# "Driver or passenger" : an integrated epidemiological and experimental perspective on the association between nontyphoidal salmonella infection and colon cancer

Duijster, J.W.

# Chapter 9

# GENERAL DISCUSSION

Worldwide, the incidence of colon cancer ranks among the highest of all cancers with 1.1 million diagnoses annually [1]. Apart from hereditary causes of colon cancer, the main risk factors are related to lifestyle and dietary habits. However, the impact of microorganisms in the initiation and progression of cancers has been the subject of study for decades. Nowadays, over 20% of all cancers is estimated to be attributable to microorganisms [2], which therefore constitute a significant risk factor for a number of malignancies. While the earliest reports of an association between viral infections and cancer date back to the beginning of the 20$^{th}$ century, the oncogenic potential of bacteria has long been neglected. This might be related to the fact that bacteria, in contrast to viruses, do not leave a genetic imprint in human cells after infection, thereby hampering causal inference. Fortunately, the oncogenic potential of pathogenic and commensal bacteria is gradually being acknowledged, as an ever expanding list of bacteria are being associated with the onset and progression of cancer [3]. Few *in vitro* and epidemiological studies suggested a role of nontyphoidal *Salmonella* in the development of colon cancer. In this thesis, we aimed to explore the role of bacteria in the onset and progression of cancers in the gastrointestinal tract, with particular focus on the association between *Salmonella* and colon cancer. Broadly, the objectives of the thesis were covered by three main themes. First, we aimed to investigate whether repeated exposure to (lower doses of) *Salmonella* yields a similar risk of colon cancer development as compared to a single severe or high-dose infection. Second, we attempted to strengthen the evidence of an association between *Salmonella* and gastrointestinal tract cancers from an epidemiological perspective by conducting two registry-based studies and reviewing the current worldwide knowledge on these associations. This was done also for other correlations between bacteria/parasites and gastrointestinal tract cancers. The third theme covers the question whether the *Salmonella* phenotype in terms of its oncogenic potential can be explained by its genotype and which mechanisms might play a role in the *Salmonella*-induced tumorigenesis. In the following paragraphs, the main findings within these themes will be summarized and discussed. Also, we put the results from this thesis in a broader perspective of existing literature. This is followed by a paragraph elaborating the overall lessons learnt, as well as implications and recommendations for further study.

## Experimental evidence for an association between repeated exposure to *Salmonella* and colon cancer is generally stronger than epidemiological data

Over a thousand culture-confirmed *Salmonella* infections are reported annually in the Netherlands, mostly representing salmonellosis patients with symptoms severe enough to require medical attention, including symptoms lasting at least 1-2 weeks, and travel-related infections [4]. In an earlier Dutch cohort study, an association was found between these reported *Salmonella* infections and proximal colon cancer, with an almost 3-fold increased risk of proximal colon cancer after infection with *S*. Enteritidis [5]. However, people acquire multiple *Salmonella* infections throughout life via consumption of contaminated food or water, contact with live animals, the environment and to a lesser extent human-to-human transmission [6, 7]. The vast majority of these infections are asymptomatic or present themselves with mild and self-limiting symptoms that do not require medical attention, thereby remaining undiagnosed and unreported. Whether the cumulative effect of multiple, a- or pauci-symptomatic infections also contributes to the initiation or progression of colon cancer, was unknown, and therefore became the subject of the studies in **Chapters 2-4**. Here, we approached this research question from both an experimental and an epidemiological angle.

In the experiments described in **Chapter 4**, mice were orally infected with either a single high dose or multiple low doses of a laboratory *S*. Typhimurium strain to assess the effect on cancer development. To this end, a frequently used mouse model, mimicking human colitis-associated colon cancer, was used in which the mice received the pro-carcinogen azoxymethane (AOM) and colitis inducing dextran sulfate sodium (DSS) to induce the formation of colon tumors. The group of mice infected with a single high dose received 10,000 colony forming units (CFU) of *Salmonella*, whereas the group receiving multiple low doses were three times infected with 10 CFU of *Salmonella* with an interval of 4 weeks. Both groups infected with *Salmonella* (high or low dose), as well as a control group that received the AOM+DSS without infection, developed colon tumors in the 16 weeks of follow-up. This was in contrast to control mice that were neither exposed to AOM+DSS nor received *Salmonella* infection, indicating that the AOM+DSS treatment was the main driver of tumor formation. Overall, no differences were observed in the number of tumors, tumor volume, number of proliferating cells or colonization with *Salmonella* between the mice that received a single high dose of infection and the mice that received multiple low doses. However, the tumors were significantly larger in the *Salmonella*-infected mice as compared to the AOM+DSS control group. In addition, the tissues in both groups of *Salmonella*-infected mice

showed high grade dysplasia and signs of invasive carcinoma, which was not observed in the AOM+DSS control group. Also, higher amounts of proliferating cells were observed in the *Salmonella*-infected mice in both the tumor tissue, as well as the adjacent tissue, whereas the degree of colonization was higher in the tumor tissues as compared to adjacent tissue. The observation that low doses of *Salmonella* are sufficient to induce an (oncogenic) effect are in line with earlier experiments in rats where a low dose of *S*. Enteritidis caused colonic lesions, even in the absence of symptoms of illness [8]. Next to the mice experiments, the effects of a low- or high-dose infection were examined in a simple cell model consisting of mouse embryonic fibroblasts (MEFs) with a tumorigenic predisposition inflicted by overexpression of the proto-oncogene c-MYC and inactivation of tumor suppressor gene TP53 (**Chapter 4**). Exposure of the MEFs to either a high (multiplicity of infection [MOI] 25) or low (MOI 5) dose of a laboratory *S.* Typhimurium strain led to transformation, as characterized by colony formation in soft agar assays. Culturing of these transformed MEFs (i.e. after the first *Salmonella* infection) and subsequent reinfection with the same high or low dose of *Salmonella* resulted in the formation of more and larger colonies. This was particularly true for the MEFs infected with a high dose of *Salmonella.* Still, the number and size of colonies was larger after reinfection of MEFs with a low dose as compared to a single infection with a high dose. Similarly, *Salmonella* showed a tropism for MEFs with the highest level of transformation. This is in line with the *in vivo* experiment, as well as prior studies in which *Salmonella* showed preferential accumulation and proliferation in tumor tissue as compared to normal tissue at a ratio of >1000:1 [9]. Hence, both *in vitro* and *in vivo* experiments showed that infections with a low dose of *Salmonella* trigger a similar tumorigenic effect as a single high-dose infection, though in MEFs a high-dose infection positively impacts the number and size of the colonies formed. All in all, the experimental evidence for an association between *Salmonella* infection and colon cancer obtained in **Chapter 4** was substantial and generally indicated that, under certain conditions that may occur realistically in nature, *Salmonella* is able to promote colon carcinogenesis.

To complement these experimental findings, we also conducted several epidemiological studies. First, we investigated whether the presumed higher levels of (repeated) exposure to *Salmonella* are reflected by a higher incidence of salmonellosis. To this end, we compared the incidence of reported salmonellosis among different occupational groups in a large nationwide population-based registry study (**Chapter 2**). We also included reported *Campylobacter* infections in this study, as this pathogen is a leading cause of gastroenteritis as well, albeit scarcely associated with cancer. We thus studied the incidence of salmonellosis and campylobacteriosis among the whole spectrum of occupations grouped into 'divisions'

according to an internationally agreed upon occupational classification system. Moreover, we defined three risk groups or divisions with a presumedly higher degree of occupational exposure to zoonotic pathogens, including *Salmonella*, due to contact with animals and products thereof. As anticipated, a significantly higher incidence of salmonellosis (1.8-fold) and campylobacteriosis (1.7-fold) was observed among people working with live animals or animal manure (e.g. farmers, veterinarians, abattoir workers) as compared to the incidence in the total employed population. Among people involved in the sale of animal-derived products (e.g. butcher's and cheese shops), the reported incidences were also significantly higher for both salmonellosis (1.6-fold) and campylobacteriosis (1.4-fold), whereas the incidences among people involved in the processing of foods of animal origin (e.g. cooks and chefs) were not significantly different from those in the total employed population. These results agree to a major extent with a previous Dutch study based on a combined analysis of microbial subtyping and epidemiological (case-control) data on human *Salmonella* infections in which occupational exposure to raw meat or animals was found to be associated with an over 6-fold increased incidence of cattle-borne human salmonellosis [6]. Excess cases of salmonellosis and campylobacteriosis (as compared to the total employed population) were observed among health care associated occupations, as well as some industrial divisions. The observed differences in incidence of reported infections might reflect a genuinely higher level of exposure to *Salmonella* and/or *Campylobacter*. However, the analysis based on reported infections only includes symptomatic cases of disease and is affected by one's propensity to seek medical care. Inequalities in the utilization of medical care are relatively minor in the Netherlands, though several studies found a significant higher probability of visiting a general practitioner (GP) among people with a lower socio-economic status (SES) as compared to those with a higher SES, also after correction for differences in overall health status between the groups [10-12]. Hence, differences in GP visitation rates between occupational groups are inevitable. Apart from the virulence determinants of the specific bacterial strain affecting its pathogenicity, the probability of developing severe gastroenteritis (i.e. requiring medical attention) also incorporates host-dependent factors related to susceptibility to infection and the ability to clear the infection before severe or long-term complaints appear. Exposure to pollutants such as chemicals, heavy metals and nanoparticles, as observed in some industrial occupations, is suggested to alter the composition of the microbiome, potentially rendering people more susceptible to (severe) *Salmonella* or *Campylobacter* infection [13]. As the propensity to use medical care and an individual's likelihood to develop a severe infection are only partially related to the extent, type and frequency of exposure to the pathogen, we analyzed other types of data, namely serological data, to compare the sero-incidence of *Salmonella* and *Campylobacter* in

a subset of the employed population (**Chapter 2**). The anti-*Salmonella*/-*Campylobacter* IgM, IgG and IgA antibody titers in an individual's serum sample served as input for calculating the time since last seroconversion (i.e. time since last exposure to the pathogen) using an established Bayesian back-calculation model [14]. The time since last seroconversion can subsequently be translated into an estimated average number of infections per person-year, i.e. the seroincidence [15]. Since the seroincidence is based on an antibody response and does not discriminate between symptomatic and asymptomatic disease, it constitutes a less biased measure of infection pressure. In our study, we observed minor variation in the seroincidences of *Salmonella* and *Campylobacter* in different occupational groups, albeit that the seroincidences were assessed at a lower hierarchical level of occupational coding due to sample size constraints. Also, the seroincidences among people with an occupation included in one of the three risk groups did not differ from the average seroincidences in the whole subset. Such a disconcordance between reported infections and seroincidence has been observed in several European countries [16, 17]. Registered type of occupation is just one proxy for the level of exposure to *Salmonella*. Yet, this covers only a part of the myriad of possible exposures in an individual's life. Consumption of raw or undercooked meat and eggs, ownership of companion animals, use of proton pump inhibitors and poor kitchen hygiene practices leading to cross-contamination have been found to be the main risk factors for salmonellosis in the Netherlands, while occupational exposure to live animals or meat was only a risk factor for cattle-associated *Salmonella* infection, with a relatively minor contribution to the total set of risk factors [6]. Moreover, other studies have found higher incidences of salmonellosis and campylobacteriosis among people living in close proximity to broiler farms or areas with a high density of dairy/cattle farms [18, 19]. Our study clearly indicated an increased risk of salmonellosis (and campylobacteriosis) in people occupationally exposed to 'the source' of the pathogen, i.e. livestock and products thereof, but the relation between occupation and frequency of exposure, as depicted by serology, did not help much disentangling further the deeper mechanistic process.

We then explored the incidence of colon cancer among different professions in order to assess whether the occupational groups with increased salmonellosis incidence were also those more prone to develop cancer. To this end, in **Chapter 3** we presented the results of another nationwide occupational registry-based study with a comparable design and occupational (risk) group classification as in the study presented in **Chapter 2**. Overall, the differences in colon cancer incidence among occupational divisions, including the pre-defined risk groups, was relatively minor. Indeed, no excess risk of colon cancer was observed among people with a history of employment in the three risk groups as compared to the general

population, despite significantly higher incidences of salmonellosis. Significantly increased risks of up to 45% were observed for some divisions including mainly 'blue-collar' and manual occupations (e.g. occupations involved in printing, manufacturing of rubber, plastics, machinery and equipment and the sale of motorcycles/-vehicles), as well as some divisions pertaining mainly to the 'white-collar' and non-manual occupations (e.g. information service and real estate activities, and (re)insurance and pension funding). Yet, the significantly higher incidence of colon cancer among people with (a history of) employment in the real estate and (re)insurance and pension funding divisions corresponded to the *Salmonella* seroincidence results, as the seroincidence among people working in these divisions was amongst the highest ones. In general, however, the study presented in **Chapter 3** helped us ascertain that occupation in itself provides little differences in colon cancer incidence and that a direct link between (occupational) exposure to *Salmonella* and (increased) colon cancer risk was not possible to make with this approach.

To overcome the limitations of using occupation as a proxy for *Salmonella* infection pressure, we then studied whether colon cancer risk is correlated to increased exposure to *Salmonella* earlier in life, regardless of occupation but still using seroincidence data (**Chapter 4**). By linking individual-level data from a Dutch serosurvey to colon cancer diagnosis data, we found 36 individuals who provided a serum sample in the survey and were diagnosed with proximal colon cancer ≥1 year later (hereafter referred to as 'cases'). In a matched case-control analysis, we then observed that, overall, *Salmonella* seroincidence did not differ significantly between cases and controls. However, upon stratification, we noted that *Salmonella* seroincidence was significantly higher among cases younger than 60 years at the time of the serosurvey as compared to controls. Similarly, a higher seroincidence was observed among cases living in a neighborhood with a high socio-economic status at time of the serosurvey. These findings suggested that the effect of (increased exposure to) *Salmonella* on colon cancer risk would be (epidemiologically) appreciable only in absence of other (and generally much stronger) known risk factors for colon cancer, such as older age and unhealthy lifestyle (which low socio-economic is a proxy for), which might thus mask the relatively smaller effect of *Salmonella* itself. A proximal colon cancer diagnosis preceded by a higher seroincidence before the age of 60 is in agreement with the results of an earlier Dutch cohort study where salmonellosis reported between 20-60 years of age was associated with a significant higher risk of proximal cancer [5].

Several factors might explain the absence of a correlation between the incidence of reported salmonellosis and colon cancer among occupational groups. The most predominant one is the fact that colon cancer is, to a large extent, attributable to lifestyle and dietary habits.

Despite substantial variations in literature concerning the magnitude of risks and population attributable fractions (PAFs), a number of well-established risk factors account for a significant portion of colon and colorectal cancer diagnoses. These include the consumption of red and processed meat (PAF 5-12%), a low intake of dietary fibers (PAF 16-18%), alcohol consumption (PAF 1-13%), low calcium intake (PAF 6-10%), excess body weight (PAF 1-17%), low levels of physical activity (PAF 5-18%) and tobacco use (9-12%) [20-23]. Although the risk of colon cancer due to (repeated) exposure to *Salmonella* could be partially contained in the PAF for the consumption of red and processed meat, this would be limited to a very minor contribution, given that a larger portion of human *Salmonella* infections were attributable to layers, eggs and broilers as compared to pigs and cattle in the study period [6]. Of note, during the last decade, the sources of human *Salmonella* infections have been changing gradually, with pigs being nowadays the main source of human infections and eggs decreasing in importance as attributable source relative to pigs [4]. Adherence to a healthy lifestyle (i.e. the absence of the aforementioned major risk factors for colon cancer) differs strongly between occupational groups. This is partially for reasons inherent to specific jobs, such as a more sedentary lifestyle for non-manual workers (i.e. occupations at a more managerial or administrative level) and exposure to chemical substances in some manual occupations [24, 25]. An example is the relatively low incidence of colon cancer observed in the risk group involving contact with live animals in **Chapter 3**, which is consistent with earlier research reporting a reduced risk of colon and colorectal cancer among farmers, presumably owing to high levels of occupational physical activity [26, 27]. On the other hand, adherence to a healthy lifestyle is to a certain extent correlated to socio-economic status unrelated to the content of the occupation itself [28, 29]. Examples hereof are an average higher intake of red/processed meat, lower levels of leisure time physical activity and higher smoking rates among people with a lower socio-economic status [30-32]. Yet, whether socio-economic status can serve as a proxy for colon cancer risk remains arguable since inconsistent results are documented in literature [33].

Both the *in vitro* and *in vivo* experiments suggested that multiple low doses of *Salmonella* infection suffice to induce a tumorigenic effect in predisposed cells/organisms. Yet, extrapolating these experimental conditions to human infections under natural conditions raises some difficulties. First, the seroincidence and the reported salmonellosis as used in the registry-based studies provide an estimation of the annual number of infections and roughly the severity and longevity of disease symptoms (requiring medical care) but lack information about the dose of the infection. Literature about the dose-response relationship for *Salmonella* in the human population is relatively scarce. Of note, the implementation

of *Salmonella* dose-response studies during foodborne outbreaks is often subject to data availability constraints, such as lack of the contaminated product consumed by the time that people visited the GP for persistent symptoms of gastroenteritis. Estimating the number of ingested bacteria is therefore practically impossible. Moreover, the size of the population exposed to the contaminated product is often unknown, as people with an asymptomatic or paucisymptomatic infection remain unidentified [8]. *Salmonella* dose-response models of outbreak data showed that the average ingested dose of bacteria that corresponds to an infection- or illness-probability of 50% (ID50) was 7 CFUs for infection and 36 CFUs for illness [34]. However, the dose-response relation strongly depends on the virulence profile of the bacterial strain, as well as on the hosts' susceptibility and immune response. Hence, the dose of infection cannot be deduced from the course of disease and a simple threshold for a low- *versus* high-dose infection is hard to establish in humans. The dose of infection can also not be estimated from the seroincidence, although the dynamics of the serological response in terms of the time until the peak of antibody production, the maximum concentrations of antibodies and the shape of the decay curve are to an unknown extent affected by the dose, as well as the serovar. Under experimental conditions, the production of IgG antibodies against *S*. Enteritidis was positively correlated with dose of infection in rats [35], whilst infection of piglets with different *S*. Typhimurium strains led to differences in onset of seroconversion [36]. Therefore, the epidemiological analyses in this thesis did not allow for disentanglement of low- *versus* high-dose infections, nor the actual frequency/degree of exposure to *Salmonella* in all groups of the population under study, nor the definition of a mild *versus* severe infection. However, we consider the contribution of *Salmonella* to colon cancer development to be the product of several factors. Ultimately, the probability that a predisposed or pre-transformed cell is infected by a *Salmonella* bacterium leading to replication of mutated cells and the development of a tumor is determined by a) the frequency of infection, b) the bacterial load as defined by the number of bacteria ingested, the number of bacteria surviving the gastric acid, bacterial replication and killing, as well as c) the duration of *Salmonella* bacteria being present in the colon before being eliminated by the hosts' immune system [37].

# Epidemiological findings from other cohorts and malignancies confirm only small effects of *Salmonella* infection

As the epidemiological evidence of a possible association between severe (nontyphoidal) *Salmonella* infection and colon cancer was based on a single registry study in a Dutch cohort [5], we aimed to substantiate these results using an independent but comparable cohort. To this end, we performed a cohort study based on linked registries from the Danish population (**Chapter 5**). The design of the study resembled the Dutch cohort study with the exception that the Danish *Salmonella* surveillance system covers virtually the whole population (in contrast to the ~64% population coverage of the Dutch surveillance system), allowing for comparison of colon cancer incidence in individuals with a history of reported culture-confirmed salmonellosis *versus* individuals without such reported infection. Cox regression revealed no overall increased risk of colon cancer (both proximal and distal) ≥1 year after *Salmonella* infection. An 1.4-fold significant risk of proximal colon cancer was observed among people with a history of infection with a serovar other than Enteritidis and Typhimurium (including its monophasic variant), which are the most frequently occurring serovars among human salmonellosis cases. Besides, the hazard ratios (HRs) for proximal colon cancer in subgroup analyses were consistently higher for the group infected with other serovars as compared to Enteritidis and Typhimurium. The incidence of distal colon cancer diagnosis before the age of 50 after infection with a non-Enteritidis/Typhimurium infection was over three-fold higher. Remarkably, the incidence of diagnosed colon cancer was over two-fold increased among individuals with a recent (i.e. <1 year interval) *Salmonella* infection as compared to those without reported infection. While a causal relationship in terms of initiation of tumor development is implausible for reported *Salmonella* infections less than one year before cancer diagnosis, we cannot rule out that *Salmonella* infection in the early (prediagnostic) phase of colon/colorectal cancer leads to enhanced tumor progression. The tropism for infecting transformed MEFs and the higher rates of colonization in murine tumor tissue compared to adjacent tissue, as observed in **Chapter 4**, support this hypothesis. As the Dutch and Danish cohort used a comparable study design, we meta-analyzed the main outcomes of both studies. Analysis of heterogeneity of the two studies showed a low between-study variation ($p=0.958$; $I^2=0.01\%$). A priori, we decided to use a random-effects model when significant heterogeneity would be observed ($p<0.1$ or $I^2>50\%$), whereas a fixed-effects model would be used if $p>0.1$ or $I^2<50$. Hence, the fixed-effect model was used to obtain a pooled study estimate. An overall pooled risk ratio (RR) of 0.81 (95%CI 0.73-0.89) was obtained with a higher weight assigned to the Danish study (74.1%) compared to the Dutch study (25.9%) (Table 1).

**Table 1**. Risk ratios of colon cancer after Salmonella infection in the Dutch and Danish cohort studies.

| | **Reported *Salmonella* infection** | | **No reported *Salmonella* infection** | | |
|---|---|---|---|---|---|
| | **Colon cancer** | **No cancer** | **Colon cancer** | **No cancer** | **RR (95%CI)** |
| Netherlands§ | 96 | 14,168 | 140,458 | 16,619,770 | 0.803 (0.658-0.980) |
| Denmark | 245 | 47,578 | 54,624 | 7,544,498 | 0.808 (0.719-0.909) |
| Pooled estimate | | | | | 0.807 (0.729-0.893) |

RR: risk ratio. § Data derived from Mughini-Gras et al. (2018) [5].

The lack of correspondence between the outcomes of the two studies is presumably attributable to several effect modifying or diluting factors not equally present in both cohorts. One such factor is the epidemiology of human salmonellosis. In both the Netherlands and Denmark, control programs have been implemented aiming to reduce the prevalence of *Salmonella* in animal production systems. In Denmark, the implementation of three major control programs in 1988, 1993 and 1997 targeting *Salmonella* reduction in broiler chickens, pigs/pork and laying hens respectively, have led to a marked decrease of human *Salmonella* infections and a shift in the distribution of serovars [38, 39]. During the study period (1994-2015) the total number of reported salmonellosis decreased from a median of 2100 infections annually in 1994-2005 to 1129 in 2006-2016. *S*. Enteritidis accounted for the largest reduction (-61.1% in 2006-2016 *vs.* 1994-2005), followed by (monophasic) *S*. Typhimurium (-36.3%) and other *Salmonella* serovars (-19.6%) [40-43]. The reduction of notified salmonellosis in the Netherlands was less pronounced during the study period of the Dutch cohort study (1999-2015). A median annual number of 1674 infections was reported in the first part of the study period (1999-2007) compared to 1264 in the second part (2008-2015). *S*. Enteritidis infections reduced by 49.1% (2008-2015 *vs* 1999-2007), whilst the reduction of infections with *S*. Typhimurium and other serovars was limited to 5.4% and 3.6% respectively. Considering that the reported infections are only the tip of the iceberg of the true burden of disease, changes in infection pressure of *Salmonella* over time are presumably not sufficiently comparable between the two countries. In 2009, the incidence of human salmonellosis based on reported infections was 38.5 per 100,000 inhabitants in Denmark *versus* 11.6 per 100,000 inhabitants in the Netherlands [44, 45]. However, data from serosurveys conducted in 2006-2007 showed a higher infection pressure in the Netherlands compared to Denmark, with respective seroincidences of 0.149 and 0.084 infections per person-year [16]. In addition to a dissimilar degree of exposure, both countries have a distinct distribution of serovars and strains/clones responsible for human

salmonellosis, partly driven by travel and import of food products. For instance, the portion of sporadic human *Salmonella* infections related to travel is considerably higher in Denmark compared to the Netherlands (30-40% *vs.* 12%) [39, 44, 46]. Both the extent of exposure to *Salmonella* as well as serovar distributions might have contributed to the differences in observed outcomes between the Dutch and Danish cohort studies. Besides distinct exposures, the differences may also have been influenced by factors directly affecting the outcome probability, such as consumption behavior, physical activity and body weight. In 2014, the percentage overweight in young adults (18-24 years) was 25% in Denmark *versus* 20% in the Netherlands, while an opposite pattern was observed among individuals aged ≥65 (48-57% in Denmark, 55-62% in the Netherlands) [47]. Likewise, the consumption of red and processed meat per capita in Denmark is about 1.5 times higher as compared to Dutch inhabitants (840 grams/week *vs.* 560 grams/week) [48, 49]. The consumption of red and processed meat can act as confounder in the two studies, given that in both Denmark and the Netherlands a substantial part of the *Salmonella* infections is attributable to pigs or pork and the heme iron in red meat can modify the microbiome in favor of *Salmonella* colonization whilst it also directly increases colon cancer risk [50].

Looking at other malignancies than colon cancer, while a significant body of literature shows an association between *S*. Typhi and gallbladder carcinoma, the association between nontyphoidal *Salmonella* infection and cancers in the biliary tract has barely been studied. In contrast to *S*. Typhi that causes a systemic disease and is frequently associated with chronic infection, nontyphoidal *Salmonella* generally causes a local inflammation of the intestine [51]. Hence, direct manipulation of the epithelial cells of the gallbladder or bile ducts by nontyphoidal *Salmonella* as part of the infectious cycle is implausible for the vast majority of infections. Nonetheless, *in vitro S*. Typhimurium infection of gallbladder organoids and MEFs induced cellular transformation and the formation of colonies in soft agar [52]. Whether this tumorigenic potential could also be observed at the population level in an epidemiological study was addressed in **Chapter 6**. Here we performed a registry-based cohort study in the Dutch population, investigating the risk of cancers of the biliary tract after reported nontyphoidal *Salmonella* or *Campylobacter* infection. Cancers in the biliary tract include the gallbladder, the proximal and distal bile ducts and the extrahepatic bile ducts. The study design resembled the designs of the Danish study (**Chapter 4**) and the earlier Dutch study assessing the risk of colon cancer after salmonellosis [5]. Biliary tract cancer was diagnosed in nine individuals with a history of reported salmonellosis and seven individuals with a history of reported campylobacteriosis. These low numbers are not surprising given that biliary tract cancer is a rare malignancy in the Netherlands, with only

~800 diagnoses annually. Although none of the outcomes reached the level of significance, a clear tendency towards increased biliary tract cancer risk was observed for people with a history of *Salmonella* infection (standardized incidence ratios [SIRs] ranging from 1.22-1.88), whilst no such tendency was observed for *Campylobacter* (SIRs ranging from 0.75-1.24). The small sample size did not allow us to analyze subgroups, such as splitting by serovar/species or age at infection. Yet, the observed higher risk of cancer after *Salmonella* infection has been corroborated by others in a Taiwanese cohort, in which a HR of 1.78 was observed for biliary tract cancer after reported salmonellosis [53]. Likewise, traces of *S*. Typhi and *S*. Typhimurium were found in 9/26 and 10/26 samples respectively of tumor and adjacent normal tissue [54].

In parallel with the start of the research activities of this thesis, we noticed a substantial increase in literature addressing the association between microorganisms and cancers owing to rapidly evolving sequencing techniques. As a consequence, the need for a summary of the current epidemiological efforts and knowledge on this topic increased as well. We therefore conducted a literature review summarizing current epidemiological reports for the association between cancer in the gastrointestinal tract and bacterial or parasitic infections (**Chapter 7**). Viruses and the bacterium *Helicobacter pylori* were not included in this review, as these have already been addressed in many studies and reviews before. The majority of the 158 included studies and abstracts, covering 10 different bacteria and three parasites, focused on colon/colorectal cancer. A total of seven publications were found that studied the association between nontyphoidal *Salmonella* and gastrointestinal cancer, including the ones that are part of this thesis. A registry study based on data from the Taiwanese population found no evidence of an increased risk of colorectal cancer after *Salmonella* infection. In contrast, two other studies revealed significant higher antibody levels against *Salmonella* flagellin in Dutch colorectal cancer patients *versus* healthy controls, and a higher abundance of *Salmonella* in colon tissue adjacent to the tumor as compared to tissue from healthy controls in the USA [50, 54].



Only a few years ago, the scientific community focused mostly on microorganisms inducing the formation of a malignancy, whereas nowadays there is accumulating evidence recognizing a role for a larger group of (predominantly) bacteria that aid the progression of tumor growth. This has been described in a so-called driver-passenger model for colorectal cancer in which bacterial species that trigger the initiation of cancer are considered 'drivers', whereas opportunistic bacteria benefitting from the altered intestinal (tumor micro) environment and facilitating tumor development are considered 'passengers' [55]. The driver-passenger model/theory demands some flexibility though, as many bacteria have

potentially carcinogenic capacities (e.g. stimulation/inhibition of an inflammatory response, production of toxins or effector proteins) leading to manipulation of cell signaling pathways and ultimately the growth and proliferation of malignant cells, while they are also enriched in advanced/late stage tumors. According to the model, Enterobacteriaceae (the family to which *Salmonella* belongs) are considered driver bacteria given their abundance in off-tumor tissue as compared to tumor tissue and their perceived resemblance to enterotoxigenic *Bacteroides fragilis* in terms of prolonged inflammation [55]. While this hypothesis is supported by the results in this thesis, as well as outcomes of prior research showing that *Salmonella* is able to induce transformation in naïve non-malignant cells, *Salmonella* also shows passenger behavior by exploiting the metabolic niche arisen in the tumor microenvironment for its survival an proliferation.

## The oncogenic potential of nontyphoidal *Salmonella* is difficult to explain from both a genotypic and phenotypic perspective

In previous *in vivo* and *in vitro* research, different *Salmonella* strains were used to assess their capacity to induce tumorigenic changes [**Chapter 4**; 52; 56]. Attempts to infect mice with three different *S*. Typhimurium and three *S*. Enteritidis isolates originating from human clinical samples (obtained from the *Salmonella* laboratory surveillance system) resulted in over 50% mortality rates in the mice for two of the Typhimurium isolates and one Enteritidis isolate (**Chapter 4**, data not shown). Moreover, the estimated risk of colon cancer after infection with *S*. Enteritidis, *S*. Typhimurium and other serovars were not consistent among the epidemiological studies [**Chapter 5**; 5]. Hence the aim of **Chapter 8** was to unravel possible virulence factors and other mechanisms responsible for the oncogenic potential of *Salmonella* and to examine whether colon tumors from patients with a reported *Salmonella* infection are different in nature to tumors from patients without a reported infection. With regard to the tumor characteristics, no significant differences were present between the two groups in terms of tumor markers, although the percentage of poorly differentiated tumors was lower in patients with a past reported *Salmonella* infection. For the other analyses, we used a matched case-control design based on 60 *Salmonella* isolates derived from patients suffering from gastroenteritis and visiting a GP. Thirty of these patients had developed colon cancer ≥1 year after the *Salmonella* infection (i.e. the 'case isolates'), whereas the other 30 did not develop cancer (i.e. 'control isolates'). Assessing the *in vitro* infectivity and capacity to induce cellular transformation of MEFs for each of the 60 isolates individually, showed a tendency towards higher infectivity and transformation capacity for case isolates as

compared to control isolates. However, subjecting the individual strains to a gastrointestinal model system resembling the human intestinal tract did not reveal a significant difference in infectivity between case and control isolates. Similarly, no consistent differences were observed in the presence of bacterial genes between case and control isolates. Analysis of the *S*. Typhimurium and *S*. Enteritidis subset revealed some bacterial genes and single nucleotide polymorphisms (SNPs) to be associated with transformation capacity, although the biological relevance of these genes/SNPs in terms of *Salmonella*'s oncogenic potential were unclear. Investigating the isolates' ability to utilize a range of different nutrient sources yielded several differences between isolates with a high *versus* a low transformation capacity. It therefore seems that the tumorigenic potential of *Salmonella* is better explained by phenotypic/metabolic differences rather than genotypic differences. Of note, transcriptional research not only revealed a distinct expression of genes associated with cell cycle, cytokine signaling and immune signaling at 2.5 *versus* 8 hours post infection, but also significant differences in the activation of (metabolic) pathways between the serovars Typhimurium and Enteritidis [57]. Hence, RNA sequencing might comprise a useful tool to examine the transcriptional signature of isolates with a high *versus* low transformation capacity [58]. Part of the isolates with a lower transformation capacity revealed a reduced ability to utilize several amino acids and peptides, whereas a contrasting utilization pattern was observed for isolates with a higher transformation capacity. Likewise, the utilization of several phosphorus and sulfur sources was more consistently low for isolates with low transformation capacity as compared to isolates with high transformation capacity which showed mixed results. Of note, both the transformation assay in MEFs and the nutrient utilization arrays are based on simple models unlike the human gut. Whether the observed variation in transformation capacity and the corresponding patterns in nutrient utilization are still valid in the complex colonic environment and confer a biological advantage on these strains in terms of oncogenic potential, remains to be elucidated. Despite this need for further research deciphering the possible biological relevance of these outcomes, we could generate some hypotheses of underlying mechanisms that may contribute to them. For instance, the development of colonic adenomas and carcinomas is accompanied by significant changes in metabolic pathways to comply with the increasing energy demand of the growing polyp/tumor allowing tumor progression and metastasis. Such metabolic changes include higher demands for nitrogen, amino acids and glucose, and a shift to aerobic glycolysis (i.e. the Warburg effect) [59]. Regarding this point, the review in **Chapter 7** summarized a myriad of studies that found a distinct presence of bacteria in tumor and off-tumor tissues as compared to tissue from healthy controls. In our previous experiments, *Salmonella* showed a tropism for transformed cells. However, this implies that the bacterium has to adjust its nutrient uptake

to the altered metabolic profile of the (early) tumor/polyp microenvironment. As for many bacteria in the gut, glucose is the preferred source of energy for *Salmonella* when available [60]. Yet, *Salmonella* has a profound adaptive response, using host- and microbiota-derived metabolites (produced as an intermediate or by-product during inflammation) to assure its metabolism even under suboptimal conditions [61, 62]. Upon invasion of the host cell, most *Salmonella* bacteria reside in the *Salmonella*-containing vacuole (SCV) without direct access to host cytosolic metabolites/nutrients. Literature suggest that *Salmonella* recruits host cell transporters to obtain nutrients such as arginine and glutamine in the SCV [63, 64]. Since developing colorectal tumors are accompanied with an accumulation of L-Arginine, the observed increased L-arginine utilization by strains with a higher transformation capacity supports the hypothesis of *Salmonella* as a passenger bacteria [65]. *Salmonella* strains with a higher capacity to utilize a broad spectrum of nutrients, as observed in **Chapter 8**, therefore have an advantage of surviving and replicating in the altered microenvironment stimulating tumor progression, particularly when it also outcompetes other bacterial species lacking such metabolic capacities or when microbe-microbe interactions play a role [66].

Several strategies have been postulated by which *Salmonella* might induce colonic tumorigenesis. Briefly, these include the upregulation of Wnt signaling and suppression of JNK and NF-kB by the *Salmonella* AvrA protein and the activation of STAT3 and MAPK/Akt pathways by the *Salmonella* effector proteins leading to epithelial cell proliferation, suppression of host immune responses and cell transformation [67]. A possible complementing strategy might include hyperactivation of the serine-threonine kinase mammalian target of rapamycin (mTOR). mTOR is part of the upstream phosphatidylinositol-3-kinase (PI3K)/Akt pathway, which is involved in a myriad of cellular functions, including metabolism, growth and survival [68]. Aberrant Akt signaling is frequently associated with cancers and constitutes one of the causes of the downstream overactivation of mTOR. On the other hand, the mTOR gene itself is identified as proto-oncogene with strong tumorigenic capacities. A myriad of pathways leading to stimulation of mTOR have been linked to tumorigenesis and proliferation of cancer cells [69, 70]. Overactivation of mTOR has been associated with cellular transformation of MEFs (tested in a soft agar assay) and rapid development of tumors after subcutaneous administration in mice [69]. mTOR consists of two complexes, activation of mTOR complex 1 (mTORC1) is mainly regulated by nutrient availability (particularly amino acid levels, including glutamine, leucine and arginine), growth factors, oxygen levels and reactive oxygen species (ROS) [71]. Under normal conditions, active mTOR complex 1 (mTORC1) induces cell growth and proliferation by the phosphorylation of the downstream protein S6 kinase (S6K) and eukaryotic translation initiation factor 4E-binding protein (4EBP1). In case

of *Salmonella* infection, a transient damage of the SCV membrane shortly after infection (1-2h), induces a rapid decline in cytosolic amino acids (mainly L-leucine and L-isoleucine), a phenomenon also observed in reaction to infection by other intracellular bacteria [72]. This decline appears to be host-driven rather than the result of consumption of amino acids by *Salmonella*. The low levels of amino acids lead to autophagy due to mTORC1 inhibition (i.e. a catabolic response of cells to cope with stress conditions) [72]. However, *Salmonella* triggers a rapid normalization of the amino acid levels in the cytosol (3-4h post infection), as well as relocation of mTOR to the maturing SCV [72]. The mechanism behind the rapid restoration of amino acid levels during *Salmonella* infection as compared to other intracellular bacterial infections is not yet known. Nonetheless, by reactivating mTOR, *Salmonella* escapes from autophagy, thereby favoring its growth and replication within the host [72].

Another route of mTOR activation involves ROS production by mitochondrial activity. Upon infection, mitochondria produce higher levels of ROS as part of the innate host defense against pathogens [73]. Yet, the amount of mitochondrial ROS (mtROS) produced was shown to be dependent on the *Salmonella* serovar [57]. Infection of human intestinal organoids (HOI) with *S*. Enteritidis revealed upregulated gene expression of mitochondrial related processes (including mitochondrial translation, protein import and oxidative phosphorylation) as compared to infection by *S*. Typhimurium. Consistently, the increase in mtROS production between 1 and 24 hours post infection was significantly higher for Enteritidis compared to Typhimurium [57]. Remarkably, enhanced mitochondrial activity has been observed in transformed predisposed MEFs (i.e. harboring $Arf^{-/-}$ and c-MYC mutations) with a history of *S*. Typhimurium infection as compared to predisposed MEFs without a prior *Salmonella* infection. This mitochondrial activity appeared crucial for maintaining the transformed state of the MEFs as inhibition of mitochondrial activity led to abolishment of the *Salmonella*-induced transformation [D.M. van Elsland, personal communication, December 5$^{th}$, 2019]. These results suggest that the induction of enhanced mitochondrial activity (and the subsequent production of mtROS) is not restricted to *S*. Enteritidis and might vary within serovars. How *Salmonella* reprograms the mitochondria towards sustained activity even when bacterial infection is cleared, remains to be elucidated. Mitochondrial dysfunction has been associated with a multitude of human pathologies, including cancers [74]. Aberrations in mitochondrial metabolism play a role in the oncogenesis cascade from malignant transformation to tumor progression [75]. The overactivated mitochondria continuously produce ROS. As a consequence, the activity of the tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome 10) is restrained due to oxidation by ROS [76], which subsequently leads to activation of the Akt pathway and the downstream mTOR

pathway [68]. Remarkably, the effect of ROS on mTORC1 is dose-dependent, as low doses are associated with mTORC1 activation whereas high-dose or long-term ROS exposure leads to decreased mTORC1 activity (as a result of AMPK-induced phosphorylation of Raptor) [77]. Whether mTOR is one of the missing links in the causality of *Salmonella*-induced cancer progression, warrants further study but it is found activated in human gallbladder carcinoma samples from Indian patients that have been chronically infected by *S*. Typhi.

## Association between bacteria and gastrointestinal cancer – lessons learned and a way forward

This thesis aimed to contribute to the existing knowledge about the association between *Salmonella* and colon cancer. Overall, the outcomes show a mixed picture. On the one hand, the *in vitro* and *in vivo* experiments showed results clearly supporting an association between *Salmonella* and the development of colon cancer. On the other hand the epidemiological outcomes were less consistent and challenging to interpret. Amongst the possible reasons are the lack of data concerning other possible risk factors, effect modifiers and confounders that warrant attention in the analyses, as well as the inherent risk of (left and right) truncation of observations in the relative short study periods. Moreover, since people are estimated to acquire, on average, a *Salmonella* infection every ~7 years (in the Netherlands), a truly unexposed population does not exist [16]. This implies that epidemiological studies using indicators on a more continuous scale, such as abundance of bacterial DNA in tumor *versus* off-tumor tissue or concentrations of anti-*Salmonella* antibodies, rather than the dichotomous (and sometimes indirect) design of our registry studies, might be recommended approaches for future research, particularly when combined with a multiyear follow-up period. Ideally, one would aim for the implementation of a large-scale prospective cohort study with a long-term follow-up, such as in the LifeLines cohort in the northern part of the Netherlands to assess phenotypic, environmental and genomic parameters related to development of chronic diseases and healthy aging by following people for ≥30 years [78]. The design of such cohort study, including repeated follow-up questionnaires and collection of biomaterials (e.g., blood, feces), as well as linkage to medical/environmental registries, allows for the assessment of the risk of cancer(s) in relation to diet, physical activity, body weight, occupational and environmental exposures, medication use, and (fluctuating) microbiome compositions. Although this might provide better insights into the correlations between risk factors and their relation with microbiome, such study would be costly, time consuming and is highly dependent on the perseverance of the participants. Alternatively, future research can aim at using existing data sources and the application of

machine learning techniques to reveal patterns/associations in the tremendous amount of data generated from high-throughput sequencing methods worldwide.

Overall, the epidemiological outcomes of this thesis seem to indicate the contribution of *Salmonella* infection to the burden of colon cancer in the Netherlands (North West Europe) as negligible, yielding no urgent implications for colon cancer screening policies or *Salmonella* control programs. However, the increased incidence of colon cancer diagnosis within one year after *Salmonella* infection, as well as the elevated rates of proliferation in tumors compared to adjacent normal tissue, and the propensity of *Salmonella* to infect cells with the highest level of transformation all confirm that *Salmonella* has a strong tropism for infecting transformed/malignant cells. This implies that, regardless of the magnitude of *Salmonella* infection as driver for cancer induction at the population level, the bacterium might substantially enhance or accelerate tumor growth with the inherent risk of cancer being diagnosed in a late stage. Therefore, *Salmonella* could be added to the list of microorganisms, including *F. nucleatum* and *Clostridium hathewayi* amongst others, warranting further study into their potential as biomarker for (early) colon cancer diagnosis [79]. Owing to the chemotaxis of *Salmonella* towards tumors, attenuated avirulent *Salmonella* strains are a topic of interest in several animal models and clinical trials to assess their applicability in bacterial-mediated cancer therapy [9, 80]. The mechanisms by which *Salmonella* exerts the anti-tumor effects remain elusive [9]. Yet, despite the contrasting roles of *Salmonella* as oncogenic *versus* therapeutic agent, we can continuously learn from the gained knowledge in the therapeutic discipline, particularly concerning the mechanisms by which *Salmonella* exerts its effects on tumor cells and the interaction with the immune system [9].



The pathogenesis of colon cancer is driven by a tripartite relationship between the microbiome, the mucosal immune balance in the colon and the colonic epithelial cells [81]. However, the vast majority of research on the association between microorganisms and cancer focused on the role of a single microorganism in cancer development or a snapshot of the distinct abundances of bacterial phyla/families in relation to cancer stage. Presence and magnitude of microbe-microbe interactions with either counteracting or amplifying effects on cancer development have hardly been studied. Several studies observed cooperative or antagonistic behaviors between bacterial species or between gut bacteria and the gut virome, such as protection of *S*. Typhimurium against β-lactam antibiotics by *Staphylococcus aureus*, *Bacteroides* species and resistant *E. coli* [82-84], and the inflammation-induced SopEΦ bacteriophage transfer to *S*. Typhimurium, which fosters disease progression [85-87]. Likewise, influenza virus infection has been associated with enhanced *Salmonella* colonization in the gut through the action of type I interferons and an

altered gut microbiome composition [88], highlighting the interplay between microbiome compartments and the immune response. Despite the obvious complexity, future research could aim at deciphering polymicrobial interactions between microbes (in the gut) that potentially exert synergistic effects on tumor formation.

As for many intestinal bacteria, research on the association between *Salmonella* and colon cancer is still in its infancy, and the studies in this thesis were rather explorative in nature. The absence of obvious genetic or metabolic markers or dose-dependent effects explaining the tumorigenic potential of *Salmonella*, suggests a more subtle contribution of *Salmonella* when certain conditions or prerequisites are met. Next steps could involve experimental research focusing on these possible conditions, such as the comparison of transcriptional profiles between serovars/strains and the effects of gene silencing on cell transformation potential. In this thesis, the experimental *in vivo* and *in vitro* research was performed in mice, MEFs and Caco-2 cells. Innovations such as intestines-on-a-chip and cell models with a higher resemblance to the human colon such as HOI might offer an opportunity to capture more realistically the putative tumorigenic role of *Salmonella* in the complex intestinal environment [89]. Moreover, the multitude of bacteria being associated with gastrointestinal cancer initiation and progression suggests a plethora of pathways involved that remain to be unraveled in the coming decades.

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