Influence of dietary protein sources on putative *in vitro* and *in vivo* colon cancer biomarkers

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Voor mijn vader en mijn moeder
die ieder op geheel eigen wijze
aan de basis van dit proefschrift
hebben gestaan

Contents

Chapter 1

General introduction

1.1 Framework of the research presented

Each year millions of people die from cancer worldwide. Epidemiological studies reveal that the diet probably accounts for one third of these cases. Although it is unclear which dietary components either induce or protect against cancer, macronutrients like fat, fibre and protein may play an important role. The research presented in this thesis concerns the question whether different dietary protein sources have a different influence on colon (large intestinal) cancer risk. Through this research more insight is provided in the relationship between food and cancer, thereby providing links to reduce colon cancer incidence on the long term. To contribute to an answer to this question both *in vitro* studies and *in vivo* animal studies were performed as a part of a four year PhD study. The research was performed at the Toxicology Group in close cooperation with the Product Design and Quality Management (PDQ) Group, both of Wageningen University.

1.2 Scope and approach

In literature, relatively little is known about the relationship between dietary protein and colon cancer, as compared to other major dietary constituents such as fat and fiber. Interpretation of especially the epidemiological data available is difficult because of many confounding and influential factors such as fat and energy content of the diet. From previous laboratory studies by our groups and the available literature, there were strong indications that different dietary protein sources have a different effect on colon cancer risk (1-4). Therefore, the main objective of the studies described was to investigate the influence of dietary protein sources on colon cancer risk and related parameters. The experiments performed focussed on comparing casein and other protein sources *in vitro* and *in vivo*. Research questions were:

1. Is there a difference between casein and other dietary protein sources in their *in vitro* carcinogen scavenging capacity? (Chapter 3 and 4).
2. Is there a different effect between casein, other dietary protein sources or their constituent amino acids, on early *in vivo* colon cancer biomarkers, such as cell proliferation, aberrant crypt foci and intestinal polyps? (Chapter 5, 6, 7 and 8).
3. Is there a different effect between casein or different dietary protein sources on potential fecal colon cancer biomarkers such as the amount of fecal fat, magnesium, fatty acids, bile acids or fecal enzyme activities such as β-glucuronidase, β-glucosidase, alkaline phosphatase or the pH and cytolytic activity of the fecal water fraction? (Chapter 5, 6, 7 and 8).

Chapter 2 provides an overview of the available literature on the relationship between dietary protein intake and colon cancer risk. After this literature overview, the background of the methods used in the experiments described, is summarized. In Chapter 3 - 8 the results of the research performed, are reported. In Chapter 3 the research on the influence of the protein type and digestion on protein interaction with the highly reactive nitrosamide (MNNG) is reported. Chapter 4 describes the interaction between dietary proteins and a non-reactive dietary carcinogen benzo[a]pyrene. In Chapter 5-8, various animal experiments are described. In all animal studies fecal samples were collected to determine several potential colon cancer biomarkers. In Chapter 5 the influence of methionine and soy non-nutrients on fecal biomarkers for colon cancer was investigated. An *in vitro* study on the interactions between bile acids and soy non-nutrients is also described. In Chapter 6 the effect of casein and soy protein on colonic cell proliferation was described. In Chapter 7, research on the influence of casein, soy protein isolate and wheat gluten proteins on the occurrence of colonic aberrant crypt foci (ACF) in rats is reported. Finally in Chapter 8, the influence of casein, soy flour, soy protein isolate and meat on the formation of intestinal polyps in the Apc^{Min} mouse model was studied. In Chapter 9, the overall influence of different dietary protein sources on all potential colon cancer biomarkers tested, is discussed.

Chapter 2

Background information

2.1 Diet and colon cancer

Cancer is a disease in which a normal body cell transforms in a malignant tumor, often causing severe illness or death. In 1996 more than 10 million new cases of cancer occurred and more than 7 million people died from cancer worldwide. Colorectal cancer is the fourth most common cancer in the world. In 1996 an estimated 875,000 new cases were diagnosed world wide, accounting for 8.5% of all new cancers (5). In The Netherlands, 2516 males and 2793 females were registered as new colon cancer patients in 1994. In that same year, 1518 males and 1692 females died of colon cancer (6). The exact mechanism behind the development of colon cancer has not been clarified yet. In 1988 Vogelstein has provided a molecular basis for the development of colon cancer by describing the complex multi-stage process in which cells accumulate alterations of genes that control cell growth and differentiation (7). In Figure 2.1 a genetic model for colon carcinogenesis is shown, based on the Vogelstein model (8). Although this model has undergone some modifications and elaborations, the model as proposed by Vogelstein still holds (9).

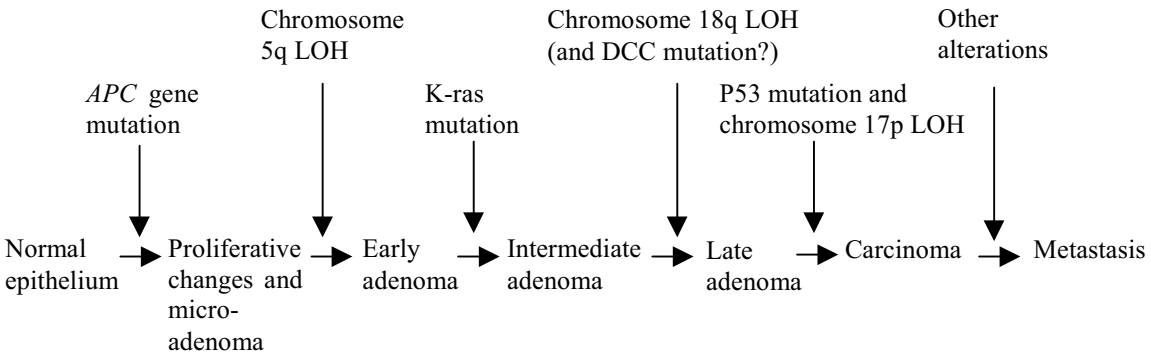

Figure 2.1 A genetic model for colon carcinogenesis, according to Fearon and Jones (8). *APC* = adenomatous polyposis coli, DCC = deleted in colon cancer, LOH = loss of heterozygosity.

The influence of the environment, including the diet, on the risk of colon cancer was established in the early sixties (10) using migrant studies. For colon cancer the ultimate conclusion from these migrant studies was that colon cancer is largely due to environmental and especially dietary practices. This percentage was approximately 90% for colon cancer. Although Dole and Peto (11) were the first to admit that these figures were estimates, these data still stand. In 1997, the World Cancer Research Fund (WCRF) together with the American Institute for Cancer Research (AICR), still estimated that one-third of all cancer deaths are caused by the diet (7). Besides the many food components that may cause colon cancer it is hypothesized that many other components show a protective effect against colon cancer risk. Mechanisms for both risk inducing and protective effects of the diet are still under

debate. Therefore, there is still no answer to the question: “what components from food cause colon cancer and what exactly do I have to eat to reduce colon cancer risk?”.
Compared to fat, fiber and minerals, such as calcium, relatively little information is available on the relationship between dietary protein and colon cancer risk. In the next sections the available information on several fields of study on protein and colon cancer will be discussed. Both protective and risk inducing data on protein and colon cancer will be mentioned.

2.2 Dietary protein and colon cancer

2.2.1 Fields of study in research on dietary proteins and colon cancer

The research on dietary protein and colon cancer can be subdivided in research areas. Each area offers possible explanations for both protective and risk inducing effects of dietary protein sources on colon cancer. Six different areas on dietary protein and colon cancer will be discussed:

1. proteins as carcinogen scavengers
2. amount of protein intake and colon cancer
3. differences in amino acid composition
4. differences in protein digestibility
5. presence of non-protein components associated with proteins
6. presence of bioactive peptides

2.2.2 Proteins as carcinogen scavengers

Dietary carcinogens probably play a role in colon cancer development (12, 13). Often mentioned in relation to colon cancer are heterocyclic amines such as 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) (14). Dietary proteins can scavenge these carcinogens thereby making them unavailable for uptake into the body. Depending on the type of binding this may result in either a protective or a colon cancer risk inducing effect. Protein-carcinogen interactions have been studied for several decades (15-19), because it was thought that carcinogens caused cancer through the interaction with cellular proteins (20). Despite this interest in protein-carcinogen interactions, very little is known about the bioavailability of carcinogens and their direct interactions with various food components such as proteins. This is also true for compounds that need bioactivation by for example the liver to become mutagenic and/or carcinogenic.

Dietary carcinogens enter the body as a part of the food matrix. Before a carcinogen can be harmful, it has to pass the gastro-intestinal epithelium. However, the food matrix may have a major influence on the bioavailability of the carcinogen. The protein component from the food matrix can influence the fate of the dietary carcinogens in several ways. First, the carcinogen can be covalently bound to the protein thereby making the carcinogen unavailable and harmless. Covalent binding takes place by reactive amino acids. A second option is a non-covalent binding between the protein and the carcinogen. This way the protein can be used as a transporter for the carcinogen, taking the carcinogen along the gastro-intestinal tract. When the protein is rapidly digested, the non-covalent binding may end and the carcinogen will be released in the stomach or small intestine after all. When the protein-carcinogen binding is intact throughout the small intestine the protein-carcinogen complex will enter the colon. Here, the protein is most likely fermented and the carcinogen will become available (21, 22).
Much work on this subject has been performed in our own group. Already in 1987, Jongen and colleagues showed that proteins from cheese had a strong interaction with carcinogens formed by incubating vicia faba beans with nitrate (23). Proteins were shown to exhibit the strongest interaction with the carcinogens in comparison to the other dietary components. In further studies more proteins and more carcinogens were tested on protein-carcinogen interactions, using the Ames-test (1), the sister chromatide exchange test (24), the *E. coli* DNA repair test and the host-mediated assay (3, 25). From these studies it was concluded that protein-carcinogen interactions can be very strong and can have major influences on the mutagenicity or carcinogenicity of a compound. Also, protein-carcinogen interactions differ between dietary protein sources.

In this thesis two studies are dedicated to protein-carcinogen interactions (Chapter 3 and 4). To study the effect of protein digestion, experiments were performed using a gastro-intestinal simulator.

2.2.3 Amount of protein intake and colon cancer

Early research on protein and cancer was focussed on the influence of the *amount* of protein in the diet rather than protein *source*. Several reviews discuss the issue of protein concentration and colon cancer risk, both from an epidemiological as from an animal experimental perspective. Studies cited in the report of the National Research Council (26) either show no effect of dietary protein concentration or a positive correlation between protein intake and colon cancer risk (mostly epidemiological studies). Because of the many confounding factors it is difficult to distinguish between the effects of dietary protein, dietary fat and energy intake. Because of the assumed influence of dietary fat and energy intake (27), the possible effect of dietary protein could not be estimated. In his review, Visek (28) does

not give a summary of *all* relevant studies performed, but the studies described also suggest a positive correlation between dietary protein intake and colon cancer risk. It is noted that a low protein intake generally inhibits spontaneous and chemically induced tumor growth. This might be due to the limiting presence of amino acids essential for the growth of the animal and the tumor. Also more recent reviews indicate a positive correlation between dietary protein intake and colon cancer risk (29, 30). Some animal studies describe a protective effect against colon cancer by low (10%) or very low (5%) protein diets (31). In 1997 WCRF (7) repeated the conclusion of the NRC that there was no judgement possible on the question whether increasing protein gave an increased or decreased risk of colon cancer.

A likely mechanism for the relationship between dietary protein and colon cancer risk is shown in animal studies. High protein intake is associated with increased colonic cell proliferation. It is hypothesized that this is due to a higher ammonia concentration in the large intestine (32). However, some animal studies have shown that a high protein intake lowers colonic cell proliferation (33), indicating that both the effect of high protein intake as well as possible mechanisms are still under debate.

In this thesis no research is described on the influence of protein concentration on colon cancer risk. All studies focussed on protein source or amino acid composition.

2.2.4 Differences in amino acid composition

The amino acid composition of each protein is different. For example, acid casein contains only one third of the amount of cystine that soy protein isolate contains, but has more than twice the amount of methionine (34). Because amino acids have an essential function in the biochemistry of cells, a variation in their dietary presence may exert various effects. Examples of amino acids that may be influential are the sulfur containing amino acids methionine and cysteine. It is thought that these amino acids can function as a sulfur donor for important metabolic routes such as glutathion metabolism (35). Methionine is an important methyl donor and might therefore influence DNA (36, 37) and nuclear protein methylation (38). DNA methylation in turn influences DNA transcription and therefore possibly colon cancer (39).

The limited presence of an essential amino acid can restrict growth, especially in young animals or children. Because the amino acid composition determines the nutritional value of a dietary protein, several parameters were defined to express protein quality. An often used parameter is the protein digestibility corrected amino acid score (PDCAAS) (40). The PDCAAS does not only take into account the essential amino acid content of a protein, but also considers protein digestibility. In Table 2.1 the PDCAAS-values for several dietary

protein sources used, are shown. When animals are fed proteins with a low PDCAAS, this means they can not fully grow compared to animals fed a reference protein such as casein (PDCAAS=1). When animals stay behind in growth, their energy intake will also be different. Because energy intake is an important risk factor for colon cancer (27) a different PDCAAS can explain differences found in colon cancer risk between different proteins.

In this thesis the research on several dietary proteins is described, thereby automatically comparing the effect of the different amino acid compositions. Research on the influence of a single amino acid (methionine) is described in Chapter 5.

Table 2.1 Protein digestibility corrected amino acid score (PDCAAS) values for several dietary protein sources used (41).

	PDCAAS
Casein	1.00
Egg white	1.00
Beef	0.92
Soybean protein concentrate	0.99
Soybean protein isolate	0.92
Wheat gluten	0.25

2.2.5 Differences in protein digestibility

Not all dietary proteins are digested (hydrolyzed) to the same extent. When the digestibility between two proteins differs, different amounts of protein or peptides enter the colon. This way differences in protein digestibility may have the same effect as a difference in dietary protein intake. The FAO/WHO reported high digestibility for several dietary protein sources (egg 97%, casein 95%, soy protein isolate 95%, soy flour 86% and wheat gluten 99%). However, digestibility reported for protein depends on the methods used (41). Recent studies using stable isotope techniques report markedly lower digestibilities than the digestibilities reported by the FAO/WHO. The true human ileal digestibility for raw egg white is 51 ± 10% and for cooked egg white is 91 ± 1% (42). For pea protein the digestibility is 89 ± 1% (43). This means considerable amounts of dietary proteins escape small intestinal digestion and absorption and enter the colon. In the colon protein can be fermented producing for example ammonia (44, 45). The substantial amount of undigested protein entering the colon also supports the hypothesis mentioned earlier, that dietary protein can be used as a transport mechanism for dietary carcinogens to enter the colon.

2.2.6 Presence of non-protein components associated with proteins

Most dietary protein sources contain more than just protein. Besides water, some common minerals, low amounts of fat and carbohydrate or non-protein components may be present. Many non-protein components belong to the group of so-called non-nutrients. Several non-protein components will be more thoroughly discussed in the following sections: calcium in milk protein (section 2.3.2), heme in meat protein preparations (section 2.3.6) and isoflavones and saponins in soy protein preparations (section 2.3.3). These non-protein components can have a major influence on digestibility, nutrient and non-nutrient bioavailability and carcinogenesis in general. A problem for the estimation of the effect of some non-nutrient components (isoflavones, saponins) is their variable concentration in protein sources.

In this thesis the effect of an ethanolic extract of soy protein isolate was tested on several fecal parameters Chapter 5. The ethanolic extract contained soy isoflavones and saponins.

2.2.7 Presence of bioactive peptides

Dietary protein digestion can result in the release of bioactive peptides. These peptides are usually small sequences bound in the protein that have a specific biological activity once released. Milk proteins are the most thoroughly investigated with regard to these bioactive peptides (46, 47). Most work on bioactive peptides was performed *in vitro*. A major question concerning bioactive peptides is whether they can pass the small intestinal barrier. There are indications that this is indeed possible. Information on absorption of intact proteins and peptides was reviewed in 1994 (48).

In this thesis no experiments were dedicated to bioactive peptide research because the results described in literature so far, seem to be of little relevance for colon carcinogenesis.

2.3 Protein sources used

2.3.1 General

In the research described in this thesis, several of the above mentioned issues were investigated to obtain a broad view on the influence of dietary protein on colon cancer risk. Because the several dietary protein sources play such an important role in this research, the following sections will define the various protein sources and their non-protein components. Also a summary of the literature will be given on the effects of the protein source or the non-protein component, on colon cancer risk.

2.3.2 Casein

Milk proteins are divided into two major groups of proteins: casein (78.5 g / 100 g milk protein) and whey proteins (21.5 g / 100 g whey protein). Casein is defined as the protein precipitating from milk near pH 4.6. It is present in milk in large aggregates, called micelles, which also contain calciumphosphate. On acidification the calcium phosphate dissolves. Proteins that do not precipitate at pH 4.6 are called whey proteins, which will be discussed in section 2.3.5. Depending on the way of preparation, whole casein, acid casein or for example Na-caseinate can be obtained. For the preparation of acid casein, used in several animal studies described, milk is acidified thereby precipitating the casein micelles. Calcium and calciumphosphate present in the milk will be contained in the whey fraction (49). The effect of calcium on several colon cancer parameters has been demonstrated (50, 51). Therefore casein preparations containing relatively low amounts of calcium were used in the studies described in this thesis.

Many animal studies have been performed, using casein as the sole or major protein source. However, in few of these studies casein was compared to other dietary protein sources. In a study of Govers (52), acid casein was compared to soy protein isolate for its effect on colonic cell proliferation and several fecal parameters. The main conclusion of this study was that soy protein compared to casein, damaged the colonic epithelium and therefore induced colonic cell proliferation. In a later study by McIntosh (2), it was shown that casein also resulted in less 1,2- dimethylhydrazine (DMH)-induced colonic tumors in rats, compared to soy protein. However, McIntosh did not use a soy protein isolate, but used a defatted soybean meal to test the effects of soy proteins. In later studies McIntosh (53) showed that there was a significant lower incidence of large intestinal tumors in rats fed a casein based diet relative to those fed a chickpea protein based diet.

To prevent discussing articles twice, all other studies comparing casein with another dietary protein source are discussed in the corresponding sections.

In the present PhD study, casein was the protein of main interest for several reasons. Casein is often used as a reference protein and can therefore be used to compare different studies. Furthermore, casein is a common source of protein in the human diet. In all studies described in this thesis casein was included.

2.3.3 Soy protein

A soybean roughly contains 30% protein, 30% fat, 30% fibre and 10% of other components. Soy protein is available in many different preparations. Soybean flour is obtained after grinding soybeans. Defatted soybean flour is sometimes called soybean meal. Defatted soybean flour used in several studies described in this thesis is referred to as soy flour (Soy-f). In animal feeding a soy protein concentrate is often used. This is obtained by washing the soybean meal with a 70% ethanol solution, washing out both soy fibre and soy non-nutrients such as saponins and isoflavones. Soy protein concentrate contains approximately 50% protein. When a higher protein percentage (90%) is required, soy protein isolate is used. Soy protein isolate is prepared from soybean meal by iso-electric precipitation. In contrast to soy protein concentrate, soy protein isolate still contains relatively high concentrations of non-nutrients (54).

Research on the effects of soy protein sources on colon cancer is complicated by the fact that soy protein preparations often contain variable amounts of non-nutrients. For each of these non-nutrients, data are available on the possible effects on colon cancer risk (55, 56). For example, soybeans contain lectins. These are glycoproteins that are able to bind to the luminal surface of epithelial cells in the intestine. It is reported that peanut lectin stimulates colonic cell proliferation (57), but there is no information available on the effect of soy derived lectins. Phytate possibly influences colon carcinogenesis by reducing colonic cell proliferation (58, 59). In recent years a lot of attention was focussed on soybean isoflavone research. For colon cancer there is little support for a protective effect of soybean isoflavones (60). Less attention has been paid to saponin research, partly because this group of compounds is very hard to detect. Saponins in general are known for their lytic potential, thereby being able to damage the colonic epithelium. There is little specific information on soy saponins, but soy saponins do not appear to be toxic. They might even have anticarcinogenic activity (61).

Several reviews have been written on the relationship between soy and colon cancer (60, 62, 63). There is no consensus on the effect of soy protein on colon cancer risk (60). Especially in experimental animal studies there is a tendency for an increase in colon cancer risk after feeding soy protein (2, 4). Thiagarajan found differences on several aberrant crypt foci parameters between soy flakes, soy flour and soy concentrate (64). In a later study comparing the effect of soy flour and soy concentrate on tumor formation the tumor incidence for the soy flour fed rats was decreased only little (65). Hakkak found a small protective effect of soy protein compared to casein on azoxymethane induced colon tumors (66). Wang (67) found no difference between soy concentrate and casein on intestinal polyp formation in Apc^{Min} mice. Thiagarajan (68) found a reduction in the proliferative capacity in colonic crypts of human subjects after consuming 39 grams of a soy protein isolate supplement per day for one year.

McIntosh found a significant increase on the occurrence of aberrant crypt foci (≥ 5 aberrant crypts per focus) in rats after feeding soybean meal compared to whey protein concentrate (69). Gee found more ACFs (≥ 3 crypts per focus) after feeding soy protein isolate compared to casein (70). This effect was only found when soy feeding took place before the first DMH injection. When soy was fed after the second DMH injection, no effect was found (70).

Soy protein isolate was used in all animal studies described in this thesis (Chapter 5, 6, 7 and 8) and in the research on protein N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) interactions (Chapter 3). Soy flour was used in the *Apc*^{Min} study (Chapter 8). In Chapter 5 an ethanolic extract of soy protein isolate was used to study the effects of saponins. This extract contained both soy isoflavones as well as saponins.

2.3.4 Egg white protein

Both the egg white and the egg yolk contain considerable amounts of protein. Egg white consists mostly of water (88%) and protein (10%). Egg yolk consists of water (49%), protein (16.7%) and fat (32%) (71). Egg white contains several proteins: ovalbumin (54%), ovotransferrin (12%), ovomucoid (11%), lysozyme (3.4%), avidin (0.05%) and other proteins (± 20%) (72). Because eggs need to contain all nutrients for the developing chick embryo, the protein quality is very high.

There are few animal experiments performed with egg white as a dietary protein source. This is possibly due to the fact that egg white is relatively expensive. Egg white contains a so-called anti-vitamin protein called avidin. Avidin specifically binds biotin, an essential B vitamin, resulting in a low biotin bioavailability (73, 74). It was thought that the avidin activity could rapidly be reduced by cooking the egg, and thereby denaturing the avidin protein (75, 76). For this reason raw eggs are thought to be unhealthy in comparison to cooked eggs.

While performing experiments with cooked egg white preparations, we noticed that the biotin-avidin interaction was probably still present, causing impaired reproduction in our *Apc*^{Min} mice study. After some thorough literature investigation we concluded that the general perception that avidin activity is strongly reduced by cooking the egg, is a misunderstanding. A short paper was written about this subject and is included in the Appendix. Ovalbumin, a part of dietary egg white was used in both *in vitro* studies on protein-carcinogen interactions (Chapter 3 and 4).

2.3.5 Whey protein

Whey proteins are the group of milk proteins that do not precipitate during cheese making or iso-electric precipitation. There are two major whey proteins, α-lactalbumin and β-lactoglobulin. Some proteins are less abundant, such as several immunoglobulins, serum albumin and lactoferrin. Whey proteins contain a high percentage of the sulfur containing amino acid cysteine (α-lactalbumin 0.56 mol/kg protein, β-lactoglobulin 0.27 mol/kg protein and serum albumin 0.25 mol/kg protein), as compared to whole casein (0.02 mol/kg protein) (49).

Important research on whey protein and cancer was performed by Bounous who reviewed the literature in 1991 (77). Often the effects of whey proteins on the immune system were included in their research. In two animal studies (78, 79) whey proteins were more protective against DMH induced colon tumors than casein or a control diet. McIntosh also found a reduction in tumor incidence and tumor burden for whey protein fed animals compared to soy and meat fed animals (2). In a later study McIntosh found a non-significant reduction of tumor incidence and tumor burden for whey protein compared to barbecued beef. The same article also reports a reduction in aberrant crypt foci (ACF) for whey protein or soybean meal with either lactoferrin or beta-lactoglobulin compared to soybean meal alone (69). In 1997 Sekine found that bovine lactoferrin protected against azoxymethane initiated colon tumors (80). Hakkak (81) found fewer tumors for a whey protein fed group compared to a casein fed group.

Because the research described in this thesis focussed on the comparison between casein and soy protein, whey proteins were only investigated to a minor extent. In Chapter 4 the interaction between whey protein and benzo[a]pyrene was studied. Currently, a study is being performed by our group on the comparison of whey protein and soy protein isolate on several liver and colon markers.

2.3.6 Meat protein

Meat is usually defined as muscle tissue. Meat is often more specifically referred to as beef, pork (red meat) or chicken (white meat). Lean raw meat roughly consists of water (70%), protein (20%), fat (5%) and other components like minerals (5%) (82). The two major proteins in meat are myosin and actin. A non-protein component in meat possibly influencing colon cancer risk, is iron. Iron is present as a part of the heme group. Sesink (83) showed that dietary heme is specifically able to increase colonic cell proliferation in rats.

The debate on the influence of meat on colon cancer is inconclusive so far. The WCRF (1997) (7) concludes that: 'diets containing substantially high amounts of red meats probably increase the risk of colorectal cancer'. Meat consumption is often highly correlated with fat and protein intake, which makes it often difficult to distinguish between these dietary factors. The assumed positive correlation between meat consumption and colon cancer risk is entirely based on epidemiological studies. Giovannucci (84) showed a relative risk of 3.57 for the consumption of beef, lamb or pork as a main dish five or more times a week in a large American cohort study. In a Dutch cohort study published in 1994 (85), an increased risk due to the consuming of processed meat was found. In this Dutch cohort study no trends for the relative rates of colon cancer were detected for the intake of energy from total protein or the intake of protein from meat. Consumption of total fresh meat, beef, pork and some other meats were not associated with an increased risk.

Relatively few animal studies were performed to confirm the epidemiological data or to investigate a possible mechanism. Most animal studies showed no effect of meat products on colon cancer risk compared to soy protein (2, 86, 87). Some found a protective effect of beef protein compared to casein (88, 89). Pernaud found no difference between meat and casein on ACF-multiplicity in rats (90).

In the present PhD study, meat was only used in the Apc^{Min} study (Chapter 8).

2.3.7 Wheat protein

In many diets wheat is a major source for carbohydrates. Wheat also contains proteins that can be divided into several subgroups according to their solubility: albumins, globulins, prolamins and glutelins. A whole grain contains 10% to 18% of protein (% dry weight) (91, 92). During mixing of wheat dough, gliadins and glutelins absorb a certain amount of water. The hydrated constituents are then transformed into a coherent protein mass, called gluten. Wheat gluten have an important role in bread baking. The nutritional quality of wheat gluten is low, because of its limiting lysine content. Methionine and tryptophan content are also relatively low. In combination with soy protein, wheat gluten display a higher nutritional value than each of the proteins does separately.

Not much is known about the health effects of wheat proteins on the colon. McIntosh (53) investigated the effect of a partial wheat protein diet in comparison to casein as a protein source, on colon tumor incidence, but observed no effect. In a study by Azuma (93) casein, a high molecular fraction (HMF) of soy protein isolate, egg yolk protein, wheat gluten and codfish meat were compared on tumor incidence following 34 weeks on a deoxycholic acid containing diet. Respective tumor incidences were 67%, 33%, 17%, 44% and 50%

implicating that wheat gluten show a mild protective effect compared to casein. Jenab showed that phytic acid in wheat bran influences early biomarkers of colon carcinogenesis (94).

In the study on aberrant crypt foci (Chapter 7) wheat gluten proteins were used. Some casein protein was added to the diet to prevent lysine deficiency.

2.3.8 Specific amino acids

Amino acids are the building blocks for all proteins. Some amino acids are called essential because they cannot be synthesized by the human body and have to be provided by the diet. As mentioned in section 2.2.4 the nutritional value of dietary protein is often dependent on one or two limiting amino acids. The explanation of the health effect of a dietary protein can be found by focussing on the effect of single amino acids.

In a summary on the influence of individual dietary amino acids on carcinogenesis Hawrylewicz reviewed methionine, tryptophan, tyrosine, phenylalanine, leucine and isoleucine. For none of the amino acids discussed, any influence on *colon* carcinogenesis is mentioned or suggested (30). In 1996 a study was published in which it was shown that L-arginine, given as a 1% solution of drinking water, inhibits DMH induced tumors in rats. The authors hypothesized that the reduction in colorectal tumor incidence was due to a non-specific stimulation of the host immune system by L-arginine (95). In the same year Grossie showed that arginine in parenteral feedings significantly increased tumor weights in rats bearing a Ward colon tumor (96). Wargovich reported a reduction in aberrant crypt foci after feeding a 0.5% arginine diet (97). Giovannucci correlated low dietary methionine intake with increased risk of colon cancer in men (36). Duranton studied the effect of dietary methionine on the promotion of intestinal carcinogenesis in rats (98). After twelve weeks on a 1% methionine supplemented diet, the turnover rate of ilio-epithelial cells was stimulated indicating enhanced crypt cell proliferation compared to a control diet. Furthermore in this group a twofold increase in the number of aberrant crypts was noted. Average crypt multiplicity was not mentioned.

In the present PhD study, the effect of the amino acid methionine was investigated as described in Chapter 5.

2.4 Methods used for the *in vitro* studies

2.4.1 Liquid suspension assay (LSA)

The mutagenicity of a compound can be measured in several ways. Traditionally the Ames test is performed, using histidine deficient *Salmonella typhimurium* strains (99). However, when protein mixtures or more specifically protein hydrolysates are used, free histidine may present in the growth medium. Therefore the histidine deficient *Salmonella* strains can still grow without exposure to a mutagen. To overcome the problem of the histidine presence, a liquid suspension assay (LSA) using two strains of *E. coli* was used instead of the Ames test (100-102). LSA and the comparable *in vivo* host mediated assay (HMA) were used earlier to study the influence of casein and soy protein isolate on *in vitro* and *in vivo* mutagenicity (3). Some different results that are possible in the LSA are shown in Figure 2.2.

Research using LSA is described in Chapter 3.

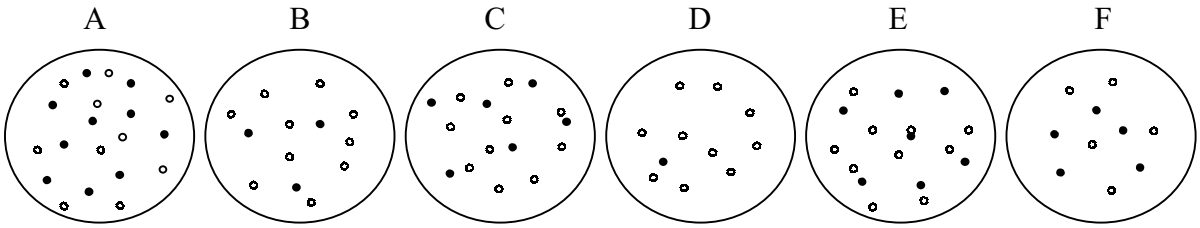

Figure 2.2 A schematic representation of different possibilities in the liquid suspension assay testing a sample on mutagenicity with or without the model mutagen MNNG. Large circles represent petri dishes. Small dark spots represent DNA repair deficient red -/753 colonies. Small open spots represent DNA repair sufficient white -/765 colonies. [A] Blank (no sample, no MNNG), [B] positive control (no sample, MNNG), [C] mutagenic sample (sample, no MNNG), [D] co-mutagenic sample (sample, MNNG), [E] anti-mutagenic sample (sample, MNNG), [F] cytotoxic (sample, no MNNG).

2.4.2 Protein digestion models

Because protein digestion already starts early in the digestive tract, it is important to investigate the interaction between protein hydrolysates and dietary carcinogens. There are several ways in which the protein hydrolysates can be obtained. The easiest way is to make a protein solution and add a digestive enzyme. However, to obtain a situation more relevant for the human physiological situation, more sophisticated models are used. Proteins can be digested by using a multi step pH-STAT method (103). First a 30 minute pepsin digestion is performed at a regulated pH to simulate gastric digestion. To simulate small intestinal protein digestion the pH is raised to 8.0 and several pancreatic enzymes are added. This digestion takes 2 hours. An even more sophisticated digestion model is represented by the gastro-intestinal simulator (Figure 2.3). Four compartments represent several sections of the gastro-

intestinal tract. Many parameters such as pepsin secretion, bile acid secretion, pancreatic fluid secretion, pH regulation, dynamic features, transit time and dialyses are accounted for (104).

Research using the gastro-intestinal digestion model is described in Chapter 3, use of the pH-STAT model is described in Chapter 4.

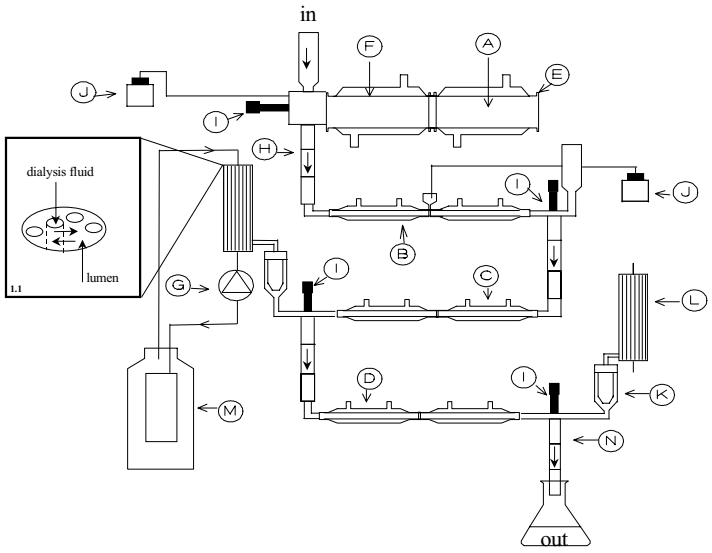

Figure 2.3 A schematic representation of the gastro-intestinal simulation model. [A] Gastric compartment, [B] duodenal compartment, [C] jejunal compartment, [D] ileal compartment, [E] glass jacket, [F] flexible wall, [G] rotary pump, [H] pyloric valve, [I] pH electrodes, [J] secretion pump, [K] pre-filter, [L] hollow fibre membrane, [M] dialysis pump, [N] ileal delivery valve, [1.1] detail of the hollow fibre membrane system (105).

2.5 Methods used for the *in vivo* studies

2.5.1 Colonic cell proliferation

Cell proliferation is often associated with carcinogenesis in rodents and humans (106-109) because it can increase mutation rates or help to fix spontaneous or induced mutations. In Figure 2.4 the possible role of increased cell proliferation as a risk factor for colon cancer is shown. Although cell proliferation is often used as an indication of cancer risk, some state cell proliferation is *not*, or only weakly, associated with carcinogenesis in rodents and humans (110, 111). As a compromise Weinstein stated that cell proliferation is essential for mutagenesis and carcinogenesis, but is probably not the only rate limiting step (112).

Colonic cell proliferation can be determined by several different methods. In this thesis ^{3}H-thymidine *in vivo* incorporation was used. In principle, radioactive labeled DNA nucleotide (^{3}H-thymidine) is injected intraperitoneally. ^{3}H-thymidine is incorporated in newly synthesized DNA throughout the body. When cell proliferation is increased, the amount of

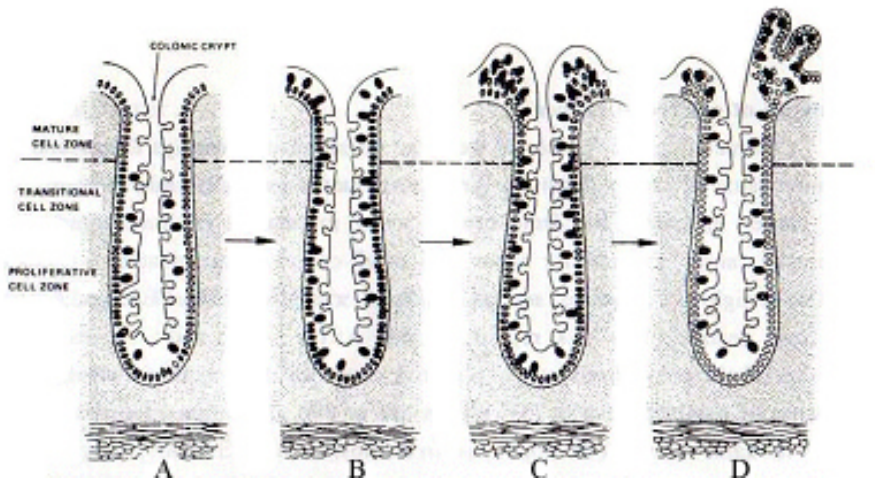

Figure 2.4 The role of cell proliferation in the development of colorectal cancer according to Lipkin (113). A) Normal colonic crypt. Dark spots represent cells undergoing DNA synthesis. B) Proliferative zone expands to higher one-third of the crypt. C) The zone of maximal proliferative activity shifts to the top of the crypt. Cells accumulate in the mucosa. D) Adenomas appear.

synthesized DNA will be increased and therefore the amount of incorporated ^{3}H-thymidine will increase. Colonic cell proliferation is measured after scraping the mucosal cell layer from the intestine. The total amount of scraped DNA is assessed in order to express cell proliferation as the amount of radioactivity incorporated per microgram of scraped DNA. It is important to measure the amount of scraped DNA in both the control and the experimental groups to check whether too many non-epithelial cells are scraped in one of the groups (114).

Research on the influence of dietary changes on colonic cell proliferation is described in Chapter 5 and 6.

2.5.2 Aberrant crypt foci (ACF)

Both the rat and the human colon consist of crypts (Figure 2.4, side view) whose main function it is to adsorb water. Some of the crypts can be distinguished by an aberrant shape. These are called aberrant crypts (AC). A cluster of aberrant crypts is called an aberrant crypt focus (ACF). It is hypothesized that colonic ACF are preneoplastic lesions indicative of colon cancer risk. ACF were first described and later reviewed by Bird (115, 116). During the last decade ACF determinations in rats have been used to determine the influence of dietary practices on colon cancer risk. Colon cancer risk can be estimated by counting the *number* of ACFs in the colon. However, many studies indicate that ACF crypt *multiplicity* (average number of aberrant crypts per focus) predicts tumor development more reliably (117, 118). ACF have also been detected in humans (119).

Rat colonic crypts can be visualized after sacrificing the animal and cutting the colon longitudinal so that the intestinal mucosa is exposed. After fixation of the colon in formaline, the crypts are visualized by coloring with methylene blue. The crypts can be distinguished as a regular pattern of small circles or spots (Figure 2.5a, top view). When animals are exposed to a colon carcinogen such as 1,2 dimethylhydrazine (DMH) or azoxymethane (AOM), aberrant crypts occur (Figure 2.5b,c). ACFs can also be initiated by known colon carcinogens present in daily food, such as heterocyclic amines. For studies on aberrant crypt foci several experimental designs are possible. Some investigators test the potential of a chemical to either initiate or promote ACFs. In the study described in this thesis a known initiator of ACFs was used (AOM). The focus of the study was to test the influence of the diet on the occurrence of ACFs. In the design used, the diet was fed prior to initiation. This way the influence of the diet on both the initiation (number of ACFs) as well as promotion (multiplicity) was tested.

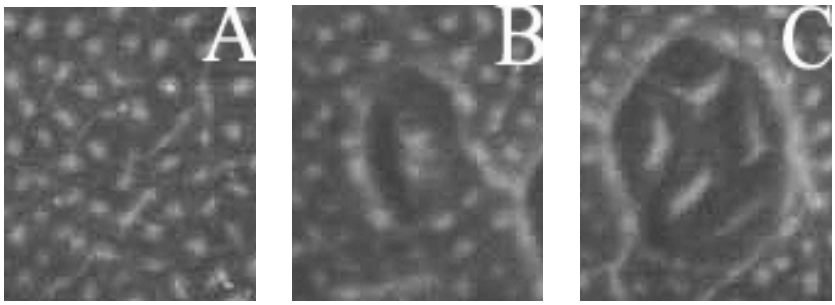

Figure 2.5 [A] Pattern of regular crypts, [B] Several aberrant crypts with multiplicity one or two, [C] Aberrant crypt focus with multiplicity six.

Research on the influence of different dietary protein sources on initiation and promotion of ACF is described in Chapter 7.

2.5.3 Intestinal polyps using the *Apc*^{Min} mice model

In contrast to chemical models (DMH, AOM or MNNG), more recently several models using genetic predisposition of intestinal tumors are described. A well known example is the *Apc*^{Min} model, first described by Moser (120) and reviewed by Shoemaker (121). The *Apc*^{Min} model is often used to test the influence of different diets on intestinal polyp formation (122). In the *Apc*^{Min} model C57BL/6J mice with a non-sense mutation at codon 850 of their *Apc* gene, spontaneously develop a large number of intestinal polyps. An *Apc* mutation is thought to be one of the first steps in the development of a normal colonic epithelial cell to a carcinoma (Figure 2.1). Most of these polyps occur in the small intestine, some of the polyps occur in the colon. The *Apc*^{Min} model closely resembles a human disease called familial adenomatous polyposis (FAP), which results in very large numbers of benign tumors in the colon of young

adults (123). Because the onset of intestinal carcinogenesis in Apc^{Min} mice is assumed to occur relatively early in life (121), exposure to the experimental diet is sometimes started during mating and is continued through weaning and lasts until animals are killed. For breeding, heterozygous male mice ($Apc^{Min/+}$) mate with female C57BL/6J mice with both *Apc* genes intact ($Apc^{+/+}$). Embryos homozygous for the in Apc^{Min} mutation fail early in development. Heterozygous animals are used for experiments but rarely live longer than 150 days of age. Their premature death is associated with secondary effects of tumor growth such as severe chronic anemia and intestinal blockage (121).

The few studies using the Apc^{Min} model relevant for this thesis involve non-protein components. It is shown that a concentrate of soybean derived Bowman-Birk inhibitor suppresses carcinogenesis in the intestines of Apc^{Min} mice (124). Sorensen demonstrated that high amounts of soy isoflavones did not protect against intestinal tumor development in the Apc^{Min} mice model (125).

Research using the Apc^{Min} mice model is described in Chapter 8 and the Appendix.

2.5.4 Fecal fat excretion

An increased fecal fat excretion might indicate an increased colon cancer risk. Total fecal fat excretion can easily be determined by extracting a 24 hour fecal sample using an organic solvent (126). Two studies describe that increasing the amount of soy protein in the diet from 10 to 40%, increases fecal lipid excretion (127, 128). Fecal lipid excretion as a percentage of intake was significantly lower when the carcinogens 2-acetylaminofluorene (AAF) or N,N-dinitrosopiperazine (DNP) were added to the diet. Female mice excrete less fecal lipid as a percentage of intake than males (127). When rats were exposed to DMH, several fecal fat components such as coprostanol and bile acids were significantly increased (129). Kritchevsky describes that fecal excretion of cholesterol is reduced in rabbits fed casein as opposed to those fed soy protein (130). In several articles about the influence of dietary protein type on colon cancer risk parameters, a positive correlation was found between the total fecal fat excretion and cell proliferation and tumor burden respectively (2, 4). Govers showed a 4.5 fold fecal fat induction for soy protein isolate compared to casein fed animals. McIntosh showed a more than 2-fold increase (2).

Research on fecal fat excretion is described in Chapter 5, 6, 7 and 8.

2.5.5 Fecal magnesium excretion

In 1991 Brink (131) showed that fecal magnesium output was increased when rats were fed soy protein isolate instead of casein as a protein source. In 1992 Brink showed (132) that this fecal magnesium increase was due to a greater excretion of endogenous excretion and not because of a disturbed uptake of magnesium. This was determined with the use of ^{28}Mg. In the 1991 study it was shown that phytate from soybean protein is also capable of increasing fecal magnesium concentrations. It was therefore hypothesized that phytate from soybean protein was responsible for the fecal magnesium increase observed after consumption of soy protein isolate. Because magnesium is present at high concentrations in colonic epithelial cells, it was hypothesized that soy protein had damaged colonic epithelium thereby releasing magnesium from epithelial cells and thus increasing fecal magnesium excretion. As a reaction to the damage of the epithelial cells it was hypothesized that cell proliferation in the colonic epithelium would increase. This hypothesis was tested in a study of Govers (4) who showed that feeding soy protein isolate compared to casein increased both fecal magnesium and colonic cell proliferation with a factor two. Because of the results of Govers, magnesium is a potential marker for damage to the colonic epithelium, and therefore maybe a very early marker for colon cancer risk in the rat.

Research on the fecal magnesium excretion is described in Chapter 5 and 6.

2.5.6 Fecal water

Fecal fat excretion and fecal magnesium excretion as described above, are determined in total feces and expressed per gram feces or per amount of feces excreted in 24 hour. Because compounds present in the fecal water fraction of feces are more likely to get in close contact with the colonic epithelium, exposure to water soluble compounds can be estimated by determining their concentration in fecal water.

Fecal water can be prepared from fresh or stored feces. In the case of mice and rat feces, no fecal water can be obtained by just centrifuging the feces because it is too dry. In the human situation some water can be obtained after homogenizing and centrifuging at high speed. Preferably, collected feces is freeze dried as soon as possible or otherwise stored at –20 °C. After freeze drying, feces is homogenized into a fine powder. Fecal water is obtained by adding demineralized water to the feces, mixing, incubating the mixture at 37 °C, centrifuging and then collecting the water fraction. Depending on the diet, the amount of water the feces can absorb, varies. This is partly dependent on the amount of fiber present in the diet.

Fecal water was used in Chapter 5, 6, 7 and 8.

2.5.7 Cytolytic activity of fecal water

Cytolytic compounds have the potential to damage the cell by disrupting its membrane, thereby causing lysis. It is known that both fecal bile acids as well as fecal free fatty acids are able to disrupt cell membrane structure causing lysis of the cell (133). Also heme is known to be highly cytolytic (83). The cytolytic potential of feces is measured by determining the cytolytic activity of fecal water. Cytolytic activity can be determined *in vitro* by measuring the lysis of erythrocytes after exposure to a test solution containing for example bile acids or fecal water. Different ions such as Fe, Na or K that are excreted once the cell is disrupted, can be determined. Often, atomic absorption spectrophotometry (AAS) is used to determine the ion release. Cell lysis using erythrocytes can also be determined spectrophotometrically by detecting heme at 540 nm. This method was used throughout this thesis. Most research on the influence of the diet on cytolytic activity and thereby on colonic cell proliferation has been performed on calcium, calcium phosphate and heme (134-136).

Research on the cytolytic activity of fecal water is described in Chapter 5, 6, 7 and 8.

2.5.8 Fecal alkaline phosphatase excretion

Alkaline phosphatase (ALP) is a dimeric zinc glycoprotein (120-140 kD), which hydrolyses organophosphates. The enzyme is located on the apical membrane of intestinal epithelial cells (137). ALP occurs throughout the body. The intestinal ALP iso-enzyme can be specifically inhibited by L-phenylalanine (138). ALP can be used as a measure of intestinal epitheliolysis (139, 140). After the disruption of an epithelial cell, ALP will be excreted into the lumen and can be measured in the feces. In cell culture studies ALP activity is used as a differentiation marker (141). ALP activity can be determined spectrophotometrically (405 nm) by measuring the release of paranitrophenol (PNP), from paranitrophenol phosphate (PNPP).

Much work on the influence of diet on fecal ALP excretion has been performed by the group of Van der Meer (134, 135) and by our own group (142, 143). Mostly ALP activity was determined in the fecal water fraction or in the water extract from the collected feces.

Research on fecal ALP excretion is described in Chapter 5, 6, 7 and 8.

2.5.9 Fecal β-glucuronidase activity

β-Glucuronidase is an enzyme present in the colonic lumen, able to deconjugate glucuronides. The majority of β-glucuronidase activity in the gut lumen is of bacterial origin (144). β-Glucuronidase specifically releases conjugates from toxic compounds detoxified in the liver and excreted in the bile (145). Since these toxic compounds can be carcinogens, the enzymatic activity of β-glucuronidase is considered as a factor able to modulate colon cancer risk. Other bacterial enzymes can also produce toxic metabolites directly from ingested material. A well-known example is β-glucosidase which can activate cycasin, a pre-carcinogen present in plants, to methyl-azoxymethanol (MAM) (146, 147). MAM is a known colon carcinogen. Still, β-glucuronidase activity is considered a better risk indicator then β-glucosidase because β-glucosidase has only few potentially toxic substrates and β-glucuronidase releases many different conjugates detoxified by the liver.

Takada showed that when β-glucuronidase is inhibited, the number of induced tumors in the rat is significantly decreased (148). This implicates that intestinal microfloral β-glucuronidase plays an important role in colonic carcinogenesis caused by AOM. Diet has a major influence on bacterial enzyme activities. Hambley (149) showed that a diet associated with a high risk for colon cancer (high fat, high sucrose, low fibre, and low calcium) increased β-glucuronidase activity 2.5 times, but reduced the β-glucosidase activity by 50%. Wise (150) fed rats an increasing amount of lactalbumin in the diet for ten days and then measured the activity of several fecal microbial enzymes. β-Glucosidase activity increased significantly. The β-glucuronidase increase was not significant.

Research on the fecal β-glucuronidase and β-glucosidase activity is described in Chapter 6, 7 and 8. Both enzyme activities were measured in fresh feces, stored feces and stored fecal water fraction.

2.5.10 Bile acids

Bile acids are surface active compounds, which dissolve lipids in the small intestine. Bile acids reaching the colon are likely to be metabolized by the microbial flora. This results in deconjugation and partial dehydroxylation from primary (cholic acid and chenodeoxycholic acid) to secondary (deoxycholic acid and lythocholic acid) bile acids. Still, primary bile acids are the major bile acids found in feces (151).

There are several hypotheses on the relationship between bile acids and colon cancer. Bile acids themselves are not mutagenic, but they do contain co-mutagenic properties and probably influence colon carcinogenesis in the promotion phase (152). Bile acids, especially

secondary bile acids, have a potential to disrupt membranes and thereby cause epithelial cell lysis (133, 151). This can cause compensatory proliferation of colonic crypt cells and this is associated with an increased risk for colon cancer (153). Calcium in the form of calciumphosphate can interact with these cytolytic bile acids and thereby prevent epithelial lysis in the colon (51, 154).
Dietary composition influences bile acid excretion (155). Soy protein isolate compared to casein increased intestinal bile acid concentration in mice (156). Substitution of soybean milk for cow milk increases bile acid excretion in young human infants (157). Some authors suggest that saponins are responsible for the increase in fecal bile acid excretion after eating soy protein (158). Others report the presence of a bile acid binding protein from soybean (159).

Research on bile acid excretion is described in Chapter 5, 6, 7 and 8. Bile acids were always determined in fecal water.

2.5.11 Free fatty acids

Free fatty acids can be subdivided into two different categories: short chain fatty acids (SCFA) and long chain fatty acids (LCFA). SCFAs are produced when fiber is fermented (160). Butyrate, one of the SCFAs, is considered to be health promoting for the colonic epithelium. Butyrate is used by colonocytes as their primary fuel source and can influence proliferation as well as apoptosis (161). LCFAs are not produced by fermentation, but are a result of lipid digestion in the small intestine. LCFAs are known to have a lytic potential (133). Remarkably, fecal LCFA content can differ after feeding diets only different in protein type (4). The mechanism for this effect is unclear.

Research on free fatty acid excretion is described in Chapter 5, 6, 7 and 8. Free fatty acids were determined in total feces as well in fecal water.

2.5.12 pH

In the rat, intracolonic pH ranges from 7.8 – 8.0 near the anus and declines to 7.4 – 7.5 at 12 centimeter from the anus (32). The pH of colonic contents is often considered a modulating factor for colon cancer development for several reasons. Intraluminal pH determines the conversion of primary to secondary bile acids. A low intraluminal pH might also represent high fiber fermentation (162), producing short chain fatty acids, such as butyrate which possibly has some healthy effects on the colonic epithelial cells (161). Also ammonia formed in the colon is protonated at lower pHs, which reduces its potential for mucosal damaging effects and encourages its uptake for protein formation by the gut flora (151). Secondary bile

acids are less soluble at lower pH, which reduces their concentrations in fecal water (163). Intracolonic pH varies with the content of protein in the diet (32). AOM exposure increases intracellular pH in isolated rat colonocytes (160).

Research on intraluminal pH is described in Chapter 5, 6, 7 and 8. The pH is always determined in fecal water.

Chapter 3

Antimutagenicity of heat denatured ovalbumin, before and after digestion, as compared to caseinate, BSA and soy protein

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ABSTRACT

Antimutagenicity of ovalbumin was investigated and compared to that of Na-caseinate, bovine serum albumin and soy protein. Antimutagenicity was measured against N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) in the *E. coli* DNA repair liquid suspension assay. Heat denatured ovalbumin showed a strong concentration dependent increase in antimutagenicity compared to undenatured ovalbumin. Antimutagenicity of heat denatured ovalbumin was superior to the antimutagenicity found for the other proteins tested. After digestion of native and heat denatured ovalbumin using the pH-stat method, antimutagenicity remained. Samples taken from a gastro-intestinal simulator for antimutagenicity measurements, showed strong comutagenic properties. This turned out to be due to comutagenicity against MNNG caused by bile acids and lipase. It was concluded that food proteins exhibit different antimutagenic properties towards MNNG, largely depending on protein treatment. Also, the choice of a particular protein digestion model can influence results to a great extent.

INTRODUCTION

Studies on the carcinogen scavenging capacity of proteins have been performed since the late nineteen forties (20). It was discovered that p-dimethylaminoazobenzene was able to bind to liver proteins in the rat. At first the binding of carcinogens to proteins was thought to be the major cause of cancer and much research was performed on this subject. For example, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), was found to react with several proteins and amino acids (15-19). Dietary proteins however, were not investigated. Nowadays, it is known that most carcinogens can not only bind to proteins but also to DNA, the latter being an important step in the development of cancer (164). Work on reactivity of mutagens and carcinogens has since focused on the interaction with DNA. Protein adducts are mostly studied in environmental toxicology as reviewed by Meyer and Bechtold (165) and Hemminki (166). The adducts are used as a biomarker of exposure to, for example, xenobiotics such as mutagens and carcinogens.

In recent years, a lot of attention has been focused on antimutagenic and anticarcinogenic properties of dietary components. In this context special attention has been paid to interactions between carcinogens and protein(food)s. Apart from caloric effects, it has been demonstrated that proteins have antimutagenic (1, 23, 167) and anticarcinogenic properties (2, 77, 168) as well as tumor inducing properties (169).

Most studies on antimutagenicity of proteins or protein containing foods focus on casein or other milk proteins and soy protein. Another interesting dietary protein is ovalbumin (a major egg protein). To our knowledge, there is only one study concerning the antimutagenicity of ovalbumin (167). These authors found ovalbumin to protect against pepper-induced mutagenicity in the Ames test. The present study was performed to investigate the antimutagenicity of the dietary protein ovalbumin towards MNNG, a well known, highly reactive, model nitrosamide (170). The effect of ovalbumin was compared to that of Na-caseinate, soy protein and bovine serum albumin (BSA). The effect of protein digestion on antimutagenicity was also tested. As a test system, the *E. coli* DNA repair assay, earlier described by Mohn (102), was chosen because of the histidine tolerance of the *E. coli* assay for testing protein hydrolysate samples. The more often used Ames test is not histidine tolerant and therefore not suited for working with protein hydrolysates.

MATERIALS AND METHODS

Chemicals and proteins Bacto-agar, bacto-peptone, bacto-tryptone (Difco, Detroit, USA), BSA, ovalbumin (Sigma), MNNG (Aldrich), Na-caseinate (DMV, Veghel, The Netherlands), pepsin (2000 FIP-U/g) and pancreatin (1400 FIP-U/g) (Merck), soy protein isolate (Loders Croklaan, The Netherlands). Chemicals used for the gastro-intestinal simulator were used as specified by Minekus (104).

E. coli LSA The *E. coli* liquid suspension assay (LSA) was performed as described earlier by Mohn (100, 102) with minor adjustments. In short, two bacterial strains were used, basically differing only in DNA-repair capacity and ability to ferment lactose (101). *E. coli* K12 strain 343/113/753 (-/753) is DNA repair deficient. The -/753 strain ferments lactose from the agar medium, which produces an acidic environment. Colonies of this strain are recognizable as red colonies on an agar plate with neutral red as a pH indicator. *E. coli* K12 strain 343/113/765 (-/765) is DNA-repair proficient and does not ferment lactose. It appears as a white colony on agar plates. When a mixture of -/753 and -/765 bacteria is exposed to a mutagen, the number of viable DNA repair deficient bacteria will decrease. After spreading an appropriate dilution out on suitable agar plates and incubation of the plates at 37 °C for two days, this decrease is quantified by a low (753/765) ratio. Also see Chapter 2, Figure 2.2.
Heat denatured ovalbumin was prepared by heating 3 ml 5.4% (w/v) ovalbumin in phosphate buffered saline (PBS) in a water bath at 100 °C for 60 seconds in test tubes. After heating, the gel formed was disrupted by sonication (Vibracell, Sonics & Materials Inc.) three times for 20 seconds to make it homogeneous. The liquid suspension assays were performed in PBS at pH=7.2 without a metabolic activation system. MNNG (0.7 μg) was pre-incubated with 900 μl of protein (hydrolysate) solution for 60 minutes at 37 °C in a shaking water bath in the dark. After adding 100 μl of bacteria mixture to the pre-incubation mixture, it was incubated for 120 minutes under the same conditions as the pre-incubation. To stop exposure of the bacteria, the mixture was diluted in PBS and spread over agar plates. The plates were incubated at 37 °C in a ventilated stove in the dark for two days and colonies were counted.
The ratio of the -/753 and the -/765 strain was taken as a measure of (anti)mutagenicity. Although the LSA is a DNA damage test, MNNG is known to induce point mutations (171).
All samples (blank, positive control, etc.) were tested with nine plate incubations per experiment. All experiments were at least performed twice.
Protection by a protein (hydrolysate) against MNNG induced mutagenicity was calculated according to the following Formula 3.1. Corrected samples (s_{corr}) were calculated to compensate for influences of e.g. protein on blank ratios.

$$P_R = \left(\frac{s_{corr} - pc}{bl - pc}\right) \times 100\% \text{, with } s_{corr} = \frac{bl}{bl_t} \times s \qquad (3.1)$$

P_R protection given by tested substance (e.g. protein)
s ratio of sample (bacteria + tested substance + mutagen)
s_{corr} ratio of corrected sample (bacteria + tested substance + mutagen)
pc ratio in positive control (bacteria + mutagen)
bl ratio in blank (only bacteria)
bl_t ratio in blank of tested substance, e.g., protein (bacteria + tested substance)

In some experiments MNNG induced mutagenicity was enhanced by substances other than protein. In those cases the following formula was used:

$$CM = \frac{(s_{corr} - pc)}{pc} * 100\% \text{, with } s_{corr} = \frac{bl}{bl_t} * s \qquad (3.2)$$

CM co-mutagenicity of the tested substance

Variance of P_R and CM were calculated with the bootstrapping technique (172). Results are expressed as $P_R \pm$ SEM and CM ± SEM. Bootstrapping (n=200) was performed using SAS/STAT version 6.

pH-stat protein digestion Protein digestion was basically performed according to the pH-stat technique first described by Jacobsen (103). A 665 Dosimat and 614 Impulsomat (Metrohm, Swiss) were used. In short, 50 ml of a 5.4% protein solution was brought at 37 °C and pH=2.00 (6 M HCl), while stirring. After a 30 minute pepsin (E:S = 0.013 : 1) digestion at pH 2.00 the reaction was stopped by raising the pH of the solution to 8.00 with 6 M NaOH. Next, a 90 min pancreatin (E:S = 0.25: 1) digestion was performed using the same solution. The pH was kept constant at all times. After digestion, samples were immediately frozen in liquid nitrogen and stored at -20 °C. From the amount of H^+ and OH^- equivalents added to keep the pH constant, the degree of hydrolysis (DH) was calculated (173).

Gastro-intestinal simulator protein digestion The gastro-intestinal simulator as described by Minekus (104) was used. Parameters were defined as listed in Table 3.1. Values were chosen to mimic adult human situation. Samples for antimutagenicity testing were taken from within the intestine after 100 minutes of digestion, frozen in liquid nitrogen and stored at -20 °C until further use.

Table 3.1 Parameters used for the gastro-intestinal simulator.

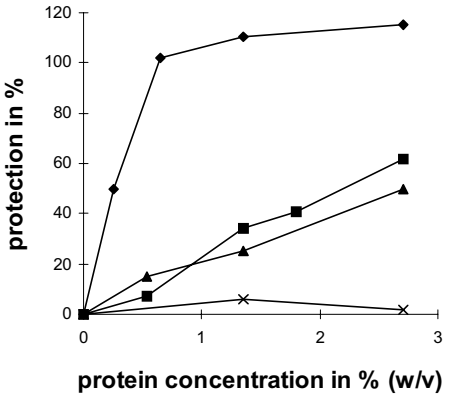
	Value
Feeding time	10 minutes
Feeding size	200 ml
Pepsin secretion	0.25 ml/min (0.2 mg/ml)
Pancreatin secretion	0.25 ml/min (7% solution extracted with 0.1 M bicarbonate)
Stomach lipase excretion	0.25 ml/min (0.25 mg/ml)
Bile secretion	0.50 ml/min (4% solution)
pH duodenum	pH=6.5
pH curve stomach	3.5 at t=0, 3.2 at t=20, 2.8 at t=40, 2.1 at t=60, 1.8 at t=90, 1.7 at t=120, 1.7 at t=360

RESULTS AND DISCUSSION

Comparison of antimutagenicity between proteins The antimutagenic capacity of ovalbumin and some other proteins towards MNNG is shown in Figure 3.1, in which various experiments are summarized. Unheated ovalbumin did not show any relevant protection against MNNG. On the contrary, when ovalbumin was heat denatured, the protection of a 1% ovalbumin solution under the given conditions was 100%. This clearly exceeded the protection given by the other proteins tested. A clear dose dependency was found for heat denatured ovalbumin, BSA and Na-caseinate. The effect of heat denatured ovalbumin was strong compared to earlier reports on antimutagenic properties of proteins (1, 167) although comparison is difficult because other test systems and conditions were used.

Difference in protection at the highest protein concentration (2.7%) was tested for significance with the Students t-test. All data points differed significantly ($p \leq 0.05$) except for the difference between BSA and Na-caseinate protein samples.

Figure 3.1 Dose-response curves of the antimutagenic capacity of unheated (**x**) and heat denatured ovalbumin (◆) in comparison to bovine serum albumin (■) and Na-caseinate (▲). Antimutagenicity was measured against MNNG in the *E. coli* liquid suspension assay. Data are shown as percentage of protection (P_R). SEM never exceeded 12.8% and was 6% on average.



Effect of heating on the antimutagenicity of ovalbumin Figure 3.2 shows that the antimutagenic capacity of ovalbumin was dependent on both the temperature and time of heating. Until 70 °C, no effect of heating was detectable on the antimutagenic capacity of ovalbumin. The effect started at a temperature between 70 °C and 85 °C. This corresponds to the denaturation temperature of ovalbumin (about 71 °C) (174) as was also indicated by turbidity measurements of the heat denatured ovalbumin solutions (data not shown). Heating resulted in denaturation and gelation of ovalbumin. Denaturation of globular protein exposes inner protein groups. It is therefore possible that the antimutagenic capacity of ovalbumin is due to hydrophobic sites or specific residues buried in the native protein. It is in this respect of interest to note that heating of casein has no effect on its antimutagenic potential (1). Casein has almost no secondary or tertiary structure and cannot be denatured by heating (175).

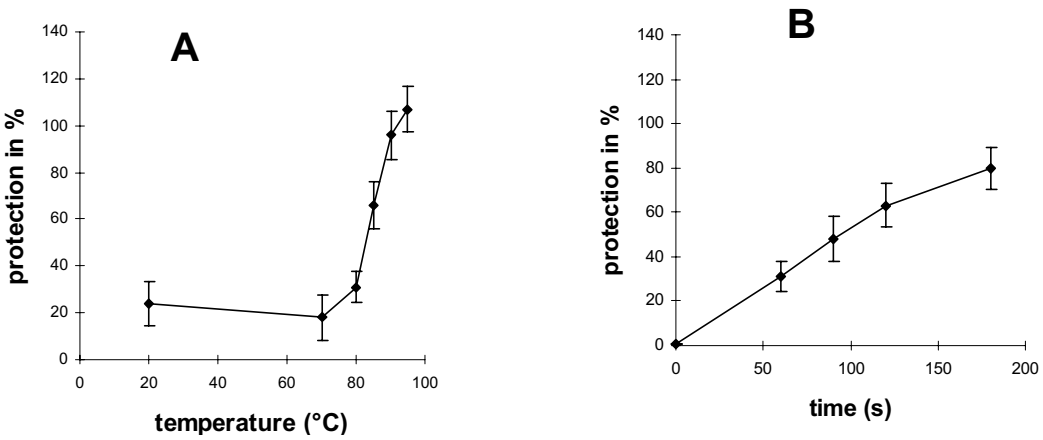
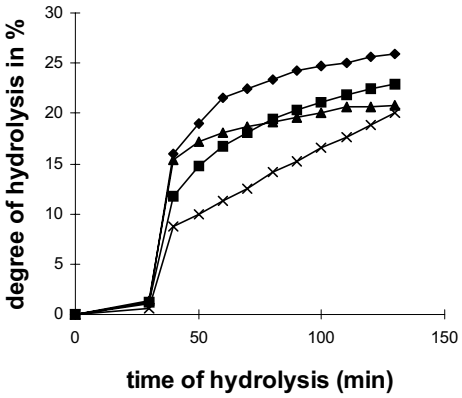


Figure 3.2 Protection by ovalbumin. A) Heated at various temperatures for 60 seconds. B) As a function of heating time at 80 °C, against MNNG in the *E. coli* liquid suspension assay. Data are shown as percentage of protection (P_R) ± SEM.

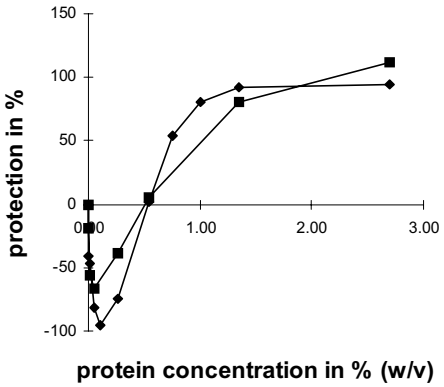
Antimutagenicity of digested proteins To investigate the effect of digestion of proteins, hydrolysates were prepared using the pH-stat technique. Figure 3.3 shows the relationship between time of hydrolysis and DH. After 30 minutes of pepsin digestion and 90 minutes of pancreatin digestion, the protein hydrolysates had approximately the same DH although there were differences in reaction kinetics.

Figure 3.3 Digestion of soy protein (◆), heat denatured ovalbumin (■), Na-caseinate (▲) and unheated ovalbumin (**x**), using the pH-stat technique. The proteins were first digested with pepsin (1: 0.013) for 30 minutes at 37 °C at pH=2.00, followed by pancreatin digestion at pH=8.00 (1: 0.25) for 90 minutes.



Ovalbumin hydrolysate showed a dose dependent antimutagenicity from 0.5 until 3.0 mg/ml protein. Hydrolyzed, unheated ovalbumin showed the same antimutagenic capacity as hydrolyzed denatured ovalbumin (Figure 3.4). So, after hydrolysis to a degree of about 22%, heating of ovalbumin before digestion did not seem to be a factor of importance any more. This can be explained by the above described hypothesis that exposure of inner protein groups increases antimutagenic capacity of ovalbumin. This means that the route, via which the inner protein groups are exposed, is unimportant.

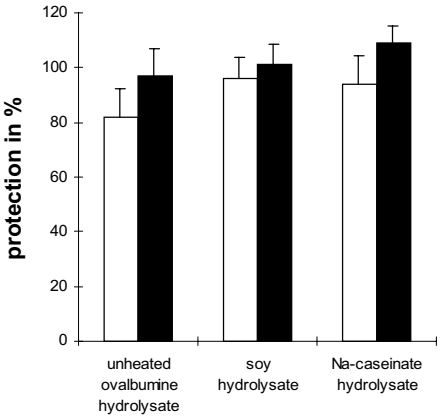
Figure 3.4 Dose effect curves for antimutagenicity of protein hydrolysates of unheated (◆) and heat denatured ovalbumin (■) against MNNG in the *E. coli* liquid suspension assay. Data are shown as percentage of protection (P_R). A negative protection indicates enhancement of the mutagenicity of MNNG. SEMs (not shown) were comparable to other experiments.



This hypothesis was investigated for other proteins by testing protein hydrolysates of soy and Na-caseinate (2.7%), also digested by the pH-stat method (Figure 3.3). Results are shown in Figure 3.5.

Because hydrolysates still contained proteolytic enzymes, samples were tested before and after heat inactivation (5 min, 100 °C) of these enzymes. When combined with Figure 3.1, Figure 3.5 shows that antimutagenicity of Na-caseinate, soy and ovalbumin (all unheated) were all increased after digestion. Undigested soy protein (2.7%) showed no antimutagenicity (3). Undigested Na-caseinate (2.7%) protected about 45% and 2.7% undigested unheated ovalbumin protected only for 15% (Figure 3.1). So antimutagenicity increased in all cases after digestion. There was no significant ($p \leq 0.05$) increase in protection found from the enzyme inactivation treatment.

Figure 3.5 Comparison between protection of three dietary proteins (2.7%) after pH-stat hydrolysis (degree of hydrolysis: about 22%) against MNNG in the *E. coli* DNA repair assay. Data are shown as a percentage of protection (P_R) ± SEM. Both samples before enzyme inactivation (□) and after enzyme inactivation (■) were tested.



The pH-stat hydrolysates exhibited comutagenicity at the lower concentrations (Figure 3.4). Therefore, the protection of undigested heat denatured ovalbumin (Figure 3.1) cannot be compared to both heated and unheated digested ovalbumins. At higher protein concentrations the comutagenic effect was apparently overruled by the presence of a high protein concentration. It was hypothesized that the comutagenicity found, was due to the presence of impurities in pancreatin. The nature of the impurities followed from experiments discussed below.

The gastro-intestinal simulator Because pH-stat digestion is only a rough estimate of the physiological situation, we used a model that comes closer to the *in vivo* situation. The gastro-intestinal simulator described by Minekus (104) is an example of such a model. Pilot experiments were performed by using protein digests from the stomach, duodenum, jejunum and ileum compartment for antimutagenicity testing. However, MNNG mutagenicity turned out to be highly affected by blank digestions (just water) (Figure 3.6). After testing all possible substances used in the gastro-intestinal simulator, bile acids and lipase turned out to be strongly comutagenic to MNNG in the *E. coli* assay used (Table 3.2). Samples with only lipase or bile showed no negative effect on the growth of -/765 and even a colony stimulating effect on -/753 (only ratios are shown).

Table 3.2 Comutagenic effects of bile acids and lipase against MNNG in the *E. coli* liquid suspension assay. Lipase was tested before and after heating (5 minutes in boiling water). Results are from two different experiments. Ratios of (753/765) are shown ± SD.

	Blank	**Positive control (MNNG)**	**Only lipase or bile**	**Sample (lipase/bile and MNNG)**	**Calculated co-mutagenicity**
Lipase 20 °C (0.025%)	0.69 ± 0.10	0.14 ± 0.03	1.28 ± 0.17	0.01 ± 0.01	100%
Lipase 100 °C (0.025%)	0.69 ± 0.10	0.14 ± 0.03	1.12 ± 0.34	0.01 ± 0.01	97%
Bile 0.05 %	0.69 ± 0.10	0.14 ± 0.03	0.63 ± 0.08	0.10 ± 0.03	23%
Bile 0.25 %	0.95 ± 0.09	0.24 ± 0.07	1.59 ± 0.18	0.03 ± 0.01	93%
Bile 0.50 %	0.95 ± 0.09	0.24 ± 0.07	1.62 ± 0.17	0.05 ± 0.02	88%
Bile 1.00 %	0.95 ± 0.09	0.24 ± 0.07	1.67 ± 0.15	0.04 ± 0.01	91%

The comutagenic capacity of bile acids confirmed previous observations by Wilpart and Roberfroid (176). No published data could be found on comutagenicity of lipase although mutagenicity tests have been performed on lipase G from penicillium camembertii. From this research it was stated that “no evidence of mutagenic potential was found” (177). Our results can explain the comutagenicity found of the protein hydrolysates as shown in Figure 3.4 because pancreatin contains lipase.

The experiments performed show that it is useful to test protein hydrolysates from systems more complicated and physiologically relevant than the pH-stat method. Besides a more realistic protein digestion, several intestinal secretions influence the antimutagenicity of the intestinal contents.

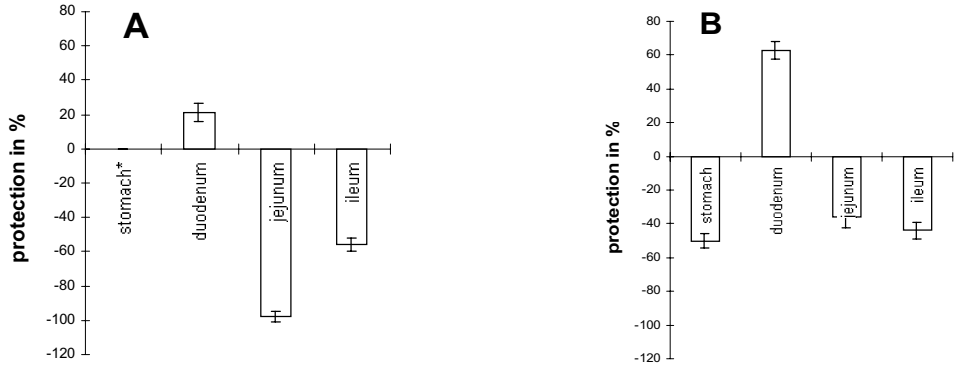


Figure 3.6 Influence of intestinal contents of the gastro-intestinal simulator on antimutagenicity against MNNG measured in the *E. coli* liquid suspension assay. A) Influence of intestinal contents after blank (= water) digestion. B) Influence of intestinal contents after digestion of 5.4% Na-caseinate solution. Data are shown as percentage of protection (P_R) ± SEM. Samples were taken after 100 minutes of digestion (* = not determined).

CONCLUSION

Heat denatured ovalbumin was found to be strongly antimutagenic against MNNG in the *E. coli* DNA repair assay compared to Na-caseinate, BSA and unheated ovalbumin. Heating of intact ovalbumin is necessary to induce antimutagenicity, which suggests that the unfolded molecule is the active species. The antimutagenicity is still present after hydrolysis (DH = about 22%) of both heated and unheated ovalbumin, so heat denaturation is then no longer required.

Several constituents of the intestine influence the (anti) mutagenicity of a diet as shown by gastro-intestinal simulator experiments. Therefore questions concerning the physiological relevance of the antimutagenicity of heat denatured ovalbumin, are still to be answered.

ACKNOWLEDGEMENTS

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Chapter 4

Influence of protein type and digestion on protein-benzo[a]pyrene interactions

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T. Johnson and G. R. Fenwick,
The Royal Society of Chemistry,
Norwich, Great Britain (2000).

ABSTRACT

There is little information on the bioavailability of dietary mutagens and carcinogens. Dietary protein is known to be able to influence the bioavailability of both nutrients and medicine. In the present study the effects of protein type and protein digestion on protein-benzo[a]pyrene interactions were studied. Protein-benzo[a]pyrene interactions were measured by determining the distribution change of benzo[a]pyrene between cellulose and protein. From the studies described it was concluded that the protein-benzo[a]pyrene interaction decreased with an increasing degree of protein-hydrolysis. Protein-benzo[a]pyrene interactions were not influenced by protein type.

INTRODUCTION

Through the years many data have been collected about dietary exposure to for instance micronutrients and, to a lesser extent, non-nutrients. Both the bioavailability and the influence of the diet matrix on this bioavailability are not entirely clear for many of these components. This lack of knowledge is especially true for non-nutrients such as mutagens and carcinogens. Dietary protein is known to be able to influence the bioavailability of both nutrients and medicine (178). Protein mutagen interactions were observed in earlier *in vitro* studies with the model mutagen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (179). In those studies protein-mutagen interactions were found to increase after protein digestion. Therefore, in the present study, the effects of protein type and protein digestion on protein-benzo[a]pyrene (B[a]P) interactions will be studied.

MATERIALS AND METHODS

In vitro binding experiments were performed by incubating protein (hydrolysate) solutions together with 10 mg of cellulose and approximately 2000 dpm (disintegrations per minute) ^{14}C labeled B[a]P (Amersham, 1.85 MBq/µmol) in a test tube. The final volume was always 1.00 ml. Influence on protein-B[a]P interactions were measured by determining the distribution change of B[a]P between cellulose and protein. After 30 minute incubation at 37 °C, test tubes were centrifuged to precipitate the cellulose fraction. Radioactivity was determined in the supernatant containing both B[a]P and protein using a liquid scintillation counter (Packard 1600TR). The experiment shown in Figure 4.4 was performed with small pieces of cellulose filter paper in stead of plain cellulose, so no centrifugation was necessary.

Protein digestion took place in the pH-stat equipment as described by Adler-Nissen (173). The degree of hydrolysis (DH) was expressed as the percentage of peptide bonds cleaved. Protein was digested for 30 minutes using pepsin (Merck, 2000 FIP/g) followed by a 90-minute pancreatin (Merck, 1400 FIP/g) digestion. All protein sources were at least 90% pure. Ovalbumin was heated until gelation and sonicated before use to disrupt gel structure.

RESULTS AND DISCUSSION

In the first experiment, reported in Figure 4.1, a dose response curve was made for the amount of cellulose in the incubation and the amount of ^{14}C B[a]P in the supernatant (protein fraction) after centrifugation. Based on this experiment further incubations were performed with 10 mg of cellulose. When casein was digested, the B[a]P binding capacity of the protein fraction decreased. This decrease was dependent on the degree of hydrolysis. At DH=17%, no protein-B[a]P interaction could be observed (Figure 4.2). Similar results were obtained when ovalbumin was hydrolyzed (Figure 4.3). To test whether there was a difference between different dietary protein types in their interaction with B[a]P, several protein sources were

compared (Figure 4.4). No obvious differences were observed between the different protein sources.

The results of above described experiments are contrary to results reported earlier with a model mutagen MNNG (179). Apparently the protein-B[a]P interaction is more dependent upon intact proteins whereas MNNG interacts with much smaller protein fractions such as present in protein hydrolysates. This difference in protein interaction may be caused by the fact that B[a]P in food is a large non-metabolized hydrophobic molecule whereas MNNG is a small and very reactive alkylating agent.

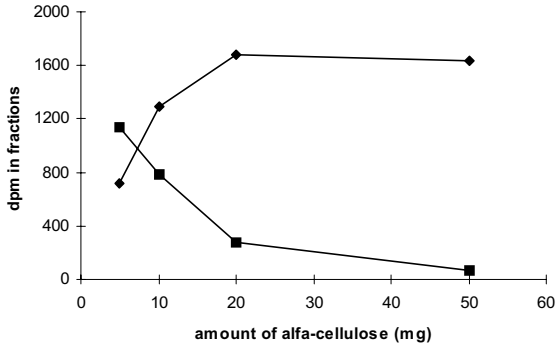

Figure 4.1 Relationship between the amount of cellulose in the incubation (1 ml, 0.1% protein) and the amount of ^{14}C B[a]P in both the cellulose and protein fraction after centrifugation. Cellulose-pellet (◆), Protein-supernatant (■).

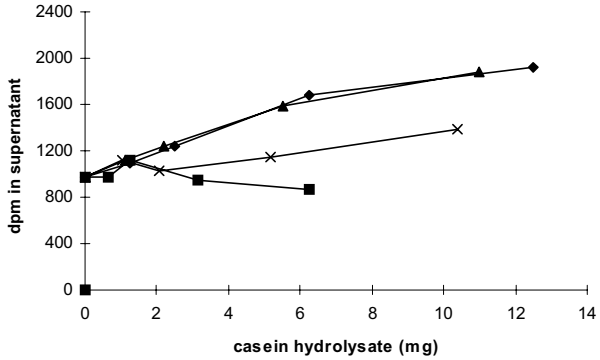

Figure 4.2 The relationship between the amount of casein hydrolysate, the degree of protein hydrolysis and the amount of ^{14}C B[a]P in the protein fraction after centrifugation. Incubation using 10 mg cellulose, total volume was 1.00 ml. Degree of hydrolyses (DH) = 0 (◆), DH = 5.1(▲), DH = 10 (**x**), DH = 17.1 (■).

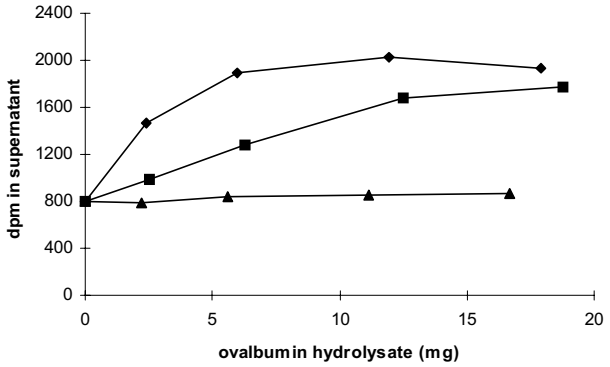

Figure 4.3 The relationship between the amount of ovalbumin hydrolysate, the degree of protein hydrolysis and the amount of ^{14}C B[a]P in the protein fraction after centrifugation. Incubation using 10 mg cellulose, total volume was 1.00 ml. Degree of hydrolyses (DH) = 0 (◆), DH = 5.3 (■), DH = 17.5 (▲).

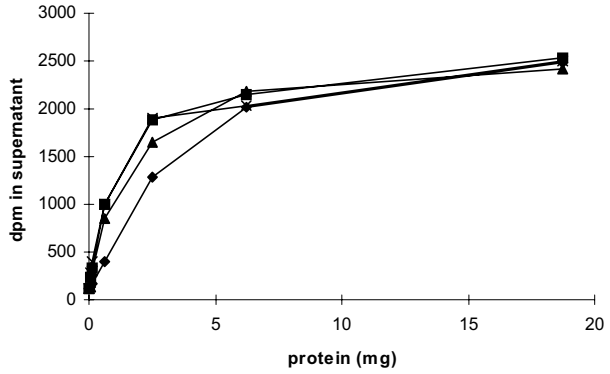

Figure 4.4 The relationship between the type of dietary protein and the amount of ^{14}C B[a]P in the protein fraction. Ovalbumin (◆), whey protein (■), BSA (▲), casein (**x**).

From the experiments described it was concluded that the protein-benzo[a]pyrene interaction decreased with an increasing degree of protein hydrolysis. Protein-benzo[a]pyrene interactions were not influenced by protein type.

Chapter 5

Possible mechanisms for the differential effects of soy protein and casein feedings on colon cancer biomarkers in the rat

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ABSTRACT

In the present studies, several hypotheses were investigated by which earlier reported differential effects of dietary proteins on colon cancer biomarkers could be explained. The studies described focussed at comparing casein and soy protein. Results of Study 1 show no influence of methionine, a limiting amino acid in soy protein, on colonic cell proliferation. From the same study it was concluded that fecal alkaline phosphatase excretion is a good marker for colonic epithelial damage whereas fecal magnesium excretion is not. In Study 2 it was shown that soy protein isolate and an ethanolic extract from soy protein isolate containing isoflavones and saponins, increased fecal fat excretion. Fecal water bile acid and free fatty acid concentrations were decreased after feeding soy protein based diets. No difference between groups in fecal alkaline phosphatase excretion was observed. In Study 3, the lytic potential of soy saponins and the interaction between saponins and some lytic bile acids, were tested *in vitro*. Data suggest a protective effect from soy saponins by reducing lytic activity of cholic acid. The overall conclusion is that soy protein, compared to casein, influences several colon cancer risk parameters indicating a protective rather than a stimulating effect on colon cancer risk.

INTRODUCTION

Colon cancer is a major health problem in many Western countries. Diet is one of the possible means of colon cancer prevention (7). Compared to fat and fibre relatively few experiments have been performed on the influence of protein concentration and protein source on colon cancer risk. Two early studies showed no difference between meat and soy protein diets on colon tumor formation (86, 87). However, more recently two studies reported stimulating effects of soy protein feeding. McIntosh (2) showed a stimulating effect of soybean meal compared to whey protein and casein on colon tumor formation in rats. Govers (4) found a stimulating effect of soy protein isolate feeding as compared to casein on several fecal colon cancer risk parameters and colonic cell proliferation in rats. Both studies found a correlation between the fecal fat excretion after feeding soy protein based diets, and respective colon cancer biomarkers.

There are several mechanisms by which a stimulating influence of soy protein isolate on colon cancer biomarkers could be explained. All dietary proteins differ in amino acid composition. Compared to casein, soy protein has a low methionine content (34). Methionine is one of the two sulfur containing amino acids that may have an influence on several colon cancer biomarkers. Methionine is an important methyl donor and might therefore influence DNA (36, 37) and nuclear protein methylation (38). DNA methylation in turn influences DNA transcription and therefore possibly colon cancer (39). The possible role of methionine was confirmed after studying two large human cohorts (36, 180). It was concluded that dietary methionine could possibly reduce incidence of colorectal cancer. However in an animal study using rats, Duranton (98) found that the addition of 1% methionine to the diet significantly increased ileal crypt cell proliferation, colonic aberrant crypts and colonic tumor incidence. Duranton (98) did not study a dose response relationship, and to our knowledge nobody has tested the effect of additional dietary methionine on colonic cell proliferation using ^{3}H-thymidine. Therefore, the effect of 0.0%, 0.2% and 1.0% methionine added to a casein based diet on the effect of colonic cell proliferation using the *in vivo* ^{3}H-thymidine assay (Study 1) was tested.

A second possible mechanism for the difference between soy protein isolate and casein is the presence of several non-protein factors within soy. Soy contains many non-nutrients such as isoflavones, lectins, phytate and saponins, each exhibiting their own biological activity (63). Saponins are known for their amphiphilic characteristics (61) and are therefore likely to interact with luminal fat. Earlier studies (2, 4) found a correlation between fecal fat and colon cancer risk. Therefore it was hypothesized that saponins present in soy protein preparations induce fecal fat excretion, thereby causing colonic damage. This might explain the positive correlation between consumption of saponin containing soy products and colon cancer biomarkers (2, 4). To test this hypothesis a second animal study (Study 2) was performed in which an ethanolic extract from soy protein isolate was added to both casein and soy protein

based diets. Ethanolic extracts from soy protein isolate contain both the isoflavones as well as the saponins present in the protein preparation.
The effect of the ethanolic extract of soy protein isolate on several fecal parameters was investigated. Fecal β-glucuronidase and β-glucosidase activities were measured as an indication of the release of liver conjugated toxicants and the colonic bacterial activity (181). Fecal fat excretion was measured, because of the earlier mentioned correlation between fecal fat excretion and several colon cancer biomarkers (2, 4). Concentrations of bile acids and free fatty acids in fecal water were measured because of their lytic potential (4, 131, 182). Cytolytic activity of fecal water was measured as an indication of the lytic potential of colonic content towards epithelial cells (183). Fecal activity of the epithelial alkaline phosphatase (ALP) iso-enzyme was measured as an indication of colonic epithelial cell damage (154). An increased fecal ALP excretion indicates a damaged colonic epithelium, thereby inducing epithelial proliferation. Fecal magnesium excretion was measured because it was hypothesized that fecal magnesium excretion induced by a soy protein diet represents colonic epithelial damage (4, 132).
A third possible mechanism for the effects of soy protein based diets on colon cancer risk is the possible interaction between saponins from soy and bile acids. Several studies report a possible correlation between fecal (water) bile acid concentration and colon cancer risk (151, 155, 184). Bile acids have a lytic potential and can therefore damage colonic epithelium. Damaged cells are replaced by induced cell proliferation. This hyperproliferation can lead to an increased colon cancer risk (106-108). Based on their comparable chemical structure, we hypothesize that soy saponins might have an interaction with bile acids, thereby influencing their lytic activity, either positively or negatively. The interaction between bile acids and saponins was investigated (Study 3) by testing the membrane disruptive (lytic) potential of separate and combined bile acid and saponin solutions on human red blood cells.

MATERIALS AND METHODS

Animals and housing (Study 1 and 2) Both experimental protocols were approved by the animal welfare committee of Wageningen University. The rats were housed individually in metabolism cages, in a room with controlled temperature (± 20 °C), relative humidity (50-60%) and a 12h/12h light cycle. Food intake and body weight were recorded three times a week.
In Study 1 the influence of dietary methionine and cholic acid (positive control) on colonic cell proliferation was tested. For this purpose 34 Wistar outbred rats (Harlan, Zeist, The Netherlands) 9 weeks of age at the start of the experiment, with a mean body weight of 205 g were used.

In Study 2 the influence of an ethanolic extract from soy protein isolate on fecal fat excretion and several fecal water parameters was tested. In this study 40 male F334 rats (Harlan, Zeist, The Netherlands) 9 weeks of age at the start of the experiment, with a mean bodyweight of 203 g were used.

Diets (Study 1 and 2) Experiments were performed using high fat, high protein diets. Calcium concentrations were kept low (25 mmol/kg diet) because calcium is known to influence colon cancer risk parameters (50, 154). Food and water was available *ad libitum*. Diet compositions are shown in Table 5.1. Acid casein (DMV, Veghel, The Netherlands) was used because it contains low amounts of calcium compared to whole casein. Soy protein isolate (Supro 500E) was purchased from Protein Technology International (Ieper, Belgium). Supro 500E is a standard soy protein isolate often used for human consumption and for preparation of specialized soy protein products. Supro 500E is produced in bulk. Supro 500E was formally known as Purina protein.

Table 5.1 Composition of the diets (Study 1 and 2). Numbers are expressed in g/kg. Diets for Study 1 were prepared in our laboratory, diets for Study 2 were prepared by Hope Farms (Woerden, The Netherlands), Met = methionine, CA = 0.35% Cholic Acid diet. [1] Acid casein, (DMV, Veghel, The Netherlands), [2] soy protein isolate (Supro 500E, Protein Technology International, Ieper, Belgium), [3,4,8] Sigma, [5] described in Table 5.2, [6,7,9,10,11] were obtained from Hope Farms, [12,13] Study 1 according to (185), Study 2 based on (34).

	Study 1				**Study 2**				
	Casein	**0.2 Met**	**1.0 Met**	**CA**	**Casein**	**Soy**	**Cas +**	**Soy -**	**Soy +**
Casein [1]	200	198	189	200	250	-	250	-	-
Soy [2]	-	-	-	-	-	250	-	250	250
DL-Methionine[3]	0	2	10	0	2	2	2	2	2
Cholic acid[4]	-	-	-	3.5	-	-	-	-	-
Ethanol extract [5]	-	-	-	-	0	0	3.3	0	3.3
Cellulose[6]	100	100	100	100	30	30	30	30	30
Dextrose[7]	450	450	450	446.5	481.7	481.7	478.4	481.7	478.4
Choline 50%[8]	5	5	5	5	4	4	4	4	4
Milkfat[9]	180	180	180	180	-	-	-	-	-
Palm oil[10]	-	-	-	-	180	180	180	180	180
Corn oil[11]	20	20	20	20	20	20	20	20	20
Vitamin mix [12]	10	10	10	10	2.5	2.5	2.5	2.5	2.5
Mineral mix [13]	35	35	35	35	29.8	29.8	29.8	29.8	29.8
Total	1000	1000	1000	1000	1000	1000	1000	1000	1000

Diets used in Study 1 were composed to resemble the diet used by Govers (4). Besides a reference casein diet, a 0.2% methionine, 1.0% methionine and a 0.35% cholic acid (positive

control) diet were used. The diets of Study 2 were based on either casein or soy protein isolate. To test the influence of soy non-nutrients on parameters tested, ethanolic extracts were prepared at room temperature from soy protein isolate and added to some diets (186). The ethanolic extract was prepared by extracting soy protein isolate with 70% ethanol. The ethanolic fraction was separated from the protein by a fine steel grid filter under vacuum. After extraction the ethanol was evaporated under an air stream. When most of the ethanol was evaporated the remaining solution was freeze dried and powdered before mixing with the diet. The amount of non-nutrients added via the powder extract was equal to the amount of non-nutrients present in the regular soy protein diet. The ethanolic extract was characterized on chemical composition as shown in Table 5.2. Five different diets were prepared: Cas, Soy, Cas +, Soy - and Soy +. The '+' or '-' denotes whether soy non-nutrients were either removed or added to the protein source. A low fibre diet, using palm oil as the major fat source, was chosen because this dietary background is often used in studies on the effects of diet on fecal parameters (52, 83).

Table 5.2 Chemical composition of the ethanolic extract from soy protein isolate (Study 2).
[1] Estimated from (187, 188).

	Weight %
Protein N	5
Non-protein N	0.3
Fat	31
Sugar	5
Minerals	10
Saponins [1]	12.5
Isoflavones	5
Unknown	31.5

Fecal analysis (Study 2) All fecal parameters were measured using feces collected at day 10 and day 11 of the experimental diet. Total fecal fat was gravimetrically determined after petroleum ether extraction (124). For the fecal magnesium determination feces was destructed by mixing 300 mg dried feces with 2.5 ml digestion mixture (7.2 gram salicylic acid and 0.35 g selenium in 100 ml 96% sulfuric acid). The mixture was heated for 2 hours at 100 °C. After cooling, 3 times 1 ml of 30% H_2O_2 was added. After mixing, the remaining solution was heated at 330 °C until the solution was colorless or light yellow. The remaining solution was diluted with a lanthanum solution and magnesium was measured in an air-acetylene flame spectrophotometer (285.2 nm).
For the bile acid determination fecal water was prepared by mixing 430 mg homogenized freeze dried feces and 1.0 ml demineralized water. For the other parameters, fecal water was

prepared by incubating 200 mg feces with 1.0 ml of water. After an incubation in a shaking water bath at 37 °C for 60 minutes, the homogenate was centrifuged for 11 minutes at 15000 g. Fecal water was removed and stored at -20 °C until further use.

Bile acid concentrations were determined after enzymatic conversion using a fluorimetric detection method (189). The free fatty acids were determined spectrophotometrically after enzymatic conversion. A kit was used (NEFA-C, Wako Chemicals) following the instructions of the manufacturer. Cytolytic activity was determined using human O-negative erythrocytes (190). When erythrocytes lyse, hemoglobin from the cytosol is excreted. After centrifugation all intact cells are pelleted and the amount of hemoglobin present in the supernatant, representing lysis, can be determined spectrophotometrically at 540 nm. Demineralized water was used as a positive control causing 100% lysis. To investigate the influence of buffered assay conditions on the cytolytic activity, experiments were performed with and without some extra 1.5 mM HEPES buffer (pH=7.4). Intestinal alkaline phosphatase (ALP) activity was measured spectrophotometrically using a kinetic protocol. L-phenylalanine was used as a specific inhibitor of the intestinal isozyme (154).

Ethanolic extract characterization (Study 2) Protein was determined using Kjeldahl by determining protein and non-protein nitrogen (191). Fat content was determined gravimetrically after petroleum ether (60-80 °C, Merck) extraction (126). Sugar content was determined according to Dubois (192). Mineral content was determined gravimetrically after ashing for three hours at 550 °C. Saponins could not be determined chemically and were therefore estimated from literature (187, 188). After extraction of soy protein isolate, isoflavone content was determined using HPLC (193, 194).

In vitro saponin/bile acid interaction (Study 3) To study the lytic potential of soy saponins and their interaction with lytic bile acids, erythrocytes were incubated with either saponin, bile acid or mixed solutions. After one hour of incubation at 37 °C, lysis was compared to demineralized water as a positive control. Cholic acid and deoxycholic acid were purchased from Sigma. Saponin solutions were prepared from two commercial preparations: Soylife100 (Soylife, Giessen, The Netherlands) containing 10% saponins and Novasoy (ADM, Rozenburg, The Netherlands) containing 40% saponins. Of both products 100 mg was extracted three times with 70% ethanol. The ethanolic fraction was centrifuged and dried under vacuum. Remaining dried material was dissolved in 0.9% saline.

Statistics Statistical analysis was performed using SPSS version 7.5. Data were analyzed using general factorial analysis of variance after testing for normality and homogeneity of variance. In case of homogeneity, the Bonferroni test of significance was used for post hoc analysis ($p \leq 0.05$). In case of non-homogeneity, data were tested on significance with the Dunnett T3 test ($p \leq 0.05$). Correlation coefficients were calculated according to Pearson (two sided). When data were not normally distributed a Kruskall Wallis test was used.

RESULTS

For Study 1 the animal weight, fecal wet weight, fecal dry weight, water percentage in feces and the food intake was equal for all three dietary groups. However, the 1% methionine group was the lowest on all these parameters (Table 5.3).

Table 5.3 Animal, food and fecal parameters (Study 1 and 2). Data are shown as average ± SD. Groups with the same letter do not significantly differ within one study. Met=methionine.

	Diet	Animal weight (g)	Fecal wet weight (g)	Fecal dry weight (g)	Water in feces (%)	Food intake (g/d)
Study 1	Casein	355±17[a]	2.9±0.6[a]	1.8±0.4[a]	14.0±2.6[a]	17.0±1.0[a]
	0.2% Met	348±10[a]	3.0±0.4[a]	1.8±0.2[a]	14.2±2.6[a]	16.2±0.9[a]
	1.0% Met	347±11[a]	2.6±0.4[a]	1.6±0.3[a]	13.3±2.1[a]	16.1±1.2[a]
Study 2	Casein	270±22[a]	1.36±0.41[a]	0.63±0.17[a]	67±7[a]	20.6±1.3 [a]
	Soy	294±24[a]	2.12±0.53[b]	0.95±0.14[b]	67±12[a]	22.7±1.8[a]
	Cas +	270±16[a]	1.50±0.40[a]	0.60±0.17[a]	65±8[a]	21.4±1.7[a]
	Soy -	283±20[a]	2.20±0.34[b]	0.97±0.22[b]	65±12[a]	22.6±2.2[a]
	Soy +	284±21[a]	2.06±0.50[b]	0.96±0.15[b]	65±8[a]	22.1±1.3[a]

The amount of DNA scraped from the proximal and total colon was equal for all dietary groups. The amount of distally scraped DNA was different between the control and the 1% methionine group. There was no difference in colonic cell proliferation (^{3}H-thymidine counts per µg of DNA) either proximally, distally or totally (Table 5.4), between the diets containing either 0%, 0.2%, or 1.0% methionine.

Table 5.4 DNA and cell proliferation data (Study 1). Data are shown as average ± SD. Groups with the same letter do not significantly differ. Met=methionine.

	Casein	0.2% Met	1.0% Met
DNA proximal (µg)	300±80[a]	321±86[a]	340±98[a]
DNA distal (µg)	273±55[a]	350±139[a,b]	347±31[b]
DNA total (µg)	570±104[a]	665±185[a]	692±85[a]
Colonic cell proliferation proximal (dpm/µg DNA)	47±14[a]	48±16[a]	48±17[a]
Colonic cell proliferation distal (dpm/µg DNA)	54±29[a]	50±21[a]	45±21[a]
Colonic cell proliferation total (dpm/µg DNA)	51±18[a]	48±15[a]	46±18[a]

In Study 2 there was no difference between the animal weights of the five dietary groups. The fecal wet weight was significantly lowered for both casein containing diets. Fecal dry weight

for both casein diets was also lower. There was no difference in water percentage in the feces. Food intake was equal for all dietary groups.
Data on fecal fat excretion are shown in Figure 5.1. Soy protein based diets show the highest fat excretion at day 16 of the experiment. In all soy protein and casein diets, fecal fat excretion increased when more soy non-nutrients were present. Differences between the diets are relatively small. There seems to be no major effect of the number of days the animals have been fed the several diets, although at day 3 of the experiment fecal fat excretions seem to be somewhat lower compared to day 7 and 16.

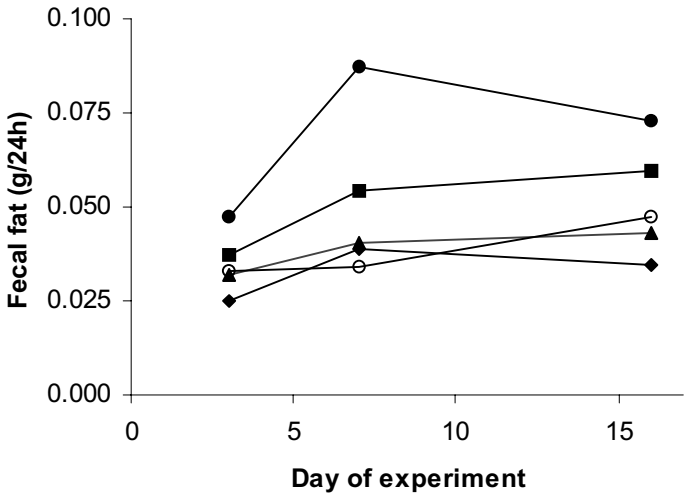

Figure 5.1 Fecal fat excretion in time after feeding different diets, containing either casein or soy protein and variable amounts of soy non-nutrients (Study 2). The coefficient of variation (CV) was approximately 30% (day 3 and 7) and 20% (day 16). Cas (◆), Soy (■), Cas + (▲), Soy - (**O**), Soy + (●).

In Table 5.5 results of the fecal parameters of Study 2 are shown. No significant differences were found for fecal ALP and Mg excretion. There was a trend towards a higher fecal water short chain fatty acid concentration in the soy fed group. The pH of the soy fecal water was significantly lowered compared to the casein control group, possibly because of the increased short chain fatty acid concentration. Fecal water bile acid and free fatty acid concentrations were significantly different between all groups. In both cases highest concentrations were found in groups on a casein protein diet.

Table 5.5 Fecal (water) parameters (Study 2). Data are shown as average ± SD. Statistically significant differences compared to the reference diet (Cas) are denoted with * (p≤0.05), ** (p≤0.01). KW means groups are significantly different using Kruskal-Wallis.

	Cas	Soy	Cas +	Soy -	Soy +
pH	8.2±0.3	7.7±0.3**	7.9±0.1	8.0±0.2	7.9±0.2
Bile acids (mM)	4.2±3.1^{KW}	0.4±0.5^{KW}	2.5±1.0^{KW}	1.3±0.6^{KW}	0.9±0.9^{KW}
Free fatty acids (µM)	540±302^{KW}	38±21^{KW}	245±231^{KW}	126±102^{KW}	5±30^{KW}
ALP (µmol/ml/min)	10.5±4.0	6.1±6.0	11.4±5.9	7.4±4.8	9.8±10.4
C2 (mg/l)	337±122	516±160	398±140	403±166	379±112
C3 (mg/l)	97±21	140±43	109±43	120±40	105±36
iC4 (mg/l)	14.3±5.0	13.4±3.9	11.0±2.1	14.1±3.6	13.0±3.3
nC4 (mg/l)	14.9±5.1	35.6±16.7**	18.7±10.2	35.3±6.4	28.7±11.2
bC5 (mg/l)	23.0±10.9	20.0±4.3	21.1±3.4	23.7±7.5	22.4±5.4
nC5 (mg/l)	4.4±7.0	10.1±6.3	13.6±4.3**	10.8±4.0	11.0±3.9
total SCFA (mg/l)	495±165	735±228	576±208	605±239	559±161
Mg (µmol/g)	67.9±9.5	68.3±8.6	67.5±8.2	70.8±7.8	70.8±7.4

In Figure 5.2 some of the results of the saponin/bile acid interactions studies are shown (Study 3). Figure 5.2a shows a dose effect curve for the lytic potential of two soy saponin preparations. The Novasoy saponin extract shows a dose dependent lytic effect. Soylife shows no lytic activity for the concentrations tested. In Figure 5.2b and 5.2c some interactive effects between bile acids and Soylife saponins are shown. Soylife saponins (Figure 5.2b) show no lytic effects, but the cytolytic activity of cholic acid increases with increasing concentration. The theoretical sum of the saponin and cholic acid mixture equals the cytolytic activity as measured for cholic acid because Soylife saponin lysis was close to 0%.

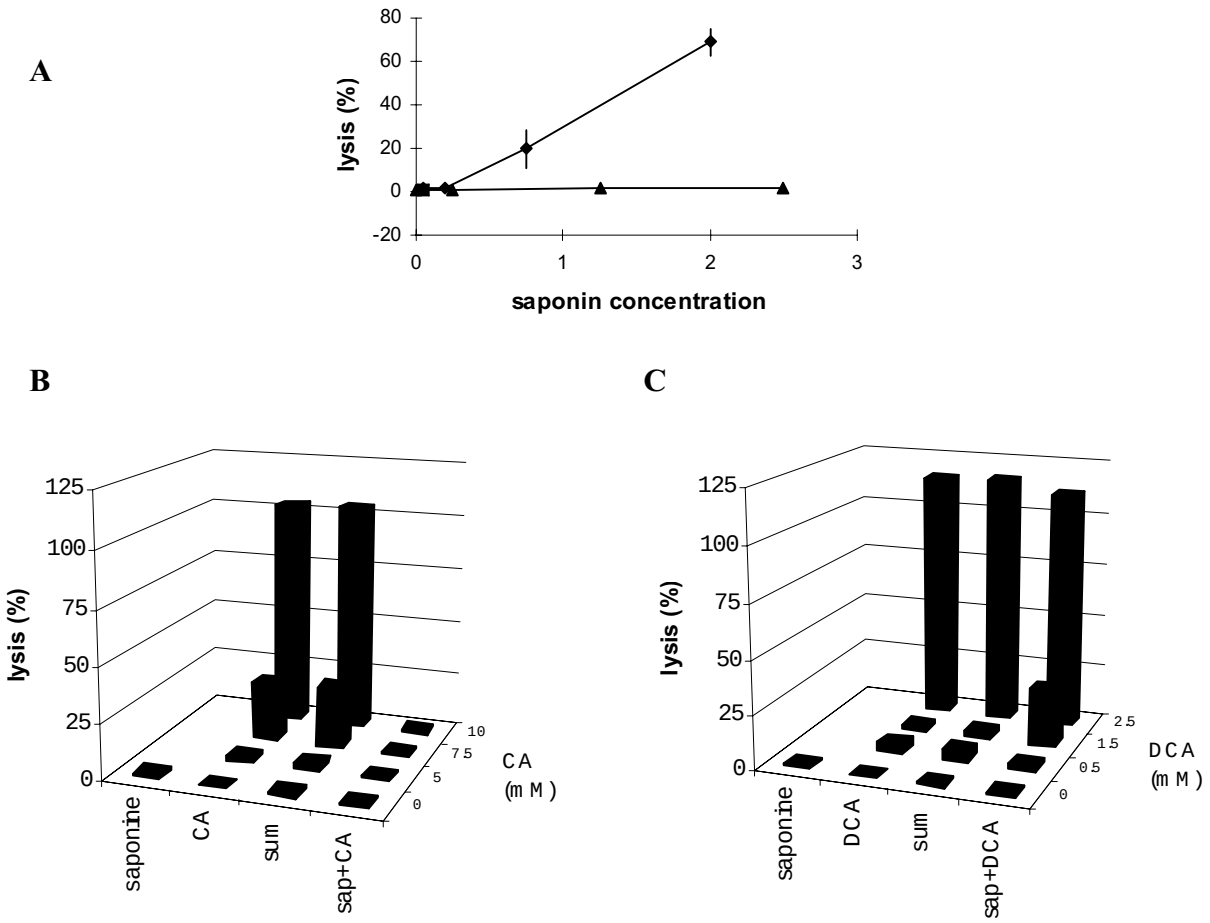

Figure 5.2 A) Lytic potential of two commercial soy saponin preparations. Data are shown as average ± SD. Novasoy (◆), Soylife (■). Saponin solutions contained 0.25 mg/ml saponins. The coefficient of variation (CV) was approximately 10% for all data shown.
B,C) Interaction between saponins and bile acids on *in vitro* cytolytic activity (Study 3). DCA = deoxycholic acid, CA = cholic acid, Sum represents the theoretical sum of the cytolytic activity from the saponin and the bile acid solution separately, Sap + (D)CA represents the cytolytic activity of a combined saponin/bile acid solution.

The results from mixing saponins and cholic acid however show that the cytolytic activity caused by cholic acid fully disappears when Soylife saponins are present. These data show that saponins inhibit the cytolytic potential of cholic acid. In Figure 5.2c deoxycholic acid is tested in stead of cholic acid. No inhibition of the cytolytic activity of the deoxycholic acid is shown, suggesting the antagonistic effect is bile acid specific. When incubating saponins together with 1.5 mM of deoxycholic acid, a small synergistic effect was observed. Bile acid/saponin interaction studies, using Novasoy saponins, showed no synergistic or antagonistic effects (data not shown).
In the first animal study (Study 1), a cholic acid group was included as a positive control on colonic cell proliferation and fecal parameters. There were no differences in animal weight or

food intake between the casein or the casein + cholic acid fed animals. Fecal dry weight was equal for both dietary groups, but the fecal wet weight was significantly increased for the cholic acid fed group, caused by a significant increase in the percentage of water in the feces (data not shown). In Table 5.6 colonic and fecal parameters are shown for the 0.35% cholic acid group. There is no difference in the amount of DNA scraped from proximal or distal colon, however the amount of DNA scraped for the total colon is significantly increased in the cholic acid group. Colonic cell proliferation is increased for proximal, distal and total colon. No differences were observed for the pH of fecal water. Cytolytic activity was only different between dietary groups after addition of a buffer. After buffer addition cytolytic activity decreased in the casein fed group but only reached significance at day 10. Fecal water ALP activity showed a strong increase in the cholic acid supplemented group. This increase significantly correlated with colonic cell proliferation (r=0.75). Total fecal magnesium excretion significantly decreased after feeding the cholic acid supplemented diet. For fecal parameters significantly different between dietary groups, the correlation between both days measured was high: ALP (r=0.95), bile acids (r=0.87), magnesium (r=0.88).

Table 5.6 Colonic and fecal (water) parameters after a 0.35% cholic acid diet compared to casein (Study 1).Data are shown as average ± SD. Statistically significant differences compared to the reference diet (Cas) are denoted with * ($p \leq 0.05$), ** ($p \leq 0.01$). KW means groups are significantly different using Kruskal-Wallis.

	Casein	**+ 0.35% cholic acid**
DNA (proximal) in µg	300 ± 80	376 ± 154
DNA (distal) in µg	273 ± 55	360 ± 194
DNA (total) in µg	570 ± 104	736 ± 178 *
Proliferation (proximal) in dpm/µg	47 ± 14	74 ± 20 *
Proliferation (distal) in dpm/µg	54 ± 29	128 ± 33 **
Proliferation (total) in dpm/µg	51 ± 18	107 ± 26 **
pH (day 10)	7.3 ± 0.5	7.6 ± 0.3
pH (day 11)	7.1 ± 0.6	7.4 ± 0.5
Bile acids (day 10) (µM)	61 ± 17	575 ± 122 **
Bile acids (day 11) (µM)	70 ± 22	798 ± 238 **
Cytolytic activity (day 10) (%)	56 ± 28	45 ± 11
Cytolytic activity (day 11) (%)	63 ± 23	47 ± 8
Cytolytic activity (buffered) (day 10) (%)	9 ± 30	49 ± 9 ^{KW}
Cytolytic activity (buffered) (day 11) (%)	12 ± 25	34 ± 22
Alkaline phosphatase (day 10) (µmol/ml/min)	2.2 ± 2.2	12.0 ± 6.8* *
Alkaline phosphatase (day 11) (µmol/ml/min)	1.4 ± 1.7	11.9 ± 6.7 **
Magnesium (day 10) (µmol/g)	80 ± 13	57 ± 6 **
Magnesium (day 11) (µmol/g)	67 ± 12	61 ± 8 **

DISCUSSION

The main purpose of the studies performed was to test several mechanisms that could explain the possible, earlier reported, stimulating effects of soy protein compared to casein on colon cancer risk biomarkers. In the several studies described, differential effects between casein and soy protein feeding on colon cancer risk parameters were shown. However, no indications were found that soy protein feeding increases colon cancer risk.

Casein and soy protein isolate differ in their methionine content (34). The results of Study 1 show that there is no effect of 1% extra dietary methionine on *in vivo* colonic cell proliferation using the ^{3}H-thymidine cell proliferation assay. This suggests that the difference in methionine content can not explain the differences on colon cancer risk biomarkers after feeding casein or soy protein isolate (2, 4). Our data are closer to the protective effects of methionine found in epidemiological studies (36, 180) than the results found by Duranton (98). Duranton found a small but significant increase in the ileal crypt cell proliferation, colonic aberrant crypts and colonic tumor incidence after feeding a 1% methionine diet. The difference between our results and those found by Duranton (98) might be explained by different methods used to determine colonic cell proliferation. To obtain a more definite answer on the influence of methionine on colon cancer risk in rats, either a study should be reproduced or a long term study on the influence of methionine on tumor formation should be performed.

The main purpose of Study 2 was to further investigate the relationship between soy protein feeding and fecal fat induction. It was hypothesized that the ethanol soluble fraction from soy protein isolate, containing saponins, was responsible for the induced fecal fat excretion found after feeding soy protein. Results showed an increased fecal fat excretion after feeding soy protein diets (1.7 fold) compared to casein at day 16. This is close to the 2 fold fecal fat induction as reported by McIntosh (2) and to several studies performed by our group recently (195, 196). An additionally increased fecal fat excretion was found after the addition of soy protein extracts. At day 16 there was an extra fecal fat induction of Cas + compared to Cas (1.23 fold), Soy compared to Soy - (1.23 fold) and Soy + compared to Soy (1.24 fold). The 4.5 fold fecal fat induction as reported by Govers (4) can not be explained by a high concentration of ethanol soluble non-nutrients. A 4.5 fold fecal fat induction seems to be more of an exception than a rule, indicating that also the increase in colonic cell proliferation found by Govers (4) after feeding soy protein might not be representative for soy protein isolates in general.

Fecal parameters measured in Study 2 do not indicate adverse colonic health effects of soy protein feeding. No significant differences in colonic epithelial cell damage (ALP) were observed. Because concentrations of lytic fecal water bile acids and free fatty acids appeared to be lower for soy protein fed animals compared to casein protein fed animals, a possible protective effect of soy protein feeding is suggested. The lack of differences between groups in fecal magnesium excretion and the small increase of fecal magnesium excretion as found

by Brink (131) again indicate that the 2 fold induction of fecal magnesium excretion found by Govers (4) can not be extrapolated to soy protein isolates in general.
In the *in vitro* saponin/bile acid interaction studies, the lytic activity of saponins, bile acids and saponin/bile acid mixtures (Study 3) were investigated. The results show that soy saponins can have some lytic potential, depending on the saponin source, varying between different commercial saponin products. The interaction studies show that soy saponins can have strong interactions with cholic acid, reducing its lytic potential. This can be interpreted as a protective effect of soy protein preparation on bile acid induced colonic epithelial damage. The absolute values for especially the fecal water bile acid concentrations and the fecal ALP excretion between Study 1 and Study 2 are different. In the case of the fecal water bile acid concentrations, this is probably due to the fact that the fecal water used to determine the bile acids was more concentrated for Study 1 than it was for Study 2 (see Materials and Methods). Difference in ALP excretion is unexplained although the different dietary backgrounds used for both studies could be an explanation.
In Study 1, a positive control (cholic acid) was included. As expected, cholic acid fed animals showed a strong increase in colonic cell proliferation, fecal ALP excretion and fecal bile acid excretion. The correlation between colonic cell proliferation and the fecal ALP excretion was high, indicating that ALP excretion is a good biomarker for colonic epithelial damage. We hypothesized that fecal magnesium excretion was also a possible biomarker for epithelial damage in the colon, because soy protein isolate stimulates both fecal (131) and endogenous (132) magnesium excretion and colonic epithelial damage (4). Because we found a consistent decrease of fecally excreted magnesium in animals showing a strong increase in colonic cell proliferation and fecal ALP excretion, we have to conclude that fecal magnesium excretion is not a marker for colonic epithelial damage. The fact that fecal magnesium excretion is consistently decreased after cholic acid feeding, remains to be explained.
Some results described in this paper show a possible protective effect of soy protein feeding compared to casein on colon cancer risk. This is confirmed by preliminary results from Thiagarajan (68) showing a possible protective effect of soy protein consumption on colonic cell proliferation in humans. Also, results from Hakkak (66) show a protective effect of soy protein isolate consumption on colon tumor incidence after feeding soy protein isolate for two generations. Therefore it is concluded that soy protein compared to casein influences several colon cancer risk parameters indicating a protective rather than a stimulating effect on colon cancer risk.

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Chapter 6

Casein and soy protein isolates differ on fecal colon cancer biomarkers but not on colonic cell proliferation in rats

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ABSTRACT

Compared to fat and fibre there is relatively little information on the influence of dietary protein on colon cancer risk. In the present study the influence of casein compared to two brands of soy protein isolate on several fecal colon cancer biomarkers and colonic cell proliferation, was tested. Compared to animals on a casein diet, soy protein fed animals showed a significantly increased fecal fat excretion, significantly decreased fecal water bile acid concentrations and 10-100 fold induced intestinal β-glucuronidase and β-glucosidase activities. The intestinal alkaline phosphatase excretion indicating colonic epithelial damage was significantly decreased after soy protein feeding. However, no difference in colonic cell proliferation, either proximally or distally, was found. This result was supported by the lack of difference between casein and soy protein diets on other fecal parameters, such as cytolytic activity and fecal magnesium excretion. An additional animal study was performed to test the influence of changing dietary vitamin and mineral composition on the influence of casein and soy on colonic cell proliferation. The lack of a differential effect between casein and soy protein was confirmed. In summary it is concluded that, compared to casein, dietary soy protein isolate influences several fecal colon cancer risk parameters, but does not influence colonic cell proliferation.

INTRODUCTION

Colon cancer is a major health problem in many Western countries. Dietary habits appear to have a major influence on colon cancer incidence. Already a few decades ago it was estimated that the diet is responsible for up to 90% of all colon cancer cases (11). Because diet has a major influence on colon cancer incidence, consuming a healthy diet offers a possibility for colon cancer prevention (7). Data on protein and colon cancer mostly concern the relationship between *amount* of dietary protein and colon cancer risk (26, 29, 30). Little is known about the influence of dietary protein *type* on colon cancer risk, possibly because some early studies showed no difference between for example meat protein and soy protein (86, 87). However, more recently Govers (4) showed that soy protein isolate induced fecal colon cancer biomarkers and colonic cell proliferation compared to the milk protein casein. Cell proliferation is often associated with carcinogenesis in rodents and humans (106-108). McIntosh (2) showed a negative effect of soybean meal as compared to the dairy proteins casein and whey in the dimethylhydrazine (DMH) induced rat colon cancer model. McIntosh (69) also showed that rats on a soy protein diet had more aberrant crypt foci in their colon than did whey protein fed rats.

Besides several colonic markers such as cell proliferation, aberrant crypt foci or presence of tumors, several colon cancer risk parameters can also be measured in feces. These fecal markers include both biomarkers of exposure (bacterial enzymes activities, fecal fat excretion, lytic activity and composition of fecal water) as well as biomarkers of effect (alkaline phosphatase (ALP) excretion and possibly magnesium excretion). In Study 1 several fecal parameters were included. Fecal β-glucuronidase and β-glucosidase activities were measured as an indication of the release of liver conjugated toxicants and the colonic bacterial activity (181). Fecal fat excretion was measured because in earlier studies fecal fat excretion correlated with colonic epithelial cell damage and DMH-induced colonic tumors respectively (2, 4). Concentrations of bile acids and free fatty acids in fecal water were measured because of their lytic potential (4, 131, 182). Cytolytic activity of fecal water was measured as an indication of the lytic potential of colonic content towards epithelial cells (183). Fecal activity of the epithelial ALP iso-enzyme was measured as an indication of colonic epithelial cell damage (154). An increased fecal ALP excretion indicates a damaged colonic epithelium, possibly resulting in induced epithelial proliferation. Fecal magnesium excretion was measured because it was hypothesized that fecal magnesium excretion induced by a soy protein diet represents colonic epithelial damage (4, 131, 132).

For the interpretation of studies on soy protein and colon cancer, different problems arise. First of all, different authors use different kinds of protein preparations. Thiagarajan (64) showed that different soy protein preparations result in differences on colon cancer biomarkers. For example, Govers (4) used a soy protein isolate, whereas McIntosh (2) used a soybean meal containing large amounts of soy fibre. Another problem is the exact composition of the dietary protein sources. An important variable in casein preparations is the

amount of calcium present. Calcium is known to influence colon cancer risk (50, 154) and should therefore be kept constant. In the present study acid casein was therefore used, containing low amounts of calcium. Soy protein preparations usually also contain many non-nutrients such as phytate, lectins, isoflavones and saponins in variable amounts. Each of these components can have differential effects on colon cancer risk parameters (63). Because of the variable amounts of soy non-nutrients we hypothesize that results using soy protein isolates vary between batches and/or soy brands. To test this hypothesis, all parameters were determined for two different soy protein isolates. Another batch from one of these isolates was used in earlier research by others (4).

In this paper the results of two animal studies are described (Study 1 and Study 2). The first objective of Study 1 was to compare two different soy protein isolate brands on several fecal colon cancer risk markers. The second objective was to compare two different soy protein isolate brands on the influence of colonic cell proliferation. The third objective of Study 1 was to more precisely localize the possible effect of casein and soy protein feeding to the proximal or distal colon.

Because in Study 1 our vitamin mineral mixtures deviated somewhat from standard AIN-recommendations, an extra animal study was performed to exclude the possibility that this strongly influenced the results of Study 1. Therefore, the aim of Study 2 was to test whether changing vitamin and mineral mixtures modulates the influence of casein and soy protein isolate on colonic cell proliferation.

MATERIALS AND METHODS

Animals and housing The experimental protocols were approved by the animal welfare committee of Wageningen University. For both studies male Wistar outbred rats from Harlan (Zeist, The Netherlands) were used. For Study 1, 30 animals, 9 weeks of age at the start of the experiment with a mean bodyweight of about 210 g, were used. The rats were housed individually (n=10 per group) in metabolism cages. For Study 2, 18 animals, 9 weeks of age at the start of the experiment with a mean bodyweight of about 205 g, were used. Rats were also housed individually (Casein n=10, Soy n=8) in metabolism cages. All rats were housed in a room with controlled temperature (± 20 °C), relative humidity (50-60%) and a 12h/12h light cycle. Food and water were available *ad libitum*. Food intake and body weight were recorded three times a week.

Diets For Study 1, rats were fed three different protein diets: Casein, Soy-1 and Soy-2. For the Casein diet, acid casein was used (DMV, Veghel, The Netherlands). For the Soy-1 diet, Soy-Prot-S was used as a soy protein isolate (Loders Croklaan B.V., Wormerveer, The Netherlands). For the Soy-2 diet, Supro 500E was used as a soy protein isolate (Protein Technology International, Ieper, Belgium). Supro 500E was formally known as Purina

protein. This was the soy isolate brand used earlier by Govers (4). Both soy protein preparations are used for human consumption.
Diets will be referred to as Casein, Soy-1 and Soy-2. The composition of the diets was comparable to those used by Govers (4), except for the vitamin mixture used and for the 0.2% methionine added to the Casein diet. The final composition of the diets is shown in Table 6.1.

Table 6.1 Ingredient composition of experimental diets (Study 1). [1] Acid casein, [2] Soy-prot-S, [3] Supro 500E.

	Casein	**Soy-1**	**Soy-2**
	g/kg		
Casein [1]	200	-	-
Soy-1 [2]	-	200	-
Soy-2 [3]	-	-	200
Milk fat	180	180	180
Corn oil	20	20	20
Dextrose	462	462	462
Cellulose	100	100	100
Vitamin mix	2.5	2.5	2.5
Trace-element mix	2.5	2.5	2.5
Salt mix	23.0	23.0	23.0
$CaHPO_4 \cdot 2H_2O$	4.3	4.3	4.3
Methionine synth.	2.0	2.0	2.0
Choline Cl 50%	4.0	4.0	4.0
Total	1000	1000	1000

Diets were presented as mixtures of 25.0 g of dry powder and 11.7 g of demineralized water. Dietary calcium concentrations were kept low because it is known that calcium interferes with colon cancer risk (50, 154). Hope Farms (Woerden, The Netherlands) prepared the diets. After 5 days of acclimatization, animals were fed the diets for 3 weeks prior to dissection.
For Study 2 casein and soy protein diets were prepared as described above. A difference between the diets for Study 1 and Study 2 was their vitamin composition. For Study 1 a commercial vitamin mixture based on NRC recommendations (34) from Hope Farms was used. For Study 2, both vitamin and mineral mixtures were according to AIN-76 (185) as used by Govers. (4). The commercial Hope Farms vitamin mixture differed from AIN recommendations in a way that all the B-vitamins, vitamin A and vitamin D in the commercial diet were 2-3 fold higher than AIN recommendations. Supro 500E was again used as a soy protein source. After 5 days of acclimatization, animals were fed the diets for 2 weeks prior to dissection.

Dissection and parameters studied in the colon In both studies animals obtained an intraperitoneal injection containing 100 μCi/kg ^{3}H-thymidine after feeding the diets for 2-3 weeks. After two hours, rats were anesthetized using CO_2 and the colon was removed. After rinsing with saline, the colonic epithelium was scraped using a spatula. The scraping was used for DNA determination and ^{3}H-thymidine count. DNA was determined colorimetrically using diphenylamine (197) with calf thymus DNA as a reference. ^{3}H-thymidine count (198) was determined using a 1600 TR liquid scintillation analyzer (Packard, Groningen, The Netherlands). Ultima Gold-AB (Packard, Groningen, The Netherlands) was used as a scintillation liquid. Radioactivity was expressed as disintegrations per minute (dpm).

Fat and Mg in feces Fat and magnesium determinations were only performed for Study 1. Total fat concentrations in feces were gravimetrically determined after an extraction with petroleum benzine (60-80 °C, Merck) (126). Fecal fat excretion was expressed both as mg fat/g wet weight as well as mg fat/day. For the fecal magnesium determination feces was destructed by mixing 300 mg dried feces with 2.5 ml digestion mixture (7.2 gram salicylic acid and 0.35 g selenium in 100 ml 96% sulfuric acid). The mixture was heated for 2 hours at 100 °C. After cooling, 3 times 1 ml of 30% H_2O_2 was added. After mixing, the remaining solution was heated at 330 °C until the solution was colorless or light yellow. The remaining solution was diluted with a lanthanum solution and magnesium was measured in an air-acetylene flame spectrophotometer (285.2 nm).

Determinations in fecal water Determinations in fecal water were only performed for Study 1. Fecal water was prepared by incubating homogenized freeze dried feces with water at 37 °C as described earlier (198). Fecal water was obtained after 11 minutes of centrifugation at 15000 g and stored at –20 °C until further use. Fecal water was prepared on a 16.7% dry weight basis because at 35% (198) no fecal water could be obtained. A second fecal water extraction was made to test the suitability of a second extraction for the several parameters measured.

Free fatty acids in fecal water were enzymatically determined using a colorimetric assay (NEFA-C, Wako Chemicals). Bile acids were determined as described by Mashige (189). Human O-negative erythrocytes for the determination of the cytolytic activity of fecal water, were prepared according to Coleman (190). Determination of cytolytic activity of fecal water was based on Govers (52). In short, 50 μl of fecal water was incubated with 5 μl washed and diluted O-negative human erythrocytes. Lysis was measured at OD 540 nm using the appropriate blanks and demineralized water as a positive control.

β-Glucosidase and β-glucuronidase activities were determined spectrophotometrically (405 nm) by measuring paranitrophenol (PNP)-β-D-glucuronide and paranitrophenol-β-D-glucoside (Sigma) hydrolysis respectively (199). The reaction was performed in a 96 wells plate, containing 75 μl of five times diluted fecal water, 25 μl of a 0.1% PNP-glucuronide or

PNP-glucoside solution and 25 µl 50 mM phosphate buffer pH 6.5. PNP was used for preparing a calibration curve.
Intestinal ALP activity was measured using L-phenylalanine as a specific inhibitor of the intestinal isozyme (154).
Statistical analysis Statistical analysis was performed using SPSS version 7.5. Data were analyzed using general factorial analysis of variance after testing for normality and homogeneity of variance. No data transformation was necessary. In case of homogeneity, the Bonferroni test of significance was used for post hoc analysis ($p \leq 0.05$). In case of non-homogeneity, data were tested on significance with the Dunnett T3 test ($p \leq 0.05$). Extreme values, as shown by box-plot analysis, were excluded from analysis. Correlation coefficients were calculated according to Pearson (two sided).

RESULTS

For Study 1 animal weights and fecal wet weights did not differ between dietary groups throughout the experiment (Table 6.2).

Table 6.2 Animal weights, food intake and fecal parameters (Study 1). Values are means ± SD. Values are significantly different from C (Casein), S1 (Soy-1) or from S2 (Soy-2) at $p \leq 0.05$. [1] Animal weight, food intake and fecal weight were determined on the same day, a few days prior to autopsy. [2] Data on food intake represent total intake (dry powder mixed with water). Data are representative for all other data obtained. [3] Fecal fat and magnesium were determined in fecal batches obtained one week prior to autopsy.

	Animal weight (g) [1]	**Food intake (g/d) [1, 2]**	**Fecal wet weight (g/d) [1]**	**Fecal dry weight (g/d) [3]**	**Fecal fat (mg/g) [3]**	**Fecal fat (mg/d) [3]**	**Fecal Mg (µmol/d) [3]**
Casein	300±16	26.5±2.6	3.77±0.83	1.90±0.33	9.2±2.2$^{S1, S2}$	30.1±7.6$^{S1, S2}$	1.40±0.40
Soy-1	299±16	26.4±3.2	3.97±0.58	2.13±0.14	27.3±4.0$^{C, S2}$	90.4±21.3^{C,S2}	1.61±0.36
Soy-2	307±16	26.8±2.4	3.62±0.72	1.99±0.24	18.0±2.4^{C,S1}	59.8±11.1^{C,S1}	1.49±0.27

There was a trend towards a lower fecal dry weight for the Casein group compared to the Soy-1 group (Table 6.2). Food intake tended to be lower for the Casein group compared to the Soy-2 group in the beginning of the experiment (data not shown). In the second half of the experiment food intake data were equal for all groups (Table 6.2). There were no differences between the diets in the amount of DNA scraped from the proximal, distal and total colon (Table 6.3), permitting the analysis of the cell proliferation data. No significant differences were found for the amount of incorporated ^{3}H-thymidine in the proximal, distal and total colon (Table 6.3).

Table 6.3 Amount of DNA and colonic cell proliferation (Study 1). Values are means ± SD. Values are significantly different from C (Casein), S1 (Soy-1) or from S2 (Soy-2) at p≤0.05. Dpm = disintegrations per minute.

	Amount of DNA			Colonic cell proliferation		
	proximal (µg)	distal (µg)	total (µg)	proximal (dpm/µg DNA)	distal (dpm/µg DNA)	total (dpm/µg DNA)
Casein	329±178	315±137	644±182	20.0±7.0	29.5±11.0	26.5±10.0
Soy-1	288±121	325±151	614±244	23.1±11.0	23.4±10.7	23.3±9.4
Soy-2	348±132	368±134	716±240	25.8±11.7	27.3±16.7	26.5±13.0

There were no differences in the total amount of fecally excreted magnesium (Table 6.2). Total fecal fat excretion showed marked differences between groups (Table 6.2). Results for all fecal water parameters measured are shown in Table 6.4.

Table 6.4 Fecal water parameters (Study 1). Values are means ± SD. Values are significantly different from C (Casein), S1 (Soy-1) or from S2 (Soy-2) at p≤0.05.

	Casein	Soy-1	Soy-2
pH	7.8±0.5	8.0±0.4^{S2}	7.5±0.3^{S1}
pH (2nd extraction)	7.7±0.4	7.9±0.4	7.5±0.3
Bile acids (mM)	1.81±0.46$^{S1, S2}$	0.52±0.12$^{C, S2}$	0.64±0.06$^{C, S1}$
Free fatty acids (mM)	0.08±0.03	0.05±0.02^{S2}	0.09±0.05^{S1}
Free fatty acids (mM) 2nd extraction	0.06±0.02	0.03±0.01	0.06±0.02
Cytolytic activity (%)	42±13	48±12	41±12
Cytolytic activity (%) 2nd extraction	45±12	50±10	41±9
ALP (µmol/ml/min)	4.10±3.20^{S1,S2}	0.42±0.23$^{C, S2}$	1.26±0.67$^{C, S1}$
β-Glucosidase (µmol/ml/min)	0.08±0.24^{S1,S2}	1.94±0.68^{C}	2.66±1.26^{C}
β-Glucuronidase (µmol/ml/min)	0.45±0.44$^{S1, S2}$	18.13±8.52^{C}	16.83±9.63^{C}

There was a significant difference in pH of fecal water between Soy-1 and Soy-2. A strong decrease in fecal water bile acid concentration was found for both soy diets compared to the Casein group. Concentration of free fatty acids in fecal water differed only significantly between Soy-1 and Soy-2. Soy-1 showed the lowest concentration of free fatty acids in fecal water. The cytolytic activity of fecal water towards human erythrocytes *in vitro* was equal for all groups. Fecal water β-glucosidase and β-glucuronidase activity showed a strong increase for both soy protein diets compared to the Casein diet. Fecal water ALP activity showed a decrease for both soy diets. Measurements of pH, free fatty acid concentration and cytolytic activity, using fecal water from the second extraction, resembled the results of experiments

using the first extraction of fecal water (Table 6.4). The differences in pH and free fatty acid concentration were no longer significant.
Results of Study 2 are shown in Table 6.5. There were no significant differences observed in animal weight, amount of DNA scraped from the colon or in the colonic cell proliferation (dpm/µg DNA).

Table 6.5 Results from Study 2.Values are means ± SD. Groups did not differ significantly at $p \leq 0.05$. Dpm = disintegrations per minute.

	Casein (AIN-76)	**Soy-2 (AIN-76)**
Animal weight (g)	355±17	365±8
DNA (µg)	570±104	623±120
Colonic cell proliferation (dpm/µg DNA)	51±18	55±12

DISCUSSION

The purpose of the study described was to investigate the influence of casein and soy on fecal colon cancer risk parameters and colonic cell proliferation in rats. Results show several differences in fecal colon cancer risk parameters between dietary groups fed casein or soy protein isolate as a protein source. However, fecal parameters indicating (risk on) colonic epithelial cell damage show no difference between casein and soy. This is confirmed by the fact that no difference was found in colonic cell proliferation between groups, neither proximally nor distally. Many observed results are in contrast to the results reported by Govers (4), who found very strong increases in several fecal parameters and a significant doubling of the colonic cell proliferation for the soy protein group. Minor differences between our experiment and that of Govers was the methionine content of the diet. This is of special relevance since Duranton (98) showed that methionine might be able to influence colonic cell proliferation. Experiments described by Vis (195) show that the addition of 0.2% or 1.0% methionine to the diet does not influence intestinal cell proliferation using the ^{3}H-thymidine model. In Study 2, using AIN-76 vitamin and mineral mixtures, there was again no difference in colonic cell proliferation, either proximally or distally (data not shown), between the casein and the soy protein isolate groups (Table 6.5). This confirms the cell proliferation data of Study 1. It is therefore concluded that no difference in colonic cell proliferation exists after feeding either casein or soy protein isolate. The difference in results between our study and that of Govers (4) can not be explained by the minor differences in dietary methionine or vitamin composition. Fecal water data of Study 1 support the lack of difference between the dietary groups. We found no difference in cytolytic activity of fecal water and no increase of the fecally excreted ALP activity for the soy groups. These data suggest that there is no difference between the cytolytic potential of the fecal waters. Therefore no increase in

damage of the epithelium for the soy protein diets tested (ALP) may be expected. ALP data even suggest a decrease of epithelial damage after soy protein feeding. In both soy diets there was a consistent decrease of the bile acid concentration in fecal water. For Study 1 a decrease in fecal water free fatty acid concentration was shown. The decrease in both fecal water bile acid and free fatty acid concentrations indicate a protective effect from soy protein feeding by reducing the concentration of lytic compounds in the fecal water fractions of rats.

The lack of difference in colonic cell proliferation between animals fed either casein or soy is also confirmed by the lack of difference in fecal magnesium excretion. Our data closely resemble data reported by Brink (131). Several results from our group also confirm the lack of difference in fecal magnesium excretion (195). Therefore it is concluded that there is no difference in fecal magnesium excretion after feeding either casein or soy protein isolate.

Results of Study 1 show an increase in total fecal fat excretion after soy protein feeding. The 2-3 fold fecal fat induction confirms results reported by McIntosh (2) who found a 2.1 fold fecal fat induction. The 4.5 fold fecal fat induction reported by Govers (4) could not be reproduced. Because Soy-2 used in Study 1 was exactly the same protein brand as the soy protein used by Govers, this might again confirm the large variability in the composition of the soy protein isolates. Whatever caused the extremely high fecal fat excretion (4.5 fold) in the Govers study might explain the increase found in colonic cell proliferation in the same study.

The strongly increased β-glucuronidase activities in fecal water after soy protein feeding imply an increased exposure of the colonic epithelium to glucuronidated conjugates of toxic components from the liver. This is mainly relevant for studies using DMH or azoxymethane (AOM) to chemically induce colonic lesions. The induction of tumor burden as reported by McIntosh after feeding soy protein compared to casein (2) could be explained by the induction of β-glucuronidase activity found in Study 1. DMH and AOM are both metabolized in the liver by glucuronidation. A high intestinal β-glucuronidase activity could lead to an increased exposure of the colonic epithelium to deglucuronidated reactive DMH/AOM species. When feeding soy protein, intestinal β-glucuronidase activities increase, possibly resulting in the release of reactive agents causing mutations and finally tumors in the colon.

As mentioned in the introduction it was hypothesized that experiments on soy protein isolate would show variable results because of the variable composition of soy products. Our results confirm this hypothesis. The differences between our results and those reported by Govers confirm the highly variable nature between soy protein batches of the same brand. This problem might be hard to overcome because the difference is probably in fecal fat inducing factors such as saponins, which are very hard to detect. Soy protein isolates are usually only standardized on isoflavone content because of its suggested biological activity (60). The fact that there are differences between Soy-1 and Soy-2 confirms our hypothesis that brands of soy

protein isolate differ in effect and probably in composition of soy non-nutrients. The interpretation of results using soy protein isolate should therefore be done with care.
Although our results differ from results reported earlier (4) there are several reasons to believe that our data are more representative for soy protein isolates in general. Our data on fecal magnesium and fat excretion confirm results of others (2, 131) and data from our own group (195). Fecal data match our data on colonic cell proliferation very well. In contrast to the study reported by Govers (4) our results were obtained using different soy protein isolates and were reproduced in two separate animal studies. Our results are close to recent studies published by others. Thiagarajan (68) showed a decrease of colonic cell proliferation in humans after daily consumption of a soy protein supplement. Hakkak (66) showed a decrease of colonic tumor incidence after feeding soy protein isolate compared to casein. Wang (67) found no influence on soy protein compared to casein on intestinal tumorigenesis in *Apc*^{Min} mice. Therefore the overall conclusion is that casein and soy protein isolate have different effects on several fecal parameters but no differences occur on colonic cell proliferation.

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Chapter 7

Influence of casein, soy and gluten protein on fecal markers and colonic aberrant crypt foci in rats

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ABSTRACT

This paper describes two animal studies in which the influence of dietary protein sources on colonic aberrant crypt foci (ACF) in the rat was investigated. In Study 1 animals were fed diets containing either casein, soy protein isolate or wheat gluten as a protein source. The number of ACF and the average foci multiplicity were calculated after 10 weeks on the experimental diet. No significant differences were shown in the number of foci between dietary groups. However, there was a small but significant difference between groups in the multiplicity (Soy < Casein < Gluten). Both fecal and cecal samples were collected to determine β-glucuronidase and β-glucosidase activity as markers of gut flora metabolizing capacity. Both β-glucuronidase and β-glucosidase activities were increased in the order Casein < Soy < Gluten. Fecal alkaline phosphatase (ALP) excretion, a measure of colonic epithelial cell damage, showed a decrease as a result of the soy protein feeding. Because of earlier reported influence of soy protein feeding on thyroid hormone levels in blood plasma, total triiodothyronine and total thyroxine and free thyroxine were measured. Soy protein feeding caused a significant increase in the plasma total triiodothyronine levels compared to the gluten group. In Study 2 the influence of casein and soy flour (in contrast to soy isolate) on the occurrence of ACF was tested. Again fecal and cecal samples were collected for enzyme activity determinations. No difference in the number or multiplicity of ACF, either proximally or distally, was observed after 8 weeks on the experimental diet. Results of enzyme activity experiments confirmed results as found in Study 1. From both studies performed it was concluded there are no major differences between casein and either soy protein isolate or soy flour on colonic ACF, despite a consistent increase in β-glucuronidase and β-glucosidase activities and a decrease in fecal ALP activity for the soy and gluten protein diets.

INTRODUCTION

Colon cancer is a major health problem, especially in countries consuming a Western style diet. The exact cause of colon cancer is uncertain but the diet seems to be a major risk factor (7, 11). To investigate the role of diet on colon cancer, several animal models have been used. Two of these models showed a stimulating effect of soy protein on colon cancer risk compared to the milk protein casein. McIntosh (2) reported a 4-fold increase of large intestinal tumor burden after dimethylhydrazine (DMH) induction. Govers (4) reported a significant doubling of colonic cell proliferation after feeding of soy protein isolate for 9 days. However, in several reviews written on the relationship between dietary soy protein and colon cancer risk, no consensus is reported (60, 62, 63). Because little is known about the influence of different dietary protein sources on colonic aberrant crypt foci (ACF) and above mentioned reviews show no consensus, two animal studies were performed to test the influence of especially soy protein and casein on colonic ACF in the rat. Colonic ACF are putative preneoplastic lesions indicative of colon cancer risk. ACF were first described and later reviewed by Bird (115, 116). ACF need to be chemically induced by a colon carcinogen like DMH or by its metabolite azoxymethane (AOM). In Study 1 casein, soy protein isolate and wheat gluten were tested. In Study 2, casein and soy flour (in contrast to soy isolate) were investigated. In both studies fecal and cecal enzyme activities were determined because of their potential influence on DMH and AOM metabolism (148, 200). Fecal and cecal alkaline phosphatase (ALP) excretion was determined because it was shown earlier that fecal ALP excretion is a marker for colonic epithelial damage (154). For all three enzymes the effect of storage of the samples (-20 °C) on enzyme activity determinations, was tested. By several authors it is hypothesized (2, 4) that the fecal fat induction by soy protein causes an increase in colon cancer risk. Therefore, total fecal fat and total free fatty acids excretion were measured in Study 1. Bile acids, free fatty acids, short chain fatty acids (SCFA), pH and cytolytic activity were measured in fecal water because several studies find correlations between these parameters and colon cancer risk (4, 32, 131, 132, 160, 181-183). Thyroid hormones were determined in blood plasma because they were earlier reported to be influenced by soy protein feeding (201).

MATERIALS AND METHODS

Animals and housing The experimental protocols were approved by the animal welfare committee of Wageningen University. Room temperature (± 20 °C), relative humidity (50-60%) and light-dark cycle (12h/12h) were controlled. All animals were housed in pairs on sawdust in plastic Macrolon™ type III cages. Food and water was available *ad libitum*. Animals were kept in isolation for at least 5 days after the AOM injection. A time schedule for the experiments is shown in Figure 7.1.

		Study 1	Study 2
- 2	←	animal arrival	
- 1	←		animal arrival
0	←	experimental diet start isolation period	experimental diet
1	←		start isolation period
2	←	first AOM injection	first AOM injection
3	←	second AOM injection	second AOM injection
4	←		end isolation period
5	←		
6	←	end isolation period	
7	←		
8	←	acclimatization wire bottom	dissection
9	←	24h feces collection	
10	←	dissection	

Figure 7.1 Time schedule for Study 1 and 2 in weeks.

For Study 1, 30 male Fischer 334 rats were used (Charles River, Someren, The Netherlands), 6 weeks of age and with a mean body weight of 95 g at arrival. Food intake and body weight were recorded weekly. After 2 and 3 weeks on the experimental diet the rats received a subcutaneous injection of AOM (Sigma) at 15 mg/kg in NaCl (0.9%, sterile) to initiate ACF. During the feces collection period to obtain feces for fecal water preparation, animals were individually placed on a wire bottom for two weeks. Fresh feces for enzyme activity determinations was collected as described in the *fecal enzyme activities* section. During dissection, two weeks later, blood plasma was obtained.

For Study 2, 20 male Fischer 334 rats were used (Charles River, Someren, The Netherlands), 3-4 weeks of age with a mean body weight of 28 g at arrival were used. For two separate weeks, food intake was recorded daily. Body weight was recorded three times a week. After 2 and 3 weeks on the experimental diet the rats received a subcutaneous injection of 15 mg/kg AOM (Sigma). Fresh feces for enzyme activity determinations was collected as described in the *fecal enzyme activities* section.

Diets For both studies, animals had an acclimatization period of at least one week after arrival before switching to the experimental diets. For Study 1, rats were divided into three dietary groups. Each group consumed a diet containing a different protein source: Na-caseinate (88% protein, DMV, Veghel, The Netherlands), soy protein isolate (92% protein, Soy-Prot-S, Loders Croklaan B.V., Wormerveer, The Netherlands) and wheat gluten (80%

protein, Protinax 140, AVEBE Latenstein BV, Nijmegen, The Netherlands). Diets will be referred to as Casein, Soy-i and Gluten respectively. The ingredients of the diets are shown in Table 7.1A. Vitamin and mineral mixtures were prepared based on Nutrient Requirements of Laboratory Animals (34), except for calcium, which was low at 25 mmol/kg diet because calcium is known to influence colon cancer risk (50, 154). Diets were prepared by Hope Farms (Woerden, The Netherlands).

Table 7.1A Composition of experimental diet (Study 1). Values represent g/kg. [1] Acid casein was added to the gluten diet to exclude a possible lysine deficiency. [2] The inert material Bentonite (Poortershaven, Rotterdam, The Netherlands) was used to adjust the different amounts of inert material in the diets.

	Casein	**Soy-i**	**Gluten**
Na-caseinate	200	-	-
Soy protein	-	203	-
Gluten protein	-	-	183
Acid Casein	-	-	50.0[1]
Methionine	2.0	2.0	2.0
Soy oil	63.3	65.0	64.3
Glucose	533	531	518
Corn starch	100	100	100
Cellulose	27.3	27.3	27.3
Inert material[2]	19.4	16.7	0.0
Vitamins	2.5	2.5	2.5
Trace elements	2.5	2.5	2.5
Minerals	46	46	46
Choline Cl 50%	4.0	4.0	4.0

For Study 2 rats were divided into two dietary groups: acid casein (88% protein, DMV, Veghel, The Netherlands) and defatted soy flour (54% protein, Cargill, Amsterdam, The Netherlands). Diets will be referred to as Casein and Soy-f respectively. The ingredients of the diets are shown in Table 7.1B. Vitamin and mineral mixtures were prepared according to AIN-93G (202). Diets were prepared by TNO Nutrition and Food Research Institute (Zeist, The Netherlands).

Table 7.1B Composition of experimental diet (Study 2). Values represent g/kg.

	Casein	Soy-f
Casein	250	-
Soy-f	-	407
Corn oil	250	250
Starch	376	280
Cellulose	61.8	0.7
L-cysteine	3.7	3.7
Choline bitartrate	2.5	2.5
Minerals	43.2	43.2
Vitamins	12.4	12.4

Dissection After 10 weeks (Study 1) or 8 weeks (Study 2) on the experimental diet, dissection took place. The rats were anesthetized using CO_2. In Study 1, blood was collected from the aorta. For both studies cecal content was collected for analysis of fresh β-glucuronidase, β-glucosidase and ALP activity. After rinsing with saline, the colon was fixed flat between two pieces of filter paper and fixated in 10% formaline for several days. After a 1 minute methylene blue (0.5%) staining, ACF were scored at an 80x magnification using a binocular. Both foci incidence and crypt multiplicity were scored by one observer. Average multiplicity was calculated as the number of crypts divided by the number of foci of one animal. For Study 1, the number of foci with crypts of a fixed number (e.g. m=1, m=2, m=3, etc.), was expressed as a percentage of the total number of foci in that animal. This calculation was used to correct for the large difference in the number of foci, between animals within one group, but also between animals of different dietary groups. Expressing the number of foci of certain multiplicity as a percentage and not as an absolute number, strongly decreases the coefficient of variation calculated as shown in Table 7.2.

Table 7.2 Coefficients of variation (%) for the number of ACF of a certain multiplicity expressed as the total number or as a percentage of the total number of foci (Study 1). Coefficient of variation was calculated as (SD/average)*100%. Coefficients of variation are calculated based on the absolute number of ACF (abs.) or as the percentage of the total number of ACF (rel.). m = multiplicity of a fixed number.

	m = 1		**m = 2**		**m = 3**		**m = 4**		**m = 5**		**m = 6**	
calculation method	abs.	rel.	abs.	rel.	abs.	rel.	abs.	rel.	abs.	rel.	abs.	rel.
Casein	38%	24%	26%	15%	29%	15%	41%	23%	57%	40%	89%	83%
Soy-i	43%	15%	36%	10%	28%	17%	48%	43%	46%	47%	82%	116%
Gluten	37%	27%	22%	11%	20%	19%	32%	33%	35%	26%	81%	80%

Fecal enzyme activities (Study 1 and Study 2) Fecal enzyme activities were determined in fresh feces collected directly from the animals colon at week 10 (Study 1) and week 2, 5, and 7 (Study 2). Cecal samples were collected during dissection in both studies. Both samples were collected in 0.9% NaCl and homogenized using an ultra turrax (Ultra Turrax T25, Janke & Kunkel). The homogenate was centrifuged at 2000 g during 15 minutes. The supernatant was used to determine enzyme activities. β-Glucuronidase and the β-glucosidase activities were spectrophotometrically determined at 405 nm by measuring p-nitrophenol (PNP) release from PNP-glucuronide (Sigma) and PNP-glucoside (Sigma) respectively (203). ALP activity was determined as described by Lapré (154). In short, PNP release was spectrophotometrically measured in time before and after the addition of L-phenylalanine. L-phenylalanine is a specific inhibitor of intestinal ALP. Intestinal ALP activity was calculated by subtracting the enzyme activity before and after L-phenylalanine addition.

Besides using fresh fecal and cecal samples, enzyme activities were also determined in stored fractions (-20 °C) to study the effect of storage on enzyme activities. In Study 1, enzyme activities were determined in fecal water, which was prepared as described below. In Study 2, enzyme activities were determined in stored supernatant prepared as described above.

Determinations in feces (Study 1) The percentage of water in fresh feces was gravimetrically determined after drying freshly collected feces for 30 hours in a 70 °C oven. Total fecal fat concentrations were gravimetrically determined after extraction with petroleum ether as described earlier (4). Total free fatty acids were enzymatically determined using a colorimetric assay measuring non-esterified fatty acids (NEFA-C, Wako Chemicals).

Fecal water preparation (Study 1) Fecal water was prepared by mixing 200 mg of homogenized freeze-dried feces with 1.0 ml of water for 30 minutes at 37 °C. The mixture was centrifuged for 15 min at 15000 g. Approximately 200 µl of supernatant was obtained and stored at -20 °C. Fecal water prepared on a 35% dry weight basis (198) was not possible because no supernatant was obtained after centrifugation.

Fecal water determinations (Study 1) Free fatty acids in fecal water were determined as described in the determinations in feces section. Bile acid concentration in fecal water was fluorimetrically determined using an enzymatic method based on Mashige (189). Cytolytic activity was measured by determining the lysis of human erythrocytes (52) after incubation with fecal water. Erythrocytes for the determination of the cytolytic activity of fecal water were prepared according to Coleman (190). Cell lysis was determined by measuring the release of hemoglobin at 540 nm. Blank samples were included to compensate for the color of the fecal water. ALP, β-glucuronidase and β-glucosidase activities were determined as described in the fecal enzyme activity section. All fecal water parameters were measured with the same fecal water batch. Short chain fatty acids determination was based on Kortekaas (204). In short, samples were measured gaschromatographically (Hewlett Packard 5890A,

Palo Alto, USA) after diluting 25 µl of fecal water in 100 µl formic acid and centrifuging 4 minutes at 15000 g.

Thyroid hormone analysis (Study 1) Thyroid hormones were determined using the Amerlite chemiluminescense kits (Amersham) according to the protocol of the supplier. Total triiodothyronine (TT3), total thyroxine (TT4) and free thyroxine (FT4) concentrations were determined in blood plasma obtained during dissection.

Statistics Statistical analysis was performed using SPSS version 7.5. Data were analyzed by using general factorial analysis of variance. In case of homogeneity of variance, the Bonferroni test of significance was used ($p \leq 0.05$). The non-homogeneic data were tested on significance with the Dunnett T3 test ($p \leq 0.05$). Extreme values for the enzyme activities, as shown by box-plot analysis, were excluded from analysis. Correlation coefficients were calculated according to Pearson. When data were not normally distributed, the Kruskal-Wallis test was used to determine significant differences among all groups.

RESULTS

Animal growth curves for Study 1 are shown in Figure 7.2.

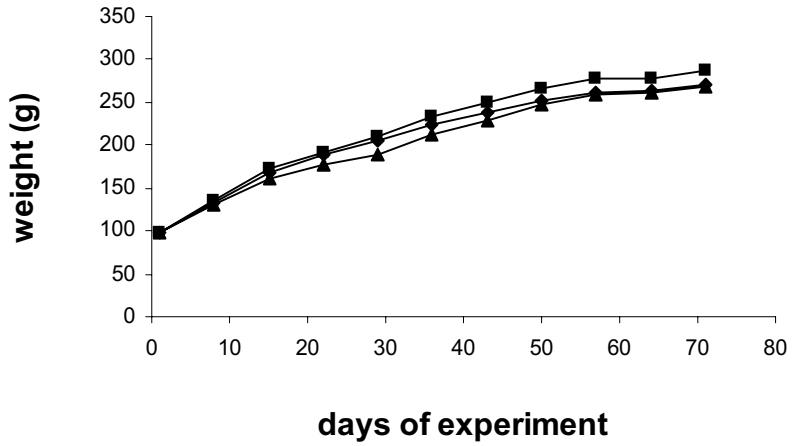

Figure 7.2 Animal growth curves (Study 1). Casein (◆), Soy-i (■), Gluten (▲).

Animals from the Soy-i diet group had a significantly higher body weight (compared to Casein and Gluten) at week 10 of the experiment. The liver weight relative to the body weight tended to be lower for the Gluten diet group ($p \leq 0.1$). No differences between the food intake by the Casein- and Soy-i diet groups were observed. Food intake for the Gluten group was generally lower (data not shown). The results of the foci determinations are shown in Figure 7.3. As multiplicity increased, the number of foci in the Soy-i diet group decreased. This

resulted in a lower percentage of foci for all cut off points and a lower total multiplicity for the Soy-i fed animals.

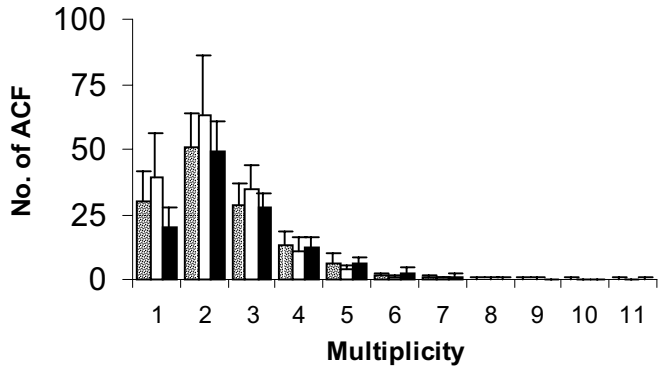

Figure 7.3 Percentage of aberrant foci for all multiplicities for each diet (Study 1). Number of ACF is expressed as a percentage of the total number of ACF (%) ± SD. Casein (spotted bars), Soy-i (open bars) and Gluten (closed bars).

No significant differences were observed for the total number of ACF, although there was a trend towards a higher number of foci for the Soy-i diet (Table 7.3). Results of the fresh fecal and cecal enzyme activity determinations are shown in Table 7.4.

Table 7.3 Percentage of ACF with a multiplicity ≥ x for each diet (Study 1). Values are means ± SD. Significantly different from C (Casein), S (Soy-i) or from G (Gluten) at $p \leq 0.05$.

	ACF	**% ≥ 2**	**% ≥ 3**	**% ≥ 4**	**% ≥ 5**	**% ≥ 6**	**Multiplicity**
Casein	131± 37	77± 5^{G}	38± 5	16± 5^{S}	6.2± 3.0	1.8± 1.6	2.41± 0.16^{SG}
Soy-i	153± 50	75± 4^{G}	34± 4^{G}	11± 3^{CG}	3.7± 1.8^{G}	1.2± 0.8	2.25± 0.10^{CG}
Gluten	119± 20	83± 5^{CS}	42± 5^{S}	18± 3^{S}	8.1± 2.9^{S}	3.1± 2.5	2.57± 0.13^{CS}

Table 7.4 Influence of dietary protein type on fresh fecal and cecal enzyme activities (Study 1). Values are means ± SD. Within parenthesis the number of animals is shown. Significantly different from C (Casein), S (Soy-i) or from G (Gluten) at $p \leq 0.05$.

	β-Glucuronidase (µmol PNP/g sample/hour)		
	Feces (day 65)	*Feces (day 66)*	*Cecum (day 72)*
Casein	15.0± 7.8 (9)^{SG}	14.4± 8.4 (10)^{SG}	1.3± 0.7 (6)
Soy-i	36.0± 5.4 (5)^{C}	44.4± 8.4 (10)^{CG}	3.7± 3.7 (9)
Gluten	28.8± 5.4 (6)^{C}	28.2± 13.2 (9)^{CS}	1.4± .5 (7)
	β-Glucosidase (µmol PNP/g sample/hour)		
	Feces (day 65)	*Feces (day 66)*	*Cecum (day 72)*
Casein	0.8± 0.5 (8)^{SG}	1.0± 0.5 (9)^{SG}	0.3± 0.2 (3)
Soy-i	3.1± 0.8 (4)^{C}	2.6± 1.5 (10)^{CG}	1.2± 1.4 (5)
Gluten	4.4± 1.6 (6)^{C}	5.6± 2.8 (9)^{CS}	0.4± 0.1(3)
	ALP (µmol PNP/g sample/hour)		
	Feces (day 51)	*Feces (day 65)*	*Cecum (day 72)*
Casein	57.6± 37.2 (9)^{G}	39.0± 24.0 (9)	51.0± 41.4 (5)
Soy-i	23.4± 11.4 (10)	27.0± 10.8 (4)	30.6± 12.6 (4)
Gluten	12.0± 7.8 (7)^{C}	78.6± 93.0 (8)	15.6± 12.0 (4)

The β-glucuronidase and β-glucosidase activities in feces from the Casein diet group were significantly lower when compared to the Soy-i and Gluten diet groups for both feces collection days measured. For the cecum, β-glucuronidase and β-glucosidase enzyme activities were the highest for the Soy-i group, although no significant differences could be observed between the dietary groups. For Soy the ALP activity in feces and cecum compared to Casein was decreased. No consistent results were obtained for the Gluten group.
In Table 7.5 results of the fecal fat parameters measured, are shown. The total amount of fat and free fatty acid in feces was significantly higher for the Soy-i diet group compared to the other dietary groups. The results of the fecal water analysis are also shown in Table 7.5.

Table 7.5 Influence of dietary protein type on fecal (water) parameters (Study 1). Values are means ± SD. Significantly different from C (Casein), S (Soy-i) or from G (Gluten) at p≤0.05.

		Unit	Casein	Soy-i	Gluten
Total feces	Total fat	mg/g	20.9±1.3	58.5±7.5	41.4±6.6
	Total fat	mg/24h	30.2 ± 5.4^{SG}	80.2 ± 16.6^{CG}	43.4 ± 5.2^{CS}
	Free fatty acids	µmol/g	21.2 ± 9.2^{SG}	44.6 ± 9.7^{CG}	33.1 ± 5.2^{CS}
	Free fatty acids	µmol/24h	30.6 ± 16.6^{S}	60.2 ± 16.6^{CG}	29.3 ± 10.7^{S}
Fecal water	pH		8.3±0.1	8.3±0.1	8.3±0.1
	Free fatty acids	mM	4.7 ± 2.5^{G}	7.1 ± 2.8^{G}	16.7 ± 7.1^{CS}
	Bile acids	mM	0.4 ± 0.0^{S}	0.3 ± 0.0^{CG}	0.5 ± 0.2^{S}
	Cytolytic activity	%	36.0 ± 19.0^{G}	19.0 ± 15.0^{G}	2.0 ± 3.0^{CS}
	ALP	µmol/ml/hour	3.3 ± 2.2^{K}	8.5 ± 3.6^{K}	42.2 ± 63.6^{K}
	β-Glucosidase	µmol/ml/hour	0.01 ± 0.04^{SG}	0.29 ± 0.14^{CG}	0.49 ± 0.08^{CS}
	β-Glucuronidase	µmol/ml/hour	1.3 ± 0.6^{K}	3.5 ± 1.5^{K}	4.2 ± 0.7^{K}
	Total SCFA	mM	13.1 ± 5.7^{G}	13.4 ± 2.1^{G}	30.1 ± 15.3^{CS}
	C2	mM	9.9 ± 4.0^{G}	10.4 ± 1.6^{G}	23.5 ± 11.2^{CS}
	C3	mM	1.1 ± 0.74^{G}	1.1±0.4	3.2 ± 2.1^{S}
	i-C3	mM	0.2 ± 0.1^{G}	0.3±0.1	0.7 ± 0.4^{S}
	n-C3	mM	1.0±0.5	1.0±0.2	1.1±0.7
	b-C4	mM	0.5±0.2	0.4±0.1	0.8±0.5
	n-C4	mM	0.3±5.7	0.3±2.1	0.6±15.3

There were no significant differences between the pH of the three types of fecal water. The (long chain) free fatty acids were significantly increased for the Gluten group. For some short chain fatty acids there was a significant increase for the Gluten group compared to both the Casein and the Soy group. There was a small but significant decrease of bile acid concentrations for the Soy fed animals compared to Casein and Gluten. The cytolytic activity for fecal water was significantly decreased for the Gluten group. The fecal ALP excretion was highest for the Gluten group. Differences between groups could not be specified because the data were not normally distributed. Both the β-glucuronidase and the β-glucosidase activity were significantly increased in the order Casein < Soy-i < Gluten.

Thyroid hormone data are shown in Table 7.6. Soy-i caused a significant increase in the TT3 concentration in plasma compared to Gluten. No significant differences between the diets were shown for the TT4 and FT4 concentrations in plasma.

Table 7.6 Influence of dietary protein type on plasma thyroid hormone levels (Study 1). Values are means ± SD. Significantly different from G (Gluten) or S (Soy-i) at p≤0.01.

	Triiodothyronine	Thyroxine	Free thyroxine
	nmol/l		
Casein	1.55 ± 0.19	33.2 ± 3.2	10.8 ± 1.3
Soy-i	1.71 ± 0.24^{G}	32.9 ± 3.7	11.2 ± 2.0
Gluten	1.33 ± 0.23^{S}	31.4 ± 3.5	11.0 ± 1.5

In Study 2 no differences in body weight and food intake were observed (data not shown). The water percentage of the feces was significantly different between the Casein (51 ± 8%) and Soy-f (69 ± 7%) group. The results of the ACF determinations are shown in Table 7.7. No differences between Casein and Soy-f were observed for the total number of foci, the percentage of foci larger than two, three, four, or five or the overall multiplicity. This is true for results calculated proximally, distally or totally.

Table 7.7 Data on ACF (Study 2). Values are means ± SD.

	Casein			Soy-f		
	proximal	**distal**	**total**	**proximal**	**distal**	**total**
Number of ACF	38.5±19.1	77.5±27.0	116±41	38.1±19.5	62.3±19.7	100±32
Number of ACF ≥ 2	26.0±12.4	55.8±21.5	81.1±30.0	26.2±14.2	47.3±13.0	73.5±22.1
Number of ACF ≥ 3	10.0±4.6	28.9±15.9	38.9±17.9	10.2±4.8	25.6±9.3	35.8±9.8
Number of ACF ≥ 4	4.2±2.0	10.0±8.2	14.2±9.0	3.7±1.9	10.3±4.0	14.0±4.4
Number of ACF ≥ 5	0.9±0.9	2.7±3.1	3.6±3.1	1.0±0.9	2.9±1.4	3.9±1.9
Multiplicity	2.12±0.17	2.25±0.27	2.20±0.19	2.18±0.36	2.43±0.21	2.31±0.20

Results of the fecal and cecal enzyme activity determinations are shown in Table 7.8. β-Glucuronidase activity showed a constant increase of a factor 2-3 for the Soy-f group compared to the Casein fed animals. Despite the consistency of the difference it was not always significant because of the high variation. The results for the β-glucosidase activities were more variable. Sometimes an increase comparable to that of the glucuronidase activities was found, sometimes no difference between groups was shown. The fecal ALP excretion was consistently decreased after feeding Soy-f although there was a large difference in the absolute values between several experiments. Only for β-glucuronidase the stored samples correlated strongly with the fresh samples (r=0.95). For β-glucosidase the correlation between stored and fresh samples varied strongly between the days. Cecal enzyme activities were only measured with stored samples so no correlation analysis for fresh and stored samples could be performed. In the cecum, β-glucuronidase and β-glucosidase showed a consistently higher

enzyme activity for the Soy-f fed animals. The results for the cecal ALP content were inconsistent.

Table 7.8 β-Glucuronidase, β-glucosidase and ALP enzyme activities (Study 2). Values are means ± SD. * Significantly different at p≤0.01 or ^{KW} using Kruskal-Wallis (p≤0.05). [1] Cecal enzyme activity expressed per g cecum weight. [2] Cecal enzyme activity expressed per total cecum weight.

	Cecal/fecal	Day of collection	Day of measurement	Casein	Soy-f	Induction factor
β-Glucuronidase	fecal (fresh)	8	8	6.5±3.1	12±10	1.9
(μmol PNP/g/hour)	fecal (fresh)	30	30	14±4.7	36±15*	2.5
	fecal (fresh)	31	31	13±6.0	34±37	2.5
	fecal (fresh)	45	45	19±13	39±29	2.0
	fecal (stored)	8	71	3.9±1.4	10±9.9	2.6
	fecal (stored)	30	78	7.7±3.3	25±11*	3.3
	fecal (stored)	31	73	3.8±1.9	14±18	3.6
	cecal (stored)[1]	57	80	2.1±0.4	4.4±1.5^{KW}	2.1
	cecal (stored)[2]	57	80	3.5±1.0	14±5.7^{KW}	3.9
β-Glucosidase	fecal (fresh)	30	30	8.6±4.3	7.3±4.4	0.8
(μmol PNP/g/hour)	fecal (fresh)	31	31	15±6.7	30±43	2.0
	fecal (fresh)	45	45	12±8.0	8.2±8.3	0.7
	fecal (stored)	8	71	2.1±2.1	4.7±6.5	2.2
	fecal (stored)	30	78	4.8±2.4	4.1±2.5	0.9
	fecal (stored)	31	73	2.7±1.6	2.5±3.7	0.9
	cecal (stored)[1]	57	80	0.8±0.4	2.3±1.5^{KW}	2.9
	cecal (stored)[2]	57	80	1.4±0.9	6.7±3.6^{KW}	4.8
ALP	fecal (fresh)	45	45	100±79	15±11^{KW}	0.2
(μmol PNP/g/hour)	fecal (stored)	8	71	312±389	54±71	0.2
	fecal (stored)	30	78	69±34	19±13^{KW}	0.3
	fecal (stored)	31	73	69±50	11±5.0^{KW}	0.2
	cecal (stored)[1]	57	80	47±21	27±3^{KW}	0.6
	cecal (stored)[2]	57	80	75±30	81±103	1.1

DISCUSSION

The purpose of the studies described was to investigate the influence of soy protein compared to casein on intestinal bacterial enzyme activities, fecal colon cancer biomarkers and colonic aberrant crypt foci in rats. Despite differences in fecal parameters, neither Study 1 nor Study 2 showed any stimulating effect of soy protein feeding compared to casein on rat colonic ACF. This appears to be in contrast to earlier reports on stimulating effects of soy protein on colonic

cell proliferation and DMH induced colonic tumors (2, 4). A possible explanation for the difference in results between Study 1 and McIntosh (2) is that we used a soy protein isolate and McIntosh used soybean flour. It is known that different types of soy protein can result in differences on colon cancer biomarkers (64). The use of a different dietary soy protein source resulted in the presence of different types of fibre in the respective diets. The diets used in Study 1 contained some cellulose (2.7%) as a fibre source, whereas the fibre source in the diet described by McIntosh (2) contained (5%) soybean derived fibre. To test whether this difference in soy protein source was the explanation for the difference found in ACF and tumors respectively, Study 2 was performed. Although soybean flour was used as both a protein and a fibre source, this study showed again no negative effects of soybean flour on ACF in the rat colon compared to casein. It is therefore concluded that there is no difference between casein and soy protein isolate on colonic aberrant crypt foci despite earlier differences found in DMH induced colonic tumor incidence. The difference between these studies is not due to the difference in dietary protein source and therefore possibly reflects a difference in composition of the basal diet or the methodologies used. The latter hypothesis is supported by the fact that several authors find a lack of correlation between colonic aberrant crypt foci and intestinal tumor formation (205-207) despite other reports that such a correlation exists (97, 119, 208).

Colonic aberrant crypt foci and tumors in rats need to be chemically induced. This is often done by intraperitoneal injection of either AOM or DMH. DMH or AOM metabolites are glucuronidated in the liver and excreted into the intestinal lumen. In the colon these metabolites can be deglucuronidated thereby finally releasing reactive alkylating species (148, 200). An increase in β-glucuronidase activity could lead to the release of more reactive AOM species in the colon, causing more epithelial lesions, eventually leading to more crypts or tumors. However, despite the increase in β-glucuronidase activity after feeding soy protein, both isolate and soy flour, no increase in the number of foci or the multiplicity was found. There were no significant correlations between the total β-glucuronidase activity and the amount of ACF found per animal. Therefore, we conclude that the β-glucuronidase activity in the colon is already so high that an additional induction does not lead to an increase in the exposure of the alkylating DMH or AOM metabolites. Only dietary constituents strongly inhibiting the β-glucuronidase activity might influence colonic aberrant crypt foci or tumor formation in a beneficial way as shown before (148).

In both animal studies, enzyme activity measurements were performed in both fresh and stored samples to investigate the predictive value of stored samples for freshly measured samples. Enzyme activity measurements from stored samples only showed high correlations with the freshly measured samples for β-glucosidase in Study 1 and for β-glucuronidase in Study 2. Although the predictive value from stored samples for freshly determined enzyme activities is sometimes high, results are variable between experiments. Therefore it is

concluded that enzyme activities measured in stored samples can sometimes give a good indication of enzyme activities in freshly measured samples but are not a reliable alternative.
Our data on fecal fat excretion were close to the 2.1 fold fecal fat induction reported by McIntosh (2). The increase in fecal fat excretion after soy protein feeding was not as strong as reported by Govers (4). Govers reported a 4.5 fold total fecal fat induction and an 8 fold fecal free fatty acid induction. The difference in fecal fat induction possibly explains the fact that in Study 1 no difference between casein and soy in the cytolytic activity of fecal water was measured. Data from Study 1 on cytolytic activity confirm the lack of a differential effect between casein and soy protein on ACF as found in Study 1 and Study 2.
The difference in result of fecal data between our study and that of Govers could be explained by the fact that soy protein isolates contain variable amounts of saponins. Saponins are known for their amphiphilic characteristics (61) and are therefore likely to interact with luminal fat and influence fecal fat excretion. It appears likely that the soy protein isolate batch used by Govers (4) contained a high amount of saponins, finally resulting in an increased colonic cell proliferation.
The lack of a negative health effect on colon cancer after feeding soy protein was confirmed by preliminary results from several authors. Wang (67) found no difference between soy concentrate and casein on intestinal polyp formation in *Apc*^{Min} mice. Thiagarajan (68) even found a reduction in the proliferative capacity in colonic crypts of human subjects after consuming 39 grams of soy protein isolate supplement per day for one year. Hakkak (66) reports a reduction in colonic tumors in rats after feeding soy protein isolate compared to casein. Also several yet unpublished studies from our own group show little evidence of negative health effects on colon cancer after soy protein feeding. Therefore, it is concluded that there are no major differences between casein and either soy protein isolate or soy flour on colonic ACF in rats, despite a consistent increase in β-glucuronidase and β-glucosidase activities and a decrease in fecal ALP excretion.

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Chapter 8

Modulation of intestinal polyp formation and fecal colon cancer biomarkers by dietary proteins in *Apc*^{Min} mice

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ABSTRACT

The purpose of the study described was to investigate the influence of casein, soy protein isolate, soybean flour or red meat protein diets on fecal colon cancer risk biomarkers and intestinal polyp formation in male and female *Apc*^{Min} mice. Several fecal parameters were determined in the fecal water fraction of collected feces. A decrease of the fecal water pH was found for the soy flour fed animals compared to the other dietary groups. Fecal water bile acid concentrations were decreased after feeding soy protein diets compared to casein. A strong increase in β-glucuronidase and β-glucosidase activity was observed for the soy isolate and the soy flour groups, compared to the casein and meat fed animals. No significant differences were observed in the number of small intestinal polyps. A doubling of the number of large intestinal polyps became apparent only after pooling the data for casein and meat (animal proteins) and soy protein isolate and soybean flour (plant proteins). In summary it is concluded that casein, soy protein isolate, soybean flour and meat protein show different results on several fecal parameters, but no consistent differences on small and large intestinal polyp formation.

INTRODUCTION

Colon cancer is a worldwide problem, especially in western countries, consuming a western style high-energy, high-fat diet (7). The knowledge on colon cancer etiology and ways of preventing colon cancer by dietary means is still insufficient. Colon cancer arises through a gradual series of histological changes, each of which are accompanied by a specific genetic alteration (209). From the genes involved, the adenomatous polyposis coli (*APC*) gene can be regarded as the gatekeeper gene for colon (210). Germ-line mutations in the *APC* gene result in familial adenomatous polyposis (FAP), one of the principal hereditary predispositions to colon cancer (209). Somatic *APC* mutations are also found in most sporadic colon tumors (211). At present, various mouse models with specific *Apc* mutations have been characterized and genotype-phenotype correlations have been described. One of the *Apc* deficient mice, the so called Apc^{Min} mouse, is often used as a model for both mechanistic studies on intestinal cancer as well as experiments using different diets (122). The Apc^{Min} mice have a non-sense mutation in their *Apc* gene at codon 850, resulting in a truncated APC polypeptide. As a result, heterozygous *Apc* mice ($Apc^{Min/+}$) on a C57BL/6 background, develop a large number of intestinal polyps (120, 121).

Protein is a major dietary component and several studies suggest there is a difference between different dietary protein sources on intestinal cancer risk (2-4). Studies using the Apc^{Min} model showed no effects of soy isoflavones on intestinal tumor development in the Apc^{Min} mouse (125). However, a decreased number of intestinal polyps after feeding soybean derived Bowman-Birk inhibitor has been reported (124). In the current study milk, soy and meat protein were compared in the Apc^{Min} mouse model. Casein and soy protein isolate were compared because of an earlier reported difference on colonic cell proliferation in rats (4). Soybean flour was included in the study because it was reported to induce large intestinal tumor burden in rats compared to casein in a tumor model using chemical induction (2). Red meat was included in the study because several articles suggest an influence on colon cancer risk (84, 85). Because recent studies indicated that heme from red meat causes intestinal damage (83), several parameters relating to this hypothesis were included.

Besides several colonic markers such as cell proliferation, aberrant crypt foci or presence of tumors, several putative colon cancer risk parameters can also be measured in feces. Fecal β-glucuronidase and β-glucosidase activities were measured as an indication of the release of liver conjugated toxicants and the colonic bacterial activity (181). Bile acids and free fatty acids in fecal water were measured because of their lytic potential (4, 131, 182). Cytolytic activity of fecal water was measured as an indication of the lytic potential of colonic content towards epithelial cells (183). Fecal activity of the epithelial ALP iso-enzyme was measured as an indication of colonic epithelial cell damage (154). An increased fecal ALP excretion indicates a damaged colonic epithelium, possibly resulting in induced epithelial proliferation.

In summary, the aim of the study described was to determine the influence of four different dietary protein sources on several fecal colon cancer risk biomarkers and intestinal polyp formation in the *Apc*^{Min} mouse. Furthermore the hypothesis that heme from meat causes a high cytolytic activity of fecal water, was also tested.

MATERIALS AND METHODS

Animals and housing The experimental protocol was approved by both the animal welfare committee of Wageningen University and the RIVM (National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands). Our protocol closely resembled a study performed earlier by our group (122). The mice were bred at the RIVM. Using DNA isolated from mouse tales, progeny was genotyped before weaning by an allele specific PCR for the non-sense mutation at codon 850 (123) as described by Jacoby (212). Mice were obtained from breeding couples, each containing one male (*Apc*$^{Min/+}$) and two female (C57BL/6) mice. After weaning, mice were transported to the Laboratory Animal Center (Wageningen, The Netherlands), housed in groups of three to seven animals of the same sex per cage of the same diet under controlled environmental conditions (temperature, relative humidity, 12h/12h light dark cycle). One hundred and twenty *Apc*^{Min} male and female mice were divided over four dietary groups (n=15 per group, per sex).

Diets Diets were already fed to parents before mating. Animals had free access to food and tap water. The rats were divided over four different dietary protein groups: casein (88% protein, DMV, Veghel, The Netherlands), soy protein isolate (90% protein, Protein Technologies International, Ieper Belgium), soybean flour (54% protein, defatted soy flour 200/20, Cargill, Amsterdam, The Netherlands) and minced pork meat obtained from a local butcher. Meat was microwave heated until done. Diets will respectively be referred to as Casein, Soy-i, Soy-f and Meat. The ingredients of the diets are shown in Table 8.1. The final composition of the diets was 20% protein, 20% fat, 10% fiber, 40% fermentable carbohydrates and 10% salts, vitamins and minerals (100 mmol/kg calcium).

Dissection At the age of 99±1 days animals were sacrificed and the entire intestine was removed. The small intestine was cut into six separate pieces of equal length. The colon was taken out separately. All intestinal parts were split open longitudinally and cleaned carefully. Then all intestinal parts were fixed flat between two pieces of filter paper and fixed and stored in 4% formaldehyde solution until further analysis.

Analysis of polyps Intestinal polyps were counted for the separate intestinal parts after a 30 second staining with 0.5% methylene blue. All polyps were counted by one person using a binocular at a 14 times magnitude.

Table 8.1 Global composition of experimental diets (g/100g). [a] Soy flour contains more starch and cellulose and less protein than other protein sources. [b] Meat contains a higher amount of fat than other protein diets.

	Casein	Soy isolate	Soy flour	Pork meat
Casein	23.3	--	--	--
Soy isolate	--	22	--	--
Soy flour	--	--	36.67[a]	--
Meat	--	--	--	22.7
Corn starch	20	20	12.33[a]	20
Cellulose	10	10	4[a]	10
Vitamin mix	0.28	0.28	0.28	0.28
Trace-elements mix	0.28	0.28	0.28	0.28
Lard	18	18	18	17.3[b]
Corn oil	2	2	2	2
Glucose	21.2	22.6	21.6	22.6
$CaHPO_4$ *$2H_2O$	1.67	1.67	1.67	1.67
KH_2PO_4	0.80	0.80	0.80	0.80
KCl	0.80	0.80	0.80	0.80
NaCl	0.34	0.34	0.34	0.34
$MgSO_4$*$7H_2O$	0.45	0.45	0.45	0.45
MgO	0.23	0.23	0.23	0.23
Methionine	0.23	0.28	0.28	0.28
Choline Cl 50%	0.35	0.353	0.353	0.353
Total	100	100	100	100

Feces collection Feces was collected a few weeks prior to the end of the experiment. Feces was collected after placing a wired bottom in each animal cage for two weeks. The first week was used for acclimatization, the second for feces collection. Feces was collected for three days and freeze-dried afterwards. Before homogenization, hairs and food crumbs were removed.

Determinations in fecal water Fecal water was prepared by incubating homogenized freeze dried feces with water at 37 °C as described earlier (198). Fecal water was obtained after 11 minutes of centrifugation at 15000 g and stored at –20 °C until further use. Fecal water was prepared on a 16.7% dry weight basis because with 35% (198) no fecal water could be obtained.

Free fatty acids in fecal water were enzymatically determined using a colorimetric assay (NEFA-C, Wako Chemicals). Bile acids were determined as described by Mashige (189). Human O-negative erythrocytes for the determination of the cytolytic activity of fecal water were prepared according to Coleman (190). Determination of cytolytic activity of fecal water was based on Govers (52). In short, 50 µl of fecal water was incubated with 5 µl washed and

diluted O-negative human erythrocytes. Lysis was measured at OD540 nm using the appropriate blanks and demineralized water as a positive control.

β-Glucosidase and β-glucuronidase activities were determined spectrophotometrically (405 nm) by measuring paranitrophenol (PNP)-β-D-glucuronide and paranitrophenol-β-D-glucoside (Sigma) hydrolysis respectively (199). The reaction was performed in a 96 wells plate, containing 75 µl of five times diluted fecal water, 25 µl of a 0.1% PNP-glucuronide or PNP-glucoside solution and 25 µl 50 mM phosphate buffer pH 6.5. PNP was used for preparing a calibration curve. Intestinal ALP activity was measured using L-phenylalanine as a specific inhibitor of the intestinal isozyme (154).

Statistics Statistical analysis was performed using SPSS version 7.5. Data were analyzed by using general factorial analysis of variance. In case of homogeneity of variance, the Bonferroni test of significance was used ($p \leq 0.05$). The non-homogeneic data were tested on significance with the Dunnett T3 test ($p \leq 0.05$). Extreme values, as shown by box-plot analysis, were excluded from analysis. When data were not normally distributed the Kruskal-Wallis test was used. All data were checked for litter dependency. If data were dependent on the litter then the litter was used as the statistical unit. All data were analyzed per sex.

RESULTS AND DISCUSSION

In Figure 8.1 animal growth curves are shown for both male and female *Apc*^{Min} mice. Data were litter dependent. There were no significant differences for either start or end weight between diets for both males and females.

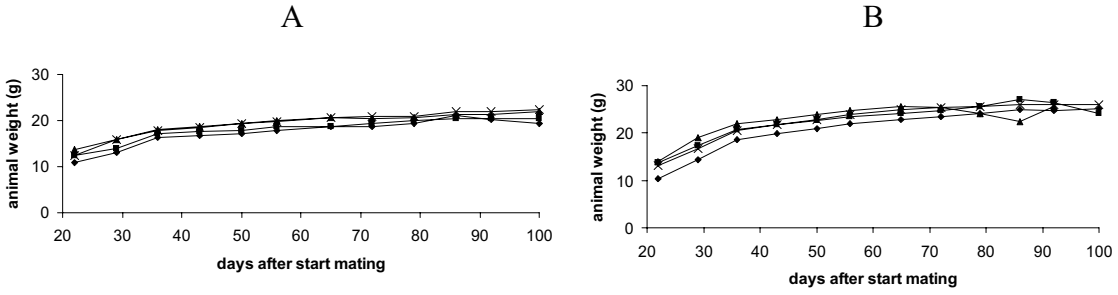

Figure 8.1 Animal weight curves. Casein (♦), Soy-i (■), Soy-f (▲) and Meat (**x**). Averages are shown. A) Females. B) Males.

In Figure 8.2 to 8.6 all the fecal water parameters are shown. Compared to the other protein diets, fecal water pH from the Soy-f group was decreased for both male and female animals (Figure 8.2). This is probably due to the fiber type of the Soy-f diet, which is apparently better fermentable than cellulose present in the other diets.

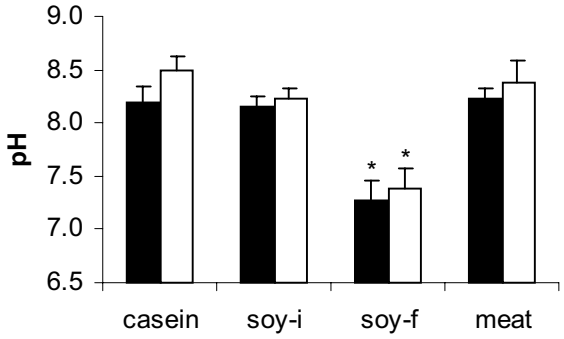

Figure 8.2 pH of fecal water. Values are shown as mean ± SD. (■) Females, (□) Males.

Some differences were found in lytic components of fecal water (Figure 8.3). Fecal water bile acid concentrations were decreased for both soy protein diets compared to casein and meat protein. For Soy-f this could be expected because secondary bile acids are less soluble at lower pH, which reduces their concentration in fecal water (151).

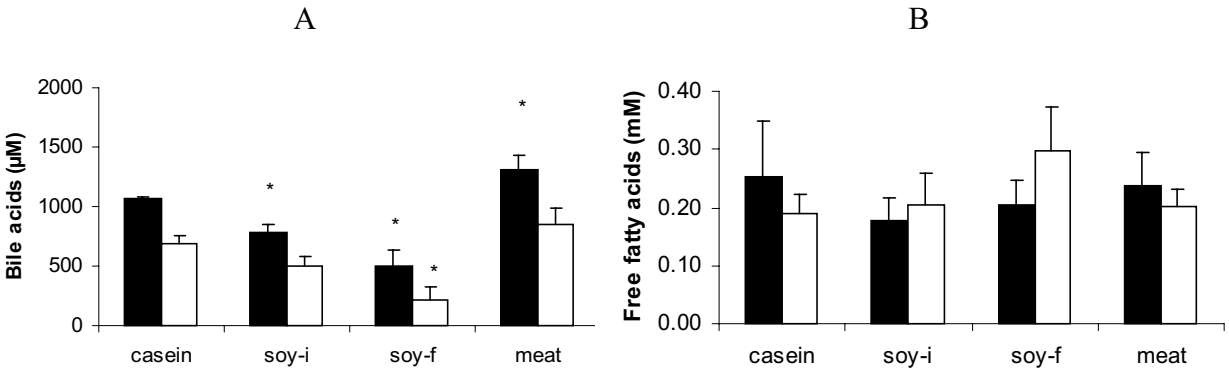

Figure 8.3 Lytic compound concentrations in fecal water. A) Bile acids. B) Free fatty acids. Values are shown as mean ± SD. (■) Females, (□) Males.

Again the Soy-f diet showed the strongest effect. Male fecal water bile acid concentrations were consistently lower than female fecal water bile acid concentrations. There were no significant differences in the fecal water free fatty acid concentrations between diets or sexes. Despite the presence of lytic free fatty acids and bile acids no cytolytic activity of fecal water was found for each diet. This is especially remarkable for the meat protein containing diet, because heme from red meat is reported to be highly cytolytic (83) (Figure 8.4). Our results suggest that the cytolytic potential of pure heme (83) is blocked by the presence of the meat proteins, meaning that only pure heme, but not red meat is a risk factor for colon cancer.

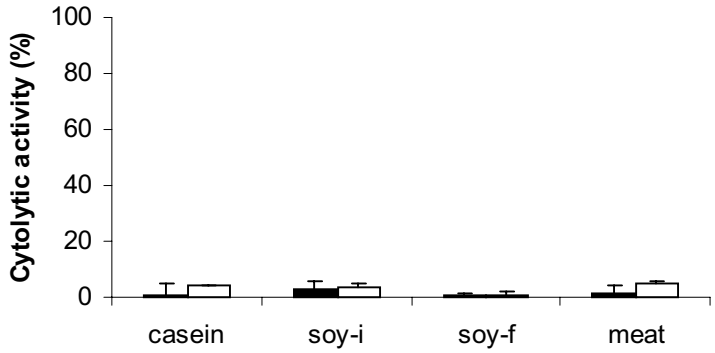

Figure 8.4 Cytolytic activity of fecal water. Values are shown as mean ± SD. (■) Females, (□) Males.

Fecal water alkaline phosphatase activity (ALP) as a marker of colonic epithelial damage was equal for all groups except for the Soy-f group that showed an unexplained increase in fecal ALP excretion. This increase in ALP excretion was specifically clear for the female group on the Soy-f diet (Figure 8.5).

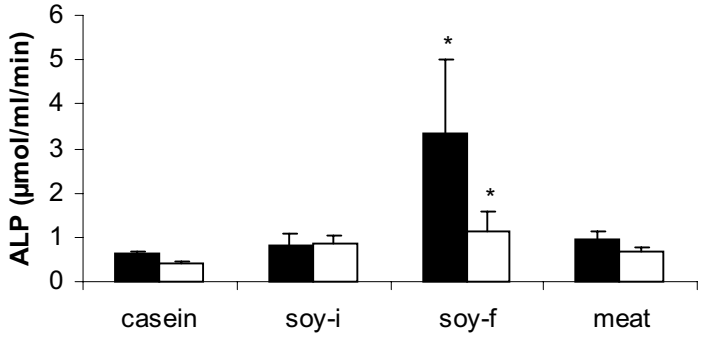

Figure 8.5 Fecal water alkaline phosphatase activity. Values are shown as mean ± SD. (■) Females, (□) Males.

The fecal water β-glucuronidase activity was significantly increased for the females on Soy-i and both Soy-f diets compared to the Casein diet. Fecal water β-glucosidase activities were also increased for both Soy-f and Soy-i diets (Figure 8.6). Soy-f showed a stronger increase than the Soy-i group for both enzymes. The difference in induction compared to Casein was much smaller than for the β-glucosidase activity. According to our results, the reported (213) increased β-glucuronidase activity and the decreased β-glucosidase activity found after switching from a grain based to a beef based diet, appears not to be caused by the protein component of beef. For all four diets fecal water from male mice showed a somewhat higher enzyme activity than the fecal water from female mice. Overall conclusion for the fecal parameters is that parameters influenced by fiber fermentation (pH, β-glucosidase) show large differences between diets but parameters linked to lytic exposure or effects are not or less influenced.

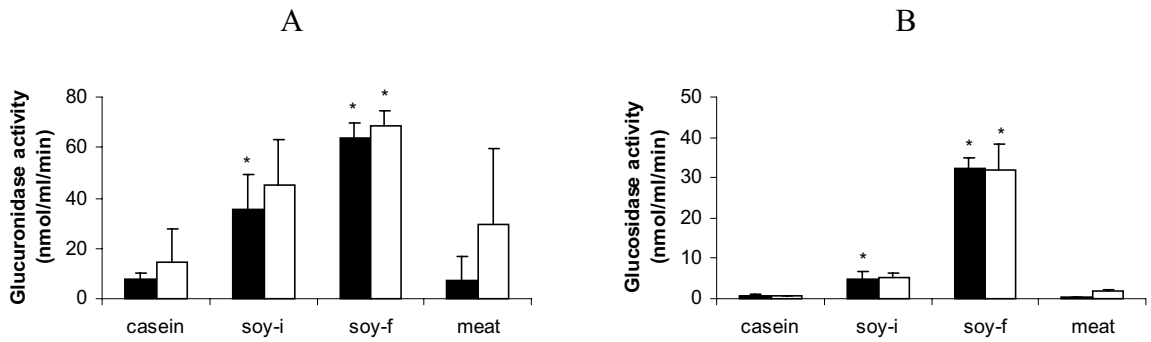

Figure 8.6 Fecal water A) Glucuronidase, B) Glucosidase activity. Values are shown as mean ± SD. (■) Females, (□) Males.

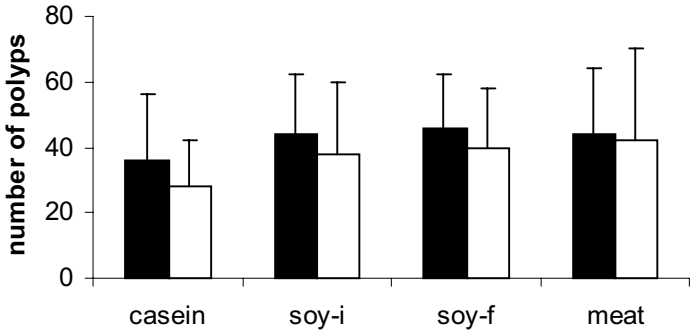

Figure 8.7 Number of polyps in the small intestine. Values are shown as mean ± SD. (■) Females, (□) Males.

Intestinal polyps were counted for both the small and large intestine. There were no significant differences in the number of small intestinal polyps between the diets (Figure 8.7). As shown in Figure 8.8A there was no significant difference in the number of colonic polyps between dietary groups or sexes. Only when data were pooled (Figure 8.8B) to plant (soy) and animal (casein and meat) proteins, a significant increase in the number of colonic polyps for the animal protein sources was found, for males only.

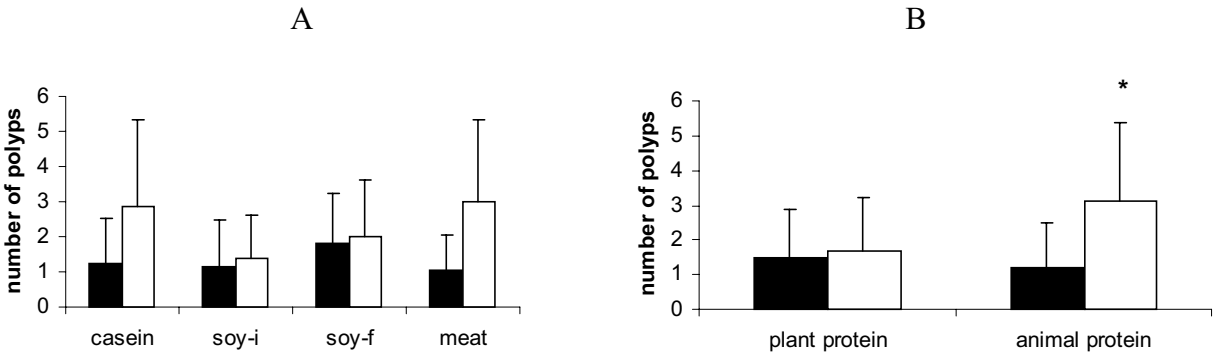

Figure 8.8 Number of large intestinal polyps A) For Casein, Soy-i, Soy-f, Meat respectively. B) For pooled data of Soy-i and Soy-f (plant protein) and casein with meat (animal protein). Values are shown as mean ± SD. (■) Females, (□) Males.

In summary, there are no clear and consistent effects of the dietary protein sources tested on both small and large intestinal polyp formation. These results are in contrast to earlier reports (2, 4), where a protective effect of casein was found compared to soybean proteins on colon cancer risk parameters in animal studies. On the other hand, more recent reports suggest a more protective effect of soy protein compared to casein (64, 66-68, 81). A possible explanation for the lack of difference between the dietary protein groups, is the relatively high amount of calcium in the diet compared to other studies. Because in this study diets were fed during breeding of the animals, a physiological amount of calcium had to be present to enable healthy growth of the young animals. Because calcium is a putative protective agent for colon carcinogenesis (50, 51), this physiological amount of calcium may have been responsible for the lack of difference between the diets. This is consistent with our findings that no cytolytic activity of fecal water was found. Possibly many of the lytic compounds were not available because they were precipitated by calcium.

Few studies include both male and female animals. This study shows that most parameters exhibit comparable effects for males and females, although some parameters reveal a consistent but small difference in the absolute values measured. Furthermore, this study shows that the type of soy protein preparation is very important when using soy protein in an animal experiment. Together with the variable presence of many soy non-nutrients (54, 55), this possibly explains why experiments reported in literature, using soy protein show such variable results.

In summary it is concluded that casein, soy protein isolate, soybean flour and meat protein show different results on several fecal parameters. These differences do not consistently lead to differences in the number of small or large intestinal polyps although the number of large intestinal polyps is significantly increased in males when plant proteins are compared to animal proteins.

ACKNOWLEDGEMENTS

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Chapter 9

General discussion

Overall

The main objective of the studies described was to investigate the influence of dietary protein sources on colon cancer risk and related parameters. This objective was mainly based on earlier observations that casein fed in animal studies showed a protective effect on several colon cancer biomarkers compared to soy protein (2-4). The overall conclusion from the studies performed is that many significant differences occur between casein and other dietary protein sources on parameters related to the carcinogen scavenging capacity of proteins and on fecal parameters such as fecal bile acid, ALP and β-glucuronidase excretion. However, no consistent *in vivo* protective effect of casein exists on colonic cell proliferation, aberrant crypt foci formation and polyp formation.

Carcinogen scavenging capacity of proteins *in vitro*

The aim of the *in vitro* studies was to more thoroughly investigate the carcinogen scavenging capacity of different dietary proteins. Results showed that although protein type influenced the scavenging capacity, protein digestion, heat treatment and carcinogen type were more important. Differences in scavenging capacity between proteins that were observed in test tube experiments disappeared or became inconsistent when using a more physiologically relevant gastro-intestinal simulator. Therefore, the relevance of the carcinogen scavenging experiments lies mainly in the illustration of important principles in the bioavailability of carcinogens present in food. The studies performed did not only show the importance of physiologically relevant models, but also showed the influence of many parameters such as type of carcinogen, food matrix, intestinal digestion conditions, reactivity of the carcinogen and lipophilicity of the carcinogen. These basic principles account not only for mutagens and carcinogens, but also for substances like vitamins or other bioactive compounds. Overall, the results illustrate that the presence of a carcinogen in food does not necessarily mean that the compound will reach the target in the body in an active form.

Fecal parameters

In the various animal studies performed, fecal parameters were measured. In Table 9.1 and Table 9.2 an overview of several parameters measured in feces is shown. When comparing animal studies, only fecal fat induction, β-glucuronidase activity, β-glucosidase activity and fecal water bile acid concentration showed a consistent difference between casein and soy. After soy protein feeding, fecal fat excretion was consistently doubled, enzyme activities were increased and the fecal water bile acid concentration was decreased. The decrease of the bile acid concentration can be interpreted as a protection. This protection is enhanced by the finding that soy saponins protect against cholic acid induced cytolytic activity.

The pH of the fecal water after feeding either casein or soy appeared to be constant around pH=8. It was hypothesized in Chapter 2 that increased fecal magnesium excretion indicates an increased colonic epithelial magnesium release. However in Chapter 5 (Study 2) and Chapter 6 (Study 1) no increase of fecal magnesium excretion after feeding of different brands of soy protein isolate was observed. In Chapter 5 (positive control of Study 1) we even showed that when colonic epithelial damage was induced by dietary cholic acid suppletion, fecal magnesium excretion decreased instead of increased. Therefore it is concluded that fecal magnesium excretion is not an indicator for colonic epithelial damage. For all other parameters, absolute values strongly differed between animal experiments. Because of this variability, no consistent conclusion could be drawn on the influence of different dietary protein sources on fecal water free fatty acid concentration. The various animal studies on fecal ALP excretion showed both significant decreases and increases after feeding soy protein isolate compared to casein. Because ALP was shown to be a good predictor of colonic cell proliferation, this confirms the inconsistency of the difference between casein and soy protein. Throughout the studies, no differences were found in the cytolytic activity of fecal water. This is especially remarkable for the mice fed red meat protein in the *Apc*^{Min} study, because heme present in meat is known to be highly cytolytic (83). Apparently heme in the form of meat protein is not nearly as lytic as heme added to the diet in the pure form.

Results of the fecal parameters clearly show that when comparing effects of casein and soy protein isolate, results are often variable between animal experiments. A likely influential parameter causing differences in absolute and relative results between casein and soy is the different dietary composition of the diets used for the different experiments. For example, the amounts of fiber, fat and minor dietary constituents differ between experiments. An advantage of such variability is that consistent effects of different dietary protein sources on for example the number of ACFs can be specified to those protein sources irrespective of dietary background. However, in this case it is concluded that the effect caused by some protein sources is possibly also partly dependent on interaction with other dietary constituents. Another permanent source of variability in the animal experiments described is the variable composition of non-protein components in especially the soy protein preparations. Soybean isoflavones and saponins are known to be present in highly variable concentrations in both the soybean and soy protein preparations (54). Although the same brand of soy protein isolate was used for the various experiments, the soy protein used was not from one single batch. Even if one single batch had been used, a possible effect of storage could have occurred (214). This knowledge combined with the experimental results described in literature and in this thesis, leads to the conclusion that one should be very cautious in the interpretation of studies in which soy protein preparations are used.

Colonic parameters

Colonic cell proliferation, aberrant crypt foci and intestinal polyps were measured because they represent early stages of colon cancer. It is concluded that there is only little or no effect of casein compared to soy protein feeding on these colonic parameters. As mentioned in several chapters, this is in agreement with recent preliminary publications from several independent research groups (66-68). The validity of earlier reported effects of soy protein feeding compared to casein on these parameters is probably limited to the protein batch used (4), or the model system used in the experiments (3) or a combination of both (2). The fact that we did not find soy protein feeding to increase colon cancer risk was confirmed by several fecal parameters measured. The induction of the total fecal fat excretion after feeding soy protein isolate compared to casein was consistent but limited to a factor two. The concentration of lytic bile acids and free fatty acids was not increased after soy feeding. Cytolytic activity of fecal water between soy and protein diets was equal and the fecal ALP excretion indicating colonic epithelial damage was even decreased after soy feeding. A possible explanation for the lack of difference on colonic parameters was that all animal experiments were performed using diets containing high amounts of dietary protein (± 20% w/w). The high amounts of dietary protein were a result of the choice to feed the animals human based diets. It is possible that would the same experiments have be performed using low protein diets (6%), some differences might have occurred, because under limiting conditions possible differences may be more pronounced.

Correlation between fecal and colonic parameters

Irrespective of the influence of dietary protein, the correlation between fecal and colonic parameters determines the predictive value of fecal parameters for colon cancer risk. From the studies performed it is concluded that the fecal parameters tested do not consistently predict the outcome of the colonic parameters measured. At first sight, fecal water pH, free fatty acid concentration and cytolytic activity were good predictors for the results of the colonic parameters, because the colonic parameters and these fecal parameters did not show any difference between casein and soy protein. However, a good predictive value can only be obtained with variables showing a clear difference between groups. Fecal parameters showing a large difference between casein and soy, such as ALP, β-glucuronidase and β-glucosidase activity, did not predict the outcome of proliferation, crypt formation or polyp formation. Apparently these parameters do not influence rate limiting steps in the development of colon cancer. Although fecal parameters have proven to be useful in mechanistic studies in which the focus is on only one aspect of colon cancer, they failed to predict early stages of colon cancer. However, because the need for valid colon cancer risk predictors remains high, research on these early predictive biomarkers other than tumors remains very important (215). In this respect, research and development in genomics (216) and in the future maybe on

proteomics (217) are promising because much information can be obtained with highly sophisticated techniques. Because these techniques can be used both in cell culture, animal and human situations they provide information on the predicting values of *in vitro* and animal system for human health risks.

Overall discussion and implications

The results described do not support an advice on consuming either more or less casein or soy protein containing products. These results suggest that no further research on the beneficial effects of casein compared to other dietary protein sources on the colon cancer risk is needed. However, many questions remain in the field of protein and colon cancer research. For example, the influence of dietary whey proteins remains to be elucidated and quantified. Promising results have been reported on the influence of whey proteins on both the immune system and the biotransformation system (77). As a follow up of the project described in this thesis, studies are currently performed by our group focussing on the effect of whey protein compared to soy protein isolate on both liver and colonic biotransformational enzyme activities and colonic cell proliferation. In view of growing markets for food supplements, functional foods, neutraceuticals and developments in the area of novel protein foods, this type of research will remain important in the years to come.

Table 9.1 Overview of some results for fecal parameters measured in the various animal studies described in this thesis. All parameters were measured in the fecal water fraction of the feces collected. ALP, β-glucuronidase and β-glucosidase activities were also expressed per gram feces. Soy-i = soy protein isolate, Soy-2 = soy protein isolate (a different brand), Soy-f = defatted soybean flour, Meat = red pork meat. Data are shown as average ± SD.

Parameters	Animal study	Chapter (study)	Unit	Casein	Soy-i	Soy-2 or Soy-f	Meat
pH	soy extracts	5 (2)		8.2±0.3	7.7±0.1		
	cell proliferation	6 (1)		7.8±0.5	8.0±0.4	7.5±0.3	
	ACF	7 (1)		8.3±0.1	8.3±0.1		
	Apc (male)	8		8.5±0.1	8.2±0.1	7.4±0.2	8.4±0.2
	Apc (female)	8		8.2±0.2	8.2±0.1	7.3±0.2	8.3±0.1
Bile acid	soy extracts	5 (2)	mM	4.2±3.1	0.4±0.5		
	cell proliferation	6 (1)	mM	1.8±0.5	0.5±0.1	0.6±0.1	
	ACF	7 (1)	mM	0.4±0.0	0.3±0.0		
	Apc (male)	8	mM	0.7±0.1	0.5±0.1	0.2±0.1	0.9±0.1
	Apc (female)	8	mM	1.1±0.0	0.8±0.1	0.5±0.1	1.3±0.1
Free fatty acid	soy extracts	5 (2)	mM	0.5 ± 0.3	0.04±0.02		
	cell proliferation	6 (1)	mM	0.08±0.03	0.05±0.02	0.09±0.05	
	ACF	7 (1)	mM	4.7±2.5	7.1±2.8		
	Apc (male)	8	mM	0.19±0.03	0.21±0.05	0.30±0.08	0.20±0.03
	Apc (female)	8	mM	0.25±0.10	0.18±0.04	0.20±0.04	0.24±0.06
Cytolytic act.	cell proliferation	6 (1)	%	42±13	48±12	41±12	
	ACF	7 (1)	%	36±19	19±15		
	Apc (male)	8	%	4.3±4.4	3.4±3.1	0.9±0.7	4.9±2.9
	Apc (female)	8	%	0.7±0.2	2.5±1.5	1.0±1.3	1.6±0.9
ALP	soy extracts	5 (2)	µmol/ml/h	11±4.0	6.1±6.0		
	cell proliferation	6 (1)	µmol/ml/h	246±192	24±12	78±4.2	
	ACF	7 (1)	µmol/ml/h	3.3±2.2	8.5±3.6		
	ACF	7 (1)	µmol/g/h	58±37	23±11		
	ACF	7 (2)	µmol/g/h	100±79		15±11	
	Apc (male)	8	µmol/ml/h	24.6±1.2	51.6±12	66±28.8	39±6.0
	Apc (female)	8	µmol/ml/h	38.4±3.0	48.6±15	198±102	56.4±10.2
β-Glucuronidase	cell proliferation	6 (1)	µmol/ml/h	30±24	1086±510	1008±576	
	ACF	7 (1)	µmol/ml/h	1.3±0.6	3.5±1.5		
	ACF	7 (1)	µmol/g/h	15.0±7.8	36.0±5.4		
	ACF	7 (2)	µmol/g/h	19±13		39±29	
	Apc (male)	8	µmol/ml/h	0.86±0.79	2.7±1.1	4.1±0.38	1.8±1.8
	Apc (female)	8	µmol/ml/h	0.49±0.11	2.1±0.85	3.8±0.38	0.43±0.57
β-Glucosidase	cell proliferation	6 (1)	µmol/ml/h	4.8±12	114±42	162±78	
	ACF	7 (1)	µmol/ml/h	0.01±0.04	0.3±0.14		
	ACF	7 (1)	µmol/g/h	0.8±0.5	3.1±0.8		
	ACF	7 (2)	µmol/g/h	15±6.7		30±43	
	Apc (male)	8	µmol/ml/h	0.04±0.0	0.32±0.05	0.19±0.39	0.1±0.02
	Apc (female)	8	µmol/ml/h	0.05±0.01	0.30±0.10	1.91±0.16	0.02±0.01

Table 9.2 Overview of some results for fecal parameters measured in the various animal studies described in this thesis. All were measured in the total feces collected (not in the fecal water fraction). Soy-i = soy protein isolate, Soy-2 = soy protein isolate (a different brand), soy+ = soy protein isolate with extra soy non-nutrients. Data are shown as average ± SD.

Parameters	Animal study	Chapter (study)	Unit	Casein	Soy-i	Soy-2 or Soy+
Magnesium	cell proliferation	6 (1)	µmol/d	1.40±0.40	1.61±0.36	1.49±0.27
	soy extracts	5 (2)	µmol/g	67.9±9.5	68.3±8.6	70.8±7.4
Total fat	cell proliferation	6 (1)	mg/g	9.2±2.2	27.3±4.0	18.0±2.4
	cell proliferation	6 (1)	mg/d	30.1±7.6	90.4±21.3	59.8±11.1
	soy extracts:					
	day 3	5 (2)	mg/d	25±13	37±19	47±18
	day 7	5 (2)	mg/d	39±19	54±16	87±20
	day 16	5 (2)	mg/d	35±8	59±13	73±16
	ACF	7 (1)	mg/g	21±1	59±8	
		7 (1)	mg/d	30±5	80±17	
Free fatty acid	ACF	7 (1)	µmol/g	21±9	45±10	
		7 (1)	µmol/d	31±17	60±17	

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Summary and concluding remarks

Colon cancer (cancer of the large intestine) is a worldwide problem in especially Western countries. The diet might be responsible for up to 90% of these colon cancer cases. This means that decreasing colon cancer risk should be possible by changing the diet. The research presented in this thesis concerns the question what the influence is of dietary protein sources on colon cancer risk. As described in **Chapter 1**, casein was compared to other dietary protein sources (mainly soy protein) for its influence on:

1. the carcinogen scavenging capacity of proteins,
2. early stages of colon cancer,
3. fecal colon cancer biomarkers.

Chapter 2 provides information on various fields of study in the research on dietary proteins and colon cancer, including background information on the methods used. It is explained how dietary proteins can act as carcinogen scavengers by binding to carcinogens in the gastro-intestinal lumen. The carcinogen can be transported along the intestinal tract and be taken up by the body. If a carcinogen is not taken up, it will be excreted with the feces. Furthermore, the influence of the *amount* of protein intake in the diet on colon cancer risk is discussed. It is concluded that either no or a positive correlation between the amount of protein intake and colon cancer risk, exists. Another important research area is protein digestibility because a low protein digestibility results in a high amount of protein in the colon. Furthermore, the amino acid composition of a protein is important for two reasons. When an essential amino acid is present in a limiting amount, growth of both the individual and the tumor is inhibited. Secondly, some amino acids are needed for specific biological functions. For example, sulfur containing amino acids can stimulate the biotransformational system. The last important factor discussed in the research area of proteins on colon cancer risk is the presence of various non-protein components. As an example, saponins and isoflavones present in many soy proteins and known to be biologically active, are discussed.

In Chapter 3 and 4 it is described how dietary proteins can scavenge carcinogens *in vitro*. In **Chapter 3** it is shown that carcinogen scavenging is influenced by many factors, such as heat treatment of the protein, degree of protein digestion and presence of bile acids or lipase. Protein type did not have a major influence so no effect of feeding different dietary protein sources on colon cancer risk is expected.

In **Chapter 4** it is shown that the carcinogen scavenging capacity of proteins is also very much dependent on the type of carcinogen. Benzo[a]pyrene, a large non-reactive pro-

carcinogen, interacted only with intact dietary proteins, whereas MNNG, as described in Chapter 3, strongly interacted with both intact as well as hydrolyzed proteins. Again no difference between dietary protein sources was shown, suggesting no difference in colon cancer risk.

In **Chapter 5** the first animal experiments are described. In Study 1, the influence of the addition of 1% methionine to the diet on colonic cell proliferation was tested. Methionine is an important methyl donor in many metabolic routes and is present in different amounts in casein and soy protein preparations. Colonic cell proliferation was used as an indication of colon cancer risk, because cell proliferation is an important phase in the process of colon cancer. The study showed no influence of methionine on colonic cell proliferation. In Study 2 rats were fed different amounts of soy non-nutrients to test the influence of these non-nutrients on several parameters in the feces. Most important fecal parameter was the fecal fat percentage, because earlier studies reported a correlation between high fecal fat and colon tumors. Study 2 showed that fecal fat excretion in rats is dependent on the amount of soy non-nutrients in the diet. However, the difference in fecal fat excretion between casein and soy protein was smaller (factor 2) than reported in literature. Therefore no major effect on colon cancer risk is expected. The influence of soy non-nutrients on processes in the colon was shown in Study 3. In this *in vitro* study, soy non-nutrients strongly reduced bile acid induced cell damage, suggesting a small protective effect of dietary soy protein.

In **Chapter 6** two animal studies on the influence of casein and soy protein on colonic cell proliferation are described. The studies showed no difference in colonic cell proliferation between these two protein sources. However different results for the casein and soy protein diets were obtained for several parameters that were measured in the feces of the animals. These fecal parameters were measured because they show how important steps in the process of colon cancer development are affected. A high fecal fat percentage correlates with a high number of tumors in the colon. Fecal alkaline phosphatase (ALP) activity represents damage to cells of the colonic epithelium because epithelial cells exhibited a high ALP activity. Bile acids and free fatty acids can damage the colonic epithelium because of their lytic potential. The cytolytic activity measures this damaging capacity, because it measures the lytic potential of the colonic contents towards cells in an *in vitro* test. The pH of the fecal contents was measured because a low pH represents a healthy colonic environment. Fecal β-glucuronidase and β-glucosidase activities were measured because they represent the capacity of the colonic contents to liberate toxicants in the colonic lumen that were earlier detoxified by the liver.
Results on cytolytic activity confirmed the lack of difference between casein and soy on colonic cell proliferation because there was no difference between diets. Fecal fat excretion was doubled after feeding soy protein compared to casein. After feeding soy protein, fecal

water bile acid concentrations and ALP activity were decreased. However, β-glucuronidase and β-glucosidase were increased 10-100 fold. Overall, no consistent conclusion on fecal parameters was possible.

In **Chapter 7** the influence of casein, soy protein isolate and soy flour on colonic aberrant crypt foci (ACF) is tested. ACFs are considered to be early stages of colonic tumors. Overall no consistent difference of casein and soy protein on aberrant crypt foci was detected. Again β-glucuronidase, β-glucosidase and ALP activities were measured in feces. β-Glucuronidase and β-glucosidase activities were significantly increased after feeding soy protein suggesting an increased risk after soy feeding.

In **Chapter 8** the influence of casein, soy protein isolate, soy protein flour and red meat is tested on the occurrence of intestinal polyps in the Apc^{Min} model. In this model, mice with a defect *Apc* gene spontaneously develop polyps in both small and large intestine. The four diets showed no difference in the occurrence of polyps. Again several differences were observed in the fecal parameters. β-Glucuronidase and β-glucosidase were strongly increased after soy protein compared to the other diets. The pH of the colonic contents was decreased after soy flour feeding indicating fermentation of fibers present in soy flour preparations has a major influence. Furthermore it was shown that heme in the form of meat protein is not nearly as lytic as heme added to the diet in the pure form. No differences between the diets were found for free fatty acids. The concentrations of bile acids in fecal water were decreased for especially the soy protein diets. Overall, fecal parameters showed marked differences, but again no consistent protective or risk inducing effects.

In **Chapter 9** all studies performed are compared and discussed with an emphasis on the animal studies. Overall, fecal fat excretion was consistently doubled after feeding soy protein. β-Glucuronidase and β-glucosidase, able to release toxicants, were consistently increased after feeding soy protein. Bile acids in fecal water, able to damage colonic epithelium, were consistently decreased after soy protein feeding. Other fecal parameters either showed no difference between diets (pH, cytolytic activity) or variable results (ALP, free fatty acids). It was shown that fecal magnesium excretion has no predictive value for colonic cell proliferation. Fecal β-glucuronidase, β-glucosidase, ALP, bile acids and free fatty acids showed large differences in absolute values between animal experiments. Possibly the effect caused by some protein sources on fecal parameters, is also partly dependent on interaction with other dietary constituents such as fat and fiber or non-protein components. Because no differences on cell proliferation, aberrant crypt foci and intestinal polyps were found, a difference in colon cancer risk after consumption of casein or soy protein is unlikely. Correlation studies revealed that none of the fecal parameters tested consistently predicted the

outcome of the colonic parameters tested, stressing the need for further research in this area. Because of the variable composition and results obtained with soy, it was concluded that one should be very cautious concerning the interpretation of studies in which soy protein preparations are used.

The main conclusion from the studies performed is that many significant differences occurred between casein and other dietary protein sources such as soy. Differences specifially occurred on parameters related to the carcinogen scavenging capacity of proteins and on fecal parameters such as fecal bile acid, ALP and β-glucuronidase excretion. However, no consistent *in vivo* protective effect of casein occurred on colon cancer, based on markers as colonic cell proliferation, aberrant crypt foci formation and polyp formation. Therefore results do not support an advice on consuming either more or less casein or soy protein containing products.

Samenvatting

Dikke darmkanker is een mondiaal probleem in voornamelijk Westerse landen. Er wordt geschat dat voeding voor ongeveer 90% verantwoordelijk zou kunnen zijn voor deze dikke darmkanker gevallen. Dit betekent dat de kans op dikke darmkanker verminderd zou kunnen worden door de voeding ten goede te veranderen. Het onderzoek dat in dit proefschrift wordt beschreven gaat over de vraag wat de invloed van verschillende eiwitbronnen in de voeding op het dikke darmkankerrisico is. Zoals wordt beschreven in **Hoofdstuk 1** is de invloed van caseïne (een belangrijk melkeiwit) vergeleken met andere eiwitbronnen (voornamelijk soja) op:

1. het wegvangen van kankerverwekkende verbindingen,
2. voorstadia van dikke darmkanker,
3. biomarkers voor dikke darmkanker in de ontlasting.

Hoofdstuk 2 geeft een overzicht van verschillende onderzoeksvelden op het gebied van voedingseiwitten en dikke darmkanker. In hetzelfde hoofdstuk wordt ook achtergrondinformatie over de gebruikte methoden verschaft. Er wordt uitgelegd hoe voedingseiwitten kankerverwekkende verbindingen kunnen wegvangen door aan deze carcinogenen te binden in het lumen van het maagdarmkanaal. De kankerverwekkende stof kan tot ver in het darmkanaal worden meegenomen en daar door het lichaam worden opgenomen. Wanneer de kankerverwekende verbinding niet wordt opgenomen, zal het worden uitgescheiden in de ontlasting. Verder wordt de invloed van de *hoeveelheid* eiwit in het dieet op het risico voor dikke darmkanker bediscussieerd. De uiteindelijke conclusie luidt dat er vaak geen of een positieve correlatie tussen de hoeveelheid eiwit in het dieet en de kans op kanker bestaat. Een ander belangrijk onderzoeksveld is de eiwitvertering omdat een slecht verteerbaar eiwit een verhoogde hoeveelheid eiwit in de dikke darm tot gevolg heeft. Daarnaast is de aminozuursamenstelling van een eiwit om twee redenen belangrijk. Wanneer een essentieel aminozuur in beperkende mate aanwezig is, wordt de groei van zowel het individu als ook het gezwel geremd. Ten tweede zijn sommige aminozuren nodig voor specifieke biologische functies. Zwavelhoudende aminozuren stimuleren bijvoorbeeld het biotransformatie systeem dat giftige verbindingen onschadelijk kan maken. Het laatst bediscussieerde onderwerp van het onderzoek naar eiwit en dikke darmkanker is de aanwezigheid van verschillende niet-eiwit factoren. Bij wijze van voorbeeld worden saponinen en isoflavonen uit soja, waarvan bekend is dat ze biologisch actief zijn, bediscussieerd.

In Hoofdstuk 3 en 4 wordt beschreven hoe kankerverwekkende stoffen door voedingseiwitten kunnen worden weggevangen (*in vitro*). In **Hoofdstuk 3** wordt aangetoond dat het wegvangen van kankerverwekkende stoffen wordt beïnvloed door een veelheid aan factoren. Voorbeelden hiervan zijn de hittebehandeling van het eiwit, de mate van eiwitvertering en de aanwezigheid van galzouten of het enzym lipase. Het type eiwit had weinig invloed. Op basis van deze waarnemingen wordt niet verwacht dat het type eiwit van grote invloed is op dikke darmkankerrisico.

In **Hoofdstuk 4** wordt aangetoond dat het wegvangen van kankerverwekkende verbindingen door eiwit sterk afhankelijk is van het type kankerverwekkende verbinding. Benzo[a]pyreen, een grote, niet-reactieve verbinding, vertoont alleen interactie met intacte voedingseiwitten. MNNG, een kleine, reactieve verbinding, daarentegen werd weggevangen door zowel intacte als verteerde eiwitten, zoals staat beschreven in Hoofdstuk 3. Opnieuw werd er geen verschil aangetoond tussen verschillende soorten eiwitten. Dit bevestigt de conclusie uit Hoofdstuk 3 dat de invloed van het soort voedingseiwit op het wegvangen van kankerverwekkende stoffen waarschijnlijk slechts een beperkte invloed heeft op het dikke darmkankerrisico.

In **Hoofdstuk 5** worden de eerste dierexperimenten beschreven. In Studie 1 staat het onderzoek beschreven naar de invloed van 1% extra methionine in het dieet op celproliferatie van epitheel in de dikke darm. Methionine is een belangrijke methyldonor in vele metabole routes. De hoeveelheid methionine in caseïne en soja-eiwit verschilt sterk. De celproliferatie van dikke darmcellen is gebruikt als een marker voor dikke darmkankerrisico omdat celproliferatie een belangrijke fase in de ontwikkeling van darmkanker is. De studie toonde aan dat er geen invloed van methionine op celproliferatie is. In Studie 2 zijn ratten op diëten met verschillende hoeveelheden non-nutriënten uit soja gezet om de invloed van deze non-nutriënten op verschillende parameters in de ontlasting te testen. De belangrijkste parameter was de hoeveelheid vet in de ontlasting omdat eerdere studies een correlatie tussen een hoog vetpercentage in de ontlasting en dikke darmtumoren hebben aangetoond. In Studie 2 is aangetoond dat de hoeveelheid vet in de ontlasting toeneemt door de aanwezigheid van non-nutriënten uit soja. De hoeveelheid fecaal vet na het sojadieet was een factor 2 hoger dan na het caseïnedieet. Deze fecaal vet inductie was aanzienlijk lager dan de factor 5 die op basis van literatuurgegevens werd verwacht. Op basis van deze gegevens wordt geen groot effect op dikke darmkankerrisico verwacht. De invloed van non-nutriënten uit soja op processen in het dikke darmlumen is in Studie 3 aangetoond. In deze *in vitro* studie reduceerde non-nutriënten door galzouten geïnduceerde celschade, hetgeen een klein beschermend effect van soja-eiwitten suggereert.

In **Hoofdstuk 6** worden twee dierstudies beschreven waarin de invloed van caseïne en soja op celproliferatie in de dikke darm wordt onderzocht. De resultaten laten geen verschil in

celproliferatie tussen beide eiwitbronnen zien. Er werden echter wel verschillen gevonden tussen caseïne en soja voor de verschillende parameters die in de ontlasting van de dieren zijn gemeten. Deze parameters maken inzichtelijk maken hoe het dieet op verschillende stappen in de ontwikkeling van dikke darmkanker invloed kan hebben. Een verhoogde hoeveelheid vet in de ontlasting correleert met een verhoogd aantal tumoren in de dikke darm. Alkaline fosfatase (ALP) activiteit in de ontlasting geeft een indicatie van celschade in het dikke darmepitheel, omdat beschadigde epitheelcellen ALP uitscheiden. Galzouten en vrije vetzuren kunnen het dikke darmepitheel beschadigen vanwege hun lytische karakter. De cytolytische activiteit is een maat voor dit lytische karakter omdat hiermee bepaald wordt in hoeverre cellen kapot gaan wanneer ze in een *in vitro* test worden blootgesteld aan de waterige fractie van de darminhoud. De pH van de waterfractie van de ontlasting is bepaald omdat een lage pH vaak een teken is van een gezonde darmomgeving. Fecale β-glucuronidase en β-glucosidase activiteiten zijn bepaald omdat deze een beeld geven van het vermogen van de darminhoud om door de lever onschadelijk gemaakte gifstoffen in de darm wederom vrij te maken.
Resultaten van de cytolytische activiteitsbepaling bevestigden het gebrek aan verschil in celproliferatie bij caseïne en soja omdat er geen verschil was tussen de diëten. De hoeveelheid uitgescheiden vet in de ontlasting was verdubbeld na het sojadieet in vergelijking met het caseïnedieet. Dieren op het sojadieet vertoonden lagere concentraties galzouten in de waterfractie van de ontlasting. Ook was de ALP activiteit verminderd. De β-glucuronidase en β-glucosidase activiteit was een factor 10 tot 100 verhoogd. Samenvattend gaven de fecale parameters geen consistent beschermend of risico verhogend beeld.

In **Hoofdstuk 7** staat de invloed van caseïne, soja-isolaat en sojameel op het ontstaan van aberrante crypt foci (ACF) in de dikke darm centraal. ACF's kunnen worden gezien als een vroeg stadium van een dikke darmtumor. De geteste diëten verschilden niet consistent in hun invloed op het ontstaan van ACF's. Net als in voorgaande dierexperimenten zijn de β-glucuronidase, β-glucosidase en ALP activiteiten in de ontlasting bepaald. β-Glucuronidase en β-glucosidase activiteiten waren significant verhoogd voor de dieren op de sojadiëten hetgeen een verhoogd dikke darmkankerrisico voor soja suggereert.

In **Hoofdstuk 8** wordt een *Apc* studie beschreven waarin de invloed van caseïne, soja-isolaat, sojameel en rood vlees is getest op de aanwezigheid van darmpoliepen in het Apc^{Min} model. In dit model ontwikkelen muizen met een defect *Apc* gen spontaan poliepen in zowel dunne als dikke darm. Er is geen verschil in het aantal poliepen tussen de verschillende diëten aangetoond. Opnieuw zijn er wel verschillen gevonden voor de gemeten fecale parameters. β-Glucuronidase en β-glucosidase activiteit was sterk verhoogd voor de dieren op sojadiëten. De pH van de darminhoud was verlaagd na het sojameel dieet, hetgeen vermoedelijk

veroorzaakt is door het type vezel dat aanwezig is in het sojameel. Verder is aannemelijk gemaakt dat heem als onderdeel van vleeseiwit nauwelijks beschadigend is voor darmepitheelcellen ten opzichte van heem in een pure vorm. Er zijn geen verschillen gevonden voor de vetzuren. De galzoutconcentratie in het fecale water was verlaagd voor de sojadiëten. Samenvattend zijn er aanmerkelijke verschillen in fecale parameters gevonden tussen de diëten, maar is er wederom geen verschil gevonden in poliepen die het uiteindelijke risico op dikke darmkanker het beste aangeven.

In **Hoofdstuk 9** zijn alle uitgevoerde studies vergeleken en bediscussieerd, met de nadruk op de dierstudies. Voor de verschillende studies was de fecale vet excretie consistent verdubbeld na het sojadieet. β-Glucuronidase en β-glucosidase waren consistent verhoogd na de sojadiëten. Galzouten in fecaal water, in principe in staat om het dikke darmepitheel te beschadigen, waren consistent verlaagd na sojadiëten. Voor de andere fecale parameters werd geen verschil gevonden tussen de diëten (pH, cytolytische activiteit) of werden variabele resultaten gevonden (ALP, vrije vetzuren). Verder is aangetoond dat fecale magnesium excretie geen voorspellende waarde heeft voor celproliferatie in de dikke darm. β-Glucuronidase, β-glucosidase, ALP, galzouten en vrije vetzuren vertoonden grote verschillen in de absolute waarden tussen de verschillende dierexperimenten. Mogelijk is het effect dat nu is toegeschreven aan specifieke eiwitbronnen ook gedeeltelijk afhankelijk van de interactie met andere voedingscomponenten zoals vet, vezel of niet-eiwit componenten. Omdat er geen verschillen tussen de diëten zijn gevonden voor celproliferatie, ACF's en intestinale poliepen, is het onwaarschijnlijk dat er een verschil in dikke darmkankerrisico bestaat tussen caseïne en soja-eiwit. Uit correlatiestudies bleek dat geen van de fecale parameters een goede voorspeller was voor de uiteindelijke voorstadia van dikke darmkanker, hetgeen de behoefte aan verder onderzoek op dit vakgebied onderstreept. Omdat de resultaten na het voeren van soja vaak variabel waren en de sojapreparaten een variabele samenstelling hadden, is geconcludeerd dat de interpretatie van studies waarin soja gebruikt is, over het algemeen met grote voorzichtigheid moet plaatsvinden.

De eindconclusie van de beschreven studies luidt dat er vele verschillen zijn tussen caseïne en andere eiwitbronnen zoals soja. Dit geldt voornamelijk voor parameters die gerelateerd zijn aan het wegvangen van kankerverwekkende stoffen en voor parameters die worden gemeten in de ontlasting, zoals galzouten, ALP en β-glucuronidase activiteit. Echter, gebaseerd op markers zoals celproliferatie van dikke darmcellen en het voorkomen van ACF's of intestinale poliepen, is er geen consistent *in vivo* beschermend effect van caseïne voor dikke darmkanker. De resultaten geven daarom geen aanleiding voor een advies om meer dan wel minder caseïne of sojabevattende producten te consumeren.

Abbreviations

-/753	*E. coli* K-12 343/113/753
-/765	*E. coli* K-12 343/113/765
AAF	2-acetylaminofluorene
AAS	atomic absorption spectrophotometry
AC	aberrant crypt
ACF	aberrant crypt focus
AIN	American Institute of Nutrition
ALP	alkaline phosphatase
AOM	azoxymethane
Apc	adenomatous polyposis coli
B[a]P	benzo[a]pyrene
BSA	bovine serum albumin
CA	cholic acid
CV	coefficient of variation
DCA	deoxycholic acid
DH	degree of hydrolysis
DMH	dimethylhydrazine
DNA	deoxyribonucleic acid
DNP	N,N-dinitrosopiperazine
E.coli	*Escherichia coli*
E:S	enzyme substrate ratio
FAP	familial adenomatous polyposis
FT4	free thyroxine
GSH	glutathion-SH
GST	glutathion-S-transferase
LCFA	long chain fatty acids
LSA	liquid suspension assay
m	multiplicity
Met	methionine
Min	multiple intestinal neoplasia
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PNP	paranitrophenol
PNPP	paranitrophenolphosphate
SCFA	short chain fatty acids
SD	standard deviation
SEM	standard error of the mean
Soy-i	soy isolate
Soy-f	soy flour
TT3	total triiodothyronine
TT4	total thyroxine

Dankwoord

Zo daar licht ei dan. Dat het af schoonmaak, waren nota getwijfeld. Wanneer dat zou zijn ze hele tijd de vraag geweest ook hebben een heel lang afgevraagd goed uit zo zien, welke data willen niet mee zou kunnen nemen, doc artikelen en willen niet in zo'n staan, en hoeveel tijd gaande achtergrondinformatie te kunnen besteden. Ook de op maken is lang een bron van zorg geweest. Als ik iets aan het Proefschrift het overgehouden is een levenslange allergie op een voet het teksten. Maar ja, daar niet meer over getreurd. Het is nu tijd voor het krukje dat wel voor iedereen lees worden. Ik heb hard geprobeerd om zoveel ogen namen te doen om vooral populieren overkomen. Klik met een Misschien is het over een statement over het leven zonder handen. Trefwoorden creatief, lastig, bij tijd en wijle grappig en uiteindelijk met een beetje moeite een inspanning West de folteringen. De goede verstaander weet dat dit Feitelijk is deze dit deel van de dankwoord is het enige stuk wat er stem alleen gedaan. Deze alinea dient dan ook de ondersteuning van in samenstellingen. En van de weinige deel van het proefschrift vervangen van hard Hope dan maar weinigen zijn dat begrijp. OK, voor de duidelijkheid en voor mensen die nog niet was gevallen, bovenstaande is met spraakherkenning gedicteerd. Zie mijn stellingen. Veel succespunt

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wie deze leerstoelgroep niet kent, het is een afgeleide van de voormalige Vakgroep Zuivel van de voormalige Landbouw Universiteit. Daarna ook wel genoemd Vakgroep Geïntegreerde Levensmiddelentechnologie en Fysica van wat eerst heette het Departement Levensmiddelentechnologie en Voedingswetenschappen, vervolgens het Departement Agrotechnologie en Voedingswetenschappen en nu weer Kenniseenheid Agrotechnologie en Voeding. Voor de duidelijkheid. In elk geval bedankt: Addie, Carline, Ruud, Riet, Francesca, Sara, Matthijs, Martin, Marco, Ana, Frans, Anita, Pieternel, Gerrit M, Marjolein, Henk, Hugo, Hanneke, Pieter en Katja. En ook de mensen bij wiens naam ik me even geen gezicht voor de geest kon halen, maar die door mijn beperkte geheugen hier onterecht niet vermeld staan. Als laatste van dit rijtje wil ik Wim Jongen in het bijzonder bedanken, voor het ooit beginnen met anti-mutageniteitsstudies en voor de jarenlange begeleiding op het project. Oh ja, en natuurlijk alle mensen van het Frankrijk-Zwitserland reisje over voeding en schuimbeton.

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Curriculum vitae

Eric Harold Vis was born in Voorburg in 1972. After high-school graduation (VWO) in The Netherlands in 1990 he spent a year in the United States of America, where he obtained his high-school diploma in 1991. From 1991 to 1996 he studied Environmental Hygiene at Wageningen University. He took part in two graduate projects: one at the Laboratory of Biochemistry (prof dr ir IMCM Rietjens), the other at the Toxicology Group (dr ir DC Morse, prof dr A Brouwer). His practical period was fulfilled at NIZO Food Research in Ede, at the Genetics Group (dr M Kleerebezem, prof dr WM de Vos). Since May 1996 he worked on a PhD project entitled 'antimutagenic and anticarcinogenic properties of milk proteins' (dr GM Alink, prof dr ir MAJS van Boekel, prof dr JH Koeman). The project was performed at the Toxicology Group in close cooperation with the Product Design and Quality Management Group. Both groups are part of the Department of Agrotechnology and Food Sciences of the Wageningen University. The project was funded by the Dutch Dairy Foundation on Nutrition and Health. The PhD project was part of the Graduate School Food Technology, Agrobiotechnology, Nutrition and Health Sciences (VLAG). During the PhD project several courses were attended for the Postdoctoral Education in Toxicology (PET) program. Also several courses and workshops organized by the graduate school VLAG, were followed.

Eric Harold Vis is in 1972 in Voorburg geboren. Na het behalen van zijn middelbare school diploma (VWO) in 1990, bracht hij een jaar door in Amerika, waar hij zijn 'High school'-diploma behaalde in 1991. Van 1991 tot 1996 studeerde hij Milieuhygiëne aan Wageningen Universiteit. Hij rondde twee afstudeervakken af: één bij de Laboratorium voor Biochemie (prof dr ir IMCM Rietjens), de ander bij de leerstoelgroep Toxicologie (dr ir DC Morse, prof dr A Brouwer). Zijn stage bracht hij door bij NIZO Food Research te Ede bij de Genetica groep (dr M Kleerebezem, prof dr WM de Vos). Sinds mei 1996 heeft hij op de leerstoelgroep Toxicologie aan een AIO-project met de titel 'anti-mutagene en anti-carcinogene eigenschappen van melkeiwitten' gewerkt (dr GM Alink, prof dr ir MAJS van Boekel, prof dr JH Koeman). Dit project werd uitgevoerd op de leerstoelgroep Toxicologie, in samenwerking met de leerstoelgroep Productontwerpen en Kwaliteitskunde (PDQ). Beide leerstoelgroepen maken deel uit van het Departement Agrotechnologie en Voedingswetenschappen van de Wageningen Universiteit. Het project werd gesubsidieerd door de Stichting Zuivel Voeding en Gezondheid. Het AIO-project maakte deel uit van de onderzoeksschool Voeding, Levensmiddelen- en Agrobiotechnologie (VLAG). Gedurende het AIO-project zijn verschillende cursussen gevolgd als onderdeel van de Postdoctorale Opleiding Toxicologie (POT). Daarnaast zijn verschillende cursussen en workshops van de onderzoeksschool VLAG gevolgd.

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Vis EH, Rijntjens E, Van Kranen HJ, Van Boekel MAJS and Alink GM, Heated egg white as a dietary protein source leads to infertility in mice, *Submitted as short communication to J Fd Chem Tox*.

Appendix

Heated egg white as a dietary protein source leads to infertility in mice

Eric Vis
Eddy Rijntjes
Henk van Kranen
Tiny van Boekel
Gerrit Alink

Submitted to:
Journal of Food and Chemical Toxicology
(Brief communication)

ABSTRACT

Unheated dietary egg white is known to induce biotin deficiencies in many animal species. It is often mentioned in literature that when egg white is heated, this toxic effect disappears. In the experiment described, male and female mice were fed diets containing 20% protein from casein, soy-isolate, soy-flour, red meat or heated egg white. Before preparing the diets, the egg white protein was heated for 60 minutes in a stainless steel jar placed in a boiling water bath. After one week on the experimental diet, animals were housed in couples for mating purposes. The 18 mating couples on the heated egg white diet showed no progeny indicating a heavily impaired reproduction. For the other dietary groups the percentage of couples obtaining progeny varied between 72% and 100%, indicating normal reproduction efficiency. When animals were switched from a casein based diet to a heated egg white protein based diet at the age of four weeks, no differences in growth curves between casein and egg white fed animals occurred. This indicates that the adverse health effects caused by heated egg white protein only occur when fed to the mothers during pregnancy. It is concluded that, in contrast to textbook believes, heated dietary egg white can cause heavily impaired reproduction *in vivo*.

INTRODUCTION

Raw egg white feeding is known to cause biotin deficiencies in many animal species and humans (1-5). Egg white induced biotin deficiencies are caused by binding of biotin to avidin, an egg white protein (6, 7). In some experiments raw egg white is deliberately used to induce biotin deficiency (1, 2). It is often assumed that when egg white is heat denatured, the biotin binding capacity of avidin is reduced (8,9). This is to be expected in view of the results of Donovan and Ross (10), who demonstrated that avidin denatures at 85 °C. Heat denatured dietary egg white is therefore used to prevent biotin deficiency when egg white is used as the major protein source.

Biotin is a vitamin, important in several processes like lipogenesis, gluconeogenesis and catabolism of amino acids (11,12). A biotin deficiency can cause symptoms like dermatitis, anorexia, glossitis, depression and peripheral neuropathy in humans (1). Some of these symptoms are also present in animals (3-5, 13). In rodents it is shown that a marginal biotin deficiency can be teratogenic before specific signs of biotin deficiency are present (2, 14, 15). Zempleni and Mock (16) conclude that a marginal biotin deficiency may be teratogenic in humans.

The purpose of this communication is to show that, in contrast to what is generally accepted, heated egg white can still cause impaired reproduction or infertility in mice. The results were obtained in a study that originally intended to investigate the influence of 5 different dietary protein sources on polyp development in both small intestine and the colon of the *Apc*^{Min} mouse (17). The *Apc*^{Min} mouse model (18, 19) can be used to study the effects of diet and diet composition on intestinal polyp formation (20, 21). One of the protein diets contained heated egg white as a dietary protein source. Egg white was heated because it was assumed this would eliminate the risk for biotin deficiency, as explained above. However, our results showed the contrary which, together with two additional studies, is described in the following paper. Both these results and the implications for using heated egg white as a dietary protein source, are discussed.

MATERIALS AND METHODS

Animals and housing The original *Apc*^{Min} experiment was described earlier (17). The experimental protocol was approved by the animal welfare committee of Wageningen University. For the research on the influence of dietary protein source on reproduction efficiency (Study 1), male and female C57BL/6 *Apc*^{Min} mice were kept at the National Institute of Public Health and Environmental Protection (Bilthoven, The Netherlands). During mating two female mice were housed with one male for two weeks. At least 18 mating couples were used per diet. Progeny was counted three and a half weeks after the mating period. Statistical differences for the number of successful mating couples between the different diets, were tested using Chi-square analysis (SPSS, version 7.5). When no progeny

was observed in the heated egg white group, mating couples from the casein and the egg white groups were housed together for another two weeks. After three and a half weeks, progeny was counted again (Study 2). To further investigate the influence of a heated egg white diet on growth and development (Study 3), the progeny from mating couples on the casein diet was moved to the Center for Small Laboratory Animals (Wageningen, The Netherlands). Directly after weaning (age 22 days), half of the casein raised weanlings were kept on their casein diet, the other half was fed the heated egg white protein diet. Animal body weight for both groups was followed during several months.

Diets Mice were fed five different protein diets: casein, soy-isolate, soy-flour, red meat or heated egg white protein. Dietary compositions are shown in Table 1. Before preparing the egg white diet, one part egg white protein was mixed with three parts water. The mixture was heated for 60 minutes in a stainless steel jar placed in a boiling water bath. Egg white temperature was at least 85 °C at several time points during protein preparation as measured using a thermometer. During the heating process the liquid egg white water mixture changed into a firm gel. Before mixing through the diet, the heated egg white was freeze-dried and powdered. A diet comparable to human Western consumption was used (Hope Farms, Woerden, The Netherlands). Biotin was added as part of the vitamin mixture to a final concentration of 350 µg/kg. The diets were pelleted. Food and water were available *ad libitum.*

RESULTS

There were no differences observed between dietary groups in growth of the female mice during pregnancy. Three-and-a-half week after the mating period there was no progeny in the animal group on the experimental egg white diet, which suggests that the heated egg white diet caused infertility (Study 1). The difference in reproduction success between the protein diets was significant using Chi-square analysis ($p \leq 0.006$). At least 72% of the mice couples on the other diets had progeny (Table 2), showing the reproduction problem was due to heated egg white protein. No signs of abortion or cannibalism were noted in the egg white group. After the second mating period there was no progeny in the dietary egg white group either (Study 2), confirming the results of the first reproduction experiment. When the casein or egg white protein diets were fed after weaning to mice raised on a casein diet, the male and female progeny showed no growth differences between the diets (Study 3). Figure 1 shows the growth curves of the male mice on the casein and egg white diets. Growth curves of the female mice were comparable (data not shown). The fact that growth curves for casein and egg white fed animals were alike, indicates that problems using heated dietary egg white protein only occur during reproduction and not when fed after weaning.

Table 1 Diet composition in g/100g. [a] Casein (acid casein, 88% protein, DMV Veghel, The Netherlands), [b] soy-isolate (Supro 500E, 90% protein, Protein Technologies International, Ieper, Belgium), [c] soy-flour (defatted soy flour 200/20, 54% protein, Cargill, Amsterdam, The Netherlands), [d] red meat (raw minced pork meat (ham), 88% protein, obtained from the local butcher, microwave heated until done and freeze dried) or [e] heated egg white protein (pasteurized chicken egg white powder, min. 87% protein NIVE, Nunspeet, The Netherlands), [f] higher soy meal content because of lower protein concentration, [g] amounts lower than other dietary groups because of protein source composition.

	Casein	Soy-isolate	Soy-meal	Red meat	Egg white
Casein [a]	23.33	-	-	-	-
Soy isolate [b]	-	22	-	-	-
Soy meal [c]	-	-	36.67[f]	-	-
Red meat [d]	-	-	-	22.67	-
Egg white [e]	-	-	-	-	25.33
Starch	20	20	12.33[g]	20	20
Cellulose	10	10	4[g]	10	10
Vitamin mix	0.28	0.28	0.28	0.28	0.28
Trace-elements mix	0.28	0.28	0.28	0.28	0.28
Lard	18	18	18	17.33[g]	18
Corn oil	2	2	2	2	2
Glucose	21.24	22.57	21.57	22.57	19.24
Salt mix	4.29	4.29	4.29	4.29	4.29
Methionine synth.	0.227	0.277	0.277	0.277	0.277
Choline Cl 50%	0.353	0.353	0.353	0.353	0.353
Total	100	100	100	100	100

Table 2 Reproduction efficiency (Study 1).

	No. of mating couples	No. of mating couples with progeny after 3½ weeks	%
Casein	33	25	76
Soy isolate	18	17	94
Soy flour	18	18	100
Pork meat	18	13	72
Egg white	18	0	0

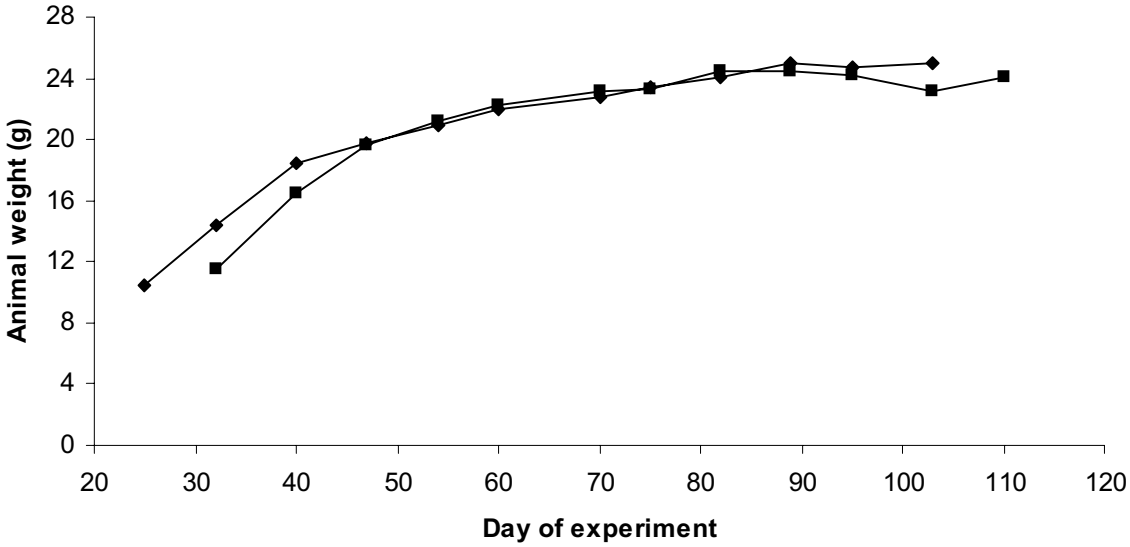

Figure 1 Growth curves of male casein and egg white fed mice (◆ Casein ■ Egg white).

DISCUSSION

To our knowledge we are the first to show that heated dietary egg white protein can cause infertility *in vivo*. So far there were only indications that heated egg white could cause marginal biotin deficiencies (13). Although it is known that biotin deficiencies can lead to reproduction problems (2, 14, 15), so far no studies show that the severity of the biotin deficiency caused by heated dietary egg white protein could actually cause severe reproduction problems or even infertility. A probable explanation for the effect found can be given on the basis of the results reported by Durance and Wong (22). This article showed that when egg white is heated in the normal way (e.g. 15 minutes in boiling water) egg white protein, including avidin, denatures but the biotin binding capacity of avidin remains intact.

It can not be excluded that the biotin binding capacity of heated egg white protein causes adverse effects on human health, especially for pregnant women. However, humans seem to be at lower risk than the animals used in this experiment for several reasons. First, humans will not likely limit themselves to eating egg white protein as their sole protein source (20%). Secondly, egg white protein is often consumed as part of a whole egg. Egg yolk contains relatively high levels of biotin (0.52 μg/kg) and can partly compensate for the biotin binding capacity of heat denatured avidin. Therefore, our findings seem to be mainly relevant for other animal studies in which dietary egg white is used as a main protein source. It is of little use to heat the egg white for 15 minutes (or for 60 minutes as in our study) to prevent biotin deficiency. If possible, dietary egg white should be avoided as a sole protein source, especially in reproduction studies, or otherwise steps have to be taken to avoid biotin deficiency by for example adding extra biotin.

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Omslagfoto door Hans van den Berg. Microscoopopname van een aberrante crypt focus (ACF) uit de dikke darm van een rat na methyleen blauwkleuring.

Drukwerk door Ponsen & Looijen bv.

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