Impact of the human intestinal microbiota on the metabolism and toxic properties of the meat contaminant 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine

ir. Lynn VANHAECKE

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FACULTEIT BIO-INGENIEURSWETENSCHAPPEN

Promotor:

Prof. dr. ir. W. Verstraete
Department of Biochemical and Microbial Technology, Ghent University

Members of the examination committee:

Prof. dr. S. Knasmüller
Institute of Cancer Research, University of Vienna, Austria

Dr. F. Le Curieux
Laboratory of Genetic Toxicology, Pasteur Insitute of Lille, France

Dr. ir. H. Jacobs
Technical & Nutritional Services, Cosucra groupe Warcoing NV

Prof. dr. M. Bracke
Department of Radiotherapy and Nuclear Medicine, Ghent University

Prof. dr. C. Janssen
Department of Applied Ecology and Environmental Biology, Ghent University

Prof. dr. ir. N. Boon
Department of Biochemical and Microbial Technology, Ghent University

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Department of Food Safety and Food Quality, Ghent University

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Department of Organic Chemistry, Ghent University

Dean:

Prof. dr. ir. H. Van Langenhove

Rector:

Prof. dr. P. Van Cauwenberge

ir. Lynn VANHAECKE

IMPACT OF THE HUMAN INTESTINAL MICROBIOTA ON THE METABOLISM AND TOXIC PROPERTIES OF THE MEAT CONTAMINANT 2-AMINO-1-METHYL-6-PHENYLIMIDAZO[4,5-*B*]PYRIDINE

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IMPACT VAN DE HUMANE INTESTINALE MICROBIOTA OP HET METABOLISME EN DE TOXISCHE EIGENSCHAPPEN VAN DE VLEESCONTAMINANT 2-AMINO-1-METHYL-6-FENYLIMIDAZO[4,5-*B*]PYRIDINE

Cover illustration: Volcanic grilled chicken in Timanfaya National Park, Lanzarote.

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3-HPA: 3-hydroxypropionaldehyde

4,8-DiMeIQx: 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline

7,8-DiMeIQx: 2-amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline

7-OH-IQ: 2-amino-3,6-dihydro-3-methyl-*7H*-imidazo[4,5-*f*]quinoline-7-one

AIAs: aminoimidazo-azaarenes

AOs: antioxidative agents

AαC: 2-amino-9H-pyrido[2,3-*b*]indole

BHA: butylated hydroxyanisole

BHT: butylated hydroxytoluene

CE: capillary electrophoresis

CFU: colony forming units

CYP: cytochrome P450 enzyme

DAD: diode array detection

DAPI: 4',6-diamidino-2-phenylindole

DEPT: distortionless enhancement by polarization transfer

DGGE: denaturing gradient gel electrophoresis

DiMeIQx: 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline

DMEM: Dulbecco's modified Eagle's medium

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

DNPH: 2,4-dinitrophenyl hydrazine

ED: electrochemical detector

EDTA: ethylenediamine tetraacetic acid

ELISA: enzyme-linked immuno sorbent assay

ESI: electrospray ionization

FACS: fluorescence-activated cell sorting

FAFLP: fluorescent amplified fragment length polymorphism

FBS: fetal bovine serum

FOS: fructooligosaccharides

GC: gas chromatography

gCOSY: gradient enhanced correlation spectroscopy

GF: germ-free

gHMBC: gradient enhanced heteronuclear multiple bond correlation

gHSQC: gradient enhanced heteronuclear single quantum correlation

GIP: glucose-dependent insulinotropic polypeptide

GLP-1: glucagon-like peptide-1

GOS: galactooligosaccharides

GST: gluthatione S-transferase

gTOCSY: gradient enhanced total correlation spectroscopy

HCA: heterocyclic aromatic amine

HFA: human fecal microbiota-associated

HPA: 3-hydroxypropionaldehyde and aqueous derivates

HPLC: high-performance liquid chromatography

IARC: International Agency for Research on Cancer

IBD: inflammatory bowel disease

IC_{30}: 30% inhibition concentration

IC_{50}: 50% inhibition concentration

IFP: 2-amino-1,6-dimethylfuro[3,2-*e*]imidazo[4,5-*b*]pyridine

IQ: 2-amino-3-methylimidazo[4,5-*f*]quinoline

IQx: 2-amino-3-methylimidazo[4,5-*f*]quinoxaline

LAB: lactic acid bacteria

LC-MS/MS: liquid chromatography coupled with tandem mass spectrometry

LC-MS: liquid chromatography coupled with mass spectrometry

LDH: lactate dehydrogenase

LMW: low molecular weight

LOD: limit of detection

LOQ: limit of quantification

m/z: mass to charge ratio

MDR: multiple drug resistance

MeAαC: 2-amino-3-methyl-9H-pyrido[2,3-*b*]indole

MeIQ: 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline

MeIQx: 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoxaline

MMS: methyl methanesulfonate

MS: mass spectrometry

MTT: methyl tetrazolium

NAT: N-acetyl transferase

NHL: non-Hodgkin's lymphoma

OTM: olive tail moment

PAH: polycyclic aromatic hydrocarbons

PBS: phosphate buffered saline

PCR: polymerase chain reaction

PFG: pulsed field gradient

PG: propyl gallate

PhIP: 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine

PhIP-M1: 7-hydroxy-5-methyl-3-phenyl-6,7,8,9-tetrahydropyrido[3',2':4,5]imidazo[1,2-*a*]pyrimidin-5-ium chloride

PI: propidium iodide

PPD: 1,3-propanediol

PUFA: polyunsaturated fatty acids

RNA: ribonucleic acid

rRNA: ribosomal ribonucleic acid

SCFA: short chain fatty acid

SCGE: single cell gel electophoresis

SD: standard deviation

SDS: sodium dodecyl sulphate

SE: standard error

SHIME: Simulator of the Human Intestinal Microbial Ecosystem

SPE: solid phase extraction

SRB: sulforhodamine B

SULT: sulfotransferase

TBE: trypan blue exclusion

TD_{50}: 50% tumorigenic dose

THBQ: *tert*-butylhydroquinone

TMS: tetramethylsilane

Trp-P-1: 3-amino-1,4-dimethyl-*5H*-pyrido[4,3-*b*]indole

Trp-P-2: 3-amino-1-methyl-*5H*-pyrido[4,3-*b*]indole

UDPGT: UDP-glucuronosyl transferase

UTP: U.S. National Toxicology Program

UV-VIS: ultraviolet-visible

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CHAPTER 1

Literature review:
How cooked meat consumption may increase the risk for cancer?

CHAPTER 1

Literature review: How cooked meat consumption may increase the risk for cancer?

1. Diet and cancer: assessing the risk

1.1. The global burden of cancer

Cancer afflicts all communities. Worldwide, the burden of this disease impinges of the lives of tens of millions annually. Based on the most recent incidence and mortality data available, there were 10.1 million new cases, 6.2 million deaths and 22.4 million persons living with cancer in the year 2000 (Ferlay *et al.*, 2001) (Figure 1.1). This represents an increase of around 19% in incidence and 18% in mortality since 1990.

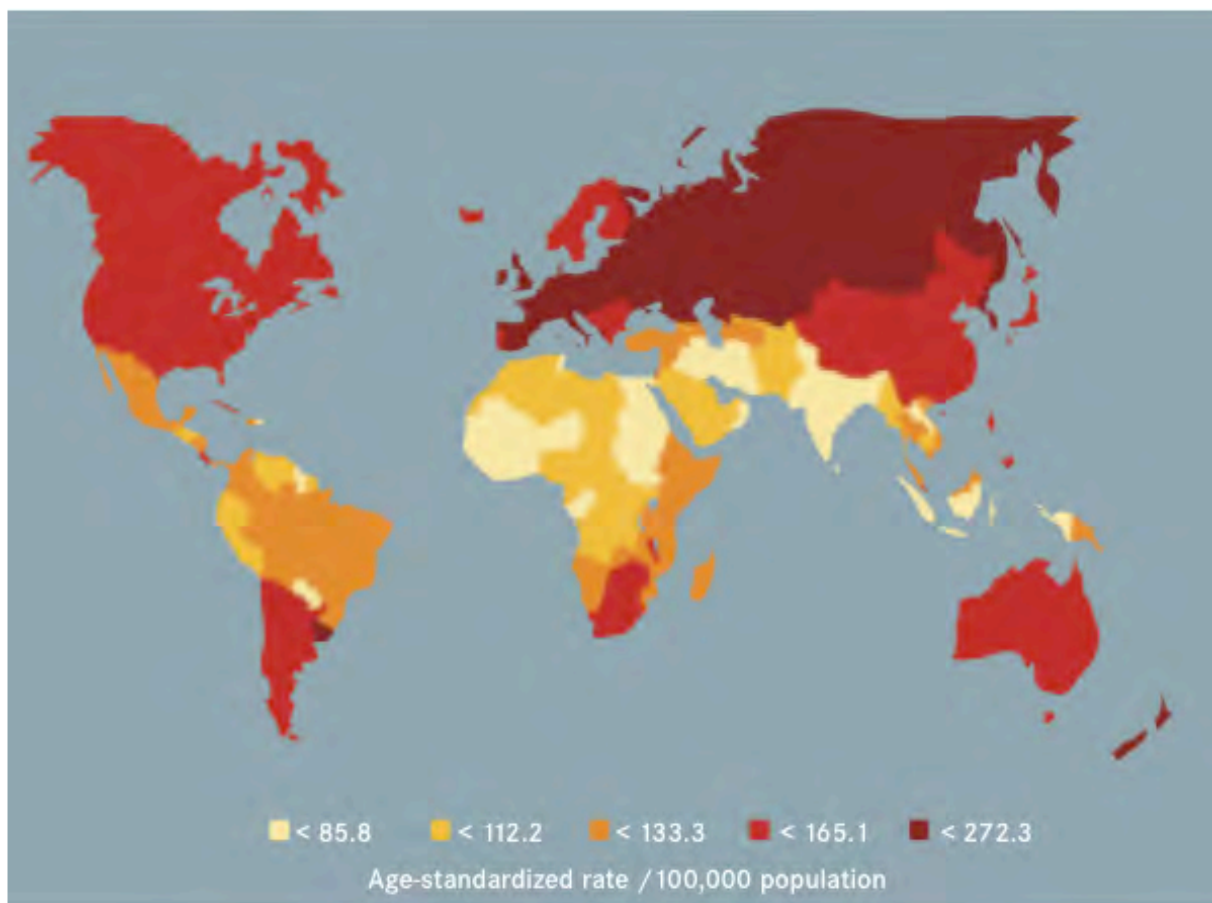

Figure 1.1 Mortality rates in men for all cancer sites combined (after: Ferlay *et al.*, 2001).

Taking in account of the growth and ageing of the world's population, based on various assumptions regarding trends in cancer risk, by 2030 it could be expected that there will be 20 to 25 million incident cases of cancer and 13 to 16 million cancer deaths annually (IARC, 2007).

In terms of incidence, the most common cancers worldwide are lung (12.3% of all cancers), breast (10.4%) and colorectal (9.4%) cancers. In terms of mortality, however, the ranking is as follows: cancers of the lung (17.8% of all cancer deaths), stomach (10.4%) and liver (8.8%). The burden of cancer is distributed unequally between the developing and the developed world, with particular cancer types exhibiting different patterns of distribution (Figure 1.2 and 1.3).

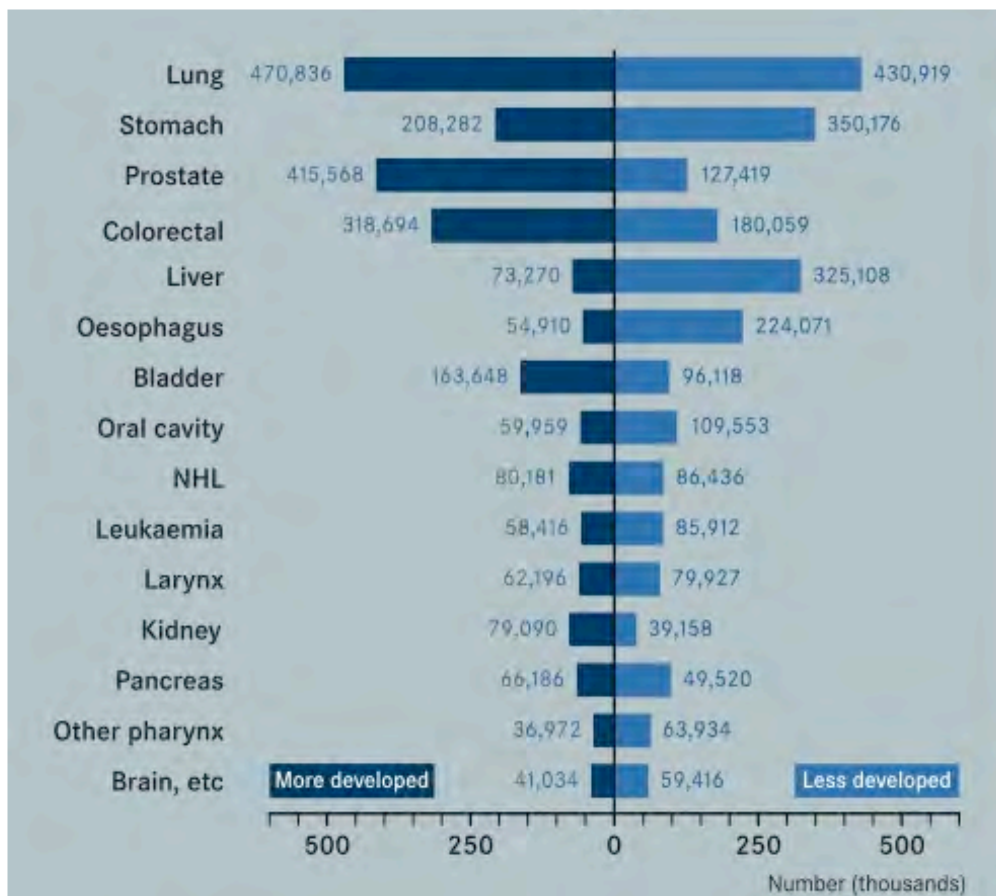

Figure 1.2 Comparison of the most common cancers for males in more and less developed countries in 2000. NHL = Non-Hodgkin lymphoma. (after: Ferlay *et al.*, 2001).

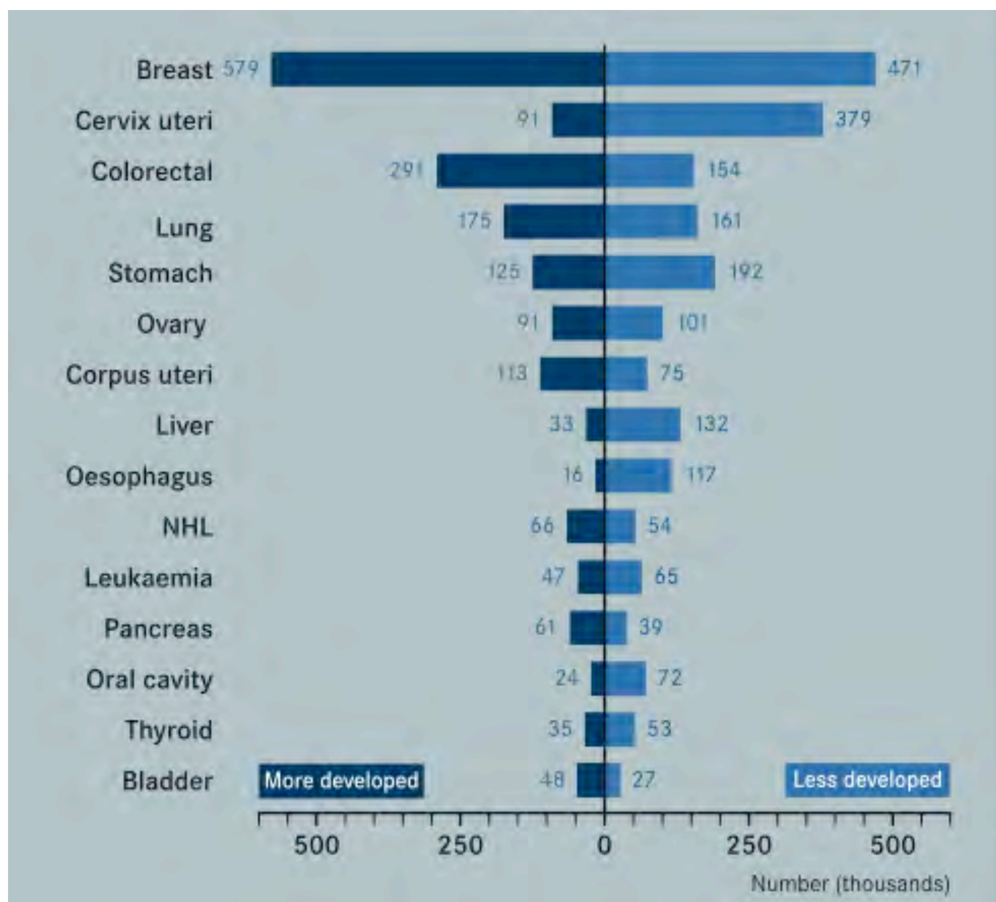

Figure 1.3 Comparison of the most common cancers for females in more and less developed countries in 2000. NHL = Non-Hodgkin lymphoma. (after: Ferlay *et al.*, 2001).

The most conspicuous feature of the distribution of cancer between the sexes is the male predominance of lung cancer. Stomach, esophageal and bladder cancer are also much more common in males. For other tumor types, including cancers of the colorectum and pancreas, there is little difference between the sexes.

1.2. Early interpretations

In 337 BC, the father of modern medicine, Hippocrates, stated, 'Let food be your medicine and medicine be your food'. Yong-He Yan, living in the Song Dynasty (960 - 1279 ad), thought that poor nutrition was a cause of the condition we would now know as cancer of the esophagus. Wiseman (1676) suggested that cancer might arise from 'an error in Diet, a great acrimony in the meats and drinks meeting with a fault in the first Concoction' (digestion) and he advised abstention from 'salt, sharp and gross meats'. Howard (1811)

proposed that constipation was an important factor in cancer, based on his 40 years of clinical practice. Lambe (1815), warned in his treatise on diet, cancer and other chronic diseases, against the danger of excess consumption of food in general, and meat in particular. Bennett (1849), author of medical textbooks, wrote that 'the circumstances which diminish obesity and a tendency to the formation of fat, would seem, a priori, to be opposed to the cancerous tendency'.

By the early twentieth century, similar views were commonplace. Williams (1908), concluded that 'probably no single factor is more potent in determining the outbreak of cancer in the predisposed, than excessive feeding' and proposed that 'many indications point to the gluttonous consumption of proteins - especially meat - which is such a characteristic feature of the age, as likely to be specifically harmful in this respect'. He also identified 'deficient exercise and probably lack of sufficient vegetable food'. During the first half of the twentieth century, two influential hypotheses on the causes of cancer were developed. The first focused on occupational causes, notably exposure of workers to carcinogenic agents (Hueper, 1942). The second general theory focused on diet. The medical statistician and epidemiologist, Frederick Hoffman, a founder of the American Cancer Society (ACS) and the US National Cancer Surveys, undertook a systematic review of the then current literature on diet and cancer (Hoffman, 1937). He concluded that 'excessive nutrition if not the chief cause is at least a contributory factor of the first importance'. He identified fatty, sugary foods, white bread and meat as possible risk factors.

In the second half of the twentieth century, theories of the dietary origins of cancer tended to be increasingly discounted, in favor of alternative theories that cancer is either the result of random genetic error, exposure to viruses or exposure to specific chemical carcinogens. Laboratory research began to concentrate on the investigation of cellular and, ultimately, molecular carcinogenesis, as well as on the effectiveness of surgery, radiotherapy and chemotherapy, as cancer treatments. The index of the fifth edition of the standard textbook Human Nutrition and Dietetics (Davidson *et al.*, 1972) included no reference to diet and cancer and its text included only cursory reference to evidence that cancers of some sites may have some relationship with diet.

However, rates of incidence and death from various cancers continued to rise in industrialized countries (compare, for instance, Park, 1899 and Parkin *et al.*, 1988) and epidemiological investigation indicated that this trend was not just a function of ageing. Further, studies of variations in cancer incidence from country to country and in successive generations of people who migrated from one part of the world to another, strongly suggested that cancers are largely environmental in origin. In the second half of the twentieth century, a new body of experimental and epidemiological work (Doll, 1967) began to indicate that diet was indeed a major environmental factor affecting the incidence of cancers of a number of sites.

1.3. Emerging consensus

Interest in nutritional causes of cancer began to revive in the 1970s, at first in the USA. This was partly because overall cancer rates remained obstinately high while costs of treatment accelerated; partly because of the new evidence on diet and cancer; and partly because 'winning the war against cancer' was perceived as a national goal equivalent in importance to the earlier achievement of putting a man on the moon (Proctor, 1994).

A review by Wynder and Gori (1977) proposed that, for both men and women, the 'preventive potential' for all cancers was 80 - 90% and that diet accounted for 40% of all male cancers and 60% of all female cancers. It was suggested that key dietary causes of cancer, in general, included overeating, fat and meat. The fact that incidence of stomach cancer varies inversely with the incidence of breast and of colon cancer was interpreted as suggesting that high-fat, low-carbohydrate diets might protect against stomach cancer.

By the mid-1970s, descriptive, ecological and analytical epidemiological studies were providing a growing body of evidence on links between diet and cancer. Doll and Peto's review (1981), which helped to reset the agenda for thinking on food, nutrition and cancer, included estimates of the extent to which cancer in general and specific cancers, can be avoided by changes in diet. Doll and Peto concluded that environmental carcinogens, other than those in tobacco and diet, are relatively unimportant causes of cancer. This conclusion was based partly on ecological data, which showed no coherent pattern (across various countries and regions) between cancer trends and the degree of external pollution.

The report anticipated that results of further research ‘may well be’ as follows: ‘Diet will be shown to be a factor in determining the occurrence of a high proportion of all cancers of the stomach and large bowel as well as of the body of the uterus (endometrium), gall bladder and (in tropical countries) of the liver’. ‘Diet may also prove to have a material effect on the incidence of cancers of the breast and pancreas and, perhaps through the anti-carcinogenic effects of various micronutrients, on the incidence of cancers in many other tissues’. ‘If this is so, it may be possible to reduce cancer death rates by practicable dietary means by as much as 35% (for specific sites their estimates were: stomach and large bowel, 90%; endometrium, gallbladder, pancreas and breast, 50%; lung, larynx, bladder, cervix, mouth, pharynx and esophagus, 20%; other types of cancer, 10%)’.

Aspects of diet mentioned in Doll and Peto’s report as possibly protective against cancer included antioxidant vitamins, vegetables, such as carrots and leafy greens that are rich in these compounds and bioactive microconstituents such as indoles and protease inhibitors. Fiber, or rather foods that make feces bulky, were also cited as important. Aspects of diet mentioned as possible causes of cancer were overconsumption (cancers of the uterus and gallbladder in women), fat (cancers of the breast, colon and rectum) and meat (cancers of the colon and rectum). Those considered to be relatively unimportant causes of cancer were food additives (including colors and sweeteners), contaminants (apart from aflatoxin in relation to liver cancer) and methods of food preparation and storage that create carcinogens.

Over the past 20 years, many epidemiological studies, particularly case-control studies and, more recently, large cohort studies, have investigated the role of habitual diet in relation to the risk of developing different cancers. The most consistent finding on diet as a determinant of cancer risk is the association between consumption of vegetables and fruit and the reduced risk of cancers of the pharynx, larynx, lung, esophagus, stomach and cervix uteri, while only vegetables, but not fruit, seem to protect against cancers of the colon and rectum. During the last 30 years over 250 epidemiological studies have been conducted around the world and about 80% of these found a significant protective effect of overall consumption of vegetable and/or fruit (WCRF, 1997).

The recent body of evidence has also reduced the importance of specific nutrients, for example, fat, in favor of foods, for example, meat. Epidemiological studies on meat

consumption and cancer risk support the existence of a specific association with colorectal, pancreatic, breast and prostate cancer risk (Norrish *et al.*, 1999; Anderson *et al.*, 2002; Dai *et al.*, 2002; Norat *et al.*, 2005; Larsson and Wolk, 2006). This association is however more consistent for red meat (beef, lamb and pork) and processed meat (ham, salami, bacon and other charcuterie) (Norat *et al.*, 2005).

1.4. Meat and human cancer

Several hypotheses have been developed to explain the association between colorectal cancer risk and meat. The fat content of meat could influence colon cancer risk by increasing excretion of bile acids, whose products may act as tumor promotors (Reddy, 1981). Other products of fat digestion, such as diacylglycerides, could selectively induce mitogenesis of adenomas and some carcinoma cells. The meat fat-hypothesis is consistent with the finding that lean beef did not promote colon carcinogenesis in rats and that high consumption of beef could increase the concentration of secondary fecal bile acids. Nevertheless, epidemiological studies have failed to show a consistent relationship between fat intake and colorectal cancer (Norat *et al.*, 2002).

Another mechanism that could explain this association is increased colonic protein metabolism due to increased protein intake from high meat diets (Blaut and Clavel, 2007). Products of colonic protein degradation and metabolism include ammonia, phenols, indoles and amines which have been shown to exert toxic effects *in vitro* and in animal models. There is, however, very limited evidence that protein *per se* increases colorectal cancer risk and some epidemiological studies have even reported a protective association between dietary protein and colon cancer.

Red meat has a higher iron content than white meat. Dietary iron enhances lipid peroxidation in the mouse colon and augments dimethylhydrazine-induced colorectal tumors in mice and rats but the results of epidemiological studies are still insufficient (Norat *et al.*, 2002).

Red meat intake also enhances the production of endogenous promoters and possible carcinogens such as N-nitroso compounds, which have been shown to induce the formation of DNA adducts in human colonocytes (Bingham *et al.*, 1996). The same effect has not been

observed with white meat. N-nitroso compounds are also formed endogenously because the amines and amides produced primarily by bacterial decarboxylation of amino acids can be N-nitrosated in the presence of a nitrosating agent. Nitrosamines have been detected in foods with added nitrates or nitrites, including salt-preserved fish and meat and in food processed by smoking or direct-fire drying.

A mechanism that has attracted particular attention is the formation of heterocyclic amines (HCA) and polycyclic aromatic hydrocarbons (PAH) carcinogens in meat when it is cooked at high temperature for a long time or over an open flame. HCAs have been shown to be potent mutagens in bacteria (Sugimura, 1977) and cultured cells (Miura *et al.*, 1993; Fan *et al.*, 1995; Zhu *et al.*, 2000), and carcinogens in mice and rats (Ito *et al.*, 1991; Imaida *et al.*, 1996; Ito *et al.*, 1997; Shirai *et al.*, 1997; Snyderwine *et al.*, 2002). Mechanistic data show that, even at low doses, heterocyclic amines form DNA adducts in rodents, primates and humans. If HCAs were also to be established as important human carcinogens, meat could potentially be made "safer" to eat by being cooked in a way that does not lead to HCA formation (Forman, 1999).

Early epidemiological studies conducted in the 1980s suggested an association between meat-cooking techniques and cancer risk. Today the majority of epidemiological studies performed generally supports the hypothesis that high-temperature cooking techniques and doneness level increase the risk for human cancers at various sites, particularly colorectal cancer. Table 1.1 summarizes human studies that have investigated the relationship between meat doneness and cancer at different sites. More than 80% of these studies show a positive correlation between cancer incidence and well-done meat consumption.

Table 1.1. Human studies investigating well-done meat and cancer risk.

Study	Result[a]	Cancer Site	N[c] (Age if given)
Han *et al*., 2004	OR = 2.38	Breast	635
Dai *et al*., 2002	OR = 1.92	Breast	3015 (25–64)
Zheng *et al*., 2002	OR = 3.4	Breast	683 (postmenopause)
Balbi *et al*., 2001	OR = 2.66 (bbq)	Bladder	720 (40–89)
	OR = NA (fried)		
Zheng *et al*., 2001	OR = 2.0	Breast	488 (55–69)
Delfino *et al*., 2000	NA	Breast	394 (>39)
Sinha *et al*., 2000	OR = 1.9	Breast	930 (56–67)
Zheng *et al*., 1998	OR = 4.6	Breast	930 (55–69)
Butler *et al*., 2003	OR = 2.0	Colon	1658 (40–80)
Kampman *et al*., 1999	OR = 1.4 (men only)	Colon	3402 (30–79)
Sinha *et al*., 1999	OR = 1.85/10 g meat	Colon	374
Augustsson *et al*., 1999	NA	Colon, rectum, bladder, kidney	1565 (56–80)
Schiffman and Felton, 1990	OR = 3.5	Colon	146
Barrett *et al*., 2003	OR = 1.97	Colon/rectum	2164 (45–80)
Tiemersma *et al*., 2004	NA	Colon/rectum	864
Le Marchand *et al*., 2002	OR = 8.8	Colon/rectum	1454
Nowell *et al*., 2002	OR = 4.36	Colon/rectum	460 (20–88)
Sinha *et al*., 2001	OR = 1.29	Colon/rectum	374
Gunter *et al*., 2005	NA	Colon/rectum	565 (50–70)
Navarro *et al*., 2004	OR = 4.57	Colon/rectum	893 (23–80)
Terry *et al*., 2003	NA	Esophagus	1004 (<80)
	NA	Gastric cardia	1077 (<80)
	OR = 2.4	Esophagus	982 (<80)
Bosetti *et al*., 2002	OR = 1.89	Larynx	1824 (31–79)
Sinha *et al*., 1998b	OR = 1.8	Lung	1216 (52–79)
Zhang *et al*., 1999[b]	RR = 2.2	NHL[d]	88410 (48–74)
Anderson *et al*., 2002	OR = 2.19	Pancreas	867 (20–65+)
Norrish *et al*., 1999	Positive trend	Prostate	787
Nowell *et al*., 2004	OR = 8.27	Prostate	923
Murtaugh *et al*., 2004	OR = 1.33	Rectum	2157
Ward *et al*., 1997	OR = 2.4	Stomach	678 (~67–82)
	OR = 2.0	Esophagus	645 (~67–82)

[a] OR = odds ratio; RR = relative risk; NA = no association.

[b] Prospective study; all other studies were case-control.

[c] N = number of subjects.

[d] NHL = Non-Hodgkin lymphoma.

2. HCAs: formation, occurrence and intake

2.1. HCAs: classification, structural features and chemical properties

In 1977 it was found that particles of smoke, produced by cooking proteinaceous foodstuffs contained significant quantities of mutagens (Sugimura *et al.*, 1977). Subsequently, Sugimura and coworkers demonstrated the presence of high mutagenic activity in the charred surface of beef and fish, grilled over a naked flame or charcoal (Nagao *et al.*, 1977). Since then, more than 20 highly mutagenic heterocyclic aromatic amines have been isolated and the structures of these fully elucidated (Table 1.2).

Table 1.2. Chemical names and abbreviations of the different HCAs commonly found in cooked foods.

Quinolines	
IQ	2-Amino-3-methylimidazo[4,5-*f*]quinoline
MeIQ	2-Amino-3,4-dimethylimidazo[4,5-*f*]quinoline
Quinoxalines	
IQx	2-Amino-3-methylimidazo[4,5-*f*]quinoxaline
8-MeIQx	2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline
4,8-DiMeIQx	2-Amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline
7,8-DiMeIQx	2-Amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline
4,7,8-TriMeIQx	2-Amino-3,4,7,8-tetramethylimidazo[4,5-*f*]quinoxaline
4-CH_2OH-8-MeIQx	2-Amino-4-hydroxymethyl-3,8-dimethylimidazo[4,5-*f*]quinoxaline
7,9-DiMeI*g*Qx	2-Amino-1,7,9-trimethylimidazo[4,5-*g*]quinoxaline
Pyridines	
PhIP	2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
4'-OH-PhIP	2-Amino-1-methyl-6-(4-hydroxyphenyl)imidazo[4,5-*b*]pyridine
DMIP	2-Amino-1,6-dimethylimidazo[4,5-*b*]pyridine
TMIP	2-Amino-1,5,6-trimethylimidazo[4,5-*b*]pyridine
Furopyridines	
IFP	2-Amino-1,6-dimethylfuro[3,2-*e*]imidazo[4,5-*b*]pyridine
Pyridoimidazoles and indoles	
Trp-P-1	3-Amino-1,4-dimethyl-*5H*-pyrido[4,3-*b*]indole
Trp-P-2	3-Amino-1-methyl-*5H*-pyrido[4,3-*b*]indole
Glu-P-1	2-Amino-6-methyldipyrido[1,2-*a*:3',2'-*d*]imidazole
Glu-P-2	2-Aminodipyrido[1,2-*a*:3',2'-*d*]imidazole
AαC	2-Amino-9H-pyrido[2,3-*b*]indole
MeAαC	2-Amino-3-methyl-9H-pyrido[2,3-*b*]indole
Norharman	β-Carboline
Harman	9-Methyl-β-carboline

According to Miller (1985), a heating temperature of about 300 °C is a critical boundary for the formation of different classes of mutagens from proteinaceous food, such as meat and fish. Those formed above 300 °C are protein pyrolysates and characterized as 2-amino-pyridine-mutagens (or amino-carbolines) (Sugimura, 1986; Wakabayashi *et al.*, 1997), while those formed at moderate temperatures (below 300 °C) are 2-amino-imidazole-type mutagens (or aminoimidazo-azaarenes (AIAs)) (Furihata and Matsushima, 1986; Jägerstad *et al.*, 1998). Kataoka (1997) further divided pyrolytic mutagens into five groups, pyridoindoles, pyridoimidazoles, phenylpyridines, tetraazafluoranthrene and benzimidazole; and the AIAs into three groups, quinolines, quinoxalines and imidazopyridines. Based on polarity (Figure 1.4), HCAs can also be divided into polar, which are mainly of the IQ- and IQx-type as well as the imidazopyridine type, and non-polar which have a common pyridoindole or dipyridoimidazole moiety (Murkovic, 2004). All HCAs have at least one aromatic and one heterocyclic structure. Most of them have an exocyclic amino group, except for the β-carbolines, harman and norharman (Jägerstad *et al.*, 1998). The amino groups or nitrogen atoms may have different pKa values. This together with different positions and number of the ionizable moieties, will, therefore, affect the behavior of HCAs during chromatographic separation.

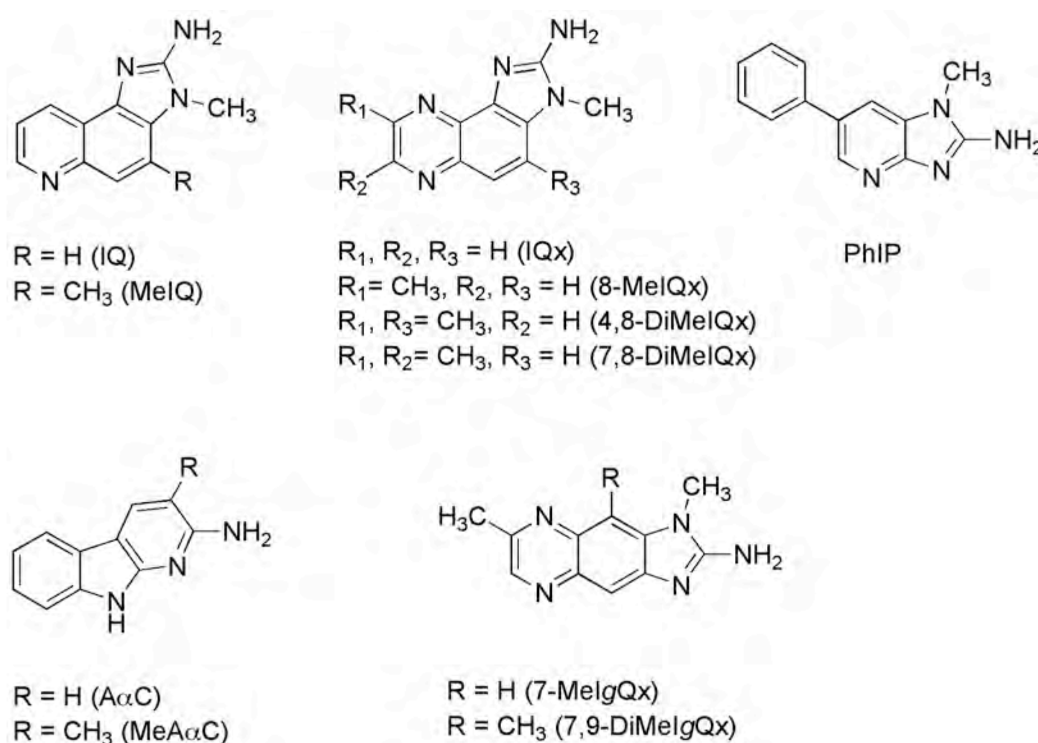

Figure 1.4 Chemical structures of representative polar and non-polar HCAs.

2.2. *Formation of heterocyclic aromatic amines*

2.2.1 Identification of the precursors

The first published model systems where mutagenic compounds were identified, were pyrolysis reactions of amino acids and proteins. Other food constituents such as nucleic acids, starch or oil did not form mutagenic substances during pyrolysis (Nagao *et al.*, 1977). Heating of single amino acids also resulted in the formation of mutagenic substances that were identified as heterocyclic aromatic amines. In general, these products of pyrolysis were assigned as the non-polar HCAs. In contrast, the polar HCAs were formed at normal cooking temperatures. These mutagens were identified in fried meat and fish (Sugimura *et al.*, 1977). They were also found in meat products, e.g. in meat extract that is extracted at comparably low temperatures but using longer times for processing (Murkovic, 2004). The precursors responsible for these polar HCAs were identified when the chemical structures of the first compounds from cooked fish (Kasai *et al.*, 1980; Kasai *et al.*, 1981a) and beef (Kasai *et al.*, 1981b; Felton *et al.*, 1986b) were determined. The aminoimidazo structure of the heterocyclic compounds suggested that creatine or creatinine was involved in the reactions. Early work in adding creatine to meat before cooking showed that it increased mutagenic activity (Jägerstad *et al.*, 1983b). Övervik *et al.* (1989) showed that free amino acids were involved in the formation of mutagenic activity. Jägerstad *et al.* (1983a) developed a system for heating the precursors in diethylene glycol and this work was followed by many studies investigating heterocyclic amine precursors (Skog and Jägerstad, 1993) and kinetics (Arvidsson *et al.*, 1997) in a sealed-tube aqueous model. Reaction intermediates were identified that lead to the formation of PhIP (Zöchling and Murkovic, 2002). Simple dry heating of heterocyclic amine precursors also forms similar relative amounts and types of heterocyclic amines as are seen in cooked meats. Table 1.3 shows amino acid, creatine and glucose content of beef, chicken breast and codfish. When these components are combined and heated for 30 minutes at 225 °C, a family of HCAs is formed. These vary with the mixture composition. The model systems in Table 1.3 show that arginine, glutamic acid, leucine and phenylalanine are greatly reduced in codfish compared to beef or chicken. Phenylalanine, a known precursor for PhIP, is highest in the chicken model system, and formed from tyrosine and isoleucine, which are also highest in chicken (Johansson *et al.*, 1995).

When using meat juice for model systems, the complexity increases since several polar and non-polar HCAs are formed (Arvidsson *et al.*, 1999; Borgen *et al.*, 2001). Since the composition of the precursors (amino acids, glucose, creatine) simulates the chemical

environment in the meat much better than a solution of single amino acids in diethylene glycol, the results are more relevant but much more complicated to interpret. Depending on the type of meat from which the juice is derived, the amino acid composition and the glucose and creatine content vary to a great extent.

Table 1.3. Concentration (mg/g meat wet weight) of free amino acids, creatine and glucose in three kinds of meats (after: Pais *et al.*, 1999).

	Beef	**Chicken Breast**	**Codfish**
L-Alanine	0.14	0.21	0.12
L-Arginine	1.07	1.19	0.03
L-Aspartic acid	0.02	0.13	0.01
L-Glutamic acid	0.09	0.23	0.02
L-Glycine	0.06	0.08	0.05
L-Histidine	0.14	0.18	0.03
L-Isoleucine	0.05	0.08	0.02
L-Leucine	0.07	0.13	0.02
L-Lysine	0.07	0.14	0.18
L-Methionine	0.06	0.08	0.04
L-Phenylalanine	0.05	0.08	0.01
L-Proline	0.10	0.10	0.14
L-Serine	0.05	0.12	0.02
L-Threonine	0.28	1.63	0.69
L-Tyrosine	0.06	0.10	0.03
L-Valine	0.06	0.10	0.04
Creatine	6.33	3.54	7.06
Glucose	7.03	0.47	0.21

2.2.2 Chemistry of HCA formation

At the beginning of the last century, Maillard proposed the browning reaction to account for the brown pigments and polymers produced from the reaction of the amino group of an amino acid and the carbonyl group of a sugar (Maillard, 1912). The chemistry underlying the Maillard reaction is very complex. It encompasses not one reaction pathway but a whole network of various reactions. The original comprehensive reaction scheme of Hodge (1953) has been improved continuously since that time. At some stages of the browning reaction, e.g. pyrazines (Hwang *et al.*, 1994), quinoxalines (Morita and Takagi, 1990) and pyrido[3,4-*d*]imidazoles (Gi and Baltes, 1994) are formed that are involved in the formation of HCAs. The involvement of these *N*-heterocycles derived from the Maillard reaction in the formation of HCAs is depicted in Figure 1.5. It can be seen that methylated pyridines and pyrazines are precursors in this reaction pathway. Aldehydes that are also

products resulting from the high temperature reaction are necessary as well as creatinine that forms the imidazo moiety of all polar HCAs (Jägerstad *et al.*, 1983).

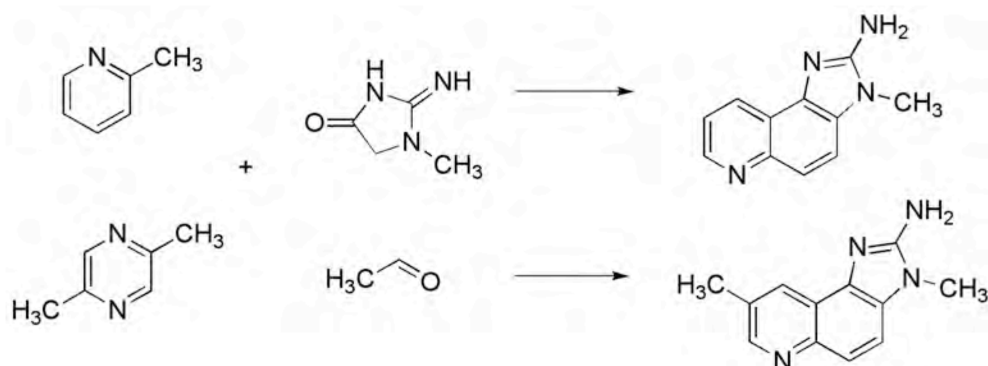

Figure 1.5 Formation of imidazoquinolines and imidazoquinoxalines from products of the Maillard reaction (2-methylpyridine, 2,5-dimethylpyrazine) with acetaldehyde and creatinine.

2.2.3 Formation of PhIP

It has been convincingly demonstrated that phenylalanine and creatinine are precursors of PhIP by dry heating of ^{13}C-labelled phenylalanine and creatinine (Felton and Knize, 1991). PhIP may also be produced from creatine heated together with leucine, isoleucine and tyrosine. Accordingly, glucose seems not to be a necessary precursor using dry heating conditions (Skog *et al.*, 1998).

However, glucose was found to have a considerable influence, either enhancing or inhibiting, depending on its concentration, the yield of PhIP produced from phenylalanine and creatine in a liquid model system (Skog and Jägerstad, 1990) and during dry heating (Felton and Knize, 1991). Manabe *et al.* (1992) reported that a tetrose (erythrose) is the most active in PhIP formation, when phenylalanine and creatinine dissolved in water are heated at temperatures of 37 and 60 °C. This author found PhIP in heated mixtures of creatinine, phenylalanine and aldehydes (Manabe *et al.*, 1992), as well as in mixtures of phenylalanine, creatinine and nucleic acids (Manabe *et al.*, 1993a). The 4-hydroxy derivative of PhIP was found in an analogous reaction using tyrosine instead (Wakabayashi *et al.*, 1995).

The formation of PhIP in a simple model system with just phenylalanine and creatinine as precursors starts with the formation of the Strecker aldehyde phenylacetaldehyde (Figure 1.6). The second step is an aldol condensation of the aldehyde with creatinine and subsequently a dehydration. Both products, the addition [A] as well as the condensation [B] product, were identified in the model system and in heated meat as well (Zöchling and Murkovic, 2002). The origin of the nitrogen forming the pyridine moiety of PhIP is at least twofold. First it can be the amino group of creatinine that reacts with the oxo group of the intermediate and second the amino group of phenylalanine or even free ammonia. The origin of the carbon atoms 5, 6 and 7 in PhIP was identified by the use of ^{13}C-labelled phenylalanine (labeled at C-2 and C-3, respectively) and analyzing the formed PhIP by NMR (Murkovic *et al.*, 1999). Combining these results a mechanism for the formation of PhIP was formulated (Figure 1.6).

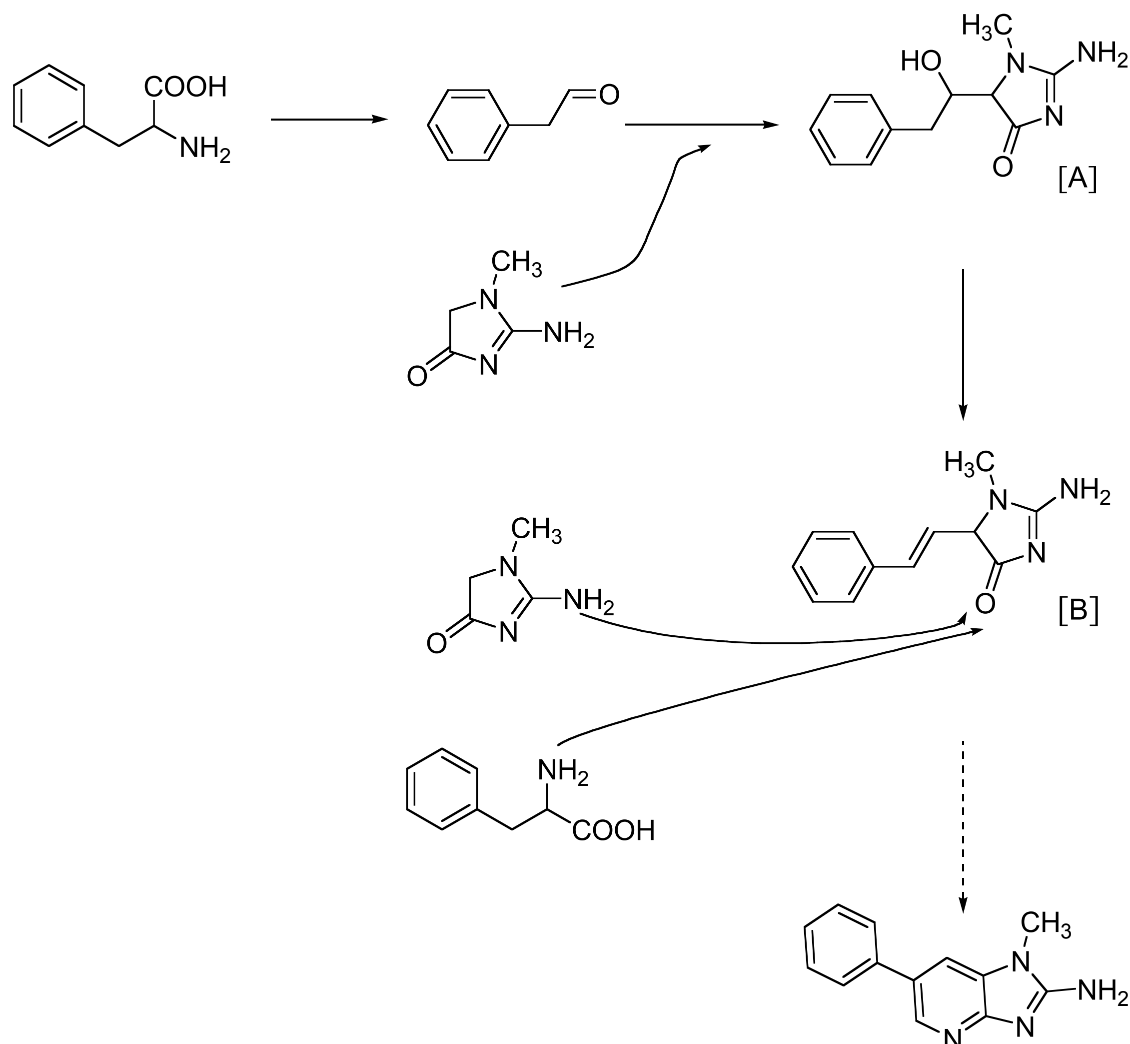

Figure 1.6 Formation of PhIP with identified intermediate reaction products.

2.2.4 Factors affecting the yields of HCAs

2.2.4.1 Cooking temperature, time, water content and cooking methods

Many studies suggest that both time and temperature have a strong impact on the formation of HCAs (Bjeldanes *et al.*, 1983; Arvidsson *et al.*, 1997) and that the amounts of HCAs generally increase with increasing temperature and time (Gröss and Grüter, 1992; Skog *et al.*, 1997). Effect of temperature was especially important according to a function developed by Bjeldanes *et al.* (1983), where the temperature term was raised to a much higher exponent than the time factor. The temperature dependence of the formation of PhIP has been investigated in several studies. Using liquid model systems, the yield of PhIP from phenylalanine, creatinine and glucose was shown to increase when the temperature was increased from 180 to 225 °C (Skog and Jägerstad, 1991). Similar results were obtained by Knize *et al.* (1994).

On the other hand, heating at much lower temperature (~100 °C), may also give rise to HCA formation if the duration of heat treatment is prolonged (Jägerstad *et al.*, 1998). For example, Manabe *et al.* (1992 and 1993a) reported the presence of PhIP in a meat juice system heated at 37 and 60 °C for 4 weeks. Although there have no conclusions been drawn on the definite effect of temperature, it would be prudent to maintain low cooking temperatures, avoid sudden increments in temperature and avoid unnecessary prolonged heat treatment in terms of minimizing mutagen formation (Skog *et al.*, 1998). One such strategy could be mixing of ground meat with water binding compounds, such as soy protein, starch or other polysaccharides, which will help lower surface temperature and hinder transport of water soluble precursors (Skog *et al.*, 1998; Shin *et al.*, 2003; Kikugawa, 2004).

Variations between food samples indicate that heat processing has a marked effect on the levels of HCAs in the products. Modifying the cooking processes could probably reduce the amounts of HCAs. Frying beefburgers using specially designed frying equipment with a thermostatically controlled hotplate produced 10-fold fewer HCAs than frying using a standard frying device (Johansson and Jägerstad, 1994; Johansson *et al.*, 1995). Oven-roasting is another cooking method that produces fewer HCAs than pan-frying (Skog *et al.*, 1997), due to the less efficient heat transfer in air than when the product is in direct contact with a frying pan. Also, oven-roasted meat usually has a lower surface area relative to its mass than, for example, beefburgers, and since HCAs are formed predominantly in the crust, the amounts of

HCAs per portion are lower. When chicken and beefburgers were cooked in a convection oven, less mutagenic activity was formed in the presence of steam, which affected the heat transport and decreased the surface temperature of the products (Skog *et al.*, 2003). Some minutes of microwave pretreatment of meat before frying has been suggested as another way to decrease HCA formation due to loss of HCA precursors with the meat juice (Felton *et al.*, 1994a).

2.2.4.2 Content and types of fat and free radical reactions

Fat has been reported to physically affect the amount of mutagens formed during cooking due to efficient heat transfer in fat (Barnes and Weisburger, 1983; Holtz *et al.*, 1985). Fat has also been reported to affect the formation of HCAs by dilution of the precursors in meat (Knize *et al.*, 1985). However, fat may be involved chemically in the formation of HCAs, by generating free radicals via lipid oxidation or by participating in the Maillard reaction. These reactions may result in an enhanced yield of certain Maillard reaction products, for instance pyrazines and pyridines, which are assumed to be involved in the formation of HCAs (Murkovic, 2004). Despite the possible involvement of lipid/fatty acids-derived radicals, their exact effects on mutagen formation in meats and fish have been controversial: while some studies have reported enhancing effects (Nilsson *et al.*, 1986; Johansson *et al.*, 1993; Felton *et al.*, 2000), others reported no effects (Johansson *et al.*, 1993) or even inhibitory effects (Barnes and Weisburger, 1983; Knize *et al.*, 1985). It was observed that fats have an enhancing effect on the yield of HCAs in model systems, probably by free radicals formed during thermally induced fat oxidation (Felton *et al.*, 2000). Addition of Fe^{2+} or Fe^{3+} to a model system containing creatinine, glycine and glucose almost doubled the amount of MeIQx formed, probably due to iron-catalyzed lipid peroxidation, and thus, formation of free radicals (Felton *et al.*, 2000). Surprisingly, Johansson and Jägerstad (1993) found that the degree of oxidation of fat did not affect the yield of MeIQx. Further studies are demanded before specific recommendations can be made on the types and concentrations of fat/fatty acids to be added during cooking.

2.2.4.3 Presence/addition of inhibitors

Despite the possibility that HCA formation during cooking and thus mutagenic activity in food products may be reduced via manipulation of some food components, it is strongly conceivable that more effective inhibition can be accomplished through addition of potent

inhibitors at certain stages of the heating process. Desirable inhibitors should fulfill the following criteria: (a) capable of causing significant reduction in total HCA content at low doses; (b) do not lead to formation of new HCAs (based on analysis of HCA profiles); (c) do not lead to formation of new or more potent mutagens (Cheng *et al.*, 2006).

Phytogenic inhibitory agents

A wide range of natural agents have been tested for their effects on formation of HCAs in model systems and in real food. However, few data on those isolated from dietary plants are available and the mechanism of inhibition has been abridged to antioxidation (Kikugawa, 1999). These phytochemicals mainly include antioxidative (AO) vitamins, phenolic compounds and carotenoids.

Vitamin C and α-tocopherol did not demonstrate consistent effects when added to real food systems (Kikugawa *et al.*, 2000; *Tai et al.*, 2001). Results for different phenolic compounds have also been divergent as well as their roles in the formation of different types of HCAs. Lee *et al.* (1992) found that flavones inhibited IQ-type mutagen formation in simple model systems. In a later study employing different kinds of phenolic AOs, contradictory observations were obtained for ellagic acid, syringic acid and nordihydroguaiaretic acid (Oguri *et al.*, 1998). Active principles from spice plants have also been targets in several studies (Persson *et al.*, 2003). In particular, curcumin exhibited dose-dependent inhibition of mutagenic Maillard products in model systems. Tea phenolics, especially green tea catechins, (-)-epigallocatechin gallate and caffeic acid have also been reported to inhibit IQx-type HCA formation (Weisburger, 1994; Oguri *et al.*, 1998). Apart from adding pure phytochemicals, addition of plant extracts/tissues is another approach to derive benefits from purported inhibitors. Vitaglione *et al.* (2002) reported that carotenoid extracts from tomato reduced IQx-type HCA formation in both chemical and meat juice systems. Addition of soy protein prior to high temperature heating can be bifunctional. Apart from serving as a layer of physical insulator, its phenolic components may also interfere with HCA formation (Vitaglione and Fogliano, 2004). A number of spices such as thyme, marjoram and rosemary have shown to exert diverse effects on HCA formation (Vitaglione and Fogliano, 2004). As an example, Murkovic *et al.* (1998) reported that application of dried rosemary, thyme, sage and garlic to the surface of meat prior to heating resulted in a significant reduction in HCA content, but to various extents with respect to different HCAs. On the other hand, extracts from many of

these spices were shown to exert an enhancing effect on formation of PhIP in model systems (Zöchling *et al.*, 2002). The large discrepancy in the effects of AO phytochemicals on HCA formation further emphasizes their ability to switch between an AO and a pro-oxidant role, depending on their specific chemical environments.

Synthetic antioxidative agents (AOs)

Synthetic antioxidative agents that have been extensively tested for inhibition of HCA formation include butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and *tert*-butylhydroquinone (TBHQ). In real food systems, BHA, PG and TBHQ were found to reduce formation of HCAs at a concentration of 100 ppm (Cheng *et al.*, 2006). In another study using a real food system at boiling temperature, it was found that BHT had a net minor inhibitory effect (Lan *et al.*, 2004), whereas in simple model systems constituting of pure HCA precursors, these synthetic AOs and BHT exerted opposite effects, particularly TBHQ, which increased MeIQx formation by more than 200% at 100 ppm (Johansson and Jägerstad, 1996).

Organosulfur compounds

Organosulfur compounds are another group of compounds that are receiving increasing attention in view of the finding that they may be effective in inhibiting non-enzymatic browning reaction (Cheng *et al.*, 2006). The most well studied sulfur-containing compound in relation to inhibition of HCA formation is sodium bisulfite ($NaHSO_3$). Addition of $NaHSO_3$ was demonstrated to significantly inhibit the formation of HCAs in canned foods (Krone *et al.*, 1986). Inhibitory effects of some organosulfur compounds such as diallyl sulfide, dipropyl disulfide and diallyl disulfide on HCA formation have also been evidential based on model systems (Shin *et al.*, 2002).

2.3. Occurrence of heterocyclic aromatic amines

2.3.1 Quantitative and qualitative analysis of HCAs

For preliminary studies and for facilitating measurements, levels of HCAs formed in model systems can be boosted. However, the amount of HCAs formed may still be in the nanogram per gram order. Therefore, efficient and robust analytical techniques are essential, especially when analyzing complex real food matrices, which contain lots of interfering

substances, which may comprise detection limits and complicate spectral interpretation. Prior to quantitative and/or qualitative analysis, liquid-liquid extraction (Murray *et al.*, 1988), or solid-phase extraction (SPE) (Gross, 1990) has been regarded as a critical step, both for cleaning and concentration of target chemical species. Blue cotton or blue rayon extraction methods have also been employed (Bang *et al.*, 2002).

Several methods have been used for the identification and quantification of HCAs (Pais and Knize, 2000). Table 1.4 summarizes the most commonly used methods and detectors for the determination of HCAs in cooked foods. LC has been the most popular chromatographic technique for separation of HCAs following extraction/purification and reconstitution in compatible solvents. Moreover SPE-LC has been compared and validated through interlaboratory studies (Santos *et al.*, 2004). Subsequent identification of known compounds can be achieved by coupling LC to UV photodiode array (DAD)-detector (Gröss and Grüter, 1992; Warzecha *et al.*, 2002), in addition to confirmation by their retention time. Quantification of fluorescent HCAs (non-polar and PhIP) can be achieved by simultaneous programmable fluorescence detection, which has been found to be 100-400 times more sensitive than UV detection (Schwarzenbach and Gubler, 1992). These detectors are satisfactory for samples taken from simple chemical model systems. With applications of electrochemical detectors (ED) (Billedeau *et al.*, 1991) and mass spectrometers (MS) (Turesky *et al.*, 1988), the challenge of ultracomplicated spectra arising from complex sample matrices has been largely overcome by increased sensitivity and selectivity.

HPLC in combination with mass spectrometry (LC–MS) was used, by Gross *et al.* (1993) to analyze highly complex extracts of bacon and later to analyze HCAs in beef extract with atmospheric pressure ionization (Pais *et al.*, 1997a) and with electrospray ionization (Pais *et al.*, 1997b). MS seems to be a valuable tool in obtaining reliable results in the analysis of complex matrices. There are also reports on the use of LC–MS/MS for the analysis of HCAs (Guy *et al.*, 2000; Busquets *et al.*, 2007; Ni *et al.*, 2008). GC has also been used for separation of HCAs. Despite the requirement of a derivatization step, its simplicity, separation efficiency, sensitivity and specificity when coupled to MS have granted it another valuable analytical tool for HCAs, especially in laboratories where more sophisticated techniques like LC-MS/MS are not accessible (Barcélo-Barrachina *et al.*, 2005).

Table 1.4. Methods for the identification and quantification of heterocyclic amines (HCAs) in cooked foods (after: Pais and Knize, 2000).

Method	Detector	Detection limit (ng/g)	Advantages	Disadvantages
HPLC	UV-DAD	0.02-50	Peak identity, homogeneity	
	Fluorescence	0.03-2	High sensitivity	No peak confirmation, only the less polar HCAs
	ED	0.05-2	Good selectivity and sensitivity, columns with smaller diameter give good separation at low flow rates	No peak confirmation, isocratic conditions
	MS	0.01-2	High sensitivity and specificity	
CE	UV, ED, MS	35-50	High separation efficiency, low operation cost	Sample preparation with high enrichment
GC	MS	0.01	Capillary GC gives high separation efficiency	Derivatization is usually needed
ELISA		1	Simple	Monoclonal antibodies only available for a limited number of HCAs

2.3.2 Levels of HCAs in cooked foods

The first quantitative data on HCAs in various meat and fish products, based on HPLC or GC-MS analysis, were published in the late 1980s. Earlier literature data on HCA levels in foods consists mainly of amounts estimated from the mutagenic activity according to the Ames/*Salmonella* assay. The complex food matrix, the low amounts of HCAs present and the need for several isolation steps makes accurate quantification difficult, but in the last decade several new methods for extraction, purification and detection have been developed (Kataoka, 1997; Pais and Knize, 2000; Cardenes *et al.*, 2004; Barcélo-Barrachina *et al.*, 2005; Martin-Calero, 2007). Studies of the amounts of heterocyclic amines produced in foods as a result of regional cooking practices have been reported for Great Britain (Murray *et al.*, 1993), Sweden (Johansson and Jägerstad, 1994; Skog *et al.*, 1997; Borgen and Skog, 2004), Spain (Busquets *et al.*, 2004; Toribio *et al.*, 2007), Japan (Wakabayashi *et al.*, 1993), Singapore (Wu *et al.*, 1996) and the United States (Knize *et al.*, 1995; Knize *et al.*, 1998). Some typical amounts of HCAs formed in cooked foods are displayed in Table 1.5.

Table 1.5. HCA levels in processed foods (after: Skog *et al.*, 1998; Felton *et al.*, 2000).

Food	**MeIQx**	**PhIP**
	ng/g meat wet weight	
Beef burger, fried	0-7	0-32
Meat balls, fried	0-0.08	0-0.06
Chicken, fried	0-3	0-480
Salmon, fried	0-5	0-23
Beef burger, pan residue	0-6	0-13
Meat extract	0-80	0-4
Beef flavor	0-20	0-4
Beef stock cube	0-0.6	0-0.3

In most cases, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine is the moss mass abundant HCA, detected in amounts up to 480 ng/g (Sinha *et al.*, 1995). The amounts of the other HCAs are generally lower, ranging from undetectable to tens of ng/g for IQ, MeIQ, MeIQx and 4,8-DiMeIQx, with the exception of the IQ content in a fish dish called 'otak-otak' (up to 87 ng/g) (Wu *et al.*, 1996). HCAs are primarily found in the crust of cooked meat and fish, but small amounts of HCAs may be present in the inner parts of fried meat (Skog *et al.*, 1995).

Commercially cooked food products generally contain very low or undetectable amounts of HCAs, with a few exceptions. The levels of HCAs in pan residues after frying different meat and fish products are generally the same as in the corresponding food products, but in some cases, the amounts are considerably higher. In some countries, pan residues are used to make gravy, which may result in a substantial contribution of HCAs to the diet. Bouillon cubes contain very low or undetectable amounts of HCAs, and the dietary intake of HCAs may thus be reduced simply by discarding the pan residue after frying, and preparing gravy and sauces using commercial products such as bouillon cubes.

HCAs have also been detected in fumes formed during the cooking of meat. Vainiotalo *et al.* (1993) found 13.7 pg MeIQx and 7.3 pg DiMeIQx per g fried meat in the fumes from frying meat, and Thiébaud *et al.* (1994) found MeIQx, DiMeIQx and PhIP in fried meat and in smoke condensate in which the concentration of these HCAs amounted to about 6% of that in the meat. PhIP has also been detected in cigarette smoke condensate (Manabe *et al.*, 1991) and in wine and beer (Manabe *et al.*, 1993b; Richling *et al.*, 1997).

2.4. Dietary intake of HCAs

Accurate assessments of the consumption of HCAs are essential for the evaluation of human cancer risks. Studies of HCA intake have relied on various methods of dietary assessment to determine intake of meat (beef and pork), chicken and fish; the primary sources of HCA in the diet. Human exposure to HCAs has been estimated to range from ng/day to μg/day, depending on dietary habits and cooking practices (Table 1.6).

Table 1.6. Estimated daily intake of HCAs (mean values).

Intake of HCAs (μg/person.day)	**HCAs included**	**Reference**
0.1-13.8	PhIP	Wakabayashi *et al.*, 1992
0.1-1.3	MeIQx	Wakabayashi *et al.*, 1993
0.8-8.4	Not specified	Eisenbrand and Tang, 1993
0.04-7.0	MeIQx, DiMeIQx, PhIP	Johansson and Jägerstad, 1994
1.8	PhIP>AαC>MeIQx>DiMeIQx>IQ	Layton *et al.*, 1995
0-12.0	MeIQx, DiMeIQx, PhIP	Skog *et al.*, 1995; 1997
0.976	Sum of 9, PhIP>AαC>MeIQx>IQ> DiMeIQx	Thomson *et al.*, 1996
0.4	IQ, MeIQ, MeIQx, DiMeIQx, PhIP	Zoller *et al.*, 1997
0-1.8	PhIP = MeIQx>DiMeIQx>IQ	Augustsson *et al.*, 1997
0.16	MeIQx, DiMeIQx, PhIP	Byrne *et al.*, 1998
0.32-0.51	IQ, MeIQ, MeIQx, DiMeIQx, PhIP, AαC, MeAαC, Trp-1, Trp-2	Sinha *et al.*, 2001
0.1-0.15	MeIQx, DiMeIQx, PhIP	Kobayashi *et al.*, 2002
0.26-0.36	MeIQx, DiMeIQx, PhIP, Trp-1, MeIQ	Nowell *et al.*, 2002
0.1	MeIQx, DiMeIQx, PhIP	Rohrmann *et al.*, 2002
0.8-1.4	MeIQx, DiMeIQx, PhIP	Keating and Bogen, 2004
0.01-0.1	MeIQx, DiMeIQx, PhIP, IFP	Wong *et al.*, 2005
0.6-0.8	MeIQx, DiMeIQx, PhIP	Ericson *et al.*, 2007

A common method of estimating the intake of dietary components, such as HCAs, is to administer a food frequency questionnaire that includes commonly consumed foods and the amount consumed on a daily (or other regular) basis. The consumption of the specific dietary component is then assessed by multiplying the quantity of food consumed by the concentration of the component of interest. Sinha and coworkers (1997) developed a 100-item food frequency questionnaire that included an evaluation of meat cooking practices (e.g. pan frying, broiling, grilling). This food-frequency questionnaire has been coupled with a database for three HCA components, MeIQx, DiMeIQx and PhIP, developed by the same

research group for meats prepared by different cooking techniques and to different doneness levels. The database contains information derived from publications on beef (Sinha *et al.*, 1998c), pork (Sinha *et al.*, 1998a), chicken (Sinha *et al.*, 1995) and fast-food meat products (Knize *et al.*, 1995). In those publications, the authors reported the HCA concentrations in different types of meats cooked by different methods and to varying degrees of doneness. Photographs were made of the cooked meats to show the internal coloring and external browning; the negative photographs were used in conjunction with the food-frequency questionnaire to standardize the responses of individuals assessed.

3. Bioavailability of HCAs

3.1. Concept of bioavailability

In human health risk assessment, the total amount of an ingested contaminant (intake) does not always reflect the fraction that is available to the body. Bioavailability is a term used to describe the proportion of the ingested contaminant that reaches the systemic circulation. Studies in animals and humans have shown that oral bioavailability of compounds from food can be significantly different depending on the food source, food processing or food preparation (van het Hof *et al.*, 2000). Thus, a better insight in the effect of the matrix on the oral bioavailability of a contaminant will lead to a more accurate risk assessment.

Oral bioavailability of a contaminant can be seen as the resultant of three different processes:

1. Release of the compound from its matrix into the gastrointestinal tract (bio-accessibility).
2. Transport across the intestinal epithelium and throughout the body (absorption and distribution).
3. Transformation of the compound in the liver or intestine and elimination out of the body (metabolism and excretion).

Information related to the potential mutagenic and carcinogenic effects of HCAs is contained in ample literature. Quite extensive research has also been performed to estimate the so-called relative cancer risk index. However relative scarce attention has been paid to investigate the bioavailability of this group of food-borne mutagens, which presumably being variable for

different HCAs, may significantly influence their ultimate effective concentrations in target physiological sites.

3.2. *Absorption and distribution of HCAs*

Both *in vitro* and *in vivo* studies indicate that a significant fraction of HCAs is absorbed from the gastrointestinal tract in humans (Lang *et al.*, 1999; Malfatti *et al.*, 1999; Krul *et al.*, 2000; Kulp *et al.*, 2000; 2004) and experimental animals (Turteltaub *et al.*, 1992; 1993). Volunteers given [2-^{14}C]PhIP (70 to 84 µg) by capsule excreted 50% to 90% of the administered dose in the urine during the first 24 hours (Lang *et al.*, 1999; Malfatti *et al.*, 1999). However, Kulp *et al.* (2000; 2004) found that the total urinary excretion of PhIP and PhIP metabolites varied widely (4% to 53% dose excreted within 24 hours) in volunteers that consumed 200 g of cooked chicken. The authors suggested that the absorption and bioavailability of PhIP might be different when consumed as part of the normal diet in a meat matrix rather than in a gelatin capsule. The absorption of several HCAs (IQ, PhIP, MeIQ and MeIQx) given as a mixture was modeled using a computer-controlled *in vitro* system that mimicked the physiological conditions of the human stomach and small intestine (Krul *et al.*, 2000). Comparable to an *in vivo* human model, all four HCAs were readily absorbed in the *in vitro* system, with approximately 50% of the total dose recovered in the dialysate after 2 hours and 95% after 6 hours. The recovery from the jejunal and ileal compartments represented 94% of the total recovery. The remaining 5 ± 1.5% of the starting material was recovered in the solution at the end of the small intestine segment.

Studies in experimental animals show that HCAs are rapidly distributed throughout the body and that tissue concentrations change rapidly with time (Watkins *et al.*, 1991; Turteltaub *et al.*, 1992; 1993; Snyderwine *et al.*, 1994; Dragsted *et al.*, 1995; Mauthe *et al.*, 1998). When male F344 rats were given [2-^{14}C]PhIP by gavage, the highest concentrations of radioactivity at 12 hours post-dose were found in the colon and cecum, while the highest concentrations at 24 hours and later were detected in the kidney and liver (Watkins *et al.*, 1991). Turteltaub *et al.* (1992) administered [^{14}C]PhIP by intubation at a dose of 41 ng/kg, which was considered to be equivalent to a human dietary dose, to six- to eight-week old C57BL/6 male mice. At 30 minutes and 1 hour, tissue levels of radiolabel were highest in intestine, stomach and adipose tissue, followed by liver, kidneys, pancreas, lung and spleen. By 96 hours post-dose, only liver, pancreas, muscle, spleen and lung contained a significant amount of radioactivity. In a

follow-up study, peak tissue levels were reached within 3 hours, with the greatest concentration of radioactivity in the gastrointestinal tract (GI), liver, kidney, pancreas and thymus (Turteltaub *et al.*, 1993). Following a 1 mg/kg dose of [^{3}H]PhIP administered by oral intubation to male Wistar rats, the highest total radioactivity was found in the stomach, small intestine and bladder after 2 hours. At 24 hours post-dose, the highest residual radiolabel concentrations were detected in the kidney and liver (Dragsted *et al.,* 1995). Snyderwine *et al.* (1994) administered oral doses of PhIP (20 mg/kg) as either a single dose or nine daily doses to male and female cynomolgus monkeys (*Macaca fascicularis*). PhIP-DNA adducts were detected in all 28 tissues examined, with the exception of fat and bone marrow.

3.3. Liver metabolism of HCAs

Most HCAs are not mutagenic/carcinogenic in their native form but acquire their biological activity after metabolic activation (Kato, 1986). *In vitro* studies with MeIQx and PhIP using liver microsomal preparations from rats, mice and rabbits showed that at least two oxidative metabolites were formed from each compound, a ring-hydroxylated product and the *N*-hydroxy derivative (Gooderham *et al.*, 1987; Turteltaub *et al.*, 1989; Watkins *et al.*, 1991; Turesky *et al.*, 1991, 1998). In addition, both the parent amines and their primary oxidative metabolites can be further biotransformed to a variety of phase II metabolites including glucuronides (Kaderlik *et al.*, 1994), sulfate esters (Chou *et al.*, 1995) and acetylated products (Lin *et al.*, 1995). As an example, the routes of PhIP metabolism are shown in Figure 1.7.

Examination of the primary oxidative metabolites in a mutagenicity assay such as the Ames *Salmonella typhimurium* test showed that the N-hydroxy metabolites were direct-acting mutagens, whereas the ring-hydroxylated products were not (Zhao *et al.*, 1994). Analysis of HCA metabolism by human liver microsomes showed N-hydroxylation to be the primary oxidative route of metabolism of HCAs with a K_m value of 55 μM for PhIP, with little if any aromatic hydroxylation (Zhao *et al*., 1994). Clearly, there are species differences in the oxidative metabolism of HCAs since experimental animals are able to both activate and detoxify these amines, whereas humans convert them predominantly to their reactive genotoxic metabolite.

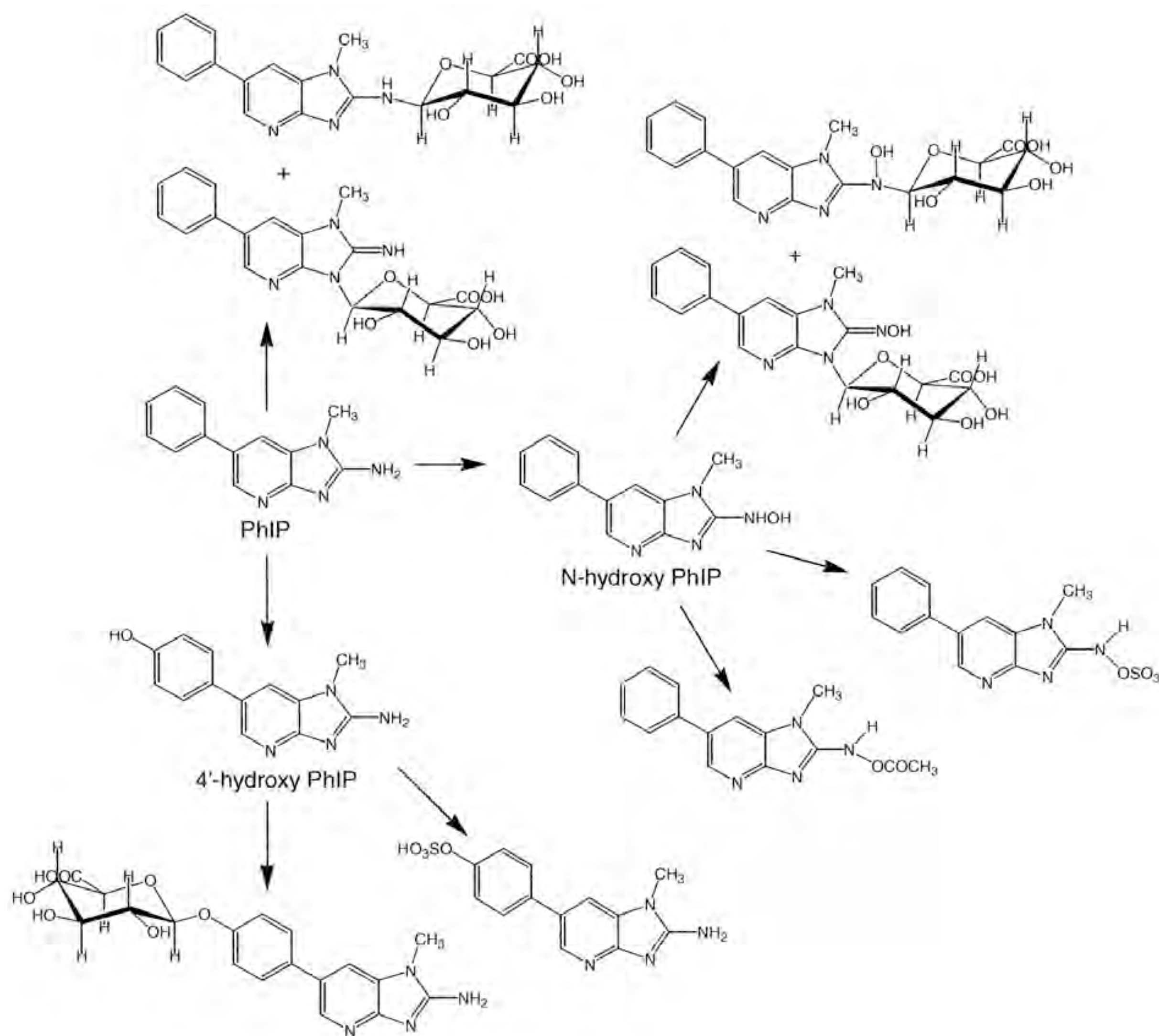

Figure 1.7 Major biotransformation pathways of PhIP.

Studies using a variety of different approaches have shown that the genotoxic N-hydroxylation pathway of these amines involves primarily members of the CYP1A2 subfamily (McManus *et al.*, 1989; Turteltaub *et al.*, 1989; Turesky *et al.*, 1991; Watkins *et al.*, 1991; Zhao *et al.*, 1994; Crofts *et al.*, 1998; Da Fonseca *et al.*, 2003). However, contribution by other cytochromes P450, including CYP1A1, CYP1B1, CYP2A6, CYP3A4, CYP2C9, CYP2C10 and CYP2A3, should by no means be neglected when assessing the total risk (McManus *et al.*, 1989; Edwards *et al.*, 1994; Crofts *et al.*, 1997; 1998; Schut and Snyderwine, 1999; Williams *et al.*, 2000; Josephy *et al.*, 2001). All of these P450 isoforms are less active toward HCA substrates than CYP1A2. Levels of CYP1A2 in human liver can vary considerably (Sesardic *et al.*, 1990); thus, hepatic metabolism of heterocyclic amines such as PhIP will vary within the human population. CYP1A2 expression is almost exclusively

hepatic, whereas CYP1A1 and CYP1B1 have been detected in a variety of extrahepatic organs, usually after exposure of inducing agents. Hence, the hepatic oxidative metabolism of HCAs will be CYP1A2-dependent, whereas in extrahepatic tissues metabolism is likely to be supported by CYP1A1 and to a lesser extent by CYP1B1. Again, variations in expression of these extrahepatic enzymes will contribute to variation in the overall disposition and toxicity of these compounds.

It has been shown that human urinary metabolites of the HCAs MeIQx and PhIP include glucuronides and sulfate esters (Turesky *et al.*, 1998; Malfatti *et al.*, 1999). Indeed, *N*-hydroxy-PhIP-N^2-glucuronide is thought to be the major urinary metabolite of PhIP accounting for about 50% of the dose (Malfatti *et al.*, 1999). At least five glucuronides of PhIP have been reported, the N^2-glucuronide, the N^2-hydroxy glucuronide, the N^3-glucuronide, the N^3-hydroxy glucuronide and the 4'-hydroxy glucuronide. There is evidence from reconstitution and tissue culture studies that N-hydroxy PhIP can also be sulfated (Buonarati *et al.*, 1990; Chou *et al.*, 1995; Lewis *et al.*, 1998). Three of the sulfotransferases (SULTs 1A2, 1A3 and 1E1) have been shown to sulfate heterocyclic amines and their hydroxylamine derivatives (Buonarati *et al.*, 1990; Lewis *et al.*, 1998; Turesky *et al.*, 1998). The latter isoform, SULT1E1, is known to be hormonally regulated and readily inducible by progesterone. This suggests that sulfation activity could vary, for example during the luteal phase of the menstrual cycle when there is a surge in progesterone levels and SULT1E1 activity may be elevated (Lewis *et al.*, 1998). Since the sulfoxy ester of N-hydroxy-PhIP is an unstable product, its detection in biological samples is likely to be very difficult. However, the 4'-hydroxy-PhIP-sulfate ester, a detoxification product, has been detected in humans, thus demonstrating the involvement of sulfotransferase in PhIP metabolism (Malfatti *et al.*, 1999). N-Hydroxylation of the HCAs, the primary metabolic pathway in humans, is also the primary route of HCA genotoxicity. For some HCAs, the N-hydroxy metabolite reacts poorly with DNA, but it can be converted to highly reactive derivatives by esterification. Other mammalian phase II enzymes that have been identified are N-acetyltransferase (NAT), prolyl tRNA synthetase and phosphorylase which produce N-acetoxy, N-prolyloxy, N-phosphatyl ester derivates, respectively (Schut and Snyderwine, 1999). Among these, NAT, expressed both in rodents and humans (predominantly in the liver), appears to play a dominant role, at least in phase II bioactivation of IQ, MeIQx and PhIP (Minchin *et al.*, 1993). It was suggested that rapid acetylator individuals might be more susceptible to HCA toxicity (Minchin *et al.*,

1993). Studies with bacterial strains that are deficient, proficient and overexpress acetyltransferase enzymes show the importance of this esterification reaction in the metabolic activation of the heterocyclic amines to bacterial mutagens (Gooderham *et al.*, 2001). Like the *N*-hydroxy sulfate esters, the acetyl esters of the *N*-hydroxy heterocyclic amines are extremely reactive and readily damage DNA.

3.4. Metabolism of HCAs by human intestinal microbiota

3.4.1 Microbial biotransformation activity

For many years, it was believed that the main purpose of the large intestine was the resorption of water and salt by the body and the facilitated disposal of waste material. However, the human large intestine harbors a highly complex microbial ecosystem of about 200 g living cells, at concentrations of 10^{12} microorganisms per gram gut content, the highest recorded for any microbial habitat (Whitman *et al.*, 1998). The use of culture-independent approaches (Zoetendal *et al.*, 2004; Gill *et al.*, 2006) and new ecological theories about evolutionary forces shaping the microbial community in the intestine (Bäckhed *et al.*, 2005; Dethlefsen *et al.*, 2006; Ley *et al.*, 2006) have given more insight in the structure of this ecosystem. Although 55 and 13 divisions have been described of respectively bacteria and archaea, the gut microbiota are dominated by only two bacterial divisions, the Bacteroidetes (bacteroides) and Firmicutes (clostridia, eubacteria, …), with lower levels of Actinobacteria (bifidobacteria) and by one member of the Archaea, *Methanobrevibacter smithii*. At this level, the intestinal communities of all humans therefore appear quite similar. However, within these divisions, a limited number of lineages terminate in broad, shallow radiations comprising hundreds of species and thousands of strains, making the microbiota of an individual as personalized as a fingerprint (Bäckhed *et al.*, 2005; Ley *et al.*, 2006).

Driven by selection forces at both microbial and host levels, this microbial community has coevolved in a mutualistic relation with the human host with important implications for health and disease. This involves the stimulation of the gut immune system (Salminen *et al.*, 1998), the regulation of cell proliferation (Dethlefsen *et al.*, 2006), the synthesis of vitamins K and B (Conly and Stein, 1992), energy salvation (Bäckhed *et al.*, 2004) and pathogen resistance (Hopkins and Macfarlane, 2003). On the other hand, the specific microbial community assemblage may also be seen as a risk factor contributing to a state of disease

(Ley *et al.*, 2006). This is shown by recent reports linking intestinal bacteria with diseases ranging from allergies (MacDonald and Monteleone, 2005) to bowel inflammation (Elson *et al.*, 2006) and obesity (Bäckhed *et al.*, 2007).

But the intestinal community has also another important role. Taking together the genomes of all these bacteria, the microbiome has a coding capacity that vastly exceeds that of the human genome and encodes biochemical pathways that humans not have evolved (Egert *et al.*, 2006). Thus, the intestinal microbiota can be regarded as a separate organ within the human host, that is capable of at least as many conversions than the human liver (Table 1.7). Most resident colon microbiota typically perform fermentation of carbohydrates and proteins, but it has become clear that many bacterial groups are also capable of transforming xenobiotics (Illet *et al.*, 1990). Numerous findings show that intestinal microorganisms and lactobacilli contained in dairy products play a key role in the activation and detoxification of various classes of DNA-reactive carcinogens such as nitrosamines, aflatoxins, polycyclic aromatic hydrocarbons, azo compounds, nitroarenes and glycosides (Rowland and Grasso, 1975; Oatley *et al.*, 2000; Wang *et al.*, 2004; Van de Wiele *et al.*, 2005).

In contrast to the oxidative and conjugative reactions from the phase I and II enzymes in the enterocytes and hepatocytes, the bacterial metabolism is more reductive, hydrolytic and even of degradative nature with great potential for both bioactivation as detoxification of xenobiotics (Illet *et al.*, 1990). Additionally, the intestinal microbiota also interfere with the human biotransformation process through enterohepatic circulation of xenobiotic compounds. Compounds that have been absorbed in the intestine and subsequently detoxified are usually conjugated with polar groups in the liver prior to excretion in the bile (Illet *et al.*, 1990). Once released in the intestinal lumen, these conjugates may be hydrolyzed again by bacterial enzymes such as β-glucuronidases, sulfatases and glucosidases. McBain and MacFarlane (1998) estimated that 10^{10}-10^{12} bacteria/mL intestinal content produce β-glucosidase and 10^{7}-10^{11} produce β- glucuronidase, showing the importance of intestinal bacteria in this deconjugation process.

Table 1.7. Metabolic potency of the human gastrointestinal microbiota (after: Illet *et al.*, 1990).

Reactions	Enzyme	Microbiota
Hydrolysis		
Glucuronides	β-glucuronidase	*E. coli*
Glycosides	β-glucosidase	*Enterococcus faecalis, Eubacterium rectale, Clostridium sphenoides*
Amides	Amide hydrolase	*E. coli, Bacillus subtilis, Bacillus mycoides*
Esters	Deacetylase	*Enterococcus faecalis*
Sulphamates	Arylsulfotransferase	Clostridia, enterobacteria, enterococci
Reductions		
Azo-compounds	Azoreductase	Clostridia, lactobacilli
Unsaturated lacton	Unsaturated glycoside hydrogenase	*Eubacterium lentum*
Aliphatic double bounds	Unsaturated fatty acid hydrogenase	*Enterococcus faecalis*
Nitro-compounds	Nitroreductase	*E. coli,* Bacteroides
N-oxides	N-oxide reductase	Human colon
S-oxide	Sulfoxide reductase	*E. coli*
Ketones	Hydrogenase	Rat cecum
Hydroxylamines	Nitroreductase	Rat GIT
Dehydroxylation		
Demethylation	Demethylase	Enterococci, lactobacilli, clostridia
N-demethylation	N-demethylase	Clostridia, bacteroides
Deamination	Deaminase	*E. coli,* bacteroides, clostridia
Decarboxylation	Decarboxylase	*Enterococcus faecalis*
Dehydrogenation	Dehydrogenase	*Clostridium welchii*
Dehalogenation	Dehalogenase	*E. coli, Aerobacter aerogenes*
Synthetic reactions		
Esterification	Acetyltransferase	*E. coli*
N-nitrosation		*Enterococcus faecalis, E. coli*
Other reactions		
Oxidation	Oxidase	*E. coli, Enterococcus faecalis*
Isomerization	Isomerase	*Eubacterium rectale, Clostridium sphenoides*
Fission aliphatic	Tryptophase	*E. coli, Bacillus alvei*
Fission ring	C-S lyase	Pig GIT, *Eubacterium aerofaciens*

3.4.2 Conversion of HCAs by intestinal microorganisms

A few studies have highlighted the crucial impact of the intestinal microbiota in the genotoxicity of HCAs. Following IQ administration, DNA adducts have been observed in mice harboring their native or a human-originating microbiota while adducts were extremely low or absent in germ-free animals (Hirayama *et al.*, 2000). Similarly, the extent of IQ-induced DNA damage in colonocytes and hepatocytes, measured with the comet assay, is 2- to 3-fold higher in human fecal microbiota-associated (HFA) rats and 4- to 5-fold higher in conventional rats, than in germ-free counterparts (Kassie *et al.*, 2001). Using HFA rats, it has also been demonstrated that the intestinal microbiota are essential to the induction of DNA damage by PhIP (Hollnagel *et al.*, 2002). The different mechanisms by which the intestinal microbiota may affect the genotoxic and carcinogenic effects and thus metabolism of HCAs are depicted schematically in Figure 1.8 and will be discussed in the following paragraphs.

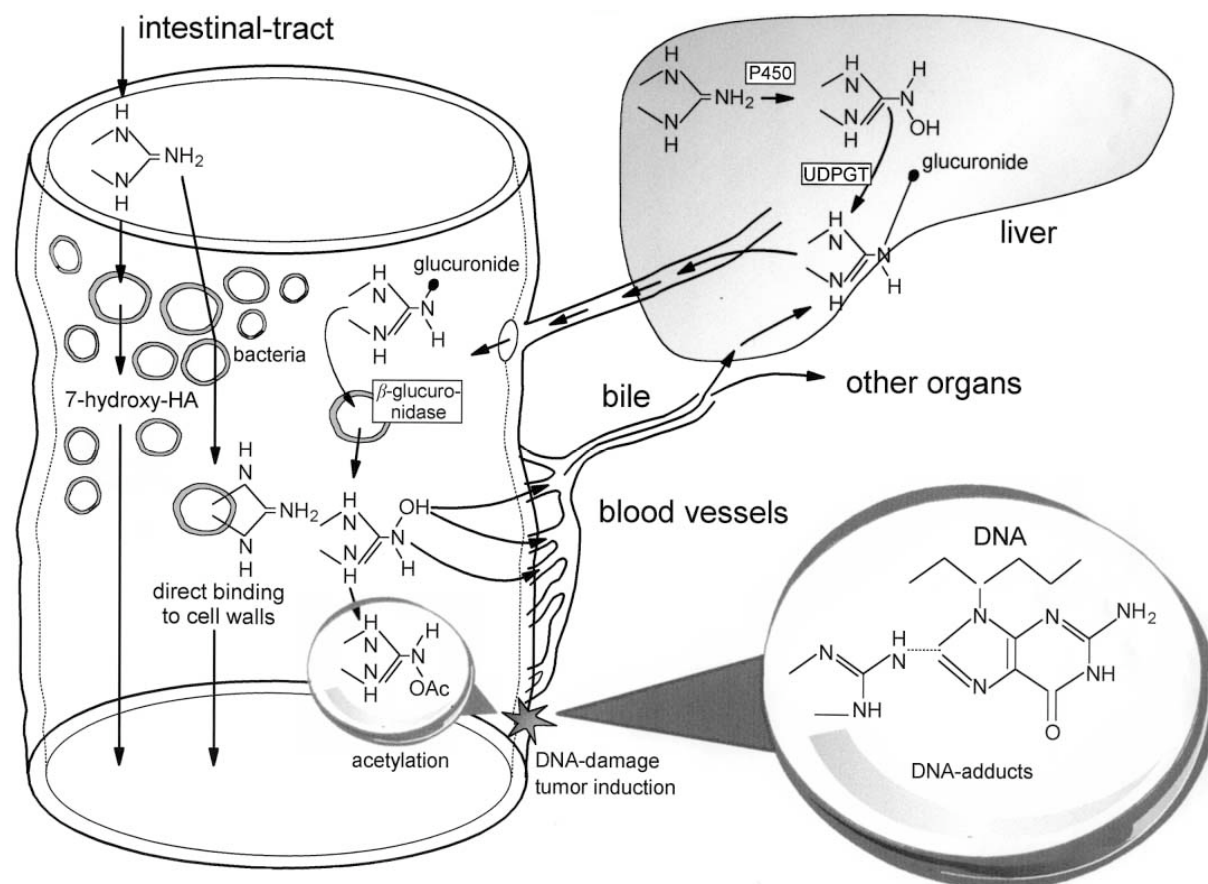

Figure 1.8 Schematic representation of the interactions between intestinal bacteria and HCAs (after: Knasmüller *et al.*, 2001).

3.4.2.1 Detoxification of HCAs by lactic acid bacteria

Lactic acid bacteria (LAB) are commonly found in the gastrointestinal tract and are utilized in many fermented dairy, meat and cereal products. Some LAB strains are termed ‘probiotics’ as they contribute to the maintenance of health (Salminen *et al.*, 1998). These specific probiotic strains may enhance the host’s immune response, remove potential carcinogens or alter the metabolic activity of the intestinal microbial community and the action of bile salts (Rafter, 1995). A number of studies have been published which describe the detoxification of HCAs by lactic acid bacteria (Orrhage *et al.*, 1994; Bolognani *et al.*, 1997; Lankaputhra and Shah, 1998; Sreekumar and Hosono, 1998; Tavan *et al.*, 2002; Turbic *et al.*, 2002; Zsivkovits *et al.*, 2003).

The exact mechanism of antimutagenicity is unclear and appears to vary with different strains. Direct binding of heterocyclic amines by LAB has been proposed (Orrhage *et al.*, 1994). Lankaputhra and Shah (1998) however suggested that living bacteria might produce metabolites or catalyze reactions, which lead to detoxification of amines. Zsivkovits *et al.* (2003) also proposed the involvement of indirect mechanisms in the antimutagenicity of LAB towards HCAs. It has been shown that lactobacilli adhere to intestinal mucosa cells *in vitro* and *in vivo* and it is conceivable that this feature may as well affect the uptake of HCAs through the intestinal barrier (Zsivkovits *et al.*, 2003).

3.4.2.2 Formation of direct-acting hydroxy-derivates

In 1987, Bashir *et al.* (1987) reported that incubation of IQ with human fecal microbiota results in the formation of the stable hydroxy-metabolite 2-amino-3,6-dihydro-3-methyl-*7H*-imidazo[4,5-*f*]quinoline-7-one (7-OH-IQ). This compound was subsequently detected in human feces following consumption of fried meat (Van Tassel *et al.*, 1990). Carman *et al.* (1988) showed that this metabolite is formed by *Eubacterium* and *Clostridium* strains, the most effective producer identified in human feces being *Eubacterium moniliforme*. Recent research identified 10 bacterial strains able to perform the IQ to 7-OH-IQ transformation: *Bacteroides thetaiotaomicron* (n = 2), *Clostridium clostridiiforme* (n = 3), *Clostridium perfringens* (n = 1) and *Escherichia coli* (n = 4) (Humblot *et al.*, 2005). 7-OH-IQ is a very potent direct acting mutagen in the *Salmonella typhimurium* strain TA98, whereas a negative result was obtained in the SOS Chromotest (Carman *et al.*, 1988; Van Tassel *et al.*, 1990). It was hypothesized that similar metabolites might also be formed from other structurally

related amines (Carman *et al.*, 1988) and indeed a hydroxy-metabolite of MeIQ could be isolated (Van Tassel *et al.*, 1990). Till 1994, evidence for mutagenic effects of the bacterial metabolites was restricted to results obtained in *Salmonella*/microsome assays. The first data from experiments with mammalian cells came from Weisburger (1994), who carried out DNA-repair assays with 7-OH-IQ in primary rat hepatocytes. Under all conditions of test negative results were obtained. The same report contains the results of carcinogenicity studies with male F344 rats and newborn CD-1 mice. The rats were treated intrarectally either with IQ or with the hydroxy-derivative, the mice were treated by intra-peritoneal injection followed by long-term dietary supplementation. In the IQ groups and this was true both for the rats and the mice, pronounced induction of colon tumors was found, whereas no such effect was seen in the 7-OH-IQ groups. The authors concluded that the presence of 7-OH-IQ in the intestinal tract of humans on a Western diet is unlikely to account for an increased colon cancer risk in individuals consuming IQ and related amines.

3.4.2.3 Formation of indirect-acting mutagens of HCAs

An important detoxification pathway for HCAs is the conjugation with glucuronic acid, which takes place mainly in the liver. These glucuronidated derivates are partly excreted via the bile into the digestive lumen. It has been hypothesized that representatives of the intestinal microbiota might hydrolyze glucuronide conjugates of HCAs. Alexander *et al.* (1991) incubated primary rat hepatocytes with PhIP and found that one of the major metabolites 2-*N*-ß-D-glucuronopyranosyl-(hydroxyamino)-1-methyl-6-phenylimidazo[4,5-*b*]pyridine is splitted by ß-glucuronidase. Recently, Humblot *et al.* (2007) constructed a β-glucuronidase-deficient isogenic mutant from a wild-type *E. coli* strain carrying the gene *uidA* encoding this enzyme and compared the genotoxicity of IQ in gnotobiotic rats monoassociated with the wild-type or the mutant strain. The comet assay performed on colonocytes and hepatocytes showed that the presence of β-glucuronidase in the digestive lumen dramatically increased (3-fold) the genotoxicity of IQ in the colon. These results clearly indicate that bacterial β-glucuronidase plays a pivotal role in the ability of IQ to induce DNA damage in colonocytes. Moreover, they are consistent with observations suggesting that IQ cannot induce DNA damage in the colonocytes of germ-free rodents (Hirayama *et al.*, 2000; Kassie *et al.*, 2001) and help to elucidate the chemoprotective effects of dietary compounds capable of lowering β-glucuronidase activity in the colon (Humblot *et al.*, 2004). One might speculate whether the central role of β-glucuronidase in the colonic genotoxicity of IQ may apply to other HCAs,

since this family includes molecules with very diverse chemical structures. Ligation of the biliary duct in rat does not alter the genotoxic effect of PhIP (Kaderlik *et al.*, 1994), suggesting that the involvement of bacterial β-glucuronidase in the metabolic fate of PhIP has no influence on its bioactivity. Hirayama *et al.* (2000) investigated the effect of human intestinal microbiota on DNA adducts induced by 2-amino-9H-pyrido[2,3-*b*]indole and found a higher level of damage in germ-free mice than in mice with microbiota. Therefore, the impact of β-glucuronidase would vary depending on the animals' exposure to different chemicals. This could arise from different susceptibilities of HCA glucuronoconjugates to β-glucuronidase hydrolysis. For example, Styczynski *et al.* (1993) showed that PhIP glucuronide originating from conjugation by human enzymes was a substrate for bacterial β-glucuronidase, whereas PhIP-glucuronide from rabbit did not undergo β-glucuronidase-catalyzed hydrolysis.

4. HCA mutagenesis and carcinogenesis

4.1. DNA binding of HCAs

HCAs must be metabolically activated to N-hydroxy-HCA derivates and undergo phase II conjugation to form N-acetoxy or N-sulfonyloxy esters to obtain their genotoxic activity. These highly reactive esters may undergo heterolytic cleavage to generate the nitrenium ion, which represents the ultimate carcinogenic species (Kato, 1986). The major DNA adducts formed with these reactive esters occur at the C-8 position of deoxyguanosine (dG) (Figure 1.9) (Turesky, 2002). In addition to these dG-C8-HCA adducts, a second adduct was reported to form at the N_2 position of dG and the C-5 atoms of IQ and MeIQx, indicating charge delocalization of the incipient nitrenium ion at this location (Turesky, 2002). These dG-HCA adducts are believed to be responsible for the mutagenicity of HCAs.

Conformational changes in DNA are induced by aromatic amine-purine base modifications and are important determinants of the adduct's biological activity and propensity to provoke base pair deletions and substitutions during translesional synthesis of DNA (Beland and Kadlubar, 1985). The conformation of the glycosidic linkage of the carcinogen adducted to DNA is also an important factor in adduct persistence (Beland and Kadlubar, 1985). Adducts that preferentially exist in the *syn* form may induce a greater distortion of the DNA helix at the site of carcinogen adduction than adducts that exist in the

normally occurring *anti* form of DNA, resulting in more facile recognition and enzymatic removal of the adduct (Beland and Kadlubar, 1985).

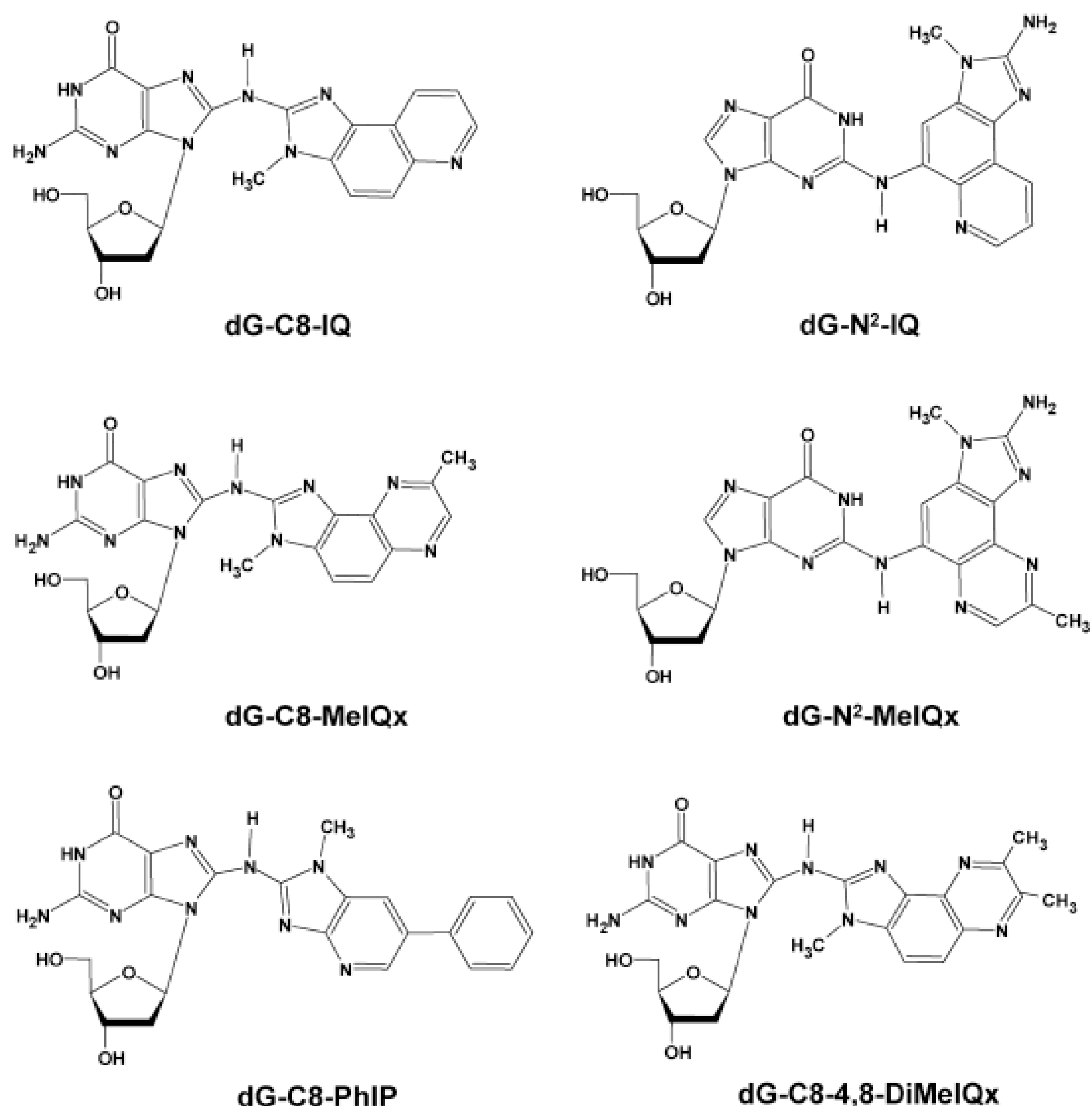

Figure 1.9 DNA adducts of several HCAs. The dG-C8 adducts have been drawn in the *syn* conformation and the dG-N^2 adducts are present in the *anti* conformation (after: Turesky, 2002).

The increased frequency of base pair deletions that occurs in DNA modified with aromatic amines and HCAs, particularly when G or T is present at the 5’ flanking position of the modified base may also be explained by adducts existing in the *syn* conformation. The

structural alterations induced by the DNA adduct may allow the formation of a frameshift intermediate, resulting in deletion of a base pair during translesional synthesis. More recently, the solution structure of dG-C8-PhIP adduct present as an 11-mer duplex was reported and the dG-C8-PhIP lesion was observed to exist in the *syn* form, which may help to explain the biological effects of this mutagen (Brown *et al.*, 2001).

4.2. Bacterial mutagenicity

The Ames mutagenicity assay, developed in the 1970s (Ames, 1973) was the first test used to assess the *in vitro* genotoxicity of HCAs in prokaryotic cells. Requirement for metabolic activation of HCAs led to modification of the method to incorporate a liver extract, conventionally called S9 mix (Sugimura, 1997). IQ, 8-MeIQx and MeIQ are amongst the most potent bacterial mutagens ever tested in the Ames assay employing several strains of genetically modified *Salmonella typhimurium* (Sugimura and Sato, 1983). Other HCAs, including PhIP and AαC are respectively, 200- and 1000-fold weaker in potency (Table 1.8).

Many of these HCA-DNA lesions can be repaired since the mutagenic potencies of several HCAs are 100-fold less active in the *uvrB+* proficient *Salmonella typhimurium* strain (Felton *et al.*, 1994b). HCAs preferentially induce frameshift mutations in *Salmonella typhimurium,* but point mutations also occur (Sugimura, 1997). The high response in frameshift mutations in the *Salmonella typhimurium* strains TA98 and TA1538 is attributed to a preference for some HCAs to react about 9 base pairs upstream of the original CG deletion in the *hisD*$^{+}$ gene in a run of CG repeats (Fuscoe *et al.*, 1988). This "hotspot" is consistent with the presence of dG-HCA adducts, which may lead to CG deletions during translesional DNA synthesis. The relatively high potency of several HCAs in the Ames assay may also be attributed to the O-acetyltransferase (OAT) enzyme expressed in *Salmonella typhimurium.* OAT efficiently activates the promutagenic N-hydroxy-HCAs produced by exogenously added P450 enzymes to form the highly reactive N-acetoxy intermediates, which readily bind to DNA within the cell. Consistent with this observation, the mutagenic potencies of several HCAs, including IQ and MeIQx, which are activated by OAT are significantly diminished in *Salmonella typhimurium* TA98/1,8DNP6, a strain deficient in OAT (McCoy *et al.*, 1983). Conversely, the *Salmonella typhimurium* tester strain YG1024, which contains elevated levels of OAT is significantly more sensitive to the genotoxic effects of several HCAs activated by this enzyme (Watanabe *et al.*, 1990).

Table 1.8. Mutagenicity of HCAs in *Salmonella typhimurium* TA98 and TA100 with S9.

	Revertants/µg	
	TA98	**TA100**
MeIQ	661000	30000
IQ	433000	7000
DiMeIQx	183000	8000
7,8-DiMeIQx	163000	9900
MeIQx	145000	14000
Trp-P-2	104200	1800
4-CH_2OH-8-MeIQx	99000	3000
IQx	75400	1500
Glu-P-1	49000	3200
Trp-P-1	39000	1700
Glu-P-2	1900	1200
PhIP	1800	120
AαC	300	20
MeAαC	200	120

The mutagenicity of HCAs in other bacterial genes such as the *lacZ, lacZa* and *lacI* of *E. coli* also reveal that mutations occur primarily at GC pairs. Other studies have examined the genotoxicity of aromatic amines and HCAs in *E. coli* that have been genetically engineered to simultaneously express human P4501A2, NADPH cytochrome P450 reductase and N-acetyltransferase. Consequently, the bioactivation of HCAs occurs within the cell, rather than extracellular as occurs with exogenously added liver S-9 homogenates or P450 preparations. Thus, the chemically reactive metabolites are in close proximity to the target gene, which enhances the sensitivity of the mutagenicity assay (Josephy *et al.*, 1998).

Other bacterial systems have used the induction of the SOS response in *Salmonella typhimurium* NM2009 as a measure of DNA damage induced by HCAs; this system possesses high OAT activity and contains a *umuC* regulatory sequence attached to the *lacZ* reporter gene. More recently, these strains have been modified to express human P450 enzymes, NADPH-cytochrome P450 reductase and OAT (Oda *et al.*, 2001). These tester strains are highly sensitive towards some HCAs and have the advantage of being simple and fast, where data are generated within several hours. Yamazaki *et al.* (2004) newly developed 10 *Salmonella typhimurium* TA1538 strains each co-expressing a form of human cytochrome P450 together with NADPH-cytochrome P450 reductase, of which CYP1A1 and 1A2 were responsible for the mutagenic activity of PhIP.

4.3. *Mutagenicity in mammalian cells in vitro*

Several types of mammalian cells have been used to derive information relating to the mutagenicity of HCAs in eukaryotic cells. For some HCAs, completely different results were obtained. This is exemplified by PhIP, which in bacterial cells exhibited a weak mutagenicity unequal to that observed in eukaryotic cells (Turesky, 2002). The discrepancies in biological potencies of these *in vitro* assays are due to different exogenous and endogenous metabolic activation systems, gene loci endpoints, base sequence contexts and neighboring base effects on the HCA-DNA lesions, which may affect mutation frequencies. The mutagenic potencies of HCAs can be dramatically increased in mammalian cells genetically engineered to express phase II enzymes, such as NAT2 or SULT1A1, which are involved in HCA bioactivation (Glatt *et al.*, 2004).

In mammalian cells, base pair substitutions at guanine are prominent mutations; however, frameshift mutations at guanine also occur, depending upon the base sequence context. These mutational events are consistent with the notion that guanine is the principal target for HCA-DNA adduct formation (Schut and Snyderwine, 1999). The PhIP-induced mutations at the *hprt* locus in human lymphoblastoid cells have been reported to occur predominantly through GC→TA transversions (Morgenthaler and Holzhauser, 1995). GC → TA transversions have also been observed at PhIP-induced mutants in the *dhfr* genes of Chinese hamster ovary cells (Carothers *et al.*, 1994). PhIP was also shown to predominantly produce GC→TA transversions at the *hprt* locus in Chinese hamster V79 cells; however, 13% of the mutants displayed a -1G frameshift mutation in the 5'-GGGA-3' sequence (Yadollahi-Farsani *et al.*, 1996).

4.4. *Mutagenicity in vivo, activation of oncogenes and inactivation of tumor suppressor genes*

There have been several studies conducted on HCA-induced mutations in transgenic animals. PhIP was reported to induce a number of one-base deletions in the *lacI* gene of the colon mucosa of the transgenic Big Blue mice and Big Blue rats (Okonogi *et al.*, 1997a; 1997b). The characteristic guanine deletion at 5'-GGGA-3' reported in the *apc* gene of rat colon cancers induced by PhIP (Nagao *et al.*, 1996) accounted for 7 and 10% of the total mutations of this *lacI* gene in each of these experimental animal models. This mutation was also observed in mammary glands of female Big Blue rats treated with PhIP, where 6% of the

mutations displayed a GC base pair deletion at the 5'-GGGA-3' site (Okochi *et al.*, 1999). PhIP was also reported to induce GC → TA transversions and -1G frameshifts of GC in *lacI* gene of prostate of Big Blue male rats (Stuart *et al.*, 2000).

A number of genetic alterations have been reported in experimental animals during long-term feeding studies with HCAs (Nagao and Sugimura, 1993; Sugimura *et al.*, 1996; 1997 and references therein). Some of the genetic alterations are summarized in Table 1.9.

Table 1.9. Genetic alterations in tumors induced by HCAs (after: Turesky, 2002).

Species	**HCA**	**Genetic alterations**						
		Ha-*ras*	**Ki-*ras***	**N-*ras***	***p53***	***apc***	**β-catenin**	**MM**
Colon								
F344 rats	Glu-1-P	0/7	1/7	0/6	0/7			
F344 rats	IQ	0/11	0/11	0/11	0/11	2/13	5/5	
F344 rats	PhIP	0/9	0/9	0/9	0/9	4/8	4/7	7/8
Mammary gland								
F344 rats	PhIP	3/17	0/12	0/12	1/10		0/23	
SDxF344 F_1 rats	PhIP							9/15
Liver								
F344 rats	MeIQx				3/13			
CDF_1 mice	IQ	7/34						
Lung								
CDF_1 mice	IQ		49/54					
Forestomach								
CDF_1 mice	MeIQ	22/64			6/8			
Zymbal gland								
F344 rats	IQ	4/7, 5/9	3/9		4/16			
F344 rats	MeIQ	9/15						
F344 rats	MeIQx	2/6						

MM = Microsattelite mutations.

Genetic alterations in rat colon adenocarcinomas induced by IQ, PhIP and the glutamic acid pyrolysate mutagen Glu-P-1 were examined for *ras* family gene mutations. The Ki-*ras* mutations were rare and no mutations were detected in either the N-*ras* or Ha-*ras* genes for any of these tumors. Similarly, *p53* gene mutations were not detected in any rat colon tumors induced by these HCAs even though 60-70% of human colon cancers have mutations in the *p53* gene (Nagao *et al.*, 1996). Therefore, HCAs may represent suitable model compounds for

investigations in sporadic colon carcinogenesis, which do not involve mutations in the *p53* gene. However, mutations in either Ha-*ras* or Ki-*ras* and the *p53* genes were found in rat Zymbal gland tumors induced by IQ (Nagao and Sugimura, 1993 and references therein). IQ was also reported to induce mutations in the *p53* gene in 4 of 20 nonhuman primates that developed hepatocellular carcinoma during long-term feeding studies; three of the mutations contained GC → TA transversions and one possessed a GC → AT transition (Nagao *et al.*, 1997). The *apc* gene plays a major role in human colon carcinogenesis and is considered as an initial or very early event in human colon carcinogenesis.

Alterations of the *apc* gene were more prominent in PhIP-induced than in IQ-induced rat colon carcinogenesis (Kakiuchi *et al.*, 1995). Four of the eight colon tumors caused by PhIP had mutations in the *apc* gene and featured a guanine deletion from 5'-GGGA-3' sequences. Moreover, the specific GC base pair deletion in 5'-GTGGGA-3' at codon 635 of the *apc* gene was detected as an early mutation in colon of male rats exposed to PhIP for only one week when probed by the mismatch amplification mutation assay (Burnouf *et al.*, 2001). One of the hotspots of PhIP-induced mutation at the 5'-GTGGGA-3' sequence around codon 635 in the rat is conserved in the human *apc* gene and may be a signature mutation of this HCA (Sugimura *et al.*, 2004). In contrast to PhIP, mutations in the *apc* gene of IQ-induced colon tumors were detected in only two of 13 tumors and there were no specific and characteristic mutations (Kakiuchi *et al.*, 1995).

4.5. Carcinogenicity in experimental animals

Carcinogenicity of HCAs has been well documented in a wide range of organs/tissues in long-term animal studies and this led to the classification of eight HCAs (MeIQ, MeIQx, PhIP, Trp-P-1, Trp-P-2, AαC, MeαC and Glu-P-2) by IARC as possible (group 2B) and IQ as probable human carcinogen (group 2A). HCAs induce tumors at multiple organs including liver, lung, hematopoietic system, forestomach and blood vessels in mice, and colon, small intestine, prostate, mammary gland, hematopoietic system, liver, Zymbal gland, skin, clitoral gland, oral cavity and urinary bladder in rats (Ito *et al.*, 1997; Shirai *et al.*, 1997; Norrish *et al.*, 1999; Sugimura *et al.*, 2004; Knize and Felton, 2005). The TD_{50} values and targets sites of HCAs in rats and mice are presented in Table 1.10.

Table 1.10. Carcinogenicities of HCAs (after: Turesky, 2002; Sugimura *et al.*, 2004).

Chemical	Species	Dose (%) in diet	Target Organs	TD_{50} (mg/kg b.w./day)
Trp-P-1	Rats	0.015	Liver	0.1
	Mice	0.02	Liver	8.8
Trp-P-2	Rats	0.01	Liver, urinary bladder	-
	Mice	0.02	Liver	2.7
Glu-P-1	Rats	0.05	Liver, small and large intestines, Zymbal gland, clitoral gland	0.8
	Mice	0.05	Liver, blood vessels	2.7
Glu-P-2	Rats	0.05	Liver, small and large intestines, Zymbal gland, clitoral gland	5.7
	Mice	0.05	Liver, blood vessels	4.9
AαC	Rats	0.08	No tumors	-
	Mice	0.08	Liver, blood vessels	
MeαC	Rats	0.02, 0.01	Liver	6.4
	Mice	0.08	Liver, blood vessels	5.8
IQ	Rats	0.03	Liver, small and large intestines, Zymbal gland, clitoral gland, skin	0.7
	Mice	0.03	Liver, forestomach, lung, large intestine	14.7
MeIQ	Rats	0.03	Zymbal gland, Large intestine, mammary gland, skin, oral cavity	0.1
	Mice	0.04, 0.01	Liver, forestomach	8.4
MeIQx	Rats	0.04	Liver, Zymbal gland, clitoral gland, skin	0.7
	Mice	0.06	Liver, lung, hematopoietic system	11.0
PhIP	Rats	0.04	Large intestine, mammary gland, prostate, lymphoid tissue	2.2
	Mice	0.04	Small intestine, lymphoid tissue	64.4

There is particular interest in breast, colon and prostate tumors, as several epidemiological studies have revealed that frequent consumption of cooked foods containing these HCAs are associated with elevated cancer risk in these organs (Ito *et al.*, 1991; Willet, 1995; Shirai *et al.*, 1997; Snyderwine *et al.*, 2002). Macroscopic and histological features of some HCA induced tumors are shown in Figure 1.10 and Figure 1.11.

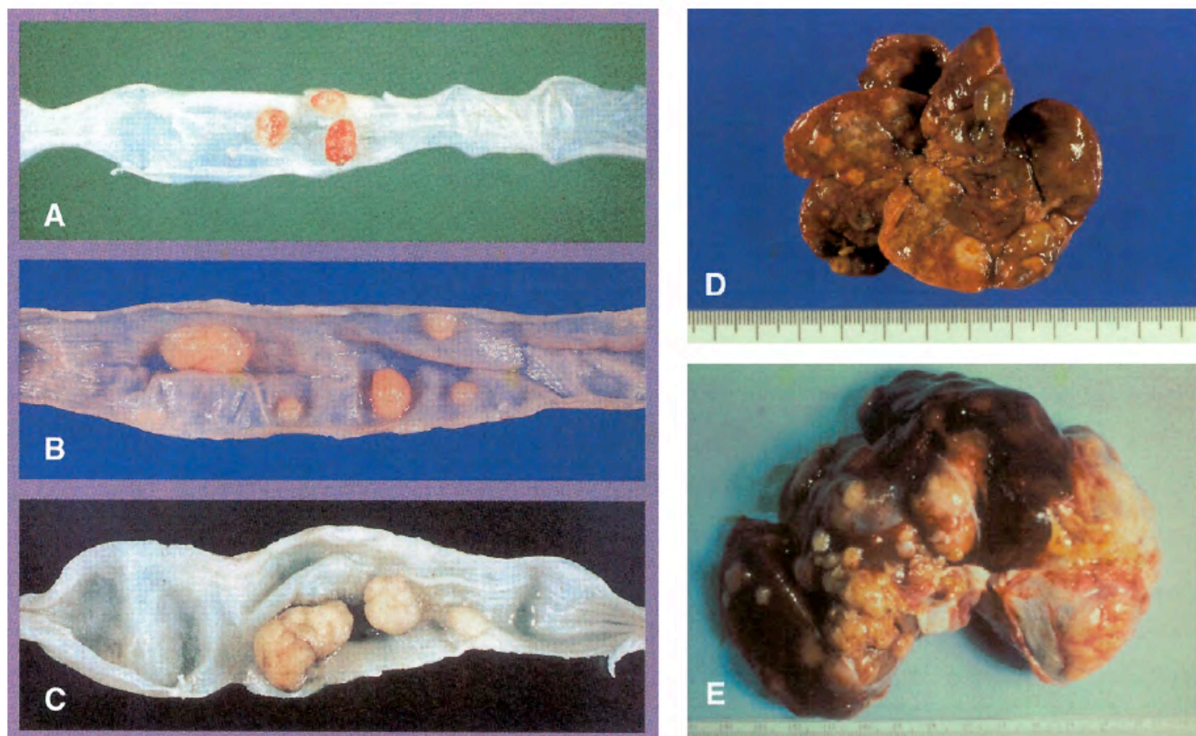

Figure 1.10 Macroscopic features of HCA-induced cancers in experimental animals. (A–C) Rat colon cancers induced by IQ (A), PhIP (B) and Glu-P-1 (C), respectively. (D and E) Liver cancers induced by MeIQx in rat (D) and by IQ in monkey (after: Sugimura *et al.*, 2004).

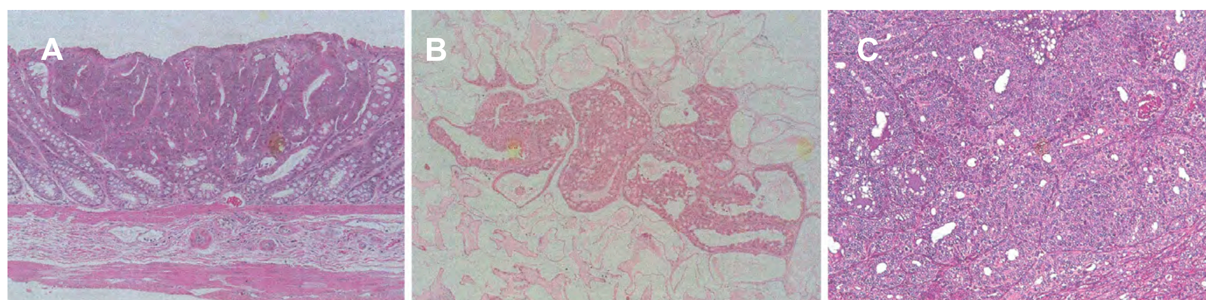

Figure 1.11 Histological features of PhIP-induced colon, prostate and mammary gland cancers in rats. (A) Colon cancer, (B) prostate cancer, (C) mammary gland cancer (after: Sugimura *et al.*, 2004).

Several studies have investigated the combined effects of HCAs in hepatocarcinogenesis. On the basis of preneoplastic foci induction by 10 HCAs, some HCAs may act in a synergistic manner and increase the effects observed over single compounds tested alone in the rat (Hasegawa *et al.*, 1996). Synergistic effects were also observed in the

small intestine and Zymbal gland, but not in other organs (Hasegawa *et al.*, 1994) Therefore, the synergism depends on the target tissue of the individual HCAs as well as the doses applied in combination. These findings may have relevance to humans since a number of HCAs are present in the diet and consumed simultaneously.

IQ, MeIQx and PhIP were assayed for carcinogenicity in cynomolgus monkeys (Adamson, 2000). IQ was reported to be a potent hepatocellular carcinogen inducing tumors in 70% of the monkeys at a dose of 10 mg/kg body weight and 100% of the monkeys at 20 mg/kg dose treated five times per week. MeIQx was also administered at both 10 and 20 mg/kg body weight but no evidence of neoplastic or preneoplastic lesions in any organs was observed. The striking difference in biological activity between these two structurally related HCAs may be attributed to the poor bioactivation of MeIQx in this species, which does not constitutively express hepatic P4501A2. Bioassays with PhIP were also conducted with the same dosing regimen. Pathological abnormalities attributed to PhIP were not observed (Adamson, 2000). Monkey liver was reported to activate PhIP to the genotoxic N-hydroxylamine metabolite and the isomeric N-glucuronide conjugates of 2-(hydroxyamino)-1-methyl-6-phenylimidazo[4,5-*b*]pyridine were detected in bile and urine of monkeys (Snyderwine *et al.*, 1997). Furthermore, significant levels of PhIP-DNA adduct formation were detected in liver and extrahepatic tissues (Snyderwine *et al.*, 1997). These biochemical data suggest that PhIP would be carcinogenic to this species if treated with the appropriate dose for a sufficient length of time (Adamson, 2000).

4.6. DNA adducts in humans

Several HCA-DNA adducts have been detected in human tissues. A GC-MS assay, based upon alkaline hydrolysis of putative dG-C8-HCA adducts to produce the parent HCAs, revealed the presence of PhIP in colorectal mucosae of several individuals at levels of up to several adducts per 10^8 DNA bases, when 100 μg DNA was used for analysis (Friesen *et al.*, 1994). Another study detected a base-labile adduct of PhIP, presumably dG-C8-PhIP, in long-lived lymphocytes of colorectal cancer subjects at levels of several adducts per 10^8 DNA bases, when 100 μg DNA was measured (Magagnotti *et al.*, 2003). This putative adduct was detected in about 30% of the population and the levels of adduct varied across a 10-fold range between the lowest and highest level, suggesting a different intake of PhIP or interindividual variation in bioactivation of PhIP. Two studies have reported the detection of DNA adducts of

PhIP in human breast tissue. The dG-C8-PhIP adduct was detected in exfoliated epithelial cells from milk of lactating mothers in 30 of the 64 samples analyzed, with a mean value of 4.7 adducts/10^7 nucleotides, through use of the ^{32}P-postlabeling method (Gorlewska-Roberts *et al.*, 2002). In another study, PhIP adducts, presumably dG-C8-PhIP, were detected, by an immunohistochemical method, in human breast tissues at levels of >1 adduct per 10^7 bases, in 82 and 71% of the normal breast tissue sections from the cancer and control patients, respectively (Zhu *et al.*, 2003).

The dG-C8-MeIQx adduct was also detected in colon and kidney DNA of several individuals at levels estimated up to several adducts per 10^9 DNA bases, by means of the ^{32}P-postlabeling assay (Totsuka *et al.*, 1996). The identities of the DNA adduct structures reported in these studies are equivocal. With the recent advances in the sensitivity of electrospray ionization mass spectrometry (LC-ESI/MS) instrumentation, it should be feasible to unambiguously characterize and quantitate HCA-DNA adducts in humans tissues at levels of modification of ~1 adduct per 10^8 DNA bases (Turesky and Vouros, 2004; Turesky, 2007).

4.7. Strategies to inhibit genotoxic and carcinogenic effects from HCAs

At present, data are available on approximately 600 individual compounds and complex mixtures that exhibit antimutagenic/anticarcinogenic effects towards HCAs. Complex mixtures include beverages, juices and homogenates from fruits and vegetables, spices and lactic acid bacteria. The individual compounds that were tested for protective properties are mainly plant-derived substances.

Many compounds and complex mixtures act in parallel at different levels. A typical example are green teas. Their chemopreventive properties towards HCAs include multiple mechanisms such as inhibition and induction of enzymes involved in the biotransformation of HCAs, scavenging of electrophilic metabolites and radicals and degradation of DNA reactive molecules (Dashwood, 2002). A schematic overview of the different modes of action that may lead to antimutagenic/anticarcinogenic effects and the most important compounds or mixtures known to exert these effects are listed in Table 1.11.

Table 1.11. Mechanisms of antimutagens and anticarcinogens.

Mechanism	Examples	References
Direct inactivation by binding and chemical reactions	Chlorophyllin and other pyrolle pigments; α-cellulose and fibers; Bacteria and their cell walls; Unsaturated fatty acids	Hernaez *et al.*, 1997; Waters *et al.*, 1996 Sugiyama *et al.*, 2002; Kato *et al.*, 1991; Kestell *et al.*, 2004 Knasmüller *et al.*, 2001 Hayatsu *et al.*, 1988 Sreekumar and Hosono, 2001
Enzymatic destruction	Peroxidases (myelperoxidase, lactoperoxidase, horseradish peroxidase, superoxide dismutase)	Hiramoto *et al.*, 1988
Inhibition of NADPH-cytochrome c reductase	Teas (green, black and decaffeinated)	Bu-Abbas *et al.*, 1996 Hasaniya *et al.*, 1997
Inhibition of CYP1A1/1A2 activity	Oleic acid; Flavonoids; Anthraquinones and anthraflavic acid; Phenethyl isothiocyanate; Retinol, β-carotene and α-tocopherol	Saito *et al.*, 1983 Bacon *et al.*, 2003 Bear and Teel, 2000 Edenharder *et al.*, 1998; 2002 Ferrer *et al.*, 2004 Mori *et al.*, 2005 Montgomery *et al.*, 2002
Reversion of the hydroxylamine to the parent compound	2,6-di-*tert*-butyl-8-hydroxy-di-benzofuran-1,4-quinone	Mizuno *et al.*, 1989
Direct inactivation of N-hydroxy-HCAs	Chlorophyllin and other pyrolle pigments; Epigallocatechin gallate; Constituents of beverages	Hayatsu *et al.*, 1988 Hernaez *et al.*, 1997 Arimoto-Kobayashi *et al.*, 1999; 2006
Induction of GST	Cafestol/kahweol palmitates and BITC;	Huber *et al.*, 1997; 2004
Inhibition of N-acetylation (NAT)	Epigallocatechin gallate; Cafestol/kahweol palmitates	Hernaez *et al.*, 1997 Huber *et al.*, 2004
Induction of MDR	Trifluoropertrazine	Ferguson and De Flora, 2005
Induction of glucuronidation	Teas	Santana-Rios *et al.*, 2001
Interaction with DNA-repair/replication	Caffeine/vanillin/coumarin; GeO_2 and $CoCl_2$	Sanyal *et al.*, 1997 Kada *et al.*, 1998
Interaction with post-initiation processes	Epigallocatechin gallate; White, green tea and caffeine	Cao and Cao, 1999 Carter *et al.*, 2007

5. Objectives of this research

In the last decades, evidence has accumulated that heterocyclic aromatic amines (HCAs), pyrolysis products of amino acids contained in meat and fish products, might play an important role in the etiology of several types of human cancers and strong efforts have been made to elucidate the metabolism and health hazards of these compounds. So far, most investigations focused on the activation and detoxification of HCAs by mammalian enzymes and several hundred biochemical studies have been carried out with mammalian cells, laboratory rodents, non human primates and man, whereas at the start of this research only a few, partly conflicting results from studies with lactobacilli and intestinal microorganisms were available. Informations on the bacterial metabolism of native heterocyclic amines were scarce and limited to some studies on the quinoline type heterocyclic amines IQ and MeIQ.

To the best of our knowledge, the aspect of microbial bioactivation potential for the pro-carcinogenic heterocyclic amine PhIP, has not yet been studied in depth. Therefore, the main objective of this work was to explore the possible role of the human intestinal microbiota in the metabolism and biological activity of PhIP. To do this, an integrated *in vitro*-*in vivo* approach has been programmed, combining fecal incubations, human studies and mammalian cell lines.

Subsequent to **Chapter 1**, which constitutes the overall scientific platform, the outline of the research can be summarized as follows:

Chapter 2 describes a first explorative study in which the *in vitro* metabolism of PhIP using batch cultures from human fecal samples is investigated. The most important finding of this study, i.e. the formation of one major PhIP derivate PhIP-M1 by the human intestinal microbiota, is then further explored in batch with focus on interindividual variability. Using a combination of LC-MS/MS, HRMS, 1D (^{1}H, ^{13}C, DEPT) and 2D (gCOSY, gTOCSY, gHMBC, gHSQC) NMR and IC analysis the complete chemical identity of the microbial PhIP metabolite is elucidated.

Chapter 3 describes the development and optimization of an analytical method using liquid chromatography tandem mass spectrometry for the detection and quantification of PhIP and its newly identified microbial metabolite PhIP-M1in human urine and fecal samples. This

method is subsequently applied on urine and feces samples from 6 human subjects that were fed 150 g of well-done chicken. In addition, the mutagenic activity of PhIP is analyzed using the *Salmonella typhimurium* strains TA98, TA100 and TA102.

Chapter 4 presents the isolation and identification of individual intestinal bacteria from human feces capable of transforming PhIP into its microbial derivate PhIP-M1. Representative culture collection strains isolated from the intestine are screened for their PhIP transformation potential and the nutritional requirements for microbial PhIP-M1 formation are clarified. In addition, the microbial and chemical mechanisms for this carcinogenic transformation are elucidated.

Chapter 5 focuses on the biological activity of the newly identified PhIP-M1 derivate. Using the epithelial intestinal Caco-2 cell line, the cytotoxic, apoptotic and genotoxic effects originating from PhIP-M1 are assessed. These cells were chosen as target since the exposure site to PhIP-M1 is the colon and because the colon is known to be one of the main target tissues for PhIP induced cancer.

Chapter 6 reports that supplementation of inulin, an extensively studied prebiotic compound, can also exert chemopreventive effects. More in particular, it will be shown that the PhIP bioactivation potency of the colon microbiota is largely inhibited by the indirect metabolic effects that inulin supplementation purports in the colon lumen.

Chapter 7 gives a general discussion of the different research chapters and delivers some take home messages. Additionally, some future research recommendations will be formulated.

CHAPTER 2

In vitro metabolism of the food associated carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine by human intestinal microbiota

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CHAPTER 2

In vitro metabolism of the food associated carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine by human intestinal microbiota

ABSTRACT

2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine is a putative human carcinogenic heterocyclic aromatic amine formed from meat and fish during cooking. Although the formation of hazardous PhIP metabolites by mammalian enzymes is well documented, nothing is known about the PhIP transformation potency of human intestinal bacteria. In this study, the *in vitro* metabolism of PhIP by human fecal samples was investigated. Following anaerobic incubation of PhIP with stools freshly collected from six healthy volunteers, we found that PhIP was extensively transformed by the human intestinal bacteria. HPLC analysis showed that the six human fecal microbiota transformed PhIP with efficiencies from 47 to 95% after 72 h incubation, resulting in one major derivative. ESI-MS/MS, HRMS, 1D (^{1}H, ^{13}C, DEPT) and 2D (gCOSY, gTOCSY, gHMBC, gHSQC) NMR and IC analysis elucidated the complete chemical identity of the microbial PhIP derivate, as 7-hydroxy-5-methyl-3-phenyl-6,7,8,9-tetrahydropyrido[3',2':4,5]imidazo[1,2-*a*]pyrimidin-5-ium chloride. At present, no information is available about the biological activity of this newly discovered bacterial PhIP metabolite. Our findings however suggest that bacteria derived from the human intestine play a key role in the activation or detoxification of PhIP, a digestive fate ignored so far in risk assessments. Moreover, the variation in transformation efficiency between the human microbiota indicates interindividual differences in the ability to convert PhIP. This may predict individual susceptibility to carcinogenic risk from this suspected dietary carcinogen.

1. Introduction

Cooked muscle meats, major components of the Western diet, contain potent genotoxic carcinogens belonging to the heterocyclic aromatic amine class of chemical compounds (Figure 2.1) (Nagao *et al.*, 1977). Of the 19 heterocyclic amines identified, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is frequently the most mass abundant heterocyclic amine produced during the cooking of beef, pork and chicken (Murray *et al.*, 1993; Sinha *et al.*, 1995; Skog *et al.*, 1997; Zimmerli *et al.*, 2001; Wong *et al.*, 2005; Busquets *et al.*, 2007). The highest levels of PhIP can be found in grilled or fried meats. In very well-done flame-grilled chicken up to 480 ng/g PhIP has been measured (Sinha *et al.*, 1995). The human intake of PhIP varies with food type and cooking conditions and is estimated to range from nanograms to tens of micrograms per day, depending on individual dietary and cooking preferences (Felton *et al.*, 1986a; Zimmerli *et al.*, 2001). Assessment studies based on rodent tumor data (Ito *et al.*, 1991; Shirai *et al.*, 1997; Norrish *et al.*, 1999; Knize and Felton, 2005) and the abundance of PhIP in the diet have indicated that this heterocyclic amine may be a risk factor in human colon, breast and prostate carcinogenesis (Imaida *et al.*, 1996; Ito *et al.*, 1991; 1997; Shirai *et al.*, 1997; Snyderwine, 2002).

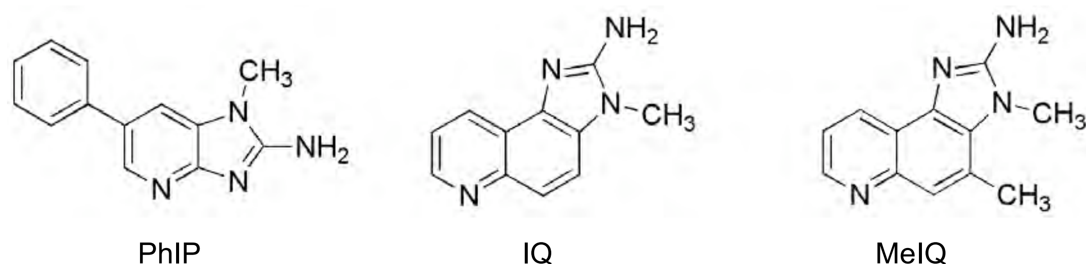

Figure 2.1 Chemical structures of heterocyclic aromatic amines.

As a means of determining the potential health risks associated with heterocyclic amines, several dietary studies have been conducted on the metabolism and disposition of these compounds in humans. So far, most investigations focused on the activation and detoxification of heterocyclic amines by mammalian enzymes. The genotoxic/carcinogenic effect of heterocyclic amines is closely related to a highly complex metabolism involving xenobiotic metabolizing enzymes generating very reactive metabolites as well as detoxified derivatives (Aeschbacher and Turesky, 1991). On the other hand, the involvement of the intestinal microbiota in the digestive fate of heterocyclic amines remains underinvestigated

(Knasmüller *et al.*, 2001). Recent research showed that the amount of PhIP metabolites excreted in the 0-24 h urine represented 17 ± 10% of the ingested PhIP in a meat matrix (Kulp *et al.*, 2004). In an earlier study with patients given PhIP in a capsule, 90% of the ingested dose was recovered in the urine (Malfatti *et al.*, 1999). This indicates that PhIP provided in capsule form is more bioavailable than PhIP ingested from meat. The non-bioavailable fraction reaches the colon intact to come there into contact with the resident microbiota. Direct binding of heterocyclic amines to the cell walls of intestinal bacteria has been reported and is currently considered as a detoxification mechanism since it prevents absorption of heterocyclic amines through the intestinal mucosa (Bolognani *et al.*, 2001; Turbic *et al.*, 2002). On the other hand, results of 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ)-induced genotoxicity assays in germ-free and conventional rodents showed that the presence of intestinal microbiota is essential for the induction of DNA-damage in the colon and liver cells (Hirayama *et al.*, 2000; Kassie *et al.*, 2001). These findings suggest that the intestinal microbiota play a significant role in the bioconversion of heterocyclic amines into harmful metabolites. Indications exist that hydrolysis of heterocyclic amine-glucuronides by bacterial β-glucuronidase may release mutagenic intermediates (Rumney and Rowland, 1992). Informations on the bacterial metabolism of native heterocyclic amines are however still scarce. Several researchers report that incubation of the heterocyclic amine IQ with mixed human feces in anaerobic conditions results in the formation of the hydroxy metabolite 7-OH-IQ (Bashir *et al.*, 1987; 1989; Carman *et al.*, 1988; Humblot *et al.*, 2005). The bacterial metabolism of the heterocyclic amine PhIP has to our knowledge not been investigated yet.

As the biological potency of PhIP-induced carcinogenicity is strongly dependent upon its digestive fate, a comprehensive understanding of the metabolism, mammalian, as well as microbial of this putative carcinogen, is essential for human risk assessment. Therefore, the focus of the present study was to investigate the role of the intestinal microbiota in the metabolism of PhIP. Interindividual differences occur with regard to the species composition and the metabolic activities of the human intestinal microbiota (Suau *et al.*, 1999). Therefore the bioconversion potential of fecal samples collected from different subjects was examined.

2. Material and methods

2.1. Chemicals

PhIP was purchased from Toronto Research Chemicals (Ontario, Canada). The constituents of the culture media, namely tryptone and yeast extract, were obtained from AppliChem (Darmstadt, Germany). All other chemicals were obtained from Sigma-Aldrich (Bornem, Belgium). The solvents for HPLC and LC-MS analysis were of HPLC grade and purchased from Acros Organics (Geel, Belgium).

2.2. Incubation Conditions

2.2.1 Collection and preparation of human fecal samples

Fecal samples were obtained from six healthy subjects (three males and three females) between the age of 20 and 35. Donors were on a Western-type diet and none had a history of digestive pathology nor had received antibiotics during 3 months prior to sample delivery. Fecal slurries of 20% (w/v) fresh fecal inocula were prepared by homogenizing the feces with phosphate buffered saline (0.1 M, pH 7), containing 1 g/L sodium thioglycolate as reducing agent. The particulate material was removed by centrifugation for 2 min at 500xg.

2.2.2 Incubation

All incubation experiments were performed in TY broth (tryptone 30 g/L, yeast extract 20 g/L, L-cysteine 0.5 g/L, pH 7.0). Fecal bacteria require anaerobic conditions (low redox potential) for growth. Therefore, resazurin (2 mg/L) was added as a redox indicator. A pink color indicated a redox potential higher then -80 mV, a colorless solution showed a redox potential below this limit, i.e. anaerobic. The redox potential in the large intestine typically ranges between -150 and -280 mV (Jonas *et al.*, 1999). The medium was autoclaved at 121 °C for 15 min. Prior to addition to the autoclaved growth medium in the incubation vessels, PhIP was dissolved in dimethyl sulfoxide (DMSO). The incubation volume was either 20 mL or 40 mL. Each batch culture consisted of 90% TY broth medium and 10% fecal inoculum in phosphate buffered saline. The batch cultures were added with PhIP dissolved in DMSO to give a final concentration of 1, 10, 100, 1000 mg/L and less than 5% DMSO (v/v). Each batch was sealed with butylrubber tops and anaerobiosis was obtained by flushing the flasks with N_2 during 15 cycles of 2 min each at 800 mbar overpressure and 900 mbar underpressure.

Cultures were incubated at 37 °C and 150 rpm for the duration of the experiment. Samples were taken at regular time intervals using syringes. All experiments were performed in triplicate. In order to assess the extent of bacterial transformation, a number of control samples were included in the experimental setup. Firstly, undosed fecal cultures were analyzed to serve as a negative control as they presumably do not contain PhIP. Secondly, an undosed fecal culture was autoclaved for 20 min at 121 °C and added with PhIP to ascertain that the disappearance of the substrate could be assigned to the metabolic activity of viable cells and not a passive adsorption on bacterial cell walls.

2.3. Chemical Analysis

2.3.1 Extraction Protocol

For HPLC and LC-MS analysis the PhIP parent component and its metabolite were extracted from the digests (1 mL sample) by performing a solid phase extraction using STRATA C_{18}-U cartridges (Phenomenex, Belgium). After centrifugation for 10 min at 7000 x g at 4 °C, the resulting supernatant was loaded onto a 200 mg C_{18}-U cartridge preconditioned with 3 mL each of acetonitrile, water and ammonium acetate (0.1 mM, pH 3.5). A vacuum manifold and an evaporation manifold (Alltech, Lokeren, Belgium) were used for manipulations with SPE cartridges and solvent evaporation, respectively. The cartridge was washed with 3 mL water and eluted with 3 mL ammonium acetate (0.1 mM, pH 3.5): acetonitrile (1:4) (v/v). The eluate obtained was dried under a N_2 stream, the residue reconstituted in 1 mL ammonium acetate (0.1 mM, pH 3.5):acetonitrile (1:4) (v/v), transferred into HPLC vials, and stored at 4 °C until analysis. The recovery of PhIP and its microbial metabolite using the latter protocol was determined in fecal digests at two concentrations, 1 and 100 mg/L and gave recoveries of 95 ± 1.3% for PhIP and its microbial metabolite. To further improve the recovery, DMSO (1.5%) was added to the ammonium acetate and acetonitrile mixture since DMSO is a very good solvent for PhIP. Although the recovery was better than using the ammonium acetate and acetonitrile mixture (approximately 99%), the evaporation of DMSO was difficult and, therefore, unsuitable for larger sample volumes or greater numbers of samples. For preparative separation and subsequent spectroscopic analysis the PhIP metabolites were extracted from the digests (40 mL sample) using a liquid-liquid extraction procedure. Prior to extraction the pH of the samples was adjusted to 9-10 with 10 mL 1 M Na_2CO_3. After extraction into ethyl acetate (3 x 25 mL), the samples were

centrifuged, and the combined organic phases were extracted with 3 x 25 mL 0.1 M HCl. PhIP and metabolites were recovered from the acidic solution by addition of 12.5 mL 1 M Na_2CO_3 and extraction with ethyl acetate (3 x 50 mL). After centrifugation and separation over a funnel to remove any remaining aqueous phases, the samples were taken to dryness at 50 °C by rotary evaporation.

2.3.2 Analytical HPLC

Samples were analyzed on a Dionex HPLC system (Sunnyvale, California, USA) comprising an autosampler ASI-100, a pump series P580 and a STH585 column oven, coupled to a UVD340S UV/VIS detector and a RF-2000 fluorescence detector. A 10 µL volume of the sample was injected and separated over a 150 x 4.6 mm i.d., 4 µm, Genesis C_{18} column (Jones Chromatography, UK). The temperature was set at 25 °C and the flow rate was maintained at 1 mL/min. Solvents were 0.01% formic acid (A) and acetonitrile (B). Solvent programming was isocratic 2% B during 2 min followed by a linear gradient to 40% B in 20 min. Absorbance was monitored at 315 nm; fluorescence was monitored at 316 nm (excitation) and 370 nm (emission). Data were collected and peaks integrated using the Chromeleon chromatography manager software (Dionex). Identification of PhIP was based on the identity of the retention time and the absorption spectrum with those of an authentic standard (Research Chemicals Inc.) and quantification was achieved using a standard curve from 1 ng/mL to 100 µg/mL. The detection limit for quantification of PhIP was 1 ng/mL for fluorescence and 1 µg/mL for absorbance detection, based on the criterion that the signal to noise ratio should be > 3 for quantification purposes. Relative productions of the microbial PhIP metabolite over time and between samples could be compared by integrating the peak areas. Quantification of the PhIP metabolite was achieved using a standard curve obtained after preparative separation and purification of the metabolite.

2.3.3 LC-MS^n

The HPLC apparatus comprised of a P4000 quaternary pump and an AS3000 autosampler (Thermo Finnigan, San Jose, CA, USA). Chromatographic separation was achieved using a 150 x 3 mm i.d., 5 µm, Zorbax SB-C3 column obtained from Agilent Technologies (Diegem, Belgium). The mobile phase consisted of a mixture of acetonitrile (A) and water with 0.01% formic acid (B). A linear gradient was run from 2% A for 2 min, increasing to 40% A over 20 min and maintaining 40% A for 8 min, and finally increasing to

100% A in the minute at a flow rate of 0.3 mL/min. The analysis was performed using a LCQ Deca ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an Electrospray Ionisation (ESI) interface. Both positive and negative ion modes were used but only the positive ion mode allowed observing PhIP and metabolite peaks. To perform MS^2 and MS^3, the precursor isolation width was set to 2 Da, the activation Q to 0.35 and the collision energy to 45%.

2.3.4 Preparative HPLC

Preparative separation was performed on a Gilson preparative HPLC system (Gilson International B.V., Middleton, United States) comprising a H322 pump system and a 206 fraction collector, coupled to a model 156 UV/VIS detector. Chromatographic separation was achieved using an Omnisphere 250 x 21.4 mm i.d., 10 µm, C_{18} column obtained from Varian (St.-Katelijne-Waver, Belgium). Compounds were eluted by an isocratic solvent mixture containing 85% water with 0.05% formic acid and 15% acetonitrile with 0.05% formic acid, the flow rate was 20 mL/min. Absorbance was monitored at 307 nm.

2.3.5 HRMS

High-resolution mass spectra (HRMS) were recorded on a Finnigan MAT 95 XP-API-GC-Trap Tandem Mass Spectrometer (Thermo Finnigan, Bremen, Germany). ESI-MS was performed in the positive mode under the following operating parameters: probe voltage, 3 kV; capillary temperature, 250 °C. The mobile phase consisted of a mixture of acetonitrile and water with 0.1% formic acid (50:50) (v/v) at a flow rate of 50 µL/min. PEG 200/300 (2.5 ng/µL sample) was used as internal standard.

2.3.6 NMR analysis

NMR spectra were recorded at 298.1 K using a Varian Mercury 300 spectrometer equipped with a 5 mm PFG-probe, observing ^{1}H at 300.0 and ^{13}C at 75.4 MHz. The compound was dissolved in 1 mL of DMSO-d_6 and transferred to a 5 mm NMR tube. All chemical shifts are expressed in ppm relative to TMS for ^{1}H spectra (δ 0 ppm) and DMSO-d_6 for ^{13}C spectra (δ 39.52 ppm). The ^{1}H NMR spectra were acquired using 128 transients, with spectral widths of 4803.1 Hz and digitized with 32 K data points. For ^{13}C NMR spectra 12000 transients were recorded and a spectral width of 18867.9 Hz digitized with 128 K points was

used. Relaxation delays were set to 1 s, and a 45° excitation pulse was used. DEPT-45°, DEPT-135°, DEPT-90° experiments were performed to distinguish methyl, methylene, methine and quaternary carbon resonances. For ^{13}C NMR spectra a line broadening of 1 Hz was applied during processing. Gradient enhanced ^{1}H - ^{1}H COSY and TOCSY correlation experiments were performed through standard pulse sequences, as suggested by the manufacturer. The gCOSY was performed using a spectral width of 4.8 kHz and 2K data points with 8 transients for each of the 200 t_1 increments. The gTOCSY was performed using a spectral width of 4.8 kHz, 2K data points and a mixing time of 80 ms with 32 transients for each of the 256 t_1 increments. Data were multiplied by a sine bell function in both dimensions and transformed into the frequency domain as a 2048 x 2048 data matrix. The one-bond ^{1}H - ^{13}C correlation experiments were acquired using the manufactures gradient HSQC pulse program with spectral width of 4.8 kHz in f_2 and 12.8 kHz in f_1 (32 transients, 2 K data points, and 512 t_1 increments). Data were multiplied by a Gaussian function in both dimensions and transformed into the frequency domain as an 8192 x 2048 data matrix. The long-range ^{1}H - ^{13}C correlation experiments were recorded using the manufactures gradient HMBC pulse sequence with spectral width of 4.8 kHz in f_2 and 18.1 kHz in f_1 (32 transients, 2 K data points, and 512 t_1 increments) and an evolution delay of 62.5 ms (J(C,H) = 8 Hz). Data were multiplied by a sine bell function in both dimensions and transformed into the frequency domain as a 2048 x 2048 data matrix.

2.3.7 IC analysis

The anionic counterpart of the microbial PhIP metabolite was determined using a Metrohm 761 Compact Ion Chromatograph (Metrohm, Herisau, Switzerland) equipped with a conductivity detector. The operational parameters were as follows: column, Metrosep A supp 5; eluent, 1.06 g/L Na_2CO_3; flow, 0.7 mL/min; sample loop, 20 µL.

3. Results

3.1. Microbial conversion of PhIP by human feces

3.1.1 Incubation of PhIP with human fecal samples

The capacity of the microbial cultures obtained from six human stool samples to transform the food carcinogen PhIP was tested by incubating the cultures with 1 mg/L PhIP for a period of 3 days (Figure 2.2).

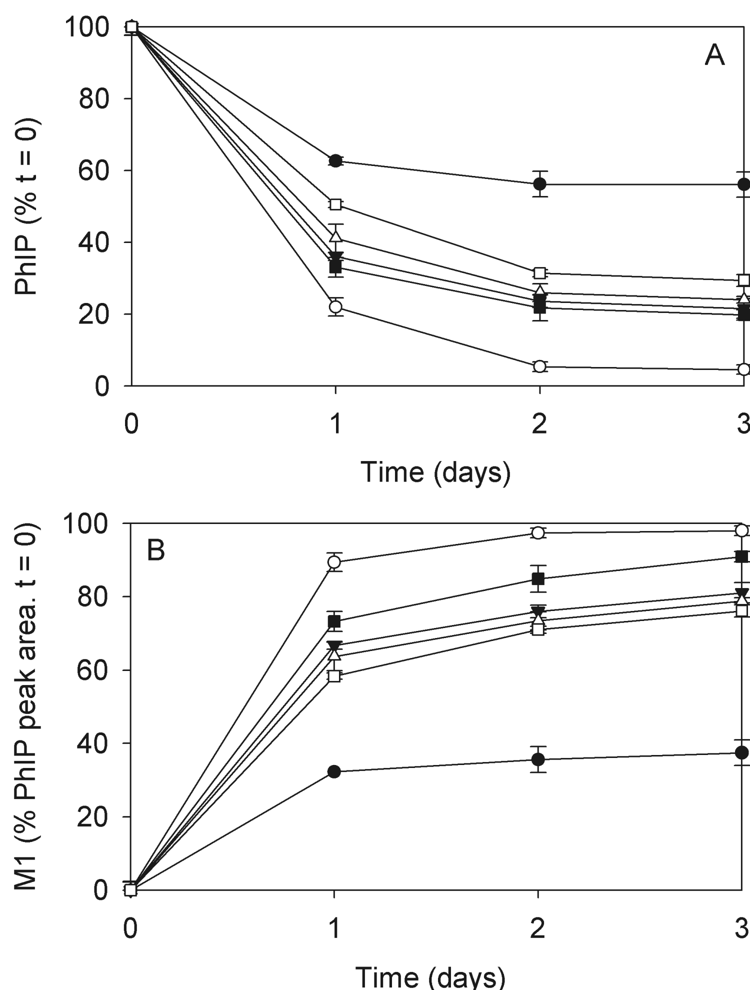

Figure 2.2 PhIP degradation (A) and formation of its microbial metabolite PhIP-M1 (B) in cell suspensions derived from six human stools (▼, □, ∇, ●, ○, ■). PhIP initial concentration was 1 mg/L. PhIP and metabolite concentrations were determined by HPLC analysis and presented as average (+SD) percentage of the PhIP peak area at day 0 (n=3).

All six human feces transformed PhIP, though with different efficiencies. Indeed the fraction of PhIP degraded over 72 h ranged from 47 to 95% of the initial quantity for the low- and high-degrading microbiota respectively. The formation of one metabolite (further referred to as PhIP-M1) accompanied PhIP degradation in each fecal incubation experiment (Figure 2.3).

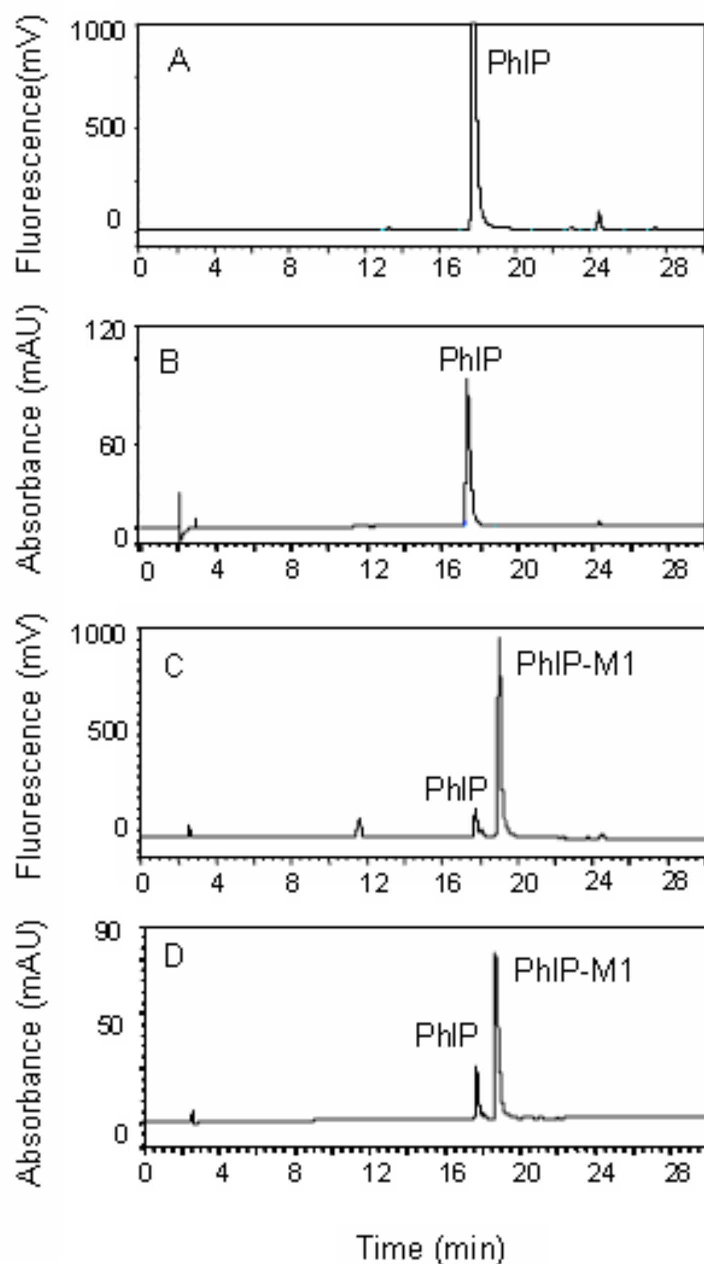

Figure 2.3 HPLC chromatograms with fluorescence (A, C) and absorbance (B, D) detection of PhIP and its metabolite PhIP-M1 produced by the human intestinal microbiota. (A, B) Standard 10 ng and 500 ng PhIP. (C, D) Metabolic products of PhIP incubated with human intestinal microbiota for 3 days. Initial incubation concentration was 10 mg/L PhIP.

This metabolite peak was not observed upon incubation of undosed fecal cultures, confirming its PhIP origin. Interindividual differences between the kinetics of metabolite formation paralleled those between the kinetics of PhIP transformation. This resulted in a

time dependent increase of 55 to 98% of the metabolite peak area relative to the initial PhIP peak area at day 0. Upon incubation of PhIP with fecal material that was inactivated prior to incubation, no decrease in PhIP concentration or metabolite formation was observed.

3.1.2 Characterization of the PhIP metabolism by human fecal cultures

The data obtained from Figure 2.2 showed that the capacity of the human microbiota to transform PhIP varied with the origin of the fecal sample. Yet, the majority of the fecal microbiota belonged to the intermediate-degrading category. Therefore further investigation of the PhIP transformation was performed with an intermediate-degrading fecal culture. To thoroughly screen for microbial PhIP metabolite production, a 12 h experiment was performed during which unprocessed incubation medium was sampled every hour and analyzed by HPLC with fluorescence and UV detection (Figure 2.4).

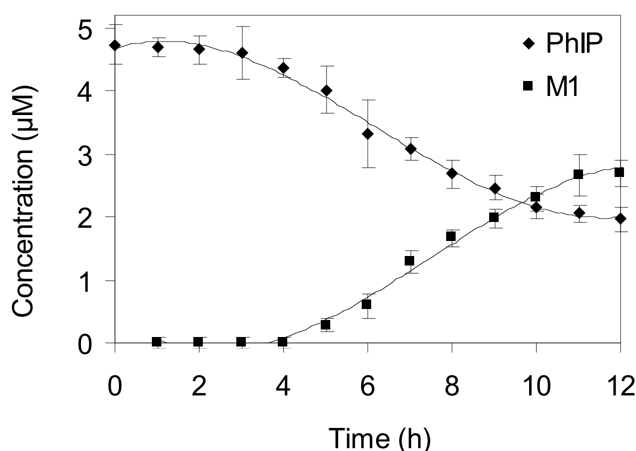

Figure 2.4 Kinetics of PhIP transformation and metabolite formation in cell suspensions derived from human feces. Results are presented as average (+SD) concentrations of PhIP and the microbial PhIP metabolite (n=3).

This approach allowed the formation of solely one transformation product to be observed. The increase in concentration of this metabolite paralleled the decrease in PhIP concentration in a time dependent manner. Subsequent experiments were conducted using five different incubation concentrations of PhIP ranging from 1 to 1000 mg/L for a period of 3 days. Again only one PhIP metabolite could be observed and the transformation occurred with a conversion efficiency of 80 ± 2% regardless of the initial concentration of PhIP.

3.2. *Chemical identification of microbial PhIP metabolite*

3.2.1 HPLC analysis of human feces incubated with PhIP

When PhIP was incubated with microbial cultures derived from human feces, one microbial PhIP metabolite could be observed by HPLC with fluorescence (Figure 2.3 B) and absorbance (Figure 2.3 D) detection. The elution profile of the metabolic products included PhIP at 17.77 min and the PhIP metabolite PhIP-M1 at 19.06. These products showed distinct absorbance maxima: PhIP (204, 227 and 316 nm), PhIP-M1 (205, 228 and 307 nm) and fluorescence excitation maxima: PhIP (316 nm), PhIP-M1 (312 nm).

3.2.2 MS^n analysis of human feces incubated with PhIP

In evaluating the chemical structure of the microbial PhIP metabolite, the LC-MS^n mass spectra in ESI positive ion mode of a 3-day incubation extract were recorded. In MS-full scan, the pseudo-molecular ions with *m/z* 225 and *m/z* 281 appeared at the respective retention times 17.77 and 19.06 min. MS^2-full scan of the pseudo-molecular ion *m/z* 225 showed the product ion with *m/z* 210. Fragmentation of this product ion gave rise to a fragment at *m/z* 183 and 168. MS^2-full scan of the pseudo-molecular ion *m/z* 281 showed the product ions with *m/z* 263 and *m/z* 225. Fragmentation of the most mass abundant product ion *m/z* 263 derived from PhIP-M1 showed fragments at *m/z* 248, *m/z* 236, *m/z* 222 and m/z 210.

3.2.3 HRMS analysis

The exact molecular formula of the PhIP metabolite PhIP-M1 was determined by recording the high-resolution mass spectrum of a sample containing 5 µg/µL of PhIP-M1, purified by preparative HPLC. A mass was measured of 281.1398 corresponding with the theoretical mass of 281.13969 and molecular formula of $C_{16}H_{17}N_4O$.

3.2.4 NMR analysis

Sufficient quantities of the PhIP metabolite PhIP-M1 for NMR analysis were obtained by incubating 20 mg PhIP in 40 mL batch culture for 5 days. Purification of the PhIP metabolite extract was achieved by preparative HPLC. Ca. 8.9 mg of the major PhIP metabolite (99.2% purity by LC-MS/MS) was obtained by this approach. For the complete and unambiguous assignment of all ^1H and ^{13}C chemical shifts and coupling constants of the

PhIP metabolite PhIP-M1, a combination of two-dimensional gCOSY, gHSQC and gHMBC experiments were acquired in DMSO-d_6. These data are summarized in Table 2.1.

Table 2.1. ^{1}H and ^{13}C NMR chemical shifts, δ(ppm), multiplicities and coupling constants, $J(^{1}H, ^{1}H)$(Hz), ^{1}H - ^{1}H and ^{1}H - ^{13}C correlations in respectively, gCOSY and gHMBC for the microbial PhIP metabolite PhIP-M1 in DMSO-d_6.

Position	δ(^{13}C)	H	δ(^{13}H)	Multi-plicity[a]	*J*(Hz)	gCOSY	gHMBC
2	147.7	-	-	-	-	-	-
13-NH	-	NH	10.81 (1H)	br s	-	H-12	-
5	141.3	5	8.63 (1H)	d	1.9	H-7	C-7,9,1’
7	116.6	7	8.38 (1H)	d	2.0	H-5	C-5,6,8,9
6	136.9	-	-	-	-	-	-
8	124.6	-	-	-	-	-	-
9	141.9	-	-	-	-	-	-
N-CH_3	29.7	CH_3	3.78 (3H)	s	-	-	C-2,8
1’	132.1	-	-	-	-	-	-
2’	127.1	2’	7.81 (1H)	d	7.3	H-3’	C-1’,3’,4’,5’,6’
3’	129.2	3’	7.54 (1H)	t	7.3	H-2’,4’	C-6,5’
4’	128.1	4’	7.52 (1H)	t	7.3	H-,3’,5’	C-2’,6’
5’	129.2	5’	7.54 (1H)	t	7.3	H-4’,6’	C-6,3’
6’	127.1	6’	7.81 (1H)	d	7.3	H-,5’	C-1’,2’,3’,4’,5’
10	34.8	10a	4.43 (1H)	ddd	12.6, 2.6, 2.6	H-10b,11b	-
		10b	4.04 (1H)	td	12.3, 4.4	H-10a,11b	
11	26.9	11a	2.18-2.27 (1H)	m	-	H-11b,12	-
		11b	1.99-2.12 (1H)	m	-	H-11a,10a,10b	-
12	71.2	12	5.37 (1H)	dd	5.3, 2.6	OH, NH, H-11a	-
12-OH	-	OH	7.00 (1H)	d	5.3	H-12	-

[a] br s: broad singlet, d: doublet, t: triplet, m: multiplet

DEPT analysis showed one methyl group, two methylene and eight methine groups; the ^{13}C-NMR spectrum revealed five quaternary carbons. These groups accounted for 15 of the 17 protons seen in the ^{1}H spectrum. The missing hydrogens, bound to hetero atoms, were identified as a hydroxyl group and a secondary amine thus being in agreement with the molecular formula of $C_{16}H_{17}N_4O$. The odd mass and the presence of four nitrogens showed that the molecule was protonated. The additional unsaturation in the PhIP metabolite must be due to the formation of an extra ring. All proton and carbon resonances of the PhIP template

could be unambiguously assigned using gCOSY, gHSQC and gHMBC and were in agreement with data reported on PhIP (Felton *et al.*, 1986a; Collins *et al.*, 2002). In PhIP-M1, the carbons at positions 2 and 9 were significantly shifted upfield from δ 158.7 and δ 157.0 to δ 147.7 and δ 141.9, respectively, suggesting that the new ring was fused to the imidazole. The alcohol (12-OH) appeared as a doublet at δ 7.00 ppm and the secondary amine (NH-13) as a broad singlet at δ 10.81 ppm. Analysis of the gCOSY spectrum showed correlation of these two signals with a methine signal at δ 5.37 (H-12), which led to the identification of a hemi-aminal. In addition three new carbon resonances were present in PhIP-M1 at δ 71.2 (C-12), δ 26.2 (C-11) and δ 34.8 (C-10), correlating with signals at δ 5.37 (H-12), δ 1.99-2.12 (H-11b), δ 2.18-2.27 (H-11a), δ 4.04 (H-10b) and δ 4.43 (H-10a). The gCOSY and gTOCSY spectra confirmed that these three groups were adjacent in the non-aromatic heterocyclic ring. This spin system terminates at one end as a hemi-aminal group and ends at the other edge at a nitrogen atom. The hemi-aminal is derived from the primary amine in PhIP and the other end of the new moiety is necessarily attached to N-3, otherwise the imidazole would be deconjugated and aromaticity would be lost.

The anionic part of PhIP-M1 was determined using ion chromatography. IC analysis of 1.6 mmol/L of the purified PhIP-M1 metabolite corresponded with an equivalent concentration of chloride. Consequently the metabolite PhIP-M1 was assigned as 7-hydroxy-5-methyl-3-phenyl-6,7,8,9-tetrahydropyrido[3’,2’:4,5]imidazo[1,2-*a*]pyrimidin-5-ium chloride as depicted in Figure 2.5.

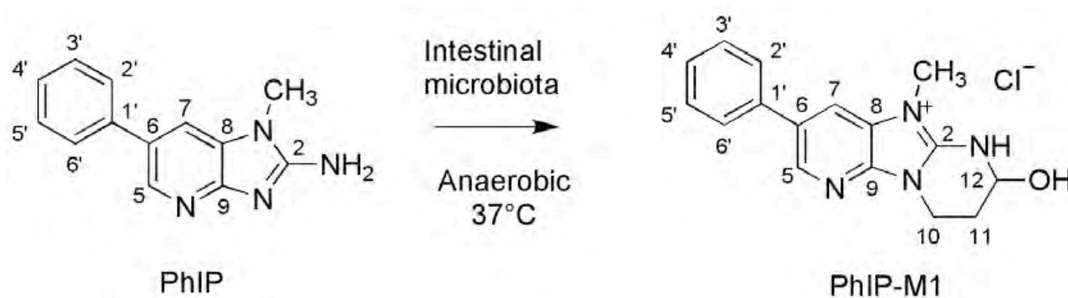

Figure 2.5 Molecular structure of PhIP and its microbial metabolite 7-hydroxy-5-methyl-3-phenyl-6,7,8,9-tetrahydropyrido[3’,2’:4,5]imidazo[1,2-*a*]pyrimidin-5-ium chloride. C-atom numbering for PhIP-M1 refers to the respective numbering of the PhIP parent compound.

4. Discussion

In the present study, we have shown that intestinal microorganisms derived from human feces actively transform the food carcinogen PhIP, resulting in the formation of one major metabolite. We elucidated the chemical structure of the microbial PhIP metabolite by a combination of mass spectrometric and NMR spectroscopic evidence. Moreover, we investigated the interindividual variation in PhIP metabolism between six human microbiota and the kinetics at different PhIP incubation concentrations.

Like many other environmental carcinogens, PhIP requires metabolic activation to exert toxic effects. Previous studies indicate that PhIP is converted into two primary products: 2-hydroxyamino-PhIP (N^2-OH-PhIP) and 4'-hydroxyamino-PhIP (4'-OH-PhIP), the former being highly mutagenic, and the latter being non-mutagenic (Crofts *et al.*, 1997; Turesky, 2002). These metabolites may subsequently be conjugated with acetyl, glucuronide, glutathione or sulphate to form secondary phase II metabolites. According to literature, the biotransformation of PhIP is highly dependent upon the cytochrome P4501A2 isozyme, mainly expressed in the liver (Crofts *et al.*, 1998). However, the liver is not the only transformation site inside the human body. The human colon contains ~10^{12} microorganisms/cm^3, with an enormous metabolic potential. Bacterial enzymes catalyze many reactions including hydrolysis, dehydroxylation, demethylation, ring cleavage and carboxylation (Ilett *et al.*, 1990). Numerous findings show that intestinal microorganisms and lactobacilli contained in dairy products play a key role in the activation and detoxification of various classes of DNA-reactive carcinogens such as nitrosamines, aflatoxins, polycyclic aromatic hydrocarbons, azo compounds, nitroarenes and glycosides (Rowland and Grasso, 1975; Oatley *et al.*, 2000; Knasmüller *et al.*, 2001; Wang *et al.*, 2004; Decroos *et al.*, 2005; Van de Wiele *et al.*, 2005). Our results confirm a similar microbial activity towards the food carcinogen PhIP, since it can be converted by the intestinal microbiota as well.

While PhIP is biotransformed into a large number of derivatives in the liver, the human intestinal microbiota selectively converted PhIP into one major metabolite. By analyzing crude incubation media by HPLC with fluorescence detection, we can assert that the PhIP derivative observed is unambiguously the only metabolite produced by bacterial conversion and rule out the possibility that other derivates have been released, yet not recovered in the extract. HPLC with fluorescence detection is a highly sensitive and powerful analytical tool

for providing quantitative information on fluorescent compounds in complex biological media (Pais and Knize, 2000; Ristic *et al.*, 2004). Synchronous absorbance and fluorescence spectroscopic analysis of PhIP and its microbial metabolite PhIP-M1 revealed a decrease in wavelength of both absorbance and fluorescence excitation maxima for the PhIP derivative compared to its precursor, indicating an alteration at the primary amine function or imidazo moiety. Crofts *et al.* (1998) measured the fluorescence intensity for PhIP and the phase I liver metabolites and observed a decrease in fluorescence excitation maxima upon hydroxylation of the primary amine, whereas hydroxylation of the phenyl substituent caused an increase in fluorescence maxima. Mass spectrometry gave a molecular ion at *m/z* 281 $[M + H]^+$ indicating that a fragment of 56 mass units had been added to PhIP (*m/z* 225 $[M + H]^+$). Loss of water from the molecule ion refered to the presence of a hydroxyl group. High resolution mass spectrometry revealed the exact molecular mass 281.1398 and molecular formula $C_{16}H_{17}N_4O$. Further elucidation of the chemical identity of the microbial PhIP metabolite was achieved by careful analysis and interpretation of the 1D and 2D NMR and IC data, assigning the metabolite as 7-hydroxy-5-methyl-3-phenyl-6,7,8,9-tetrahydropyrido[3’,2’:4,5]imidazo-[1,2-*a*]pyrimidin-5-ium chloride (Figure 2.5).

Up to now, data regarding the microbial transformation of heterocyclic amines are scarce. Only for the quinolines IQ and MeIQ (2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline) has it been reported that incubation with human fecal microbiota resulted in the formation of stable hydroxy metabolites (Bashir *et al.*, 1987; Carman *et al.*, 1988; Vantassell *et al.*, 1990). The microbial metabolism of PhIP shows however no resemblance to that of IQ and MeIQ. One possible explanation for this discrepancy is the protective effect of the phenyl substituent of PhIP, thereby impairing hydroxylation on the imidazo moiety. Several reports however emphasize the crucial role of the intestinal bacteria in the genotoxicity of heterocyclic amines (Kassie *et al.*, 2001; Knasmüller *et al.*, 2001), implying cleavage of glucuronide-conjugates as the most important mechanism by which intestinal bacteria activate heterocyclic amines. In contrast, bacteria in fermented foods and dairy products are known to detoxify these heterocyclic amines by direct binding to the cell walls (Bolognani *et al.*, 1997; Knasmüller *et al.*, 2001). Moreover, overall health effects may result from a combination of microbial interactions with multiple and perhaps additive or interfering activities. The impact of microbial transformations on the carcinogenicity of heterocyclic amines, entering the colon in their native form, remains underinvestigated. Our results indicate that microbial

transformation of PhIP causes an increase in hydrophobicity for the metabolite, thereby facilitating its absorption from the colon to exert potential biological activity inside the human body. Research has shown that the human colonic mucosa generally has a higher permeability to hydrophobic compounds than the small intestinal mucosa (Ungell *et al.*, 1998; van der Bijl and van Eyk, 2003). Further *in vivo* studies are warranted to acquire insight into the bioavailability and biological activity of this newly discovered PhIP metabolite throughout the intestine. However, as the efficiencies of the fecal samples to degrade PhIP ranged from 47 to 95%, interindividual variability in the microbial community and activity could strongly influence the individual exposure to this dietary carcinogen. Interindividual differences in microbial metabolic activities are not uncommon. A striking example is the microbial conversion of the dietary phytoestrogen daidzein (Decroos *et al.*, 2005; Wang *et al.*, 2005). Intensive research has shown that only approximately one third of humans harbour an intestinal microbiota capable of transforming daidzein into equol (Rowland *et al.*, 2000). A similar interindividual variability in microbial transformation has been shown for the group of the prenylflavonoids as well (Possemiers *et al.*, 2005).

In conclusion, by converting PhIP into 7-hydroxy-5-methyl-3-phenyl-6,7,8,9-tetrahydropyrido[3',2':4,5]imidazo[1,2-*a*]pyrimidin-5-ium chloride, human intestinal microbiota would contribute to the bioactivation or detoxification of a putative food-borne carcinogen. As a significant fraction of the daily exposure of PhIP is suggested to reach the colon in its native form, this biotransformation potency has to be considered when estimating the risks related to fried meat ingestion. Moreover, we showed interindividual differences in the microbial PhIP transformation, which may predict individual differences in susceptibility to the risks associated with this suspected dietary carcinogen.

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CHAPTER 3

Intestinal bacteria metabolize the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine following consumption of a single cooked chicken meal in humans

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CHAPTER 3

Intestinal bacteria metabolize the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine following consumption of a single cooked chicken meal in humans

ABSTRACT

2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is a carcinogenic heterocyclic amine formed in meats during cooking. Although the formation of PhIP metabolites by mammalian enzymes has been extensively reported, the involvement of the intestinal bacteria remains unclear. This study examined the urinary and fecal excretion of a newly identified microbial PhIP metabolite 7-hydroxy-5-methyl-3-phenyl-6,7,8,9-tetrahydropyrido[3',2':4,5]imidazo[1,2-*a*]pyrimidin-5-ium chloride (PhIP-M1) in humans. The subjects were fed 150 g of cooked chicken containing 0.88-4.7 µg PhIP, and urine and feces collections were obtained during 72 h after the meal. PhIP-M1 and its trideuterated derivate were synthesized and a LC-MS/MS method was developed for their quantification. The mutagenic activity of PhIP-M1, as analyzed using the *Salmonella* strains TA98, TA100 and TA102, yielded no significant response. Of the ingested PhIP dose, volunteers excreted 12-21% as PhIP and 1.2-15% as PhIP-M1 in urine, and 26-42% as PhIP and 0.9-11% as PhIP-M1 in feces. The rate of PhIP-M1 excretion varied among the subjects. Yet, an increase in urinary excretion was observed for successive time increments, whereas for PhIP the majority was excreted in the first 24 h. These findings suggest that besides differences in digestion, metabolism and diet, the microbial composition of the gastrointestinal tract also strongly influences individual disposition and carcinogenic risk from PhIP.

1. Introduction

Diet is a major risk factor in human cancer (Doll and Peto, 1981). Epidemiological studies indicate that the consumption of cooked meat and meat products predisposes individuals to neoplastic disease, particularly of the colon (Deverdier *et al.*, 1991; Doll, 1992). Dietary factors which may be important in the etiology of human cancer include heterocyclic amines (Felton *et al.*, 1986b). Of the 19 heterocyclic amines identified, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is the most mass abundant heterocyclic amine produced during the cooking of beef, pork and chicken (Felton *et al.*, 1986a; Murray *et al.*, 1993; Sinha *et al.*, 1995; Wong *et al.*, 2005). The highest levels of PhIP can be found in grilled or fried meats. In very well-done flame-grilled chicken PhIP can be found at levels up to 480 ng/g (Sinha *et al.*, 1995). The human intake of PhIP varies with food type and cooking conditions and is estimated to range from nanograms to tens of micrograms per day, depending on individual dietary and cooking preferences (Layton *et al.*, 1995; Zimmerli *et al.*, 2001). Experimentally, PhIP is a potent mutagen and genotoxin and has been shown to produce mammary gland, prostate and colon tumors in rats (Ito *et al.*, 1991; Shirai *et al.*, 1997; Sugimura, 2000). In humans, less is known about the potential role of PhIP and related heterocyclic amines in tumor development. Several studies have shown that individuals who eat well-done meat have an elevated risk of breast (Zheng *et al.*, 1998) and colorectal (Sinha, 1999; Gunter *et al.*, 2005) cancers. Not all studies have shown a positive correlation, however (Augustsson *et al.*, 1999).

Until recently, studies of human PhIP metabolism mainly focused on the activation and detoxification of heterocyclic amines by mammalian enzymes. PhIP must first be metabolized via Phase I and Phase II enzymes to exert its mutagenic and carcinogenic effect. This involves an initial cytochrome P4501A2 (CYP1A2) catalyzed N-hydroxylation step, to form N^2-hydroxy-PhIP. N^2-hydroxy-PhIP, which is mutagenic on its own, can be converted to a more biologically reactive form via Phase II metabolizing enzymes, to electrophilic *O*-sulfonyl and *O*-acetyl esters which have the capacity to bind DNA and cellular proteins (Buonarati *et al.*, 1991; Boobis *et al.*, 1994; Edwards *et al.*, 1994). Detoxification primarily involves glucuronidation. N^2-hydroxy-PhIP can form stable glucuronide conjugates at the N^2 and N^3 positions, which can be excreted or transported to extra-hepatic tissue for further metabolism (Alexander *et al.*, 1991; Kaderlik *et al.*, 1994). PhIP can also be hydroxylated at the 4’position. 4’-Hydroxy-PhIP can be conjugated by sulfation and glucuronidation to polar

compounds that are readily excreted (Watkins *et al.*, 1991; Buonarati *et al.*, 1992). In addition, the parent compound can be directly glucuronidated at the N^2 and N^3 positions. These glucuronides are not reactive and therefore considered as detoxification products (Styczynski *et al.*, 1993; Kaderlik *et al.*, 1994).

Recent research has shown that the amount of PhIP metabolites excreted in the 0-24 h urine represented 17 ± 10% of the ingested PhIP in a meat matrix (Kulp *et al.*, 2004). In an earlier study with patients given PhIP in a capsule, 90% of the ingested dose was recovered in the urine (Malfatti *et al.*, 1999). This indicates that PhIP provided in capsule form is more bioavailable than PhIP ingested from meat. The non-bioavailable fraction reaches the colon in an intact form to come into contact with the resident microbiota. Direct binding of heterocyclic amines to the cell walls of intestinal bacteria has been reported and is currently considered as a detoxification mechanism since it prevents absorption of heterocyclic amines through the intestinal mucosa (Bolognani *et al.*, 1997; Turbic *et al.*, 2002). However, little has been done to characterize PhIP metabolism by the human intestinal microbiota, although our early work examined the *in vitro* transformation of PhIP by human fecal cultures (Vanhaecke *et al.*, 2006). The latter study identified one major microbial PhIP metabolite, namely 7-hydroxy-5-methyl-3-phenyl-6,7,8,9-tetrahydropyrido[3',2':4,5]imidazo[1,2-*a*]pyrimidin-5-ium chloride (PhIP-M1) (Figure 3.1).

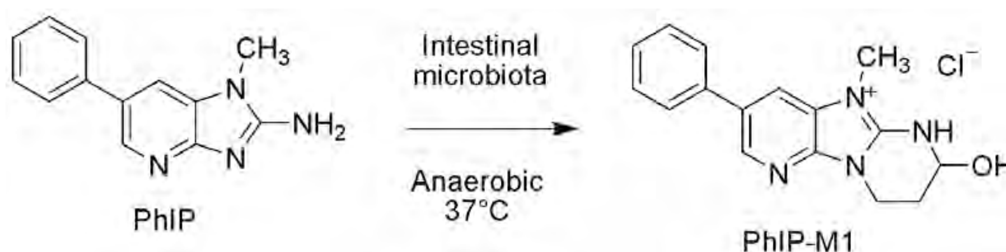

Figure 3.1 Metabolite of PhIP formed by the human intestinal microbiota: 7-hydroxy-5-methyl-3-phenyl-6,7,8,9-tetrahydropyrido[3',2':4,5]imidazo[1,2-*a*]pyrimidin-5-ium chloride.

Currently, there is no information available about the biological activity and *in vivo* formation of this newly discovered bacterial PhIP metabolite. Therefore the focus of the present study was to investigate the role of the intestinal microbiota in the metabolism of PhIP, following consumption of a single cooked chicken meal in humans. A solid phase

extraction LC-MS/MS method was developed for quantifying PhIP and PhIP-M1 in human urine and feces. We applied this method to characterize microbial PhIP metabolism in six healthy adults receiving a known dose of naturally produced PhIP. In addition, the mutagenic activity of PhIP-M1 was analyzed using the Ames test.

2. Material and methods

2.1 Synthesis of PhIP-M1 and its trideuterated derivate

7-hydroxy-5-methyl-3-phenyl-6,7,8,9-tetrahydropyrido[3',2':4,5]imidazo[1,2-*a*]pyrimidin-5-ium chloride (PhIP-M1) and its trideuterated derivate [$^{2}H_{3}$]PhIP-M1 were synthesized using procedures modified from previous studies (Vanhaecke *et al.*, 2006). Briefly, incubation mixtures consisted of 25 mg/L PhIP or 5 mg/L [$^{2}H_{3}$]PhIP in TY broth (tryptone 30 g/L, yeast extract 20 g/L, L-cysteine 0.5 g/L, pH 7.0) supplemented with 10% (v/v) fecal inoculum in phosphate buffered saline (0.1 M, pH 7) in a final volume of 50 mL. Each sample was sealed with a butyl rubber top and anaerobiosis was obtained by flushing the flasks with N_2 during 15 cycles of 2 min each at 800 mbar overpressure and 900 mbar underpressure. Cultures were incubated at 37 °C and 150 rpm for 5 days. After incubation, PhIP-M1 or [$^{2}H_{3}$]PhIP-M1 were extracted from the digests using a previously published liquid-liquid extraction procedure (Vanhaecke *et al.*, 2006). The yield of PhIP-M1 from PhIP and [$^{2}H_{3}$]PhIP-M1 from [$^{2}H_{3}$]PhIP was ~ 90%.

Purification was obtained by preparative high-performance liquid chromatography on a Gilson preparative HPLC system (Gilson International B.V., Middleton, United States) comprising a H322 pump system and a 206 fraction collector, coupled to a model 156 UV/VIS detector. Chromatographic separation was achieved using a 10 µm 21.4 x 250 mm Omnisphere C_{18} column obtained from Varian (St.-Katelijne-Waver, Belgium). Compounds were eluted by an isocratic solvent mixture containing 85% water with 0.05% formic acid and 15% acetonitrile with 0.05% formic acid, at a flow rate of 20 mL/min. Absorbance was monitored at 307 nm. The identities of the microbial PhIP metabolites were confirmed by their LC-MS/MS fragmentation pattern (see below). The peaks corresponding to PhIP-M1 and [$^{2}H_{3}$]PhIP-M1 were collected and evaporated to dryness under nitrogen gas. Purity of PhIP-M1 and its deuterated derivate was 97 ± 0.8% as determined by LC-MS/MS. Isotopic purity of [$^{2}H_{3}$]PhIP-M1 was 99%.

2.2 Study design

The study protocol was reviewed and approved by the Ethics Committee of the Ghent University Hospital (EC UZG 2005/404). Informed consent was obtained from each subject prior to beginning the study. The six individuals participating were recruited from the local workforce, were all male, between 20 and 30 years old, in good health, non-smokers and of normal weight. None had a history of digestive pathology nor had received antibiotics during 3 months prior to the study.

2.3 Meat preparation and controlled dietary period

Boneless, skinless chicken breasts were cut into ~ 2.5 cm pieces and fried in a non-stick coated pan, sprayed with a non-stick cooking spray, for 25-35 min. Pan temperature was recorded every 5 min, averaging 180 °C for the cooking period. At the end of the cooking time the chicken was white with some browning. A representative chicken sample was removed for heterocyclic amine analysis using previously published methods (Knize *et al.*, 1995). Total PhIP dose depended on the exact cooking time and was different for each of the three batches of chicken cooked. The PhIP content in the various batches ranged from 4.4 to 39 ng/g. The two first study subjects (A, B) were provided chicken containing 39 ng/g PhIP along with other non-meat foods and beverages. The total PhIP dose was 4.7 µg PhIP. The next two study subjects (C, D) were given chicken containing 4.4 ng/g, for a total dose of 0.88 µg. The remaining two subjects (E, F) received chicken containing 18 ng/g PhIP, for a total dose of 2.7 µg. The subjects were all provided with 150 g of chicken.

Subjects were asked to abstain from meat consumption for 3 days prior and 3 days after eating the well-done chicken breast. There were no other dietary restrictions. Control urine and feces samples were received before eating the chicken and all urine and feces was collected for 3 days afterwards, in 8 h increments for urine and 24 h increments for feces. Fecal slurries of 20% (w/v) fresh fecal inocula were prepared by homogenizing the feces with phosphate buffered saline (0.1 M, pH 7). Samples were coded, the volume recorded and stored frozen at -20 °C until analysis.

2.4 Analysis of PhIP and PhIP-M1 in human feces and urine

Urine samples (5 mL) and fecal slurries (5 mL) were spiked with 100 μL internal standard containing 125 μg/L [$^{2}H_{3}$]PhIP and [$^{2}H_{3}$]PhIP-M1 in dimethylsulfoxide (DMSO), added with 0.5 mL of 6 M NaOH and mixed with 5 g of diatomaceous earth. The mixture was placed into an empty Extrelut-20 cartridge and extracted with 30 and 60 mL of dichloromethane for the urine and fecal samples, respectively. The eluate was directly passed through an Oasis MCX (30 and 60 mg) cartridge, preconditioned with either 1 or 2 mL of dichloromethane. After washing the cartridges with 1 mL of 0.1 M HCl and 1 mL of acetonitrile, heterocyclic amines were eluted with 6 or 12 mL of 10% NH_3 in acetonitrile for the urine and fecal samples, respectively. Finally, the extracts were evaporated to dryness under a stream of nitrogen, redissolved in 100 μL of acetonitrile-5 mM formic acid (75:25) and injected into the LC-MS/MS in a volume of 20 μL.

Acid hydrolysis of urine was carried out by adding 0.5 mL of 1 M HCl to 5 mL of urine and heating at 90 °C for 1 h. For fecal samples 0.5 mL of 6 M HCl was used. After hydrolysis was completed, 0.5 mL of 1 and 6 M of sodium hydroxide was added to the urine and feces, respectively, to obtain a basic medium. Subsequently the samples were processed using the optimized clean-up procedure mentioned above.

Chromatography was carried out on a Thermo Finnigan HPLC system (San Jose, CA, USA) comprising a P4000 quaternary pump and an AS3000 autosampler, equipped with a 5 μm 2.1 x 150 mm Symmetry C_{18} column obtained from Waters (Milford, MA, USA). Metabolites were eluted at a flow rate of 300 μL/min using a mobile phase of 98% A (0.01% aqueous formic acid) and 2% B (acetonitrile) for 2 min, increasing linearly to 60% B at 22 min, maintaining 60% B for 8 min, and finally increasing to 100% B in the minute.

Analytes were detected with a LCQ Deca ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) in the MS/MS positive ion mode using an Electrospray Ionisation (ESI) interface. A capillary temperature of 240 °C, a source voltage of 4.5 kV and sheath gas of 70 units with no auxiliary gas were used.

Alternating scans were used to isolate $[M + H]^{+}$ ions at masses 225 and 281 for PhIP and PhIP-M1, respectively and 228 and 284 for the deuterated internal standards. The

precursor isolation width was set to 2 Da, the activation Q to 0.35 and the collision energy to 45%. Daughter ions were detected at appropriate masses: 210 $[M + H\text{-}CH_3]^+$ from 225 for PhIP, 263 $[M + H\text{-}OH]^+$ and 225 $[M + H\text{-tetrahydropyridine-}OH]^+$ from 281 for PhIP-M1, 210 $[M + H\text{-}CD_3]^+$ from 228 for $[^2H_3]$PhIP, 266 $[M + H\text{-}OH]^+$ and 228 $[M + H\text{-tetrahydropyridine-}OH]^+$ from 284 for $[^2H_3]$PhIP-M1. The overall recovery of PhIP and PhIP-M1 was determined by spiking each urine and feces sample with known amounts of their deuterated analogues. Final PhIP and PhIP-M1 concentrations were adjusted based on recovery of the internal standard. The effect of the urine or fecal matrix on the overall recovery of PhIP and PhIP-M1 was determined by spiking increasing amounts of the internal standard in 5 mL of water and comparing these recoveries to the recovery of the internal standard in 5 mL urine or fecal slurry. Replicate analyses of several different urine and fecal samples were made during the course of the study to determine the precision of the assay.

2.5 Salmonella mutagenicity assay

The mutagenic activity of the purified extract of PhIP-M1 in DMSO (100 ng/µL for TA98 and 2 µg/µL for TA100 and TA102) was determined using the standard plate incorporation assay described by Ames *et al.* (1973), with *Salmonella typhimurium* strains TA98, TA100 and TA102 (gifts of Professor Bruce Ames, University of California, Berkeley) and tested in 5, 10, 25, 50 and 100 µL volumes. Aroclor-induced rat liver S9 protein (2 mg per plate) was used for metabolic activation. As a positive control, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) was used. DMSO was the negative control (spontaneous revertant counts). Dose-response curves of the mutagenic activity were calculated using the method of Moore and Felton (1983). A minimum of four dose points from duplicate platings was used, and the linear portion of the curve was used to calculate the number of revertants per µg of PhIP-M1 extract.

3. Results

3.1 Method development, urine and feces analysis

The goal of this study was to develop and apply a method that reliably quantifies PhIP and its newly identified microbial metabolite PhIP-M1 in urine and feces samples of healthy individuals administered a known dose of PhIP. The initial step of the method utilized an acid

hydrolysis to release phase II conjugates. Strickland *et al.* (2001) found that the optimal conditions for releasing PhIP from urine conjugates was incubation at 90 °C for 60 min at a final HCl concentration of 0.05-0.1 N. For fecal samples a final HCl concentration of 0.5-0.6 N is required for optimal hydrolysis (unpublished data). During the next step of the method liquid-liquid extraction was applied to eliminate macromolecules from the urine and fecal matrices. In order to avoid problems due to emulsions and manipulation of the sample, the contact was increased between both liquids by the addition of a solid support of diatomaceous earth. After this initial purification, secondary purifications were designed to exploit the protonation of the heterocyclic nitrogen atoms common to PhIP and PhIP-M1 in an Oasis MCX cartridge, combining reversed-phase silica and cation-exchange mechanisms. During this final step, the removal of uncharged interference and concentration of the compounds was achieved.

Because of the complexity of the urine and fecal extracts and the overlapping retention times of the analytes and the internal standards, UV or fluorescence detection could not be used. Due to co-elution of hundreds of compounds into the mass spectrometer, detection of a signal above the background with single-ion monitoring MS for the parent masses was only possible for a limited amount of samples (data not shown). Therefore multiple MS detection was necessary for these analyses. An authentic standard of PhIP and a synthesized standard of PhIP-M1 were used to optimize the HPLC separation and fragmentation. The LC-MS/MS peak areas were linear over the range 0.25-100 µg/L with R^2 values of 0.999 and 0.997 for PhIP and PhIP-M1, respectively. The method developed in this study using LC-MS/MS detects peaks for PhIP, the microbial metabolite PhIP-M1 and the deuterated internal standards in a single chromatographic run (Figure 3.2) and has been successfully applied for urine as well as feces. Since other ion peaks are sometimes present in the chromatograms that are not PhIP or PhIP-M1 (Figure 3.2), expected peak retention times were compared with the internal standards and calibration standards to identify PhIP and PhIP-M1. PhIP typically exhibits a sharp peak and a good signal-to-noise ratio (Figure 3.2 A). The internal standard [$^2H_3$]PhIP elutes at the same time as the non-labeled product (Figure 3.2 B). PhIP-M1 is separated in time from PhIP and fragments into two daughter ions with masses 225 and 263. The sum of those two peaks is used for quantification (Figure 3.2 C). The internal standard [$^2H_3$]PhIP-M1 shows a similar profile as the natural product (Figure 3.2 D).

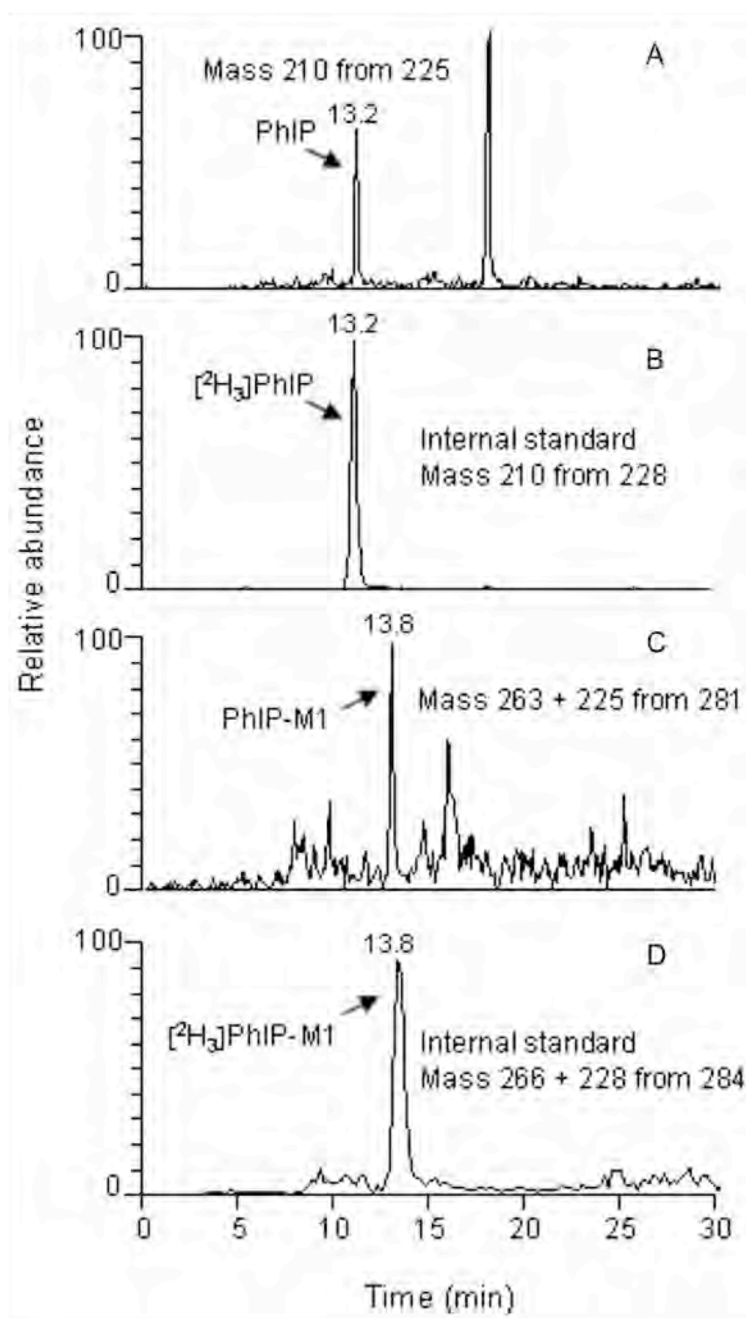

Figure 3.2 Ion plots of PhIP, the microbial metabolite PhIP-M1 and the deuterated internal standards from hydrolyzed urine of subject B 8 to 16 h after consuming the well-done chicken. (A) Mass 210 peak plot after fragmenting mass 225, representing PhIP. (B) Mass 210 peak plot after fragmenting mass 228, representing the internal standard [$^2H_3$]PhIP. (C) Sum of masses 225 and 263 after fragmenting mass 281, representing PhIP-M1. (D) Sum of masses 228 and 266 after fragmenting mass 284, representing the internal standard [$^2H_3$]PhIP-M1.

3.2 Recovery and reproducibility

Spiking human urine and feces samples with increasing concentrations of [$^2H_3$]PhIP and [$^2H_3$]PhIP-M1 allowed us to determine the recovery of the compounds while optimizing the extraction protocol. Typical recoveries ranged from 74 to 83% for [$^2H_3$]PhIP and 51 to 86% for [$^2H_3$]PhIP-M1 in urine samples and from 51 to 59% for [$^2H_3$]PhIP and 24 to 31% for [$^2H_3$]PhIP-M1 in fecal samples (Table 3.1). Recovery of the internal standards was obviously better in water (81 to 100%) compared to urine and feces, indicating that the complexity of the urine and fecal matrices interferes with the efficiency of the solid phase extraction columns or lowers the sensitivity of the mass spectrometer through ion suppression.

Table 3.1. Percent recovery of [$^2H_3$]PhIP and [$^2H_3$]PhIP-M1 spiked into water, urine or feces upon extraction.

Compound	Spike (ng)	Water	Urine	Feces
[$^2H_3$]PhIP	0.5	95.7 ± 3.1	77.7 ± 5.1	50.7 ± 15.6
	2.5	91.8 ± 12.8	74.0 ± 2.1	59.2 ± 29.4
	10	99.4 ± 3.6	82.8 ± 8.9	52.0 ± 0.9
[$^2H_3$]PhIP-M1	0.5	97.0 ± 1.2	85.9 ± 3.4	24.0 ± 12.0
	2.5	80.9 ± 12.5	51.1 ± 2.6	30.9 ± 3.8
	10	93.6 ± 3.3	57.8 ± 9.7	29.7 ± 3.1

Recovery using the optimized method for the kinetic samples was quantified by spiking each urine or fecal sample with the deuterium-labeled internal standards [$^2H_3$]PhIP and [$^2H_3$]PhIP-M1. Final PhIP and PhIP-M1 concentrations in each sample were adjusted based upon recovery of the internal standards in that sample. Because of the small peak sizes in our assay, there is variation inherent in the mass spectrometry detection. To account for this variation, each extract was injected three times and the peak areas averaged.

3.3 Microbial PhIP metabolite quantification

Control urine and feces samples were collected from each of the six volunteers the day before the consumption of the well-done chicken, during the period that they abstained from eating cooked meat. PhIP was detectable in one of six control urine samples (72 ng/L) and in all six control feces samples (593 ± 342 ng/L). PhIP-M1 was detectable in two of six control urine samples (18 ± 14 ng/L) and in four of six control fecal samples (28 ± 9.7 ng/L). Because of the low concentrations detected in the control urine samples compared to the urine after

chicken consumption, these background concentrations were not taken into account for quantification. The fecal pre-feeding concentrations were however a factor 10 higher. Therefore a correction was made by subtracting the volume corrected pre-feeding values from the respective post-feeding amounts. Total urine and feces excreted after chicken consumption were collected for 72 h in 8 h increments for urine and 24 h increments for feces. Values shown are corrected for the total volumes of urine and feces.

Figure 3.3 shows the absolute dose percentages of PhIP and the microbial metabolite PhIP-M1 recovered in urine and feces for the six subjects. These varied from 12 to 21% for PhIP and 1.2 to 15% for PhIP-M1 in urine, and from 26 to 42% for PhIP and 0.9 to 11% PhIP-M1 in feces. No significant differences in absolute PhIP or PhIP-M1 dose percentage excreted could be observed for the different PhIP doses administered.

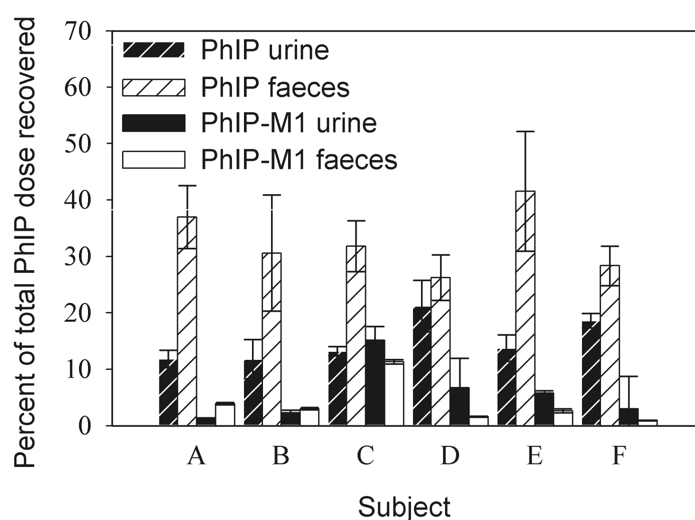

Figure 3.3 Total 72 h excretion of urinary and fecal PhIP and PhIP-M1 for six individuals after ingesting a well-done chicken meal. The recovery-corrected sum of the amount of PhIP and PhIP-M1 (mean ± SD) detected in hydrolyzed fecal and urine samples are shown (n=3).

Figure 3.4 shows the rate of excretion of PhIP and the microbial PhIP metabolite for the respective time periods collected. Our results demonstrate that excretion rates for PhIP and PhIP-M1 vary among volunteers, but that most urinary PhIP (Figure 3.4 A) was excreted during the first 24h, while for the microbial metabolite (Figure 3.4 B) the urinary excretion

increased throughout time with a maximum between 48 and 72 h. Subject A however excreted only 1.2% of PhIP-M1 in urine.

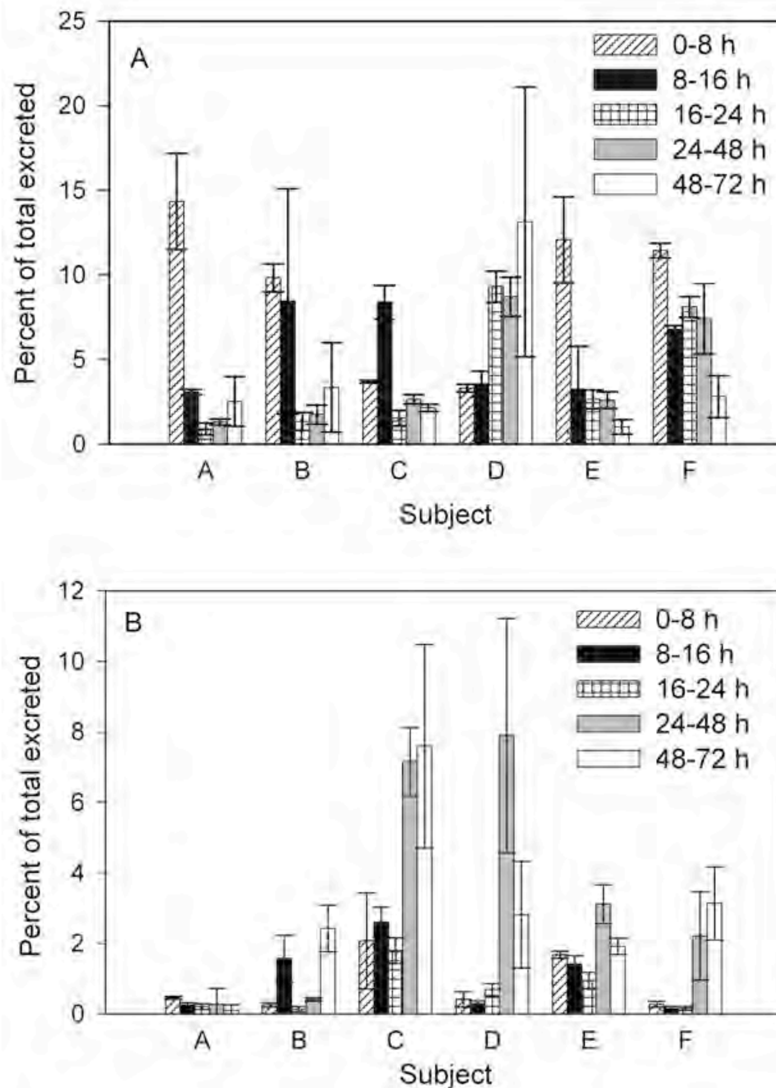

Figure 3.4 Rate of excretion of PhIP and its microbial metabolite PhIP-M1 in human urine from six volunteers. Time increments shown are 0-8 h, 8-16 h, 16-24 h, 24-48 h and 48-72 h after consuming well-done chicken. Data represent the percentage of the total PhIP or PhIP-M1 excreted (mean ± SD) during the designated time intervals (n=3). (A) PhIP recovered from hydrolyzed urine samples. (B) PhIP-M1 recovered from hydrolyzed urine samples.

Fecal PhIP excretion (Figure 3.5 A) was the highest during the 24-48 h period for subjects A and E, whereas subjects C and F excreted most in the 48-72 h period. Subject's B

fecal PhIP excretion was almost equal all three days. Subject D excreted most PhIP during the first 24 h. Fecal PhIP-M1 excretion (Figure 3.5 B) was the highest during the 24-48 h period for subjects A, C and E; subject B excreted more during the first 24 h; subject F excreted most during the 48-72 h period. Subject D excreted almost equally all three days.

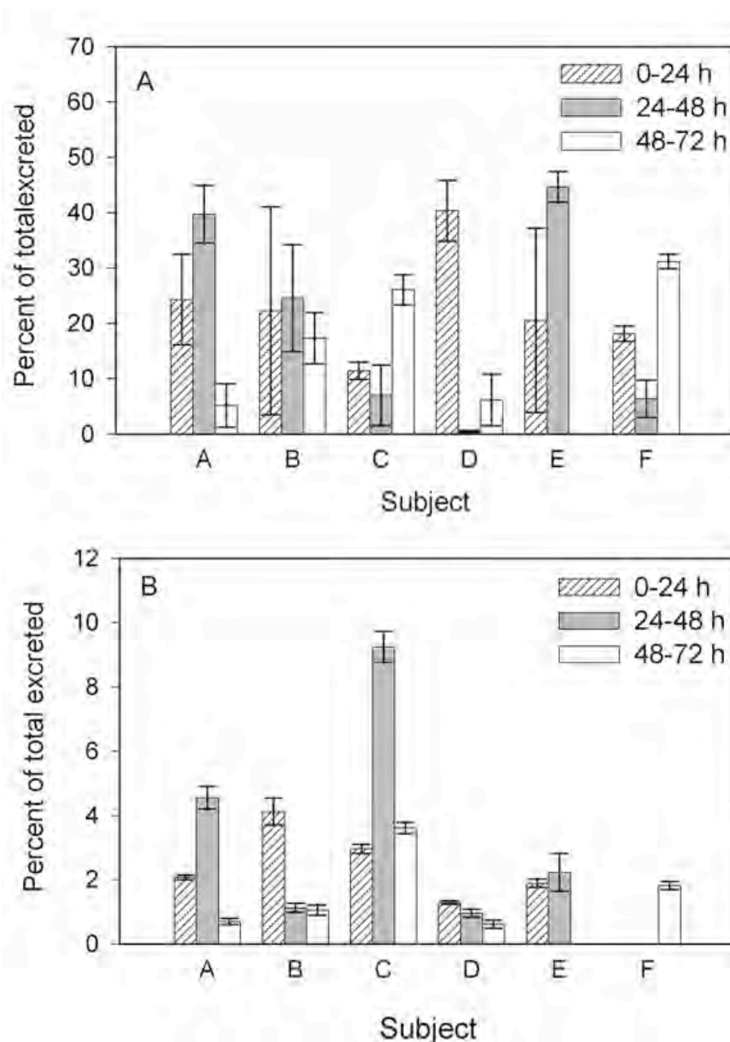

Figure 3.5 Rate of excretion of PhIP and its microbial metabolite PhIP-M1 in human feces from six volunteers. Time increments shown are 0-24 h, 24-48 h and 48-72 h after consuming well-done chicken. Data represent the percentage of the total PhIP or PhIP-M1 excreted (mean ± SD) during the designated time intervals (n=3). (A) PhIP recovered from hydrolyzed feces samples. (B) PhIP-M1 recovered from hydrolyzed feces samples.

3.4 Salmonella mutagenicity data

As a positive control, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) gave 800-1130 revertants per 5 ng dose for TA98, 1300-1400 revertants per 0.2 µg dose for TA100 and 500-600 revertants per 1 µg dose for TA102. DMSO gave TA98 values of 20-40 revertant colonies per plate, TA100 values of 140-170 revertant colonies per plate and TA102 values of 260-300 revertant colonies per plate.

Analysis of the mutagenic activity of PhIP-M1 using the Ames test with strains TA98, TA100 and TA102 without metabolic activation gave no positive result. S9-mediated analysis, gave a positive response (a positive slope for the dose-response curve) for strain TA98 and strain TA100 (Table 3.2). For each strain the revertant colonies per Petri plate were plotted against the mass equivalents of PhIP-M1 extract. The slope of this line was used to determine the mutagenic response. Yet, a mutagenic potency for the microbial metabolite was measured of about 2-4% of that of PhIP (Table 3.2).

Table 3.2. Comparison of the mutagenic activity of PhIP (Felton and Knize, 1990) and its microbial metabolite PhIP-M1.

***Salmonella* strain**	**Mutagenic response (revertants/µg)**	
	PhIP	**PhIP-M1**
TA98	1700	45.9 ± 1.99
TA100	140	6.46 ± 0.85
TA102	Not positive	Not positive

4. Discussion

The metabolism of PhIP has been well characterized in animal species (Buonarati *et al.*, 1992; Davis *et al.*, 1994) and several studies have been undertaken to examine the disposition of PhIP in humans (Malfatti *et al.*, 1999; Kulp *et al.*, 2000; Kulp *et al.*, 2004). Yet, little is known about the contribution of the intestinal microbiota to the overall metabolism of PhIP. The present study is the first to detect the excretion of a microbial PhIP metabolite in human urine and feces. The variation in microbial PhIP metabolism between six healthy human subjects, the kinetics of PhIP microbial metabolite excretion and the mutagenic activity of this newly identified microbial PhIP metabolite, are reported.

Optimizing a solid phase extraction procedure for PhIP and its microbial metabolite encountered some difficulties due to the complexity of the urine and fecal matrices. The Oasis MCX brand was selected because of its dual nature in retaining heterocyclic amines and was found superior in recovery compared to the various brands of C_{18} and cation exchange supports. Diatomaceous earth extract proved a suitable substrate for eliminating emulsion and manipulation problems and increasing contact between analytes and solvent (Galceran *et al.*, 1996). Subsequent liquid-liquid extraction with dichloromethane achieved a significant decrease in matrix interferences without completely ruling out co-extracted impurities in the final sample. To retain as much analyte as possible, further washing steps were minimized and a satisfactory procedure was devised meeting our goal to quantify PhIP and PhIP-M1 in both urine and fecal samples. Urine and fecal samples were heated with acid prior to analysis in order to hydrolyze phase II conjugates (Reistad *et al.*, 1997; Stillwell *et al.*, 1997). A large increase (7-10 fold) in the amount of PhIP detected following this acid treatment has been reported for urine (Lynch *et al.*, 1992; Stillwell *et al.*, 1997; Strickland *et al.*, 2001) and indicates that acid-labile PhIP metabolites represent a major proportion of the PhIP in human urine. This has been confirmed in recent studies on the metabolism of ingested PhIP indicating that PhIP-N^2-glucuronide, N^2-OH-PhIP-N^2-glucuronide and N^2-OH-PhIP-N^3-glucuronide are common metabolites in human urine (Kulp *et al.*, 2004). Overall, the acid treatment enhances the amount of free PhIP and should provide an estimate of total mammalian PhIP metabolites excreted, without having to analyze each liver metabolite separately. Analysis of urine and feces samples as such have shown that acid hydrolysis does not affect the recovery of PhIP-M1 (data not shown), implying that PhIP-M1 is not conjugated by mammalian enzymes.

Well-done chicken is the best source of PhIP exposure because at high temperatures and long cooking times chicken breast preferentially forms more PhIP and less of the related heterocyclic aromatic amines as compared with beef. Formation of PhIP seems to be favored by higher amounts of the amino acids phenylalanine, isoleucine, leucine and tyrosine and lower amounts of glucose that are present in chicken (Pais *et al.*, 1999). Both the amounts of chicken consumed by our volunteers and the PhIP levels were comparable with consumption levels measured in households or restaurants.

It is unlikely that PhIP-M1 was formed *de novo* during hydrolysis in the urine or feces from PhIP. We spiked PhIP and PhIP-M1 in baseline urine and fecal samples and no production of PhIP-M1, respectively PhIP, was measured. Numerous publications describe the incubation of PhIP with liver hepatocytes or enzymes and none of them report the detection of a metabolite resembling PhIP-M1 (Zhao *et al.*, 1994; Crofts *et al.*, 1998; Turesky *et al.*, 2002), whereas incubation of PhIP with specific intestinal bacterial species in the presence of glycerol and a protein-rich feed source, does give rise to the formation of this metabolite (Vanhaecke *et al.*, 2008b). Therefore our results confirm that the intestinal microbiota contribute to the overall metabolism and disposition of PhIP *in vivo*, although a high degree of interindividual variation in the urinary and fecal excretion exists. The percentage of the PhIP dose excreted in the 0-72 h hydrolyzed urine varied from 12 to 21% with an average of 15 ± 3.9% for PhIP and from 1.2 to 15% with an average of 5.7 ± 5.1% for its microbial metabolite PhIP-M1. Our findings for PhIP are comparable with data previously obtained by Strickland *et al.* (2001), where the average 24 h urinary excretion of PhIP (unchanged plus acid-labile conjugates) from individuals fed a uniform diet containing high-temperature cooked meat, amounted 17 ± 7.4%. The percentages of the total PhIP dose excreted in this study as PhIP (26-42%) and PhIP-M1 (0.9-11%) in feces were surprisingly high and could explain the relatively low PhIP dose percentages measured in urine in previous metabolism studies of human subjects given PhIP in a meat matrix (Strickland *et al.*, 2001; Kulp *et al.*, 2004). The total percentage of the PhIP dose accounted for in the 72 h urine and feces as PhIP and PhIP-M1 varied among individuals from 49 to 71% with an average of 51 ± 8.8%. When N-OH-PhIP-N^2-glucuronide, the major human N-oxidation metabolite of PhIP is hydrolyzed under acidic conditions, the deaminated product 2-OH-PhIP is formed. This derivate was not quantified during this study, but Stillwell *et al.* (2002) measured 2-OH-PhIP in urine collected from 66 subjects after ingestion of a meat-based meal and reported that 25 ± 8.4% of the ingested PhIP dose was excreted as 2-OH-PhIP in the 0-24 h urine. The formation of this hydroxylated derivate might explain the deficit in dose percentage encountered in this study. The variability in PhIP-M1 excretion can be explained by the interindividual variability in microbial community composition and activity between test subjects (Eckburg *et al.*, 2005). *In vitro* incubation of PhIP with intestinal bacteria derived from stools freshly collected from healthy volunteers confirms these results, measuring PhIP transformation efficiencies from 37 to 90% within the first 24 h of incubation (Vanhaecke *et al.*, 2006). Interindividual differences in microbial metabolic activities are not uncommon. A

striking example is the microbial conversion of the dietary phytoestrogen daidzein (Decroos *et al.*, 2005).

The kinetics of PhIP excretion in our study are similar to those previously observed for humans on a meat based diet (Stillwell *et al.*, 1997; Strickland *et al.*, 2001). Our results demonstrate that excretion times vary among the volunteers, but that 72 ± 27% of total PhIP excretion takes place in the first 24 h. Malfatti *et al.* (1999) is to our knowledge the only paper in which the kinetics of PhIP, in this particular case [^{14}C]PhIP, were examined over a period of 72 h. In the latter study the subjects were hospitalized elderly cancer patients who were given PhIP in a gelatine capsule. This route of administration resulted in a recovery of 90% of the ingested dose in the urine and in all subjects the majority of the dose was excreted in the first 12 h. Our study consisted of younger men on their normal diet, which was unrestricted except for refraining from meat consumption for the 72 h prior to dosing and during the course of the study. It is probable that the PhIP when formed in a meat matrix, is not as bioavailable as PhIP in capsule form. In addition, the interaction with additional foods and the resident microbiota in the gastrointestinal tract influences the absorption, distribution and as demonstrated here, the metabolism and excretion as well. The kinetics of microbial PhIP metabolite excretion showed a significant interindividual variability as well. Compared to PhIP, the microbial metabolite excretion was shifted in time, 35 ± 18% was excreted in the first 24 h, 33 ± 19% during the 24-48 h period and 32 ± 18% during the 48-72 h period. Microbial metabolites have indeed the tendency to appear later in excretion profiles of plasma and urine (Watanabe *et al.*, 1998; Li *et al.*, 2006).

In a final part of this study, we assessed the microbial genotoxicity of the newly identified PhIP metabolite. A weak activity was measured upon S9 activation amounting up to 2.7 ± 0.2% of the original PhIP mutagenic potency for TA98 and 4.5 ± 0.6% for TA100. As the PhIP-M1 extract was, despite of the preparative separation, not entirely pure (97 ± 0.8%), a residual fraction of PhIP in this extract might explain the weak mutagenic activity measured after S9 activation. Based on these results, the microbial transformation of PhIP may be considered as a detoxification. Further studies will focus on determining the *in vitro* and *in vivo* mammalian toxicology of this microbial PhIP derivate.

In summary, we have developed a method for quantifying PhIP and its newly identified microbial metabolite PhIP-M1 in urine and feces utilizing solid phase extraction and LC-MS/MS. This method allowed to detect PhIP and PhIP-M1 in urine and fecal samples collected from six volunteers following ingestion of a natural dose of PhIP. These findings suggest that besides individual differences in digestion, metabolism and diet, the microbial composition of the gastrointestinal tract also strongly influences individual disposition and carcinogenic risk from PhIP.

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CHAPTER 4

Isolation and characterization of human intestinal bacteria, capable of transforming the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine

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CHAPTER 4

Isolation and characterization of human intestinal bacteria, capable of transforming the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP)

ABSTRACT

2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is a carcinogenic heterocyclic aromatic amine formed in meat products during cooking. Although the formation of hazardous PhIP metabolites by mammalian enzymes has been extensively reported, research on the putative involvement of the human intestinal microbiota in PhIP metabolism remains scarce. In this study, the *in vitro* conversion of PhIP into its microbial derivate 7-hydroxy-5-methyl-3-phenyl-6,7,8,9-tetrahydropyrido[3',2':4,5]imidazo[1,2-*a*]pyrimidin-5-ium chloride (PhIP-M1) by fecal samples from eighteen human volunteers was investigated. HPLC analysis showed that all human fecal samples transformed PhIP, but with efficiencies ranging from 1.8 to 96% after 72 h incubation. Two PhIP transforming strains PhIP-M1-a and PhIP-M1-b were isolated from human feces and identified by FAFLP™ and *pheS* sequence analyses as *Enterococcus faecium*. Some strains from culture collections belonging to the species *Enterococcus durans*, *Enterococcus avium*, *Enterococcus faecium* and *Lactobacillus reuteri* were also able to perform this transformation. Yeast extract, special peptone and meat extract supported PhIP transformation by the enriched *Enterococcus faecium* strains, while tryptone, monomeric sugars, starch and cellulose did not. Glycerol was identified as a fecal matrix constituent required for PhIP transformation. Abiotic synthesis of PhIP-M1 and quantification of the glycerol metabolite 3-hydroxypropopionaldehyde (3-HPA) confirmed that the anaerobic fermentation of glycerol via 3-HPA is the critical bacterial transformation process responsible for the formation of PhIP-M1. Whether it is a detoxification is still a matter of debate, since PhIP-M1 has been shown to be cytotoxic towards Caco-2 cells, but is not mutagenic in the Ames assay.

1. Introduction

Diet is a major risk factor in human cancer (Doll and Peto, 1981). Epidemiological studies indicate that the consumption of cooked meat and meat products predisposes individuals to neoplastic disease, particularly of the colon (Doll, 1992). Cooked muscle meats contain potent genotoxic carcinogens belonging to the heterocyclic aromatic amine (HCA) class of chemical compounds (Nagao *et al.*, 1977). Of the 19 heterocyclic amines identified so far, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is the most mass abundant heterocyclic amine produced during the cooking of beef, pork and chicken (Felton *et al.*, 1986a; Sinha *et al.*, 1995). Experimentally, PhIP is a potent mutagen and genotoxin and has been shown to produce mammary gland, prostate and colon tumors in rats (Ito *et al.*, 1997; Shirai *et al.*, 1997). In humans, less is known about the potential role of PhIP and related heterocyclic amines in tumor development. Several studies have shown that individuals who eat 'well-done' meat have an increased risk of breast (Zheng *et al.*, 1998) and colorectal cancers (Gunter *et al.*, 2005).

To determine the potential health risks associated with heterocyclic amines, several dietary studies have been conducted on the metabolism and disposition of these compounds in humans. So far, most investigations focused on the activation and detoxification of heterocyclic amines by mammalian enzymes. The genotoxic/carcinogenic effect of heterocyclic amines is closely related to a highly complex metabolism involving xenobiotic-induced enzymes generating very reactive metabolites as well as detoxified derivatives (Aeschbacher and Turesky, 1991). On the other hand, the involvement of the intestinal microbiota in the digestive fate of heterocyclic amines remains poorly investigated (Knasmüller *et al.*, 2001). Recent research showed that PhIP metabolites excreted in the 0-24 h urine represented 17 ± 10% of the ingested PhIP in a meat matrix (Kulp *et al.*, 2004). In an earlier study with patients administered with PhIP in capsules, 90% of the ingested dose was recovered in the urine (Malfatti *et al.*, 1999), indicating that PhIP provided in capsule form is more bioavailable than via meat ingestion. The non-bioavailable PhIP fraction reaches the colon in an intact form and is there in contact with the resident microbiota. Direct binding of heterocyclic amines to the cell walls of intestinal bacteria has been reported and is currently considered as a detoxification mechanism since it prevents absorption of heterocyclic amines through the intestinal mucosa (Bolognani *et al.*, 1997; Turbic *et al.*, 2002). However, results of IQ (2-amino-3-methylimidazo[4,5-*f*]quinoline)-induced genotoxicity assays in germ-free

and conventional rodents showed that the presence of intestinal microbiota is essential to the induction of DNA-damage in colon and liver cells (Hirayama *et al.*, 2000; Kassie *et al.*, 2001). These findings suggest that the intestinal microbiota play a significant role in the bioconversion of HCAs into harmful metabolites. Indications exist that hydrolysis of HCA-glucuronides by bacterial β-glucuronidase may release mutagenic intermediates (Rumney and Rowland, 1992).

Information on the bacterial metabolism of native HCAs is still scarce. Nevertheless researchers have shown that incubation of the heterocyclic amine IQ with mixed human feces under anaerobic conditions results in the formation of the hydroxy-metabolite 7-OH-IQ (Carman *et al.*, 1988; Bashir *et al.*, 1989) and recent research identified 10 bacterial strains able to perform the IQ to 7-OH-IQ transformation: *Bacteroides thetaiotaomicron* (n = 2), *Clostridium clostridiforme* (n = 3), *Clostridium perfringens* (n = 1) and *Escherischia coli* (n = 4) (Humblot *et al.*, 2005). Little has however been done to characterize PhIP metabolism by human intestinal microbiota, although our early work examined the *in vitro* transformation of PhIP by human fecal microbiota (Vanhaecke *et al.*, 2006). In this study one major microbial metabolite of PhIP (PhIP-M1) was identified using ESI-MS/MS and 1D and 2D NMR as 7-hydroxy-5-methyl-3-phenyl-6,7,8,9-tetrahydropyrido[3’,2’:4,5]imidazo[1,2-*a*]pyrimidin-5-ium chloride. This compound was subsequently detected in human urine and feces following consumption of well-done chicken meat and showed no mutagenic potency in the Ames test (Vanhaecke *et al.*, 2008a).

This study presents the isolation and identification of individual intestinal bacteria from human feces capable of transforming PhIP into its microbial derivate PhIP-M1. Representative culture collection strains isolated from the intestine were screened for their PhIP transformation potential and the nutritional requirements for microbial PhIP-M1 formation were clarified. In addition, the microbial and chemical mechanisms for this carcinogenic transformation were elucidated.

2. Material and methods

2.1 Chemicals

PhIP was purchased from Toronto Research Chemicals (Ontario, Canada). For incubation purposes, it was dissolved in dimethyl sulfoxide (DMSO). The constituents of the culture media, namely tryptone, yeast extract and meat extract were obtained from AppliChem (Darmstadt, Germany). All other chemicals were obtained from Sigma-Aldrich (Bornem, Belgium). Acrolein was purified by distillation at 53 °C. The HPA system (3-HPA and its aqueous derivates) was produced as described by Vollenweider *et al.* (2003) using *Lactobacillus reuteri* ATCC 53608. The solvents for HPLC and LC-MS analysis were of HPLC grade and purchased from Acros Organics (Geel, Belgium).

2.2 Collection and preparation of human fecal samples and matrix

Fecal samples were obtained from eighteen healthy volunteers between the age of 20 and 65. Donors were on a Western-type diet and none had a history of digestive pathology nor had they received antibiotics during 3 months prior to sample delivery. Fecal slurries of 20% (w/v) fresh fecal inocula were prepared by homogenizing the feces with phosphate buffered saline (0.1 M, pH 7), containing 1 g/L sodium thioglycolate as reducing agent. The particulate material was removed by centrifugation for 2 min at 400 x g.

Fecal matrix was prepared by autoclaving fecal slurries for 20 min at 121 °C and centrifuging for 10 min at 8000 x g.

2.3 PhIP-M1 production by inactivated human fecal microbiota

Bacterial incubations of 72 h grown fecal communities of the human volunteer with the highest PhIP transformation efficiency were subjected to several treatments to verify the involvement of the colonic bacteria and fecal matrix constituents in the transformation of PhIP. During a first treatment, the overall fecal microbiota were filtered over a 0.22 µm filter to remove the bacterial cells from the suspension but withhold the extracellular protein fraction, a treatment further referred to as FS. A second treatment consisted of a consecutive filter sterilization and pasteurization for 30 min at 60 °C in a warm water bath to achieve removal of microbial biomass and degradation of heat sensitive enzymatic activity, a

treatment further referred to as FS-PS. During the third treatment the fecal grown microbial communities were autoclaved for 20 min at 121 °C. All treatments were performed in triplicate and data were compared using Student's *t*-test.

2.4 *Effect of pH, surfactants and protease inhibitors*

The sensitivity of the active substances involved in PhIP-M1 formation to surfactants, protease inhibitors and pH was tested on cell-free supernatants of a 72 h grown mixed fecal community from a high PhIP-transforming individual, incubated at 37 °C in TY broth under anaerobic conditions. The cells were harvested by centrifugation (8000 x g, 10 min, 4 °C), and the cell-free supernatant adjusted to pH 6.0 with 6 M NaOH.

The surfactants tested were sodium dodecyl sulphate (SDS), Tween 80 and Triton X-100 at final concentrations of 0.1% (w/v). The protease inhibitor EDTA (ethylenediamine tetraacetic acid) was added to the cell-free supernatant to yield a final concentration of 5 mM.

The sensitivity of the active substance to different pH values (from 1 to 12) was tested by adjusting the cell-free supernatants from pH 1.0 to 12.0 (at increments of one pH unit) with sterile 1 M NaOH or 1 M HCl. The pH values were measured before and after the 72 h incubation and remained constant during the entire incubation period.

Untreated cell-free supernatants were used as controls. All treatments and controls were incubated anaerobically at 37 °C for 72 h. Samples were taken every 24 h for HPLC analysis. The different treatments were executed in triplicate and data were compared using Student's *t*-test.

2.5 *Effect of nutrition on microbial PhIP metabolism*

One mL of human fecal inoculum of the individual with the highest PhIP transformation capacity was transferred in 10 mL minimal medium (composition per Liter: 6 g Na_2HPO_4, 3 g KH_2PO_4, 1 g NH_4Cl, 0.5 g NaCl, 0.12 g $MgSO_4$, 0.01 g $CaCl_2$ and 0.5 g L-cysteine) supplemented with 10 g/L of different feed sources covering the main nutritional components relevant for the colon: yeast extract (YE), tryptone, special peptone (PEP), protease peptone, meat extract (ME), fibers, glucose or olive oil (Extra Virgin, Delhaize). The

suspensions were prepared in 50 mL penicillin flasks and incubated anaerobically at 37 °C and 140 rpm for 72 h in the presence of 5 μM PhIP. At the end of this incubation period samples were taken for HPLC analysis and pH measurements were made. Samples were kept at -20 °C prior to analysis. The different treatments were executed in triplicate and data were compared using Student's *t*-test.

2.6 Isolation and identification of PhIP-transforming bacteria

The fecal slurry of the two human volunteers with the highest PhIP transformation capacity was diluted in a 10-fold dilution series (10^{-1} to 10^{-8}) in TY broth supplemented with fecal matrix (10%, vol/vol) and PhIP (5 μM). Dilutions were incubated at 37 °C under anaerobic conditions for 3 days and assayed at 24 h intervals for residual PhIP and PhIP-M1 formation. At the same time intervals, samples from all dilutions were spread onto TY agar plates supplemented with PhIP (5 μM) to maintain a continuous exposure of the bacteria to the substrate. Following incubation at 37 °C under an atmosphere of nitrogen/hydrogen/carbon dioxide (84/8/8), five colonies per plate that differed, whenever possible, in size, shape, and color were picked up, subcultured in TY broth supplemented with fecal matrix (10%, vol/vol) and PhIP (5 μM) and then stored as stock cultures at -80 °C after addition of glycerol (20%, vol/vol). Identification of the biotransforming strains was performed phenotypically by microscopic examination and genetically by sequence comparison of the amplification products of the cloned 16S rRNA genes. Total DNA was extracted from 24 h cultures in TY broth by using the QIAamp DNA mini stool kit (Qiagen Benelux B.V., Venlo, The Netherlands). Denaturating gradient gel electrophoresis (DGGE), using universal bacterial primers, was performed according to Possemiers *et al.* (2004). The entire 16S rRNA gene of the isolated strains, amplified using the primers 63r and 1378f (Boon *et al.*, 2000), was cloned using a TOPO-TA cloning kit (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions. Sequencing of the 16S rRNA gene fragments was performed by ITT Biotech (Bielefeld, Germany). Analysis of DNA sequences and sequence identity searches were completed with standard DNA sequencing programs and the BLAST server of the National Center for Biotechnology Information using the BLAST algorithm and the BLASTN program for the comparison of a nucleotide query sequence against a nucleotide sequence database (Altschul *et al.*, 1997).

Using the former approach, identification of the bacterial strains was achieved at the genus level. Identification at species level was obtained by fluorescent amplified fragment length polymorphism (FAFLP™) and partial *pheS* sequence analysis. DNA was prepared according to Gevers *et al.* (2001). FAFLP^{TM} is a PCR based technique for whole genome DNA fingerprinting via the selective amplification of restriction fragments (Vos *et al.*, 1995) and was performed as described by Vancanneyt *et al.* (2006), except that the BioNumerics software package version 4.61 (Applied Maths, Belgium) was used. A fragment of the *pheS* gene was amplified and sequenced following the protocol of Naser *et al.* (2005) using an ABI Prism® 3130XL Genetic Analyzer (Applied Biosystems, USA). Sequence assembly was obtained via the AutoAssembler™ program (Applied Biosystems, Foster City, CA, USA). Phylogenetic analysis was performed using the BioNumerics software package, version 4.61 after alignment of the consensus *pheS* sequences with in-house determined *pheS* sequences of reference strains of lactic acid bacteria taxa currently covered by the database of the Laboratory of Microbiology, BCCM/LMG Bacteria Collection.

2.7 Strains from culture collections

Six strains from the collection of 'Unité d'Ecologie et de Physiologie du Système Digestif' (INRA, Jouy-en-Josas, France) were kindly provided by Sylvie Rabot. All of them originated from human feces or intestinal contents and were isolated locally. The strains were strictly anaerobic Gram-negatives belonging to *Bacteroides* or Gram-positives belonging to *Clostridium*, *Eubacterium* and *Bifidobacterium*. A further fourteen strains from human origin were selected from the BCCM/LMG Bacteria Collection. They were micro-aerophilic Gram-positives belonging to the lactic acid bacteria *Enterococcus*, *Pediococcus* and *Lactobacillus*. One *Lactobacillus reuteri* strain from human origin was purchased from the ATCC culture collection (Table 4.1). The cells were stored at -80 °C in physiological solution (8.5 g/L NaCl) supplemented with sterile glycerol (20%, vol/vol).

2.8 Incubation conditions for isolates and culture collection strains

All strains were inoculated in penicillin flaks containing 50 mL autoclaved TY broth supplemented with 0.5 g L-cysteine/L and incubated for 24 h at 37 °C. Subsequently 9 mL of the 24 h grown cultures were transferred to a penicillin flask containing 1 mL fecal matrix and 5 µM PhIP. The flasks were incubated anaerobically at 37 °C while shaking at 140 rpm for 72

h; daily samples were taken for HPLC analysis. All strains were incubated in triplicate. A negative control, 1 mL fecal matrix incubated in 9 mL TY broth supplemented with 5 µM PhIP, was included to exclude that physico-chemical interactions of the fecal matrix components are at the origin of the disappearance of PhIP.

2.9 Effect of nutrition on PhIP metabolism by Enterococcus faecium PhIP-M1-a

Fifty µL of thawed *Enterococcus faecium* PhIP-M1-a stock was transferred in 10 mL of minimal medium supplemented with 10 g/L yeast extract (YE), tryptone, special peptone (PEP), protease peptone, meat extract (ME), fibers, sugars (glucose, dextrose, lactose, sucrose, maltose, mannose, ribose and fructose), carbohydrates (starch and cellulose), olive oil or combinations thereof. The suspensions were prepared in 50 mL penicillin flasks, the headspace replaced by nitrogen gas and incubated at 37 °C while shaking at 140 rpm for 72 h in the presence of 5 µM PhIP. Then 1 mL was sampled from each flask for HPLC analysis and incubation continued for 72 h upon supplementation of 10% (vol/vol) fecal matrix. At the end of this incubation period samples were taken for HPLC analysis and pH measurements were made. Samples were kept at -20 °C prior to analysis. The different treatments were executed in triplicate and data were compared using Student’s *t*-test.

2.10 Elucidation of fecal matrix constituents

The fecal slurry of the human volunteer with the highest PhIP transformation capacity was diluted using serial 10-fold dilutions (10^{-2} to 10^{-4}) in 10 mL of TY broth supplemented with 10 g/L of glucose, dextrose, lactose, sucrose, maltose, mannose, ribose, fructose, starch, cellulose, glycerol, fumarate, succinate or pyruvate and 5 µM PhIP. In addition, an amount of 50 µL thawed *Enterococcus faecium* PhIP-M1-a or *Lactobacillus reuteri* ATCC 53608 was transferred in 10 mL of TY broth added with the same supplements. Dilutions and pure cultures were incubated in triplicate in penicillin flasks at 37 °C, while shaking at 140 rpm under anaerobic conditions for 72 h. Every 24 h, samples were taken for PhIP and PhIP-M1 analysis and pH measurements were made. Samples were kept at -20 °C prior to analysis. The highest PhIP transforming fecal dilution (10^{-4}) and *Lactobacillus reuteri* ATCC 53608 were subsequentely incubated in 50 mL of 10 g/L meat extract supplemented with 10 g/L glycerol

at 37°C and 140 rpm under anaerobic conditions for 72 h. Every 24 h, samples were taken for 3-HPA analysis and derivatized as described below.

2.11 *Abiotic synthesis of PhIP-M1*

The potential glycerol metabolites or derivates of interest: i.e. the HPA system and acrolein were supplemented in concentrations of 0.01, 0.1, 1, 10 and 100 mM to penicillin flasks containing 10 mL of 10 g/L meat extract and 5 µM of PhIP. Flasks were incubated at 37 °C while shaking at 140 rpm for 36 h and samples were taken every 12 h for 3-HPA and PhIP-M1 analysis. Incubations were performed in triplicate.

2.12 *Acrolein and 3-HPA analysis*

The concentration of the HPA system (3-HPA and derivates) during synthesis was determined by using a colorimetric method containing tryptophan adapted from Circle *et al.* (1945) by Vollenweider *et al.* (2003). The concentration of acrolein and 3-HPA during batch incubation experiments was determined by preparing the more stable 2,4-dinitrophenyl hydrazine (DNPH) derivates. DNPH derivatization was carried out according to literature (Zwiener *et al.*, 2003) by adding 500 µL DNPH reagent solution to 5 mL of bacterial medium or bacterial medium dilution. The reagent solution was prepared by dissolving 20 mg DNPH in 15 mL HCl/water/acetonitrile 2:5:1 (vol/vol) according to literature (Kieber and Mopper, 1990). The reaction time was at least 12 h at room temperature. The acidified samples were exctracted and pre-concentrated by SPE on Oasis HLB cartridges (60 mg sorbent, Waters, Milford, MA, USA). The cartridges were preconditioned with methanol (3 mL), acetonitrile (3 mL) and MilliQ water (4 mL). For extraction the acidified samples (5 mL) were sucked through the preconditioned sorbent at a flow rate of approximately 5 mL/min. After sample extraction the adsorbent was washed with MilliQ water (1 mL) and the adsorbed compounds were eluted with acetonitrile (3 x 2 mL). Before measurement the samples were evaporated to dryness with a gentle stream of nitrogen and the residue was dissolved in acetonitrile/MilliQ (50:50) (vol/vol). Pre-concentration factors of 1 to 25 were achieved. HPLC analysis was performed on a Dionex system (Sunnyvale, California, USA) comprising an autosampler ASI-100, a pump series P580 and a STH585 column oven coupled to a UV-VIS detector UVD340S. A 20 µL volume of the sample was injected and separated over a Genesis C_{18} column (150 mm x 4.6 mm, 5 µm) (Jones Chromatography). The temperature was set at 35

°C and the flow rate was maintained at 1 mL/min. Solvents were A: water/acetonitrile/tetrahydrofuran/iso-propanol (59:30:10:1) and B: acetonitrile/water (65:35). The elution gradient was 100% A at 0 min to 60% A at 12 min, to 40% A at 17 min and back to 100% A at 20 min. Absorbance was monitored at 365 nm. Linear calibration curves for acrolein and 3-HPA spiked in 10 g/L meat extract, extracted with SPE and redissolved in an equal amount of acetonitrile/MilliQ, were obtained in the concentration range 0.75-90 mg/L.

2.13 PhIP and PhIP-M1 analysis

One hundred µL of each sample was diluted 10-fold with acetonitrile-0.01% formic acid (75:25), vortexed rigorously and centrifuged (10,000 x g, 2 min). The supernatant was transferred to a HPLC vial and stored at 4 °C until analysis. PhIP and PhIP-M1 analyses were performed on a Dionex HPLC system (Sunnyvale, California, USA) (Vanhaecke *et al.*, 2006).

3. Results

3.1 PhIP-M1 production by human fecal microbiota

The capacity of mixed microbial cultures obtained from 18 human stool samples to transform the food carcinogen PhIP was tested by incubating the obtained overall human fecal microbiota with 5 µM PhIP for a period of 3 days (Figure 4.1).

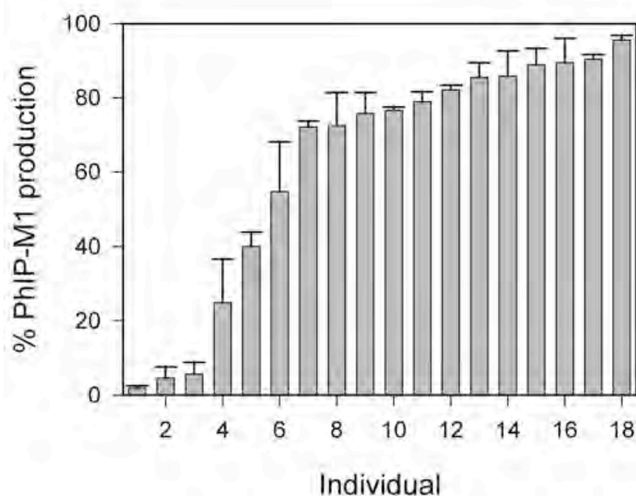

Figure 4.1 Conversion of PhIP into PhIP-M1 by intestinal bacteria from 18 different humans incubated during 72 h with 5 µM PhIP. The individuals were arranged by increasing PhIP-M1 production. Values are means ± SD (n = 3).

All human fecal samples transformed PhIP, though with different efficiencies ranging for the produced PhIP-M1 from 1.8 to 96% for the lowest and highest transforming microbiota, respectively. Based on these results two high PhIP-converting microbiota were selected for elucidation of the nature of PhIP metabolism and isolation and identification of the PhIP-transforming species.

3.2 PhIP metabolism by inactivated human fecal microbiota

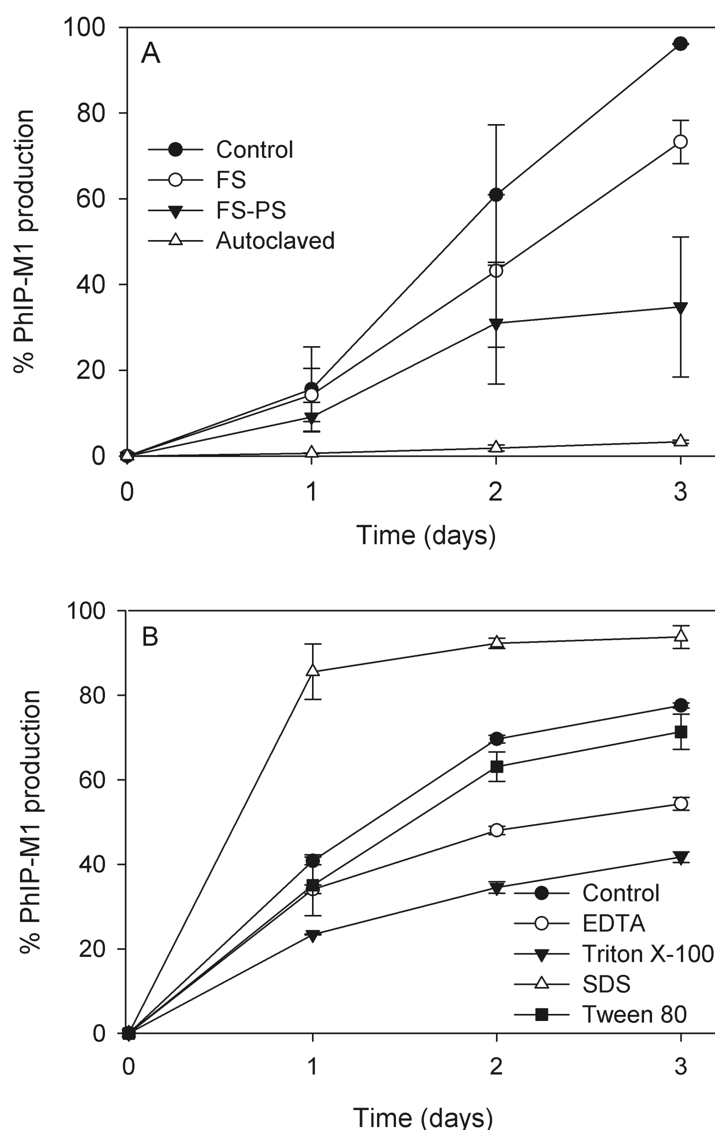

Figure 4.2 Conversion of PhIP into PhIP-M1 by (A) 72 h grown fecal microbiota exposed to different inactivating treatments, (B) 72 h grown fecal community cell-free supernatants exposed to enzyme inhibitors and surfactants. Data are presented as means ± SD (n = 3).

The production of PhIP-M1 during 3 days following different inactivating conditions is presented in Figure 4.2 A. The control reached an average PhIP-M1 formation of 96 ± 0.1% after three days, which decreased significantly ($p < 0.05$) to 73 ± 5.0% and 35 ± 16%, upon filter sterilization alone or combined with pasteurization, respectively. The difference in PhIP-M1 formation between FS and FS-PS treatments was however not significant. After autoclaving of the bacterial suspension, only a very limited PhIP-M1 production was detected.

3.3 Effect of surfactants, protease inhibitors and pH

The production of PhIP-M1 following 3 days of incubation of supernatants prepared from overall fecal microbiota and supplemented with different surfactants and protease inhibitors is depicted in Figure 4.2 B. The control incubation revealed an average PhIP-M1 production of 78 ± 0.6% after 72 h, while with SDS a significant ($p < 0.01$) increase in PhIP-M1 formation up to 94 ± 2.7% was observed. Treatment with EDTA (54 ± 1.5%) and Triton X-100 (42 ± 1.2%) significantly ($p < 0.01$) decreased the PhIP-M1 production. No significant ($p > 0.05$) effects could be observed upon Tween 80 addition.

The transformation of PhIP into PhIP-M1 measured at different pH values ranging from 1 to 12 revealed a maximum efficiency of 93% at pH 6 and no PhIP-M1 production below pH 2 and above pH 9.

3.4 Effect of nutrition on PhIP metabolism by mixed cultures

The capacity of the highest PhIP transforming mixed fecal microbiota to transform the food carcinogen PhIP under different nutritional conditions was tested by incubating 5 µM PhIP for a period of 3 days in the presence of minimal medium supplemented with different protein sources, glucose, starch, cellulose, fibers and olive oil. It was observed that supplementation of protein-rich feed sources such as meat extract, yeast extract and special peptone containing also traces of sugars and carbohydrates, lead to a significant production of PhIP-M1 (Figure 4.3 A), while protein-rich feed sources such as tryptone and protease peptone containing exclusively amino acids and peptides did not support PhIP-M1 formation. Glucose supplementation however drastically decreased the PhIP transformation efficiency ($p < 0.01$) (Figure 4.3 A). The carbohydrates starch and cellulose and the fiber-rich medium did

not sustain any PhIP-M1 formation (data not shown). Supplementation of olive oil allowed intermediate transformation efficiency (Figure 4.3 A). Concomitant supplementation of yeast extract and glucose, yeast extract and carbohydrates and yeast extract and fibers did not significantly ($p > 0.05$) affect the transformation efficiency observed after yeast extract supplementation (data not shown).

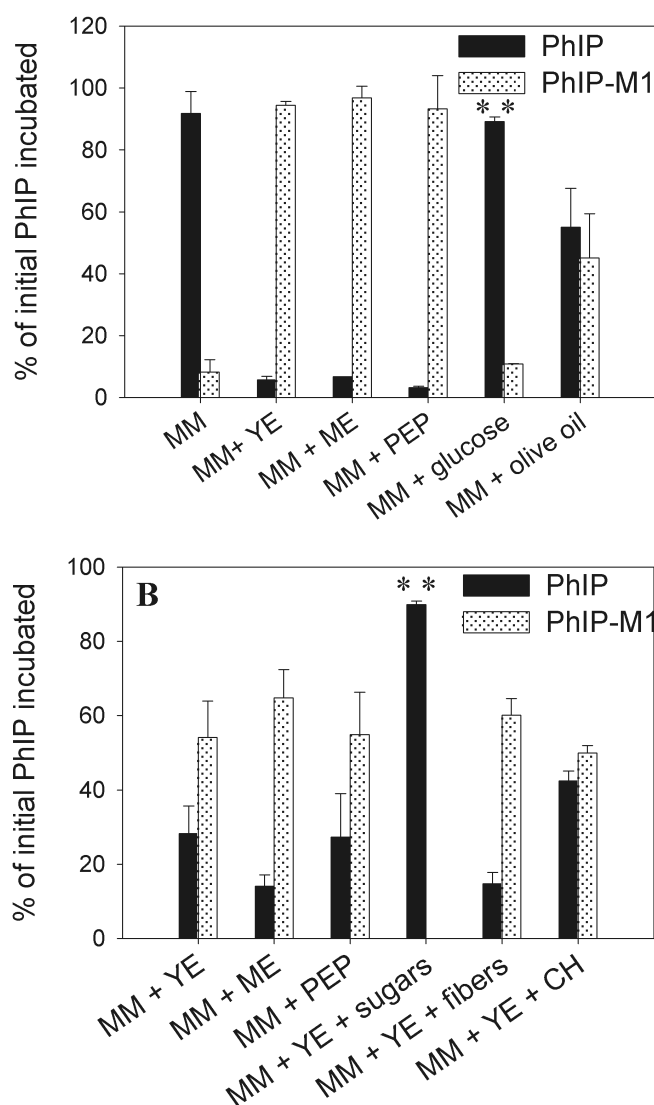

Figure 4.3 Conversion of PhIP into PhIP-M1 (A) by mixed fecal microbiota and (B) *Enterococcus faecium* PhIP-M1-a, grown under different nutritional conditions for 72 h, supplemented with 10% (vol/vol) fecal matrix and incubated for another 72 h. Values are means ± SD (n = 3). MM = minimal medium, YE = yeast extract, ME = meat extract, PEP = special peptone, CH = carbohydrates. Significantly different from MM + YE, * $p < 0.05$; ** $p < 0.01$.

3.5 *Isolation and characterization of PhIP transforming bacteria*

When incubating PhIP with serial 10-fold dilutions in TY broth of the highest PhIP-converting fecal bacterial community, only 10^{-1} and 10^{-2} concentrations demonstrated PhIP transformation up to 95% and 84%, respectively. Because it did not seem probable that bacteria are present in this low order of magnitude in fecal suspensions, the assumption was made that diluting the fecal inoculum lead to the concurrent dilution of an unidentified fecal matrix component, essential for sustaining microbial PhIP metabolism.

Therefore, new fecal dilution series of the two most efficient PhIP-converting individuals were again tested, but with additional supplementation of 10% cell-free fecal matrix (vol/vol). Co-supplementation of this fecal matrix allowed PhIP-M1 formation to occur at lower dilutions (until 10^{-5}), confirming our hypothesis (data not shown). Therefore the enrichment procedure was performed in the presence of fecal matrix. Among the 65 colonies picked from plates on which the serial dilutions of the PhIP-M1 producing fecal microbiota was plated, two colonies were retrieved that transformed PhIP upon subculturing, as measured by HPLC analysis of culture supernatants (Table 4.1).

Table 4.1. Abilities of individual bacterial strains originating from the human digestive tract to convert PhIP to PhIP-M1[a].

Bacterial species and strain	Origin	Source or reference	% initial PhIP converted[b]
Enterococcus durans LMG 20231	Human feces	LMG	93
Enterococcus durans LMG 16891	Human blood	LMG	65
Enterococcus faecium LMG 8147	Human feces	LMG	2.4
Enterococcus avium LMG 10744	Human feces	LMG	90
Enterococcus fecalis LMG 7937	Human feces	LMG	0.0
Enterococcus faecium PhIP-M1-a	Human feces	This study[c]	91
Enterococcus faecium PhIP-M1-b	Human feces	This study[c]	86
Lactobacillus reuteri LMG 13557	Human feces	LMG	97
Lactobacillus reuteri ATCC 53608	Human feces	ATCC	96

[a] For incubation, cell suspensions of the strains in TY broth were supplemented with 5 μM PhIP (anaerobic conditions, 37 °C, 140 rpm).

[b] At the end of the incubation (72 h), the PhIP and PhIP-M1 concentrations were determined by HPLC analysis.

[c] Among the 65 strains isolated from the human fecal samples and assayed for PhIP transformation, only the 2 biodegradative strains are indicated in this table.

The identity of the isolated strains was confirmed by comparing the sequence of the 16S rRNA gene of each strain within a database. Both isolates were shown to have a 100% sequence similarity with the genus *Enterococcus*. Partial sequence of the 16S rRNA has been deposited at GenBank under accession numbers EF373550 for *Enterococcus sp.* PhIP-M1-a and EF373551 for *Enterococcus sp.* PhIP-M1-b. Definite identification of the isolates at species level was achieved by fluorescent amplified fragment length polymorphism (FAFLP™) and partial *pheS* sequence analysis. Clusteranalysis of the FAFLP™ profiles of these strains with FAFLP™ profiles of reference strains of lactic acid bacteria taxa (including bifidobacteria), identified both strains as *Enterococcus faecium*. Clusteranalysis of the consensus *pheS* sequences of these strains with *pheS* sequences of reference strains of lactic acid bacteria taxa also identified both strains as *Enterococcus faecium.* Distinct profiles were however observed for the PhIP-M1-a and PhIP-M1-b strains and this for both the FAFLP™ and *pheS* sequence phylogenetic fingerprints (data not shown).

3.6 PhIP metabolism by bacterial strains of fecal origin

As the new biodegradative strains were identified as members of the genus *Enterococcus*, a selection of strains belonging to the genus *Enterococcus* and family of *Lactobacillaceae* were tested for their PhIP-transforming capacity (Table 4.1). Among the twenty collection strains that were assayed in the present experiment, six were able to produce PhIP-M1 as shown by HPLC analysis with fluorescence detection (Table 4.1). Most of them belonged to the genus *Enterococcus*, two *Lactobacillus* strains were capable as well.

3.7 Effect of nutrition on PhIP metabolism by Enterococcus faecium PhIP-M1-a

The percentual transformation of PhIP into PhIP-M1 after incubation of *Enterococcus faecium* PhIP-M1-a under different nutritional conditions is presented in Figure 4.3 B. Incubation of the strain in minimal medium did not result in PhIP-M1 formation. Supplementation of the medium with a protein-rich feed source, such as yeast extract, meat extract and special peptone, low in sugar content resulted in a significant PhIP-M1 production. In the absence of a protein-rich feed source or in the presence of protein sources not containing traces of sugar no transformation could be observed (data not shown). Co-supplementation of yeast extract with easily degradable sugars, such as glucose, sucrose,

mannose, maltose etc. completely ($p < 0.01$) inhibited the microbial metabolite formation. Addition of carbohydrates or fibers to the yeast extract containing medium did not significantly alter the microbial PhIP-M1 production ($p < 0.05$).

3.8 Elucidation of fecal matrix constituents

Supplementation of different diet-relevant components or systemic metabolites to the most efficient PhIP transforming fecal community, *Enterococcus faecium* PhIP-M1-a or *Lactobacillus reuteri* ATCC 53608 in TY broth showed that glycerol allows a significant PhIP transformation.

No other supplement sustained the microbial PhIP-M1 production. PhIP-M1 formation was detected in mixed microbial cultures and a clear increase in PhIP transformation could be observed with increasing fecal dilution (Figure 4.4 A). Upon incubation of *Enterococcus faecium* PhIP-M1-a in a glycerol enriched medium, only a limited percentage of PhIP-M1 conversion was measured while *Lactobacillus reuteri* ATCC 53608 showed an intermediate transformation efficiency (Figure 4.4 A) as compared to its high transformation efficiency after supplementation of fecal matrix (Table 4.1).

Incubation of the highest PhIP transforming fecal dilution (10^{-4}) and *Lactobacillus reuteri* ATCC 53608 in the presence of glycerol gave rise to the formation of 3-HPA (Figure 4.4 B). The 3-HPA concentration however decreased with longer incubation durations.

3.9 Abiotic synthesis of PhIP-M1

Incubation of *Lactobacillus reuteri* ATCC 53608 in 200 mM of glycerol, lead to the formation of 3-HPA and its aquatic derivates (HPA system), as measured colorimetrically with the method of Circle *et al.* (1945). Supplementation of the HPA system to PhIP in a protein-rich matrix gave rise to the formation of PhIP-M1 for a HPA concentration ranging from 0.1 to 100 mM (Table 4.2). Addition of acrolein also significantly induced PhIP-M1 production for the same concentrations (Table 4.2). During the acrolein synthesis experiments, no detectable amounts of acrolein could be measured. Equivalent concentrations of 3-HPA were however detected. During incubation with 3-HPA significant decreases in the 3-HPA concentration could be observed. After 24 h of incubating 100 mM of 3-HPA in 10

g/L of meat extract supplemented with PhIP only 11 ± 1.7% of the initial 3-HPA dose could be detected. Incubating 10 mM of 3-HPA during 24 h resulted in the detection of only 3.3 ± 0.4% of the initial 3-HPA dose.

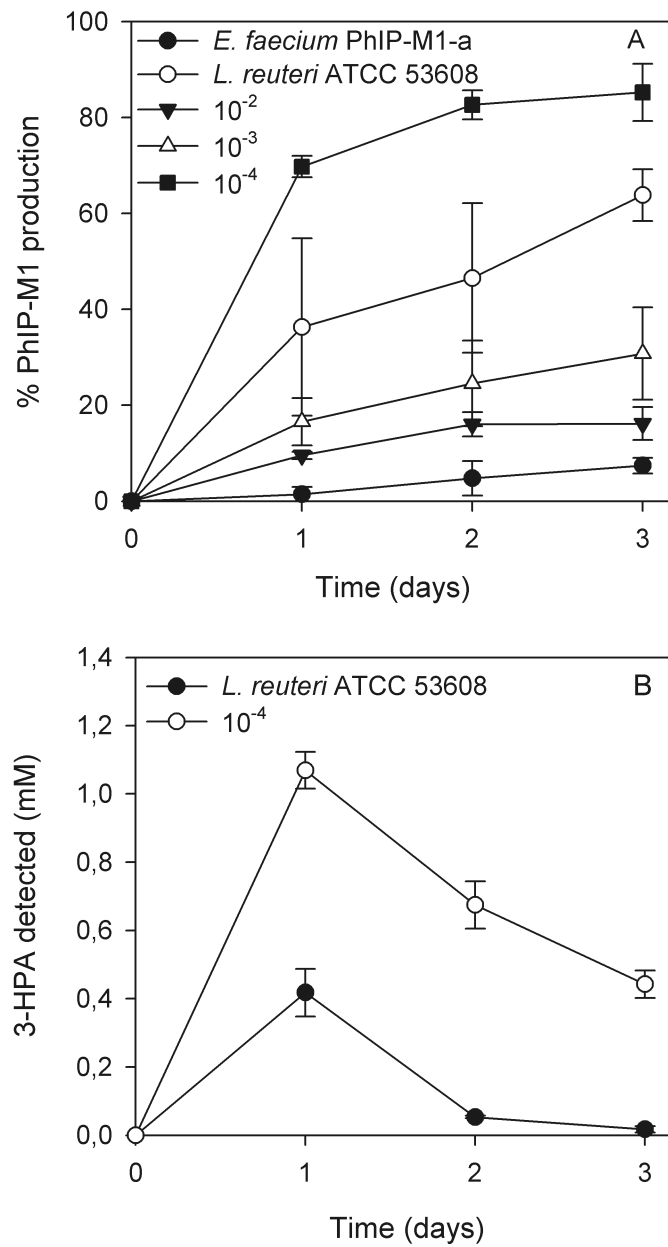

Figure 4.4 Formation of (A) PhIP-M1 and (B) 3-HPA by 10-fold dilutions of mixed fecal microbiota, *Enterococcus faecium* PhIP-M1-a and *Lactobacillus reuteri* ATCC 53608 supplemented with 10 g/L glycerol. Values are means ± SD (n = 3).

Table 4.2. Abiotic synthesis of PhIP into PhIP-M1 by addition of HPA or acrolein to the sterile bacterial growth medium containing 5 μM of PhIP.

Concentration (mM)	% PhIP-M1 production after 24 h	
	Acrolein	HPA
0.1	1.5 ± 0.8	1.2 ± 0.8
1	11.3 ± 0.9	8.6 ± 1.3
10	69 ± 0.6	71 ± 0.5
100	89 ± 5.3	78 ± 0.8

4. Discussion

In this study, we have isolated two individual strains, capable of transforming the food carcinogen PhIP into its derivate PhIP-M1, from human fecal samples and examined the production of PhIP-M1 upon inoculation of the isolated strains under different nutritional conditions. Moreover, we investigated the interindividual variation in PhIP metabolism between eighteen human gut microbiota. In addition, we contributed to the mechanistic basis for this transformation (Figure 4.5) by incubating mixed fecal microbiota under different inactivating conditions and identifying the nutritional requirements for PhIP conversion.

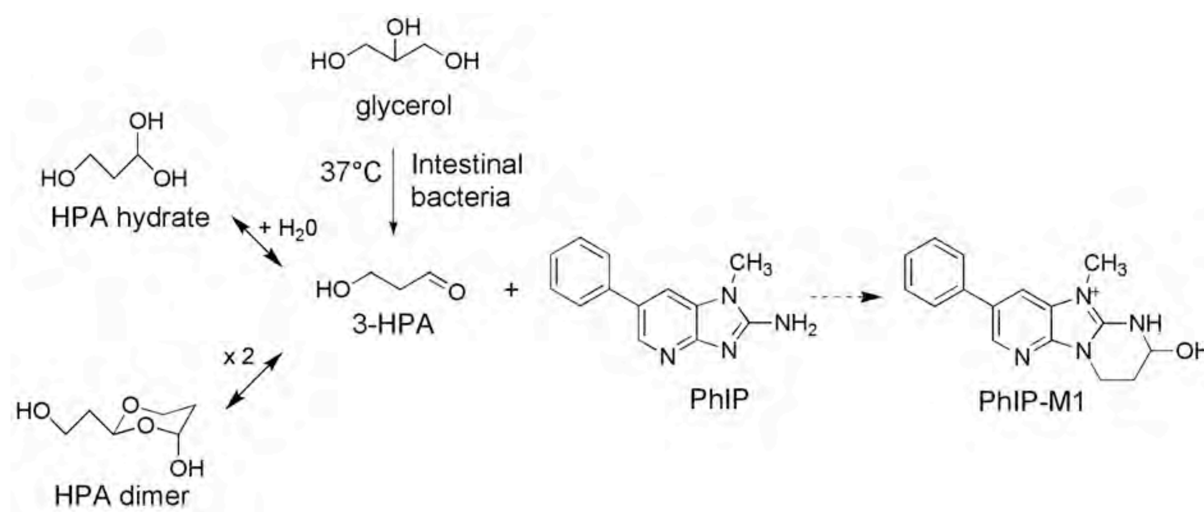

Figure 4.5 Reaction mechanism for microbial PhIP-M1 formation through fermentation of glycerol to 3-HPA by the isolated human intestinal bacteria *Enterococcus faecium* PhIP-M1-a and PhIP-M1-b. → Enzymatic reaction; ↔Equilibrium reactions; ---> Chemical reaction.

Like many other environmental carcinogens, PhIP requires metabolic activation to exert toxic effects. Previous studies indicate that PhIP is converted into two primary products: 2-hydroxyamino-PhIP (N^2-OH-PhIP) and 4'-hydroxyamino-PhIP (4'-OH-PhIP), the former being highly mutagenic, and the latter being non-mutagenic (Crofts *et al.*, 1998; Turesky *et al.*, 2002). These metabolites may subsequently be conjugated with acetyl, glucuronide, glutathione or sulphate to form secondary phase II metabolites. While PhIP is biotransformed into a large number of derivatives in the liver, the human intestinal microbiota selectively convert PhIP into one major metabolite (Vanhaecke *et al.*, 2006). Strong individual variations however occur between the eighteen human fecal samples, screened in this study, with regard to their PhIP-transforming capabilities. Such metabolic variations can be attributed to commonly encountered interindividual differences in microbial community activity and structure. A striking example is the microbial conversion of the dietary phytoestrogen daidzein (Rowland *et al.*, 2000; Decroos *et al.*, 2005). Intensive research has shown that only approximately one third of humans harbour an intestinal microbiota capable of transforming daidzein into equol (Rowland *et al.*, 2000). In addition, as our experimental results have shown, the nutritional composition and concentration of required cofactors for transformation by the individual human feces might greatly influence the individual PhIP metabolism. The differences in PhIP transformation capacity may thus well be linked with individual diet and gastro-intestinal absorption, metabolism and excretion.

Until now, the metabolic nature leading from PhIP to PhIP-M1 was unknown. Liver cytochrome P450 in humans and rats is able to perform several hydroxylations and subsequent glucuronidations of the PhIP molecule. The addition of a ring substituent as observed with PhIP-M1 is however unseen. Our results have clearly shown that this metabolite cannot be produced in the absence of intestinal bacteria, i.e. upon autoclaving of the incubation suspension. Filter sterilization and pasteurization of the fecal slurry significantly decreased the metabolite formation, confirming the role of actively fermenting bacteria in PhIP-M1 formation and this by production of an extracellular substance through an enzymatic process. Reduction of the PhIP-M1 formation after supplementation of the enzyme inhibitor EDTA and the surfactant Triton X-100 may be explained by the involvement of an enzymatic reaction in the PhIP transformation process. Moreover, we have observed that PhIP-M1 production only takes place in the presence of a nitrogen-rich food source containing trace amounts of sugars and carbohydrates. These nutritional requirements were

shown for mixed fecal microbiota as well as for the *Enterococcus faecium* PhIP-M1-a transforming strain. This underlines the importance of a specific bacterial fermentation pattern for PhIP-M1 formation to occur. Besides the nutritional composition of the bacterial medium, additional components or cofactors, present in the fecal matrix, not influenced by autoclaving, are required for PhIP transformation to take place. These fecal constituents, identified during our study as glycerol and its fermentation products and the potential cofactors required by enterococci to perform the glycerol fermentation reaction, are not generally included in culture media for intestinal bacteria. From a nutritional point of view, glycerol may be considered as a relevant colonic nutritional constituent since it is liberated from dietary fat (triglycerides) in the intestinal tract (Matsson and Volpenhein, 1964). Glycerol is a small hydrophilic solute and until recently, it was generally believed to be absorbed mainly by paracellular passive transport from the intestine. Recent research however shows that glycerol absorption is saturable in the rat small intestine *in situ* (Yuasa *et al.*, 2003) and in the HCT-15 human colon cancer cell line (Fujimoto *et al.*, 2006) and involves carrier mediated transport (Kato *et al.*, 2005; Fujimoto *et al.*, 2006). This creates the opportunity for intra-luminal glycerol, depending on the fat intake of the individual, to become available for intestinal microbial metabolism by fermenting strains or fecal excretion.

The 8 PhIP-M1-producing individual bacterial strains that we discovered in the mixed fecal contents of humans (n = 2) and in culture collections (n = 6) are all, except for *Lactobacillus reuteri* members of the genus *Enterococcus* and belong to 3 different species, *Enterococcus durans*, *Enterococcus faecium* and *Enterococcus avium*. All of the strains converted PhIP solely into PhIP-M1, regardless of the extent of substrate consumption (range, 2.4 to 96%). The enterococci phylogenetically belong to the clostridial subdivision of the Gram-positive bacteria and are detected in adult human feces at concentrations of 6.1 ± 0.7 $\log_{10}$ cfu/g (Hopkins *et al.*, 2002). *Lactobacillus reuteri* is also a resident of the gastro-intestinal tract of humans and animals and is one of the dominant heterofermentative species in this ecosystem (Rodriguez *et al.*, 2003). Under anaerobic conditions, several lactobacilli among other bacterial species (*Klebsiella*, *Clostridium*, *Enterobacter*, *Citrobacter*) have been shown to use glycerol as an external electron acceptor (Schutz and Radler, 1984; Talarico *et al.*, 1988; Sauvageot *et al.*, 2000). Our study is however the first to relate bacterial species of the genus *Enterococcus* to this anaerobic pathway of glycerol dissimilation. During this fermentation glycerol is converted by a coenzyme B12-dependent dehydratase to 3-

hydroxypropionaldehyde (3-HPA). 3-HPA is normally an intracellular intermediate that does not accumulate but is reduced by an NAD^{+}-dependent oxidoreductase to 1,3-propanediol (PPD) (Daniel *et al.*, 1998; Biebl *et al.*, 1999). *Lactobacillus reuteri* is unique compared to other lactobacilli in that the glycerol metabolite 3-HPA is excreted in higher amounts than is the case for other lactobacilli forming the HPA system (3-HPA and its aqueous derivates), also known as Reuterin, a potent bacterial inhibitor (Talarico *et al.*, 1990). The regulation of the PPD pathway is dependent on the availability of fermentable carbohydrates, in particular glucose. In the absence of glucose PPD formation is the rate-limiting step and 3-HPA may accumulate. In the present study we have observed that easily degradable sugars inhibit PhIP-M1 production. This may be linked to the absence of 3-HPA and its aqueous derivates under these conditions.

Addition of the HPA system to our bacterial medium significantly enhanced PhIP-M1 formation. The 3-HPA dehydratation product acrolein was also potent in producing PhIP-M1, but was as a consequence of its instable nature in aqueous environments immediately converted to 3-HPA. Another remarkable finding was the relatively fast disappearance of 3-HPA when spiked into a protein-rich bacterial medium. This can be explained by the tendency of 3-HPA and its derivates, molecules which all have aldehyde groups, to react with amino groups in biological tissues (Sung *et al.*, 2003). This tendency of 3-HPA to react with free amino groups may thus very well be responsible for the PhIP to PhIP-M1 conversion. Incubation of a high PhIP transforming mixed fecal dilution and *Lactobacillus reuteri* strain with glycerol clearly gave rise to the formation and detection of 3-HPA, even though a large part of the total amount of 3-HPA produced by the bacteria, was as a result of interactions with cellular material and medium components, probably not detectable. Although Reuterin (the HPA system) is currently accepted as an antibiotic produced by probiotic strains such as *Lactobacillus reuteri*, the risk involved with 3-HPA and its addition potency towards biological tissue components and pro-carcinogens such as PhIP should be taken into account.

PhIP-M1 has been investigated for its potential mutagenic/genotoxic activity. It does not act as a direct mutagen in the Ames test, but a small increase in mutagenicity is observed after addition of S9 liver fraction (Vanhaecke *et al.*, 2006). On the other hand, it has been shown that the intestinal microbiota are essential to the induction of DNA damage by PhIP in human fecal flora associated rats (Hollnagel *et al.*, 2002), and recent investigations

(Vanhaecke *et al.*, 2008c) indicate that PhIP-M1 exerts cytotoxic and apoptotic effects towards Caco-2 cells *in vitro*. Such contrasting data highlight the necessity of identifying the metabolites produced by microbial processes from important known pro-carcinogens in our diet and of further evaluating their genotoxic/carcinogenic activity.

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CHAPTER 5

The microbial PhIP metabolite 7-hydroxy-5-methyl-3-phenyl-6,7,8,9-tetrahydropyrido[3’,2’:4,5]-imidazo[1,2-*a*]pyrimidin-5-ium chloride (PhIP-M1) induces DNA damage, apoptosis and cell cycle arrest towards Caco-2 cells

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The microbial PhIP metabolite 7-hydroxy-5-methyl-3-phenyl-6,7,8,9-tetrahydropyrido[3',2':4,5]imidazo[1,2-*a*]pyrimidin-5-ium chloride (PhIP-M1) induces DNA damage, apoptosis and cell cycle arrest towards Caco-2 cells

ABSTRACT

7-Hydroxy-5-methyl-3-phenyl-6,7,8,9-tetrahydropyrido[3',2':4,5]imidazo[1,2-*a*]pyrimidin-5-ium chloride (PhIP-M1) is a newly identified intestinal microbial metabolite from the food carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). Although the mutagenic potential of the endogenous N-hydroxy PhIP derivate has been reported, the risks associated with PhIP-M1 have not yet been explored. In this work, the cytotoxic and genotoxic effects originating from PhIP-M1 were assessed in the epithelial intestinal Caco-2 cell line. PhIP-M1 significantly decreased in a time- and dose-dependent manner mitochondrial dehydrogenase activity and protein synthesis, with IC_{50} values of, respectively, 180 ± 39.4 and 173 ± 20.3 μM after 24 h, and 33.8 ± 3.5 and 37.3 ± 10.9 μM after 72 h. Apoptosis within the concentration ranges of cytotoxicity was confirmed by morphological examination, DAPI nuclear staining and annexin V staining. PhIP-M1 provoked cell cycle arrest, characterized by a significant increase in the number of nucleoids in the G2/M phase. A dose-dependent increase in DNA damage, as quantified by the alkaline comet assay, was observed after 3 h in the 50-200 μM range. Because these PhIP-M1-induced genomic and cellular events may contribute to the carcinogenicity of PhIP, the potency of the colon microbiota to bioactivate PhIP must be included in future risk assessments.

1. Introduction

Diet has long been recognized as one of the major risk factors in human cancer (Doll and Peto, 1981). Epidemiological studies indicate that the consumption of meat is positively correlated with human cancer, particularly of the colon (Doll, 1992). Cooking of meat is known to generate potent genotoxic carcinogens, including the heterocyclic aromatic amine class of chemical compounds (Sugimura, 1997). The most abundant compound among these heterocyclic amines, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), has been shown to specifically induce tumors of the colon, mammary gland and prostate in rats (Ito *et al.*, 1991; Shirai *et al.*, 1997), which, co-incidentally are the three most common sites of diet-associated cancer in the Western world.

To obtain its mutagenic potential, PhIP requires metabolic activation by drug metabolizing enzymes (Aeschbacher and Turesky, 1991). In common with other genotoxic aromatic amines, PhIP is metabolically activated by oxidation of the exocyclic amino group, a reaction mediated mainly by the cytochrome P450 isoenzyme CYP1A2 (Crofts *et al.*, 1998; Turesky *et al.*, 2002). N^2-hydroxy-PhIP, which is mutagenic on its own, can be converted by Phase II metabolizing enzymes to the more biologically reactive electrophilic *O*-sulfonyl and *O*-acetyl esters, which have the capacity to bind DNA and cellular proteins (Buonarati *et al.*, 1991; Boobis *et al.*, 1994; Edwards *et al.*, 1994). Detoxification primarily involves glucuronidation. N^2-hydroxy-PhIP can form stable glucuronide conjugates at the N^2 and N^3 positions, which can be excreted or transported to extra-hepatic tissue for further metabolism (Alexander *et al.*, 1991; Kaderlik *et al.*, 1994). PhIP can also be hydroxylated at the 4' position. 4'-Hydroxy-PhIP can be conjugated by sulfation and glucuronidation to polar compounds that are readily excreted (Watkins *et al.*, 1991; Buonarati *et al.*, 1992). In addition, the parent compound can be directly glucuronidated at the N^2 and N^3 positions. These glucuronides are not reactive and therefore considered as detoxification products (Styczynski *et al.*, 1993; Kaderlik *et al.*, 1994).

While PhIP is biotransformed into a large number of derivates by mammalian enzyme systems, the human intestinal microbiota selectively convert PhIP into one major metabolite, 7-hydroxy-5-methyl-3-phenyl-6,7,8,9-tetrahydropyrido[3',2':4,5]imidazo[1,2-*a*]pyrimidin-5-ium chloride (PhIP-M1) (Vanhaecke *et al.*, 2006; Vanhaecke *et al.*, 2008b) (Figure 5.1). This compound has been detected in human urine and feces following consumption of well-done

chicken meat and did not act as a direct mutagen in the Ames test (Vanhaecke *et al.* 2008a). On the other hand, it has been shown that the intestinal microbiota are essential to the induction of DNA damage by PhIP in human fecal microbiota associated rats (Hollnagel *et al.*, 2002).

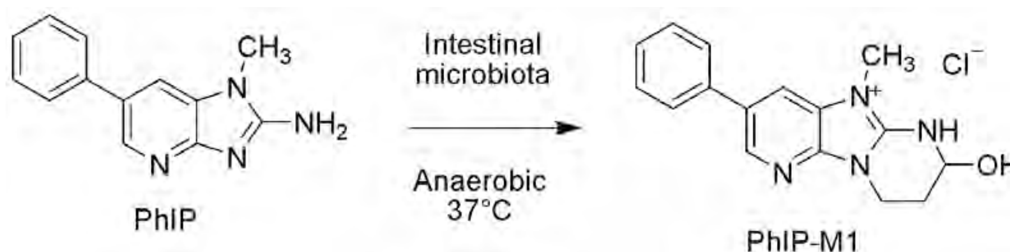

Figure 5.1 Metabolite of PhIP formed by the human intestinal microbiota: 7-hydroxy-5-methyl-3-phenyl-6,7,8,9-tetrahydropyrido[3’,2’:4,5]imidazo[1,2-*a*]pyrimidin-5-ium chloride, PhIP-M1 (Vanhaecke *et al.*, 2006).

In this context, we have performed an in *vitro* evaluation of the cytotoxic and genotoxic potential of the newly identified microbial PhIP metabolite, PhIP-M1, on the human intestinal Caco-2 cell line. These cells were chosen as target since the exposure site to PhIP-M1 is the colon and because the colon is known to be one of the main target tissues for PhIP induced cancer.

2. Materials and methods

2.1 Chemicals

PhIP was purchased from Toronto Research Chemicals (Ontario, Canada). For incubation purposes, it was dissolved in dimethyl sulfoxide (DMSO). PhIP-M1 (7-hydroxy-5-methyl-3-phenyl-6,7,8,9-tetrahydropyrido[3’,2’:4,5]imidazo[1,2-*a*]pyrimidin-5-ium chloride) was synthesized and purified as described by Vanhaecke *et al.* (2008b). Purity of PhIP-M1 was 97 ± 0.8% as determined by LC-MS. PhIP and PhIP-M1 stock solutions of, respectively, 50 mM and 100 mM and subsequent working solutions were prepared in DMSO and distributed so that the final DMSO concentration was maximum 1%. Previous research has shown that this concentration does not decrease Caco-2 cell viability (Da Violante *et al.*, 2002).

2.2 Cell culture

The human colonic carcinoma Caco-2 cell line (ATCC HTB37) was obtained from Eric Pringault (Institut Pasteur, Paris, France)(Chastre *et al.*, 1993). Cells were sub-cultured weekly in Dulbecco's modified Eagle's medium (DMEM) + Glutamax (Invitrogen, Merelbeke Belgium), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% non-essential amino acids (100x) (Invitrogen), penicillin (100 IU/mL) (Invitrogen), streptomycin (100 µg/mL) and human transferrin (5 µg/mL) (both from Invitrogen). Cells were maintained as monolayer cultures at 37 °C under a humidified atmosphere of 10% CO_2. Cells were passaged weekly, using 0.05% (w/v) trypsin and 0.02% (w/v) EDTA. For the experiments Caco-2 cell cultures were harvested at 80% confluency.

2.3 Methyl tetrazolium (MTT) cytotoxicity assay

Cell viability following PhIP-M1 or PhIP exposure was examined using an MTT assay. Metabolically active mitochondrial dehydrogenases convert the tetrazolium salt MTT to insoluble purple formazan crystals at a rate that is proportional to cell viability. The cultured Caco-2 cells (200 µL) were seeded in 96-well plates at a concentration of 1 x 10^5 cells/mL and exposed during 3, 24 or 72 h to increasing concentrations of PhIP-M1 (0-700 µM for 3 h treatment and 0-180 µM for 24 and 72 h treatments). Prior to the 3 h exposure, cells were allowed to adhere overnight. At the end of the respective incubation periods, 20 µL of a MTT solution (Sigma, St. Louis, Missouri, USA) (5 mg/mL in PBS) was added to each well and plates were returned to incubate for two additional hours at 37 °C in the dark. Subsequently, the growth medium was taken off and the formazan crystals were resuspended in 200 µL of DMSO. The 490 nm absorbance was read using a Microplate reader (Molecular Devices, Sunnyvale, CA, USA). Relative cell viability (in percentage) was expressed as (Abs_{490} treated cells/Abs_{490} control cells) x 100. The IC_{30} and IC_{50} values were estimated by means of linear regression from a graph depicting cellular sensitivity versus PhIP-M1 concentration.

2.4 Cell viability using trypan blue exclusion

Trypan blue exclusion was also performed to assess cytotoxicity of PhIP-M1 on Caco-2 cells. Cells were seeded in 6-well plates at concentrations of 3 x 10^5 cells/mL. Cells were treated with varying concentrations of PhIP-M1 (0-700 µM for 3 h treatment and 0-180 µM

for 24 and 72 h treatments) for 3, 24 or 72 h. Subsequently the medium was removed from the wells by aspiration, the cells were washed with moscona and trypsin-EDTA was added in order to detach the cells from the wells. Finally, the cells were resuspended in fresh medium, and counted under the microscope using trypan blue (Sigma) as a marker for cell viability. Relative cell viability (in percentage) was expressed as (Number of viable treated cells/Number of viable control cells) x 100. The IC_{30} and IC_{50} values were estimated by means of linear regression from a graph depicting cellular sensitivity versus PhIP-M1 concentration.

2.5 Sulforhodamine B (SRB) growth inhibition assay

The SRB assay was performed to assess the growth inhibition of PhIP-M1 or PhIP towards Caco-2 cells. Cell proliferation, measured as total protein synthesis, is a very sensitive toxicology marker. Sulforhodamine B (SRB) is an anionic dye that binds to proteins electrostatically. The fixed dye, measured photometrically (490 nm) after solubilization, correlates with the total protein synthesis rate and therefore with cell proliferation. The SRB assay was performed in 96-well plates containing 200 µL of culture medium seeded at 1×10^5 cells/mL. Prior to the 3 h exposure, cells were allowed to adhere overnight. At various times post-exposure to a broad PhIP-M1 concentration range (0-700 µM for 3 h treatment and 0-180 µM for 24 and 72 h treatments), 50 µL of a trichloroacetic acid solution (Sigma) (50%) was added (1 h incubation at 4 °C) to assure fixation of the cells. Thereafter, plates were rinsed with water, dried and stained with 200 µL of SRB (Sigma) solution per well (0.4% in 1% acetic acid). After 30 min, unbound dye was removed by rinsing with 1% ice acetic acid. Subsequently, cell bound dye was extracted with Tris buffer (200 µL, 10 mM, pH 10.5) and determined photometrically on a Microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 490 nm. Inhibition of growth was expressed as relative viability (Abs_{490} treated cells/Abs_{490} control cells) x 100. The IC_{30} and IC_{50} values were calculated by means of linear regression of concentration/response curves.

2.6 Assessment of cell injury

Lactate dehydrogenase (LDH) leakage is a means of measuring membrane integrity as a function of the amount of cytoplasmic LDH released from the cytosol into the medium. Total LDH activity measurements were performed with an automated controlled system

(Roche/Hitachi Modular Analytics SWA, Tokyo, Japan), with a lower limit for detection of 1 U/L and a coefficient of variation of < 5%. The LDH released (%) after 24 h exposure with increasing concentrations of PhIP-M1 (0-300 μM) was expressed as (LDH activity in treatment - LDH activity in control medium)/(LDH activity in total death cells - LDH activity in control medium) x 100%. Total LDH release corresponding to complete Caco-2 cell death was determined by treatment with 1% Triton X-100.

2.7 Evaluation of changes in cell morphology

Caco-2 cells were seeded at 3 x 10^5 cells/mL in 6-well plates and treated for 24 h with increasing concentrations of PhIP-M1 (0-100 μM). After treatment, the cells were observed through an inverted light microscope (Leica Microsystems, Heerbrugg, Switzerland).

2.8 Apoptosis assay by flow cytometry

Caco-2 cells were seeded at 3 x 10^5 cells/mL in 6-well plates and after 3 or 24 h of exposure to PhIP-M1 (0-300 μM), PhIP (300 μM) or DMSO solvent control, detection of apoptosis was performed using the annexin V-FITC binding assay (human annexin V–FITC Detection kit, Bender MedSystems Diagnostics, Vienna, Austria). The basis of this assay is that during apoptosis, phosphatidylserine is translocated from the inner side to the outer side of the plasma membrane, where it can be detected by conjugation with annexin V-FITC. Annexin V-FITC-positive/propidium iodine (PI)-negative cells are considered apoptotic. Briefly, cells were resuspended in binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Annexin V-FITC was added according to the product insert and incubated for 15 min at room temperature in the dark. One minute before flow cytometric analysis, PI was added at a concentration of 20 μg/mL. Cells were analyzed by a Beckman Coulter Cytomics FC500 flow cytometer (Beckman Coulter, Miami, FL, USA). About 10 000 events were accumulated per sample, quadrant settings were based on control samples.

2.9 Morphological assessment of apoptosis in cells

Caco-2 cells were seeded on coverslips (3 x 10^5 cells/mL). After 24 h of exposure to increasing PhIP-M1 concentrations (0-300 μM) cells were fixed in ice-cold methanol (-20 °C) for 15 min. After rinsing with Tris Buffered Saline, cells were stained with DAPI (0.4 μg/mL)

for 15 min in the dark and then visualized with an inverted fluorescence microscope (Leica Microsystems, Heerbrugg, Switzerland). Apoptotic cells were defined as cells showing nuclear and cytoplasmic shrinkage, chromatin condensation and apoptotic bodies.

2.10 Flow cytometry analysis of cellular cycle of Caco-2 cells

For analysis of the cell cycle distribution, the Coulter® DNA Prep™ Reagents Kit (Beckman Coulter, Fullerton, CA, USA) was used according to the manufacturer's recommendations. Caco-2 cells were seeded into T25 flasks (10^6 cells/ flask) and treated with PhIP-M1 (0-100 µM), PhIP (0-100 µM) or DMSO solvent control for 3 or 24 h. After the treatment, cells were harvested by trypsinization, washed with PBS and exposed to DNA Prep LPR for 1 min, followed by incubation with DNA Prep Stain for 15 min at room temperature in the dark. Cellular DNA content was monitored on a Beckman Coulter Cytomics FC500 flow cytometer (Beckman Coulter, Miami, FL, USA). Cell cycle fractions were quantified using WinCycle software (Phoenix Flow Systems, San Diego, CA).

2.11 Genotoxicity testing using the comet assay

Cells were seeded at 3 x 10^5 cells/mL in 96-well plates and exposed for 3 h to PhIP or PhIP-M1 concentrations (0-200 µM) determined as non-cytotoxic ($<IC_{30}$) in the previous cytotoxicity assessment step. Positive controls were performed by treating two wells per plate with 0.1 mM or 1 mM methyl methanesulfonate (MMS) for 3 h. The cell content of each treated well was collected by centrifugation at 400 x g for 5 min. Cell viability was verified by the MTT assay. Only cell suspensions exhibiting a viability of >70% were used. The comet assay was then carried out according to the procedure described by Tice *et al.* (2000) with slight modifications. Seventy-five microlitres of 0.5% low-melting point agarose containing 3 x 10^4 cells were spread on a slide previously covered with two layers of, respectively, 1.5% and 0.8% normal-melting point agarose. A coverslip was added and the agarose was allowed to solidify on ice. Then the coverslip was removed and the cells lysed immediately by immersion in a solution (pH 10) of 2.5 M NaCl, 100 mM EDTA, 10 mM TRIS, 1% Triton X-100 (v/v) and 10% DMSO (v/v) for at least 1 h at 4 °C. The slides were removed and placed on a horizontal gel electrophoresis unit, which was filled with freshly prepared alkaline buffer (1 mM EDTA and 300 mM NaOH, pH > 13). In order to reduce the variability associated with gel box slide position or multiple electrophoresis runs, slides were

randomly distributed. The cells were exposed to the alkali for 20 min to allow DNA unwinding and expression of single-strand breaks and alkali-labile sites. Next, electrophoresis was conducted for 20 min at 0-4 °C by applying an electric current of 0.7 V/cm (25 V/300 mA). All these steps were conducted sheltered from daylight to prevent the occurrence of additional DNA damage. After electrophoresis, the slides were neutralized with 0.4 M Tris (pH 7.5) and the DNA was exposed for 5 min to absolute ethanol in order to preserve the comet slides. Subsequently, the slides were air-dried and stored at room temperature until scored for DNA migration. Just prior to scoring, the DNA was stained with propidium iodide (20 µg/mL; 25 µL/slide). Slides were coded and examined at 250× magnification using a fluorescent microscope (Leica Microscopy and Scientific Instruments Group, Heerbrugg, Switzerland) equipped with an excitation filter of 515-560 nm and a 590 nm barrier filter, connected through a gated CCD camera to Comet Image Analysis System version 4.0 software (Kinetic Imaging Ltd., Liverpool, UK). For each concentration, 100 randomly selected cells (50 cells from each of the two replicate slides) were analyzed and the comet parameter retained was the Olive tail moment (OTM). The quantitative data were derived from six sets of independent experiments.

2.12 Halo assay

The halo assay, also known as the low molecular weight (LMW) DNA diffusion assay is one of the few methods for measuring apoptosis and necrosis *in vitro* that also matches the higher sensitivity of the comet assay (Godard *et al.*, 1999). In the absence of electrophoresis, cells with extensive DNA degradation associated with cell death exhibit a highly diffuse pattern of DNA, while viable cells have a condensed pattern associated with the high molecular weight DNA. To assess for the presence of cells with low molecular weight (LMW) DNA indicative of cell death, one comet slide from each sample was removed from the electrophoresis buffer (1 mM EDTA and 300 mM NaOH, pH > 13) after unwinding for 20 min and directly washed in neutralization buffer (0.4 M Tris, pH 7.5), dipped in ethanol and air-dried, without being submitted to an electric field. After staining the slides with propidium iodide, 100 cells per slide were scored visually and classified using the following criteria: Type I = mostly condensed DNA with little or no diffusion; Type IIa = mostly diffused DNA with a visible nucleus, Type IIb = completely diffused DNA with no visible nucleus.

2.13 Statistical analysis of data

The data are presented as means ± standard deviation (SD) or standard error (SE). The statistical significance for the cytotoxicity, apoptosis, cell cycle analysis and LMW DNA diffusion assays was determined using the Student's *t*-test. For the genotoxicity study, the Olive Tail Moment (OTM) was used to evaluate DNA damage. Since the OTM frequencies and other tail parameters do not follow a Gaussian distribution (Bauer *et al.*, 1998), the non-parametric Kruskall-Wallis test was used to display a possible dose-effect relationship. Moreover, the statistical significance of differences in the median values between each group versus the control was determined with the non-parametric Mann-Whitney U-test.

3. Results

HPLC analysis (Vanhaecke *et al.*, 2008b) performed on cell supernatants has shown that initial PhIP and PhIP-M1 concentrations to which Caco-2 cells were exposed, were not modified during 3, 24 or 72 h of incubation, implying that endogenic PhIP or PhIP-M1 metabolism does not take place in Caco-2 cells (data not shown).

3.1 Effect of PhIP-M1 on Caco-2 viability and growth

Trypan blue exclusion was used to determine the number and viability of Caco-2 cells following PhIP-M1 treatment. Growth inhibition was determined using the SRB assay. The cytotoxicity of the newly identified microbial PhIP derivate, PhIP-M1, was evaluated using the MTT assay. PhIP-M1 decreased the MTT reactions of Caco-2 cells and inhibited the growth in a concentration- and time-dependent manner (Figure 5.2 A-C). The addition of PhIP-M1 to Caco-2 cells resulted for the 3 h treatment in a significant decrease in cell viability as measured using trypan blue exclusion (data not shown). At the highest dose (700 μM), cell viability was reduced to 0%. For the 24 and 72 h treatments however there was very little change in cell viability (dye exclusion), but a marked inhibition of cell growth was observed (decrease in cell number recorded under the microscope), which was dose-dependent and detectable at lower concentrations (lower IC_{30} and IC_{50}) than the observed MTT and SRB responses (Table 5.1). PhIP-M1 concentrations exhibiting 30 and 50% decrease in cell viability were found to be in a very similar range when calculated with the MTT or SRB assays for the 24 and 72 h exposure durations.

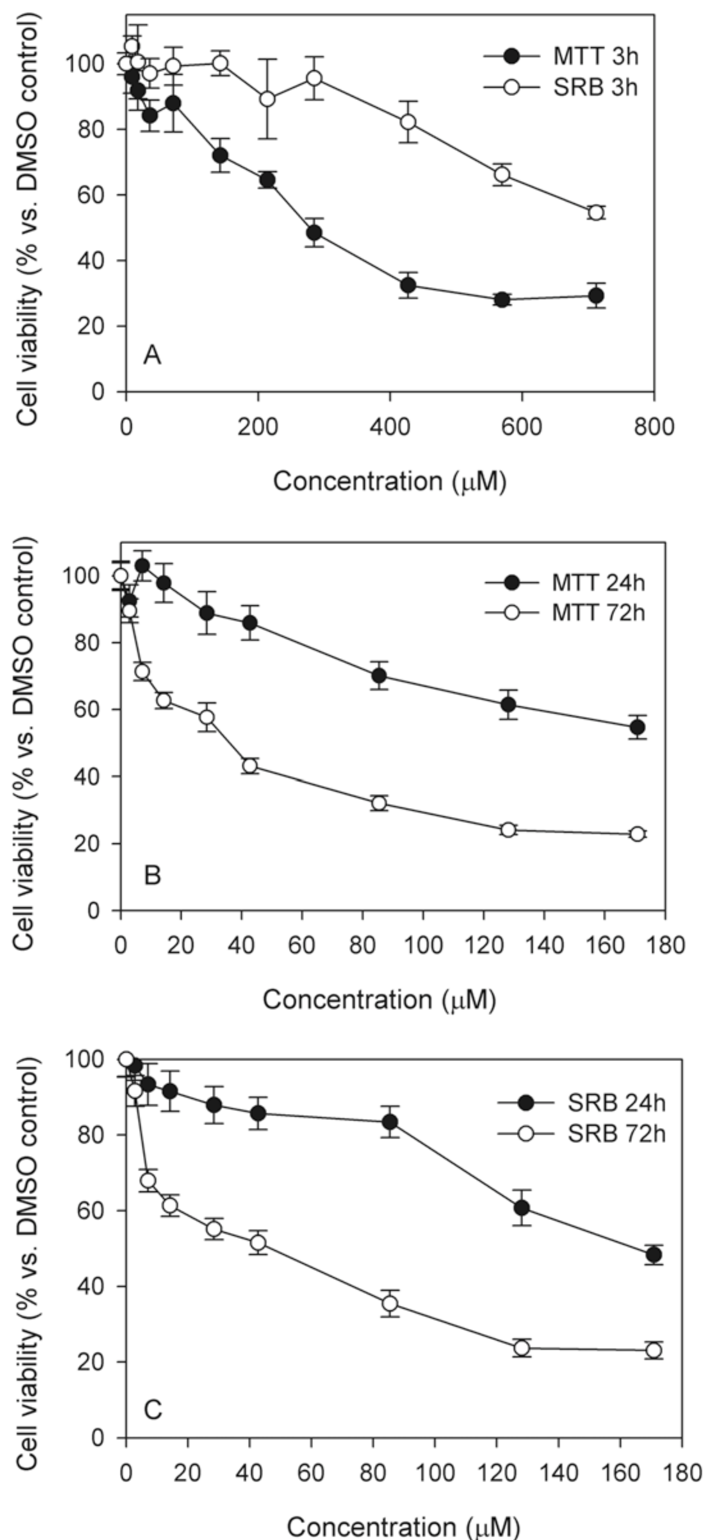

Figure 5.2 Effect of the microbial PhIP derivate PhIP-M1 on Caco-2 cell viability, (A) 3 h exposure using the MTT and SRB assays, (B) 24 and 72 h exposure using the MTT assay, (C) 24 and 72 h exposure using the SRB assay. Values are expressed as percent of control response and each value is a result of at least three independent experiments in four replicates. Bars represent SD.

An exposure time of 3 h had a lower impact on the total protein synthesis, than on mitochondrial activity and trypan blue exclusion. This might be the result of the limited growth and protein synthesis that cells experience during this short incubation period. PhIP did not exert significant cytotoxic effects at the 3 and 24 h exposure times and only minor effects compared to PhIP-M1 after 72 h exposure (Table 5.1).

Table 5.1. IC_{30} and IC_{50} values (± SD) for Caco-2 cells exposed during 3, 24 and 72 h to PhIP and PhIP-M1 as measured by the TBE (trypan blue exclusion), MTT and SRB assays (- : no data).

Treatment	IC_{30} (µM)			IC_{50} (µM)		
	TBE	**MTT**	**SRB**	**TBE**	**MTT**	**SRB**
PhIP-M1						
3 h	233 ± 9.2	198 ± 24.2	602 ± 80.9	345 ± 11.6	358 ± 48.3	> 710
24 h	21.9 ± 4.3	106 ± 26.5	101 ± 23.7	46.9 ± 4.5	180 ± 39.4	173 ± 20.3
72 h	5.4 ± 0.15	16.6 ± 2.5	17.6 ± 9.2	10.4 ± 0.46	33.8 ± 3.5	37.3 ± 10.9
PhIP						
3 h	-	> 710	> 710	-	> 710	> 710
24 h	-	> 180	> 180	-	> 180	> 180
72 h	-	153 ± 7.5	149 ± 9.3	-	> 180	> 180

To evaluate whether the observed cytotoxic effects could be attributed to PhIP-M1 induced plasma membrane damage, LDH release upon a 24 h treatment with 0-300 µM of PhIP-M1 was measured. At the highest PhIP-M1 concentrations tested (100, 200 and 300 µM), LDH releases of, respectively, 9.2 ± 1.3%, 12.6 ± 3.2% and 17.8 ± 7.4% were recorded.

In addition, light microscopic observations were made on Caco-2 cells treated for 24 h with PhIP-M1. As shown in Figure 5.3 A, only limited morphological changes were observed between the solvent controls and the 10 µM treatment group. However, in Caco-2 cells treated with 100 µM PhIP-M1, dramatic changes (Levin *et al.*, 1999) were observed: cell shrinkage and cytoplasmic condensation. Cells retracted from their neighboring cells, rounded up and eventually floated into the medium, which are indicative for apoptosis (Geng and Libby, 2002).

3.2 Evaluation of the role of PhIP-M1 in apoptosis

The morphological changes in Caco-2 cells and the LDH measurements suggested PhIP-M1 induced apoptotic cell death and only very limited cell necrosis. To substantiate this hypothesis, cells were treated for 24 h with increasing concentrations of PhIP-M1 (10-300 μM) and morphological changes in cell nuclei were examined through fluorescence microscopy after DAPI staining. Normal nuclei show normal distribution of euchromatin and heterochromatin with homogeneous fluorescence intensity. In contrast, upon PhIP-M1 exposure, various stages of the apoptotic process could be observed: chromatin condensation, characteristic of early morphological nuclear manifestations of apoptosis and nuclear fragments with membrane-bounded apoptotic bodies as the latter event of PhIP-M1-initiated apoptosis in Caco-2 cells (Figure 5.3 B). Interestingly, at the lower PhIP-M1 concentrations a significant increase in cells showing chromosome replication and chromatid segregation could be observed, providing evidence for a G2/M arrest.

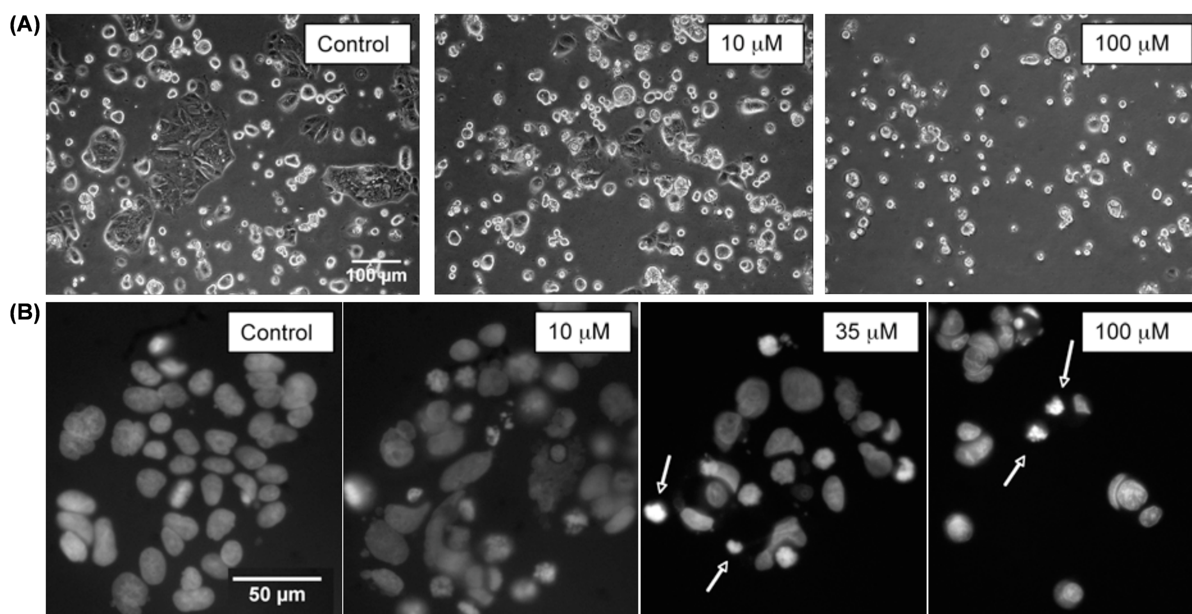

Figure 5.3 (A) Photomicrographs of the effects of PhIP-M1 on Caco-2 cells. (B) Morphological changes in cell nuclei as determined by fluorescence microscopy after DAPI staining. Arrows indicate apoptotic nuclei. Cells were treated with PhIP-M1 for 24 h at the concentrations indicated in the figure.

To further evaluate the significance of these morphological observations with respect to apoptosis, annexin V-FITC staining was performed on cells treated with PhIP-M1 (10-300

μM). The percentage of apoptotic cells after treatment with different concentrations of PhIP-M1 for 3 and 24 h are presented in Figure 5.4.

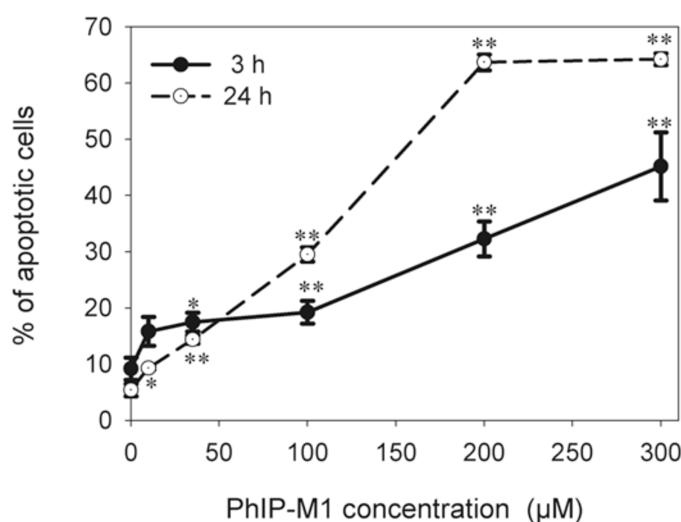

Figure 5.4 PhIP-M1-induced phosphatidylserine externalization in Caco-2 cells. PhIP-M1-induced apoptosis was assessed by determining the percentage of annexin V^+/PI^- cells after 3 h and 24 h treatment. Data are expressed as means ± SE of at least 3 independent experiments. Significantly different (Student's *t*-test) from control, * $p < 0.05$; ** $p < 0.01$.

Apoptosis in sub-confluent Caco-2 cells was significantly induced by PhIP-M1 in both a concentration-dependent and a time-dependent manner. Moreover, for the higher incubation concentrations (200-300 μM) during the 24 h exposure, a significant increase in the number of PI+ cells, which are considered late apoptotic, was observed with an average percentage of 28.7 ± 2.7 at 300 μM (Figure 5.5 A-C). However, PhIP at the highest concentration of 300 μM, did not significantly induce phosphatidylserine externalization (Figure 5.5 D).

3.3 Induction of cell cycle arrest by PhIP-M1

In order to determine whether PhIP-M1 had any effect on progression through cell cycle, Caco-2 cells were treated with increasing concentrations (< IC_{30}) of PhIP-M1 (0-100 μM) for 3 or 24 h. As depicted in Figure 5.6 B, exposure to relatively low concentrations (1-10 μM) of PhIP-M1 resulted after 24 h in a dose-dependent significant increase in the G2/M cell population, accompanied by a decrease in G0/G1 and S cell populations.

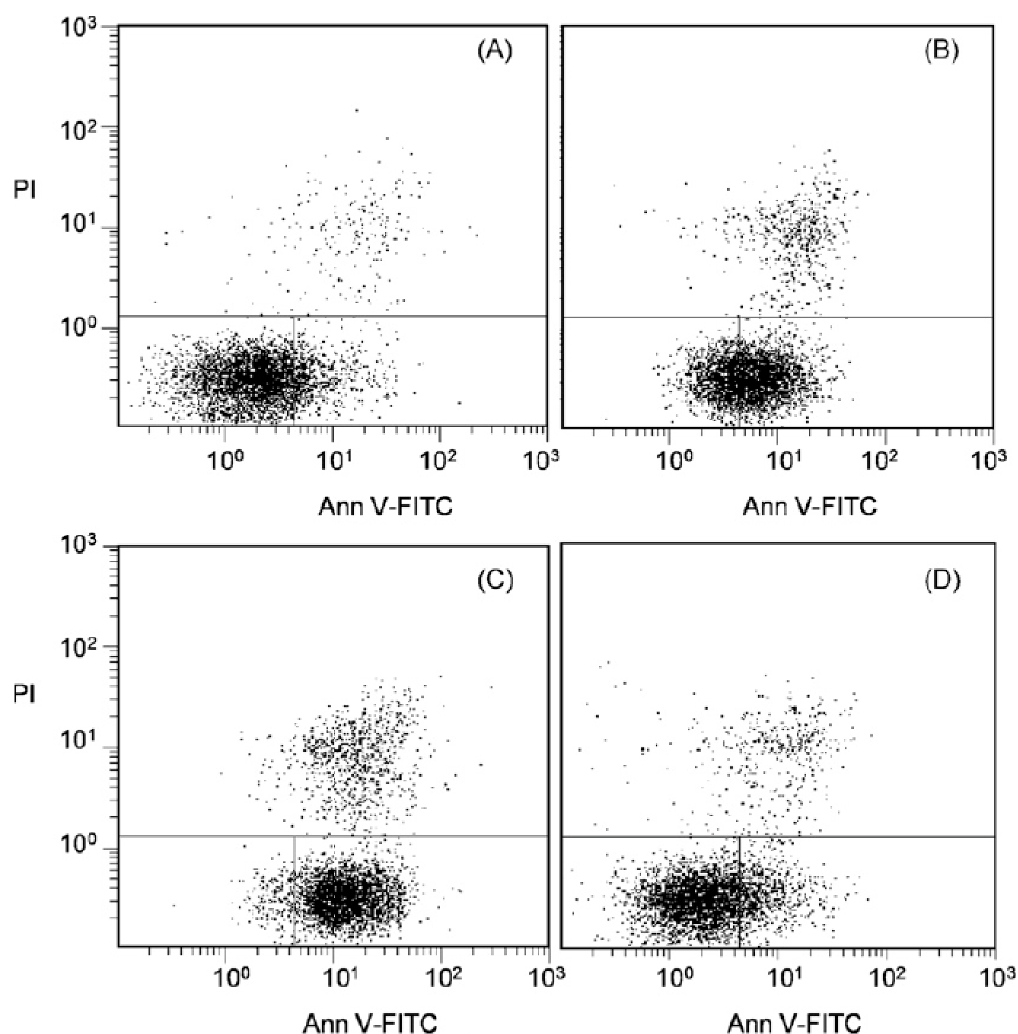

Figure 5.5 FACS analysis of apoptosis in Caco-2 cells exposed for 24 h to (A) solvent control, (B) 100 μM PhIP-M1, (C) 300 μM PhIP-M1 and (D) 300 μM PhIP.

Cells treated with concentrations higher than 35 μM showed again a decrease in G2/M cell population to eventually become even lower than the control treatment at 100 μM and result in a significant increase in S cell population. From Figure 5.6 A, it can be seen that after 3 h PhIP-M1 already affected the cell cycle. A similar increase in G2/M cell population at concentrations below 35 μM, accompanied by a decrease in G0/G1 cell population was observed. At concentrations higher than 35 μM, the G2/M population started to decrease again but at this time point no pronounced effects on the S-phase were observed, while at the highest concentration (200 μM) a significant increase in G0/G1 nucleoids was measured. At the same concentration range and exposure time, PhIP, without any metabolic activation, failed to affect the cell cycle profile.

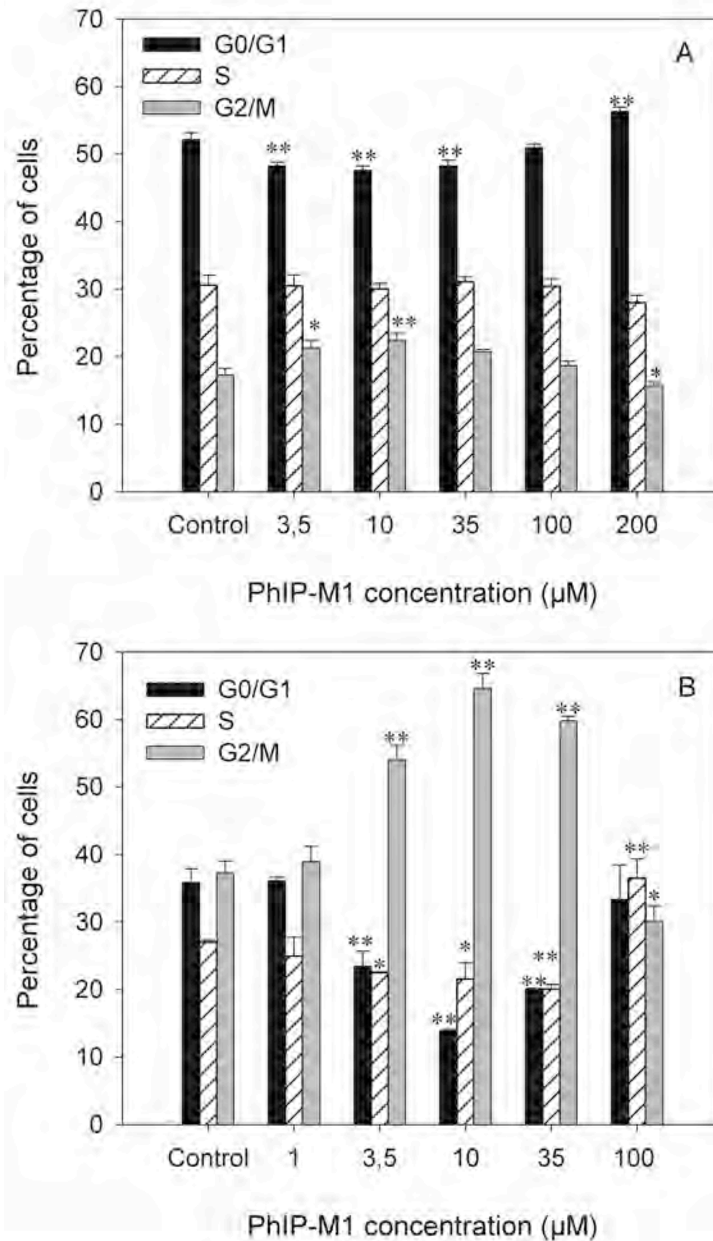

Figure 5.6 Percentage of cells in each stage of cell cycle after treatment with PhIP-M1 in Caco-2 cells for (A) 3 h and (B) 24 h. Data are expressed as means ± SD of at least 3 independent experiments. Significantly different (Student's *t*-test) from control, * $p < 0.05$; ** $p < 0.01$.

3.4 DNA damage in Caco-2 cells exposed to PhIP-M1

In accordance with the guidelines proposed by Tice *et al.* (2000), the decrease in cell viability should not be more than 30% when compared with the concurrent control. Therefore it was decided to undertake the subsequent genotoxicity study by using a PhIP-M1

concentration range peaking at 200 μM, corresponding to the lowest calculated IC_{30} (3 h exposure). In parallel with the comet assay, as a critical component in data interpretation, cytotoxicity was measured in all samples analyzed for DNA damage using the MTT assay and the halo or LMW DNA diffusion assay.

The median comet tail moments, the mitochondrial viability and the percentage of Type II cells upon exposure of Caco-2 cells to different concentrations of PhIP-M1 are shown in Table 5.2. Also the data for PhIP, without any metabolic activation, solvent and positive MMS controls are presented in Table 5.2.

Table 5.2. DNA damage, cytotoxicity and Low Molecular Weight DNA diffusion in Caco-2 cells as estimated by the median Olive Tail moment, the mean mitochondrial viability (± SD) and the mean percentage of Type II cells (± SD) in the halo assay (Type IIa: halo with nucleus, Type IIb: halo without nucleus) after 3 h exposure to various PhIP-M1 concentrations. Significantly different from control, * $p < 0.05$; ** $p < 0.01$.

Treatment	Comet assay	MTT assay	Halo assay		
	Median OTM	% viability	% Type IIa	% Type IIb	% Total Type II
PhIP-M1 (μM)					
0	0.56	100 ± 0.0	7.3 ± 0.35	5.5 ± 0.50	13 ± 0.35
10	0.59	98 ± 2.8	7.3 ± 2.5	4.0 ± 2.2	12 ± 2.8
50	0.75	93 ± 13	7.8 ± 1.8	7.5 ± 1.8	15 ± 0.35
100	1.1 *	93 ± 9.9	11 ± 3.7	7.5 ± 1.6	18 ± 2.8*
150	1.2 *	79 ± 17	17 ± 7.2	12 ± 0.35	29 ± 7.5*
200	3.0 **	72 ± 16*	27 ± 2.2	17 ± 2.0	44 ± 3.4**
PhIP (μM)					
180	0.52	84 ± 24	13 ± 2.9	8.5 ± 1.6	21 ± 2.0**
MMS (mM)					
0.1	3.2 **	91 ± 15	56 ± 16.9	9.5 ± 4.9	66 ± 12.0**
1	28 **	73 ± 12*	0.0 ± 0.0	100 ± 0.0	100 ± 0.0**

It can be seen from Table 5.2 that PhIP-M1 evoked a dose-dependent ($p < 0.01$, Kruskal-Wallis) increase in the median comet tail moment of the intestinal cells, but within the same concentration-range a dose-dependent increase in LMW DNA diffusion was observed. This significant increase in Type II cells and comet tail moment was also observed in a dose-dependent manner for the positive control MMS, while native PhIP evoked a

significant increase in LMW DNA diffusion, but did not modify the comet tail moment. The mitochondrial viability remained for the different PhIP-M1 concentrations and control samples above 70%.

The most straightforward way to interpret data from the comet assay is by presenting the distribution of the cells according to the percentage of DNA in the tail, which is positively correlated with the comet tail moment. From Figure 5.7 it can be seen that PhIP-M1 treatment caused a dose-dependent decrease in the number of cells with OTM values from 0-0.99 and 1-1.99 and a dose-dependent increase in the number of cells with OTM classes 2-9.99, 4-9.99 and >10.

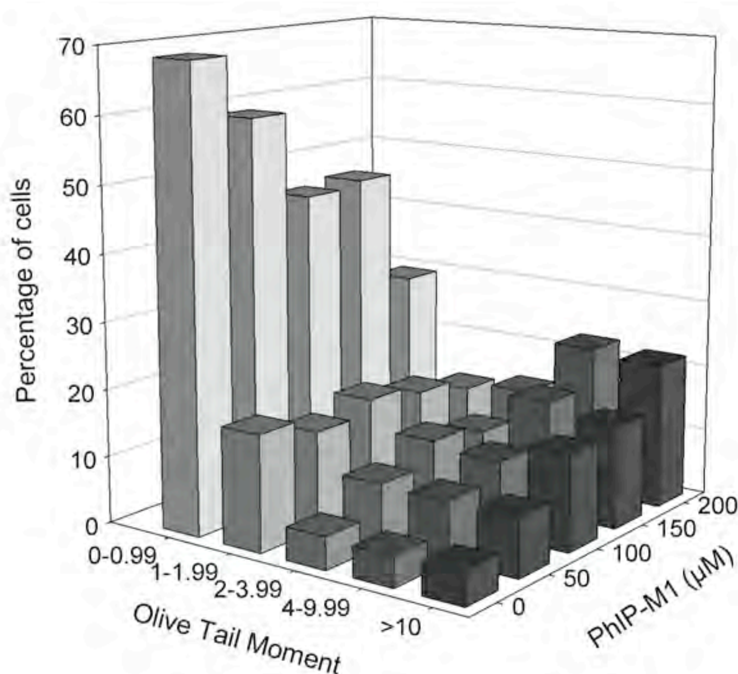

Figure 5.7 Frequency of DNA damage of Caco-2 cells exposed to various concentrations of PhIP-M1 after 3 h exposure.

4. Discussion

In the present study, we examined the cellular and genetic events caused by the newly identified microbial PhIP metabolite, PhIP-M1, on intestinal cells in the *in vitro* Caco-2 model. We have demonstrated that PhIP-M1 significantly decreases the mitochondrial dehydrogenase activity, the membrane integrity and the protein synthesis of Caco-2 cells in a time- and dose-dependent manner. Morphological examination, LDH release, DAPI nuclear

staining and annexin V-FITC staining suggested that the predominant effect of PhIP-M1 on Caco-2 cell viability was due to the induction of apoptosis. Cell cycle analysis revealed a G2/M arrest at lower concentrations and S arrest at higher concentrations as a consequence of PhIP-M1 exposure. In addition, the data presented showed that PhIP-M1 evoked a dose-dependent increase in DNA strand breaks in Caco-2 cells, as measured by the comet tail moment and LMW DNA diffusion assay.

In general, the cytotoxic or growth inhibiting effects from PhIP-M1 were similar in most assays. Gooderham *et al.* (2007) reported a PhIP IC_{30} value of ± 35 μM and IC_{50} value of ± 100 μM in MCF10A cells in co-culture with metabolically active MCL-5 cells treated with PhIP for 24 h and measured using the trypan blue dye assay. The cytotoxicity of P450-activated PhIP in MCF10A cells is thus slightly lower than that of PhIP-M1 measured in our culture system using trypan blue exclusion. This trypan blue exclusion assay, however, showed significantly lower IC_{30} and IC_{50} values at the 24 and 72 h exposure periods than the MTT and SRB assays. This could be due to apoptotic cells easily washed away by our procedure. Within the concentration range which provoked 50% of protein synthesis inhibition after a 24 h treatment (173 ± 20.3 μM), 50% of mitochondrial activity inhibition (180 ± 39.4 μM) and a 50% decrease in cell number (46.9 ± 4.5 μM), morphological changes, resembling apoptosis were indeed observed. These data are in line with the results of Martin *et al.* (1990), who observed apoptosis in human leukemia HL-60 cells when protein synthesis was inhibited by several anti-cancer drugs. One of the early events during apoptotic cell death is the translocation of phosphatidylserine from the inner side of the plasma membrane to the outer layer without loss of membrane integrity (van Engeland *et al.*, 1998). Loss of membrane integrity manifested by PI uptake in the nucleus tends to occur during the late apoptosis and during necrosis. Our data indicate that Caco-2 cells treated with PhIP-M1 die via apoptosis rather than via necrosis since low concentrations of PhIP-M1 at early time-points clearly led to an increase in annexin V+/PI- cells, and these alterations consequently resulted at a later time point or higher concentrations in an increase in PI+ stained cells. In addition, DAPI staining showed that PhIP-M1 induced typical features of apoptosis in Caco-2 cells, such as condensed chromatin and fragmented nuclei. DNA fragmentation is a typical morphological change observed during apoptosis (Allen *et al.*, 1997). This phenomenon is caused by specific endonucleases that cleave chromatin at the linker regions between nucleosomes resulting in

extensive fragmentation of DNA into subnucleosomal subunits of 180 to 200 bp (Darzynkiewicz and Traganos, 1998).

The hallmarks of apoptosis and its related DNA fragmentation can be revealed by the alkaline version of the comet assay. Therefore in parallel with the comet assay, the halo assay or LMW DNA diffusion assay was performed on each sample (Godard *et al.*, 1999). Under these conditions, we were able to quantitatively detect cells with highly fragmented DNA. Interestingly, MMS significantly induced the formation of type II cells in the halo assay, implying that excessive DNA damage, not specifically linked to the incidence of apoptosis, but related to genotoxic events, may as well lead to the diffusion of DNA during the alkaline halo assay. PhIP, without any metabolic activation, however, gave rise to a slight increase in cytotoxicity as measured by the MTT assay and the LMW DNA diffusion, but did not increase the comet tail moment, demonstrating that cytotoxicity within our assay system, did not lead to false positive results. In addition, the number of cells presenting a halo pattern upon PhIP-M1 exposure was higher than the number of apoptotic cells as detected by the annexin V-FITC staining method (44 ± 3.4 *vs.* 32 ± 6.2 for 200 μM), while it is generally accepted that the annexin V assay detects one of the early stages of the apoptotic process (Vermes *et al.*, 1995; van Engeland *et al.*, 1998) and therefore is more sensitive in the detection of early and late apoptotic cells than the halo assay (Godard *et al.*, 1999). Furthermore, apoptotic nucleosomal fragmentation of DNA leads to characteristic highly damaged figures with the comet assay, with no or very small heads and nearly all the DNA in the tail (Hartmann and Speit, 1997). Such highly damaged figures were scarce in our study. The wide distribution of the frequency of the tail moments and damage levels obtained upon treatment of Caco-2 cells with PhIP-M1 (Figure 5.7) are characteristic of a genotoxic effect. Genotoxicity usually generates varying degrees of damage in a cell population, contrasting with the bimodal distribution of damage, i.e. undamaged cells and cells with highly damaged DNA, resulting from cytotoxicity and apoptosis. The observed increase in the migration of DNA fragments of cells exposed to PhIP-M1 may therefore be linked with the ability of this newly identified microbial PhIP metabolite to induce DNA strand breaks or evoke such changes in the DNA structure that can be transformed into single or double strand breaks in the conditions of the comet assay in Caco-2 cells. Earlier research detected no direct mutagenic effect from PhIP-M1 in the Ames assay (Vanhaecke *et al.*, 2008a). The difference observed in the outcome of the Ames assay and the comet assay could be explained by

differences in tested biological systems (bacteria *vs.* eukaryotic cells), in test conditions (agar *vs.* liquid medium), in biotransformation capacities (extracellular S9-mix *vs.* intracellular endogenous metabolism), or in detected genotoxic endpoints (point mutation *vs.* primary DNA damage). This last point is particularly crucial because the Ames assay detects a mutagenic effect only if the DNA damages induced by PhIP-M1 have remained after cell division (*i.e.* are stable and have not been repaired) whereas the comet assay detects all primary DNA damages, including the ones not causing mutagenicity. Hartmann *et al.* (2001) have indeed shown that the comet assay is capable of detecting genotoxic compounds that were tested negative in the Ames assay. Edenharder *et al.* (2002) investigated the genotoxicity of PhIP in metabolically competent V79 cells using the comet assay and measured a tail moment of 25 and 50 for 9 and 17 µM PhIP, respectively, for 24 h incubation; while Pfau *et al.* (1999) measured a significant increase in median tail moment from 200 µM on in MCL-5 cells incubated for 3 h with PhIP. This is the first study that examined the genotoxic potential of PhIP-M1 in human cell lines and further research is needed to substantiate its genotoxic activity.

Although the MTT and SRB assays can be suitable for the determination of changes in the functional and metabolic capability of cells under toxic stress and the trypan blue exclusion assay gives an indication about the absolute amount of cells and their plasma membrane integrity, those do not necessarily yield specific information on the detailed events associated with cell death. Therefore flow cytometry was applied to determine the effect of PhIP-M1 on the cell cycle and mode of action of cell death in greater detail. In the presence of PhIP-M1 between 1 and 35 µM, a significant decrease in the percentage of nucleoids in G0/G1 phase was accompanied by a significant increase in the percentage of nucleoids in G2/M phase. However, at higher concentrations, the percentage of nucleoids in the G2/M phase reached its control level again and a significant increase in S cell phase was observed. CYP450-activated PhIP has been shown to induce G1 or S checkpoint in MCF10A or TK6 cells, respectively (Zhu et al, 2000; Gooderham *et al.*, 2002; Gooderham *et al.*, 2007), while PhIP-M1 in our cell system caused G2 and S phase cell cycle arrest. Arrest at G2/M phase is indicative of inhibition of the cell cycle at stages of chromosome segregation (Yuan *et al.*, 2005), while arrest at S-phase is indicative of inhibition of DNA replication (Kaufmann, 2007). This cell cycle arrest may be an adaptive process in which a surveillance mechanism delays or arrests the cell cycle when DNA damage is encountered (Shimada and Nakanashi,

2006). Other studies have demonstrated the ability of cells to delay or arrest their multiplication cycle in G0/G1, S or G2/M in order for repair to take place (Lane, 1992; Thorn *et al.*, 2001; Plesca *et al.*, 2007). DNA damage caused by numerous alkylating agents or cross-linking agents (Tokunaga *et al.*, 2000; Zhu *et al.*, 2000; Park *et al.*, 2004) in various cell types has been associated with G2/M or S phase delay or arrest. The possible initiation of repair processes may explain the moderate absolute damage levels induced by PhIP-M1 and the relative high concentrations of PhIP-M1 required to detect the incidence of DNA damage in Caco-2 cells. It is apparent that for cells with unrepairable DNA, or with too many repairable DNA lesions, the apoptotic pathway is the only option for a cell (Lowe *et al.*, 1993a; Lowe et al, 1993b). Consistent with our hypothesis, the DNA damage caused by PhIP-M1, under the incubation conditions in this study, did not only initiate cell cycle arrest in Caco-2 cells, but also induced a significant, dose-dependent increase in the percentage of apoptotic cells. These results are consistent with the three processes being related to one another. The following sequence of events may thus be proposed for PhIP-M1 induced activity: (i) cells exposed to increasing concentrations of PhIP-M1 sustain DNA damage as demonstrated by the comet assay; (ii) the DNA lesions or DNA fragmentations require repair, and the heavy demand for repair activity triggers cell cycle arrest in the G2/M phase of Caco-2 cells; and (iii) in cells for which PhIP-M1 concentrations are high enough and damage persistent enough, the repair capability is exceeded and the apoptosis pathway is initiated, leading eventually to cell death and growth inhibition. The occurrence of apoptotic cell death at a very early time point (3 h) and the relative high ratio of apoptotic cells compared to those exerting a cell cycle delay however suggests that an additional mode of action by which PhIP-M1 causes apoptosis might exist. The molecular toxicity of PhIP-M1 might be explained by a ring opening of PhIP-M1 into its aldehydic function. Many aldehydes modify DNA resulting in a mutagenic activity and are known to exert cytotoxic and oxidative effects (O'Brien *et al.*, 2005). Further research is however required to provide evidence for this ring opening of PhIP-M1 and unravel the mechanistic basis of this high apoptotic ratio of PhIP-M1 under *in vitro* conditions.

Daily human exposure to PhIP in foodstuffs and concentrations of PhIP-M1 measured in urine and fecal samples of humans consuming average amounts of meat are 2-3 orders of magnitude below the concentrations used in this study. As the molecular and cellular effects observed in different cell systems with CYP450-activated PhIP are not significantly larger

than those observed for PhIP-M1 in our test system, the potential physiological relevance of our newly identified microbial metabolite in PhIP carcinogenicity must be taken into account. Given the ability of PhIP-M1 to initiate DNA damage and disrupt the cell cycle, further assessment of the potential role of this newly identified microbial metabolite of the IARC 2B carcinogen PhIP may be desirable in the light of factors such as potential accumulation and possible additive or synergistic effects with the DNA-reactive N-OH-PhIP derivate or other dietary components.

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CHAPTER 6

Chemopreventive effects from prebiotic inulin towards microbial 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) bioactivation

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CHAPTER 6

Chemopreventive effects from prebiotic inulin towards microbial 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine bioactivation

ABSTRACT

Inulin is frequently studied for its prebiotic potential as it stimulates health-promoting bacteria in the human intestine. Inulin is also hypothesized to exert inhibitory effects towards hazardous biotransformation reactions from the resident colon microbiota. Using a Simulator of the Human Intestinal Microbial Ecosystem (SHIME), we investigated the chemopreventive potential of chicory inulin towards the *in vitro* bioactivation of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) by human intestinal microbiota. Additionally, prebiotic effects were evaluated by monitoring the metabolic activity and community structure from the microbiota in the different colon compartments. HPLC data revealed that inulin significantly decreased the formation of the genotoxic PhIP-M1 metabolite, with the highest inhibitory activity in the colon ascendens (87% decrease). Interestingly, this chemopreventive effect, correlated with alterations of bacterial community composition and metabolism in the different colon compartments. Conventional culture-based techniques and PCR-DGGE analysis on the SHIME colon suspension revealed significant bifidogenic effects during inulin treatment, whereas the overall microbial community kept relatively unchanged. Additionally, short chain fatty acid production increased with 12%, 3% and 7%, while ammonia concentrations decreased with 3%, 4% and 3% in the ascending, transverse and descending colon compartments, respectively. This indicates that the prebiotic effects from inulin may also purport protective effects towards microbial PhIP bioactivation. As the colonic microbiota may contribute significantly to the carcinogenic potential of PhIP, the search for dietary constituents that decrease the formation of this harmful metabolite, may help in preventing its risk towards human health.

1. Introduction

Dietary epidemiological studies implicate heterocyclic amines (HCAs), mutagenic/carcinogenic compounds formed from high-protein diets during cooking, as risk factors in the etiology of human cancer (Felton *et al.*, 2005). Of the 19 heterocyclic amines identified so far, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is the most mass abundant heterocyclic amine produced during the cooking of beef, pork and chicken (Felton *et al.*, 1986a; Murray *et al.*, 1993; Sinha *et al.*, 1995; Wong *et al.*, 2005). Experimentally, PhIP is a potent mutagen and genotoxin and has been shown to produce mammary gland, prostate and colon tumors in rats (Ito *et al.*, 1991; Shirai *et al.*, 1997; Sugimura, 2000). In humans, less is known about the potential role of PhIP and related heterocyclic amines in tumor development. Several studies have shown that individuals who eat well-done meat have an elevated risk of breast (Zheng *et al.*, 1998) and colorectal (Sinha, 1995; Gunter *et al.*, 2005) cancers.

In realizing its mutagenic potential, PhIP requires metabolic activation by drug metabolizing enzymes (Aeschbacher and Turesky, 1991). In common with other genotoxic aromatic amines, PhIP is metabolically activated via oxidation of the exocyclic amino group, a reaction mainly mediated by the cytochrome P450 isoenzyme CYP1A2 (Crofts *et al.*, 1998; Turesky *et al.*, 2002). While PhIP is biotransformed into a large number of derivates by mammalian enzyme systems, the human intestinal microbiota selectively convert PhIP into one major metabolite, 7-hydroxy-5-methyl-3-phenyl-6,7,8,9-tetrahydropyrido[3’,2’:4,5]-imidazo[1,2-*a*]pyrimidin-5-ium chloride (PhIP-M1) (Vanhaecke *et al.*, 2006). This compound has been detected in human urine and feces following consumption of well-done chicken meat and does not act as a direct mutagen in the Ames assay (Vanhaecke *et al.*, 2008a), but exerts genotoxic and cytotoxic effects towards the human intestinal Caco-2 cell line and is therefore considered as a toxified PhIP derivate (Vanhaecke *et al.*, 2008c).

During the last decades strong efforts have been made to identify dietary constituents, which protect against the genotoxic and carcinogenic effects from HCAs. More then 600 complex mixtures and individual compounds contained in the human diet have been studied for their chemopreventive effects towards HCAs (Schwab *et al.*, 2000). Among them, dietary fibers and lactic acid bacteria are known to bind and prevent the absorption of HCAs (Ferguson and Harris, 1996; Bolognani *et al.*, 1997), while cruciferous vegetables induce

phase I and phase II metabolism in humans. Inulin type fructooligosaccharides, which are selectively fermented by beneficial microorganisms in the colon, have been shown to exert their chemopreventive effects through alteration of bacterial metabolism in the distal gut (Humblot *et al.*, 2004) and proved more potent in comparison to oligofructose and Brussels sprouts in preventing 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ)-induced genotoxicity in colonocytes from HFA rats. Evidence on the mechanisms by which inulin may decrease the carcinogenic risk from IQ has been recently provided. Hydrolysis of heterocyclic amine-glucuronides by bacterial β-glucuronidase has shown to be essential for colonic IQ genotoxicity (Humblot *et al.*, 2007). The impact of prebiotic inulin on the bacterial bioactivation of native heterocyclic amines has however not been investigated yet.

In vivo experiments are the most representative approach for evaluating the effects of prebiotics administration, since physiological parameters and interactions with the host organism are taken into account. However, *in vivo* experiments are costly and time-consuming and - especially with human trials - they investigate fecal microbiota that do not represent the microbial community composition from the different parts of the colon. Advanced *in vitro* reactor systems that mimic both the proximal and distal regions of the human colon may therefore be useful for studying human intestinal microbiota (Macfarlane *et al.*, 1989; Molly *et al.*, 1993; Minekus *et al.*, 1999). Additionally, they give more reproducible results and allow mechanistic studies with several parameters under control. Such *in vitro* methods are therefore well suited for studying the influence of prebiotics on a specific microbial bioactivation reaction and the intestinal microbial population in terms of fermentation activity and community structure and this in the ascending, transverse and descending colon compartments, respectively.

In this study, we used the Simulator of the Human Intestinal Microbial Ecosystem (SHIME), which harbors a microbial community resembling that from the human colon both in fermentation activity and composition (Molly *et al.*, 1993; Possemiers *et al.*, 2004). The aims of the study were (i) to evaluate whether inulin supplementation could decrease the microbial bioactivation of the IARC 2B carcinogen PhIP, (ii) to assess whether possible shifts in fermentation pattern, enzymatic activity and microbial composition could be attributed to inulin and (iii) to link potential bifidogenic or prebiotic effects from inulin with its effect on PhIP microbial bioactivation.

2. Materials and methods

2.1 Chemicals

PhIP was purchased from Toronto Research Chemicals (Ontario, Canada). For incubation purposes, it was dissolved in dimethyl sulfoxide (DMSO). PhIP-M1 (7-hydroxy-5-methyl-3-phenyl-6,7,8,9-tetrahydropyrido[3',2':4,5]imidazo[1,2-*a*]pyrimidin-5-ium chloride) was synthesized and purified using a procedure from previous studies (Vanhaecke *et al.*, 2008a). Purity of PhIP-M1 was 97 ± 0.8% as determined by LC-MS. The solvents for HPLC and LC-MS analysis were of HPLC grade and purchased from Acros Organics (Geel, Belgium).

2.2 Culture system

The SHIME is a dynamic model of the human gastrointestinal tract. It consists of 5 double-jacketed vessels maintained at a temperature of 37 °C, respectively simulating the stomach, small intestine, ascending colon, transverse colon and descending colon, with a total retention time of 76 h. The colon vessels harbor a mixed microbial community and pH controllers (pH controller R301, Consort, Turnhout, Belgium) maintain the pH in the range 5.6-5.9, 6.2-6.5 and 6.6-6.9 in the ascending, transverse and descending colon simulations, respectively. There is no gas exchange between the different vessels and the headspace of the culture system was flushed once a day for 15 min with N_2 to ensure anaerobic conditions. Growth medium for the microbial inoculum consisted of a carbohydrate-based medium containing arabinogalactan (1 g/L), pectin (2 g/L), xylan (1 g/L), starch (4 g/L), glucose (0.4 g/L), yeast extract (3 g/L), peptone (1 g/L), mucin (4 g/L) and L-cysteine (0.5 g/L). Detailed information about the SHIME system can be found in Molly *et al.* (1993).

2.3 Experimental reactor setup

At the beginning of the experiment, the last three vessels of the SHIME reactor were inoculated with a fecal sample derived from a healthy adult volunteer (age 22) who had no history of antibiotic treatment in the six months prior to the fecal sample collection and was characterized as a high PhIP transformer (Vanhaecke *et al.*, 2008b). During the start-up period, the reactor was supplemented with basal feed medium, which enabled the microbial community to adapt itself to the nutritional and physico-chemical conditions that prevail in

the different colon vessels (Molly *et al.*, 1993). After two weeks, the experiment was initiated with the basal period from day 1 to day 14, under the same conditions as during the start-up period. This allowed recording the basal parameters of the system prior to altering the feed medium during the treatment period. On day 15, the treatment period was initiated, which lasted until day 35. The nutrition for the treatment period consisted of the normal compounds as described above, except that the amount of starch in the medium was reduced from 4 to 1 g/L and that native chicory inulin (Fibruline Instant, COSUCRA, Warcoing, Belgium) was added at 3 g/L. After the treatment period, a washout period from day 36 to day 49 concluded the run, during which the same basal feed medium was used as during the basal period. This allowed seeing whether the metabolic parameters, microbial carcinogen transformation and/or microbial concentrations evolved towards their initial values from the basal period.

2.4 Experimental setup PhIP transformation

During the basal, treatment and washout periods, consequently on the third day of each week and immediately after the supplemented nutrition had reached the last vessel of the colon, samples were taken from each vessel (20 mL), transferred to penicillin flasks and supplemented with 1 mg/L PhIP and 5% fecal matrix (Vanhaecke *et al.*, 2008b). Each batch was sealed with butylrubber tops and anaerobiosis was obtained by flushing the flasks with N_2 during 15 cycles of 2 min each at 800 mbar overpressure and 900 mbar underpressure. Cultures were incubated at 37 °C and 150 rpm during 96 h. Samples were taken every 24 h for HPLC analysis.

2.5 PhIP and PhIP-M1 analysis

PhIP and PhIP-M1 analyses were performed according to Vanhaecke *et al.* (2008b) on a Dionex HPLC system (Sunnyvale, California, USA) comprising an autosampler ASI-100, a pump series P580 and a STH585 column oven coupled to a fluorescence detector RF-2000. Briefly, a 10 µL volume of the sample was injected and separated over a Zorbax-Extend C_{18} column (150 mm x 4.6 mm, 5 µm) (Agilent technologies, Diegem, Belgium). The temperature was set at 25 °C and the flow rate was maintained at 1 mL/min. Solvents were A: 0.01% formic acid and B: acetonitrile. Solvent programming was isocratic: 2% B in 2 min followed by a linear gradient to 40% by 20 min. Fluorescence was monitored at 316 nm

(excitation) and 370 nm (emission). Data were collected and peaks integrated using the Chromeleon chromatography manager software (Dionex).

2.6 Metabolic activity analysis

Liquid samples were collected daily during the entire SHIME run and kept frozen at -20 °C for subsequent analysis.

2.6.1 Short chain fatty acids (SCFAs)

The SCFAs were extracted from the samples with diethyl ether and analyzed on a Di200 gas chromatograph (Shimadzu, 's-Hertogenbosch, The Netherlands) equipped with a EC-1000 Econo-Cap column (Alltech, Laarne, Belgium; dimensions: 25 m x 0.53 mm, film thickness 1.2 µm) and a flame ionization detector with a Delsi Nermag 31 integrator (Thermo Separation Products, Wilrijk, Belgium). Nitrogen was used as a carrier gas at a flow rate of 20 mL/min and the column temperature and injector temperature were set at 130 and 195 °C, respectively. 2-Methyl hexanoic acid was used as an internal standard.

2.6.2 Ammonia

Using a 1062 Kjeltec Auto Distillation apparatus (FOSS Benelux, Amersfoort, The Netherlands), ammonium in the samples was liberated as ammonia by the addition of an alkali (MgO). The released ammonia was distilled from the sample into a boric acid solution (20 g/L). This solution was subsequently titrated using a 665 Dosimat (Metrohm, Berchem, Belgium) and 686 Titroprocessor (Metrohm).

2.6.3 Proteolytic markers

Phenol and *p*-cresol were extracted from the SHIME samples with ethyl acetate and analyzed on a Dionex HPLC system (Sunnyvale, California, USA) comprising an autosampler ASI-100, a pump series P580 and a STH585 column oven coupled to a fluorescence detector RF-2000. A 10 µL volume of the sample was injected and separated over a Zorbax-SB C_{18} column (150 mm x 4.6 mm, 5 µm) (Agilent technologies, Diegem, Belgium). Solvents were A: 2% acetic acid and B: acetonitrile. The temperature was set at 25 °C and the flow rate was maintained at 1 mL/min. Solvent programming was isocratic: 2% B

in 2 min followed by a linear gradient to 60% by 20 min. Fluorescence was monitored at 260 nm (excitation) and 305 nm (emission). 4-Ethylphenol was used as an internal standard.

2.6.4 Enzyme analysis

The samples were centrifuged at 10000 x *g* for 10 min. Cell free supernatant (100 μL) was transferred into a 96-well plate, with 100 μL of a 5.0 mM substrate, prepared in 0.1 mM phosphate buffer (pH 6.5). The substrate (Sigma, Bornem, Belgium) used was *p*-nitrophenyl-β-glucuronide for β-glucuronidase. The plates were incubated at 37 °C and the absorbance at 405 nm recorded after 30 min on a multiwellreader (SunriseTM, Tecan Benelux BVBA, Mechelen, Belgium). The amount of *p*-nitrophenol released was measured based on a standard curve of *p*-nitrophenol. The results were expressed in μmol *p*-nitrophenol released.

2.7 Microbial community analysis

2.7.1 Plate counting

To assess the effect of inulin on the large groups of bacteria in the different compartments of the SHIME reactor, plate counts were performed on Brain Heart Infusion agar (total aerobes and total anaerobes), Tryptose Sulphite Cycloserin agar (clostridia), Raffinose *Bifidobacterium* agar (bifidobacteria), LAMVAB agar (lactobacilli), *Enterococcus* agar (enterococci), Mannitol Salt agar (staphylococci) and Martin agar (fungi and yeasts). Liquid samples were withdrawn from the culture system and serially diluted in saline solution (8.5 g NaCl/L). Three plates were inoculated with 0.1 mL sample of three dilutions, and incubated at 37 °C (43 °C for *E. coli*). Anaerobic incubation of plates was performed in jar with a gas atmosphere (84% N_2, 8% $C0_2$, and 8% H_2) adjusted by the Anoxomat 8000 system (Mart, Sint-genesius-Rode, Belgium).

2.7.2 PCR-DGGE

The protocol for total DNA extraction from the SHIME samples was described earlier (Boon *et al.*, 2000). Two microbial groups were analyzed: general bacteria and bifidobacteria. A nested PCR approach (Boon *et al.*, 2002) was used to amplify the 16S ribosomal RNA genes of the bifidobacteria. In brief, one μL of the DNA was amplified using the primers BIF164f-BIF662r (Satokari *et al.*, 2001). When the first PCR round gave a clearly visible band, a second amplification round with forward primer P338f (with a GC-clamp of 40 bp)

and reverse primer P518r was used (Muyzer *et al.*, 1993). The 16S rRNA of all bacteria was amplified by applying primers P338f with GC-clamp and P518r on total extracted DNA.

Denaturing gradient gel electrophoresis was performed as described earlier using the Bio-Rad D Gene System (Bio-Rad, Hercules, CA, USA) (Muyzer *et al.*, 1993). PCR fragments were loaded onto 8% (w/v) polyacrylamide gels in 1 × TAE (20 mM Tris, 10 mM acetate, 0.5 mM EDTA, pH 7.4). On each gel, a home made marker of different PCR fragments was loaded, which was required for processing and comparing the different gels (Boon *et al.*, 2002). The polyacrylamide gels were made with a denaturing gradient ranging from 45% to 60%. The electrophoresis was run for 16 h at 60 °C and 38 V. Staining and analysis of the gels was performed as previously described (Boon *et al.*, 2000). The normalization and analysis of DGGE gel patterns was done with the BioNumerics software 2.0 (Applied Maths, Kortrijk, Belgium). The calculation of the similarity matrix was based on the Pearson correlation coefficient. Clustering algorithm of Ward was used to calculate dendrograms (Ward, 1963).

2.7.3 DNA sequencing

16S rDNA gene fragments were cut out of the DGGE gel with a clean scalpel and added to 50 μL of PCR water. After 12 hours of incubation at 4 °C, 1 μL PCR water was reamplified with primer set P338f and P518r. Five μL PCR product was loaded on a DGGE gel (see above) and if the DGGE pattern only showed 1 band, it was sent out for sequencing. DNA sequencing of the ca. 180 bp fragments was carried out by ITT Biotech-Bioservice (Bielefield, Germany). Analysis of DNA sequences and homology searches were completed with standard DNA sequencing programs and the BLAST server of the National Center for Biotechnology Information (NCBI) using the BLAST algorithm (Boon *et al.*, 2002).

3. Results

3.1 *PhIP bioactivation during the SHIME run*

To evaluate whether inulin supplementation could affect the microbial PhIP bioactivation, PhIP-M1 production was monitored by incubating PhIP at a concentration of 1 mg/L in mixed microbial suspensions retrieved from the three different colon compartments of the SHIME reactor throughout the basal, treatment and washout periods. PhIP bioactivation was evaluated by measuring PhIP-M1 production over a 5 day time-window using HPLC analysis (Figure 6.1). Average basal microbial PhIP transformation efficiencies of respectively 17.7 ± 1.2, 89.7 ± 12.4 and 79.2 ± 23.2% in the colon ascendens, transversum and descendens were measured. In the colon ascendens, inulin supplementation initially led to a large increase in PhIP-M1 formation, followed by a subsequent decrease during the second and third week of inulin treatment. The PhIP transformation potency of the microbial community was however restored during the washout period, reaching basal levels again. In the colon transversum and descendens a clear decrease in PhIP transformation was observed upon inulin supplementation. This effect was more pronounced in the transverse colon, where only 2.6% of the initial PhIP dose was converted after three weeks of treatment. In both compartments the PhIP-M1 production reached their basal levels again during the washout period.

3.2 *Metabolic activity during the SHIME run*

Replacement of starch by a metabolic equivalent amount of chicory inulin in the feed of the SHIME shifted the microbial fermentation pattern in the three different colon vessels towards a more saccharolytic metabolism (Table 6.1).

This metabolic shift resulted in an increase in total SCFA production with 20%, 5.2% and 9.9% in the ascending, transverse and descending colon, respectively at the end of the three-week treatment period. It was noted that the increase in SCFA production primarily originated from an increased production of propionate and butyrate, while only in the colon ascendens a significant increase in acetate concentration was observed. This generally higher total SCFA production was only prolonged in the colon descendens during the washout period, although the significant increases in propionate and butyrate concentrations were maintained in the colon ascendens, transversum and descendens.

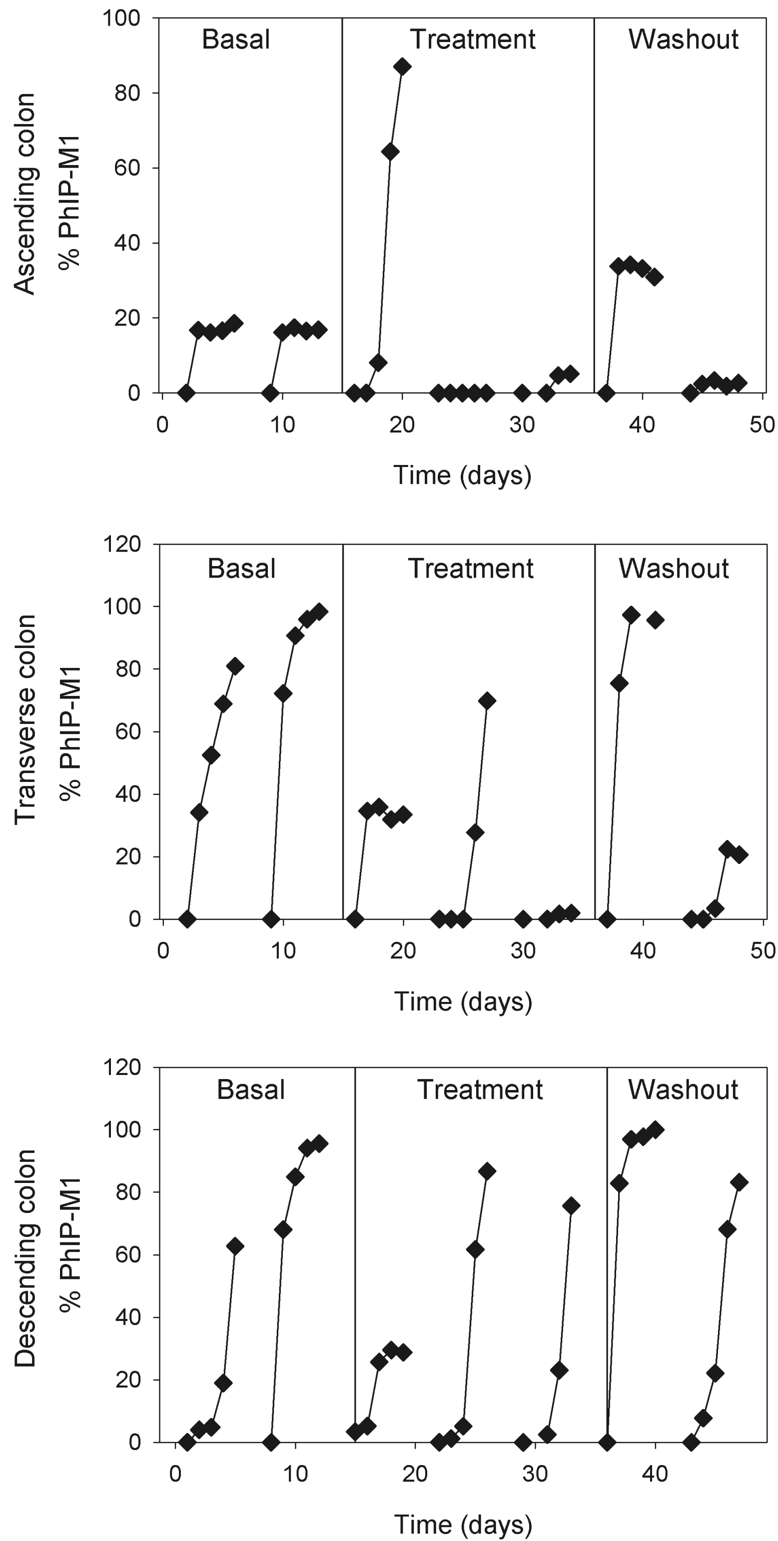

Figure 6.1 Conversion of PhIP into PhIP-M1 by mixed fecal microbiota sampled from the ascending, transverse and descending colon throughout the basal (day 1 and 8), treatment (day 15, 22 and 29) and washout (day 36 and 43) periods in which inulin was tested for its chemopreventive potential. PhIP-M1 formation upon incubation was for each sampling point monitored during 5 consecutive days.

Table 6.1. Concentration of short-chain fatty acids (SCFAs), NH_4^+ and phenol in vessels 3,4 and 5 of the SHIME during the basal (n = 4), treatment with inulin (n = 4) and washout periods (n = 4). Data are means ± SD.

Parameter	Ascending colon	Transverse colon	Descending colon
		mmol/L suspension	
Basal period			
Acetic acid	30.4 ± 2.3	33.7 ± 1.0	37.1 ± 1.9
Propionic acid	11.6 ± 1.0	14.2 ± 0.5	14.5 ± 1.5
Butyric acid	3.4 ± 0.8	4.5 ± 0.5	5.6 ± 0.5
Branched SCFAs	0.6 ± 0.1	2.4 ± 0.1	2.6 ± 0.1
Total SCFAs	46.1 ± 3.4	54.9 ± 1.4	60.1 ± 2.6
Ammonium	18.4 ± 4.1	23.0 ± 1.7	25.9 ± 1.0
Phenol	(7.1 ± 8.3) x 10^{-3}	(113.3 ± 51.0) x 10^{-3}	(136.2 ± 48.8) x 10^{-3}
Treatment period			
Acetic acid	36.2 ± 3.0**	34.7 ± 4.4	39.5 ± 1.7*
Propionic acid	15.7 ± 1.1**	15.7 ± 2.3	17.4 ± 0.3**
Butyric acid	4.6 ± 0.8*	5.2 ± 0.8	6.7 ± 0.1**
Branched SCFAs	1.0 ± 0.9	2.3 ± 0.2	2.7 ± 0.02*
Total SCFAs	57.7 ± 4.1**	58.0 ± 7.4	66.7 ± 2.0**
Ammonium	15.5 ± 2.5	19.5 ± 4.4*	23.0 ± 5.1
Phenol	(18.9 ± 7.6) x 10^{-3}	(83.8 ± 35.4) x 10^{-3}	(90.7 ± 12.5) x 10^{-3} *
Washout period			
Acetic acid	29.4 ± 1.5°°	30.9 ± 2.9	38.5 ± 1.1
Propionic acid	14.3 ± 0.8**°	16.4 ± 1.6*	17.6 ± 1.4**
Butyric acid	5.4 ± 0.6**	5.9 ± 0.7**°	7.3 ± 0.5**°
Branched SCFAs	2.2 ± 0.2**°	2.7 ± 0.2*°	2.9 ± 0.1**°°
Total SCFAs	51.3 ± 2.5*°	56.0 ± 5.4	66.8 ± 2.9**
Ammonium	20.4 ± 9.2	25.4 ± 1.7*°°	28.2 ± 2.4*°
Phenol	(20.7 ± 10.5) x10^{-3}	(36.3 ± 20.1) x 10^{-3} *°	(32.1 ± 11.2) x 10^{-3}**°°

Significantly different from the basal period: *, $p < 0.05$; **, $p < 0.01$.

Significantly different from the treatment period: °, $p < 0.05$; °°, $p < 0.01$.

During inulin administration, ammonia concentrations significantly decreased in the transverse colon vessel, whereas no significant changes were observed in the other colon vessels. In the washout period ammonia levels increased again and became significantly higher in the colon transversum and descendens than during inulin supplementation. As for

phenol, a non-significant decrease was observed in the transverse and descending colon compartments. This decrease was prolonged throughout the washout period, reaching a significant difference with the basal period in both vessels. The enzymatic β-glucuronidase activity and *p*-cresol were monitored in the respective colon compartments and did not change significantly during the entire SHIME run (data not shown).

3.3 Microbial community analysis

3.3.1 Plate count analysis

Using selective growth media, analysis of the microbial suspension from the SHIME colon compartments revealed that inulin administration had limited effects on the overall microbial composition of the SHIME community, although significant increases in the amount of total anaerobes were observed in all colon vessels (Figure 6.2). This increase was most pronounced in the colon ascendens (0.5 log CFU increase). Concentrations of the beneficial microbial group, bifidobacteria, increased in all colon vessels throughout the inulin treatment, yet only significant in the colon ascendens ($p < 0.05$). During the washout period, starch again replaced inulin in the nutrition of the SHIME reactor. The increases in number of total anaerobes and bifidobacteria were maintained after inulin supplementation. For the ascending, transverse and descending colon compartments respectively, bifidobacteria concentrations were 0.6, 0.4 and 0.5 log CFU higher during the washout than during the basal period (Figure 6.2).

3.3.2 Microbial fingerprinting and sequencing

PCR-denaturing gradient gel electrophoresis was used as a molecular fingerprint technique to monitor qualitative changes in the composition of the microbial community from the three colon compartments throughout the SHIME run. Samples were taken from every colon vessel once a week during the entire SHIME run. Thus, for the three colon compartments a total of 21 samples were collected and DGGE fingerprinting and cluster analysis were performed for general bacteria and bifidobacteria.

The global fingerprint for general bacteria showed that all samples from the colon descendens clustered in separate group, while the samples from the ascending and transverse colon clustered together in another group (Figure 6.3).

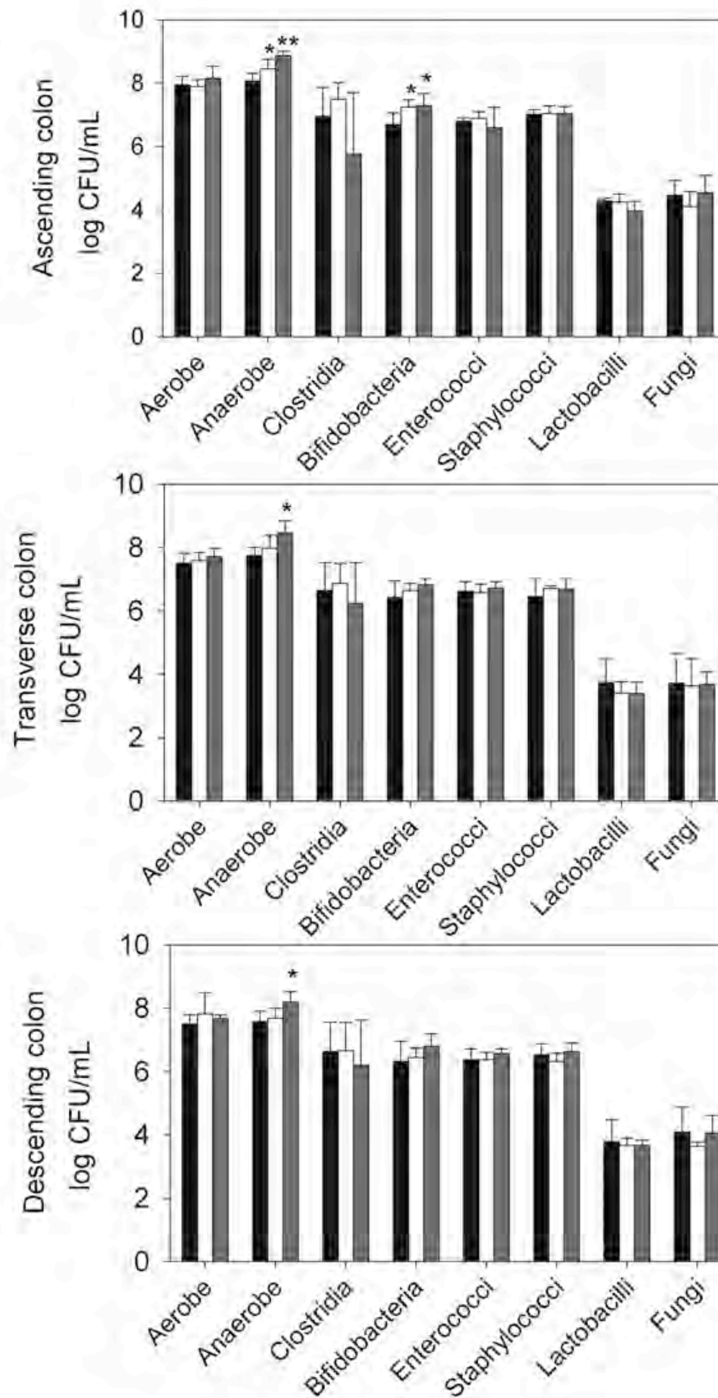

Figure 6.2 Plate count analysis of total aerobic and anaerobic bacteria, clostridia, bifidobacteria, enterococci, staphylococci, lactobacilli and fungi in the ascending, transverse and descending colon vessels of the SHIME reactor during the basal (n=4), treatment (n=6) and washout (n=4) periods. Bars represent SD values of the different replicates. ■ Basal; □ treatment; ■ washout. Significantly different from the basal period: *, $p < 0.05$; **, $p < 0.01$.

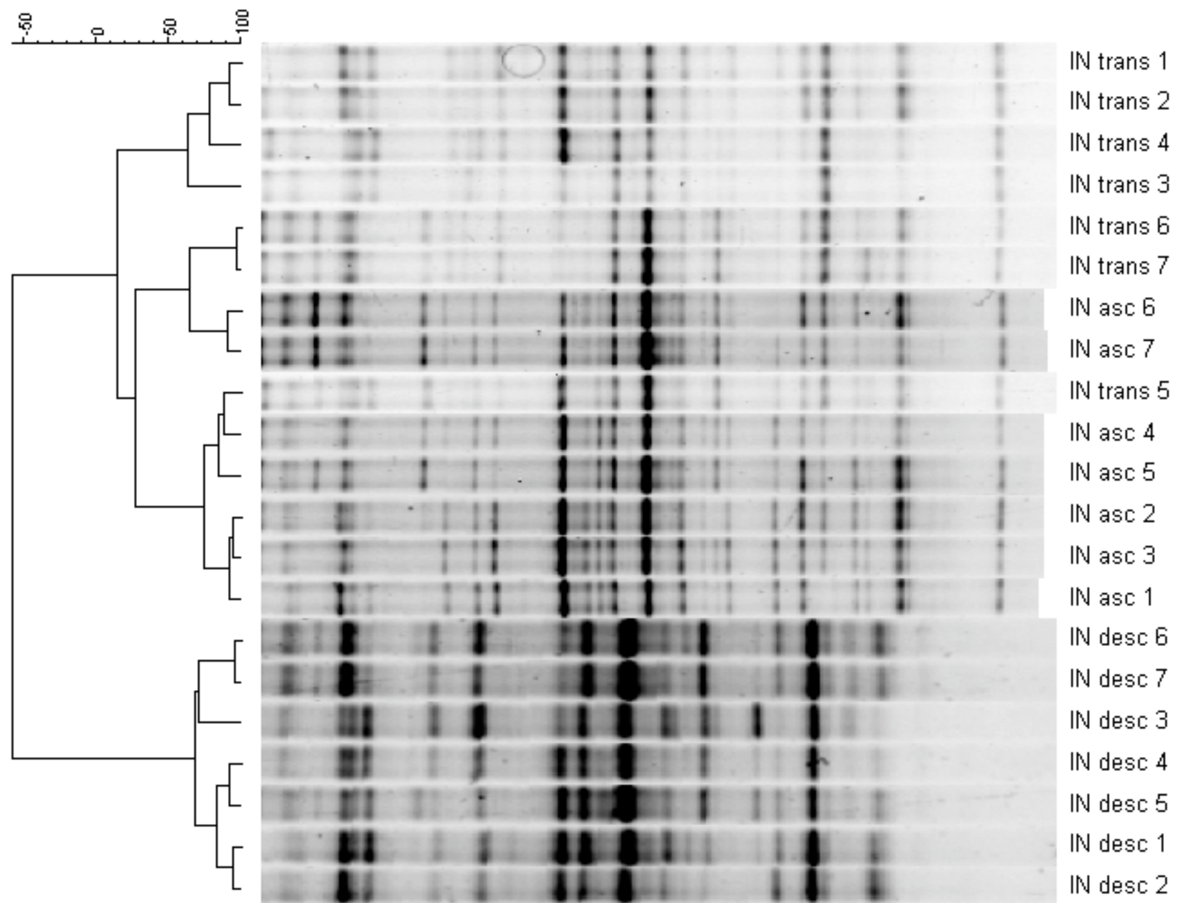

Figure 6.3 DGGE fingerprint patterns and clustering analysis for general bacteria sampled from the ascending (asc), transverse (trans) and descending (desc) colon compartments. Samples 1 and 2 were taken during the basal period, samples 3,4 and 5 were taken during the treatment period, samples 6 and 7 were taken during the washout period.

Both within the descending colon group as within the ascending/transverse colon group, the effect of inulin supplementation was observed by the separate clustering of the washout periods and the inulin treatment periods. Although this inulin effect was slightly apparent, the dominant factor for clustering was the colon compartment itself, from which the samples were taken. This roughly corresponds to the limited variations in microbial populations that were observed using conventional plating techniques. This was in contrast to the clustering analysis of DGGE patterns for the bifidobacteria. For this bacterial group, the dominant factor for clustering was the time point at which the samples were taken (Figure 6.4). In the colon descendens, a strong band appeared after one week of inulin supplementation, and although this band was weaker during the second week of inulin treatment, it regained its intensity

during the washout period. This band was also observed in the colon ascendens and descendens at the end of the inulin treatment period. The band marked 'bif' on the bifidobacteria DGGE gel revealed 98% similarity (123 out of 125 bases) to *Bifidobacterium bifidum*.

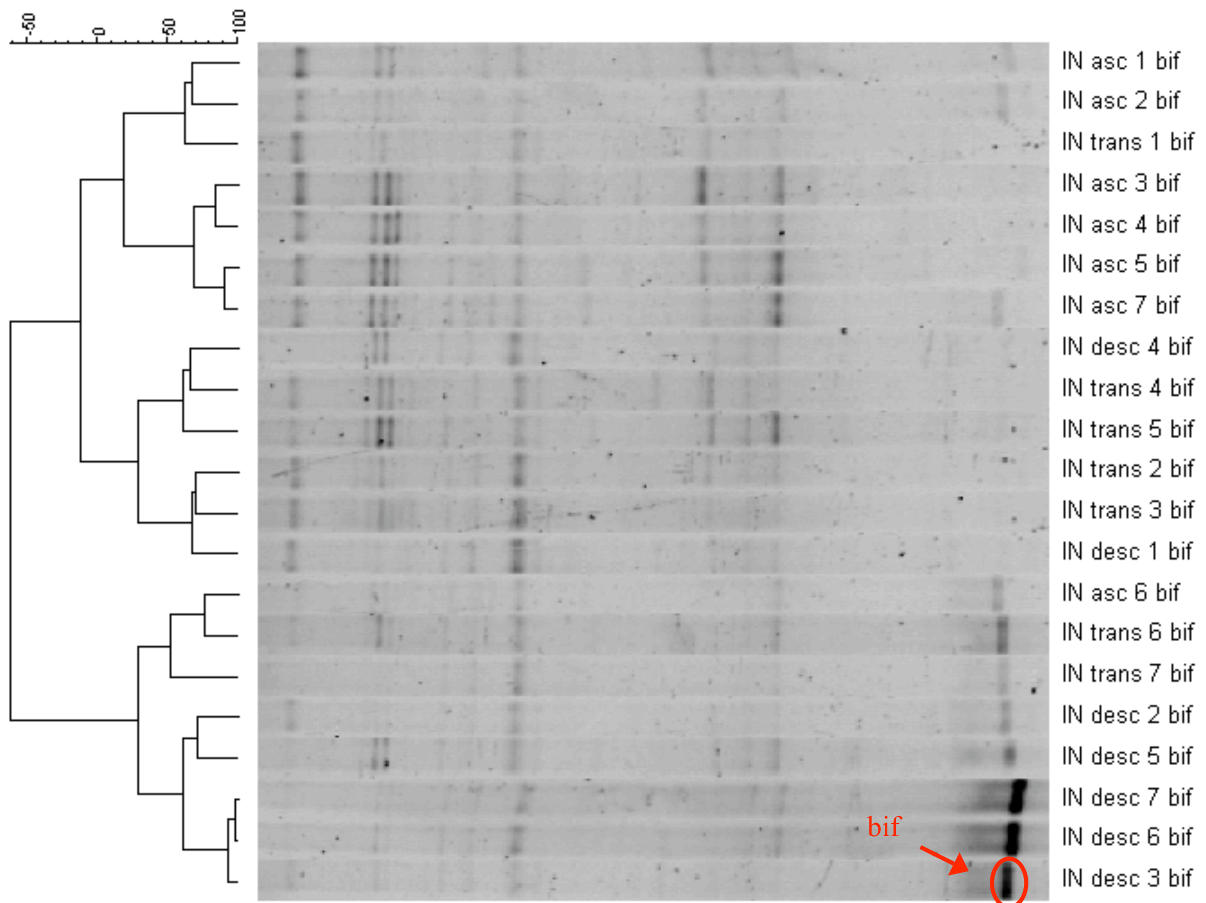

Figure 6.4 DGGE fingerprint patterns and clustering analysis for bifidobacteria sampled from the ascending (asc), transverse (trans) and descending (desc) colon compartments. Samples 1 and 2 were taken during the basal period, samples 3,4 and 5 were taken during the treatment period, samples 6 and 7 were taken during the washout period.

4. Discussion

In this study, the chemopreventive potential of chicory inulin was demonstrated towards the microbial bioactivation of the pro-carcinogenic meat component PhIP into the genotoxic PhIP-M1 derivate. In addition, a mechanistic basis for this preventive activity was proposed. PhIP-M1 production has been shown to occur under proteolytic conditions and is not supported in the presence of merely carbohydrates (Vanhaecke *et al.*, 2008b). As we amongst others (Van de Wiele *et al.*, 2004) have demonstrated, inulin exerts prebiotic effects towards the *in vitro*-cultured colon microbiota from the SHIME reactor, in particular at the level of the metabolic activity, resulting in a saccharolytic fermentation pattern and acidic environment. This fermentational shift, which is eventually a consequence of the changes in microbial community composition, might explain for the protective effects detected.

The protective activity from inulin against PhIP bioactivation was evidenced by a lower PhIP-M1 production from colon suspensions that were sampled from the SHIME reactor during 3 weeks of inulin treatment and that had been incubated with PhIP for 96 h. These chemopreventive effects occurred, in the colon transversum and descendens, already after one week of inulin administration at a dose of 3 g/day, which corresponds to an equivalent human dose of 6 g/day. This is a feasible human intake and well within the range of earlier reports investigating the effects of inulin *in vitro* and *in vivo* (Macfarlane *et al.*, 2008). Similar inhibitory effects from inulin were previously reported towards IQ-induced genotoxicity in the HFA F344 rat (Humblot *et al.*, 2004). Regarding the risk of colon cancer, inulin-type fructans have the capacity to suppress chemically induced colon carcinogenesis in both mice and rats (Pool-Zobel, 2005). Inulin-type fructans therefore are classified as negative modulators of the carcinogenic process. The mechanisms proposed to explain the chemopreventive effects towards overall carcinogenicity in the colon, or in this study, the microbial bioactivation of PhIP, can be explained by the prebiotic properties of inulin in the lumen of the gastrointestinal tract.

The successful application of inulin as a prebiotic agent implies specific changes, both in the composition and/or activity of the gastrointestinal microbiota, which confer benefits on host well-being and health (Gibson *et al.*, 2004). Administration of inulin to the nutrition of the SHIME reactor beneficially influenced the fermentation pattern of the colon microbiota

towards a significantly higher SCFA production, more in particular propionate and butyrate. This can be considered as highly positive given their beneficial effects on human health. Propionate is largely metabolized in the liver, is gluconeogenic, and may inhibit *de novo* lipogenesis (Vogt *et al.*, 2004). Butyrate, on the other hand, is the major energy source for the colonocytes and has been implicated in the prevention of colitis and colorectal cancer (Roy *et al.*, 2006). The increase in total SCFA production seems uncommon since the treatment period entailed a replacement of starch by an equivalent amount of inulin and not an addition. This additional SCFA production may be possibly explained by the additional bifidobacterial biomass, created by the bifidogenic effect from inulin. Additionally, other microbial groups in the colon suspension that are used to starch degradation may ferment alternative carbon sources from the medium to SCFA. The shift towards propionate and butyrate caused by inulin has been reported by other workers, both *in vitro* (Topping and Clifton, 2001) and *in vivo* (Uehara *et al.*, 2001). These observations do not directly point towards bifidogenic effects, since bifidobacteria are acetate and lactate producers. Other microbial groups have however been implicated in the conversion of lactate or acetate into butyrate (Louis *et al.*, 2007). Recent work by Belenguer *et al.* (2006) has shown how butyrate-producing species such as *Anaerostipes caccae* and *Eubacterium halli* can cross-feed on lactate produced by *Bifidobacterium adolescentis* growing on fructooligosaccharides, while a non-lactate utilizing, butyrate-forming *Roseburia* sp. could assimilate carbohydrate fragments formed when the *Bifidobacterium* hydrolyzed complex polymeric substrates. Similar processes in the SHIME reactor may explain the relative constant acetate concentrations during inulin treatment, whereas specific increases in bifidobacterial biomass were noted.

Inulin administration also resulted in a decrease of ammonia and phenol levels in the different colon compartments of the SHIME reactor. Ammonia is produced in the colon by bacterial hydrolysis of urea as well as by bacterial deamination of amino acids, peptides, and proteins (Vince *et al.*, 1976). Unlike carbohydrate fermentation, some of the protein degradation end products may be toxic to the host. High concentrations of ammonia in the colon have been linked to increased DNA synthesis and neoplastic proliferation (Ichikawa and Sakata, 1998). Different indoles, amines and phenols that result from amino acid fermentation have been linked to a range of pathologies including schizophrenia, migraine and hypertension (Tuohy *et al.*, 2006). Lower proteolytic activities are therefore related to health-promoting effects. This can be extrapolated to the PhIP bioactivation inhibition

observed during this study. Previous studies have demonstrated that PhIP-M1 production takes place in the presence of a nitrogen-rich food source, containing only trace amounts of sugars or carbohydrates (Vanhaecke *et al.*, 2008b). This implies that proteolytic conditions are essential for PhIP-M1 production. Interestingly, the chemopreventive effects from inulin against PhIP-M1 bioactivation were most apparent in the colon transversum, which is in fact the site where the highest significant decrease in ammonia production was recorded. Moreover, during the washout period, the recovery of microbial PhIP bioactivation was accompanied by a significant increase in ammonia production. The inhibition of inulin of these proteolytic end products and shift towards a more saccharolytic environment may thus well lie at the origin of its chemopreventive activity towards microbial PhIP bioactivation.

With regard to the effects towards the microbial community, bifidobacteria have a competitive advantage over other intestinal microorganisms in a mixed culture environment due to their β-fructofuranosidase enzyme, allowing them to break down and utilize inulin-type fructans (Kolida and Gibson, 2007). Besides their nutritional advantage, bifidobacteria have been suggested to inhibit excessive growth of pathogenic bacteria, modulate the immune system, repress the activities of rotaviruses, and restore microbial integrity of the gut microbiota following antibiotic therapy (Kolida and Gibson, 2007). Significant changes in microbial community composition following inulin administration to the SHIME nutrition, were only observed after two weeks, whereas metabolic changes were found within days. This can be explained by the faster adaptation of the microbial population towards metabolism (RNA-based) than towards their community structure (DNA-based) (Boon *et al.*, 2003). Structure analysis of the colon microbiota using PCR-DGGE confirmed these plate count data by showing that the overall microbial community kept relatively unchanged. Plate count analysis revealed that bifidogenic effects in the ascending colon vessel became significant after three weeks of supplementation, while in the transverse and descending colon compartments only non-significant increases were recorded. Several authors report a significant increase in bifidobacteria and a concomitant decrease in *Enterococcus* spp. upon inulin supplementation to humans (Kleessen *et al.*, 1997) and rats (Licht *et al.*, 2006). As our recent work has shown that *Enterococcus faecium* is one of the principal colonic species responsible for the bioactivation of PhIP (Vanhaecke *et al.*, 2008b), a decrease in *Enterococci* concentrations following inulin administration might as well play a part in the inhibition of PhIP-M1 formation. Plate counts during this SHIME run however only recorded non-

significant decreases in *Enterococci* concentrations. Further research into the effects of inulin on the growth and activity of the PhIP-M1 producing *Enterococcus* species using more specific molecular techniques such as FISH and RT-PCR, is therefore required.

The results from this study demonstrate that the lower dose of 3 g/day needs to be administered over a longer time frame to effectively induce and maintain beneficial effects. Single doses of inulin are therefore of no use. Higher levels of inulin supplementation may be considered in order to further reduce putrefactive ammonia and phenol production, increase SCFA production, sustain more pronounced bifidogenic effects and completely inhibit the microbial PhIP bioactivation potency. However, complaints of flatulence, abdominal pain and bloating have been reported in human feeding studies involving prebiotics (Macfarlane *et al.*, 2008). Evidence suggests that at a rational dose of up to 20 g/day, gas distension should not occur (Kolida and Gibson, 2007).

In summary, our study revealed beneficial effects from native inulin towards microbial carcinogen bioactivation, microbial community composition and activity. The inhibition of genotoxic PhIP metabolite formation may be considered as beneficial, since this reduces the risks that PhIP-M1 may pose towards the colon epithelium *in vivo*. Additionally, a shift in fermentation pattern was rapidly seen with an increase in SCFA production towards propionate and butyrate and a decreased ammonia production. As the typical proteolytic conditions in the distal parts of the colon are normally more detrimental to the host *in vivo*, in particular in the light of microbial bioactivation processes, these positive modifications in the metabolism and microbial community indicate that inulin is a promising chemopreventive agent.

Acknowledgements

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CHAPTER 7

General discussion & perspectives

CHAPTER 7

General discussion and perspectives

1. General research outcomes

1.1. Positioning of this research

Diet has long been recognized as one of the major factors that can influence the development of cancer (Doll and Peto, 1981). Humans are exposed to complex mixtures of compounds, and while some of them meet nutritional demands, others have been suspected as risk factors for neoplasms. Epidemiological studies suggest that consumption of meat is positively correlated with human cancer and the cooking of meat is known to generate chemical carcinogens of high genotoxic potency, including the family of heterocyclic amines. Cooking meat in the kitchen readily produces HCAs and most people are exposed to appreciable amounts of these unequivocal carcinogens. The most abundant of these heterocyclic aromatic amines, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine, has been shown to specifically induce tumors of the colon, breast and prostate in mice and rats, which, coincidentally are the three most common sites of diet-associated cancer in Western society. In realizing its mutagenic potential, PhIP requires metabolic activation by endogenous pathways.

Traditionally, the process of food digestion and nutrient and energy provision has been considered to end at the distal ileum and scant attention has been paid to the transformation of dietary constituents that enter the colon. However, from a clinical perspective, the colon was recognized as central to host health with even the earliest of observers, Hippocrates 400 BC noting that 'death sits in the bowel'. Colon cancer is the second leading cause of cancer death in Western societies and gastrointestinal infections, inflammatory diseases, such as ulcerative colitis (UC) and Crohn's disease, or functional disorders of the gut probably account for the majority of the economic cost of community health care. The human bowel is populated by a large number of bacteria which play a fundamental role in the general health status of a human subject, both positively, e.g. by providing energy for the host, educating the host's immune system, protecting against colon cancer (Tuohy *et al.*, 2003) and negatively, e.g. by the production of hazardous metabolites, the colonization of pathogens or facilitating the

onset of obesity (Bäckhed *et al.*, 2004; Cani *et al.*, 2006). A wide range of functional foods now exist which target colonic health and this has led to the growth of this industry into a multi-billion Euro market in recent years, e.g. in 2005 the European probiotic yoghurt market valued 1.25 billion Euro. Conversely, scientific findings confirm the involvement of the colon microbiota in colorectal and other diet related cancers by the production of carcinogenic metabolites from dietary constituents (Illet *et al.*, 1990; Hirayama *et al.*, 2000; Gill and Rowland, 2002; Humblot *et al.*, 2004; 2005; 2007; Van de Wiele *et al.*, 2005). Several enzymes and metabolites have been identified that are directly or indirectly related to colorectal carcinogenesis (Illet *et al.*, 1990; Gill and Rowland, 2002). Yet, there are only limited data on the potency of the colon microbiota to directly bioactivate dietary components. If the colon microbiota are capable to bioactivate chemicals that would normally be excreted through the feces and if these bioactivated metabolites significantly contribute to the risk of a certain chemical, this could have profound consequences for current human health risk assessment. Chemicals that are not absorbed in the small intestine may become available for biotransformation by the resident microbiota and may as such form an additional hazard for the colonocytes and through absorption and distribution even affect other tissues.

1.2. Bioactivation of PhIP

The goal of this research was to elucidate the possible impact of the human intestinal microbiota on the biological activity of the heterocyclic amine PhIP. Numerous studies have reported on the metabolism of heterocyclic amines and in particular IQ, MeIQ and PhIP by mammalian enzymes, whereas only a few, partly conflicting result from studies with intestinal microorganisms are available. This is clearly shown by a short search in the available literature (Figure 7.1). The majority of the limited amount of existing microbial studies has focused on the native metabolism of the heterocyclic amine IQ. For PhIP, to the best of our knowledge, no other research group ever investigated the intestinal microbial metabolism up to date. It has, however, been recently shown that the amount of PhIP metabolites excreted in the urine of humans following ingestion of PhIP in a meat matrix is significantly lower than that of patients administered PhIP in a capsule. This indicates that PhIP provided in capsule form is more bioavailable than PhIP ingested from meat. This non-bioavailable fraction reaches the colon and becomes available for biotransformation by the colonic bacteria.

Therefore, in this work an integrated approach of *in vitro* and *in vivo* research was followed to explore the PhIP bioactivation potency of the human intestinal microbiota. Starting from simple *in vitro* batch incubations with human fecal samples, the most important research findings were validated and confirmed *in vivo* in a human intervention trial. Mechanistic aspects of the microbial transformation process were further explored using in depth molecular and chemical analyses. Mammalian cell-culture based assays were finally used to determine the biological relevance of the microbial PhIP transformation process.

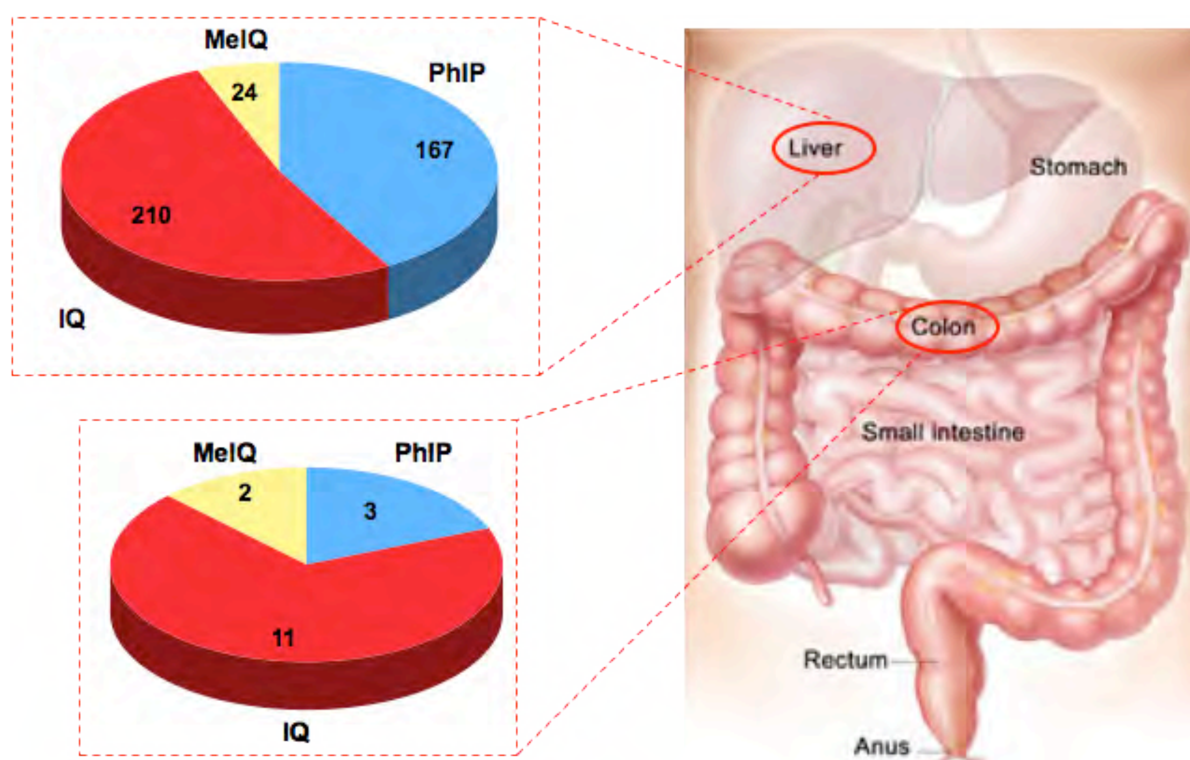

Figure 7.1 Number of hits in Web of Science when searching for PhIP, IQ and MeIQ in relation with liver and bacterial metabolism in the intestine (http://apps.isiknowledge.com).

1.3. Main research findings

The major accomplishments of this work can be summarized as follows and are schematized in Figure 7.2.

- ***In vitro* metabolism of PhIP into PhIP-M1.** For the first time, the intestinal microbial metabolism of the heterocyclic aromatic amine PhIP was investigated. Upon

in vitro incubation of human fecal samples, it was shown that intestinal microorganisms actively transform the food carcinogen PhIP, resulting in the formation of one major metabolite. By a combination of mass spectrometric and NMR spectroscopic evidence, the complete chemical configuration of this microbial PhIP derivate was identified as 7-hydroxy-5-methyl-3-phenyl-6,7,8,9-tetrahydropyrido-[3',2':4,5]imidazo[1,2-*a*]pyrimidin-5-ium chloride (**Chapter 2**).

- ***In vivo* detection of PhIP-M1.** An analytical method was developed that reliably and simultaneously quantifies PhIP and PhIP-M1 in urine and feces samples from healthy individuals administered a known dose of PhIP. Subsequently, this method was applied on pooled human urine and feces samples from 6 human subjects that were fed 150 g of well-done chicken and for the first time the excretion of a microbial PhIP metabolite in human urine and feces was observed (**Chapter 3**).

- **Interindividual variation in PhIP transformation.** As the microbial transformation of PhIP was not identical in every fecal sample tested, the production of PhIP-M1 was shown to be dependent on interindividual differences. A first explorative experiment with 6 human fecal samples demonstrated this relation (**Chapter 2**). Subsequent fecal incubations with 18 human microbiota confirmed that individuals could be separated in low, moderate and high PhIP-M1 producers (**Chapter 4**). Finally, differences in intestinal PhIP-M1 production were found to determine differences in PhIP-M1 excretion *in vivo* in humans (**Chapter 3**).

- **Isolation, identification and characterization of PhIP-M1 producing bacteria.** In the search to find bacterial species responsible for PhIP-M1 production, two individual strains were isolated from human feces and identified as *Enterococcus faecium* PhIP-M1-a and PhIP-M1-b. Some strains from culture collections belonging to the species *Enterococcus durans*, *Enterococcus avium*, *Enterococcus faecium* and *Lactobacillus reuteri* were also able to perform this transformation. Glycerol was identified as a fecal matrix constituent required for PhIP transformation. The anaerobic fermentation of glycerol via 3-HPA was determined as the critical bacterial transformation process responsible for the formation of PhIP-M1 (**Chapter 4**).

- **Biological activity of PhIP-M1.** The mutagenic activity of PhIP-M1, as analyzed using the *Salmonella* strains TA98, TA100 and TA102, yielded no significant

response (**Chapter 3**). PhIP-M1 however induced significant cytotoxic, apoptotic and DNA damaging effects towards the human colon cancer cell line Caco-2 (**Chapter 5**).

- **Chemopreventive properties of inulin against PhIP-M1 formation.** A potential added value of chicory inulin was explored. Inulin exerted strong inhibitory effects towards microbial PhIP bioactivation as measured using HPLC analysis (**Chapter 6**).

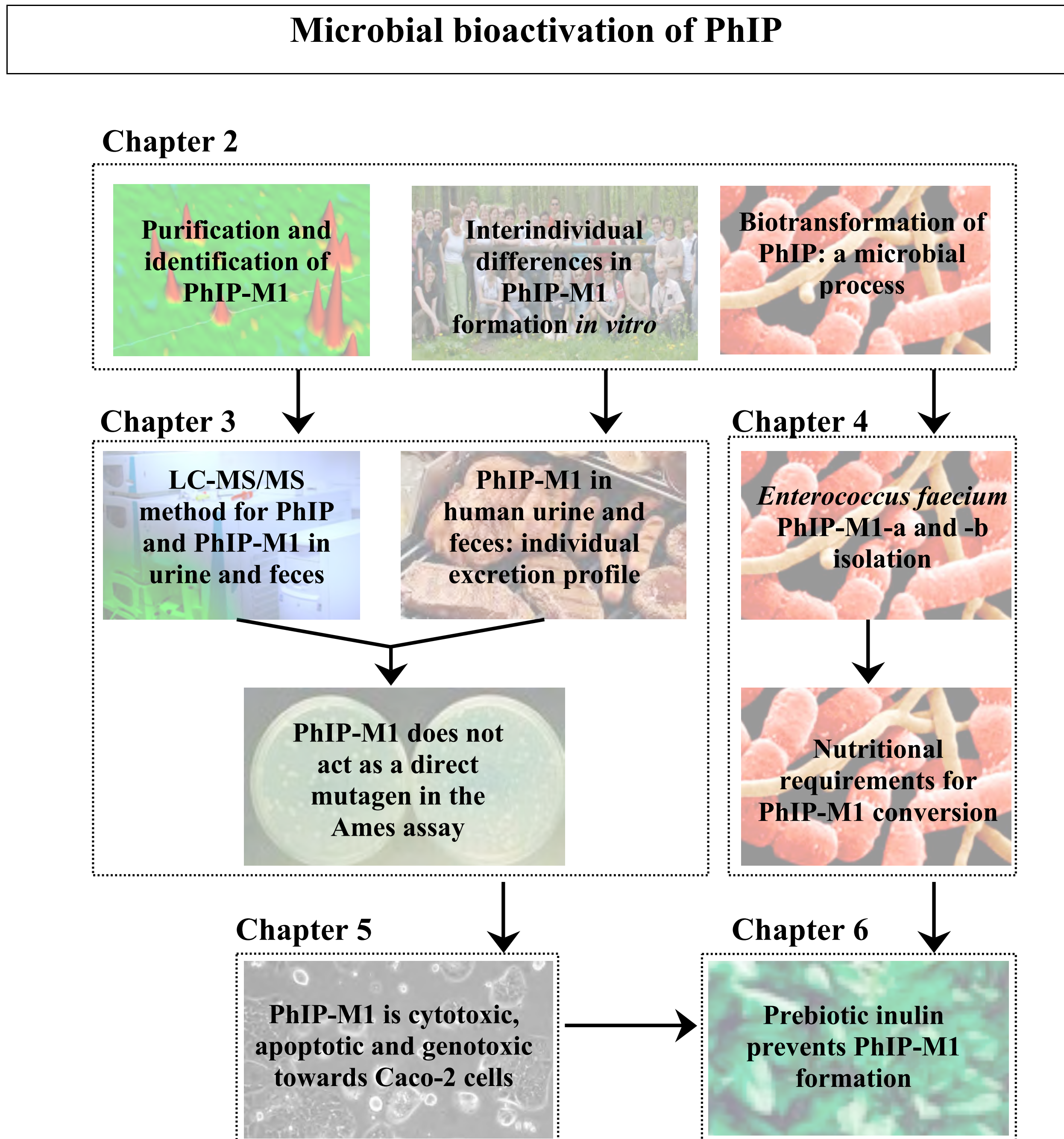

Figure 7.2 Schematic overview of the main research accomplishments of this work.

2. Contribution to scientific knowledge

2.1. PhIP-M1: a newly identified microbial PhIP metabolite

2.1.1 *In vitro* formation by fecal microbiota: purification and identification

As described in **Chapter 2**, the original goal of this dissertation was to investigate any possible metabolism of the most abundant heterocyclic aromatic amine, PhIP, by the human intestinal microbiota. In a first experiment to exploit this biotransformation potential, we incubated the microbial cultures obtained from six human stool samples with an environmentally relevant concentration of PhIP. Interestingly, all six human feces degraded PhIP and concurrently produced one metabolite, PhIP-M1. This microbial conversion proved to be concentration independent. Therefore, a strategy of anaerobic batch fermentation of a highly transforming fecal culture with a PhIP concentration reaching saturation solubility was applied to obtain large quantities of the newly discovered PhIP metabolite. Subsequently, a straightforward preparative RP-HPLC method was developed for the large-scale purification of PhIP-M1. The preparative RP-HPLC method enabled purification of PhIP-M1 in quantities of several tens of mg and allowed in depth mass spectrometric, NMR spectroscopic and IC analysis. Careful interpretation of these data led to the assignment of PhIP-M1 as 7-hydroxy-5-methyl-3-phenyl-6,7,8,9-tetrahydropyrido[3’,2’:4,5]imidazo[1,2-*a*]pyrimidin-5-ium chloride.

This newly optimized preparative RP-HPLC method will not only be useful in the development and validation of new analytical methods (**Chapter 3**) but will as well enable investigation of the biological properties of PhIP-M1 in *in vitro* bacterial bioassays (**Chapter 3**) and human cell line tests (**Chapter 5**) and *in vivo* experiments with laboratory animals, which could contribute to a better overall understanding of the potential effects of this metabolite in relation to human health. Moreover, our results demonstrate the importance of powerful high-resolution analytical techniques such as HRMS and ^{1}H and ^{13}C NMR spectroscopy in microbial metabolomics.

2.1.2 *In vivo* detection in human urine and feces: completing the PhIP mass balance

Although several analytical methods have been developed for the detection and quantification of PhIP and/or its liver metabolites in urine (Strickland *et al.*, 2001; Stilwell *et al.*, 2002; Kulp *et al.*, 2004; Malfatti *et al.*, 2006), research on the bioavailability and

biological activity of the heterocyclic amine PhIP in a meat matrix has so far been hampered by the absence of good analytical methods for the detection or quantification of PhIP in feces. In addition, the potential contribution of the intestinal bacteria to the absorption, metabolism and excretion of PhIP has so far been neglected in human intervention trials.

In this work we developed, optimized and validated a rapid and accurate solid phase extraction LC-ESI-MS/MS method for the simultaneous quantification of PhIP and PhIP-M1 in human urine and feces (**Chapter 3**). Because our method was devised to investigate the microbial involvement in PhIP metabolism and in order to enhance the recovery of PhIP, urine and feces samples were hydrolyzed with acid prior to analysis. This treatment causes the release of PhIP from putative glucuronide or sulphate conjugates (Strickland *et al.*, 2001). RP-HPLC is the most utilized technique for separating PhIP and its deconjugated metabolites in biological matrices, such as urine, milk or feces (Chen *et al.*, 2007; Scott *et al.*, 2007), although some bottlenecks emerge herewith, including the complexity of biological extracts, the overlapping retention times of analytes and deuterated standards and the ng/L concentrations at which these compounds occur in biological matrices - making conventional detection by UV absorption, fluorescence or single-ion monitoring MS impossible. The unsuitability of these detection methods was efficiently circumvented by using tandem mass spectrometry, which exhibited sharp peaks and good signal-to-noise ratios, for PhIP and its microbial metabolite in both urine and feces samples. Quantification of the newly identified PhIP-M1 metabolite was made possible as external and internal standard curves were constructed with respectively, PhIP-M1 and $[^2H_3]$PhIP-M1, purified using preparative HPLC as described in **Chapter 2**. This method can contribute to further research on the metabolism and bioavailability of PhIP.

In **Chapter 3**, we applied this method on urine and feces samples from six healthy adults, which received a known dose of naturally produced PhIP. Of the ingested dose, volunteers excreted 1.2-15% as PhIP-M1 in urine and 0.9-11% as PhIP-M1 in feces. This PhIP-M1 was not formed *de novo* from PhIP in urine and feces samples during hydrolysis. A number of studies describe the incubation of PhIP with mammalian enzymes and none of them reported the detection of a metabolite resembling PhIP-M1 (Zhao *et al.*, 1994; Crofts *et al.*, 1998; Turesky *et al.*, 2002), while incubation of PhIP with fecal bacteria does give rise to the formation of this metabolite (**Chapter 2**). Moreover, for PhIP-M1, an increase in urinary

excretion was observed for successive time increments, whereas for PhIP the majority was excreted in the first 24 h. Therefore, our results confirm that the intestinal bacteria significantly contribute to the overall metabolism and disposition of PhIP *in vivo*.

In addition, PhIP and PhIP-M1 quantification revealed that the percentages of the total PhIP dose excreted in this study as PhIP (26-42%) and PhIP-M1 (0.9-11%) in feces were surprisingly high and could explain the relatively low PhIP dose percentages measured in urine in previous metabolism studies of human subjects given PhIP in a meat matrix (Kulp *et al.*, 2000; 2004; Strickland *et al.*, 2001). The total percentage of PhIP and PhIP-M1 accounted for in the 72 h urine and feces, varied from 49% to 71%. The 2-OH-PhIP derivate, which is formed during acidic hydrolysis from the major mammalian N-oxidation metabolite N-OH-PhIP-N^2-glucuronide, was however not quantified during our study. It has been reported that 25 ± 8.4% (66 volunteers) of the ingested PhIP dose after ingestion of a meat-based meal is excreted as 2-OH-PhIP (Stillwell *et al.*, 2002). This might explain the additional deficit in PhIP dose percentage encountered in this study. Therefore, future studies should be devised to assess the urinary and fecal excretion of PhIP, 2-OH-PhIP and PhIP-M1 and to relate these percentages to microbial community composition, phase I and phase II enzyme expression and specific systemic biological effects.

2.2. PhIP-M1 formation: a mechanistic basis

2.2.1. Isolation and identification of PhIP-M1 producing bacteria

After the observation that PhIP could be metabolically converted *in vitro* and *in vivo*, the next step was to identify the bacterial species responsible for this process. We attempted to isolate a PhIP-M1 producing bacterium from the feces of two human volunteers with a high PhIP transforming potential. We applied a strategy of anaerobic culturing in the presence of fecal matrix, followed by plating and re-incubating picked up colonies. From the 65 colonies we picked, only two resulted in a culture that converted PhIP into PhIP-M1. However, as was observed by microscopic analysis, the obtained cultures consisted of morphologically identical bacterial species. Molecular techniques (16S rRNA PCR, DGGE, cloning, sequencing) confirmed the genus of the strains as *Enterococcus*. Definite identification of the isolates was achieved by FAPLP™ and partial *pheS* sequence analysis, which are now deposited as two new strains: *Enterococcus faecium* PhIP-M1-a and PhIP-M1-b. In addition,

several culture collection strains belonging to the species *Enterococcus durans*, *Enterococcus avium*, *Enterococcus faecium* and *Lactobacillus reuteri* were also capable of producing PhIP-M1 (**Chapter 4**).

2.2.2. Microbial, chemical and nutritional aspects in PhIP-M1 formation

Remarkably, the microbial transformation of PhIP showed no resemblance to that of the heterocyclic amines IQ and MeIQ, which have been reported to form stable hydroxy derivates (Vantassell, 1990; Humblot *et al.*, 2005). One possible explanation for this discrepancy, as mentioned in **Chapter 2**, might be the protective effect of the phenyl substituent of PhIP, impairing hydroxylation on the imidazo moiety. Moreover, the microbial conversion reaction, which has been discovered during this research, is unique in its kind, as nobody else ever reported the involvement of intestinal bacteria or even anaerobic bacteria in general, in this sort of three-carbon ring expansion.

Therefore, as a next step in our research, we attempted to clarify the metabolic processes behind the microbial PhIP to PhIP-M1 conversion. In **Chapter 4**, several *in vitro* PhIP incubation experiments with inactivated fecal cultures, surfactants and protease inhibitors were performed. These provided evidence for the involvement of actively fermenting bacteria in PhIP-M1 formation by the production of an extracellular substance through an enzymatic process. Subsequently, it was found that glycerol is required for the conversion of PhIP into PhIP-M1. Addition of glycerol to the growth medium of mixed fecal microbiota, *Enterococcus faecium* PhIP-M1-a or *Lactobacillus reuteri* ATCC 5360 clearly initiated PhIP-M1 production. This PhIP-M1 formation was accompanied by the detection of 3-HPA in the mixed and pure culture fermentation broths. Therefore, it could be concluded that the anaerobic fermentation of glycerol to 3-HPA is the critical bacterial transformation responsible for the formation of PhIP-M1. The addition of 3-HPA to the PhIP molecule, resulting in the three-carbon ring expansion, is however chemical, as abiotic synthesis of PhIP-M1 by addition of 3-HPA to the sterile bacterial growth medium in the presence of PhIP was successfully performed.

Under anaerobic conditions, several lactobacilli, as well as other bacterial species (*Klebsiella*, *Clostridium*, *Enterobacter* and *Citrobacter* genera) have been shown to use glycerol as an external electron acceptor (Schutz and Radler, 1984; Talarico *et al.*, 1988;

Sauvageot *et al.*, 2000), resulting in the coenzyme B_{12}-dependent dehydratase mediated conversion to 3-HPA. 3-HPA is normally an intracellular intermediate that does not accumulate but is reduced by an NAD^+-dependent oxidoreductase to 1,3-propanediol (PPD) (Biebl *et al.*, 1999). We are however the first to relate bacterial species of the genus *Enterococcus* to this anaerobic pathway of glycerol dissimilation. Surprisingly, *Enterococcus faecium* PhIP-M1-a turned out to be only a weak 3-HPA and PhIP-M1 producer under fecal matrix-poor conditions, implying that other fecal matrix constituents might be required by this strain to perform the glycerol fermentation. It could however not be determined which fecal excretion products were requisite for 3-HPA formation. Next to the fecal matrix, PhIP-M1 production also requires the presence of a nitrogen-rich food source containing trace amounts of sugars and carbohydrates. This was shown for mixed fecal microbiota as well as for the *Enterococcus faecium* PhIP-M1-a transforming strain. Lactic acid bacteria (LAB) are nutritionally fastidious microorganisms, which are, nevertheless, capable of hydrolyzing peptides down to free amino acids. Amino acid catabolism produces, in turn, a number of compounds, including ammonia, amines, aldehydes, phenols, indole and alcohols. *Enterococcus faecium* has been shown to display high dehydrogenase activity and high oxidase activity towards selected amino acids compared to other selected LAB (*Lactobacillus paracasei*, *Leuconostoc mesenteroides*, *Lactococcus lactis*) (Tavaria and Malcata, 2003). These proteolytic activities and the respective catabolism products of *Enterococcus faecium* may be indirectly related to the glycerol fermentation process, although further research is required to clarify the possible interactions and elucidate the glycerol fermentation pathway followed by *Enterococcus faecium* and phylogenetically related enterococci. In **Chapter 4**, we have observed that easily degradable sugars inhibit PhIP-M1 production. It has been reported that the regulation of the PPD pathway is dependent on the availability of fermentable carbohydrates, in particular glucose (Biebl *et al.*, 1999). In the absence of glucose, PPD formation is the rate-limiting step and 3-HPA may accumulate. The inhibition of PhIP-M1 production in the presence of easy degradable sugars may thus well be linked to the absence of 3-HPA under these conditions.

Indications from recent literature exist that enterococci may play a role in the metabolism of glycerol in a mixed microbial culture *in vitro*. Cleusix *et al.* (2008) investigated the effects of *Lactobacillus reuteri* ATCC 55730 on adult intestinal microbiota and its capacity to secrete 3-HPA in the presence of glycerol using an *in vitro* colonic model.

The addition of 100 g/L glycerol strongly modified the SCFA ratio (increased butyrate production). In addition, a marked increase in PPD production was observed**.** Among the propanediol-producers clostridia are the most commonly reported intestinal species and contrary to *Lactobacillus reuteri* and other propanediol-producers the only ones known to produce butyrate as a byproduct. Some enterococci, however, have been recently shown to posses the bacterial gene encoding butyrate kinase, present in the butyrogenic bacterium *Clostridium* (Raz *et al.*, 2007). Moreover, Cleusix *et al.* (2008) observed an increase in lactobacilli/enterococci populations upon glycerol supplementation, whereas no increase in *Lactobacilllus reuteri* or clostridial populations was detected. In light of these findings, further studies are warranted to assess the involvement of enterococci in glycerol fermentation. Therefore, more specific quantitative molecular detection techniques such as RT-PCR or FISH might be considered to evaluate the shifts in colonic microbial composition upon glycerol supplementation.

2.3. *Interindividual variability in PhIP-M1 production and excretion*

An important aspect of the health impact of the intestinal microbiota that has recently emerged is the interindividual variation in activity and composition of the gut bacteria. Each individual harbors a unique microbial community that comprises a total of ca. 10^{14} bacterial cells belonging to 500-1000 different species. As a result each individual microbial community may exert distinct health effects towards the human host and may posses a different bioactivation/detoxification potential towards dietary components, which on their turn may also influence human health. Examples of bacterial transformations, which are subjected to a high interindividual variability and may influence the hosts' health both in a positive or negative fashion, are presented in Table 7.1.

Similar observations were made for the PhIP to PhIP-M1 conversion by human intestinal bacteria. In a first preliminary *in vitro* experiment with six fecal samples, significant interindividual differences were observed in the capacity of the intestinal microbiota to produce PhIP-M1, ranging from 47 to 95% (**Chapter 2**). Expansion of the number of fecal samples to eightteen, lead to a broader transformation efficiency range (1.8 to 96%) (**Chapter 4**). Remarkably, every *in vitro* incubated fecal microbial community screened so far, has been proven capable of producing PhIP-M1 to some extent.

Table 7.1. Examples of intestinal microbial metabolites from dietary components of which the formation is subjected to a large interindividual variation.

Dietary component	Bacterial metabolite	Effect on human health	Reference
IQ	7-OH-IQ	Mutagenic in Ames assay, not carcinogenic in rodents	Humblot *et al.*, 2005
Daidzein	Equol O-DMA	Estrogenic activity, reduction of certain diseases including risk breast and prostate cancers	Atkinson *et al.*, 2005
Lignans	Enterodiol Enterolacton	Estrogenic activity, prevention breast and colon cancer, diabetes and atherosclerosis	Clavel *et al.*, 2005
Cholesterol	Coprostanol	Associated with colorectal carcinogenesis	Veiga *et al.*, 2005
Flavonoids (general)	Inactive compounds	Compounds with no estrogenic activity and thus no preventive potential	Simons *et al.*, 2005
Primary bile acids	Secondary bile acids	Risk of colon cancer and cholesterol gallstones	Kitahara *et al.*, 2004
Isoxanthohumol	8-Prenylnaringenin	Prevention bone loss, inhibition metastasis and angiogenesis, estrogenic and antiandrogenic activity	Possemiers *et al.*, 2005

Interindividual variability in PhIP-M1 production was further investigated in **Chapter 3**, in which a human intervention trial with 6 individuals was set up to investigate whether interindividual differences in PhIP-M1 production *in vitro* would also lead to differences in urinary and fecal PhIP-M1 excretion *in vivo*. Indeed, significant differences in urinary and fecal excretion were observed for PhIP-M1 (1.2–15% in urine and 0.9–11% in feces), while for PhIP, which consisted of free PhIP and acid-labile PhIP conjugates, these differences were far less pronounced (12–21% in urine and 26-41% in feces). This indicates that interindividual differences in microbial composition and metabolism may at least be equally important than differential expression and genetic polymorphisms in phase I and II endogenous enzymes, which have been considered so far as the obvious candidates responsible for individual variability in urinary excretion of PhIP metabolites following

ingestion of similar quantities of parent compound (Stillwell *et al.*, 2002). The microbial composition and metabolic activity as well as the expression of mammalian CYP1A2, SULT, UDPGT and NAT can all to some extent be attributed to environmental factors such as drugs, diet, alcohol consumption and smoking (Zevin and Benowitz, 1999; Schwab *et al.*, 2000). As we have shown in **Chapter 4,** the nutritional composition and concentration of specific cofactors in the fermentation broth strongly influenced final PhIP-M1 production by fecal microbiota *in vitro*. This may be extrapolated to human nutrition *in vivo*.

In addition, our results did not only show that intestinal activation of PhIP determines PhIP-M1 excretion *in vivo*. For some individuals the urinary *vs.* fecal excretion of PhIP-M1 was substantially elevated compared to that of other individuals. Therefore, it can be concluded that differences in intestinal production and absorption of PhIP-M1 determine the systemic exposure and possible health outcome related to consumption of PhIP-containing meat products. Future studies are however needed to assess the stability of the microbial PhIP bioactivation phenotype over longer periods and to explore the final importance of this variability towards specific activity related endpoints such as DNA adducts and chromosomal aberrations.

2.4. Bioactivation of PhIP: microbial contribution

The current focus of risk assessment for the oral exposure to food contaminants lies on human bioactivation processes by cytochrome P450 complexes in enterocytes and hepatocytes. There are however many indications that the intestinal microbiota can inactivate or bioactivate a wide variety of chemical agents from diet or biliary excretion (McBain and Macfarlane, 1998; Macfarlane and Macfarlane, 2007). Microbial bioactivation is however not covered in current risk assessment practice, but it has already been extensively discussed when reviewing the relationships between diet and cancer and the role of intestinal microorganisms (McBain and Macfarlane, 1998; Gill and Rowland, 2002; Tuohy *et al.*, 2006; O'Keefe, 2008).

It has been shown that the intestinal microbiota are essential to the induction of DNA damage by PhIP in HFA rats (Hollnagel *et al.*, 2002). Moreover, since ligation of the biliary duct in rats does not alter the genotoxic potential of PhIP (Kaderlik *et al.*, 1994), the

deconjugation of reactive glucuronides by bacterial β-glucuronidase was suggested not to influence the metabolic fate and bioactivity of PhIP (Humblot *et al.*, 2007). Therefore, it is very much conceivable that the microbial formation of PhIP-M1 contributes to the final genotoxic and carcinogenic activity of PhIP.

As was reported in **Chapter 3**, PhIP-M1 did not act as a direct mutagen in the *Salmonella*/Ames assay. The experiments conducted in **Chapter 5** however showed that PhIP-M1 induces DNA damage, cell cycle arrest, apoptosis and eventually cell death and growth inhibition towards the human intestinal Caco-2 cell line. DNA damage in Caco-2 cells was detected using the comet assay or single-cell gel electrophoresis (SCGE). This assay is recognized as a sensitive tool widely used for the evaluation of primary DNA damages at the individual cell level (Tice *et al.*, 2000), while the bacterial Ames assay only detects mutagenic effects if the DNA damage induced remained after cell division. Hartmann *et al.* (2001) have indeed shown that the comet assay is capable of detecting genotoxic compounds that were tested negative in the Ames assay. The conversion of PhIP into PhIP-M1 is therefore considered as a microbial bioactivation.

As the genomic and cellular events of CYP1A2-activated PhIP in different *in vitro* cell systems (Pfau *et al.*, 1999; Zhu *et al.*, 2000; Edenharder *et al.*, 2002; Gooderham *et al.*, 2002; 2007) are not significantly higher than those observed for PhIP-M1 in our test system, the physiological relevance of this microbial PhIP derivate in PhIP carcinogenicity may not be neglected. Extrapolation of these *in vitro* data to the *in vivo* situation must however be made with caution. Therefore, further assessment of the *in vitro* genotoxicity and *in vivo* carcinogenicity of PhIP-M1 may be desirable.

The microbial bioactivation of ingested PhIP in **Chapter 5** and the indirect bioactivation through microbial deconjugation enzymes of ingested IQ (Humblot *et al.*, 2007), indicate the important role of the colon microbiota in the generation of genotoxic compounds from HCAs. Since such bacterial transformation processes and enzymatic activities are, as mentioned previously, often diet related, it would be interesting to modulate the bioactivation potency through dietary factors. Several studies have shown that the diet strongly modulates the metabolic activity from intestinal microbiota (Louis *et al.*, 2007) and changes in the microbial community composition have been observed to influence the

metabolism of DNA-reactive carcinogens (Humblot *et al.*, 2005). It has been demonstrated that oligosaccharides such as FOS, GOS or inulin inhibit the formation of heterocyclic amines in a meat matrix (Shin *et al.*, 2003). Moreover, it has been observed that oligofructose and inulin decrease IQ-induced genotoxicity in HFA rats through inhibition of the β-glucuronidase activity (Humblot *et al.*, 2004; 2007), whereas the impact of oligosaccharides and inulin on direct microbial bioactivation processes has not been reported. Therefore, in **Chapter 6**, the use of native chicory inulin was evaluated as an inhibitory feed constituent against microbial PhIP bioactivation. HPLC analysis revealed that inulin administration significantly decreased the formation of the genotoxic PhIP-M1 derivate in PhIP incubated colon samples, specifically in the transverse and descending colon and to a lesser extent in the ascending colon. The most important effect of inulin administration towards the microbial community was the increase in bifidobacteria, as indicated by plate counts and PCR-DGGE. Inulin-type fructans are composed of β-D-fructofuranoses attached by β-2→1 linkages that are preferentially degraded by bifidobacteria, thus providing a competitive advantage over other intestinal microorganisms in the colon (Kolida and Gibson, 2007). This bifidogenic effect may suppress other microbial groups such as enterococci (Kleessen *et al.*, 1997; Licht *et al.*, 2006), which are involved in microbial PhIP bioactivation. With regard to the metabolic activity, inulin beneficially influenced the fermentation pattern of the colon microbiota towards a significantly higher SCFA production, primarily propionate and butyrate and a decrease in ammonia and phenol production, which can be considered as general indicators of lower colon cancer risk (Macfarlane *et al.*, 2008). As PhIP-M1 production has been shown to occur under proteolytic conditions, this shift towards a more saccharolytic environment may explain for the chemopreventive effects detected. These observations indicate that the prebiotic effect of inulin addition acts on several aspects, which may all lay at the origin of a decrease in PhIP-M1 formation in the colon. Future research should investigate the effects of inulin on the growth and activity of the PhIP-M1 producing *Enterococcus* species using more specific molecular techniques such as RT-PCR and flow cytometry. Additionally, other dietary inhibitors towards microbial PhIP bioactivation in general need to be explored.

3. Future perspectives

Against the background of this dissertation - microbial bioactivation of food contaminants - several future research topics can be identified that are directly or indirectly related to microbial metabolism and human health. There is expanding evidence that many colonic diseases, and in particular colon cancer risk, are determined by interactions between the diet and microbiota. Further research into the composition, characterization and metabolite activity of our microbiota may provide the key to the influence of nutrition and environment on colonic health and disease. For most practical purposes however, the large bowel is inaccessible for routine investigation, and a further exploration of high-throughput screening techniques that are still reliable with regards to bioavailability, metabolism and toxicity processes is needed.

3.1. Intestinal bacteria: metabolism and colonic health

3.1.1. Diet and nutrition

One of the fundamental properties of mucosal epithelia is their ability to directly utilize 'topical' nutrients, derived from the diet or digestion of food, without reliance on the blood flow. The two main fermentative substrates of dietary origin are non-digestible carbohydrates (10-60 g/day) and protein (~ 13 g/day) that escape digestion in the small intestine (Tuohy *et al.*, 2006). Although colonocytes do not secrete enzymes that are capable of digesting these residues, the colonic microbiota do, and in an excellent example of symbiosis, the bacteria metabolize these residues. Unfortunately, bacteria can also synthesize metabolic products that are injurious to the mucosa (Figure 7.3).

Carbohydrates (fiber and resistant starch) in the colon are fermented to SCFAs, which maintain mucosal respiration and growth, and one of them, butyrate, regulates proliferation and differentiation and reduces tumorigenesis (Roy *et al.*, 2006). Generally, a diet rich in fibers and resistant starch is believed to reduce the risk of colon cancer (O'Keefe, 2008). Unfortunately, carbohydrate fermentation can also produce toxic metabolites. Fermentation of starch produces hydrogen gas that can impair NAD regeneration and inhibit cellular metabolism (Gibson *et al.*, 1993). Interestingly, high fermenters, such as ruminants, have adapted by replacing hydrogen-producing bacteria with epithelium-sparing methane producers.

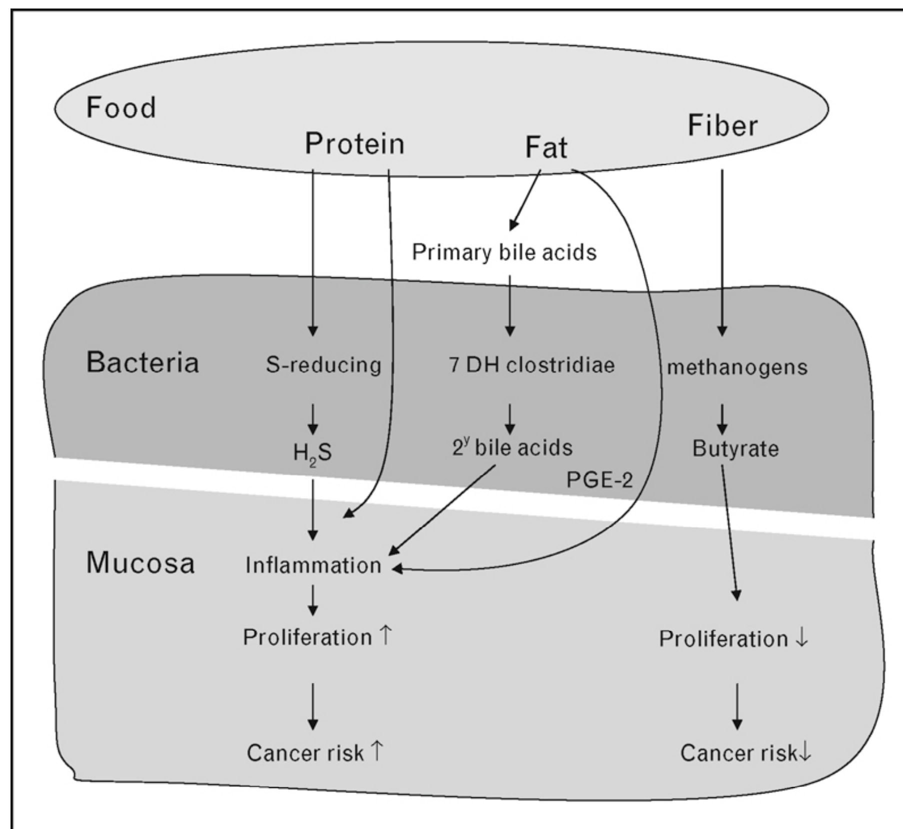

Figure 7.3 The effects of diet on colonic health and disease mediated by the colonic microbiota (O'Keefe, 2008).

Proteins and amino acids are also available for bacterial fermentation in the colon. Several of the products resulting from amino acid fermentation have some relevance to human health. Oxidative or reductive deamination leads to the formation of ammonia, which has been shown to act as a tumor promoter (Ichikawa and Sakata, 1998). Bacterial degradation of sulfur-containing amino acids promotes the growth of sulfur-reducing bacteria and outcompetes methanogenic bacteria for hydrogen to form hydrogen sulphide. Hydrogen sulphide impairs cytochrome oxidase, suppresses butyrate utilization, inhibits synthesis of mucus and DNA methylation (Christl *et al.*, 1992), has been implicated in ulcerative colitis (UC) (Roediger *et al.*, 1993) and has been shown to act as a genotoxin through the generation of free radicals (Attene-Ramos *et al*, 2007). The anaerobic fermentation of aromatic amino acids gives rise to phenols and indoles. Phenols such as *p*-cresol and its secondary metabolites have been proposed to act as pro-carcinogens in colon cancer (Blaut and Clavel, 2007). Decarboxylation of amino acids in the colon results in the formation of amines. Under acidic conditions or catalyzed by bacteria, the latter may react with nitrite to form carcinogenic N-

nitroso compounds such as nitrosamines. Many oral and intestinal bacteria are capable of reducing nitrate to nitrite. It is however not clear whether bacterial nitrite is a key agent in nitrosation. Moreover, it has been shown that different representative gut bacteria may further reduce nitrite to nitric oxide (Sobko *et al.*, 2005). Nitric oxide is a free radical with moderate reactivity compared to other species, which gives rise to a multitude of physiological and pathological events in the gastrointestinal tract. A complete understanding of the mechanisms regulating the formation of N-nitroso compounds and nitric oxide formation from nitrite by intestinal microbiota would require the isolation and characterization of the responsible bacteria and more extensive *in vivo* studies. Future studies are also needed to reveal the biological significance of these metabolic processes, in particular in the light of their potential involvement in intestinal inflammation and IBD (Kolios *et al.*, 2004).

Fat intake and in particular animal fat has long been recognized as a risk factor in colon cancer (Doll and Peto, 1981). On the other hand, experimental data have clearly demonstrated that the influence of dietary fats on cancer depends on the quantity and the type of lipids. Whereas a high intake of n-6 PUFA and saturated fat has tumor-enhancing effects, n-3 PUFA, conjugated linoleic acid and gamma-linolenic acid have inhibitory effects. Until present, identification of the underlying mechanisms of this association in relation to intestinal microbial metabolism was mainly indirect. Fat consumption stimulates the synthesis and enterohepatic circulation of the primary bile acid, cholic acid, which is mostly reabsorbed, however a fraction may reach the colon (Reddy, 1981). If the colonic microbiota contain 7α-dehydroxylating bacteria, cholic acid is converted to deoxycholic acid, a well-recognized co-carcinogen (Nagengast *et al.*, 1995). Moreover, the intestinal microbiota have recently been implicated in the regulation of fat storage and the onset of obesity. Glucagon-Like Peptide-1 (GLP-1) and Glucose-dependent Insulinotropic Polypeptide (GIP) are produced in the intestine in response to glucose intake and would play an important role in the onset of diabetes and insulin resistance. Microbial metabolites in the intestine would influence GLP-1 and GIP production, thereby influencing satiety (Cani *et al.*, 2006). Similarly, the expression of the Fasting Induced Adipose Factor (FIAF), which inhibits blood lipase activity and inhibits fat storage, would be suppressed by intestinal microbiota, leading to increased fat storage (Bäckhed *et al.*, 2004).

The direct interactions of dietary lipids and its lipolysis products in relation to colonic microbiota and human health are however largely unexplored. It has been shown that in healthy subjects, about 2-4 g of the daily dietary lipid intake reaches the colon (Hill, 1995). In addition, a significant fraction free glycerol, liberated from dietary fat in the small intestine by pancreatic lipases, may reach the colon as such, since its intestinal absorption is saturable and involves carrier-mediated transport (Yuasa *et al.*, 2003; Kato *et al.*, 2005; Fujimoto *et al.*, 2006). A few studies provide evidence for the hydrogenation of essential PUFAs by colonic microbiota (Howard and Henderson, 1999; Devillard *et al.*, 2007) and recent research explored the fermentation of glycerol in an *in vitro* colonic model (Cleusix *et al.*, 2008). In the light of the potential health effects of these and possibly other microbial processes, the bioavailability, metabolism and biological activity of dietary lipids and their degradation products with respect to the microbial community should be further investigated. This should preferably be done by a combination of *in vitro* fermentation technology and *in vivo* metabolism studies. In both cases there is a need for good biomarkers, which efficiently reflect the risks associated with certain metabolic processes.

3.1.2. Carcinogenic food contaminants

Food consumption represents an important pathway for human exposure to chemicals from a variety of sources. There are 4 primary types of potentially carcinogenic compounds (Abnet, 2007). The first are natural products that may be present in food and are unavoidable. For example, the process of creating salted fish produces carcinogens (N-nitrosodimethylamine and other N-nitroso compounds) that cannot be avoided easily. Second are natural products that might be avoided such as the contamination of grain with the carcinogenic fungal metabolite aflatoxin, which can be reduced or eliminated using best practices for grain storage. Third, anthropogenic chemicals may be present in food. For instance, 2,3,7,8-tetrachlorodibenzo-p-dioxin has been inadvertently produced during the manufacture of chlorinated hydrocarbons, but it contaminates the environment, resists degradation, and accumulates in certain foodstuffs. A fourth category of concern is anthropogenic chemicals intentionally added to foods, such as saccharin and food coloring. Given the widespread occurrence and production of carcinogenic contaminants in human nutrition, prevention of further food contamination must be a national health policy priority in every country and formal risk assessments should be routinely completed by governmental and international agencies. Two important complementary programs exist that classify

whether exposures pose a carcinogenic risk to humans. Firstly, The U.S. National Toxicology Program (NTP) produces the *Report on Carcinogens* (NTP, 2002), currently in its eleventh edition. Secondly, the International Agency for Research on Cancer (IARC) produces *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans* (http://monographs.iarc.fr). Numerous national programs in different countries also provide valuable information regarding the carcinogenicity of different agents in humans, much of which are used in the IARC and NTP evaluations.

The metabolic versatility of the diverse human intestinal microbiota is increasingly understood to act in concert with human metabolic systems to transform a range of dietary compounds, including phytochemicals, drugs and xenobiotic compounds (Macfarlane and Macfarlane, 2007). Some of these metabolic processes lead to the detoxification of potentially carcinogenic compounds, for example the direct binding of HCAs by lactic acid bacteria (Bolognani *et al.*, 1997; Zsivkovitz *et al.*, 2003). Others have been shown to produce more toxic derivates, not only by the direct conversion of xenobiotics, but also by the deconjugation of excreted phase II metabolites in the intestinal lumen (Humblot *et al.*, 2007). Important future research perspectives are the fate of food contaminants in the gastro-intestinal tract and how the interrelationship with food matrices may affect their bioavailability. Interactions of food xenobiotics with macromolecular food components may reduce their release from food matrices and subsequent intestinal absorption, but may increase the fraction that reaches the colon intact and comes into contact with the resident microbiota. Clarification of the microbial-ecological mechanisms that influence the release and bioactivation/detoxification of food contaminants to hazardous metabolites is therefore required.

3.2. Integrated approach for studying microbial transformation processes

The growing awareness of the relationship between nutrition, food contaminants and human health and the involvement of the intestinal microbiota points out the significance of conducting research in the field of human nutrition, gastrointestinal microbiology and health relevant microbial transformation processes. A range of *in vitro* and *in vivo* models of the human gastrointestinal microbiota may be applied to study the interaction between diet, food contaminants and the gut bacteria. *In vitro* systems range from simple anaerobic batch cultures to multistage continuous culture models using human feces as inoculum. Several *in*

vitro models of the human gut have been developed with varying degree of complexity (Molly *et al.*, 1993; Minekus *et al.*, 1999). For instance, the TIM model focuses mainly on the small intestine, using dialysis through semi-permeable membranes to simulate intestinal absorption, but puts less emphasis on long-term culturing of the gut microbiota (Minekus *et al.*, 1999). In contrast, the SHIME allows long-term evaluation of the interaction between food components and the intestinal microbial community (Possemiers *et al.*, 2004). There are a number of reasons one would carry out such experiments *in vitro* rather than *in vivo*. First, the *in vitro* culture system allows to determine the conversion capabilities of the gut microbiota with easy access to the metabolic end products, while such end products may be absorbed *in vivo* and remain undetected in feces. Similarly, *in vitro* culture systems employ a human inoculum, which is important considering the significant compositional and metabolic differences between the gut microbiota of humans compared to animal models. Finally, *in vitro* culture systems offer a cost-effective experimental tool for looking at the microbial conversion of food contaminants that are often only available in small quantities, and not in the quantities necessary to carry out meaningful animal or human feeding studies.

Yet, the outcome of *in vitro* studies needs to be validated *in vivo* with animal models and human studies. Gnotobiotic technologies, including ex-germ-free animals colonized with human intestinal microbiota, overcome some of the limitations of the *in vitro* systems, in that they also include a mammalian input towards metabolic conversions and absorption of end products. The health implications of microbial transformations may be more readily measured because of the availability of mucosal samples from different regions of the gut and the possibility of post-mortem examination of tissues for specific pathologies. Such studies have been central to identify the intestinal microbiota as key players in the conversion of the heterocyclic amines IQ and PhIP (Kassie *et al.*, 2001; Hollnagel *et al.*, 2002). However, they are expensive and studies on the fate of food contaminants in existing food products can proceed from initial *in vitro* screenings directly to human feeding studies.

Recent advances in the fields of microbial ecology, analytical chemistry and nutritional molecular biology may further revolutionize the way we can study interactions between diet, human metabolism (including metabolic activities of our resident microbiota) and disease susceptibility. Molecular fingerprinting and quantification techniques such as PCR-DGGE, real-time PCR and flow cytometry now allow the microbiologist to capture species diversity

and visualize population fluxes within the complex gut microbiota in a manner never possible with traditional culture based techniques (Eckburg *et al.*, 2005; Blaut and Clavel, 2007). Application of high-resolution analytical techniques (e.g. LC-MS/MS and NMR) may help to elucidate the microbial transformation processes and increase the understanding of toxicokinetics. Moreover, combining *in vitro* digestion technology such as the SHIME with the culture of various cell types opens up an additional field of research. Combination with mucus secreting HT-29 cells would allow investigation of bacterial adhesion to the intestinal cell wall in relation to specific microbial transformation processes (Hwang *et al.*, 2005). Similarly, combination with Caco-2 cells would allow the study of intestinal transport processes (Schutte *et al.*, 2008; Vasiluk *et al.*, 2008) and a screening of the biological activity of microbial metabolites, as was performed for PhIP-M1 in **Chapter 5**. Finally, combination with metabolically competent hepatocytes such as Hep-G2 cells (Hongo *et al.*, 2005) would allow the incorporation of a mammalian input towards metabolic conversions. By that approach the bacterial deconjugation of the enterohepatic circulated fraction of a contaminant, which is currently neglected, could be taken into account in future microbial metabolism studies.

Integrating *in vitro* gastrointestinal digestion technology and cell cultures of hepatocytes, enterocytes and colonocytes for measuring microbe-host interactions allows to carry out mechanistic investigations. These will allow for a better interpretation and extrapolation of results to the *in vivo* situation. In this way, a combination of *in vitro* technology with *in vivo* studies will provide a better knowledge of the underlying mechanisms behind the potentially adverse health effects of microbial transformation products of nutritional constituents and food contaminants.

Summary

Cancer is a major disease burden worldwide that accounts in countries with a Western lifestyle for 20% of the mortality rate. Epidemiological evidence suggests that diet makes a substantial contribution to the burden of human cancer. It is the consumption of meat, and in particular red meat, that has shown the strongest association with human neoplastic disease, particularly tumors of the colon and rectum. Cooking of meat is known to generate a family of promutagenic/procarcinogenic compounds, including the heterocyclic aromatic amine class of chemical compounds. Of the 19 heterocyclic amines identified so far, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is frequently the most mass abundant heterocyclic amine produced during the cooking of beef, pork and chicken. The human intake of PhIP varies with food type and cooking conditions and is estimated to range from nanograms to tens of micrograms per day, depending on individual dietary and cooking preferences. Assessment studies based on rodent tumor data and the abundance of PhIP in the diet have indicated that this heterocyclic amine may be a risk factor in human colon, breast and prostate carcinogenesis; which co-incidentally are the three most common sites of diet-associated cancer in the Western world.

As a means of determining the potential health risks associated with heterocyclic amines, several dietary studies have been conducted on the metabolism and disposition of these compounds in humans. So far, most investigations focused on the activation and detoxification of heterocyclic amines by mammalian phase I and II enzymes. In common with other aromatic amines, PhIP is metabolically activated by N-oxidation of the exocyclic amino group, a reaction mediated mainly by the cytochrome P450 isoenzyme CYP1A2. On the other hand, the involvement of the intestinal microbiota in the digestive fate of heterocyclic amines remains poorly investigated. Recent research has however shown that the amount of PhIP metabolites excreted in the urine of humans following ingestion of PhIP in a meat matrix is significantly lower than that of patients administered PhIP in a capsule. This indicates that PhIP provided in capsule form is more bioavailable than PhIP ingested from meat. The non-bioavailable fraction reaches the colon and becomes available for biotransformation by the colonic bacteria. At the start of this research only a few, partly conflicting results from studies with lactobacilli and intestinal microorganisms were available. Indications exist that the intestinal microbiota are essential to the induction of DNA damage by PhIP in HFA rats. Information on the bacterial metabolism of native heterocyclic amines is however scarce and limited to some studies on the quinoline type heterocyclic amines. Therefore, the main

objective of this work was to explore the possible role of the human intestinal microbiota in the metabolism and biological activity of PhIP. To do this, an integrated *in vitro*-*in vivo* approach was followed, combining fecal incubations, human studies and mammalian cell lines.

In the first part of this research, the microbial metabolism of PhIP was investigated. A preliminar explorative study in which PhIP was anaerobically incubated with stools freshly collected from six healthy volunteers demonstrated that PhIP was extensively transformed by the human intestinal bacteria. HPLC analysis revealed that the human fecal microbiota converted PhIP specifically into one major derivative. ESI-MS/MS, HRMS, 1D (^{1}H, ^{13}C, DEPT) and 2D (gCOSY, gTOCSY, gHMBC, gHSQC) NMR and IC analysis elucidated the complete chemical identity of the microbial PhIP metabolite, as 7-hydroxy-5-methyl-3-phenyl-6,7,8,9-tetrahydropyrido[3′,2′:4,5]imidazo[1,2-*a*]pyrimidin-5-ium chloride (PhIP-M1).

To evaluate whether this newly identified microbial PhIP metabolite could be produced by the intestinal bacteria *in vivo* as well, a human intervention trial was set up. Six human subjects were fed 150 g of cooked chicken containing 0.88-4.7 μg PhIP, and urine and feces collections were obtained during 72 h after the meal. PhIP-M1 and its trideuterated derivate were synthesized and a rapid and accurate solid-phase extraction LC-ESI-MS/MS method for the simultaneous quantification of PhIP and PhIP-M1 in human urine and feces was developed. Of the ingested PhIP dose, volunteers excreted 12-21% as PhIP and 1.2-15% as PhIP-M1 in urine, and 26-42% as PhIP and 0.9-11% as PhIP-M1 in feces. The rate of PhIP-M1 excretion varied among the subjects. Yet, an increase in urinary excretion was observed for successive time increments, whereas for PhIP the majority was excreted in the first 24 h. These findings confirmed that the human intestinal bacteria significantly contribute to the overall metabolism and disposition of PhIP *in vivo*.

After the observation that PhIP could be metabolically converted by the human intestinal bacteria *in vitro* and *in vivo*, the next step was to identify and characterize the bacterial species responsible for this process. Two PhIP transforming strains PhIP-M1-a and PhIP-M1-b were isolated from human feces and identified by a combination of microscopy, PCR-DGGE, FAFLP™ and *pheS* sequence analyses as *Enterococcus faecium*. Some strains from culture collections belonging to the species *Enterococcus durans*, *Enterococcus avium*, *Enterococcus faecium* and *Lactobacillus reuteri* were also able to perform this transformation. Glycerol was identified as a fecal matrix constituent required for PhIP

conversion. Abiotic synthesis of PhIP-M1 and quantification of the glycerol metabolite 3-hydroxypropopionaldehyde (3-HPA) confirmed that the anaerobic fermentation of glycerol via 3-HPA is the critical bacterial transformation process responsible for the formation of PhIP-M1. Although several lactobacilli, as well as other bacterial species have been shown to use glycerol as an external electron acceptor, we are the first to relate bacterial species of the genus *Enterococcus* to this anaerobic pathway of glycerol dissimilation. In addition, we have shown that PhIP-M1 production occurs under proteolytic conditions. This was true for mixed fecal microbiota as well as for the *Enterococcus faecium* PhIP-M1-a transforming strain.

The production of PhIP-M1 was shown to be dependent on interindividual differences. A first explorative experiment with six human fecal samples demonstrated this factor. Subsequent fecal incubations with eighteen human microbiota confirmed that individuals could be separated into low, moderate and high PhIP-M1 producers with transformation efficiencies ranging from 1.8 to 96%. Finally, significant differences in intestinal PhIP-M1 production were found to determine differences in urinary and fecal PhIP-M1 excretion *in vivo* in humans. This indicated that interindividual differences in microbial composition and metabolism may at least be equally important than differential expression and genetic polymorphisms in phase I and II endogenous enzymes, which have been considered so far as the obvious candidates responsible for individual variability in PhIP metabolism, bioavailability and carcinogenicity.

In the second part of this doctoral research, the impact of the intestinal microbiota on the biological activity of PhIP was evaluated. Since ligation of the biliary duct has been shown not to alter the genotoxic potential of PhIP, the deconjugation of reactive glucuronides by bacterial β-glucuronidase is most likely not to alter the metabolic fate and bioactivity of PhIP. Therefore, it was very much conceivable that the microbial formation of PhIP-M1 contributed to the final genotoxic and carcinogenic activity of PhIP.

Firstly, it was observed that PhIP-M1, as analyzed using the *Salmonella typhimurium* strains TA98, TA100 and TA102, yielded no significant mutagenic response. Subsequently, it was assessed whether PhIP-M1 could exert any cytotoxic or genotoxic effects towards a human intestinal cell line. PhIP-M1 was shown to induce DNA damage, cell cycle arrest, apoptosis and eventually cell death and growth inhibition towards the epithelial Caco-2 cell line. DNA damage in Caco-2 cells was detected using the Comet assay. This assay is recognized as a sensitive tool widely used for the evaluation of primary DNA damages at the

individual cell level, while the bacterial Ames assay only detects mutagenic effects if the DNA damage induced remained after cell division. The conversion of PhIP into PhIP-M1 was therefore considered as a microbial bioactivation. As the genomic and cellular events of CYP1A2-activated PhIP in different *in vitro* cell systems are not significantly higher than those observed for PhIP-M1 in our test system, the physiological relevance of this newly identified microbial PhIP derivate in PhIP carcinogenicity may not be neglected.

Finally, it was investigated whether addition of native chicory inulin could inhibit the extent of microbial PhIP bioactivation. Inulin is generally considered to exert prebiotic effects as it stimulates health-promoting bacteria in the human gut such as bifidobacteria. However, it is also hypothesized that it may exert chemopreventive effects by the indirect suppression of microbial groups such as enterococci that are responsible for the hazardous conversion of carcinogenic compounds such as PhIP. In addition, inulin is known to bring about prebiotic effects at the level of the metabolic activity, resulting in a saccharolytic fermentation pattern and acidic environment. Supplementation of inulin during several weeks to a full-scale SHIME reactor showed significant inhibitory effects towards PhIP bioactivation, in particular in the transverse colon compartment. Interestingly, the strongest decrease in proteolytic end products was also observed in this region of the colon, indicating an indirect relationship with the chemopreventive effects from inulin. As the typical proteolytic conditions in the distal parts of the colon are normally more detrimental to the host *in vivo*, in particular in the light of the microbial PhIP bioactivation process, these positive modifications in the metabolism and microbial community indicate that inulin is a promising chemopreventive agent.

Samenvatting

Kanker is een belangrijk en mondiaal gezondheidsprobleem dat in landen met een Westerse levenswijze verantwoordelijk is voor 20% van het sterftecijfer. Epidemiologische studies hebben uitgewezen dat de voeding een substantiële bijdrage levert tot het risico op het ontstaan van kanker bij de mens. Het is de consumptie van vlees, en in het bijzonder rood vlees die de sterkste correlatie vertoont met het voorkomen van neoplastische ziektebeelden en voornamelijk colon- en rectumtumoren bij de mens. Heterocyclische aromatische amines, een familie promutagene/procarcinogene componenten, die gevormd worden tijdens het bakken, braden of grillen van vlees en vis, blijken geassocieerd te zijn met de etiologie van kanker bij de mens. Van de 19 reeds geïdentificeerde heterocyclische amines, is 2-amino-1-methyl-6-fenylimidazo[4,5-*b*]pyridine (PhIP) het meest voorkomende heterocyclische amine geproduceerd tijdens de bereiding van kip, varkensvlees en rund. Schattingen van de dagelijkse inname van PhIP variëren van enkele nanogrammen tot tientallen microgrammen per persoon per dag en zijn afhankelijk van de individuele eetgewoonten en bereidingswijze van het vlees. Bij knaagdieren is PhIP verantwoordelijk voor de inductie van tumoren ter hoogte van de colon, de melkklieren en de prostaat en dit terwijl de overige heterocyclische amines eerder ter hoogte van de lever actief zijn. Deze site-specificiteit is intrigerend aangezien voornoemde organen eveneens de primaire sites zijn van dieetgeassocieerde kankers in de Westerse wereld.

Teneinde de mogelijke gezondheidsrisico's gerelateerd met de consumptie van heterocyclische amines te kunnen inschatten, werden reeds verschillende dieetstudies uitgevoerd met betrekking tot het metabolisme en de distributie van deze componenten in het menselijk lichaam. Tot op heden richtte het wetenschappelijk onderzoek zich voornamelijk op de bioactivatie en detoxificatie door menselijke fase I en II enzymsystemen. Heterocyclische amines en in het bijzonder PhIP worden geactiveerd tot mutagene/carcinogene derivaten door N-oxidatie van de exocyclische aminegroep. Deze reactie wordt gekatalyseerd door het cytochroom P450 isoenzyme CYP1A2. Slechts een aantal, deels tegenstrijdige gegevens zijn beschikbaar over de rol van de intestinale microbiota in de biobeschikbaarheid en activiteit van deze componenten. Recent *in vivo* onderzoek toonde aan dat de fractie aan urinaire PhIP metabolieten significant lager is bij mensen die PhIP innemen in een vleesmatrix dan wanneer deze in capsulevorm wordt toegediend. Dit betekent dat PhIP in capsulevorm meer biobeschikbaar is dan PhIP in een vleesmatrix. Deze niet-biobeschikbare fractie bereikt de colon onveranderd en treedt er in contact met de intestinale microbiota. Indicaties zijn

voorhanden dat de intestinale microbiota een cruciale rol spelen in de inductie van DNA beschadiging door PhIP in ratten, geassocieerd met humane darmbacteriën. Onderzoek naar het microbieel metabolisme van heterocyclische amines is echter schaars en beperkt zich tot enkele studies over de heterocyclische chinoline verbindingen. De belangrijkste doelstelling van dit werk bestond er dan ook in om de kennis omtrent het metabolisch potentieel van de intestinale microbiota in de bioactivatie/detoxificatie van het heterocyclische aromatische amine PhIP te verruimen. Daartoe werd *in vitro* en *in vivo* onderzoek geïntegreerd door combinatie van batch incubaties, humane studies en cellijntesten.

Tijdens het eerste deel van dit doctoraat werd het microbieel metabolisme van PhIP nader onderzocht. Een exploratieve studie waarbij PhIP anaëroob geïncubeerd werd met het fecaal materiaal van zes gezonde vrijwilligers leidde tot de vaststelling dat PhIP extensief gemetaboliseerd wordt door de intestinale microbiota. HPLC analyse toonde dat de zes humane fecale microbiota PhIP specifiek transformeerden tot één metaboliet. Met behulp van ESI-MS/MS, HRMS, 1D (^{1}H, ^{13}C, DEPT) en 2D (gCOSY, gTOCSY, gHMBC, gHSQC) NMR en IC analyse werd de volledige chemische structuur van het microbiële PhIP derivaat geïdentificeerd als 7-hydroxy-5-methyl-3-fenyl-6,7,8,9-tetrahydropyrido[3',2':4,5]imidazo-[1,2-*a*]pyrimidin-5-ium chloride (PhIP-M1).

Om de mogelijke *in vivo* productie van dit nieuw geïdentificeerde microbiële PhIP derivaat na te gaan, werd een humane interventiestudie opgezet. Daartoe werd aan zes vrijwilligers 150 g goed doorbakken kip toegediend en de urine en fecale stalen gedurende 72 u na de maaltijd opgevangen. PhIP-M1 en zijn gedeutereerde derivaat werden gesynthetiseerd en een snelle en nauwkeurige vaste fase extractie LC-ESI-MS/MS methode werd ontwikkeld voor de simultane kwantificatie van PhIP en PhIP-M1 in urine en feces. De vrijwilligers excreteerden 12-21% als PhIP en 1.2-15% al PhIP-M1 in urine en 26-42% als PhIP en 0.9-11% als PhIP-M1 in feces. De snelheid waaraan PhIP-M1 werd uitgescheiden, varieerde sterk tussen de proefpersonen onderling. Toch werd voor PhIP-M1 een toename in urinaire excretie waargenomen in functie van de tijd, terwijl voor PhIP het merendeel gedurende de eerste 24 h werd uitgescheiden. Deze bevindingen ondersteunden de hypothese dat ook *in vivo* de humane intestinale microbiota een significante bijdrage leveren tot het metabolisme en de distributie van PhIP.

Na de observatie dat PhIP zowel *in vitro* als *in vivo* gebiotransformeerd wordt door de humane colonmicrobiota, bestond een volgende stap erin om de bacteriële species

verantwoordelijk voor dit proces te identificeren en karakteriseren. Twee PhIP-transformerende stammen PhIP-M1-a en PhIP-M1-b werden geïsoleerd uit humane feces en geïdentificeerd door een combinatie van microscopie, PCR-DGGE, FAFLP^{TM} en *pheS* sequentie analyse als *Enterococcus faecium*. Enkele stammen afkomstig van cultuurcollecties behorende tot de species *Enterococcus durans*, *Enterococcus avium*, *Enterococcus faecium* en *Lactobacillus reuteri* waren eveneens in staat om deze omzetting uit te voeren. Glycerol werd geïdentificeerd als de noodzakelijke fecale matrix constituent verreist voor PhIP conversie. Abiotische synthese van PhIP-M1 en kwantificatie van de glycerol metaboliet 3-hydroxypropionaldehyde (3-HPA) bevestigden dat de anaërobe fermentatie van glycerol via 3-HPA het cruciale bacteriële transformatieproces is noodzakelijk voor de vorming van PhIP-M1. Ondanks het feit dat verschillende lactobacilli, evenals een aantal andere bacteriële species in staat zijn glycerol als externe elektron acceptor te gebruiken, is dit de eerste maal dat bacteriële species van het genus *Enterococcus* gerelateerd worden met de anaërobe glycerol dissimilatie. Daarnaast werd tevens aangetoond dat microbiële PhIP-M1 vorming enkel plaatsvindt in aanwezigheid van eiwitrijke voeding.

Tenslotte werd ook vastgesteld dat de productie van PhIP-M1 door de darmbacteriën gekarakteriseerd wordt door interindividuele verschillen. Een eerste exploratieve studie met zes humane fecale stalen toonde dit aan. Daarop volgende fecale incubaties met 18 humane inocula bevestigden dat individuen kunnen opgedeeld worden in zwakke, matige en sterke PhIP-M1 producenten met transformatie-efficiënties reikend van 1.8 tot 96%. Vervolgens werd aangetoond dat significante verschillen in intestinale PhIP-M1 productie tevens aanleiding geeft tot verschillen in urinaire en fecale PhIP-M1 excretie. Dit wijst erop dat interindividuele verschillen in de samenstelling en metabolische activiteit van de intestinale microbiota op zijn minst even belangrijk zijn dan differentiële expressie en genetische polymorfismen in fase I en II endogene enzymen, die tot op heden werden beschouwd als de voornaamste oorzaak voor individuele variabiliteit in PhIP metabolisme, biobeschikbaarheid en carcinogeniciteit.

In het tweede deel van dit doctoraal onderzoek, werd de impact van de intestinale microbiota op de biologische activiteit van PhIP geëvalueerd. Aangezien reeds aangetoond werd dat afklemmen van de galleider geen invloed uitoefent op de genotoxische activiteit van PhIP, is het waarschijnlijk dat de deconjugatie van reactieve glucuronide PhIP derivaten door bacterieel β-glucuronidase geen rol speelt in het metabolisme en de biologische activiteit van

PhIP. Daarom leek het aanneembaar dat de microbiële vorming van PhIP-M1 bijdraagt tot de finale genotoxische en carcinogene activiteit van PhIP.

Met behulp van de Ames test werd waargenomen dat PhIP-M1 geen mutagene respons veroorzaakt bij de *Salmonella typhimurium* TA98, TA100 en TA102 stammen. Vervolgens, werd nagegaan of PhIP-M1 mogelijks cyto- of genotoxische effecten kon uitoefenen ter hoogte van het intestinale epithelium. PhIP-M1 bleek DNA beschadiging, celcyclus arrest, apoptose en uiteindelijk celdood en groei-inhibitie teweeg te brengen ten opzichte van de intestinale Caco-2 cellijn. DNA beschadiging in Caco-2 cellen werd gedetecteerd met behulp van de komeettest. Deze test wordt beschouwd als een gevoelige techniek voor de evaluatie van primaire DNA beschadiging op het individuele celniveau, terwijl de Ames test enkel mutagene effecten detecteert wanneer de DNA schade behouden blijft na celdeling. De conversie van PhIP tot PhIP-M1 wordt daarom beschouwd als een microbiële bioactivatie. Aangezien de genetische en moleculaire effecten van CYP1A2 geactiveerde PhIP in verschillende *in vitro* celsystemen niet significant hoger zijn dan deze geobserveerd voor PhIP-M1 in ons testsysteem, dient de fysiologische relevantie van dit nieuw geïdentificeerde PhIP derivaat in de carcinogeniciteit van PhIP benadrukt te worden.

Tenslotte werd onderzocht of toediening van inuline de microbiële PhIP bioactivatie kon inhiberen. Inuline wordt algemeen beschouwd als een prebioticum en dit door het stimuleren van gezondheidsbevorderende bacteriën in de menselijke darm zoals bifidobacteria. Er wordt echter ook gesteld dat inuline een chemopreventieve werking heeft door de indirecte onderdrukking van microbiële groepen zoals de enterococci die verantwoordelijk zijn voor de schadelijke omzetting van carcinogene componenten zoals PhIP. Bovendien is het geweten dat inuline prebiotische effecten uitoefent op het niveau van de metabolische activiteit, resulterend in een saccharolytisch fermentatiepatroon en een zuur milieu. Toevoegen van inuline gedurende een aantal weken aan de SHIME reactor gaf aanleiding tot een significant inhiberend effect naar de PhIP bioactivatie, in het bijzonder in de colon transversum. Een interessante vaststelling hierbij was dat de sterkste afname in proteolytische eindproducten tevens in deze regio van de colon werden waargenomen, wat wijst op een indirect verband met de chemopreventieve effecten van inuline. Aangezien de typische proteolytische condities in de distale coloncompartimenten als meer schadelijk voor de gastheer worden aanzien, in het bijzonder in het kader van de microbiële PhIP bioactivatie, wijzen deze positieve modificaties in het metabolisme en de microbiële gemeenschap erop dat inuline een veelbelovend chemopreventief agens is.

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Curriculum vitae

Personalia

Name: Vanhaecke Lynn J.M.
Address: Gaversesteenweg 349
B-9820 Merelbeke
BELGIUM
Tel.: + 32 495 590750
E-mail: Lynn.Vanhaecke@ugent.be
Date of birth: 24 september 1981
Place of birth: Brugge
Civil standing: married
Nationality: Belgian

Education

1993-1999: Science mathematics, Sint-Andreas lyceum Sint-Kruis (Belgium).

1999-2004: Engineer in Environmental Technology, Faculty of Bioscience Engineering, Ghent University. Graduated with great distinction.

2003-2004 : Master thesis at the department of Biochemical and Microbial Technology, Laboratory of Microbial Ecology and Technology (LabMET) titled: 'Risks from biotransformation of persistent organic pollutants by intestinal microbiota'.

Professional Activities

2004-2008: Doctoral fellowship for PhD research granted by the Institute for the Promotion of Innovation by Science and Technology in Flanders (IWT) at the department of Biochemical and Microbial Technology, Laboratory of Microbial Ecology and Technology (LabMET) titled: 'Impact of the human intestinal microbiota on the carcinogenicity of the food contaminant 2-amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine (PhIP)'.

2004-2008: Tutor of 4 Master students in Bioscience Engineering.

2004-2006: Supervisor of practical exercises of the course 'Microbial Ecological Processes' at the Faculty of Bioscience Engineering.

15-16 nov 2004: Organizer and collaborator of the 'Soil Bioremediation course' at the Laboratory of Microbial Ecology and Technology.

2005-2008: Coordinator and collaborator of research projects in analytical chemistry, functional foods and environmental risk assessment commissioned by Janssen Pharmaceutica, Energetica Natura, Cosucra, European Space Agency and Institut Meurice.

2005-2007: Responsible for the HPLC and preparative HPLC-MS systems.

15-17 sept 2005: Laboratory stay: Northern Ireland Centre for Diet and Health (NICHE), University of Ulster, Coleraine, UK.

june-oct 2007: Research at the Laboratory of Genetic Toxicology (Prof. dr. Daniel Marzin), Pasteur Institute of Lille, Lille Cedex, France.

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Conferences, workshops, seminars

Active participation

Global Issues in Genetic Toxicology and Environmental Mutagenesis, 9th International Conference on Environmental Mutagens & 36th Annual Meeting of the Environmental Mutagen Society, San Francisco, California, USA, September 2005. *Poster presentation.*

Understanding the Role of probiotics in Health, International Yakult Symposium, Ghent, Belgium, October 2005. *Poster presentation*.

1th Intern Networking Event Food2Know, Ghent, Belgium, December 2005. *Poster presentation.*

Environmental Contaminants Workshop, Platform for Scientific Concertation: Food safety, Liège, Belgium, April 2006. *Poster presentation.*

Gut Microbiology, Research to Improve Health, Immune Response and Nutrition, Aberdeen, Scotland, June 2006. *Lecture*: 'Metabolism of the food associated carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) by human intestinal microbiota' and *poster presentation*.

8th Annual Gut Day, Groningen, The Netherlands, November 2006. *Poster presentation.*

New Methods in (Geno)Toxicology and Ecotoxicology, Joint Meeting of The Belgian Society for Toxicology and Ecotoxicology & The Belgian Environmental Mutagen Society, Leuven, Belgium, December 2006. *Poster presentation.*

Mutational and Epigenetic Mechanisms of Susceptibility and Risks for Genetic Diseases, Environmental Mutagen Society 38th Annual Meeting, Atlanta, Georgia, USA, September 2007. *Lecture*: 'Intestinal bacteria play a crucial role in the carcinogenic risk from 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine' and *poster presentation*.

Passive participation

17th Forum for Applied Biotechnology (FAB), Ghent, Belgium, September 2003.

FEVIA Workshop PRO-, PRE- AND SYNBIOTICS, Affligem, Belgium, March 2005.

Bacteria and processes in the GUT: *in vitro* and *in vivo*, mini-symposium Max Planck Institute, Germany, October 2005.

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