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Impact of different preparations on the mutagenicity of meat products during digestion as evaluated by the AMES-test

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4-HNE	4-hydroxynonenal
ATNC	Apparent total nitroso-compounds
BaP	Benzo[*a*]pyrene
CEA	Carcino-embryonic antigen
CIMP	CpG island methylator phenotype
DMSO	Dimethyl sulfoxide
FAP	Familial adenomatous polyposis
FOBT	Fecal occult blood test
HNPCC	Hereditary nonpolyposis colon cancer
hTERT	Human telomerase reverse transcriptase
hTR	Human telomerase RNA
IARC	International agency for research on cancer
MDA	Malondialdehyde
MeIQx	2-amino-3,8-dimethylimidazo[4,5-f]-quinoxaline
MSI	Microsatellite instability
NO	Nitric oxide
NOCs	N-nitroso compounds
PAH	Polycyclic aromatic hydrocarbons
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-b]-pyridine
SAs	Structure alerts
SIN	Chromosomal instability
TME	Total mesorectal excision
TP1	Telomerase-associated protein 1

Abstract

The increasing incidence of colorectal cancer may be related to the increase of meat consumption. The mutagens present in meat and meat products may play an important role on the formation of colorectal cancer. This study aims at using the Ames test as a tool to evaluate the mutagenicity of different kinds of meat products and the impacts of different pretreatments of digested meat samples. On the other hand, the impacts of antioxidants in digested meat samples were also studied on the results obtained by the Ames test.

The samples assessed in the first experiment were: cooked pork with 5% fat with and without nitrites, overcooked pork with 5% fat with and without nitrites, cooked pork with 20% fat with and without nitrites, cooked chicken and beef with 5% fat without nitrites; the added concentration of nitrite was 120mg/kg meat. Samples were fed into an *in vitro* digestion model and digested till colon stage. The samples assessed in the second experiment were: cooked beef with 20% fat, cooked beef with 20% fat with quercetin or vitamin C and pure quercetin. Quercetin or vitamin C (5mg) was added to 4.5g of beef, where after the meat was digested till duodenum stage.

In the Ames assay, no mutagenicity was detected by *Salmonella* strain *his*D3052 in cooked pork, chicken and beef, all with the same 5% fat content at colon digestion stage. Signs of mutagenicity were detected by strain *his*D3052 in pork samples which were treated with nitrites at the colon digestion stage. Longer cooking time and higher cooking temperature was also proved to promote the formation of mutagens. During the duodenum digestion stage, the beef sample itself was mutagenic which was tested by strain TA100 in the presence of S-9 metabolic system and showed signs of mutagenicity in the other strain. Quercetin acted directly and its frameshift mutagenicity and base-pair substitution increased with metabolic activation. In the presence of S-9 metabolic system, beef sample with quercetin was mutagenic in strains TA98 and TA100. In the absence of S-9 metabolic system, beef sample with quercetin was mutagenic in strain TA98 and showed signs of mutagenicity in the other strain. In the absence of S-9 metabolic system,

beef sample with vitamin C showed mutagenicity in both tester strains. In the presence of S-9 metabolic system, the added vitamin C in the beef sample resulted in toxic effect to tester strains. The formations of unstable lipid oxidants, such as malondialdehyde (MDA) and 4-hydroxynorenal (4-HNE), and of N-nitroso compounds (NOCs) probably are the key compounds leading to the positive results obtained by the Ames test. S-9 metabolic system also plays an important role on the formation of mutagens, meaning that after metabolism, more genotoxic products can be formed.

Key words: meat, mutagenicity, colorectal cancer, quercetin, Ames test, in vitro digestion

1 Introduction

Colorectal cancer is the third most common malignant tumor not only in developed countries but also in developing countries especially those emerging economic countries [1]. The increasing incidence of colorectal cancer may be related to the increase of meat consumption. The World Cancer Research Fund report has stated that the intake of meat products such as fermented sausage, cooked ham, dried ham, should be reduced, since there seems to be a positive relationship between the intake of these meat products and the development of colorectal cancer [2]. However, there is no information which type of meat (such as poultry or red meat) or which kind of processes (deep frying, fermentation, drying or salting) are responsible. The aim of this research is:

1. To use the Ames test as a tool to investigate the mutagenicity of different kinds of digested meat products;
2. To study the impact of different pretreatments of digested meat samples on the results obtained by the Ames test;
3. To study the impact of antioxidants in digested meat samples on the results obtained by the Ames test.

2 Literature review

2.1 Colorectal cancer

2.1.1 Epidemiology

Colorectal cancer is now the most common malignancy; according to the report from international agency for research on cancer (IARC) in 2002 [3], new cases of colorectal cancer occupied 9.4% from all cancer cases, the incidence in male ranked forth comparing with third in female. The total patients of colorectal cancer were second only to breast cancer [4].Colorectal cancer has significant differences in geographical distribution and these discrepancies were changed with the passage of time especially in emerging economies such as China, Latin America and part of Africa. In China, the number of morbidity and death in 2005 were 172000 and 99000 due to colorectal cancer; the incidence in male and female were 15.0 and 9.1 per 100000, respectively. These data were already much higher than that of in America [5]. World widely speaking, more male patients of colorectal cancer were observed then female patients [4], but in developed countries, such as America, the incidence rate is quite even in male and female [6]. The risk of colorectal cancer also increased with age. In developed countries, 90% of the patients were more than 50 [7].

2.1.2 Etiology

Although the real etiology of colorectal cancer is still not clear, most researchers concluded that colorectal cancer is caused by the synergistic effects of environment, diet, lifestyle and genetic factors.

2.1.2.1 Diet

WHO already pointed out that high-fat diet and over intake of animal protein, especially from red meat, are the most dangerous factors which can cause colorectal cancer [3]. The mechanisms that high-fat diet causes colorectal cancer are not clear; it is probable due to

the formation of oxidation products. On the other hand, the concentration of secondary bile acids in the colon is related to colorectal cancer. Bile acids can be oxidized by enzymes into methylcholanthrene which is considered as a carcinogen; bile acids are also considered as cancer-promoting agents which can directly attack DNA and induce epithelial hyperplasia. Fatty acids, especially in ionic state, can promote the DNA synthesis of intestinal epithelium, induce and activate ornithine decarboxylase. Fat can also promote the conversion of primary bile acids into secondary bile acids which lead to nonspecific colonic mucosa injury and hyperplasia of epithelium [8].

Dietary fiber is present in fruits, vegetables and grains which can increase the amount of feces resulting in a dilution of carcinogens in the colon; dietary fiber can also absorb bile acids. The formation of short chain fatty acids due to bacterial glycolysis can reduce the pH, which inhibit the growth of tumor cells. Greenwald [8] summarized that the average fecal weight have negative correlation with the risk of colorectal cancer. By contrast, the intake of dietary fiber has a positive correlation with the risk of colorectal cancer: 18g/d dietary fiber intake may reduce the risk of colorectal cancer.

Trace elements and vitamins, such as selenium, zinc, calcium; vitamin A, C, E, are considered playing important roles on preventing colorectal cancer. High amount of calcium intake results in a lower incidence rate in colorectal cancer; vitamin A, C, E can inhibit the reaction of radicals in order to prevent DNA damage from oxidants. Vitamin A, C, E can also reverse hyperplasia of epithelium [8].

2.1.2.2 Life style

Thune *et al.* [9] concluded that the incidence rate of colorectal cancer in active physical laborers were much lower than that of other sedentary professions. WHO also argued that physical activities conducive to reduce the incidence of colorectal cancer [3]. Thune *et al.* [9] also figured out that compared to the people who had a lower BMI (<21), the incidence rate of colorectal cancer increased 50% to people with BMI higher than 29.

2.1.2.3 Genetic factors

Besides familial adenomatous polyposis (FAP) and Gardner syndrome, hereditary nonpolyposis colon cancer (HNPCC) occupied 4.0-13.0% of the colorectal cancer. Six relative genes were already defined: hMSH2, hMSH1, hMSH6, hWSH3, hPMS1 and hPMS2 [10].

2.1.2.4 Diseases

3-5% of chronic ulcerous can be converted into colorectal cancer, 15-40% colorectal cancer originated from multiple colonic polyps. 100% of FAP patients will suffer from colorectal cancer before 50. 70% of Crohn disease can be converted into colorectal cancer [10].

2.1.3 Colorectal carcinogenesis

From polyps to sporadic colorectal cancer, there are 3 major molecular mechanisms: chromosomal instability (SIN), microsatellite instability (MSI) and CpG Island Methylator Phenotype (CIMP) [11].

Most colorectal cancer follows SIN pathway. It is characterized by broader disorder of chromosome and loss of heterozygosity which is caused by chromosome segregation and defection of telomere stability and DNA damage. Typical karyotype abnormalities with characteristic mutations of tumor suppressor genes and oncogene are the key pathways of colorectal carcinogenesis. Gene mutation of APC, MCC, gene inactivation of MMR, gene mutation of K-ras, deletion of DCC, mutation and deletion of *p53* are the classic molecular genetic models. Recent research proved that mutation of APC is the initial step of carcinogenesis; a single mutation of K-ras cannot trigger colorectal cancer. Colorectal cancer was promoted only when mutation of APC was also present simultaneously [11, 12].

MSI is a mutant phenotype which is caused by deletion of mismatch repair. Devitalization of MMR leads to MSI and causes the change of genes such as MLH1,

GARB1, BAX, MBD-4 and etc. MSI can be used for diagnosing specific colorectal cancer such as Lynch syndrome; it can also be used for tumor screening and prognosis [11].

Almost half of promoters in the human genome are implanted in CpG Island (residue cluster of cytosine-guanine). Cytosines are methylated in CpG islands and catalyzed by DNA transmethylase. Abnormal DNA methylation was observed in colorectal cancer which affects DNA mismatch repair gene including hMLH1, hMLH2, *p14* and *p16*. The abnormal methylation of these genes leads to reduction of genetic expression or even causes genetic elimination [11].

2.1.4 Diagnosis

Besides common diagnosis methods such as fecal occult blood test (FOBT) and endoscopy, non-invasive screening techniques have already been widely used in clinical diagnosis for colorectal cancer.

2.1.4.1 Tumor marker

Detection of tumor markers is one of those non-invasive screening techniques. Carcino-embryonic antigen (CEA) is an intracellular glycoprotein that presents in embryo with low concentration under normal circumstances. The level of Serum CEA is positively associated with Dukes classification of colorectal cancer. Kim *et al.* [12] argued that fecal CEA test had a higher sensitivity than that of serum CEA. On the other hand, CEA can be used as a tool to predict the prognosis; the increasing CEA after radical operation for carcinoma of colon implies recurrence and worse prognosis.

2.1.4.2 Enzyme marker

Telomerase is a ribonucleoprotein that can catalyze and extend telomere end, mainly include: human telomerase RNA (hTR), telomerase-associated protein 1 (TP1) and human telomerase reverse transcriptase (hTERT). Most of normal cells are telomerase negative, but telomerase is activated in tumor cells resulting in cell immortalization.

Gertler *et al*. [13] concluded if the length ratio of telomere between carcinoma tissues and normal tissues was greater than 0.90, it implied a relative lower survival rate; 5-year survival rate was 78.2% - 16.9% in 75% of colorectal carcinoma patients with lower length ratio of telomere (< 0.90). Hauguel *et al*. [14] proposed that telomerase inhibitor increased the sensitivity of tumor cells to DNA damage factor, which could be a new treatment for colorectal cancer.

2.1.4.3 Genetic diagnosis

As mentioned on 2.1.3, the carcinogenesis of colorectal cancer is a complex process which involves multiple mutations of tumor associated genes. Take an example of K-ras gene, the mutation of K-ras is an early molecular phenomenon of colorectal cancer. More than 40% of patients were detected with K-ras mutation and all point mutations located at 12, 13 and 61 codon, resulting in uncontrollable cell proliferation and malignant transformation [15]. Therefore, detection of K-ras mutation can be used as a clinical molecular biology method for early diagnosis and screening.

2.1.5 Treatment

Major treatments of colorectal cancer include surgery, radiotherapy and chemotherapy. Total mesorectal excision (TME) is the most classical surgery for colorectal cancer which is applicable primarily to T1 – T3 stage without distant metastasis and the tumor does not reach to placenta percreta. Marting *et al.* [16] reported that TME reduced 50% of recurrence rate and improved the prognosis. Preoperative radiotherapy can reduce the volume of tumor and can lead to extinction of cellular degeneration, fibrous tissue proliferation and circumferential margin infiltration. Postoperative radiotherapy is used for T3, T4 colorectal cancer with lymph node metastasis. 5-FU and its biologic modifiers such as lucoverin, interferon-α and trimetrexate are commonly used as the major chemotherapy.

2.2 General situation and research development on meat

Since the discovery of polycyclic aromatic hydrocarbons (PAH) and N-nitroso compounds (NOCs) in meat products in 1960's, the importance of research about the relationship between meat intake and mutagens/carcinogens and colorectal cancer is recognized. Demeyer *et al.* [2] summarized that increasing meat intake leads to an increasing risk of colorectal cancer; a dose-response relationship was also observed. Norat *et al.* [18] concluded that "do not demonstrate that high intake of red meat accompanied by high intake of vegetables is not associated with colorectal cancer risk" which is opposite from what people thought in the past. A large scale epidemiological research in the U.S. found that the hazard risk ratio for colorectal cancer incidence is 1.20 ($p<0.001$) when the intake is 63 g red meat per 1000kcal or 23 g processed meat per 1000kcal [19]. Gonzalez and Riboli [20] also reported that when people consume 100g red meat per day, the risk of colorectal cancer can increase between 17% and 19%. For processed meat, this figure reached to 49%.

2.3 Hypothesis of the carcinogenic mechanisms

2.3.1 Nitrites and nitrates

Nitrites and nitrates are often added in processed meat products to support the red color development. The nitric oxide (NO) which is formed by nitrites and nitrates can react with hemoglobin and myoglobin; finally, the color-stable nitrosyl-haemoglobin and nitrosyl-myoglobin are formed. The aim to add nitrites and nitrates to the processed meat products is on one hand to keep the color of the products, on the other hand to limit the outgrowth of certain bacteria, specifically active against *Clostridium botulinum* in meat products. As shown in Fig. 2-1, an oxygen sequestering is achieved since the NO can react with myoglobin and/or SH group amino acids, or be oxidized to NO_2 by oxygen. Due to the anaerobic environment, the development of rancidity can be put off. The disadvantages of adding nitrites and nitrates to meat products are that they can be converted into NOCs under acidic conditions which are proved as strong carcinogens [21]. Fig. 2-2 shows the reaction between amines and nitrites at high temperatures.

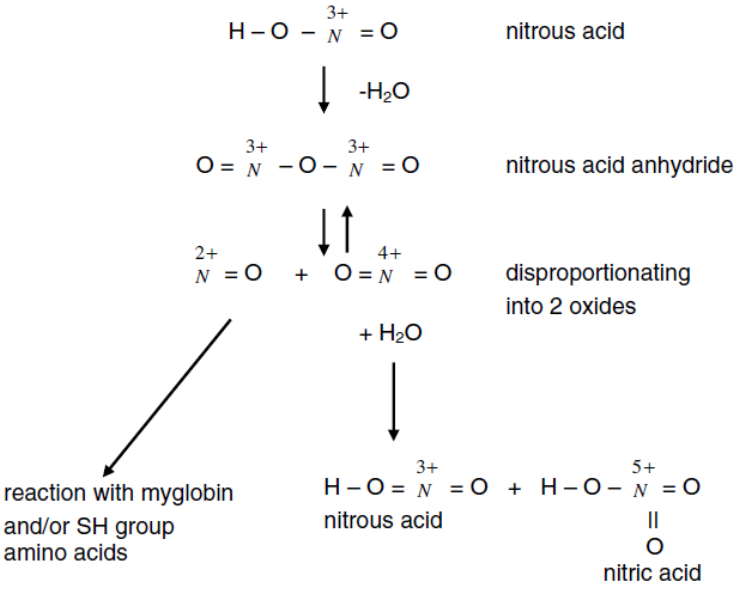

Figure 2-1. Reaction of nitrous acid in meat systems [22]

$$NaNO_2 + H^+ \rightarrow HNO_2 + Na^+$$
$$HNO_2 + H^+ \rightarrow NO^+ + H_2O$$
$$2HNO_2 \rightarrow N_2O_3 + H_2O$$
$$N_2O_3 \rightarrow NO + NO_2$$
$$NO + M^+ \rightarrow NO^+ + M$$

primary amine	RNH_2	+ NO^+	$\rightarrow$ RNH-N = O + H^+ $\rightarrow$ ROH + N_2
secondary amine	R_2NH	+ NO^+	$\rightarrow$ R_2N-N = O + H^+
tertiary amine	R_3N	+ NO+	no nitrosamine formation

Figure 2-2. Reaction between amines and nitrites at high temperatures [22]

2.3.2 Heme iron

A recent meta-analysis from Bastide *et al*. [23] reported that red meat and processed meat intake was associated with a risk of colorectal cancer. On the other hand, the World Cancer Research Fund panel suggested that the intake of red meat should be limited and the processed meat should be avoided in order to reduce the risk of colorectal cancer [24]. One of the mechanisms which can explain the relationship between the risk of colorectal cancer and the intake of red or processed meat is heme iron.

Two aspects of evidences are provided heme iron is toxic: from the meta-analysis point of view and experimental point of view. In the chemical structure of heme, an iron atom is present in the center of porphyrin. Many globulins contain heme such as hemoglobin and myoglobin. The red color present in red meat is because the high concentration of myoglobin. In processed red meat, heme iron is nitrosylated [25].

In the meta-analysis from Bastide *et al.* [23], the relative risk of colon cancer was 1.18 (95% CI: 1.03-1.32) according to five previous studies. This meta-analysis showed a strong association between high intake of heme iron and increased risk of colon cancer. During the study of Pierre *et al.* [26], strong experimental evidence was found. They directly fed the rats with two kind of meat rich in heme iron: beef and blood sausage (chicken as control group), and fed them with a low-calcium diets. This study was the first to show that dietary meat can promote colon carcinogenesis. The aim of low-calcium diets is: the calcium can inhibit heme-induced cytotoxicity and colonic epithelial hyperproliferation [27]. Another study also from Pierre *et al.* [28] concluded that heme iron in processed meat was more toxic than the heme in fresh meat. Bastide *et al.* [23] summarized that heme iron promoting carcinogenesis in rats is consistent with epidemiological evidence. Heme promotion may explain why the intake of red and processed meat is associated with a risk of colorectal cancer, but the mechanism of heme promotion is still not known. Two mechanistic hypotheses are based on the catalytic effect of heme iron: first, on the formation of NOCs and the second one is the formation of lipid oxidation endproducts.

2.3.3 N-nitroso compounds

NOCs are produced by N-nitrosation of amines and amides. NOCs are chemical compounds which are consisted by alkylating agents. Those agents, after metabolic activation by cytochrome P450 enzymes or by alkylation on the O^6-position of guanine appear to be the major mutagenic lesion and can lead to G→A transition which is a common mutation in colorectal cancer. Humans can be exposed to NOCs by both exogenous routes (certain processed meats, such as grilled bacon, discussed in 2.3.1) and endogenous routes [25]. The link between endogenous NOCs and red meat can be built

following the detection of NOCs (as assessed by apparent total NOCs (ATNC) from human faeces). The high heme content in red meat shows a strong link between fecal NOCs and heme intake. Heme can easily be converted into nitrosylated heme, which is known to act as a nitrosating agent and thus promote the formation of NOCs [29]; on the other hand, Lunn *et al*. [30] demonstrated that, in *in vitro* studies, heme and myoglobin are readily nitrosated and can further act as nitrosating agents, which will lead the nitrosation in the absence of colonic flora. During the experiments, acidified heme enhances the nitrosation under neutral conditions. Nitrosyl-haemoglobin which is formed in acidic conditions appeared as a nitrosating agent at pH6.8. Consequently, heme could be responsible for the increase in endogenous ATNC in the ileostomy output in the presence of minimal bacterial flora (Table 2-1).

Table 2-1.Ileal output and ATNC and nitrite concentration in ileal output (mean ±SEM) in response to diet [29]

	g Ileal output/ 24 h (range)	µg ATNC/kg	Nitrite concentration (µg/kg)
Control diet	639 ± 62 (233–1004)	283 ± 74	355 ± 209
PM diet	709 ± 67 (449–1145)	1832 ± 294*	845 ± 283
RM diet	684 ± 52 (239–962)	1175 ± 226**	831 ± 396***

*t-test versus control diet, $P < 0.0005$.
**t-test versus control diet, $P = 0.001$.
***t-test versus control diet, $P = 0.005$.

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Santarelli *et al.* [25] also reported that, in animal studies, processed meat intake leads to increasing fecal excretion of NOCs. Also in human studies, the red meat dramatically increases NOCs excretion in feces. They also found the formation of DNA adduct O^6-carboxymethyl guanine in colonic exfoliated cells or meat-fed volunteers. This NOC-specific alkylating DNA adducts proves that increased endogenous productions of NOCs are highly linked to colorectal cancer. Sum up, NOCs are present in most of processed meat, and formed endogenously after red and processed meat consumption. Heme is a major determinant of NOCs formation.

2.3.4 Lipid peroxidation

Lipid peroxidation starts with the attack of membrane lipids by free radicals. This reaction can be catalyzed by heme. In unsaturated fatty acids, the initial products are lipid

hydroperoxides. As they have a short life time, most of the hydroperoxides will react with metals to produce reactive aldehydes; they can be further converted into malondialdehyde (MDA) or oxidized to more reactive epoxy compounds [31] (Fig. 2-3). 4-hydroxynonenal (4-HNE) is the main toxic product of lipid peroxidation which can cause DNA damage [32]. 1,N^2-malondialdehyde-deoxyguanosine, which is a DNA adduct formed by MDA with DNA, was detected from adenoma patients during colorectal biopsies [33]. Baradat *et al*. [34] also concluded that 4-HNE can induce apoptosis. Bastide *et al*. [23] summarized that several phenolic compounds, can inhibit the lipoperoxidation, such as quercetin, vitamin E and exgrapetotal, an extract from the red wine.

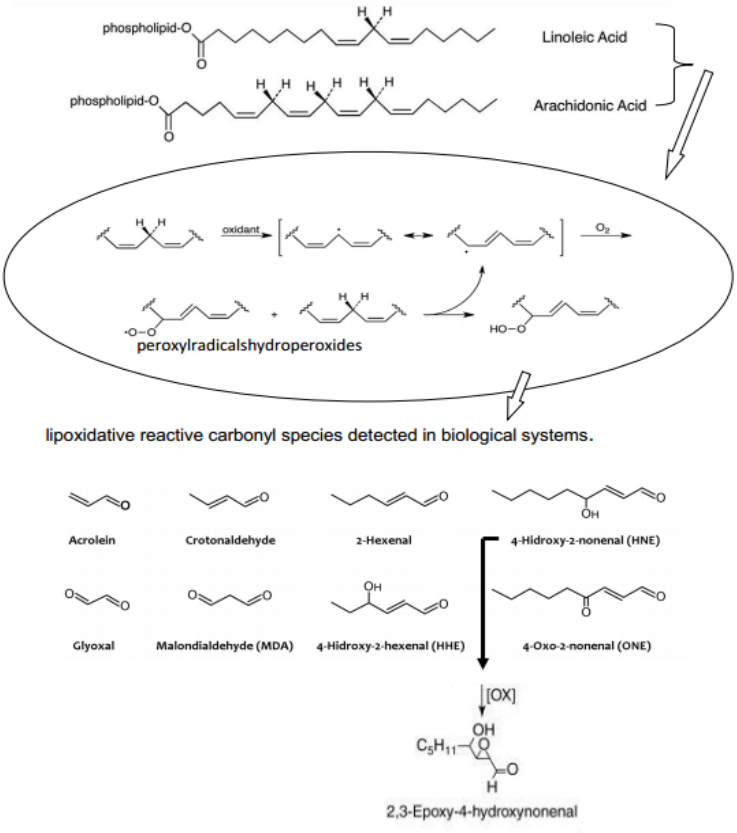


Figure 2-3.Peroxidation of polyunsaturated fatty acids [30]

2.3.5 Heterocyclic amines

Heterocyclic amines can be formed during high temperature cooking of food and some of them are carcinogenic in long-term studies [35]. Two of the most important mutagenic or

carcinogenic heterocyclic amines in cooked food are 2-amino-3,8-dimethylimidazo[4,5-f]-quinoxaline (MeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-b]-pyridine (PhIP). Maillard reaction or free radical reactions play a leading role during the formation of these compounds. Furthermore, the formation of heterocyclic amines is highly dependent on cooking time and temperature [36, 37] (Table 2-2).

Table 2-2. Heterocyclic amines contents in different meat [36]

			Heterocyclic amines in meat (ng/g)		
	Cooking method[a]		MEIQx	PhIP	Total
Beef	Fried	medium rare	1.75	0.94	2.73
		well done	3.33	5.27	8.92
	Broiled	medium rare	0.08	1.58	1.72
		well done	0.12	5.63	6.04
	Baked		0.33	1.49	2.34
Pork	Fried		2.39	9.20	13.91
	Baked		0.23	2.20	3.29
Poultry breast	Fried				
+ skin			0.23 (0.31)[b]	2.61 (2.72)	2.89 (3.13)
- skin			0.46	6.06	7.06
Fish	Baked		1.27-2.95	4.40-5.67	7.85-8.70
	Fried		2.31-3.11	9.11-10.89	13.09-16.29

[a] *Frying* = in a Teflon-coated frying pan without adding oil at a surface temperature of 204 °C. Meat was fried, turned once, and removed from the pan when the desired temperature was reached.
Broiling = Oven (convection, top and bottom heat) was preheated to 232 °C (monitored with oven thermometer). The meat was placed on a broiler pan to keep the broiled beef out of the drippings. The meat was removed when a final internal temperature (51° C medium rare - 71 ° C well done) was achieved
Baking : Oven (convection, top and bottom heat) was preheated to 177 °C (monitored with oven thermometer). The meat was placed on a baking pan. The meat was removed when a final internal temperature was achieved. [b] () = in meat and skin

2.3.6 Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAH), such as benzo[*a*]pyrene (BaP) can be formed during high temperature cooking of meat and meat products over a direct or naked fire. PAH can also be formed during the pretreatment of meat products; for instance, smoking [38]. After being activated by metabolic process PAH can be converted into reactive form, which can bind to guanine bases leading to DNA damage [38]. Gunter *et al.* [39] argued that “an increased intake of 10g barbecued red meat per day was associated with a 29% increased risk of large adenoma”. However, other kinds of food, such as fish, cereal

based food and some vegetables also contain PAH (Table 2-3), no evidence was found to support an increased risk of colorectal cancer [40].

Table 2-3.Concentration of PAH (ng/kg) in food [40]

	BaP	PAH4
Milk	60	870
Cereal based foods	270 – 720	980 – 1980
Fish and Fish products	580	2220
Dairy products	130	600
Fresh and cooked vegetables	180	2390
Meat and meat products	580	3970

2.4 Cooking methods and formation of mutagens in meat

Nowadays, increasingly food products are heat processed. People now prefer easier and faster cooking methods in the modern time which means more fried or baking methods are used than before. There is no doubt that the ways of cooking food have a strong link to human health, especially the relationship between the cooking method and the formation of mutagens. Skog *et al.* [37] concluded that the cooking method has a considerable influence on the formation of mutagenic activity. They also found the differences between processed meat and white meat (chicken); in all the cooking methods (baking in the oven, deep fry and deep-fat fryer at both low and high temperature) mutagenicity are detected from chicken; by contrast, processed meat, like hamburger, only produced mutagens when cooked in deep-fat fryer at high temperature. The results can be explained by: first, the chemical composition of the hamburger and chicken is different, for instance, the fat content and fatty acid profile. Second, some fatty acids, such as oleic and linoleic acids had already been known that they can inhibit the Ames test [41]. Third, the hamburger contains complex ingredients and different additives which may inhibit the formation of mutagenic compounds. Another significant discovery by Skog *et al.* [37] is the relationship between mutagenic activity and weight loss during cooking. Higher weight loss was related with higher mutagenic activity (Fig. 2-4). This can be interpreted that the cooking method which can minimize the weight loss of food will produce less mutagens.

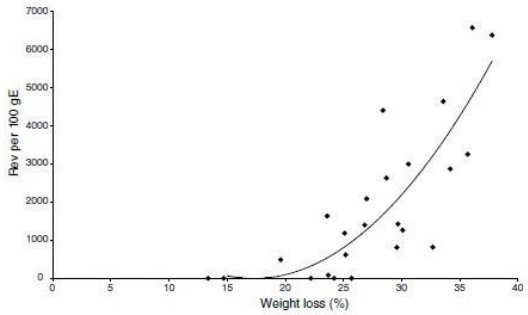


Figure 2-4. Mutagenic activity (average of duplicated determinations) vs. weight loss during cooking [36]

2.5 Structure alerts for carcinogenicity and the Salmonella assay system

Carcinogens and mutagens, which are the toxic compounds, have significant relationship with human health. For this reason, many researchers put a lot of efforts on them, several testing methods are developed. The mechanism of chemical carcinogenicity can be interpreted into theoretical mode: structure alerts (SAs) system [42] and experimental mode: the *Salmonella* assays system.

2.5.1 Structure alerts

James and Elizabeth Miller (1977, 1981) argued that most chemical carcinogens are electrophilic. Even in the early time, Millers (1960s) already found that many carcinogenic alkylating agents and acylating agents are electrophilic. After decades, Millers' hypothesis was demonstrated and improved; carcinogens are divided into genotoxic carcinogens, which directly cause DNA damage, and most of known mutagens belong to this category, and epigenetic carcinogens, which do not directly attack the DNA and are usually negative in the standard mutagenicity tests [42].

In 1985, John Ashby defined and compiled the structure alerts which followed the Millers' electrophilic theory: there is a strong link between the molecular functional groups or substructures and the carcinogenic activity of the chemicals. According to this theory, the chemicals which contain the specific groups or substructures would have high potential to cause cancer. SAs are often used for the genotoxic carcinogens testing, but not for non-genotoxic carcinogens [43]. Recently, more and more toxicity databases are built

according to the SAs [44, 45]. The new databases not only include the chemical names and chemical abstract service numbers, but also the structure which is searchable through the data and has improved sensitivity and accuracy [42].

2.5.2 Ames test

In order to create a cheaper and short term method, Bruce Ames developed the *Salmonella typhimurium*/microsome assay (*Salmonella* test; Ames test), which is an *in vitro* bacterial test for differentiating substances or compounds which can cause genetic damage that leads to gene mutations in a short-term period. Several *Salmonella* strains cannot synthesize a specific amino acid histidine, and consequently make it unable to grow and form colonies in its absence. New mutations will allow the cells to synthesize histidine. These newly mutated cells can grow without histidine and form colonies. The Ames test has a high sensitivity and correlation with rodent carcinogenicity study (77%-90%) and is widely used for screening or determines mutagenicity of new chemicals or drugs; if positive results are obtained that means they have a high potential of carcinogenicity for mammals [46].

2.5.2.1 The Ames test strains

Table 2-4 illustrates some strains which are normally used during the Ames test; all of them are histidine dependence. All strains except TA102 have deletion mutation through biotin genes leading to biotin dependence. All the strains, which process *rfa* mutation contain a lipolysaccharide defect coat on the surface, some bulky chemicals such as crystal violet can easily penetrate the membrane resulting in inhabitation of growth. Strains contain R-factor (pKM101 plasmid) can enhance mutagenesis of certain chemicals via error-prone repair system. Strain TA102, which has A-T base pairs at reversion site, is the only strain containing pAQ1 plasmid leading to more sensitive to reactive oxidants. Strain TA100 is used specific for detecting of base-pair substitution; strains TA97, TA98 and TA1538 are more sensitive to frameshifts; strain TA102, the wild type can be used for detecting transitions/transversions [46].

Table 2-4. Strains are normally used during the Ames test [46]

Genotype of the most commonly used *Salmonella* tester strains

Mutation (strain)	*bio chlD uvrB gal*	LPS defect	Plasmid
hisG46			
TA1535	Deletion mutation	*rfa*	No plasmid
TA100	Deletion mutation	*rfa*	pKM101
hisD3052			
TA1538	Deletion mutation	*rfa*	No plasmid
TA98	Deletion mutation	*rfa*	pKM101
hisC3076			
TA1537	Deletion mutation	*rfa*	No plasmid
hisD6610	Deletion mutation	*rfa*	pKM101
hisO1242			
TA97			
hisG428			
TA104	Deletion mutation	*rfa*	No plasmid
TA102	Wild type	*rfa*	pKM101, pAQ1

2.5.2.2 Conventional method of Ames test

The spot test: the spot test directly put the test substances on the center of an agar medium plate which contain *Salmonella typhimurium*. If the substance is a mutagenic compound, it will form a ring of revertant colonies (Fig. 2-5).

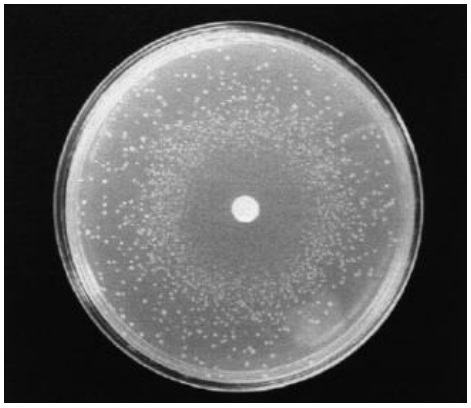
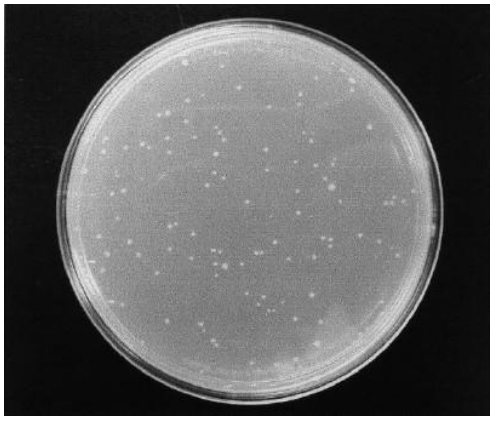
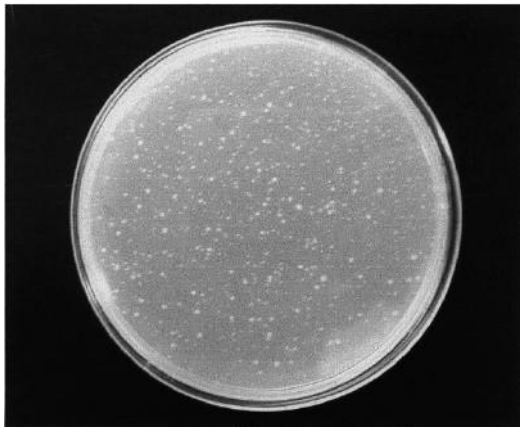


Figure 2-5. Spot test with strain TA100 and methanesulfonate (10 µl) [46]

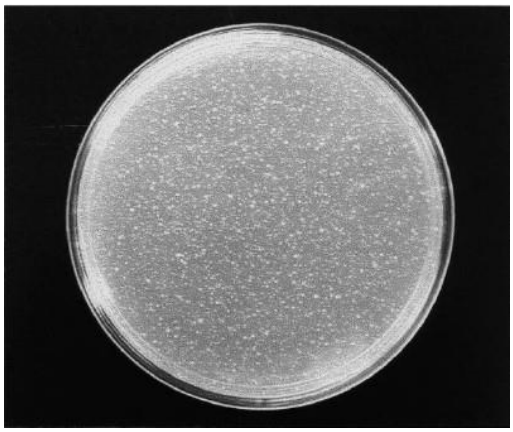
The plate incorporation test: The experimental path is: Mix the S-9 solution, the histidine dependent bacteria and test sample into a test tube with top agar which contains trace amount of histidine. The mixture is poured on the glucose minimal agar plates. When the top agar becomes solid, the plates will be putted in an inverted position in a 37℃incubator for 48h.Then the histidine revertant colonies will be counted. The principle is when a mutagen is added to the plate, the number of revertant colonies per plate will increase. The mutation values obtained can be expressed as number of mutants/plates, or number of mutants/amount of chemical added (Fig. 2-6).



Control



Dose 1



Dose 2

Figure 2-6. Mutagenic dose response with strain TA100 and sodium azide. Control: spontaneous revertants; dose 1: 2.5 μg/plate; dose 2: 5 μg/plate [46]

3 Material and methods

3.1 In vitro digestion of meat samples

All the meat samples were obtained from the Laboratory of Animal Production and Animal Product Quality (UGent) and were prepared as followed: meat samples (lean pork, beef and chicken) were manually chopped into cubes of approximately 1-2cm^3. For pork, chicken and beef samples, subcutaneous pork fat were added respectively to the chopped meat to obtain a targeted total fat content of 5% and 20%. Meat samples with added fat were first minced in a grinder equipped with a 10mm plate, followed by grinding through a 3.5mm plate. For samples with nitrites, nitrite-curing was applied by adding 20g nitrite salt/kg meat, equaled to 120mg nitrite/kg meat. Cooked meat samples were heated for 15 min after core temperature of the meat reached 65 ℃. Overcooked meat samples were heated for 30 minutes after the core temperature of the meat reached 90 ℃.

Meat samples (4.5 g) from previous treatments were sequentially incubated for 5 minutes with 6ml saliva, 2h with 12ml gastric juice, and 2h with 2ml bicarbonate buffer (1M), 12ml duodenal juice and 6ml bile (duodenal stage). After that, 22ml simulation of the human intestinal microbial ecosystem (SHIME) medium and 22ml human fecal inoculum were added to the digesta. In order to obtain an anaerobic environment, the vessels were flushed with N_2 for 30min. Subsequently, the vessels were incubated for 72h while stirring at 37 ℃.Then digestion samples were homogenized by an ultraturrax at 9500rpm. While stirring on a magnetic field in dark, samples were subdivided in 1.3ml aliquots and stored at -20 ℃ till analyses. The beef sample used for studying the impact of added antioxidants only reached to duodenal stage (4.5g meat in 40.5g digestion juice). Quercetin or vitamin C (5mg) was added to 4.5g of beef, where after the meat was digested. Frozen aliquots of all these samples were transported to the Laboratory of Food Microbiology and Biotechnology (UGent) to be analyzed with the Ames test.

3.1.1 Meat samples of experiment 1

The different meat samples used in experiment 1 are shown in Table 3-1. The objectives of these experiments were to study:

- The effect of the type of meat;
- The effect of the fat content;
- The effect of added nitrites;
- The effect of the cooking methods (internal heating temperature);

on the formation of mutagens during colon digestion, evaluated by the Ames test.

Table 3-1. Meat samples used in experiment 1

Type of meat	Treatment		
	Fat content	Nitrites (mg/kg)	Cooking method
Pork (*m. Longissimus*)	5%	0	Cooked
	5%	0	Overcooked
	5%	120	Cooked
	5%	120	Overcooked
	20%	0	Cooked
	20%	120	Cooked
Chicken (*m. Pectoralis profundus*)	5%	0	Cooked
Beef (*m. Biceps femoris*)	5%	0	Cooked

3.1.2 Meat samples of experiment 2

In Table 3-2, the meat samples are shown for experiment 2. The objective of this experiment was to study the effect of added antioxidants (quercetin or vitamin C) on the formation of mutagens during duodenum digestion as evaluated by the Ames test.

Table 3-2. Meat samples for experiment 2

Type of meat	Treatment	Cooking method
Beef with 20% fat	/	Cooked
Beef with 20% fat	Quercetin	Cooked
Beef with 20% fat	Vitamin C	Cooked

3.2 Ames test

All the meat samples and chemical compounds were conducted by Ames test. The principle of the Ames test has already been discussed in the literature review (see 2.5.2). The plate incorporation test was applied during the experiments. *Salmonella* strain *his*D3052 was used for investigating the mutagenicity of different meat products and the impact of different pretreatments of digested meat samples. Strains TA98 and TA100 were used for studying the impact of antioxidants in digested meat samples.

3.2.1 Equipment

- 37 ℃ incubator
- Analytical balance
- Autoclave
- Bunsen burner
- Centrifuge
- Glassware
  - Beakers
  - Culture flasks
  - Erlenmeyer flasks

- Flasks
- Measuring cylinders
- Reagent bottles (screw cap)
- Test tubes
- Watch glasses

- Magnetic stirrer (with heating plate) and stir bars
- Micropipettes (200µl, 1000µl) and sterile pipettes tips (200µl, 1000µl)
- Alu caps
- pH meter
- Spectrophotometer
- Centrifuge tubes (1.5ml)
- Sterile petri plates (100×15mm)
- Sterile pipettes (2ml, 10ml) and pipette pump
- Sterile syringes (5ml)
- 0.45 µm syringe-driven filters
- Vortex
- Warm water bath (with shaker)

3.2.2 Products

- Agar bacteriological No.1
- Ampicillin
- Tetracycline

- Citric acid monohydrate
- Distilled water
- Dimethyl sulfoxide (DMSO)
- D-biotin
- Glucose
- L-histidine
- Lyophilized TA98, TA100 strains, *his*D3052 strain
- HCl (0.02M)
- $MgSO_4 \cdot 7H_2O$
- K_2HPO_4
- $NaNH_4HPO_4 \cdot 4H_2O$
- NaCl
- $NaH_2PO_4 \cdot 2H_2O$
- $Na_2HPO_4 \cdot 12H_2O$
- Oxoid Nutrient broth No.2
- S-9 mix
- 2-nitrofluorene

3.2.3 Reagents and media

3.2.3.1 Vogel-Bonner (VB) medium E (50×)

The chemicals were added in the following sequence to 65ml warm distilled water (50 ℃) in 100ml flask and stirred on a magnetic stirrer, waited until the salt was totally dissolved

before adding the next one: 1g $MgSO_4 \cdot 7H_2O$, 10g citric acid monohydrate, 50g K_2HPO_4, 17.5g $NaNH_4HPO_4 \bullet 4H_2O$. The final solution was adjusted to 100ml and dispensed to 250ml reagent bottle, and further autoclaved for 20min at 121 ℃.The solution was stored at room temperature (22 ℃) in dark.

3.2.3.2 10% glucose solution

Glucose (50g) was added to 350ml distilled water in 500ml flask and stirred on a magnetic stirrer, waited until the solution became clear. The final solution was dispensed into 500ml reagent bottle, and further autoclaved for 20min at 121 ℃. The solution was stored at 4 ℃.

3.2.3.3 Glucose minimal (GM) agar

Agar (7.5g) was added to 450ml distilled water in a 500ml reagent bottle, and further autoclaved for 20min at 121 ℃. After cooling down for 25min at room temperature, 10ml sterile VB medium E solution was added to the agar. The solution was shaken well till VB medium E was totally dissolved. Then 25ml sterile 10% glucose solution was added, also mixed well. Then, 25ml agar medium was poured in each 100×15mm petri dish. All the plates were stored at 4 ℃ in sealed bags when agar medium cooled down and became solid.

3.2.3.4 0.5 mM histidine-biotin solution

To 500 ml boiled distilled water, 62mg D-biotin and 48mg L-histidine were added. When all the compounds were dissolved, the final solution was dispensed into 500ml reagent bottle and further autoclaved for 20min at 121 ℃. After cooling down the solution was stored at 4 ℃.

3.2.3.5 Top agar with histidine-biotin

Agar (1.2g) and NaCl (1.2g) were added to 180ml distilled water in 500ml reagent bottle, then 20ml 0.5mM histidine-biotin solution was also added into the bottle, and further autoclaved for 20min at 121 ℃.

3.2.3.6 Nutrient broth

Nutrient broth powder (6.5g) was added to 500ml distilled water and stirred on a magnetic stirrer until the solution became clear. Then 20 ml of the final solution was dispensed in each 100ml flask, and further autoclaved for 20min at 121 ℃.

3.2.3.7 Nutrient agar

Agar (7.5g) and 12.5g nutrient broth was added to 500ml distilled water in 500ml reagent bottle, and further autoclaved for 20min at 121 ℃. Then20ml nutrient agar was dispensed in each sterile petri plate. All the plates were stored at 4 ℃in sealed bags when agar medium cooled down and became solid.

3.2.3.8 0.1mM, pH 7.4 sodium phosphate buffer

To 500 ml distilled water, 7.8g $NaH_2PO_4 \cdot 2H_2O$ and 17.9g $Na_2HPO_4 \cdot 12H_2O$ was added in two 1l flasks, separately. Then, 60ml $NaH_2PO_4 \cdot 2H_2O$ solution and 440ml $Na_2HPO_4 \cdot 12H_2O$ solution was dispensed in a 1l flask, mixed well and the final pH of the solution was adjusted by using $NaH_2PO_4 \cdot 2H_2O$ solution to 7.40. A 500ml final solution was dispensed in a 500ml reagent bottle and further autoclaved for 20min at 121 ℃.

3.2.3.9 0.01% biotin solution

D-biotin (50 mg) was added to 500ml boiled distilled. A 100ml final solution was dispensed to a 200ml reagent bottle, and further autoclaved for 20min at 121 ℃. The solution was stored at 4 ℃.

3.2.3.10 **0.5%** ***histidine solution***

L-histidine (500mg) was added to 100ml distilled water in a 200ml reagent bottle, and further autoclaved for 20min at 121 ℃. The solution was stored at 4 ℃.

3.2.3.11 0.8% ampicillin solution

In 100ml 65 ℃ distilled water, 800mg ampicillin was dissolved, using a 0.45µm sterile syringe-driven filter to sterilize the solution. Then, 10ml of the final solution was dispensed in a sterile test tube.

3.2.3.12 0.8% tetracycline solution

Tetracycline (800 mg) was dissolved in 100ml 0.02M HCl solution, a 0.45µm sterile syringe-driven filter was used to sterilize the solution. Then, 10ml of the final solution was dispensed in a sterile test tube.

3.2.3.13 0.1% crystal violet solution

In 100 ml distilled water, 100mg crystal violet was dissolved, by using a 0.45µm sterile syringe-driven filter to sterilize the solution and stored at 4 ℃.

3.2.3.14 2-nitrofluorene

To prepare a 1mg/ml stock solution, 100mg 2-nitrofluorene was dissolved in 100ml Dimethyl sulfoxide (DMSO) and sterilized though a 0.45µm filter to a sterile flask. The stock solution was further diluted with DMSO in sterile test tubes to reach the following concentration:

- 10µg/100µl: 1ml stock solution in 9ml DMSO
- 5µg/100µl: 5ml 10µg/100µl solution in 5ml DMSO
- 2.5µg/100µl: 2.5ml 10µg/100µl solution in 7.5ml DMSO
- 1µg/100µl: 1ml 10µg/100µl solution in 9ml DMSO

3.2.3.15 Quercetin $\cdot 2H_2O$

1.35mg/ml stock solution: 135mg quercetin $\cdot 2H_2O$ was dissolved in 100ml DMSO and sterilized though a 0.45µm filter in a sterile flask. The stock solution was diluted with DMSO in sterile test tubes to reach the following concentration:

- 13.5µg/100µl: 1ml stock solution in 9ml DMSO
- 10.125µg/100µl: 7.5ml 13.5µg/100µl solution in 2.5ml DMSO
- 6.75µg/100µl: 5ml 13.5µg/100µl solution in 5ml DMSO
- 3.375µg/100µl: 2.5ml 13.5µg/100µl solution in 7.5ml DMSO
- 1.35µg/100µl: 1ml 13.5µg/100µl solution in 9ml DMSO

3.2.3.16 Physiological saline

In a 1l reagent bottle, 9g NaCl was dissolved to 1l distilled water. Then, 9ml of the final solution was dispensed in each test tube and autoclaved for 20min at 121 ℃.

3.2.3.17 Working cultures

The lyophilized TA98/TA100 products were stored at 4 ℃ temperature. One required disc was put into 100ml sterile flask with 25ml sterile nutrient broth; 78µl of 0.8% ampicillin was added in the nutrient broth in order to maintain the stability of the plasmid. Working cultures were incubated in a warm water bath with shaker (100rpm) at 37 ℃ for 18h until a density of $1\text{-}2 \times 10^9$ cfu/ml was achieved. Working cultures were stored at 4 ℃.

3.2.4 Methods

3.2.4.1 Growth curve of Salmonella

Salmonella strain *his*D3052 working culture (1ml) was dispensed to a 500ml sterile cultural flask with 100ml nutrient broth so that the flask contained $1\text{-}2 \times 10^7$CFU/ml *Salmonella* culture. The bacteria were incubated in a warm water bath with shaker

(100rpm) at 37℃. Every 2h (include 0h) the optical density (OD) was measured with a spectrophotometer at 600nm and pH value was measured by using a pH meter. A sample from the erlenmeyer was further diluted using sterile physiological saline solution. The proper diluents were spread to nutrient agar plates with 3 proper dilutions every 2h and incubated for 24h at 37℃. The value of cfu/ml was calculated by using the following formula:

CFU/ml = (average number of colonies × dilution factor) / 100µl

3.2.4.2 Genetic analysis of the tester strains

The tester strains should be checked for their genetic integrity before the experiments [46]. The genetic analysis included the following steps:

- **Histidine dependence:** A 1µl of culture was inoculated across a GM agar plate which contained 160µl 0.01% biotin solution. The plate was incubated in 37℃incubator for 24h.
- **Biotin dependence:** A 1µl of culture was inoculated across a GM agar plate which contained 160µl 0.01% histidine solution. The plate was incubated in 37℃ incubator for 24h.
- **Histidine and biotin dependence:** A 1µl of culture was inoculated across a GM agar plate which contained 160µl 0.01% biotin solution and 160µl 0.5% histidine solution. The plate was incubated in 37℃ incubator for 24h.
- ***rfa* marker:** A 1µl of culture was inoculated across a GM agar plate which contained 160µl 0.01% biotin solution and 160µl 0.5% histidine solution. A small piece of sterile filter paper was placed in the center of the plate which contained 0.1% crystal violet solution. The plate was incubated in 37℃ incubator for 24h.
- **Ampicillin resistance:** A 1µl of culture was inoculated across a GM agar plate which contained 160µl 0.01% biotin solution and 160µl 0.5% histidine solution. A small piece of sterile filter paper was placed in the center of the plate which

contained 0.8% ampicillin solution. The plate was incubated in 37℃ incubator for 24h.

- **Tetracycline resistance:** A 1μl of culture was inoculated across a GM agar plate which contained 160μl 0.01% biotin solution and 160μl 0.5% histidine solution. A small piece of sterile filter paper was placed in the center of the plate which contained 0.8% tetracycline solution. The plate was incubated in 37℃ incubator for 24h.

3.2.4.3 The plate incorporation test

The diagram of the plate incorporation assays is shown in Fig. 3-1. Fresh inocula of *Salmonella* cultures were prepared before the test: a 200μl working cultures of TA98/TA100/*his*D3052 was dispensed in a sterile 100ml erlenmeyer flask with 20ml nutrient broth, incubate the flask in a warm water bath under continuous shaking (100rpm) at 37℃ for 18h until a density of $1\text{-}2\times10^9$ cfu/ml was achieved. The GM plates were incubated overnight at 37℃ in order to eliminate an excess of moisture before starting the experiments. Meat samples (1.3ml/aliquot) were firstly centrifuged for 10min at 10000g; then the supernatant was collected and sterilized though a 0.45μm filter into a sterile 1.5ml centrifuge tube. The supernatant were further diluted to 75%, 50%, 25% and 10% with sterile distilled water, respectively; so totally 5 doses of meat samples (100μl/plate, 75μl/plate, 50μl/plate, 25μl/plate and 10μl/plate) were prepared. S-9 mix was prepared by adding 20ml cold sterile distilled water freshly before the experiment. S-9 mix is a metabolic activation mixture which can stimulate some carcinogenic compounds into active forms [46]. S-9 mix consists of supernatant fraction of rat liver homogenate and NADP cofactors. Since *Salmonella* itself do not contain P450 enzyme system (mainly exists in mammal liver), S-9 mix was added in the Ames test to detect indirect mutagenicity of certain compounds. The highest dose of quercetin $\cdot 2H_2O$ i.e. 13.5μg/100μl was chosen since 5mg pure quercetin was added per incubation (40.5ml) in the beef samples. Top agar was melt in a microwave. Then 2 ml top agar was dispensed in each sterile test tube and kept warm at 43℃-48℃. As negative control, 10% glucose was chosen for making standard curves, 2.5μg/100μl of 2-nitrofluorene was chosen as

positive control for strains TA98 and *his*D3052; 10µg/100µl of 2-nitrofluorene was chosen as positive control for strain TA100. All the experiments were performed in duplicate with 3 plates for each dose. A sample was considered having mutagenicity when a dose-related increase was observed and a 2-fold increase (MI≥2) was obtained with one or more concentrations; a sample was considered having signs of mutagenicity when a dose-related increase was observed but the revertants number was not doubled at the highest dose. The statistical software TIBCO Spotfire S+® 8.2 for windows was used to analyze the results; the data (revertants/plate) were assessed by linear regression in order to evaluate the dose-related increase.

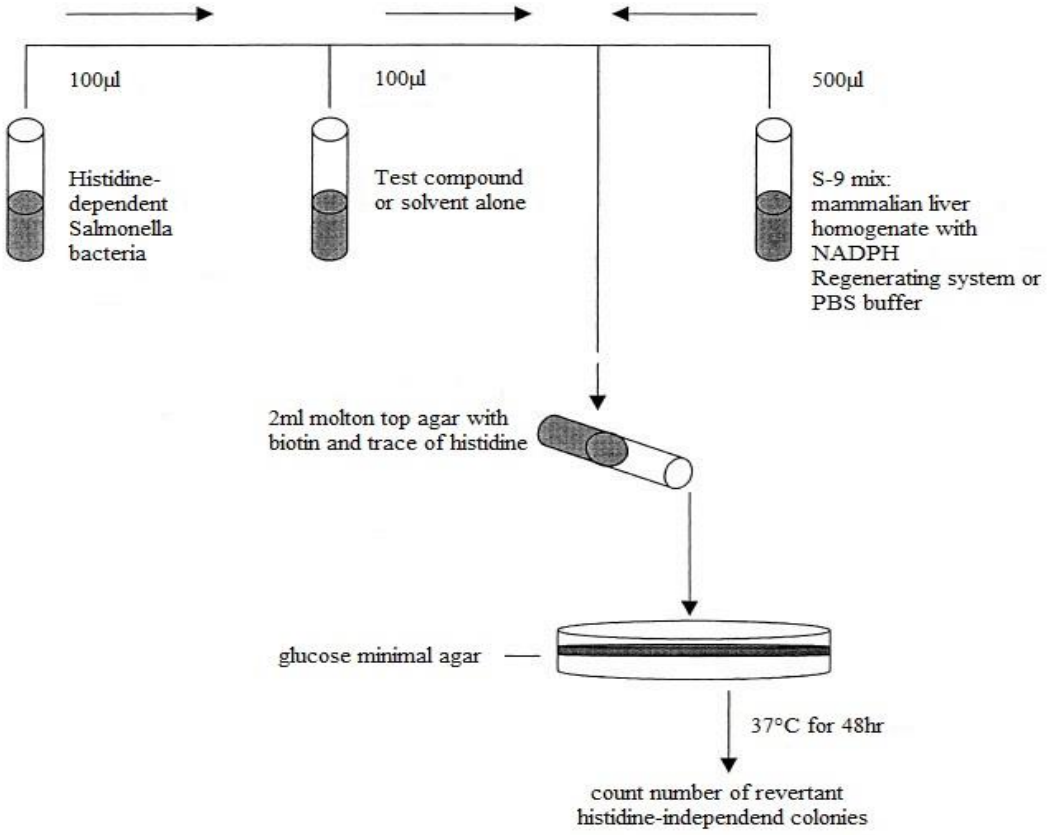


Figure 3-1. Diagram depicting the steps involved in the plate incorporation assays [46]

4 Results

4.1 Implementation of the Ames test

*4.1.1 Growth curve of Salmonella strain his*D3052

The growth curve of strain *his*D3052, which was measured by the OD_{600} and by CFU/ml, is shown in Fig. 4-1and Fig. 4-2.

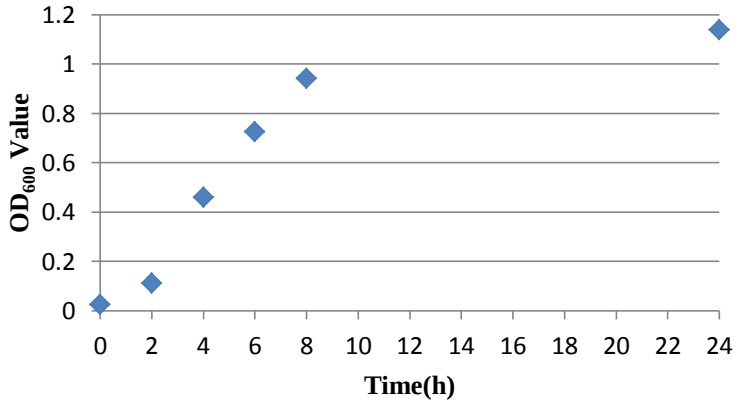


Figure 4-1. 24h OD_{600} value of strain *his*D3052 incubated in 37 ℃ warm water bath

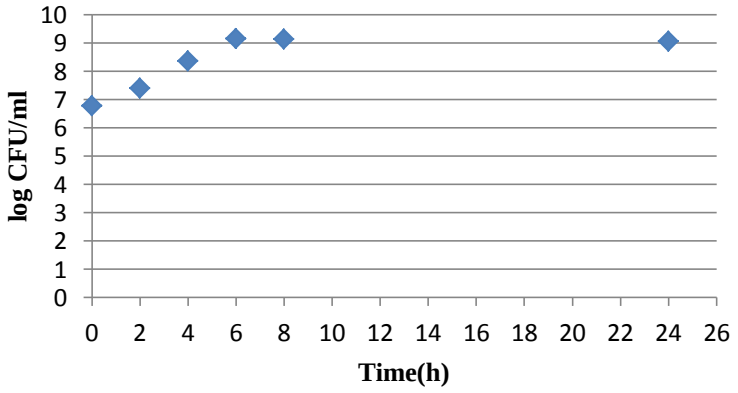


Figure 4-2. 24h growth curve of strain *his*D3052 incubated in 37 ℃ warm water bath

According to the measurements in Fig. 4-1, it can be concluded that the lag phase of *Salmonella* only last for a short period; furthermore, the transition between the exponential phase and stationary phase was not visible.

Fig. 4-2 illustrates the increasing CFU/ml relative to the incubation time. A short lag phase was also observed and the stationary phase started after 10h, but according to Mortelmans *et al*. [46], fresh inocula should be incubated for 16-18h in order to reach a density of 1-2 $\times 10^9$ cfu/ml.

4.1.2 Genetic analysis of the tester strains

Genetic analysis results of tester strain *his*D3052 are shown in Fig. 4-3 and Table 4-1.

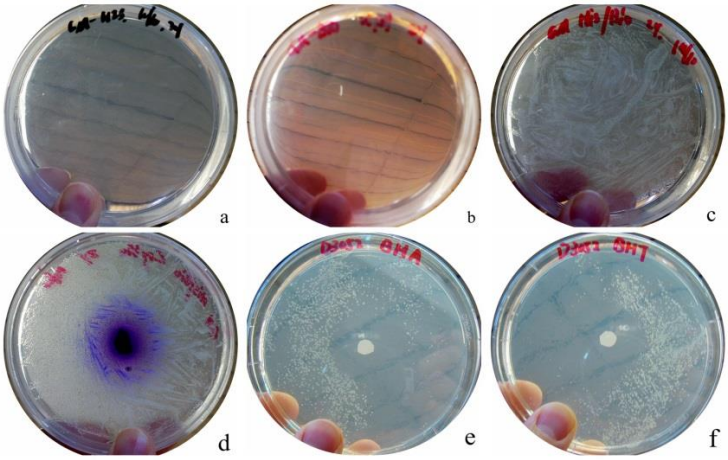


Figure 4-3. Genetic analysis of strain *his*D3052

Table 4-1. Genetic analysis result of strain *his*D3052

Sector	Medium	Diagnostic	Result
a	Histidine+, Biotin -	*Biotin*$^-$	No growth
b	Histidine -, Biotin +	*Histidine*$^-$	No growth
c	Biotin +, Histidine +	*Histidine*$^-$, *Biotin*$^-$	Growth
d	Biotin +, Histidine +, Crystal violet +	*rfa*	Zonal inhibition
e	Biotin +, Histidine +, Ampicillin +	R-factor	Zonal inhibition
f	Biotin +, Histidine +, Tetracycline +	pAQ1	Zonal inhibition

These results confirmed that this tester strain *his*D3052 was biotin and histidine dependent, had *rfa* mutation (lipopolysaccharide defect), did not contain R-factor (pKM101 plasmid) nor pAQ1 plasmid.

Genetic analysis results of tester strain TA98 are shown in Fig. 4-4 and Table 4-2.

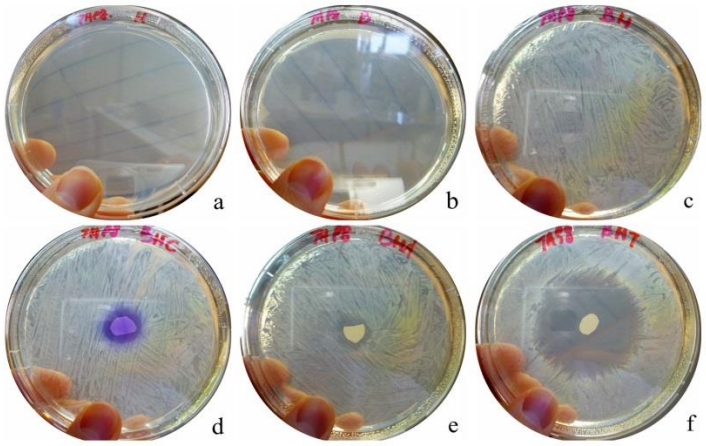


Figure 4-4. Genetic analysis of strain TA98

Table 4-2. Genetic analysis result of strain TA98

Sector	Medium	Diagnostic	Result
a	Histidine+, Biotin -	*Biotin*$^-$	No growth
b	Histidine -, Biotin +	*Histidine*$^-$	No growth
c	Biotin +, Histidine +	*Histidine*$^-$, *Biotin*$^-$	Growth
d	Biotin +, Histidine +, Crystal violet +	*rfa*	Zonal inhibition
e	Biotin +, Histidine +, Ampicillin +	R-factor	Growth
f	Biotin +, Histidine +, Tetracycline +	pAQ1	Zonal inhibition

These results confirmed that this tester strain TA98 was biotin and histidine dependent, had *rfa* mutation (lipopolysaccharide defect), contained R-factor (pKM101 plasmid) but no pAQ1 plasmid.

Genetic analysis results of tester strain TA100 are shown in Fig. 4-5 and Table 4-3.

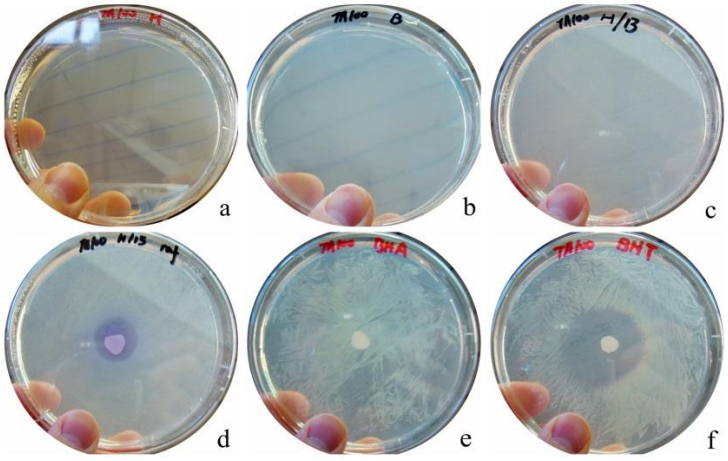


Figure 4-5. Genetic analysis of strain TA100

Table 4-3. Genetic analysis result of strain TA100

Sector	Medium	Diagnostic	Result
a	Histidine+, Biotin -	*Biotin*$^-$	No growth
b	Histidine -, Biotin +	*Histidine*$^-$	No growth
c	Biotin +, Histidine +	*Histidine*$^-$, *Biotin*$^-$	Growth
d	Biotin +, Histidine +, Crystal violet +	*rfa*	Zonal inhibition
e	Biotin +, Histidine +, Ampicillin +	R-factor	Growth
f	Biotin +, Histidine +, Tetracycline +	pAQ1	Zonal inhibition

These results confirmed that this tester strain TA100 was biotin and histidine dependent, had *rfa* mutation (lipopolysaccharide defect), contained R-factor (pKM101 plasmid) but no pAQ1 plasmid.

4.1.3 Standard curves of the tester strains

The standard curves of strain *his*D3052, TA98 and TA100 treated with 2-nitrofluorene at different doses, expressed as the mean and standard deviation of numbers of revertants per plate and R^2, are shown in Fig. 4-6 to Fig. 4-8.

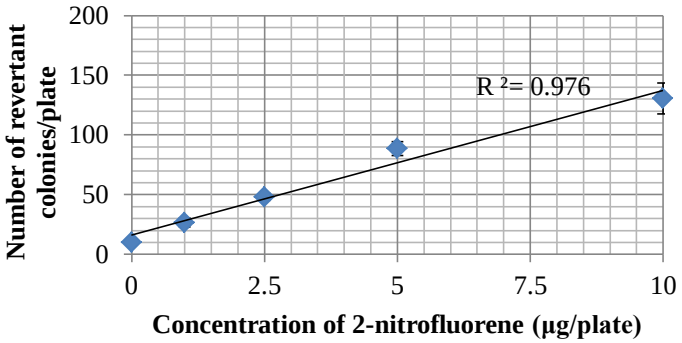


Figure 4-6. Standard curve of strain *his*D3052

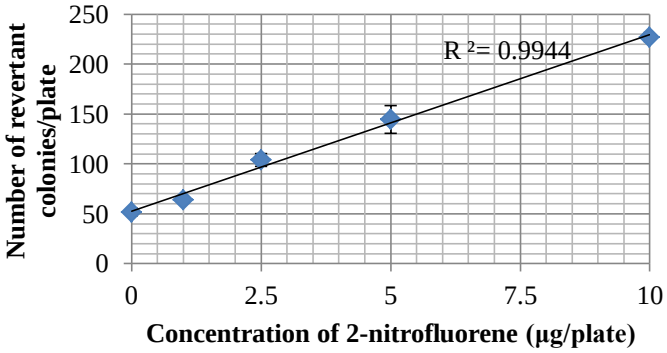


Figure 4-7. Standard curve of strain TA98

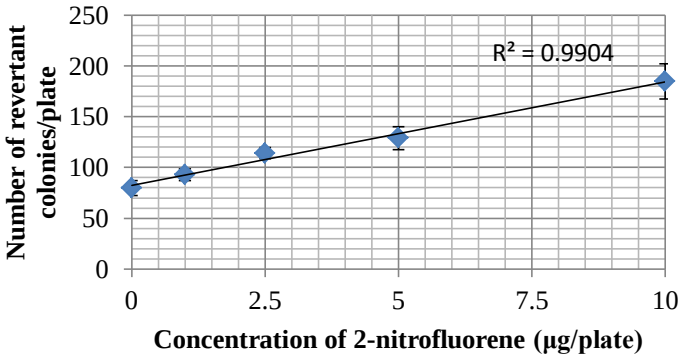


Figure 4-8. Standard curve of strain TA100

Every strain had a significant dose-related increase and a high R^2 (>0.95). At 1µg/plate, 2-fold increase was observed for strain *his*D3052. At 2.5µg/plate, 2-fold increase was observed for strain TA98; at 10µg/plate, 2-fold increase was observed for strain TA100.

The spontaneous mutant frequency for each tester strain was also checked. The range of spontaneous revertant colonies for strains *his*D3052, TA98 and TA100 were 5-15, 15-60 and 70-200, respectively.

4.2 Results of experiment 1

The mutagenicity of pork, chicken and beef samples with different fat content, nitrite treatment and cooking method (internal heating temperature) was evaluated by strain *his*D3052 and the results are shown in Table 4-4. Of the 8 meat samples, none of them reached a two-fold increase at the highest dose level. A dose-response relationship was observed in the following samples: overcooked pork with 5% fat, overcooked pork with 5% fat and treated with nitrites, cooked pork with 5% fat and treated nitrites, cooked pork with 20% fat and treated with nitrites. No dose-response manner was observed in cooked pork with 5% or 20% fat, cooked chicken or beef with 5% fat. A summary of the mutagenicity results are also shown in Table 4-5.

4.2.1 The effect of the type of meat on the formation of mutagens

No dose-response relationship was observed in cooked pork, chicken or beef with the same fat content but without nitrite treatment (Table 4-4 comparison 1). The mutagenic index in all of those samples maintained at 0.9-1.1. No mutagenicity was confirmed by strain *his*D3052 for these samples, meaning that there was no difference between types of meat on the formation of mutagens.

4.2.2 The effect of the fat content on the formation of mutagens

No dose-response relationship was observed in cooked pork with 5% or 20% fat without nitrite treatment. The range of mutagenic index was 1.0-1.1 (Table 4-4 comparison 2). It can be concluded that the addition of subcutaneous pork fat to the pork sample did not result in the formation of mutagens which was tested by strain *his*D3052.

4.2.3 The effect of added nitrites on the formation of mutagens

Compared to cooked pork with 5% or 20% fat, without nitrite treatment, cooked pork samples with the same fat content, but treated with nitrites resulted in an obvious increase of revertant colonies with increasing doses of meat homogenates (Table 4-4 comparison 3 and 4). It can be concluded that, after treated with nitrites, meat samples were considered to induce signs of frameshift mutagenicity and there was an effect of nitrites on the formation of mutagens.

4.2.4 The effect of cooking methods (internal heating temperature) on the formation of mutagens

Overcooked pork with 5% fat, without nitrite treatment induced a high number of revertant colonies at 100μl/plate relative to 0μl/plate, with a mutagenic index of 1.7, which was also the highest mutagenic index in experiment 1. This result confirmed that this sample presented signs of frameshift mutagenicity compared to cooked pork with 5% fat, without nitrites (Table 4-4 comparison 5). There was a slight difference between the mutagenicity of cooked and overcooked pork with 5% fat, with nitrite treatment, with the mutagenic index of 1.4 and 1.5 at 100μl/plate, respectively (Table 4-4 comparison 6).

Table 4-4. Mutagenicity of different meat samples at different doses during colon digestion stage, explained as the mean and standard deviation of the number of revertants/plate and the mutagenic index (MI), tested by *Salmonella* strain *his*D5032 without adding S-9 metabolic system (n = 6)

	Comparison	Treatment (μl/plate)	Number of revertants/plate as mean ±SD (MI)
			*his*D5032
Pork with 5% fat cooked	1, 2, 4, 5	0 [a]	11.3 ±0.5
		10	11.3 ±0.5 (1.0)
		25	11.5 ±0.8(1.0)
		50	11.7 ±1.7 (1.0)
		75	11.7 ±1.2 (1.0)
		100	12 ±0.9 (1.1)
		Control+ [b]	48.3 ±2.9
		Regression analysis	Slope 0.0075 [y]
Chicken with 5% fat cooked	1	0 [a]	10.7 ±2.1
		10	10.5 ±0.5 (1.0)
		25	10.3 ±3.1 (1.0)
		50	10.7 ±2.5 (1.0)
		75	10.7 ±1.9 (1.0)
		100	11.3 ±1.7 (1.1)
		Control+ [b]	59 ±2.9
		Regression analysis	Slope 0.0065 [y]
Beef with 5% fat cooked	1	0 [a]	10.3 ±0.5
		10	10.7 ±0.9 (1.0)
		25	10.7 ±0.9(1.0)
		50	12.3 ±0.5 (1.2)
		75	11.3 ±1.7 (1.1)
		100	10.7 ±0.9(1.0)
		Control+ [b]	35.3 ±2.5
		Regression analysis	Slope 0.0055 [y]
Pork with 20% fat cooked	2, 3	0 [a]	12 ±1.4
		10	12.3 ±1.4 (1.0)
		25	12.5 ±2.7 (1.0)
		50	12.3 ±1.6 (1.0)
		75	12.7 ±2.0 (1.1)
		100	13.3 ±1.5 (1.1)
		Control+ [b]	37.5 ±7.1
		Regression analysis	Slope 0.0104 [y]

Table 4-4. Cont.

	Comparison	Treatment (µl/plate)	Number of revertants/plate as M±SD (MI)
			*his*D5032
Pork with 20% fat+nitrites cooked	3	0 [a]	11.3±0.9
		10	11.7±1.5 (1.0)
		25	14.3±1.4(1.3)
		50	12.7±3.2 (1.1)
		75	14.7±3.3 (1.3)
		100	16±2.2 (1.4)
		Control+ [b]	39.5±5.4
		Regression analysis	Slope 0.0412 [x]
Pork with 5% fat overcooked	5	0 [a]	11.5±1.3
		10	10±1.7 (0.9)
		25	11.5±2.2(1)
		50	10.8±1.2 (0.9)
		75	10.8±2.0 (0.9)
		100	20±5.0 (1.7)
		Control+ [b]	46.2±7.8
		Regression analysis	Slope 0.0804 [x]
Pork with 5% fat+nitrites overcooked	6	0 [a]	11.8±2.4
		10	12.3±1.4 (1.0)
		25	13.3±1.5 (1.1)
		50	14±2.6 (1.3)
		75	16±1.9 (1.4)
		100	18±3.4 (1.5)
		Control+ [b]	39.3±5.3
		Regression analysis	Slope 0.0596 [x]
Pork with 5% fat+nitrites cooked	4, 6	0 [a]	11±0.6
		10	11.8±1.1 (1.1)
		25	12.3±1.8 (1.1)
		50	13.5±2.1 (1.2)
		75	14.3±2.1 (1.3)
		100	15±3.4 (1.4)
		Control+ [b]	40.2±3.4
		Regression analysis	Slope 0.0316 [x]

M±SD: mean ± standard deviation; MI: mutagenic index; a: negative control (100µl/plate distilled water); b: positive control (2.5µg/plate 2-nitrofluorene); x: $p<0.05$(dose-related increase); y: $p>0.05$ (no dose-related increase)

Table 4-5. Results of evaluation of mutagenicity of different meat samples

	Treatment			*his*D5032
	Fat content	Nitrites (mg/kg)	Cooking method	
Pork	5%	0	Cooked	-
	5%	0	Overcooked	+/-
	5%	120	Cooked	+/-
	5%	120	Overcooked	+/-
	20%	0	Cooked	-
	20%	120	Cooked	+/-
Chicken	5%	0	Cooked	-
Beef	5%	0	Cooked	-

-: negative; +/-: signs of mutagenicity

4.3 Results of experiment 2

4.3.1 The mutagenicity of quercetin

The mutagenicity of quercetin was evaluated by *Salmonella* strains TA98 and TA100, with and without S-9 metabolic system (Table 4-6).

Table 4-6. Mutagenicity of quercetin at different doses, explained as the mean and standard deviation of the number of revertants/plate and the mutagenic index (MI), tested by *Salmonella* strains TA98 and TA100, with and without adding S-9 metabolic system (n = 6)

Quercetin (µg/plate)	Number of revertants/plate as M ±SD (MI)			
	TA98		TA100	
	S9-	S9+	S9-	S9+
0 [a]	42.5 ±4.3	49.8 ±5.5	60.7 ±10.7	80 ±2.8
1.35	60.7 ±5.4(1.4)	97.3 ±4.1(2.0)	66.5 ±10.7(1.1)	101 ±7.4(1.3)
3.375	107 ±11.6(2.5)	188.5 ±17.9(3.8)	72.5 ±5.2(1.2)	107.7 ±10.2(1.3)
6.75	193.5 ±14.2(4.6)	311.7 ±8.9(6.3)	84.5 ±4.9(1.4)	144 ±14.6(1.8)
10.125	292.5 ±8.6(6.9)	514.8 ±26.3(10.3)	91.5 ±4.5(1.5)	192.2 ±15.9(2.4)
13.5	374.8 ±17.2(8.8)	684 ±29.8(13.7)	105.3 ±16.1(1.7)	225.5 ±9.2(2.8)
Control+	87.2 ±5.8 [b]	95 ±5.7 [b]	144.7 ±16.7 [c]	245.5 ±12.1 [c]
Regression analysis				
Slope	3.4243 [x]	6.3667 [x]	0.4292 [x]	1.4527 [x]

M ±SD: mean ± standard deviation; MI: mutagenic index; a: negative control (100µl/plate distilled water); b: positive control (2.5µg/plate 2-nitrofluorene); c: positive control (10µg/plate 2-nitrofluorene); x: $p<0.05$ (dose-related increase)

The mutagenicity of quercetin in strain TA98 increased with an obvious dose-response relationship, with and without adding S-9 metabolic system. A mutagenicity index higher than 2.0 was observed at concentrations of 3.375μg/plate or higher when S-9 metabolic system was absent. A 2-fold increase of revertant number was observed at 1.35μg/plate when the S-9 metabolic system was present. The largest mutagenic indexes with and without S-9 metabolic system were 8.8 and 13.7, respectively. The mutagenicity of quercetin in strain TA100 also increased with a dose-response relationship, with and without adding S-9 metabolic system. When S-9 metabolic system was absent, no two-fold increase was observed even at the highest dose used but when S9 was present, a mutagenic index higher than 2.0 was observed at 10.125μg/plate. According to the above results, quercetin can be considered to induce frameshift mutagenicity and base-pair substitution.

4.3.2 The effect of added quercetin or vitamin C on the formation of mutagens

The mutagenicity of cooked beef samples with 20% fat, treated with quercetin or vitamin C, was evaluated by strains TA98 and TA100, with and without adding S-9 metabolic system (Table 4-7). A dose-response relationship was observed in all samples, except the sample treated with Vitamin C in the presence of S-9 metabolic system. A 2-fold increase of revertant number relative to the negative control was observed in the following samples: beef sample tested by strain TA100 in the presence of S-9 metabolic system; beef sample treated with quercetin, tested by strain TA98, with and without adding S-9 metabolic system and beef sample treated with quercetin, tested by strain TA100, in the presence of S-9 metabolic system. A summary of the mutagenicity results are also shown in Table 4-8.

4.3.2.1 The mutagenicity of beef sample

A dose-response relationship was observed in the beef sample without added antioxidants tested by strain TA98, with and without the presence of S-9 metabolic system, but no 2-fold increase of revertant number was observed. This result confirmed that this sample showed signs to induce frameshift mutagenicity. No clear differences in the number of

revertant colonies of strain TA98 were observed with or without addition of the S-9 metabolic system. In the TA100 strain, an obvious increase of revertant colonies with increasing doses of meat homogenates in the absence of S-9 metabolic system was also observed. In the presence of S-9 metabolic system, a 2-fold increase of revertant number was observed at the highest dose. It can be concluded that the beef sample can also induce base-pair substitution.

4.3.2.2 The effect of added quercetin on the formation of mutagens

A dose-response relationship was observed in the beef sample treated with quercetin, tested by both strains TA98 and TA100, with and without the presence of S-9 metabolic system. In the TA98 strain, a 2-fold increase of revertant number was observed with a mutagenic index higher than 2.0 at the highest dose in the absence of S-9 metabolic system. In the presence of S-9 metabolic system, a 2.0 of mutagenic index was observed at 75μl/plate of meat homogenates. It can be concluded that this sample showed frameshift mutagenicity. In the TA100 strain, no 2-fold increase of revertant number was observed at the highest dose in the absence of S-9 metabolic system but in its presence, a 2.0 of mutagenic index was observed at the highest dose of meat homogenates. It can be concluded that this sample also induced base-pair substitution.

4.3.2.3 The effect of added vitamin C on the formation of mutagens

Dose-response relationships were observed in the beef sample treated with vitamin C, tested by both strains TA98 and TA100 in the absence of S-9 metabolic system and no 2-folds increase of revertant number was observed. In the presence of S-9 metabolic system, a dramatic decrease of revertant number relative to the negative control was observed in strain TA98, the revertant number was even lower than that of negative control, with a mutagenic index of 0.6 at the highest dose of meat homogenates; in the TA100 strain, too many pinpoints were present in the discs at 75μl/plate and 100μl/plate of meat sample. It can be concluded that this sample showed signs to induce frameshift mutagenicity and base-pair substitution in the absence of external metabolic system.

Table 4-7. Mutagenicity of meat samples at different doses treated with different antioxidants (5mg of antioxidants in 40.5ml incubation) at duodenum digestion stage, explained as the mean and standard deviation of the number of revertants/plate and the mutagenic index (MI), tested by *Salmonella* strains TA98 and TA100 with and without adding S-9 metabolic system (n =6)

	Treatment (µl/plate)	Number of revertants/plate as M±SD (MI)			
		TA98		TA100	
		S9-	S9+	S9-	S9+
Beef with 20% fat	0 [a]	38.2±4.5	32.7±3.2	101.5±3.8	146.5±7.9
	10	36.8±3(1.0)	37.7±2.9(1.2)	98±8.8(1.0)	171.2±12.0(1.2)
	25	39.5±2.2(1.0)	46±2.3(1.4)	105.5±14.7(1.0)	196±15.9(1.3)
	50	47.3±5.9(1.2)	42.3±6.4(1.3)	138.7±16(1.4)	222.7±16.3(1.5)
	75	48.8±5.9(1.3)	53±6.8(1.6)	148.8±8.6(1.5)	243.5±13.5(1.7)
	100	56.7±4.2(1.5)	54±4.1(1.7)	173.8±4.3(1.7)	287.7±9.4(2.0)
	Control+	72±13.4 [b]	61±1.6 [b]	178.2±7.0 [c]	240.3±12.7 [c]
	Regression analysis				
	Slope	0.1935 [x]	0.1986 [x]	0.7766 [x]	1.2988 [x]
Beef with 20% fat+quercetin	0 [a]	39.2±3	32.8±5.1	99.3±6.4	135.2±7.7
	10	38.2±2.9(1.0)	41.7±2.5(1.3)	113±6.5(1.1)	149.2±11.0(1.1)
	25	47.3±3.2(1.2)	53.8±2.3(1.6)	118.5±6.1(1.2)	171.8±15.7(1.3)
	50	51.5±6 (1.3)	56.5±4.6(1.7)	133.5±15.1(1.3)	199.5±7.0(1.5)
	75	61.7±8.9 (1.6)	63.3±6.8(2.0)	143.3±11.6(1.4)	221.2±17.3(1.6)
	100	80.8±5.5 (2.1)	88±8 (2.7)	162.8±9.2(1.6)	289±31.1(2.0)
	Control+	79±10.9 [b]	57.3±5.2 [b]	179±9.7 [c]	240.3±12.7 [c]
	Regression analysis				
	Slope	0.4 [x]	0.4798 [x]	0.5819 [x]	1.5645 [x]
Beef with 20% fat+vitamin C	0 [a]	15.8±2.1	25±2.3	188±13.2	190.8±3.6
	10	20.2±2.4(1.3)	36.8±4.9(1.5)	216.5±9.1(1.2)	248.3±12.1(1.3)
	25	23.3±3.4(1.5)	35.2±7.8(1.4)	233.7±6.7(1.2)	256±9.1(1.3)
	50	25.3±1.2(1.6)	23.3±7.7(0.9)	249±11.4(1.3)	244.5±11(1.3)
	75	26.3±0.7(1.7)	22±5.2(0.9)	273.2±11.7(1.5)	*
	100	26.2±1.6(1.7)	15.5±2.6(0.6)	329±6.4(1.8)	*
	Control+	58.8±5.1 [b]	56.5±6.8 [b]	316.3±7.9 [c]	318.6±2.3 [c]
	Regression analysis				
	Slope	0.0922 [x]	-	1.2272 [x]	-

M±SD: mean ± standard deviation; MI: mutagenic index; a: negative control (100µl/plate distilled water); b: positive control (2.5µg/plate 2-nitrofluorene); c: positive control (10µg/plate 2-nitrofluorene); * too many pinpoints; x: $p<0.05$(dose-related increase); -: data not available (toxic effect)

Table 4-8. Results of evaluation of mutagenicity of meat samples treated with antioxidants

	TA98		TA100	
	S9-	S9+	S9-	S9+
Beef with 20% fat	+/-	+/-	+/-	+
Beef with 20% fat+quercetin	+	+	+/-	+
Beef with 20% fat+vitamin C	+/-	*	+/-	*

+: positive; -: negative; +/-: signs of mutagenicity; *: toxic effect

5 Discussion

In the present study, we evaluated the mutagenicity of different meat samples which were digested in an *in vitro* digestion model during duodenum or colon stage by using the Ames test, in order to find out possible indications for the relationship between colorectal cancer and meat products. During the first experiment we investigated if a direct mutagenicity was present in the meat samples. The meat samples were already treated in a colon digestion, thus the formed metabolites during the colon digestion will not be absorbed and transported to a significant extent, but will be excreted with the faeces or will be toxic for the colon cells themselves. On the other hand, we tried to evaluate the effect of adding antioxidants in the formation of direct or indirect mutagens in meat samples during duodenum digestion and the (anti)mutagenicity of the antioxidants themselves in the second experiment.

5.1 The effect of heme iron and lipid oxidation on the formation of mutagens

According to the results from experiment 1, no mutagenicity was detected by strain *his*D3052 in cooked pork, chicken and beef, all with the same 5% fat content at colon digestion stage. The same meat samples were also analyzed by Van Hecke *et al*. [47] by using colorimetrical methods and through HPLC analysis. They concluded that cooked uncured meat samples at colon digestion stage with higher heme iron content, i.e. beef sample, resulted in the formation of higher amounts of lipid oxidation products such as MDA and 4-HNE, meaning that the promoting effect of heme iron on fat oxidation were distinct. The negative results obtained by strain *his*D3052 probably can be explained by, i.e. the low concentration of MDA (26.3nmol/ml) and 4-HNE (23.6pmol/ml) present in the beef sample [47]. Negative result was obtained in cooked pork with 20% fat, indicating that, even if the fat content was increased up to 20%, still not enough mutagens were present to stimulate the mutant reversion of strain *his*D3052. For pork sample with 20% fat which was overcooked, by means of a longer cooking time and a higher cooking temperature, signs of mutagenicity were observed since longer cooking time and higher cooking temperature dramatically increased the formation of mutagens [48]. As the exact

concentration of lipid oxidation products MDA and 4-HNE in overcooked pork sample with 20% fat is not available, we are not able to conclude that lipid oxidation products are or are exclusively responsible for the signs of mutagenicity observed. In previous research, Riggins *et al*. [49] showed that MDA is a weak frameshift mutagen when tested by strain *his*D3052 at 0-10μmol/plate. Also mutagenicity indications of MDA with other tester strains have been reported e.g. Marnett *et al.* [50] proved that MDA lead to a positive result to strains TA102 and TA104 at 0-110μmol/plate. Although no studies are available for strain *his*D3052, no mutagen effect of 4-HNE was observed as 4-HNE in a concentration range of 0-0.5μmol/plate did not show positive results to strain TA102 or TA104 [50, 51]. In experiment 2, cooked beef sample with 20% fat digested in duodenum stage already showed signs of mutagenicity which was detected by strains TA98 and TA100 in the absence of S-9 metabolic system. These results can be explained firstly by unknown mutagens, such as aldehydes, amines, present in the meat samples that were detected by strains TA98 and TA100; secondly, strains TA98 and TA100 are probably more sensitive to lipid oxidation products than the other tester strain, although no data or article was found by using strain TA98 or TA100 to detect the mutagenicity of MDA and 4-HNE. The sufficient amounts of MDA, 4-HNE and/or other unknown mutagens probably lead to mutant reversion of strains TA98 and TA100. When the external metabolic system S-9 mix was present in strain TA100, the beef sample showed mutagenicity of base-pair substitution, meaning that probably higher amount of mutagens were formed after metabolism.

5.2 The effect of added nitrites on the formation of mutagens

According to the results from experiment 1, signs of mutagenicity were detected in pork samples which were treated with nitrites at the colon digestion stage by strain *his*D3052. Nitrite itself cannot induce the mutant reversion of strain *his*D3052, as Balimandawa *et al*. [52] proved that $NaNO_2$ (1-5mg/plate) can cause revertant to strain TA100 although the reversion rate is quite low compared to strain TA1535; for strain *his*D3052, no positive result was obtained. By contrast, metabolites of nitrites, NOCs, such as N-nitrosodimethylamine, N-nitirosodiethylamine and N-nitrosodiethanolamine could be the possible mutagens, if present, in the meat samples. Several studies already concluded that

the NOCs mentioned above can induce mutant reversion in varies of *Salmonella* strains [53-57]. In 1990, Ohata *et al*. [58] applied strain TA100 to analyze different meat products including pork, beef, chicken, ham, bacon sausage and horse, Each meat sample (8.75g) was treated with 40ml of 22mM nitrites at acidic condition (pH=3). Only bacon sausage without S9 was reported to give positive result. They argued that the reasons could be: first, the NOCs were trapped in the food polymers; second, the concentration of nitrites added in the meat samples probably was too low. The limitation of their experiment was that they only chose 2 doses of the meat samples (12mg/plate and 120mg/plate), so no dose-dependent manner could be observed. According to the analysis by Van Hecke *et al*. [47], high amounts of the NOC-specific DNA adduct O^6-carboxy-methyguanine were only detected in meat samples treated with nitrites after colon digestion. This result confirmed that without the P450 enzymatic system, the formation of NOCs may result from biological catalysis by bacteria in the colon [59]. Van Hecke *et al*. [47] also demonstrated that the detection of the DNA adduct was highly dependent on the applied fecal inoculum; a wide range of bacterial enzymes such as β-glucuronidase, nitrate reductase and nitro reductase are capable of generating potentially carcinogenic metabolites in the colon.

The antioxidative effect of nitrite was observed in experiment 1. The mutagenic index of overcooked pork with 5% fat content, with and without nitrites was 1.5 and 1.7, respectively. Nitrite is a known antioxidant by sequestering oxygen and may act as a precursor of the heat-stable NO-myoglobin by which the release of Fe^{2+} during heating is inhibited [60]. Consequently, less Fe^{2+} is available to catalyze the Fenton reaction, which is responsible for the initiation of oxidation processes. Moreover, in anaerobic conditions, nitrite can react with unsaturated fatty acid radicals, yielding a variety of nitro-alkene-, dinitro- or nitro-hydroxyl lipid derivatives [61]. It is also necessary to mention that nitric oxide can also react with lipophilic peroxyl radicals, generating far more stable alkyl peroxynitrates. Consequently, it was concluded that a 1:1 ratio of nitric oxide to reactive oxygen species enhances lipid peroxidation, while an excess of nitric oxide results in inhibition [62].

5.3 The effect of antioxidants on the formation of mutagens

Quercetin is a polyphenol, widely present in fruits and vegetables [63]. The antioxidative properties of quercetin and its derivatives were already reported and related to their protective effects against cardiovascular disease and certain cancers [63]. The antioxidative effect of quercetin can be explained by their scavenging capacity of radicals. Also quercetin can inhibit carcinogenesis by modulation of the metabolism of food-born carcinogens through inhibition and/or induction of phase I and II biotransformation enzymes, and by the suppression of the abnormal proliferation of early, preneoplastic lesions. Inhibition of cell proliferation may result from inhibition of various enzymes involved in cellular responses to growth factors, including protein kinase C, tyrosine kinase and phosphatidylinositol 3-kinase. Also inhibition of cell proliferation can be caused by flavonoids due to their effect on the expression of various tumor-related genes including antioxidant protein genes or the tumor suppressor gene *p53* [64]. However, flavonoids have been claimed to be able to exert prooxidant chemistry as well, and they can show mutagenicity, which was proved by the Ames test and several mammalian cell systems [65]. According to the results from experiment 2, mutagenicity of quercetin was confirmed and the results were similar with those obtained by Resende *et al.* [66]. The mutagenicity of quercetin may be explained by the formation of mutagenic quinone-type metabolites: quinone and quinone methide which are considered as alkylating DNA-reactive intermediates [63]. The prooxidant chemistry and the formation of quinone/quinone methide are shown in Fig. 5-1 and Fig. 5-2. MacGregor *et al.* [67] demonstrated that the mutagenicity of quercetin was related to its structure feature with the presence of hydroxyl groups on the B ring, free hydroxyl group at the 3-position, a double bond at the 2,3-position and a keto-group at the 4-position. A metabolic activating system, especially the presence of cytochrome P450 monooxygenase system can promote the formation of quinone and quinone methide; moreover it can catalyze non-mutagenic flavonoids into mutagenic forms [68]. This can explain the higher mutagenic indexes when the S-9 metabolic system was present during experiment 2.

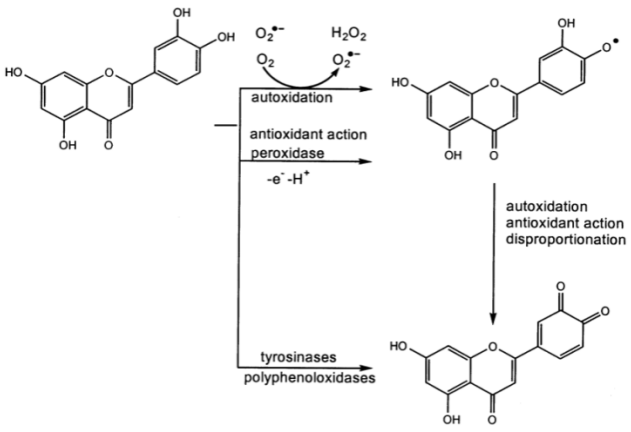


Figure 5-1. Prooxidant chemistry of catechol-type flavonoids [64]

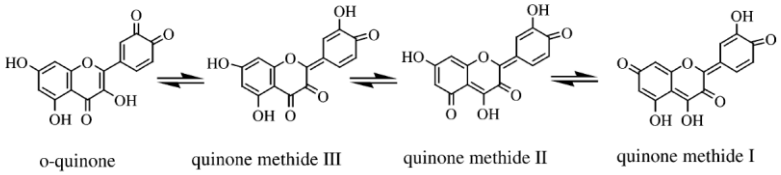


Figure 5-2. Quinone/quinone methide isomerization of quercetin [64]

Van Hecke *et al.* [69] demonstrated that after adding quercetin in the beef sample, the concentration of MDA and 4-HNE dramatically decreased by 40% and 80%, respectively compared to control sample. Compared to the beef sample without adding quercetin, the mutagenic indexes of beef sample with quercetin evaluated by strain TA98 were not decreasing but increased; the mutagenic index of beef sample with quercetin evaluated by strain TA100 slightly decreased in the absence of S-9 metabolic system but in its presence, the mutagenic index was equivalent (MI=2.0) to that of the beef sample without quercetin. This indicates that after adding quercetin, the meat sample still showed mutagenicity. This can be explained by the reaction between the added quercetin and unstable lipid oxidation products, such as MDA and 4-HNE, but the residuals of quercetin which were present in the meat homogenates still resulted in the increase of revertant colonies. In conclusion, since quercetin itself can induce mutation for *Salmonella* tester strains, the Ames test is not an optimal assay to evaluate the antioxidant effect of quercetin and the effect on the mutagenicity of meat samples supplemented with quercetin.

Vitamin C (ascorbic acid) is well-known for its prevention and cure of curvy [70]. On the other hand, vitamin C can scavenge reactive oxygen and nitrogen species, e.g. super-oxide, hydroperoxyl radicals and nitroxide radicals [71].The antioxidative effect of vitamin C can be explained as follows [72]; first, low reduction potentials of ascorbate (converted from vitamin C) and its one-electron oxidation product, ascorbyl radical can react with oxidant; second, the stability and low reactivity of the ascorbyl radicals formed during scavenging of a reactive oxygen or nitrogen species by ascorbate. The ascorbyl radicals can further be converted into ascorbate and dehydroascorbic acid. Vitamin C can also act as a prooxidant depending on the concentration used, the environment and conditions [71].For instance, Fe^{3+} can be reduced to Fe^{2+} by vitamin C leading to the formation of reactive hydroxyl radicals by reacting with hydrogen peroxide, which is known as Fenton chemistry. The antioxidant and prooxidant chemistry of vitamin C is shown in Fig. 5-3. Lipid hydroperoxides may also react with Fe^{2+} resulting in the formation of lipid alkoxyl radicals that can "initiate and propagate the chain reactions of lipid peroxidation" [72].

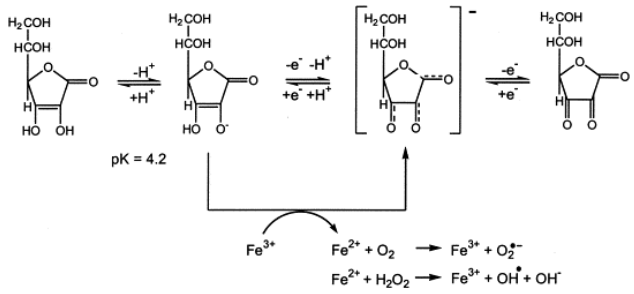


Figure 5-3. Antioxidant and prooxidant chemistry of vitamin C [72]

The prooxidant effect of vitamin C when added to meat sample was observed in experiment 2. Although vitamin C itself cannot cause mutant reversion to any *Salmonella* tester strains [73], beef sample treated with vitamin C had higher mutagenic indexes in both strains TA98 and TA100 compared to the beef sample without added vitamin C in the absence of S-9 metabolic system. Van Hecke *et al*. [69] also demonstrated that after adding vitamin C to the beef sample, MDA and 4-HNE increased by approximately 130% compared to untreated sample, indicating that vitamin C promoted the formation of MDA and 4-HNE. Toxic effects were observed in the presence of S-9 metabolic system in both

tester strains: for strain TA98, thinning of the background lawn was observed accompanying with a decrease in the number of revertant colonies to levels below the spontaneous reversion level of a dose of 50μl/plate; for strain TA100, many pinpoints were observed in conjunction with an absence of background lawn of a dose of 75μl/plate. Mortelmans *et al*. [46] concluded that the partial toxic compounds cannot kill or inhibit all the plated bacteria, but that the surviving bacteria can still form some microcolonies but not densely packed ones. On the other hand, when higher amount of toxic compounds are present in the plates, histidine-independent bacteria were killed leading to more histidine being available for the survival of the histidine-dependent bacteria, meaning that those bacteria can still grow until the histidine was depleted. Paolini *et al*. [74] demonstrated that vitamin C can induce P4502E1-linked monooxygenases leading to the formation of large amounts of the anion radical superoxide. These reactive radicals probably caused the toxic effect when S-9 metabolic system was present.

5.4 The evaluation of the Ames test

Since the supernatant of meat samples probably contained certain amounts of histidine or histidine-related precursors, it is necessary to be aware of possible interference of histidine on the results obtained by Ames test. Aeschbacher *et al*. [75] and Bruch *et al*. [76] already reported that the presence of histidine and histidine-related precursors in biological samples can give false-positive results in the Ames test. For instance, the average content of histidine in beef, *m. Biceps femoris* was 41mg/100g fresh muscles [77]; this means that in experiment 2, the concentration of histidine in beef meat samples was approximately 4μg/plate at the highest dose. Although Jin *et al*. [78] demonstrated that less than 5μg/plate of histidine did not result in a significant increase of revertant number, some peptides, especially histidine-containing dipeptides can be decomposed to histidine during in vitro digestion; on the other hand, the histidine and its precursors are probably partly trapped in the meat matrix, so the accurate content of histidine in the test supernatant was unknown. We tried to use a colorimetric method to analyze the content of histidine in the test supernatant, but we failed since the interference of the complicated content of proteins and amino acids in the samples, especially due to the presence of tyrosine [79]. Khandoudi *et al*. [80] even reported that the presence of arginine may also

cause false positive results in the Ames test since the histidine transport component is also able to transport arginine, meaning that arginine probably can be used as substrate for histidine dependent *Salmonella*. They demonstrated that arginine induced a significant increase in the revertant number to strains TA98 and TA100 from 0.4 to 8mg/plate, 1.2 to 8mg/plate, respectively. The average content of arginine in beef was 21.8mg/100g fresh muscles [77], meaning that the concentration of arginine in beef meat samples was approximately 2.18μg/plate at the highest dose, which means we could not have interference from arginine which is present in the meat samples.

Several modified methods were built in order to minimize the influence of histidine and other factors on the Ames test. One method that was built by Cornor *et al*. [81] successfully evaluated urine samples without interference from histidine and its precursors, but during the extraction process, part of the mutagens or active compounds may be lost, which is a drawback of this modification. Jin *et al*. [78] established another type of modified Ames test assay. Tester strains and test samples were firstly transferred into test tubes which contained liquid Lysogeny broth (LB) medium and incubated for 6-12h; after that, the bacterial cells were washed and finally accessed to the GM plates. After 48h incubation, the number of revertants was counted. During the preincubation period, tester strains had sufficient time to contact with LB medium which contained much higher amounts of histidine, arginine and their precursors than that of in the test samples, meaning that all the histidine dependent bacteria reached to their maximum growth before they were transferred to GM plates. If the test samples contained mutagen, they can still induce those histidine dependent bacteria into histidine independent and form colonies in the GM plates. All the modified methods mentioned above lack however, international guidelines.

The pretreatment of meat samples before adding to the plates can also affect the results of the Ames test. We use centrifugation method to collect the supernatant, this treatment may result in the excess of histidine present in the samples and/or part of compounds can be trapped in the precipitate. Extraction procedures are widely used to treat biological samples before the experiment [58, 82, 83], but as discussed before, this procedure can cause the loss of certain compounds and cannot promise the elimination of histidine in

the samples; however, there is no literature to discuss about the effect of pretreatment of meat samples.

5.5 Conclusion

By using the Ames test, the study evaluates the mutagenicity of different meat samples, with different pretreatments during the colon digestion stage and the (anti)mutagenicity of antioxidants (quercetin and vitamin C) when they added to beef samples during duodenum or colon digestion stage. The main findings from this study are described as follow. During colon digestion stage, there was no difference between different types of meat on the formation of mutagens; the addition of subcutaneous fat did not result in the formation of mutagens; meat samples treated with nitrites can induce signs of mutagenicity; longer cooking time and higher cooking temperature promoted the formation of mutagens. During the duodenum digestion stage, the beef sample itself can induce base-pair substitution in the presence of S-9 metabolic system; quercetin is a strong mutagen which can induce frameshift mutagenicity and base-pair substitution; beef samples with quercetin or vitamin C still induced mutagenicity; the added vitamin C in the beef samples even resulted in toxic effect to tester strains in the presence of S-9 metabolic system.

5.6 Further research

Further research would be recommended to improve the study:

1 A better modified Ames test should be built up to eliminate the interference caused by histidine and its precursors.

2 Structure-activity relationship of phenolic compounds and their (anti)mutagenic and antioxidative activity should be evaluated, meaning that different phenolic compounds should be conducted by the Ames test and more *Salmonella* strains should be employed such as strains TA102 and TA104.

3 Inhibition of mutagen formation due to lipid and protein oxidation by adding phenolic compounds with no mutagenic effects in a simulated *in vitro* digestion model should be investigated by the Ames test.

4 Interaction between nitrite and its derivatives and phenolic compounds should also be studied in order to understand if new nitrosylated phenolic compounds with antioxidative and anti-mutagenic effects are formed and how phenolic compounds and nitrite interact in limiting oxidation processes.

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