2003) and that monocytes can take up DNA through absorptive endocytosis (Yi et al., 1998). However, the exact mechanisms responsible for uptake are not fully known.

Macrophages

Macrophages are multifunctional cells with phagocytic and secretory properties that recognize, ingest and kill invading microorganisms (> 0.3 μm). Primitive macrophages arise from a common precursor in the bone marrow of mammals and circulate in the blood as

monocytes before they migrate into tissues throughout the body to transform into various types of tissue macrophages (e.g microglia, Kupffer and sinus histocytes). They are found in especially high numbers in connective tissue, in the submucosal layer of the gastrointestinal tract, in the lung, along certain blood vessels in the liver, called sinusoids (where they are known as Kupffer cells), and throughout the spleen (Naito et al., 1997). Since they are the most important cell population responsible for *in vivo* clearance of plasmid DNA different studies have tried to demonstrate the uptake and explain the mechanism how the plasmid DNA is taken up by these cells (Stacy et al., 1996; Takakura et al., 1999; Yamane et al., 2005). Stacy et al. (Stacy et al., 1996) demonstrated that plasmid DNA is taken up by bone marrow-derived macrophages and activates inflammatory gene induction. Further, Takakura et al. reported that macrophages in mice were able to take up plasmid DNA by an endocytic uptake mechanism mediated by a receptor similar to the scavenger receptor (Takakura et al., 1999).

Scavenger endothelial cells

The scavenger endothelial cells represent an important part of the innate immune system, where the main function is to remove soluble waste macromolecules from the circulation and also material absorbed from the gut by receptor-mediated endocytosis via clathrin coated pits (Smedsrød, 2004). To carry out the scavenger function, liver endothelial cells (LSEC) express at least 4 types of specific receptors for endocytosis of major physiological waste products (Smedsrød, 2004; Malovic et al., 2007). The class of scavenger receptors (SR) involved in uptake of DNA is not well studied but studies on hepatic uptake and degradation of naked plasmid DNA after intravenous injection in rats have shown that plasmid DNA is rapidly eliminated from the circulation and taken up by the liver, where the LSECs contributed to most of the hepatic uptake of plasmid DNA (Hisazumi et al., 2004). In general, the scavenger receptors (SRs) are cell surface glycoproteins which were originally defined in macrophages. Other cells such as certain endothelial cells and myeloid cells (macrophages and dendritic cells) also exhibit SR activity. The receptors have been categorized into different classes of their structure (Gough et al., 2000), and bind and internalize micro-organisms and their products including Gram-positive bacteria, Gram-negative bacteria, intracellular bacteria and CpG DNA (Peiser et al., 2002).

Hepatocytes

Hepatocytes constitute roughly 70-80 % of the mass of the liver and are involved in a number of metabolic, endocrine and secretory functions like protein synthesis, protein storage and transformation of carbohydrates, synthesis of cholesterol, bile salts and phospholipids, and detoxification, modification and excretion of exogenous and endogenous substances. Studies have demonstrated that high levels of plasmid DNA expression in hepatocytes can be obtained by tail vein injection when large volumes are rapidly injected (Zhang et al., 1999). However, by using normal delivery during i.v administration (low administration volume and normal pressure) to mice, the plasmid DNA is rapidly removed from the circulation and taken up by the liver, predominantly by the liver non-pharencymal cells (Hisazumi et al., 2004; Kobayashi et al., 2004).

Myocytes

A myocyte (also known as a muscle cell) is the type of cell found in muscles. They arise from myoblasts (a type of stem cell that exists in muscles), and each myocyte contains myofibrils, which are long chains of sarcomeres, the contractile units of the cell. Plasmid DNA has also shown to be expressed in mammalian myocytes *in vitro* after i.m injection (Wolff et al., 1990; Bureau et al., 2004; Wang et al., 2004). The mechanisms of entry have not fully been understood but possible uptake mechanisms like some cell membrane transporter has been discussed (Wolff, 1992; 1997; Danko et al., 1997; Bureau et al., 2004; Wang et al., 2004).

Keratinocytes

The skin barrier is composed mainly of the epidermidis, which is constantly renewed by the mitotic activity of the stem cells in the basal layer, which provides new keratinocytes constituting 95% of the cells found there (Basner-Tschakarjan et al., 2004; Lippens et al., 2009). From studies on human gene therapy and DNA vaccines, it has been shown that naked plasmid DNA can be taken up and expressed in human, pig and mouse epidermal keratinocytes. The exact mechanism by which keratinocytes internalize and transport plasmid DNA remains unknown but the main mechanism seems to be macropinocytosis (Hengge et al., 1995; 1996; Fan et al., 1999; Basner-Tschakarjan et al., 2004). Fan et al. (Fan et al., 1999) demonstrated that by applying naked plasmid DNA directly onto the skin induced specific immune responses. This again, also emphasizes the reason to follow good laboratory practice when handling recombinant DNA since the skin may be exposed and cells may be able to take up and express foreign recombinant DNA (Udvardi et al., 1999).

Uptake of DNA by bacterial cells in the gastrointestinal tract

The numbers of bacteria within the mammalian gastrointestinal tract (GIT) differs and the bacterial density gradually increases from the stomach to the large intestine containing up to 10^{14} bacteria per gram colonic content (McCracken and Lorenz, 2001; O`Hara and Shanahan, 2006). Analysis of microbial communities colonizing the GIT by culture and molecular methododologies have revealed that the number of different bacterial species colonizing the mammalian GIT range from 500-1000, where the composition can be quite different between different mammalian species (Tannock et al., 2001; Wilson, 2005).

Several bacterial species that normally reside in the GIT have been shown to develop competence for natural transformation *in vitro* (Lorenz and Wackernagel, 1994; Mercer et al., 1999 a, b). The GIT, and the colon in particular, is likely to be one of the most important environments for gene transfer, because of its high density of microorganisms and nutrients (Mercer et al., 1999 a, b; 2001). However, the knowledge about the mechanisms and the capacity of these bacteria to develop competence *in vivo* is limited. The release of genetically modified organisms such as transgenic plants has raised concerns about the potential impact of recombinant DNA and also HGT from transgenic plants into bacteria. Several experimental studies have demonstrated that some bacterial species can take up fragments of plant transgenes under highly optimized conditions (Gebhard and Smalla, 1998; De Vries et al., 2001). Whether bacteria in the GIT are able to take up fragments of DNA from plants remains unknown (Nordgård et al., 2007).

Experimental studies examining the uptake of DNA in bacteria derived from/residing the gastrointestinal tract

Few studies have been performed on the possible uptake of DNA by bacteria in the GIT and most studies have been focused on *in vitro* studies with oral bacteria (Table 2). The oral cavity is the first place feed-derived DNA is entering the GIT and therefore most likely to receive the highest amount of intact DNA entering with the diet. Mercer et al. (Mercer et al., 1999 a, b; Mercer et al., 2001) examined the fate of free DNA and transformation of the oral bacterium *Streptococcus gordonii* DL1 by plasmid DNA in human saliva. Plasmid DNA that had been exposed to human saliva was still biological active and capable of transforming the naturally competent *Streptococcus gordonii* DL1, although the transforming activity decreased rapidly. Similar stability results were obtained by Duggan et al. who examinated

the biological activity of plasmid DNA in the ovine oral cavity (Duggan et al., 2003). When plasmid DNA had been exposed to degradation by ovine saliva it was still capable of transforming electro competent *E. coli* cells after up to 8 min in the oral cavity (Duggan et al., 2003).

In a study by Mercer et al. (Mercer et al., 1999) the possible transformation of the rumen bacterium *Streptococcus bovis* JB1 was investigated. The bacterium was shown to be naturally transformable *in vitro*, but no transformation was observed in the presence of rumen fluid. However, Duggan et al. (Duggan et al., 2000) demonstrated that plasmid DNA was capable of transforming electro competent *E. coli* cells *in vitro* in rumen fluid *in vitro* after less than 1 min, demonstrating the stability of the plasmid DNA. A few studies on gene transfer have been investigating the digestive activity and the possible *in vivo* transformation in the different GI compartments of germfree rats and mice. Kharazmi et al. (Kharazmi et al., 2003) did not detect the donor DNA or any transformants in the GIT *in vivo* but were able to detect transformants from partially degraded plasmid DNA from saliva *ex vivo* up to 6h after incubation. A study by Shedova et al. using the same strains as Kharamzi et al. (Kharazmi et al., 2003) demonstrated that cow saliva rendered *S. godonii* cells competent for DNA uptake *in vitro* (Shedova et al.2009).

Wilcks et al. (Wilcks et al., 2004) demonstrated the persistence of DNA in the different GIT compartments and in one of the experiments plasmid DNA could be recovered throughout the GIT and DNA isolated from these intestinal samples was able to transform electro-competent *E. coli*. The transformation frequency was low indicating that the concentration of intact DNA was reduced in the different GI compartments. Later, Nordgård et al (Nordgård et al., 2007) investigated the ability of *Acinetobacter baylyi* colonizing germ-free mice and rats for potential *in vivo* transformation after feeding DNA. No transformants were detected *in vivo* or *in vitro*.

Studies regarding natural transformation in the human gastrointestinal tract are few. As mentioned Mercer et al. demonstrated that DNA exposed to human saliva is able to transform the naturally competent oral bacterium *Streptococcus gordonii in vitro* (Mercer et al., 1999). There is one study investigating the possibility of natural transformation in samples from 7 human ileostomists fed transgenic plant DNA (Netherwood et al., 2004). A small proportion of the transgene was recovered in all seven ileostomists, whereas the transgene did not survive passage through the intact gastrointestinal tract of human subjects fed transgenic plant

DNA. Three out of these seven ileostomists showed evidence of low-frequency gene transfer from transgenic plant to the microflora of the small bowel, but this appeared to have occurred before feeding the experimental meal. No microbes containing the transgene could be cultured from the feces from humans with an intact GIT.

Table 2: Studies of bacterial transformation in the GIT and under GIT-simulating conditions

Strain	**Environmental situation**	**Genetic marker**	**Reference**
*Streptococcus bovis JB*1	Ovine saliva and rumen fluid	*Ery*^{R}	Mercer et al., 1999
Streptococcus gordonii DL1	Human saliva	*Em*^{R}, *Cm*^{R}	Mercer et al., 1999
Endogenous avian microflora	Avian GIT	*Ery*^{R}	Netherwood et al., 1999
Streptococcus gordonii DL1	Human saliva	*Tet*^{R}, *gfp*	Mercer et al., 2001
Streptococcus gordonii LTH 5597	Germfree rats, saliva and GIT	*Km*^{R}, *Ery*^{R}	Kharazmi et al., 2003
Microflora of the small bowel	Human GIT	*epsp*	Netherwood et al., 2004
Human fecal flora, *E.coli MS15979, B.subtilis 168,E.coli* Aw200,	Germfree rats, GIT	*Cam*^{R}, *Amp*^{R}, *Km*^{R}	Wilcks et al., 2004
A. baylyi BD413	Germfree mice and rats, GIT	*Km*^{R}	Nordgård et al., 2007
Streptococcus gordonii NCTC7868 *Streptococcus gordonii LTH 5597*	Cattle saliva and rumen liquid	*Km*^{R}, *Ery*^{R}	Shedova et al., 2009
GIT flora of rats	WISTAR rats, GIT	*Km*^{R}, *Str*^{R}	Nordgård et al., unpublished

GIT: gastrointestinal tract, Amp^R: ampicillin resistance, Cam^R: choloramphenicol resistance, Ery^R: erythromycin resistance, Km^R: kanamycin resistance, Tet^R: tetracycline resistance, gfp: green fluorescence protein, *epsps*: transgene from GM soya.

Suggested mechanisms of DNA uptake in bacteria

Bacteria can acquire new genetic information by three major mechanisms which are: conjugation, transduction and natural transformation. During conjugation DNA is transferred directly from one bacterium to another, whereas in transduction, the DNA is carried by bacteriophages. Natural transformation differs from conjugation and transduction because the transfer of genes occurs via free DNA and is initiated by the recipient cell. Only transformation is relevant when it comes to possible transfer of DNA from plants to bacteria (Nielsen et al., 1998). The capability of natural transformation is widespread among bacteria of diverse metabolism and habitat and from a wide phylogenetic range. Today more than 80 naturally transformable species have been identified (Lorenz and Wackenagel, 1994; De Vries and Wackernagel, 2004). Recent studies can show that this cellular uptake of free DNA is not restricted to bacterial DNA but also involve also uptake of plant DNA into bacterial cytoplasm (De Vries et al., 2001).

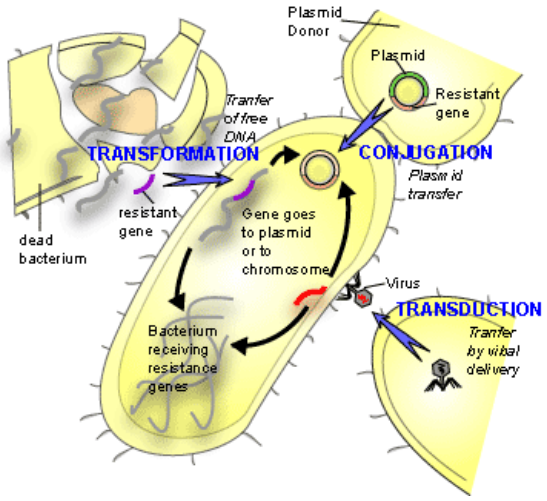


Figure 3: The mechanisms of horizontal gene transfer (HGT) in bacteria. From http://bioinfo.bact.wisc.edu/themicrobialworld/HorizontalTransfer.gif, reprinted with permission.

The main four steps involved in natural transformation are:

i) development of bacterial competence,

ii) DNA binding,

iii) DNA uptake into the cell,

iv) establishment of the DNA by integration into the recipient genome.

Development of bacterial competence

The ability of a cell to take up free DNA from the surrounding medium through the cell membrane is defined as genetic competence (Lorenz and Wackernagel, 1994; Dubnau, 1999; Averhoff and Friedsrich, 2003; Chen and Dubnau, 2004; Chen et al., 2005). The process of naturally genetic competence is a highly regulated physiological state in which bacteria can bind and internalize naked DNA. It has been found that 20-50 proteins are involved in this process. In most naturally transformable bacteria, such as *Bacillus subtilis* and *Streptococcus pneumoniae*, the competence is transient and time-limited in response to specific environmental conditions (Zawadzki and Cohan, 1995; Majewski and Cohan, 1999; Li et al., 2001). *Neisseria gonorrhoea* is an exception, where competence is constitutive and DNA can be taken up during all phases of growth (Goodal et al., 1982; Hamilton and Dillard, 2006). *Acineteobacter baylyi* strain BD413, the model organism used in this study, reaches maximum competence for DNA uptake in early exponential phase (Palmen et al., 1993).

DNA binding

The difference in cell surface of gram-positive and gram-negative bacteria leads to differences in the DNA uptake route. DNA must pass through the cell wall and the cytoplasmatic membrane in gram-positive bacteria (the *Streptococcus-Bacillus* model) and in gram-negative bacteria, DNA must also pass the outer membrane (the *Haemophilus-Neisseria* model). The first step in both models is binding of double-stranded (ds) DNA to the cell surface by a process that is not completely understood (Lorenz and Wackernagel, 1994; Chen and Dubnau, 2004, Thomas and Nielsen, 2005). Competent bacteria have specific binding sites where extracellular DNA binds non-covalently to sites present on the cell surface (Thomas and Nielsen, 2005), ranging from 30 to 80 in *S. pneumonia* and *A. baylyi* respectively. The majority of bacteria binds extracellular DNA nondiscriminately while the gram-negative bacteria *H. influenza* and *N. gonorrhoeae*, successful DNA binding and translocation requires a specific DNA uptake sequence (DUS) of approximately 10 bp. *Acinetobacter sp.*, another

gram-negative bacteria, does not require an uptake sequence and is able to take up DNA from any source (Lorenz and Wackernagel, 1994; Chen et al., 2005; Thomas and Nielsen, 2005).

DNA uptake into the cell

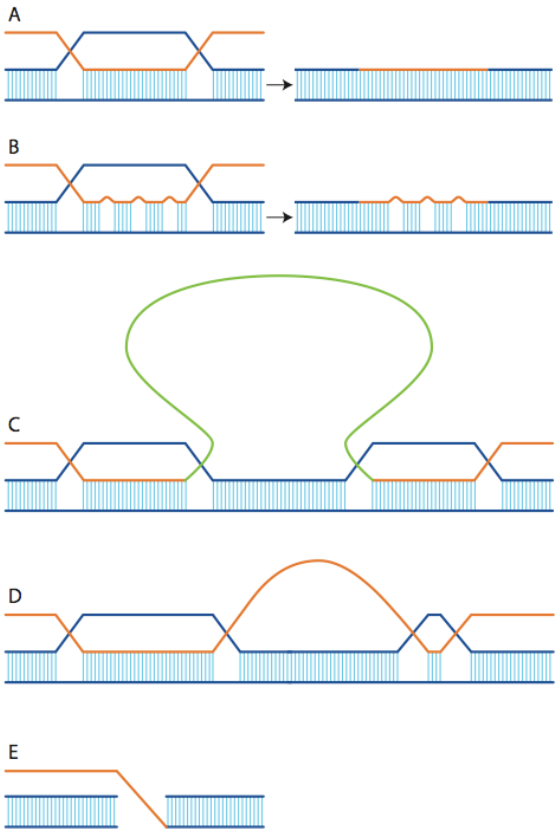
DNA is transported linearly into the cytoplasma, and a free end must be present in the DNA molecule for transport to begin, providing evidence for an endonuclease which degrades one strand during DNA uptake with in, or associated with, the translocation apparatus (Dubnau and Provvedi, 2000; Chen and Dubnau, 2004). Gram-positive and gram-negative bacteria use related proteins to import DNA where the DNA is thought to be translocated into the cytoplasma through a pore-forming multimeric complex that spans the inner membrane/periplasm/outer membrane in gram-negative bacteria and the inner membrane/cell wall in gram-positive bacteria. This machinery is working in a nucleoside-triphosfate (NTP)-dependent manner (Averhoff and Friedrich, 2003), with the exception of *H. influenza*, in which DNA is translocated in double stranded form in membrane-bound vesicles (Goodgal, 1982). Translocated DNA in most cases enters the cytoplasma in single-stranded (ssDNA) form, providing evidence for an endonuclease with in, or associated with, the translocation apparatus that degrades one strand during DNA uptake.

Integration

The internalized ssDNA strands can be integrated into the bacterial chromosome. This process is *RecA*-protein dependent, and requires sequence similarity between the incoming DNA and the bacterial chromosome (Lorenz and Wackernagel, 1994). In general, the incoming DNA must contain regions of minimum 25-200 bp in length of high similarity to the recipient genome for homologous base-pairing to occur (Thomas and Nielsen, 2005). The stability of the heteroduplex molecule depends on the degree of similarity between the donor and the recipient DNA (Zawadski et al., 1995; Majewski and Cohan, 1999). As an example, the minimal length for DNA similarity required in *E. coli* for initiating homologous recombination is approximately 20 bp (Shen et al., 1986).

In cases where donor DNA and recipient DNA are identical across their entire lengths, integration of the donor DNA will not result in detectable genetic or phenotypic change (*homogamic substitutive recombination*, Fig. 4A). If there is some dissimilarity between donor and recipient and if this region is flanked by homologous regions, the donor DNA can replace recipient DNA. This is referred to as *substitutive heterogamic recombination* (Fig. 4B) (Singer et al., 1982; Lorenz and Wackernagel, 1994; Worth et al., 1994). Another type of

integration is acquisition of DNA through *additive integration* (Schwesinger, 1977) (Fig 4C). This occurs when the DNA sequence present only in the donor is flanked on both sides by sequences common to both donor and recipient. Additive integration can also happen when there is high DNA similarity on one side of the invading DNA and random microhomology (3-12 nt) on the other, in a mechanism referred to as homology facilitated illegitimated recombination (HFIR) (de Vries and Wackernagel, 2002) (Fig 4D).



Figur 4: Various recombination types in bacteria. Light blue vertical lines indicate base-pairing A) Homogamic substitutive recombination in which donor DNA (red line) is identical to the recipient (blue line), leading to no change in the recipient. (B) Heterogamic recombination occurs when donor and recipient DNA are similar, but some mismatches exist. (C) Additive integration where a unique DNA sequence (green line) is integrated into the recipient through homogamic/heterogamic recombination at the sides (D) HIFR, integration of donor DNA occurs by a stretch of homology on one side (left) and microhomology on the other. (E) Hypothetical model of illegitimate recombination in which donor DNA invades recipient DNA at double strand breaks. (Figure: Jessica L. Ray, PhD thesis. Reprinted with permission)

AIM OF STUDY

Develop a detailed understanding of the fate of feed-derived DNA in the gastrointestinal tract of mammals by using rodent models.

The major objectives were to determine:

- the stability of food ingested DNA (plasmid) in the gastrointestinal tract of rodents, and the host tissue distribution and persistence of plasmid DNA after feeding in young actively growing rats and pregnant rats and also their foeti and pups (paper I),

- if natural transformation occurs in the gastrointestinal tract of different rat and mice models (paper II and III),

- if food-ingested DNA in gut content remains biologically active through the gastrointestinal tract in *ex vivo* natural transformation assays (paper II),

- whether human PBMCs (peripherial blood mononuclear cells) are naturally competent for uptake of foreign DNA in an *ex vivo* whole blood experimental model (paper IV).

SUMMARY OF PAPERS

Paper I

The extent and significance of DNA uptake in bacteria or mammalian cells in the gastrointestinal tract (GIT) in mammalian organisms is controversial. We therefore studied the intestinal uptake and tissue distribution of linear and circular plasmid DNA (pDNA) added to semisynthetic, DNA devoid feed in actively growing rats, as well as in pregnant rats and their foeti and offspring. The young rats received one pDNA containing meal (50 μg pDNA) by gavaging. Individuals were killed and blood, organ and tissue samples were harvested 2 h, 6 h and 3 days post feeding (p.f). The pregnant females were fed pellets containing pDNA (100 μg) every day for defined periods, starting at day 5 after establishing pregnancy. Females and foeti were killed at days 7 and 14 of gestation, and born pups were killed at the time of weaning. Genomic DNA was analyzed by PCR followed by Southern blot and quantitative real-time PCR. A 201 bp target sequence was detected in mesenteric lymph nodes, spleen, liver and pancreas samples from young actively growing rats two hour p.f. Six hours p.f. target DNA was detectable in the kidney from one half of the individuals. Three days p.f. the liver of one half of the individuals contained target DNA. A tendency towards more efficient uptake of linear than circular DNA was observed. Target DNA was neither detected in tissues or blood samples of pregnant rats, nor from their foeti or pups.

Paper II

Biological risk assessment of food containing recombinant DNA has exposed knowledge gaps related to the general fate of DNA in the gastrointestinal tract (GIT). In this study, we present a series of experiments designed to determine if genetic transformation of the naturally competent bacterium *Acinetobacter baylyi* BD413 occurs, with feed-introduced bacterial DNA containing a kanamycin resistance gene (*npt*II), in the GIT mice and rats. Strain BD413 was found in various gut locations in germfree mice at 10^3-10^5 CFU per gram GIT content 24 h after inoculation. However, subsequent DNA exposure of the colonized mice did not result in detectable bacterial transformants with a detection limit of 1 transformant per 10^3-10^5 bacteria. Further attempts to increase the likelihood of detection by introducing weak positive selection with kanamycin of putative transformants arising *in situ* during a 4 weeks long feeding experiment (where the mice received DNA and the recipient cells regularly) did not yield transformants either. Moreover, the *in vitro* exposure of actively growing *A. baylyi* cells to gut contents from the stomach, small intestine, cecum or colon contents of rats (with a normal microbiota) feed either purified DNA (50 µg) or bacterial cell lysates did not produce bacterial transformants. The presence of gut content of germfree mice was also highly inhibitory to transformation of *A. baylyi* indicating that microbially-produced nucleases are not responsible for the sharp 500 to 1 000 000-fold reduction of transformation frequencies seen. Finally, a range of isolates from the genera *Enterococcus, Streptococcus* and *Bifidobacterium* spp. that are frequently found in the GIT was examined for competence expression *in vitro* without yielding any transformants. In conclusion, model choice and methodological constraints severely limit the sample size and, hence, transfer frequencies that can be measured experimentally in the GIT. Our observations suggest that the contents of the GIT shields or adsorbs DNA, preventing detectable exposure of feed-derived DNA fragments to competent bacteria.

Paper III

Lack of DNA sequence similarity preventing homologous recombination to occur has been identified as a major barrier to interspecies transfer of chromosomal DNA. In general, the incoming DNA must contain regions of minimum 25-200 bp in length of high similarity to the recipient genome for the homologous base-pairing to occur (Zawadski et al., 1995; Majewski and Cohan, 1999; Thomas and Nielsen, 2005). In this study we investigated if regions of high DNA similarity between indigenous bacteria in the GIT and feed derived DNA could lead to recombination and additive integration of an inserted antibiotic resistance gene by homologous recombination. The feed introduced DNA was a plasmid with two antibiotic resistance marker genes with flanking recombination sites with high DNA similarity to 16S rRNA and 23S rRNA genes to bacteria normally present in the GIT. The nucleotide-sequence similarity between the donor DNA and the recipient genome of bacteria in the GIT could facilitate homologous recombination. Six Wistar rats harboring a normal microbiota were fed pellets added plasmid DNA daily for a period of four days before sampling of contents from the different GI compartments (stomach, small intestine, cecum and colon). In addition, two rats were included as negative controls and did not receive plasmid DNA in their food pellets. Colonies emerged on selective media after plating samples from the different sites in the GIT. DNA was isolated and the presence of the plasmid was checked by PCR. The PCRs targeted ligation sites between different areas in the plasmid to make sure that the possible positive PCR results originated from our plasmid and not from antibiotic resistant bacteria inhabiting the digestive tract. Our results indicated that ingestion of the two different plasmids (pM2 and pM3) does not confer increased proportions of Km/Strep resistance.

Paper IV

Whether human PBMCs (peripheral blood mononuclear cells) are naturally competent for uptake of foreign DNA is a controversial and important question with potential impacts for evolutionary processes, pathogenetic principles and medical as well as food/feed/agricultural applications of genetic engineering and synthetic biology. In the present study, plasmid DNA was added to an *ex vivo* human whole blood model with PBMCs that were either LPS (lipopolysaccharide) stimulated or not. After a 4 or 18 hours incubation period, the PBMCs were isolated and analysed for uptake of foreign DNA. At both time points it was demonstrated that human PBMCs under these whole blood experimental conditions had taken up, or were intimately associated with, plasmid DNA sequences that were at least 995 bp long, irrespective of LPS stimulation. Expression of a potentially active *gfp* reporter gene carried by pDNA was not detected. Finally, it was demonstrated that fluid phase, not cell-associated, pDNA in the whole blood model might persist in fragments of at least 755 bp for 18 hours or more.

DISCUSSION

With the introduction of genetically modified plants (GMP) to the European market during the last few years, a public discussion came up concerning the safety and advantage of food production for humans or farm animals produced in this way (Dale, 1999; Hug, 2008). Concerns have been raised regarding the possibility that DNA introduced into genetically modified crops could be transferred into mammalian cells or into bacteria harboring the gastrointestinal tract in the animals that eat these crops, and whether there might be any risks associated with such transfer (Traavik, 1995; Nielsen et al., 1998; Gasson, 2001; EFSA 2009). In order to assess the impact of the transfer of GMP-DNA on food safety, it is necessary to understand the gene transfer processes occurring in nature and the mechanisms behind them, including their occurrence at different stages along the food chain (Jonas et al., 2001; Lipp et al., 2001; Kharazmi et al., 2003; van den Eede et al., 2004; Heritage, 2005). Defined foreign DNA molecules can be introduced into organisms under different regimes to determine the resistance of DNA to physical and chemical treatments, the DNA dynamics, the integrity of DNA in food and through the gastrointestinal tract and also possible unintended biological effects of uptake of feed-derived DNA into prokaryotic cells or across the intestinal barrier (Flachowsky et al., 2005; EFSA, 2008; EFSA, 2009).

For potential expression of genes on plasmid DNA or other foreign macromolecules, cellular entry and transport into the nucleus is necessary. Here DNA can be translated into RNA which subsequently can be transcribed into a protein (Dean et al., 1997; Belting et al., 2005; Wolff and Budker, 2005). However, the fate and possible consequences of foreign DNA entering cells can be several and may have unanticipated side effects, e.g. in terms of degradation and / or integration of the DNA, chromatin changes, genome instability, unexpected protein products from the transgene, and influence on overall gene expression patterns (Kurth et al., 1998, Freese and Schubbert, 2004). It is difficult to predict the potential consequences of intended *in vivo* chromosomal integration of e.g. transgenes, however international methods of safety assessment are designed to assess this (Dale, 1999; Freese and Schubbert, 2004).

There are different ways to study the fate of foreign DNA in mammalian systems, e.g. in different model systems such as cell cultures, in gnotobiotic animals and by performing animal feeding studies. The different model systems may have severe imitations in their

ability to calculate horizontal transfer rates to other species and this cannot be ignored by the investigator. As for example, the characteristics of the gastrointestinal tract vary greatly between different species (Arganzio, 1993; Dyce et al, 1987; Kryvi and Totland, 1997) and this will affect the persistence and degradation of foreign DNA which means that the results from studies involving different animals species may not be directly comparable to humans. This suggest that the value of the different ways to study the fate of foreign DNA depends upon a range of critical determinants which includes clear objectives, study design, dose level selection, sensitivity, protocol, data analysis and science-based interpretation (EFSA, 2008; EFSA, 2009). Further, highly sensitive molecular and histological tools can be applied to answer some of the potential uptake scenarios in mammalian systems. In independent studies, listed in Table 1 and 2, mixed diets were given to different mammals for different periods and the possible survival, persistence and uptake of DNA into mammalian and bacterial cells were investigated. The most applied method for detection of dietary DNA uptake in animals is PCR and Southern blot hybridization. In addition also *in situ* hybridization (ISH) and real-time PCR have been applied. Other animal studies have been performed to get insight into other safety aspects as nutritional, toxicity and allergenicity of newly expressed proteins and different parameters like body weight, feed consumption, blood chemistry, organ weights, histopathology, morbidity, mortality etc. (D'Agnolo, 2005; MacKenzie et al., 2007; Séralini et al., 2007; EFSA, 2008; He et al., 2008; EFSA, 2009).

The current study (paper I-IV) confirms and extends the previous findings in rodents regarding uptake of foreign DNA into mammalian cells or into bacteria harboring the gastrointestinal tract. The results are discussed in detail in paper I-IV, but some selected topics are discussed further here.

DNA stability and host cell uptake in mammalian systems

The increasing use of GMO`s worldwide has lead to in several studies investigating the fate and potential uptake of dietary DNA in animals. In different feeding trials, animals have been fed plasmid-, phage and plant products. The results from studies in rodents, fish, poultry, pigs, sheep, cattle, wild animals and humans (Table 1) suggest that feed-derived DNA persists to a certain degree in fragmented form in the gastrointestinal tract and that the gastrointestinal tract is not an absolute barrier against the uptake of macromolecules that persist through the GIT after feeding.

Paper I aimed to describe the possible persistence and uptake of food ingested plasmid DNA in young actively growing rats and in adult pregnant rats and their foeti under different feeding regimes. The results from our study revealed possible uptake by organ tissue and degradation over time after the plasmid DNA entered the animal. The possible route of uptake from the gastrointestinal tract and possible expression was not investigated in the current study. While the oral route of delivery has generally proven to be rather ineffective in studies with DNA vaccination, a report from 1991 show strong expression of foreign DNA after injection into fish muscle (Hansen et al., 1991). In paper I, fragments of orally ingested plasmid DNA could be detected in different organs like mesenteric lymph nodes, spleen, liver and pancreas in the study with young actively growing rats and a tendency towards more efficient uptake of linear than circular DNA was observed. This is in contrast with other studies where circular plasmid DNA is believed to be the most stable topoform (Prazeres et al., 1999; Walter et al., 2003). However, *in vivo* studies with mice, demonstrated that cleaved recombinant plasmid- or phage vector DNA, with polyoma viral DNA sequences, is more **infectious** than circular recombinant DNA (Israel et al., 1979; Chen et al., 1979).

The results in Paper I, do not clarify if our findings of fragments of plasmid DNA are located intracellular or extracellular within the tissues. This has been investigated in previous studies in mice where phage DNA and plasmid DNA was shown to be accumulated in the cell nucleus of the intestinal wall, spleen and liver (tissues of the immune cells) (Schubbert et al., 1997; 1998). In paper I we also observed degradation of plasmid DNA over time in the different organs after oral administration. This was shown by real-time PCR where we were able to detect more foreign DNA in several organs in our study with young actively growing rats killed 2 h compared to 6 h and 3 days after the last feeding. We were not able to detect

foreign DNA in any organs in the feeding experiment involving the pregnant rats, or more precisely fragments of feed-derived DNA were below the present limit of detection. These rats were sacrificed 24 hours after the last feeding and Doerfler and Schubbert (Doerfler and Schubbert, 1998) demonstrated in their studies that fragments of DNA could be detected up to 18 hours after feeding in contents from the gastrointestinal tract of mice and up to 24 hours in spleen and liver cells, but not later. Neither could we see any indication of germline transmission of orally ingested DNA as previously reported by Schubbert et al. (Schubbert et al., 1998) where a limited transplacental transmission to the fetus when pregnant mice were fed foreign DNA were demonstrated. Phage DNA or plasmid DNA fragments were detected by FISH in cell clusters in several organs, both in fetuses and in newborn mice. However, in a follow-up study by the same group (Hoelweg and Doerfler, 2001) germline transmission in mice could not be detected after feeding foreign DNA for generations.

In paper I, we could not detect any fragments of plasmid DNA in blood samples from the young actively growing rats or the pregnant female rats. Once plasmid DNA enters the blood it may be eliminated from the circulation (Liu et al., 2007). Previous studies have reported that plasmid DNA is degraded quickly by nucleases in the blood and blood could therefore represent a barrier of delivery of plasmid DNA to peripherally located tissues. Furthermore, plasmid DNA has been shown to be rapidly removed from the circulation by liver uptake, after intravenous administration into mice (Kawabata et al., 1995; Yoshida et al., 1996; Hisazumi et al., 2004). Even though purified DNA is rapidly hydrolyzed or eliminated when incubated in blood (Shaw et al., 1991) a certain lower concentration may be maintained in the blood and can then reach tissues (Schubbert et al., 1994; 1997). There is one published study proving this phenomenon in humans (Forsman et al., 2003), by demonstrating the transfer of partially degraded or non-degraded alimentary DNA across the intestinal mucosa and into the circulation, where target DNA fragments were present in plasma as well as in PBMCs (pheriepheral blood mononuclear cells) from both subjects participating in the experiments. The authors suggested that the DNA was gradually taken up into PBMCs by a scavenger mechanism. A few studies on foreign DNA uptake in human PBMCs have been published so far and most have been based on PBMCs related to cell lines or primary cell cultures that separate and independently study different PBMC derived cell types (Fukuhara et al., 2007; Yoshida et al., 2009). In paper IV an *ex vivo* whole blood system more akin to *in vivo* conditions (Østerud, 2000) was used to study the potential uptake and expression of plasmid DNA in human PBMCs after 4 and 18 h incubation. At both time points it was demonstrated

that human PBMCs had taken up or where intimately associated with plasmid DNA fragments that were at least 995 bp long, suggesting that PBMCs can act as vectors or transport vehicles of foreign DNA to tissues and organs. However, our study suggests that further investigation is needed.

The results of our two feeding experiments in paper I differ and there are some possible explanations. First, the different feeding regimes might influence the distribution of DNA and the difference in our results between the two experiments. By gavage feeding in the experiment with the young actively growing rats, plasmid DNA avoids the possible degradation in the oral cavity and the amount of DNA reaching the gastrointestinal tract will be higher compared to the experiment with oral ingested plasmid DNA. A recent (Duggan et al., 2003) demonstrated that approximately 70% of plasmid DNA was extensively degraded after one min. incubation *in vivo*, in the ovine oral cavity. Another reason may be that the young actively growing rats are more active in uptake of food-ingested DNA compared to the adult pregnant rats in our study. Studies have shown that the uptake and transport of macromolecules may be influenced by the age, where mammals (e.g. rat, mice, hamsters, guinea pigs, calves, dog, monkeys and man) are especially susceptible for uptake of macromolecules early in life in the neonatal period (Udall and Walker, 1982; Vallenga et al., 1985).

In general, the detection of ingested DNA is influenced by a number of factors**,** including gene copy-number, feed processing, resident time of the DNA in the gastrointestinal tract, degree of DNA fragmentation, DNA isolation method, sample type, the presence of inhibitors in the PCR assay and the limit of detection of amplicons. Most of the accumulated data in this field (Table 1) report detection of DNA fragments from high copy-number molecules (e.g from chloroplast DNA) compared to detection of DNA fragments from single copy genes (e.g from recombinant DNA). This may lead to the assumption that foreign DNA fragments can be detected in some animals if the amount/copy-number of starting material is high enough (e.g chloroplast DNA). Feed processing and DNA degradation stage of DNA at the place of uptake is also affecting the chance of detecting foreign DNA fragments. A nonuniformity of results between different experiments and across the different subsamples which been observed and may be due to unequal distribution of plasmid DNA within the organ tissues. To detect foreign DNA entering the organism through food, highly sensitive methods are needed

because the amount of recombinant DNA an animal consumes per day is very small compared to the total amount of DNA consumed.

To summarize, the data presented in paper I, together with those from the other feeding experiments with rodents (Schubbert et al., 1994; 1997; 1998; Hoelweg and Doerfler, 2001; Palka-Säntini et al., 2003), demonstrate that foreign DNA is not completely degraded in the gastrointestinal tract and that the intestinal epithelia are not a complete barrier against host uptake of foreign DNA fragments in mice and rat models. Small amounts of the feed-introduced plasmid DNA can reach several hosts organ systems, via the intestinal epithelia and the blood and / or lymph circulation, perhaps because they are protected in DNA-protein complexes. Whether PBMCs are the transport vehicles of plasmid DNA to tissues and organs needs further evaluation (Paper IV). For the detection of foreign DNA in tissues and blood, the use of sensitive methods is of major importance. However, even with sensitive methods it may be difficult to detect the target DNA if it is present only in a few copies and in a few cells, due to the large size of the sample material.

Future work in this field should try to make a comprehensive understanding of how the fragment size distribution of DNA present in various food sources and digestive compartments in mammals varies, how the intracellular location and protein interactions may affect DNA stability and degradation and also how the macromolecules overcome the barrier of the intestinal wall and the possible routes for macromolecule transfer from the intestinal lumen to the blood and tissues. It will also be interesting to determine more precisely whether the cells in the intestinal wall that incorporate DNA from the lumen of the gut are a random set of cells in the intestinal surface or if it is special cells that exhibit spesific properties. These knowledge gaps are not specifically linked to the consumption of recombinant DNA, but encompass the fate of all DNA molecules that enter the gastrointestinal tract.

Uptake of DNA by bacterial cells in the gastrointestinal tract

The frequent use of antibiotic resistance genes as markers in genetically modified plants has put forward the question whether such resistance genes can transform pathogenic bacteria present in the gastrointestinal tract (Nielsen et al., 1998; Netherwood et al., 1999; Thomson, 2001; EFSA, 2009). Despite the importance of horizontal gene transfer, studies of natural transformation have mostly been performed under optimized conditions and relatively few studies have been done on the possibility of natural transformation to occur in the human/animal gut microbiota. To our knowledge, no experimental studies have been able to demonstrate *in vivo* uptake of extracellular DNA by bacteria in the gastrointestinal tract, with the exception of the mouth. For instance it has been reported that the naturally competent oral bacterium *Streptococcus gordonii* could be transformed by plasmid DNA in saliva (Mercer et al., 1999; Mercer et al., 2001). Despite degradation, a certain amount of plasmid DNA survived and was capable of transforming the naturally competent *Streptococcus gordonii*, although the transforming activity decreased rapidly. Similar stability results were obtained by Duggan et al. (Duggan et al., 2000; 2003) who examinated the biological activity of plasmid DNA after *in vitro* and *in vivo* incubation in ovine saliva. Plasmid DNA exposed to ovine saliva up to 8 min incubation in the oral cavity was still shown to be capable of transforming electro-competent *E. coli* cells to ampicillin resistance.

A series of experiments were designed, summarized in paper II an III, and conducted to determine the possibility of natural transformation to occur in the gastrointestinal tract of different rat and mice models. The intention in paper II was to establish an *in vivo* model system for transformation using different gnotobiotic mice and rat models where we could determine to what extent genetic transformation of the naturally competent bacterium *Acinetobacter baylyi* strain BD413 could occur in the gastrointestinal tract. Further, in paper III, we wanted to determine to what extent bacterial members of indigenous bacteria developed competence for uptake of feed-derived DNA by investigating if regions of high DNA similarity between indigenous bacteria and feed-derived DNA could lead to genomic integration of a selectable marker gene. In both papers, natural transformation processes in the gastrointestinal tract were below the detection limit.

The presence of competent bacteria, naked DNA and DNA sequence similarities are the most important parameters for natural transformation to occur. However, despite series of experiments to optimize conditions for genetic transformation of the naturally competent

bacterium *Acinetobacter baylyi* strain BD413, we concluded that this model species is not detectably transformable in the GIT of rodents (Paper II). Our colonization studies showed that this strain persisted in the GIT in gnotobiotic rats at only low levels, excluding this animal model for further studies. In gnotobiotic mice, colonization occurred at a comparable higher level, possible due to improved oxygen delivery, due to higher mucosal surface per unit content in the mice. This strain is originally derived from soil and may not be adapted to the bile and partially anaerobic conditions in the GIT of mice. The main advantage using this strain in our study is the exceptional high level of competence achievable during normal growth. Selecting another bacterial inoculum with the ability to colonize at higher levels will improve the likelihood of detecting transformants if the competence level is equally high and easily inducible; however, most bacteria express competence less efficiently than *A. baylyi* (Lorenz and Wackernagel, 1994). *A. baylyi* is non-pathogenic to humans, but a relative, *A. baumanii*, is a cause of infection in immuno-compromised hosts. However, lately Chen et al. (Chen et al., 2008) reported on six patients with bacteremia due to *A. baylyi*, indicating that this strain can be a potential human pathogen that can cause nosocomial infections in immunocompromised patients. These new findings may also increase the relevance to calculate transfer rates of this strain that may also be meaningful for humans.

In paper III, we investigated if regions of high DNA similarity between indigenous bacteria and feed-derived DNA could lead to recombination (and additive integration) of a selectable marker gene, but no microbes containing the added plasmid DNA could be detected from the content from the various gastrointestinal part, indicating no transformants or transformation occurring below detection limit. For bacteria in the gastrointestinal tract to take up and integrate diet-derived DNA, the stability of DNA in all regions of the digestive system is important. The digestive activity of the various gastrointestinal compartmental fluids is known to affect the persistence of DNA and is probably not optimal for the development of competence in bacteria (Nordgård et al., 2007). Although a continuous supply of plasmid DNA was ensured by daily administration of high amount of DNA in our experiments, the digestive activity of the various gastrointestinal compartmental fluids may result in lack of available DNA substrates at relevant concentrations in the GIT and difficulties to observe any uptake by natural transformation in bacteria harboring the gastrointestinal tract.

Most studies have looked at DNA persistence in a qualitative way, but quantitative studies are needed to be able to estimate foreign DNA sequences available for the flora in the gut.

Alternatively, it may also be possible that the inability to culture microorganism (s) on agar media contributed to the lack of detection of possible transformats in our model system. De Vries and Wackernagel (De Vries and Wackernagel, 2004) list the presently known (almost 90) species that comprise only about 2% of the identified, culturable prokaryotic species that are transformable *in vitro*. Clearly many bacteria have not been examined for natural transformation and the transfer frequency among unculturable bacteria is mostly unknown. The true sequence distribution of competent bacteria or lack of competence-expressing bacterial cells in the gastrointestinal tract is still not known. In addition, if new genetic material is acquired by natural transformation, it will most likely only be retained in the transformants at detectable levels if it has a selective advantage over its competitors that favors' its survival and reproduction rates over time. This means that a lack of a selective advantage of the horizontally-transferred DNA may result that rare bacterial transformants never mulitiply and are not detected in investigations working with limited gut sample sizes.

Overall, the available studies do not suggest natural transformation to be a frequent event in the gastrointestinal tract, despite that numerous conditions have been applied to facilitate natural transformation to happen in the gastrointestinal tract. The naturally competent bacterium *Acinetobacter baylyi* strain BD413, was not detectably transformable in the GIT of rodents (paper II), the presence of gut contents was inhibitory to transformation of *Acinetobacter baylyi* strain BD413 *in vitro* and only purified DNA added to cecum and large intestine content samples was able to transform strain BD413 *in vitro* (paper II). Further, regions of high DNA similarity between indigenous bacteria and feed-derived DNA did not lead to any detectable transformants in various parts of gastrointestinal tract of rats (paper III).

To summarize, available information and methodology do not allow a clear confirmation or elimination of HGT processes relevant to bacterial evolutionary processes in the GIT (Nielsen and Townsend, 2004). Careful consideration of recipient inoculum, donor DNA, population levels, competence level and requirements is therefore needed in future studies of natural transformation in the gastrointestinal tract. With improved experimental techniques, gene transfer will possibly be detected in specific habitats where detection efforts are absent or have failed so far.

REFERENCE LIST

Aeschbacher K, Messikommer R, Meile L, Wenk C (2005) Bt176 Corn in poultry nutrition: physiological characteristics and fate of recombinant plant DNA in chickens. Poult Sci 84: 385-394

Agodi A, Barchitta M, Grillo A, Sciacca S (2006) Detection of genetically modified DNA sequences in milk from the Italian market. Int J Hyg Environ Health 209: 81-88

Alexander TW, Sharma R, Okine EK, Dixon WT, Forster RJ, Stanford K, McAllister TA (2002) Impact of feed processing and mixed ruminal culture on the fate of recombinant EPSP synthase and endogenous canola plant DNA. FEMS Microbiol Lett 214: 263-269

Alexander TW, Sharma R, Deng MY, Whetsell AJ, Jennings JC, Wang Y, Okine E, Damgaard D, McAllister TA (2004) Use of quantitative real-time and conventional PCR to assess the stability of the *cp4 epsps* transgene from Roundup Ready canola in the intestinal, ruminal, and fecal contents of sheep. J Biotechnol 112: 255-266

Alexander TW, Reuter T, Okine E, Sharma R, McAllister TA (2006) Conventional and real-time polymerase chain reaction assessment of the fate of transgenic DNA in sheep fed Roundup Ready rapeseed meal. Br J Nutr 96: 997–1005

Arganzio RA (1993) Digestion, absorption and metabolism, p 325-335. In Swenson MJ and Reece WO (eds), Dukes`physiology of domesticated animals. Comstock publishing, Ithaca

Ashkar AA, Rosenthal KL (2002) Toll-like receptor 9, CpG DNA and innate immunity. Curr Mol Med 2: 545-556

Averhoff B, Friedrich A (2003) Type IV pili-related natural transformation systems: DNA transport in mesophilic and thermophilic bacteria. Arch Microbiol 180: 385-393

Basner-Tschakarjan E, Mirohammadsadegh A, Baer A, Hengge UR (2004) Uptake and trafficking of DNA in kerationocytes: evidence for DNA-binding proteins. Gene Therapy 11: 765-774

Bauer F, Hertel C, Hammes WP (1999) Transformation of *Escherichia coli* in foodstuffs. Syst Appl Microbiol 22: 161-168

Bauer T, Weller P, Hammes WP and Hertel C (2003) The effect of processing parameters on DNA degradation in food. Eur Food Res Technol 217: 338-343

Bauer T, Hammes WP, Haase NU, Hertel C (2004) Effect of food components and processing parameters on DNA degradation in food. Environ Biosafety Res 3: 215-223

Beagle JM, Apgar GA, Jones KL, Griswold KE, Radcliffe JS, Qiu X, Lightfoot DA, Iqbal MJ (2005) The digestive fate of *Escherichia coli* glutamate dehydrogenase deoxyribonucleic acid from transgenic corn in diets fed to weanling pigs. J Anim Sci 84: 597-607

Beever DE, Kemp CF (2000) Safety issues associated with DNA in animal feed derived from genetically modified crops. A review of scientific and regulatory procedures. Nutrition abstracts and Reviews. Series B: Livestock Feeds and Feeding 70: 175-182

Belting M, Sandgren S, Wittrup A (2005) Nucelar delivery of macromolecules: barriers and carriers. Adv Drug Deliv 28: 505-527

Bertheau Y, Helbling JC, Fortabat MN, Makhzami S, Sotinel I, Audéon C, Nignol AC, Kobilinsky A, Petit L, Fach P, Brunschwig P, Duhem K, Martin P (2009) Persistence of plant DNA sequences in the blood of dairy cows fed with genetically modified (Bt176) and conventional corn silage. J Agric Food Chem 57: 509-516

Bijsterbosch MK, Manoharan M, Rump ET, De Vrueh RL, van Veghel R, Tivel KL, Biessen EA, Bennett CF, Cook PD, van Berkel TJ (1997) *In vivo* fate of phosphorothioate antisense oligodeoxynucleotides: predominant uptake by scavenger receptors on endothelial liver cells. Nucleic Acids Res 25: 3290-3296

Bräutigam M, Hertel C, Hammes WP (1997) Evidence for natural transformation of *Bacillus subtilis* in foodstuffs. FEMS Microbiol Lett 155: 93-98

Brayden DJ, Jepson MA, Baird AW (2005) Keynote review: intestinal Peyer's patch M cells and oral vaccine targeting. Drug Discov Today 10: 1145-1157

Bronk JR, Hastewell JG (1987) The transport of pyrimidines into tissue rings cut from rat small intestine. J Physiol 382: 475-488

Budker V, Budker T, Zhang G, Subbotin V, Loomis A, Wolff JA (2000) Hypothesis: naked plasmid DNA is taken up by cells *in vivo* by a receptor-mediated process. J Gene Med 2: 76-88

Bureau MF, Naimi S, Torero IR, Seguin J, Georger C, Arnould E, Maton L, Blanche F, Delaere P, Scherman D (2004) Intramuscular plasmid DNA electrotransfer: biodistribution and degradation. Biochim Biophys Acta 1676: 138-148

Bushman F (2002) Lateral DNA Transfer. Cold Spring Harbor Lab. Press, New York, NY

Calsamiglia S, Hernandez B, Hartnell GF, Phipps R (2007) Effects of corn silage derived from a genetically modified variety containing two transgenes on feed intake, milk production, and composition, and the absence of detectable transgenic deoxyribonucleic acid in milk in Holstein dairy cows. J Dairy Sci 90: 4718-4723

Castillo AR, Gallardo MR, Maciel M, Giordano JM, Conti GA, Gaggiotti, Quaino O, Gianni C, Hartnell GF (2004) Effects of feeding rations with genetically modified whole cottonseed to lactating Holstein cows (2004) J Dairy Sci 87: 1778-1785

Chainark P, Satoh S, Hirono I, Aoki T, Endo M (2008) Availability of genetically modified feed ingredient: investigations of ingested foreign DNA in rainbow trout *Oncorhynchus mykiss*. Fisheries Sci 74: 380-390

Chan HW, Israel MA, Garon CF, Rowe WP, Martin MA (1979) Molecular cloning of polyoma virus DNA in *Escherichia coli*: lambda phage vector system. Science 203: 887-892

Chambers PA, Duggan PS, Heritage J, Forbes JM (2002) The fate of antibiotic resistance marker genes in transgenic plant feed material fed to chickens. J Antimicrob Chemother 49: 161-164

Chan HW, Israel MA, Garon CF, Rowe WP, Martin MA (1979) Molecular cloning of polyoma virus DNA in *Escherichia coli*: lambda phage vector system. Science 203: 887-892

Chen I, Dubnau D (2004) DNA uptake during natural transformation. Nat Rev Microbiol 2: 241–249

Chen I, Christie PJ, Dubnau D (2005) The ins and outs of DNA transfer in bacteria. Science 310: 1456-1460

Chen TL, Siu LK, Lee YT, Chen CP, Huang LY, Wu RC, Cho WL, Fung CP (2008) *Acinetobacter baylyi* as a pathogen for opportunistic infection. J Clin Microbiol 46: 2938-2944

Chiter A, Forbes JM, Blair GE (2000) DNA stability in plant tissues: implications for the possible transfer of genes from genetically modified food. FEBS Lett 481: 164-168

Chowdhury EH, Kuribara H, Hino A, Sultana P, Mikami O, Shimada N, Guruge KS, Saito M, Nakajima Y (2003a) Detection of corn intrinsic and recombinant DNA fragments and *Cry1Ab* protein in the gastrointestinal contents of pigs fed genetically modified corn Bt11. J Anim Sci 81: 2546-2551

Chowdhury EH, Mikami O, Nakajima Y, Hino A, Kuribara H, Suga K, Hanazumi M, Yomemochi C (2003b) Detection of genetically modified maize DNA fragments in the intestinal contents of pigs fed StarLink CBH351. Vet Hum Toxicol 45: 95-96

Chowdhury EH, Mikami O, Murata H, Sultana P, Shimada N, Yoshioka M, Guruge KS, Yamamoto S, Miyazaki S, Yamanaka N, Nakajima Y (2004) Fate of maize intrinsic and recombinant genes in calves fed genetically modified maize Bt11. J Food Prot 67: 365-370

Coelho-Castelo AAM, Santos RR, Bonato VLD, Jamur MC, Oliver C, Silva CL (2003) B-lymphocytes in bone marrow or lymph nodes can take up plasmid DNA after intramuscular delivery. Hum Gene Ther 14: 961-966

D'Agnolo G (2005) GMO: human health risk assessment. Vet Res Commun 2: 7-11

Dale PJ (1999) Public concerns over transgenic crops. Genome Res 9: 1159-1162

Deaville ER, Maddison BC (2005) Detection of transgenic and endogenous plant DNA fragments in the blood, tissues, and digesta of broilers. J Agric Food Chem 53: 10268-10275

de Vries J, Meier P, Wackernagel W (2001) The natural transformation of the soil bacteria *Pseudomonas stutzeri* and *Acinetobacter* sp. by transgenic plant DNA strictly depends on homologous sequences in the recipient cells. FEMS Microbiol Lett 195: 211–215

de Vries J, Wackernagel W (2002) Integration of foreign DNA during natural transformation of *Acinetobacter* sp. by homology-facilitated illegitimate recombination. Proc Natl Acad Sci USA 99: 2094–2099

de Vries J, Wackernagel W (2004) Microbial horizontal gene transfer and the DNA release from transgenic crop plants. Plant and Soil 266: 91-104

Doerfler W, Schubbert R (1998) Uptake of feed-derived DNA from the environment: The gastrointestinal tract and the placenta as portals of entry. Wien Klin Wochenschr 110: 40-44

Dubnau D (1999) DNA uptake in bacteria. Annu Rev Microbiol 53: 217-244

Dubnau D, Provvedi R (2000) Internalizing DNA. Res Microbiol 151: 475-480

Duggan PS, Chambers PA, Heritage J, Forbes MJ (2000) Survival of free DNA encoding antibiotic resistance from transgenic maize and the transformation activity of DNA in ovine saliva, ovine rumen fluid and silage effluent. FEMS Microbiol Lett 191: 71-77

Duggan PS, Chambers PA, Heritage J, Michael Forbes J (2003) Fate of genetically modified maize DNA in the oral cavity and rumen of sheep. Br J Nutr 89: 159-166

Dyce KM, Sack WO, Wensing CJG (1987) Textbook of veterinary anatomy, p 121-130. Saunders WB Company, Philadelphia

EFSA GMO Panel Working Group on Animal Feeding Trials (2008) Safety and nutritional assessment of GM plants and derived food and feed: the role of animal feeding trials. 46: S2-70

EFSA (2009) Statement of EFSA on the consolidated presentation of opinions on the use of antibiotic resistance genes as marker genes in genetically modified plants. The EFSA Journal 1108, 1034-1035

Einspanier R, Klotz A, Kraft J, Aulrich K, Poser R, Schwägele F, Jahreis G, Flachowsky G (2001) The fate of forage plant DNA in farm animals: a collaborative case-study investigating cattle and chicken fed recombinant plant material. Eur Food Res Technol 212: 129-132

El Sayed RM, El-Sanhoty S, Desoky A, El- Maged ABD, Ramadan MF (2006) Safety assessment of genetically modified potato spunta: degradation of DNA in gastrointestinal tract and carryover to rat organs. J Food Biochem 30: 556-578

Fan H, Lin Q, Morrissey GR, Khavari PA (1999) Immunization via hair follicles by topical application of naked DNA to normal skin. Nat Biotechnol 17: 870-872

Flachowsky G, Chesson A, Aulrich K (2005) Animal nutrition with feeds from genetically modified plants. Arch Anim Nutr 59: 1-40

Forsman A, Ushameckis D, Bindra A, Yun Z, Blomberg J (2003) Uptake of amplifiable fragments of retrotransposon DNA from the human alimentary tract. Mol Genet Genomics 270: 362-368

Freese W, Schubert D (2004) Safety testing and regulation of genetically engineered foods. Biotechnol Genet Eng Rev 21: 299-324

Gasson MJ (2000) Gene transfer from genetically modified food. Curr Opin Biotechnol 11: 505-508

Gebhard F, Smalla K (1998) Transformation of *Acinetobacter sp*. strain BD413 by transgenic sugar beet DNA. Appl Environ Microbiol 64: 1550-1554

Gil A (2002) Modulation of the immune response mediated by dietary nucleotides. Eur J Clin Nutr 56: S1-S4

Goodgal SH (1982) DNA uptake in *Haemophilus* transformation. Annu Rev Genet 16: 169-192

Gough PJ, Gordon S (2000) The role of scavenger receptors in the innate immune system. Microbes Infect 2: 305-311

Gouli Z, Mingguang Z, Zhijiang Z, Hongsheng O, Qiang L (1999) Establishment and application of a polymerase chain reaction for the identification of beef. Meat Science 51: 233-236

Guertler P, Lutz B, Kuehn R, Meyer HHD, Einspanier R, Killermann B, Albrecht C (2007) Fate of recombinant DNA and *Cry1Ab* protein after ingestion and dispersal of genetically modified maize in comparison to rapeseed by fallow deer (*Dama dama*). Eur J Wildl Res 54: 36-43

Guertler P, Paul V, Albrecht C, Meyer HHD (2009) Sensitive and highly specific quantitative real-time PCR and ELISA for recording a potential transfer of novel DNA and *Cry1Ab* protein from feed into bovine milk. Anal Bioanal Chem 393: 1629-1638

Hamilton HL, Dillard JP (2006) Natural transformation of *Neisseria gonorrhoeae*: from DNA donation to homologous recombination. Mol Microbiol 59: 376-385

Hansen E, Fernandes K, Goldspink G, Butterworth P, Umeda PK, Chang KC (1991) Strong expression of foreign genes following direct injection into fish muscle FEBS Lett 290: 73-76

Harris G, KuoLee R, Chen W (2006) Role of Toll-like receptors in health and diseases of gastrointestinal tract. World J Gastroenterol 14: 2149-2160

He XY, Huang KL, Li X, Qin W, Delaney B, Luo YB (2008) Comparison of grain from corn rootworm resistant transgenic DAS-59122-7 maize with non-transgenic maize grain in a 90-day feeding study in Sprague-Dawley rats. Food Chem Toxicol 46: 1994-2002

Heeg K, Dalpke A, Peter M, Simmermann S (2008) Structural requirements for uptake and recognition of CpG oligonucleotides. Int J Med Microbiol 298: 33-38

Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, Matsumoto M, Hoshino K, Wagner H, Takeda K, Akira S (2000) Toll-like receptor recognizes bacterial DNA. Nature 7: 740-745

Hengge UR, Chan EF, Foster RA, Walker PS, Vogel JC (1995) Cytokine gene expression in epidermidis with biological effects following injection of naked DNA. Nature 10: 161-166

Hengge UR, Walker PS, Vogel JC (1996) Expression of naked DNA in human, pig, and mouse skin. Dermatol 97: 2911-2916

Heritage J (2005) Transgenes for tea? Trends Biotechnol 23:17-21

Hisazumi J, Kobayashi N, Nishikawa M, Takakura Y (2004) Significant role of liver sinusoidal endothelial cells in hepatic uptake and degradation of naked plasmid DNA after intravenous injection. Pharm Res 21: 1223-1228

Ho MW, Ryan A, Cummins J (2000) Cauliflower mosaic viral promoter – a recipe for disaster? Microb Ecol Health Dis 11: 194-197

Hohlweg U, Doerfler W (2001) On the fate of plant or other foreign genes upon the uptake in food or after intramuscular injection in mice. Mol Genet Genomics 265: 225-233

Hubbard AL (1989) Endocytosis. Curr Opin Cell Biol 1: 675-683

Hug K (2008) Genetically modified organisms: do the benefits outweigh the risks? Medicina 44: 87-99

Hupfer C, Hotzel H, Sachse K, Engel KH (1998) Detection of the genetic modification in heat-treated products of Bt maize by polymerase chain reaction. Z Leben Unters Forsch 206: 203-207

Israel MA, Chan HW, Rowe WP, Martin MA (1979) Molecular cloning of polyoma virus DNA in *Escherichia coli*: plasmid vector system. Science 203: 883-837

Israel MA, Chan HW, Martin MA, Rowe WP (1979) Molecular cloning of polyoma virus DNA in *Escherichia coli*: oncogenicity testing in hamsters. Science 205: 1140-1142

Jennings JC, Albee LD, Kolwyck DC, Surber JB, Taylor ML, Hartnell GF, Lirette RP, Glenn KC (2003) Attempts to detect transgenic and endogenous plant DNA and transgenic protein in muscle from broilers fed YieldGard Corn Borer Corn. Poult Sci 82: 371-380

Jennings JC, Kolwyck DC, Kays SB, Whetsell AJ, Surber JB, Cromwell GL, Lirette RP, Glenn KC (2003) Determining whether transgenic and endogenous plant DNA and transgenic protein are detectable in muscle from swine fed Roundup Ready soybean meal. J Anim Sci 81: 1447-1455

Jennings JC, Whetsell AJ, Nicholas NR, Sweeney BM, Klaften MB, Kays SB, Hartnett GF, Lirette RP, Glenn KC (2004) Determining whether transgenic or endogenous plant DNA is detectable in dairy milk or beef organs. Bulletin 383/2003 of the International Dairy Federation, p 41-46

Jonas DA, Elmadfa I, Engel KH, Heller KJ, Kozianowski, König A, Müller D, Narbonne JF, Wackernagel W, Kleiner J (2001) Safety consideration of DNA in food. Ann Nutr Metab 45: 235-254

Kawabata K, Takakura Y, Hashida M (1995) The fate of plasmid DNA after intravenous injection in mice: involvement of scavenger receptors in its hepatic uptake. Pharm Res 12: 825-830

Kaiserlian D, Etchart N (1999) Entry sites for oral vaccines and drugs: A role for M cells, enterocytes and dendritic cells? Semin Immunol 11: 217-224

Keese P (2008) Risks from GMOs due to horizontal gene transfer. Environ Biosafety Res 7: 123-149

Kelly BG, Vespermann A, Bolton DJ (2009) Gene transfer events and their occurrence in selected environments. Food Chem Toxicol 47: 978-983

Kharazmi M, Bauer T, Hammes WP, Hertel C (2003) Effect of food processing on the fate of DNA with regard to degradation and transformation capability in *Bacillus subtilis*. Syst Appl Microbiol 26: 495-501

Klotz A, Mayer J, Einspanier R (2002) Degradation and possible carry over of feed DNA monitored in pigs and poultry. Eur Food Res Technol 214: 271-275

Kobayashi N, Hirata K, Chen S, Kawase A, Nishikawa M, Takakura Y (2004) Hepatic delivery of particulates in the submicron range by a hydrodynamics-based procedure: implications for particulate gene delivery systems. J Gene Med 6: 455-463

Krieg AM (2002) CpG motifs in bacterial DNA and their immune effects. Annu Rev Immunol 20: 709-760

Kumagai Y, Takeuchi O, Akira S (2008) TLR9 as a key receptor for the recognition of DNA. Adv Drug Deliv Rev 29: 795-804

Kucharzik T, Lügering N, Rautenberg K, Lügering A, Schmidt MA, Stoll R, Domschke W (2000) Role of M cells in intestinal barrier function. Ann N Y Acad Sci 915: 171-183

Kurth R (1998) Risk potential of chromosomal integration of feed-derived DNA. Dev Biol Stand 93: 45-56

Kyd JM, Cripps AW (2008) Functional differences between M cells and enterocytes in sampling luminal antigens. Vaccine 26: 6221-6224

Li YH, Lay PCY, Lee JH, Ellen RP, Cvitkovitch DG (2001) Natural genetic transformation of *Streptococcus mutans* growing in biofilms. J Bacteriol 183: 897-908

Lindahl T (1993) Instability and decay of the primary structure of DNA. Nature 362: 709-714

Lipp M, Bluth A, Euquem F, Kruse L, Schimmel H, Van den Eede G, Anklam E (2001) Validation of a method based on polymerase chain reaction for the detection of genetically modified organisms in various processed foodstuffs. Eur Food Res Technol 212: 497-504

Lippens S, Hoste E, Vandenabeele P, Agostinis P, Declercq W (2009) Cell death in the skin. Apoptosis 14: 549-569

Lorentz MG, Wackernagel W (1994) Bacterial gene transfer by genetic transformation in the environment. Microbiol Rev 58: 563-602

MacKenzie SA, Lamb I, Schmidt J, Deege L, Morrisey MJ, Harper M, Layton RJ, Prochaska LM, Sanders C, Locke M, Mattsson JL, Fuentes A, Delaney B (2007) Thirteen week feeding study with transgenic maize grain containing event DAS-Ø15Ø7-1 in Sprague-Dawley rats. Food Chem Toxicol 45: 551-562

Majewski J, Cohan FM (1999) DNA sequence similarity requirements for interspecific recombination in *Bacillus*. Genetics 153: 1525-1533

Malovic I, Sørensen KK, Elvevold KH, Nedredal GI, Paulsen S, Erofeev AV, Smedsrød BH, McCourt PA (2007) The mannose receptor on murine liver sinusoidal endothelial cells is the main denatured collagen clearance receptor. Hepatology 45: 1454-1461

Martin-Orue SM, O'Donnell AG, Arino J, Netherwood T, Gilbert HJ, Mathers JC (2002) Degradation of transgenic DNA from genetically modified soya and maize in human intestinal simulations. Br J Nutr 87: 533-542

Master CI, Miles CA, Mackey BM (1998) Survival and biological activity of heat damaged DNA. Lett Appl Microbiol 27: 279-282

Maturin L, Curtiss R (1977) Degradation of DNA by nucleases in intestinal tract of rats. Science 196: 216-218

Mazza R, Soave M, Morlacchini M, Piva G, Morocco A (2005) Assessing the transfer of genetically modified DNA from feed to animal tissues. Transgenic Res 14: 775-784

McAllan AB (1980) The degradation of nucleic acids in, and the removal of breakdown products from the small intestines of steers. Br J Nutr 44: 99-112

McAllan AB (1982) The fate of nucleic acids in ruminants. Proc Nutr Soc 41: 309-317

McCracken VJ, Lorenz RG (2001) The gastrointestinal ecosystem: a precarious alliance among epithelium, immunity and microbiota. Cell Microbiol 3: 1-11

Mercer DK, Melville CM, Scott KP, Flint HJ (1999 a) Natural genetic transformation in the rumen bacterium *Streptococcus bovis* JB1. FEMS Microbiol Lett 179: 485-490

Mercer DK, Scott KP, Bruce-Johnson WA, Glover LA, Flint HJ (1999 b) Fate of free DNA and transformation of the oral bacterium *Streptococcus gordonii* DL1 by plasmid DNA in human saliva. Appl Environ Microbiol 65: 6-10

Mercer DK, Scott KP, Melville CM, Glover AL, Flint HJ (2001) Transformation of an oral bacterium via chromosomal integration of free DNA in the presence of human saliva. FEMS Microbiol Lett 200: 163-167

Naito M, Hasegawa G, Takahashi K (1997) Development, differentiation, and maturation of Kupffer cells. Microsc Res Tech 15: 350-364

Nemeth A, Wurz A, Artim L, Charlton S, Dana G, Glenn K, Hunst P, Jennings J, Shilito R, Song P (2004) Sensitive PCR analysis of animal tissue samples for fragments of endogenous and transgenic plant DNA. J Agric Food Chem 52: 6129-6135

Netherwood T, Bowden R, Harrison P, O'Donnell AG, Parker DS, Gilbert HJ (1999) Gene transfer in the gastrointestinal tract. Appl Environ Microbiol 65: 5139-5141

Netherwood T, Martin-Orue SM, O'Donnell AG, Gockling S, Graham J, Mathers JC, Gilbert HJ (2004) Assessing the survival of transgenic plant DNA in the human gastrointestinal tract. Nat Biotechnol 22: 204-209

Nielsen CR, Berdal KG, Bakke-McKellup AM, Holst-Jensen A (2005 a) Dietary DNA in blood and organs of Atlantic salmon (*Salmo salar L.*) Eur Food Res Technol 221: 1-8

Nielsen CR, Holst-Jensen A, Løvseth A, Berdal KG (2006) Persistence and distribution of intravenously injected DNA in blood and organs of Atlantic salmon (*Samo salar L.)* Eur Food Res Technol 222: 258-265

Nielsen KM, Bones AM, Smalla K, vanElsas JD (1998) Horizontal gene transfer from transgenic plants to terrestrial bacteria – a rare event? FEMS Microbiol Rev 22: 79-103

Nielsen KM, Berdal KG, Kruse H, Sundsfjord A, Mikalsen A, Yazdankhah S, Nes I (2005 b) An assessment of potential long-term health effects caused by antibiotic resistance marker genes in genetically modified organisms based on antibiotic usage and resistance patterns in Norway. VKM-Report, Norwegian Scientific Committee for Food Safety, Oslo, Norway.

Nielsen KM, Townsend JP (2004) Monitoring and modeling horizontal gene transfer. Nat Biotechnol. 22: 1110-1104

Nielsen KM, Johnsen PJ, Bensasson D, Daffonchio D (2007) Release and persistence of extracellular DNA in the environment. Environ Biosafety Res 6: 37-53

Nordgaard L, Nguyen T, Midtvedt T, Benno Y, Traavik T, Nielsen KM (2007) Lack of detectable uptake of DNA by bacterial gut isolates grown in vitro and by *Acinetobacter baylyi* colonizing rodents *in situ*. Environ Biosafety Res 6: 149–160

O'Hara AM, Shanahan F (2006) The gut flora as a forgotten organ. EMBO Rep 7: 688-693

Ogawa Y, Yoshinaga T, Yasuda K, Nishikawa M, Takakura Y (2005) The uptake and degradation of DNA is impaired in macrophages and dendritic cells from NZB/W F (1) mice. Immunol Lett 101: 32-40

Owen RL (1999) Uptake and transport of intestinal macromolecules and microorganisms by M cells in Peyer's patches--a personal and historical perspective. Semin Immunol 11: 157-163

Pääbo S, Gifford JA, Wilson AC (1988) Mitochondrial DNA sequences from a 7000-year old brain. Nucleic Acids Res 16: 9775-9787

Palka-Santini M, Schwarz-Herzke B, Hösel M, Renz D, Auerochs S, Brondke H, Doerfler W (2003) The gastrointestinal tract as the portal of entry for foreign macromolecules: fate of DNA and proteins. Mol Genet Genomics 270: 210-215

Palmen R, Vosman B, Buijsman P, Breek CK, Hellingwerf KJ (1993) Physiological characterization of natural transformation in *Acinetobacter calcoaceticus*. J Gen Microbiol 139: 295-305

Pauli U, Liniger M, Zimmermann A, Schrott M (2000) Extraction and amplification of DNA from 55 foodstuffs. Mitt Lebensm 91: 491-501

Peiser L, Mukhopadhyay S, Gordon S (2002) Scavenger receptors in innate immunity. Curr Opin Immunol 14: 123-128

Phipps RH, Deaville ER, Maddison BC (2003) Detection of transgenic and endogenous plant DNA in rumen fluid, duodenal digesta, milk, blood, and feces of lactating dairy cows. J Dairy Sci 86: 4070-4078

Phipps RH, Jones AK, Tingey AP, Abeyasekera S (2005) Effect of corn silage from an herbicide-tolerant genetically modified variety on milk production and absence of transgenic DNA in milk. J Dairy Sci 88: 2870-2878

Pirondini A, Marmiroli N (2008) Environmental risk assessment in GMO analysis. Riv Biol 101: 215-246

Poms RE, Hochsteiner W, Luger K, Glossl J, Foissy H (2003) Model studies on the detectability of genetically modified feeds in milk. J Food Prot 66: 304-310

Pontiroli A, Simonet P, Frostegard A, Vogel TM, Monier JM (2007) Fate of transgenic plant DNA in the environment. Environ Biosafety Res 6: 15-35

Prazeres DMF, Ferreira GNM, Monteiro GA, Cooney CL, Cabral JMS (1999) Large scale production of pharmaceutical-grade plasmid DNA for gene therapy: problems and bottlenecks. Trends Biotechnol 17: 169-174

Reuter T, Aulrich K (2003) Investigations on genetically modified maize (Bt-maize) in pig nutrition: fate of feed-ingested feed-derived DNA in pig bodies. Eur Food Res Technol 216: 185-192

Rizzi A, Brusetti L, Arioli S, Nielsen KM, Tamagnini I, Tamburini A, Sorlini C, Daffonchio D (2008) Detection of feed-derived maize DNA in goat milk and evaluation of the potential of horizontal transfer to bacteria. Eur Food Res Technol 227: 1699-1709

Roberts TL, Idris A, Dunn JA, Kelly GM, Burnton CM, Hodgson S, Hardy LL, Garceau V, Sweet MJ, Ross IL, Hume DA, Stacey KJ (2009) HIN-200 proteins regulate caspase activation in response to foreign cytoplasmic DNA. Science 20: 1057-1060

Rossi F, Morlacchini M, Fusconi G, Pietri A, Mazza R, Piva G (2005) Effect of Bt corn on broiler growth performance and fate of feed-derived DNA in the digestive tract. Poult Sci 84: 1022-1030

Sanden M, Bruce IJ, Rahman MA, Hemre GI (2004) The fate of transgenic sequences present in genetically modified plant products in fish feed, investigating the survival of GM soybean DNA fragments during feeding trials in Atlantic salmon, *Salmo salar L*. Aquaculture 237: 391-405

Sanden M, Berntssen MHG, Hemre GI (2007) Intracellular localization of dietary and naked DNA in intestinal tissue of Atlantic salmon, *Salmo salar L*, using *in situ* hybridization. Eur Food Res Technol 225: 533-543

Schubbert R, Lettmann C, Doerfler W (1994) Ingested foreign (phage M13) DNA survives transiently in the gastrointestinal tract and enters the bloodstream of mice. Mol Gen Genet 242: 495-504

Schubbert R, Renz D, Schmitz B, Doerfler W (1997) Foreign (M13) DNA ingested by mice reaches peripheral leukocytes, spleen, and liver via the intestinal wall mucosa and can be covalently linked to mouse DNA. PNAS 94: 961-966

Schubbert R, Hohlweg U, Renz D, Doerfler W (1998) On the fate of orally ingested feed-derived DNA in mice: chromosomal association and placental transmission to the fetus. Mol Gen Genet 259: 569-576

Schwesinger MD (1977) Additive recombination in bacteria. Bacteriol Rev 41: 872-902

Séralini GE, Cellier D, de Vendomois JS (2007) New analysis of a rat feeding study with a genetically modified maize reveals signs of hepatorenal toxicity. Arch Environ Contam Toxicol 53: 596-602

Seternes T, Sørensen K, Smedsrød B (2002) Scavenger endothelial cells of vertebrates: a nonperipheral leukocyte system for high-capacity elimination of waste macromolecules. Proc Natl Acad Sci U S A 99: 7594-7597

Sharma R, Alexander TW, John SJ, Forster RJ, McAllister TA (2004) Relative stability of transgene DNA fragments from GM rapeseed in mixed ruminal cultures. Br J Nutr 91: 673-681

Sharma R, Damgaard D, Alexander TW, Dugan ME, Aalhus JL, Stanford K, McAllister TA (2006) Detection of transgenic and endogenous plant DNA in digesta and tissues of sheep and pigs fed Roundup Ready canola meal. J Agric Food Chem 54: 1699-1709

Shaw JP, Kent K, Bird J, Fishback J, Froehler B (1991) Modified deoxyoligonucleotides stable to exonuclease degradation in serum. Nucleic Acids Res 25: 747-750

Shedova E, Albrecht C, Zverlov VV, Schwarz WH (2009) Stimulation of bacterial DNA transformation by cattle saliva: implications for using genetically modified plants in animal feed. World J Microbiol Biotechnol 25: 457-463

Shen P, Huang HV (1986) Homologous recombination in *Escherichia coli*: dependence on substrate length and homology. Genetics 112: 441-457

Singer BS, Gold L, Gauss P, Doherty DH (1982) Determination of the amount of homology required for recombination in bacteriophage T4. Cell 31: 25-33

Smedsrød B (2004) Clearance function of scavenger endothelial cells. Comp Hepatol 14: S22

Stacey KJ, Sweet MJ, Hume DA (1996) Macrophages ingest and are activated by bacterial DNA. J Immunol 157: 2116-2122

Sun D, Zhang Y, Mei Y, Jiang H, Xie Z, Liu H, Chen X, Shen P (2006) *Escherichia coli* is naturally transformable in a novel transformation system. FEMS Microbiol Lett 265: 249-255

Takakura Y, Takagi T, Hashiguchi M, Nishikawa M, Yamashita F, Doi T, Imanishi T, Suzuki H, Kodama T, Hashida M (1999) Characterization of plasmid DNA binding and uptake by peritoneal macrophages from class A scavenger receptor knockout mice. Pharmac Res 16: 503-508

Tannock GW (2001) Molecular assessment of intestinal microflora. Am J Clin Nutr 73: 410S-414S

Tamkovich SN, Vlasov VV, Laktionov PP (2008) Circulating deoxyribonucleic acids in blood and their using in medical diagnostics. Mol Biol 42: 12-23

Thomas CM, Nielsen KM (2005) Mechanisms of, and barriers to, horizontal gene transfer between bacteria. Nat Rev Microbiol 3: 711-721

Thomson JA (2001) Horizontal transfer of DNA from GM crops to bacteria and to mammalian cells. J Food Sci 66: 188-193

Tonheim TC, Leirvik J, Lovoll M, Myhr AI, Bogwald J, Dalmo RA (2007) Detection of supercoiled plasmid DNA and luciferase expression in Atlantic salmon (*Salmo salar L.*) 535 days after injection. Fish Shellfish Immunol 23: 867-876

Tonheim TC, Bøgwald J, Dalmo RA (2008) What happens to the DNA vaccine in fish? A review of current knowledge. Fish Shellfish Immunol 25: 1-18

Tony MA, Butschke A, Broll H, Grohmann L, Zagon J, Halle I, Dänicke S, Schaauzu M, Hafez HM, Flachowsky G (2003) Safety assessment of Bt176 maize in broiler nutrition: degradation of maize-DNA and its metabolic fate. Arch Anim Nutr 57: 235-252

Traavik T (1995) For tidlig kan være for sent. Økologiske faremomenter ved anvendelse av nakent DNA. Direktoratet for naturforvaltning, Trondheim. ISBN 82-7072-181-6

Traavik T, Nielsen KM, Quist D (2007) Genetic engineering of living cells and organisms. In: Traavik T, Lim LC eds., Biosafety First: Holistic approaches to risk and uncertainty in genetic engineering and genetically modified organisms, Tapir Academic Press, Trondheim, 2007

Tsume Y, Taki Y, Sakane T, Nadai T, Sezaki H, Watabe K, Kohno T, Yamashita S (1996). Quantitative evaluation of the gastrointestinal absorption of protein into the blood and lymph circulation. Biol Pharm Bull 19: 1332-1337

Tudisco R, Lombardi P, Bovera F, d`Angelo D, Cutrignelli MI, Terzi V, Avallone L, Infascelli F (2006) Genetically modified soya bean in rabbit feeding: detection of DNA fragments and evaluation of metabolic effects by enzymatic analysis. Anim Sci 82: 193-199

Tyrer PC, Ruth Foxwell A, Kyd JM, Otczyk DC, Cripps AW (2007) Receptor mediated targeting of M-cells. Vaccine 25: 3204-3209

Udall JN, Walker WA (1982) The physiologic and pathologic basis for the transport of macromolecules across the intestinal tract. J Pediatr Gastroenterol Nutr 1: 295-301

Udvardi A, Kufferath I, Grutsch H, Zatloukal K, Volc-Platzer B (1999) Uptake of exogenous DNA via the skin. J Mol Med 77: 744-750

van den Eede G, Aarts H, Buhk HJ, Corthier G, Flint HJ, Hammes W, Jacobsen B, Midtvedt T, van der Vossen J, von Wright A, Wackernagel W, Wilcks A (2004) The relevance of gene transfer to the safety of food and feed derived from genetically modified (GM) plants. Food Chem Toxicol 42: 1127-1156

Vacik J, Dean BS, Zimmer WE, Dean DA (1999) Cell-specific nuclear import of plasmid DNA. Gene Ther 6: 1006-1014

Vellenga L, Mouwen JM, van Dijk JE, Breukink HJ (1985) Biological and pathological aspects of the mammalian small intestinal permeability to macromolecules. Vet Q 7: 322-332

Vlassov VV, Laktionov PP, Rykova EY (2007) Extracellular nucleic acids. Bioessays 29: 654-667

Walther W, Stein U, Voss C, Schmidt T, Schleef M, Schlag PM (2003) Stability analysis for long-term storage of naked DNA: impact on nonviral *in vivo* gene transfer. Anal Biochem 318: 230-235

Wang Z, Troilo PJ, Wang X, Griffiths TG, Pacchione SJ, Barnum AB, Harper LB, Pauley CJ, Niu Z, Denisova L, Follmer TT, Rizzuto G, Ciliberto G, Fattori E, Monica NL, Manam S, Ledwith BJ (2004) Detection of integration of plasmid DNA into host genomic DNA following intramuscular injection and electroporation. Gene Ther 11: 711-721

Weiss J, Ros-Chumillas M, Pena L, Egea-Cortines M (2007) Effect of storage and processing on plasmid, yeast and plant genomic DNA stability in juice from genetically modified oranges. J Biotechnol 128: 194-203

Wiedemann S, Lutz B, Kurtz H, Schwarz FJ, Albrecht C (2006) *In situ* studies on the time-dependent degradation of recombinant corn DNA and protein in the bovine rumen. J Anim Sci 84: 135-144

Wiedemann S, Lutz B, Albrecht C, Kuehn R, Killermann B, Einspanier R, Meyer HHD (2008) Fate of genetically modified maize and conventional rapeseed, and endozoochory in wild boar (*Sus scrofa*). Mamm Biol 74: 191-197

Wilcks A, van Hoek AHAM, Joosten RG, Jacobsen BBL, Aarts HJM (2004) Persistence of DNA studied in different *ex vivo* and *in vivo* rat models simulating the human gut situation. Food Chem Toxicol 42: 493-502

Wilson M (2005) Microbial Inhabitants of Humans: Their Ecology and Role in Health and Disease. Cambridge University Press. Cambridge, UK.

Wolff JA, Malone RW, Williams P, Chong W, Acsadi G, Jani A, Felgner PL (1990) Direct gene transfer into mouse muscle *in vivo*. Science 23: 1465-1468

Wolff JA, Dowty ME, Jiao S, Repetto G, Berg RK, Ludtke JJ, Williams P, Slautterback DB (1992) Expression of naked plasmids by cultured myotubes and entry of plasmids into T tubules and caveolae of mammalian skeletal muscle. J Cell Sci 103: 1249-1259

Wolff JA (1997) Co-transplantation of plasmid-transfected myoblasts and myotubes into rat brains enables high levels of gene expression long-term. Cell Transplant 6: 445

Worth L Jr, Clark S, Radman M, Modrich P (1994) Mismatch repair proteins *MutS* and *MutL* inhibit *RecA*-catalyzed strand transfer between diverged DNAs. Proc Natl Acad Sci U S A 91: 3238-3241

Yamane I, Nishikawa M, Takakura Y (2005) Cellular uptake and activation characteristics of naked plasmid DNA and its cationic liposome complex in human macrophages. Int J Pharm 305: 145-153

Yi AK, Tuetken R, Redford T, Waldschmidt M, Kirsch J, Krieg AM (1998) CpG motifs in bacterial DNA activate leukocytes through the pH-dependent generation of reactive oxygen species. J Immunol 160: 4755-4761

Yonemochi C, Ikeda T, Harada C, Kusama T, Hanazumi M (2003) Influence of transgenic corn (CBH 351, named Starlink) on health condition of dairy cows and transfer of *cry9C* protein and *cry9C* gene to milk, blood, liver and muscle. Animal Sci J 74: 81-88

Yoshinaga T, Yasuda K, Ogawa Y, Takakura Y (2002) Efficient uptake and rapid degradation of plasmid DNA by murine dendritic cells via a specific mechanism. Biochem Biophys Res Commun 299: 389-394

Zawadzki P, Cohan FM (1995) The size and continuity of DNA segments integrated in *Bacillus* transformation. Genetics 141: 1231-1243

Zhang G, Budker V, Wolff JA (1999) High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA. Hum Gene Ther 10: 1735-1737

Zhu Y, Li D, Wang F, Yin J, Jin H (2004) Nutritional assessment and fate of DNA of soybean meal from roundup ready or conventional soybeans using rats. Arch Anim Nutr 58: 295-310

Ziv E, Bendayan M (2000) Intestinal absorption of peptides through the enterocytes. Microsc Res Tech 49: 346–352