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## THE ROLE OF STREPTOCOCCUS GALLOLYTICUS SUBSPECIES GALLOLYTICUS IN COLON CANCER DEVELOPMENT

Jennifer L. Herold

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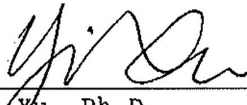
THE ROLE OF *STREPTOCOCCUS GALLOLYTICUS* SUBSPECIES

*GALLOLYTICUS* IN COLON CANCER

DEVELOPMENT

by

Jennifer Lynn Herold



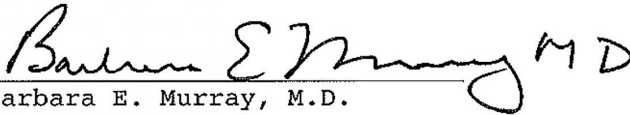
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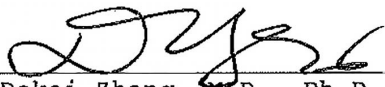
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THE ROLE OF *STREPTOCOCCUS GALLOLYTICUS* SUBSPECIES

*GALLOLYTICUS* IN COLON CANCER

DEVELOPMENT

A

DISSERTATION

Presented to the Faculty of  
The University of Texas  
Health Science Center at Houston  
and  
The University of Texas  
MD Anderson Cancer Center  
Graduate School of Biomedical Sciences  
in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

Jennifer Lynn Herold  
Houston, Texas

December 2016

## **DEDICATION**

To my father, Howell Smith, who is no longer physically here, but will live forever in my heart and soul. You were my motivation, my unending source of support, and my hero.

To my mother, Polly Smith, and husband, Chris Herold. I would not be where I am today without your love, patience, and hope in the future.

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Last, but not least, I would like to thank all of my family and friends who have been there for me through the ups and downs of obtaining a Ph.D. My husband and my

parents have always been my biggest supporters and I would not be where I am today without them.

## **ABSTRACT**

### **THE ROLE OF *STREPTOCOCCUS GALLOLYTICUS* SUBSPECIES *GALLOLYTICUS* IN COLON CANCER DEVELOPMENT**

**Jennifer Lynn Herold**

**Supervisory Professor: Yi Xu, Ph.D.**

Colorectal cancer (CRC) is the third most common cancer in men and women and is also the third most common cause of cancer death. A large body of evidence points towards the possibility that bacteria can have a significant impact on the development of cancer. It has been suggested that *Streptococcus gallolyticus* subsp. *gallolyticus*, a group D streptococci, may play a role in the development of CRC. *Sg*, formerly referred to as *S. bovis* biotype I, has been shown to be highly associated with CRC. In observing patients with either *Sg* bacteremia or endocarditis it was found that 25-80% of patients with *Sg* bacteremia had tumors and 18-62% of patients with *Sg* endocarditis had colonic neoplasias. However, other closely related Streptococcal strains, such as *S. pastorianus* and *S. infantarius*, have not been shown to have this strong association with CRC. In fact, it has been shown that biotype I is more often associated with CRC (94%) as compared to biotype II (18%). This knowledge has important

clinical implications, and yet little is known about the role of *Sg* on CRC and the underlying mechanisms. Here we show that mice treated with *Sg* had significantly more tumors, higher tumor burden and dysplasia grade, and increased cell proliferation and  $\beta$ -catenin level in colonic crypts compared to mice treated with control bacteria. *Sg* strains that promoted proliferation were also more efficient at adhering to CRC cell lines and colonizing a mouse model. Additionally, in human patients *Sg* was highly prevalent in CRC patients and tumor tissues had an increased *Sg* burden in comparison to normal adjacent tissues. These results provide exciting new information and establish a tumor-promoting role of *Sg* that involves specific bacterial and host factors.

## Table of Contents

APPROVAL SHEET.....	i
TITLE PAGE.....	ii
DEDICATION.....	iii
ACKNOWLEDGEMENTS.....	iv
ABSTRACT.....	vi
TABLE OF CONTENTS.....	viii
LIST OF FIGURES.....	xii
LIST OF TABLES.....	xv
ABBREVIATIONS.....	xvi
<b>I: Introduction.....</b>	<b>18</b>
<i>Streptococcus gallolyticus</i> subsp. <i>gallolyticus</i> .....	18
Changes in nomenclature .....	18
The development of CRC .....	19
Microbes and cancer .....	21
<i>Sg</i> bacteremia/endocarditis association with CRC .....	23
Cause or consequence .....	25
<i>Sg</i> genomes .....	26
The pili operon and gut colonization .....	27
Binding to extracellular matrix components .....	29
Other factors that may be involved in the colonization of the gut .....	30
<b>II: <i>Sg</i> promotes colorectal tumor development.....</b>	<b>33</b>

Introduction .....	33
Experimental Procedures .....	49
Results .....	54
-Sg adheres to both responsive and unresponsive cells .....	54
-Sg promotes tumor growth in a xenograft model .....	58
-Sg promotes colon tumor development in an AOM-induced mouse model of CRC .....	58
-Sg promotes colonic crypt cell proliferation in vivo .....	64
-Sg does not induce strong inflammatory responses .....	64
-Sg abundance in the colon correlates with tumor multiplicity and tumor burden .....	68
-Sg abundance in tumor vs. normal tissues .....	72
-Immunofluorescence detection of <i>Sg</i> within tumor tissues in the mouse colon .....	72
Discussion .....	76
<b>III: Prevalence of <i>Sg</i> in human patient samples....</b>	<b>83</b>
Introduction .....	83
Experimental Procedures .....	85
Results .....	88
-Establishing a protocol for specific detection	



of <i>Sg</i> in colon tissues .....	88
-Detection of <i>Sp</i> in colon tumor and normal tissues from CRC patients .....	88
- <i>Sg</i> is present in the majority of CRC patients and preferentially associates with tumor tissues .....	91
Discussion .....	91
<b>IV: <i>Sg</i> adherence correlates with cell</b>	
<b>proliferation.....</b>	<b>95</b>
Introduction .....	95
Experimental Procedures .....	96
Results .....	99
-The ability of <i>Sg</i> to adhere to cell lines correlates with its ability to promote cell proliferation .....	99
-Involvement of $\beta 1$ integrin in mediating <i>Sg</i> adherence to colon cancer cells .....	99
-TX20005 is more efficient at colonizing mice colons than TX20008 .....	101
- <i>Sg</i> TX20008 does not promote tumor growth in a xenograft model .....	104
-Investigation of the potential of TX20008 to promote tumor growth in the AOM-model .....	104
Discussion .....	106

<b>V: Discussion.....</b>	<b>111</b>
<i>Sg</i> promotes tumor development .....	111
Prevalence of <i>Sg</i> in CRC patients .....	117
Polymorphism in <i>Sg</i> strains.....	118
Implications to CRC prevention, diagnosis, and treatment .....	120
Conclusion and future directions .....	122
References.....	124
Vita.....	149

## List of figures

<i>Number</i>	<i>Page</i>
1. <i>Sg</i> stimulates cell proliferation in responsive colon cancer cell lines .....	35
2. <i>Sg</i> promotes cell proliferation, but does not affect apoptosis .....	37
3. Detection of cleaved caspase 3 in cells treated with <i>Sg</i> .....	39
4. Promotion of cell proliferation requires <i>Sg</i> -specific factors and depends on bacterial growth phase and direct contact with CRC cells .....	40
5. <i>Sg</i> culture supernatant had no effect on cell proliferation .....	42
6. <i>Sg</i> increases the level of $\beta$ -catenin and c-Myc in HCT116 and HT29 cells .....	44
7. $\beta$ -catenin knockdown .....	46
8. <i>Sg</i> promotes cell proliferation in a $\beta$ -catenin dependent manner .....	47
9. Adherence of <i>Sg</i> to different cell lines .....	56
10. Adherence of <i>Sg</i> and closely related strains to HCT116 cells .....	57
11. <i>Sg</i> treatment promotes tumor growth in a xenograft model .....	59

12.Representative mouse colons .....	60
13. <i>Sg</i> promotes colon tumor development in an AOM-induced mouse model of CRC .....	61
14. <i>Sg</i> promotes colon tumor development in an AOM-induced mouse model of CRC .....	63
15. <i>Sg</i> promotes colon tumor development in an AOM-induced mouse model of CRC .....	65
16.TUNEL assay of colon sections from mice treated with AOM and bacteria .....	66
17. <i>Sg</i> increases $\beta$ -catenin levels, but does not promote inflammation in a mouse model .....	67
18. <i>Sg</i> does not induce strong inflammatory responses .....	69
19. <i>Sg</i> does not induce strong inflammatory responses .....	70
20.Correlation of bacterial burden with tumor number and burden .....	71
21. <i>Sg</i> abundance in tumor vs. normal tissues ....	73
22.Specificity of rabbit 1 anti- <i>Sg</i> serum .....	74
23.Specificity of rabbit 2 anti- <i>Sg</i> serum .....	75
24.Establishing a protocol for specific detection of <i>Sg</i> in colon tissues .....	90
25.Detection of <i>Sp</i> in colon tumor and normal tissues from CRC patients .....	92

26.	<i>Sg</i> is present in the majority of CRC patients and preferentially associates with tumor tissues .....	93
27.	<i>Sg</i> 's ability to adhere to cell lines correlates with its ability to promote cell proliferation .....	100
28.	Involvement of $\beta$ 1 integrin in mediating <i>Sg</i> adherence to colon cancer cells .....	102
29.	Colonization of mice by <i>Sg</i> .....	103
30.	TX20005 promotes tumor growth in a xenograft model .....	105
31.	Presence of Pil3 locus in TX20005 and TX20008 .....	109

## **List of tables**

<i>Number</i>	<i>Page</i>
1. Nomenclature changes .....	20
2. Patient Characteristics .....	87
3. Colon cancer survival rate, by stage .....	121

## ABBREVIATIONS

AOM	Azoxymethane
ACF	Aberrant crypt foci
APC	Adenomatous polyposis coli
BrdU	Bromodeoxyuridine
CFU	Colony forming units
CRC	Colorectal cancer
CT	Cycle threshold
ECM	Extracellular matrix
H&E	Hematoxylin and eosin
HlpA	Histone-like protein A
IBD	Inflammatory Bowel Disease
IHC	Immunohistochemistry
ISH	<i>in situ</i> hybridization
qPCR	Quantitative polymerase chain reaction
SBSEC	<i>Streptococcus bovis</i> / <i>Streptococcus equinus</i> complex

<i>Sg</i>	<i>Streptococcus gallolyticus</i> subsp. <i>gallolyticus</i>
<i>Sm</i>	<i>Streptococcus gallolyticus</i> subsp. <i>macedonicus</i>
<i>Sp</i>	<i>Streptococcus gallolyticus</i> subsp. <i>pasteurianus</i>
<i>Si</i>	<i>Streptococcus infantarius</i> subsp. <i>infantarius</i>
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling



## Chapter 1:

### INTRODUCTION

#### ***Streptococcus gallolyticus* subsp. *gallolyticus*.**

*Streptococcus gallolyticus* subsp. *gallolyticus* (Sg) belongs to the *Streptococcus bovis*/*Streptococcus equinus* complex (SBSEC) and was previously known as *S. bovis* biotype I [1, 2]. This organism is a Gram-positive, opportunistic pathogen that causes bacteremia and endocarditis in humans. It has also been shown to strongly associate with colorectal cancer (CRC), however the role Sg plays in the development is unclear [3-19].

**Changes in nomenclature.** *S. bovis* belongs to the group D streptococci. Since the 1970's *S. bovis* has undergone several changes in nomenclature, beginning with the reclassification of *S. bovis* into biotypes, based on bacteriological plating assays. Biotype I was classified by its ability to ferment mannitol, biotype II/1 was mannitol negative and  $\beta$ -glucuronidase negative, and biotype II/2 was mannitol negative and  $\beta$ -glucuronidase positive [20]. In the early 2000s, molecular techniques consisting of DNA homology, whole-cell protein extracts, and *sodA* gene sequencing were developed that led to a further reclassification of *S. bovis*. *S. bovis* biotype I is now *S. gallolyticus* subsp. *gallolyticus*, *S. bovis*

biotype II/1 is now *S. infantarius* subsp. *infantarius* or subsp. *coli*, and *S. bovis* biotype II/2 is now *S. gallolyticus* subsp. *pasteurianus* (Table 1)[1, 2]. Unfortunately, these changes have not been fully embraced in the literature and have led to some discrepancies in *S. bovis* identification.

**The development of colorectal cancer (CRC).** CRC is the second to third most common cancer in the world and a leading cause of cancer-related death [21, 22]. Approximately 134,490 new CRC cases and 49,190 deaths are estimated to occur in the United States in 2016 [23]. Worldwide, ~ 1.4 million CRC cases were diagnosed and ~ 694,000 deaths occurred in 2012 [24]. The classical model for the development of CRC is a multi-stage, multi-factor process, involving the accumulation of a series of mutations over ~20-40 years as illustrated in the well-known “Vogelgram” [25, 26]. The first step involves mutations in the adenomatous polyposis coli (*apc*) gene, which leads to aberrant activation of  $\beta$ -catenin and the development of early adenoma. Mutations in Ras and p53 drive the progression from early to late adenoma, and from late adenoma to carcinoma, respectively. Each of these stages is also accompanied by a number of other genetic and epigenetic alterations including loss of heterozygosity and changes in DNA methylation. Studies performed in the last decade or so have added new components to the picture, and highlighted the contribution of the tumor

**Table 1:**

<b>Original Nomenclature</b>	<b>Later Nomenclature</b>	<b>Current Nomenclature</b>
<i>S. bovis</i> biotype I	<i>S. gallolyticus</i>	<i>S. gallolyticus</i> subsp. <i>gallolyticus</i>
<i>S. bovis</i> biotype II/1	<i>S. infantarius</i>	<i>S. infantarius</i> subsp. <i>infantarius</i>
	<i>S. infantarius</i> subsp. <i>coli</i>	<i>S. lutetiensis</i>
<i>S. bovis</i> biotype II/2	<i>S. pasteurianus</i> <i>S. macedonicus</i>	<i>S. gallolyticus</i> subsp. <i>pasteurianus</i> <i>S. gallolyticus</i> subsp. <i>macedonicus</i>

The milestone of the taxonomy of *S. bovis/gallolyticus* and the closely related members of Group D Streptococci.

microenvironment. These new components include inflammatory responses, abnormal metabolic activities [27] and microbes [4, 28, 29]. Inflammatory responses produce bioactive molecules that can affect several hallmark capabilities [27]. For example, inflammatory mediators can supply growth, survival, and proangiogenic factors, ECM-modifying enzymes, and invasion and metastasis signals to the tumor microenvironment. Alterations in metabolism lead to reprogramming of metabolic activities that allow for sustained proliferation and tumor growth. Both of these components can be modulated by the presence of microbes.

**Microbes and cancer.** It is estimated that ~15-20% of cancers are linked to infectious agents [30]. This percentage is generally higher in developing countries and lower in developed countries, such as the United States. The link between microbes and cancer was first established in viruses. One example is human papilloma virus (HPV), which causes nearly all cervical cancers. HPV integrates into the host chromosome and interferes with cell cycle control and apoptosis through the overexpression of HPV oncoproteins E6 and E7 [31]. *Helicobacter pylori* was the first bacterial pathogen linked to cancer. Colonization of *H. pylori* in the gastrointestinal tract significantly increases the risk for gastric cancer and elimination of it from the gut reduces the

risk [6, 32]. *H. pylori* contributes to cancer development through many mechanisms, but most notably by increasing inflammation, producing DNA-damaging toxins, and altering  $\beta$ -catenin signaling [33-37].

The colon is frequently exposed to  $10^{14}$  microorganisms. This collection of microbes – the microbiome – has increasingly been recognized as an active participant in shaping the development of immune responses, modulating metabolic activities and nutrient acquisition, and thus contributing to the health and disease status of the gut [3]. A number of bacterial species have been linked to CRC through either epidemiological studies and/or analyses of the gut microbiota and have led to a newly coined term– oncomicrobes [28]. They include – Enterotoxigenic *Bacteroides fragilis*, *Fusobacterium nucleatum*, polyketide synthase positive (*pks+*) *Escherichia coli*, *Enterococcus faecalis*, etc. The mechanisms utilized by these bacteria to promote tumorigenesis are diverse, including producing toxins that damage DNA [38-44], aberrant activation of  $\beta$ -catenin signaling [45-51], or triggering inflammatory responses that favor tumor growth [28, 45, 52-58]. *Enterococcus faecalis* promotes tumorigenesis through a bystander effect by activating macrophages, which in turn produce DNA damaging clastogens [59].

The recognition that microbial agents are intimately involved in the health and disease status of the gut [3] and that specific microbes can drive colon tumorigenesis [41, 48, 50, 60-62] further raise hope that we may be able to exploit knowledge about specific tumor-promoting microbes to improve cancer diagnosis and treatment by incorporating microbes into clinical strategies [4-7, 28, 63]. For example, specific microbial antigens may be useful as a diagnostic biomarker for CRC. The presence of specific tumor-promoting microbes in the colon may require optimized therapeutic regimens that take the microbes into consideration.

***S. gallolyticus* subsp. *gallolyticus* bacteremia/endocarditis association with CRC.** Although the association of *S. bovis* infections with CRC was first reported in 1951, this association was not fully recognized until 1974 when reported by Keusch *et al.*[64]. Numerous case reports and case series have documented elevated risks for CRC among patients infected with *S. bovis* or *Sg.* In a more recent meta-analysis by Boleij *et al.*, 52 case reports and 31 case series published in PubMed from 1970 to 2010 were reviewed [10]. The analysis found a median prevalence of CRC of approximately 39% among patients with *S. bovis* infections. However, not all patients in these cases underwent colonoscopies, which may have resulted in undetected lesions or small polyps and a subsequent

underestimation of the actual prevalence of CRC within the patient population. This possible underestimation of patients with CRC appears to be supported by further analysis investigating patients who were both infected with *S. bovis* and also underwent colonoscopy. In these cases, the prevalence of CRC among these patients increased to 60% [10]. Additionally, *S. bovis* bacteremia has been shown to be associated with other malignancies, such as tumor lesions in the duodenum, gallbladder, pancreas, ovary, uterus, lung, and hematopoietic system [14].

Boleij *et al.* also evaluated studies that distinguished *Sg* (*S. bovis* biotype I) from other biotypes within the *S. bovis* group [10]. They found that *Sg* bacteremia has a 71% association with CRC, while the association between *S. bovis* biotype II bacteremia with CRC is only 17%. Patients with *S. bovis* biotype I infections were more likely to have CRC (33–71% prevalence) in comparison to the normal population (10–25%). Additionally, a recent prospective study on 203 colonoscopy patients found a clear relationship between patients positive for *S. bovis* in the colonic suction fluid and presence of malignant tumors and large polyps in the colon [65]. Specifically, all 17 malignant tumors diagnosed in this cohort were *S. bovis* positive. Further, there is also evidence that a substantial proportion of CRC patients are “silently”

infected with *Sg*. For example, Abdulamir *et al.* studied 52 CRC patients without symptoms of bacteremia and found that approximately 33% of tumors and 23% of matched normal colon tissues to be *Sg*-positive when a conventional PCR method was used for detection of *Sg* [66].

**Cause or consequence.** Although the association between *Sg* and CRC has long been recognized, there is not much known concerning the role *Sg* plays in the development of CRC. Some studies have suggested that *Sg* is merely a consequence of preferential colonization of the tumor environment [67], while others have suggested that *Sg* plays an etiological role in tumorigenesis [8, 66, 68]. However, some epidemiological studies provide hints at a possible active role of *Sg* in CRC development. First, the strong association of patients with *S. bovis* biotype I bacteremia/endocarditis with CRC is striking and much higher than that observed with other *S. bovis* biotypes [66]. It was also found that patients with *S. bovis* biotype I, in the absence of bacteremia/endocarditis, have a higher incidence of CRC and tumor tissues from these patients were more readily colonized [10, 66]. Additionally, patients with bacteremia/endocarditis due to *S. bovis* developed significantly more colonic neoplastic lesions in subsequent years (2 to 4 years) compared to patients with bacteremia/endocarditis due to enterococci. This suggests a



role of *Sg* in early stages of tumor development [69, 70]. Overall, despite the strong epidemiological evidence for an association between *Sg* and CRC, the role *Sg* plays in the development of CRC is unclear.

***Sg* genomes.** In general, *Sg* strains possess a single, circular chromosome of approximately 2.3 Mbps. Eleven strains have been sequenced to date with the majority of analytical data available for strains BAA-2069, UCN34, TX20005, and ATCC 43143 [71-73]. Genomic analyses have shown a similar gene arrangement between the genomes of BAA-2069 and UCN34 and an 87% commonality in open reading frames (ORFs). An extrachromosomal plasmid has been identified in BAA-2069, but not UCN34, TX20005, or ATCC 43143. This 20,765 base pair (bp) plasmid, pSGG1, confers tetracycline resistance and may also be important for conjugation.

*Sg* strains demonstrate other notable features, including structural genes that encode capsules and bacterial pili. These particular structures are important for bacterial persistence within the host under certain conditions. For example, a 12-gene operon that encodes the extracellular capsule results in increased bacterial resistance to host innate immunity and mechanisms of bacterial clearing by complement and phagocytosis [74, 75].

Further, *Sg* strain UCN34 also possesses three pilus operons. These pili have been shown to function as adhesins and bind collagens and mucins. Homologous sequences to these pili genes are also seen in TX20005 and ATCC 43143. Numerous studies demonstrate bacterial attachment to host tissues by pili as a first step in pathogenesis. Additionally, there are 29 predicted LPXTG motif proteins in ATCC 43143, 21 in BAA-2069, and 18 in UCN34. The predicted function of many of these is to aid in bacterial adherence.

In addition to the genes that encode capsules or pili, *Sg* strains often express genes that result in the production of unique enzymes facilitating survival within the intestinal tract of the host. For example, *Sg* produces a tannase enzyme, which has the ability to degrade tannins (plant materials toxic to many bacteria) and to hydrolyze bile salts (conferring resistance to detergents). In addition to these enzymatic activities, *Sg* is predicted to encode 25 efflux proteins, critical in bacterial detoxification. All of these properties allow for survival of *Sg* in the gastrointestinal tract [71, 73]. Currently, there are no known *Sg* secreted toxins.

**The pili operon and gut colonization.** Pili are filamentous structures that play an important role in bacterial adhesion in many pathogens. Genome sequencing of *Sg*

UCN34 found the presence of three pilus loci, *pil1*, *pil2*, and *pil3* [73]. Earlier work by Danne, *et al.* showed that Pil1 mediated *Sg* adherence to collagen in a rat model of experimental endocarditis [76]. More recently, Pil3 has been identified as an important mediator in colon colonization with specific regard to mucus attachment. The *pil3* locus consists of genes encoding 2 structural pilin subunits (*gallo\_2040* and *gallo\_2039*), a sortase C enzyme, a type 1 signal peptidase, and a small open reading frame of unknown function [77]. The two structural pilin subunits, Pil3A and Pil3B, are the adhesin and major pilin, respectively. Initial bioinformatics analysis of Pil3A was of particular interest in this study due to putative mucus-binding domains. Pil3 expression is regulated through a phase variation mechanism in which additions or deletions of an upstream GCAGA repeat results in transcriptional read through or termination [78]. A longer upstream repeat allows for transcription of the *pil3* locus and subsequent Pil3 expression. This mechanism of Pil3 regulation results in heterogeneous expression of Pil3 on bacterial cells. Since Pil3 is heterogeneously expressed on UCN34 cells this study used a Pil3 overexpression strain and a  $\Delta pil3$  mutant to demonstrate the important role of Pil3 in its association with colon mucus in *Sg* colonic cell attachment. Currently, it is unclear if all *Sg* strains express Pil3, and

more specifically, pilin surface expression levels are unknown.

*Sg* pili have also been shown to be common antigens expressed in vivo, which could aid in the early detection of CRC. Boleji *et al.* identified 4 antibodies to pili that could serve as an assay for the detection of *Sg* in patients, with a sensitivity of 16-43% [79]. Butt *et al.* found Pil3B and Pil1 to be most significantly associated with an increased risk of CRC [40]. Furthermore, when antibodies to these two pili proteins were detected simultaneously this strengthened the association. Gonzalez *et al.* identified 22kd and 30kd proteins that have a strong association with CRC, but the identity of these proteins has not been revealed [80]. It would be interesting to know if these are pilus proteins as well.

**Binding to extracellular matrix components.** One of the first steps in bacterial pathogenesis is microbial attachment to host tissues. This attachment is often facilitated by a bacterial factor, such as a surface protein, that binds to specific host molecules or receptors on host tissues. *Sg* is known to colonize both the intestinal tract and heart valves. A study by Sillanpaa *et al.* evaluated 17 *Sg* clinical isolates from patients with endocarditis and their ability to bind to extracellular matrix (ECM) proteins [81]. Of these isolates, 76% adhered to collagen type I, followed by 53% that adhered

to collagen type IV, 47% adhered to fibrinogen, and 35% adhered to collagen type V and fibronectin. There were three isolates that adhered to all ECM proteins investigated and three isolates that did not adhere to any ECM proteins. The *Sg* strains in this study were also analyzed by pulse-field electrophoresis and over 50% of the isolates were genetically diverse. Due to the diversity among strains and differences in *Sg* adherence, this suggests different *Sg* isolates express different surface proteins to colonize patients. This genetic diversity and the differences in *Sg* strain adherence to the various ECM components suggest that individual *Sg* isolates express different surface proteins with which the bacteria bind to host tissues. These differences in adherence also suggest that heterogeneous expression of bacterial surface adhesion factors may account for the disparate ECM binding results and that surface adhesin expression may be a highly regulated event. Vollmer *et al.* reported adherence of *Sg* to endothelial cells [82]. They also found that *Sg* adhered highly to collagen I, II, and IV, followed by fibrinogen, tenascin, and laminin. Interestingly, only 9 of the 23 isolates evaluated possessed Pil3, again suggesting other factors are involved in *Sg* adherence and colonization.

**Other factors that may be involved in the colonization of the gut.** Several bacterial factors have been shown to mediate

*Sg* adherence. For example, Boleij *et al.* reported the role of histone-like protein A (HlpA) in mediating adherence to HCT116 and HT29 colon cancer cell lines [83]. Another class of proteins called MSCRAMMS (Microbial Surface Components Recognizing Adhesive Matrix Molecules) are also known to promote bacterial adherence of Gram-positive bacteria to host tissues [84]. MSCRAMMS are proteins attached to the bacterial cell wall by the enzyme, sortase, which recognizes an LPXTG anchoring motif within the target MSCRAMM to mediate covalent attachment and bacterial surface expression of the adhesion. When Sillanpaa *et al.* analyzed *Sg* TX20005, they identified 11 putative proteins with LPXTG motifs. Additional bioinformatics analysis predicted that these 11 proteins possessed binding domains to ECM components, such as collagen. Assays using recombinant protein of these binding domains demonstrated adherence to the ECM component, collagen. These data provide further evidence that pili are not the only factors on the surface of *Sg* strains mediating bacterial adherence [85].

Not only is adherence important in *Sg* colonization, but the ability of *Sg* to grow in the colonic tumor environment. Boleij *et al.* also investigated the ability of *Sg* to grow in spent media from malignant colonocytes, which mimics the colonic tumor microenvironment [86]. *Sg* had a growth advantage

in comparison to other bacteria, suggesting the tumor environment provides a specialized niche for *Sg* growth.

## **Chapter 2: *Sg* promotes colorectal tumor development.**

### **INTRODUCTION**

*Sg* belongs to the SBSEC group, which also includes a number of closely related species such as *S. pasteurianus* (*Sp*), *S. macedonicus* (*Sm*), and *S. infantarius* (*Sp*) [1, 20]. Among the different species within SBSEC, *Sg* infection has the strongest association with CRC (~ 7 fold higher risk compared to infections caused by the other species), suggesting the existence of a *Sg*-specific mechanism(s) that promotes the association between the pathogen and CRC. Despite the well-documented strong association between *Sg* and CRC, the role of *Sg* in CRC development, *i.e.* whether it drives colon tumorigenesis or merely colonizes the colon tumor environment, was unknown.

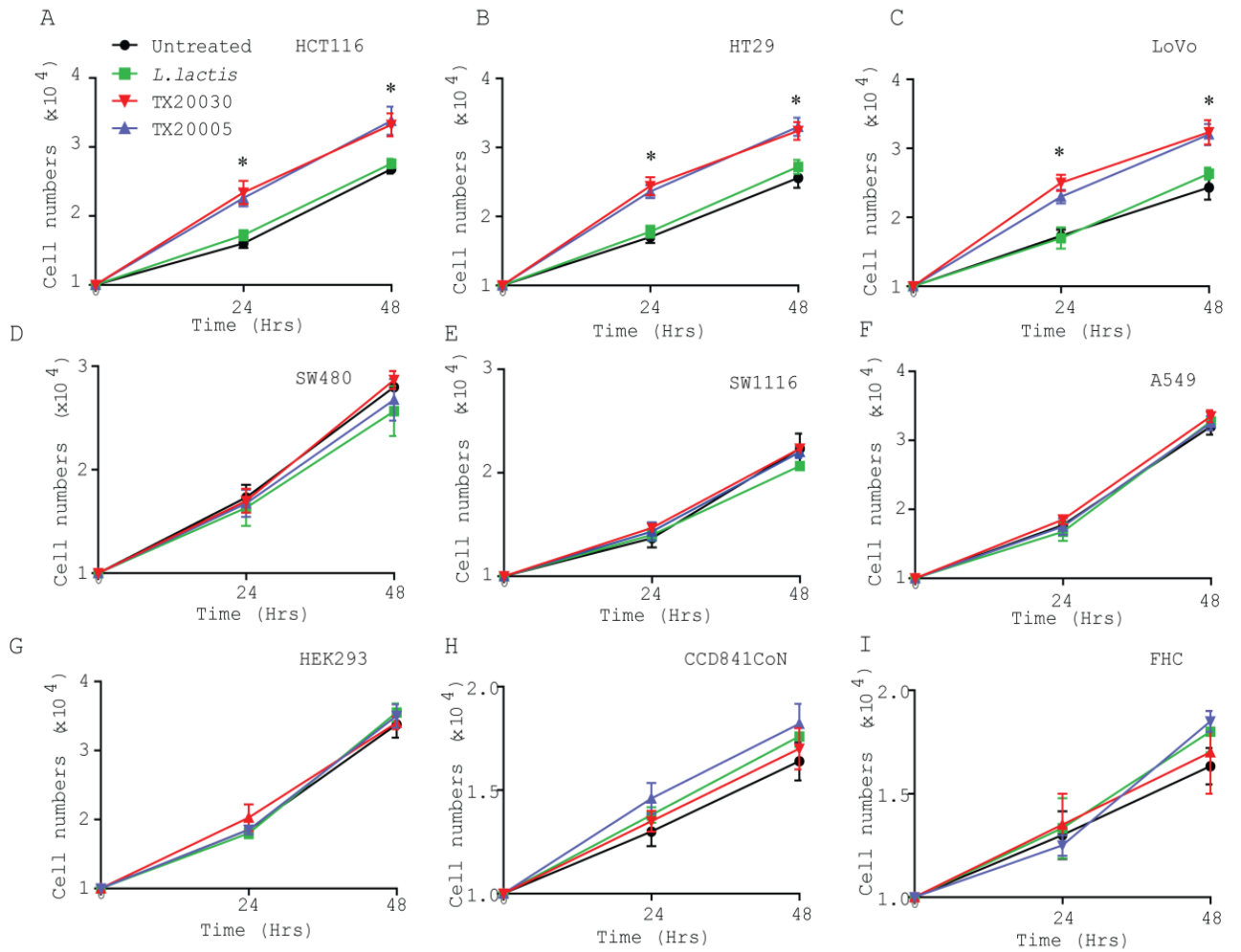
**Pertinent studies.** Unpublished studies from our laboratory demonstrate that *Sg* promotes colon cancer cell proliferation in a manner requiring specific interactions between *Sg* and colon cancer cells. As these studies are highly pertinent, they are summarized below.

**1) *Sg* promotes colon cancer cell proliferation.** The overall effect of *Sg* on cell growth and proliferation was examined using a variety of cell lines. Human colon cancer cell lines HCT116, HT29, LoVo, SW480, SW1116, normal human



colon epithelial cell lines FHC and CCD 841 CoN, human kidney epithelial cell HEK293 and human lung cancer cell line A549 were co-cultured with *Sg* strains TX20005 and TX20030, and *Lactococcus lactis* MG1363 (used as a negative control bacterial strain). The number of viable cells was counted after 24 and 48 hours of incubation. We found that, in the presence of the *Sg* strains, HCT116, HT29 and LoVo had significantly more viable cells than the respective untreated or *L. lactis*-treated colon cancer cells (~ 50-60% more at 24 hours and ~ 20-30% more at 48 hours) (Fig. 1a-1c). Interestingly, we did not observe any increase in cell numbers for the other cell lines tested including the colon cancer cell lines SW480 and SW1116 (Fig. 1d-1i). These results suggest that *Sg* strains TX20005 and TX20030 promote colon cancer cell growth in a cell context-dependent manner. Therefore, we refer to HT29, HCT116 and LoVo hereafter as “responsive” colon cancer cells, and the others as unresponsive cells.

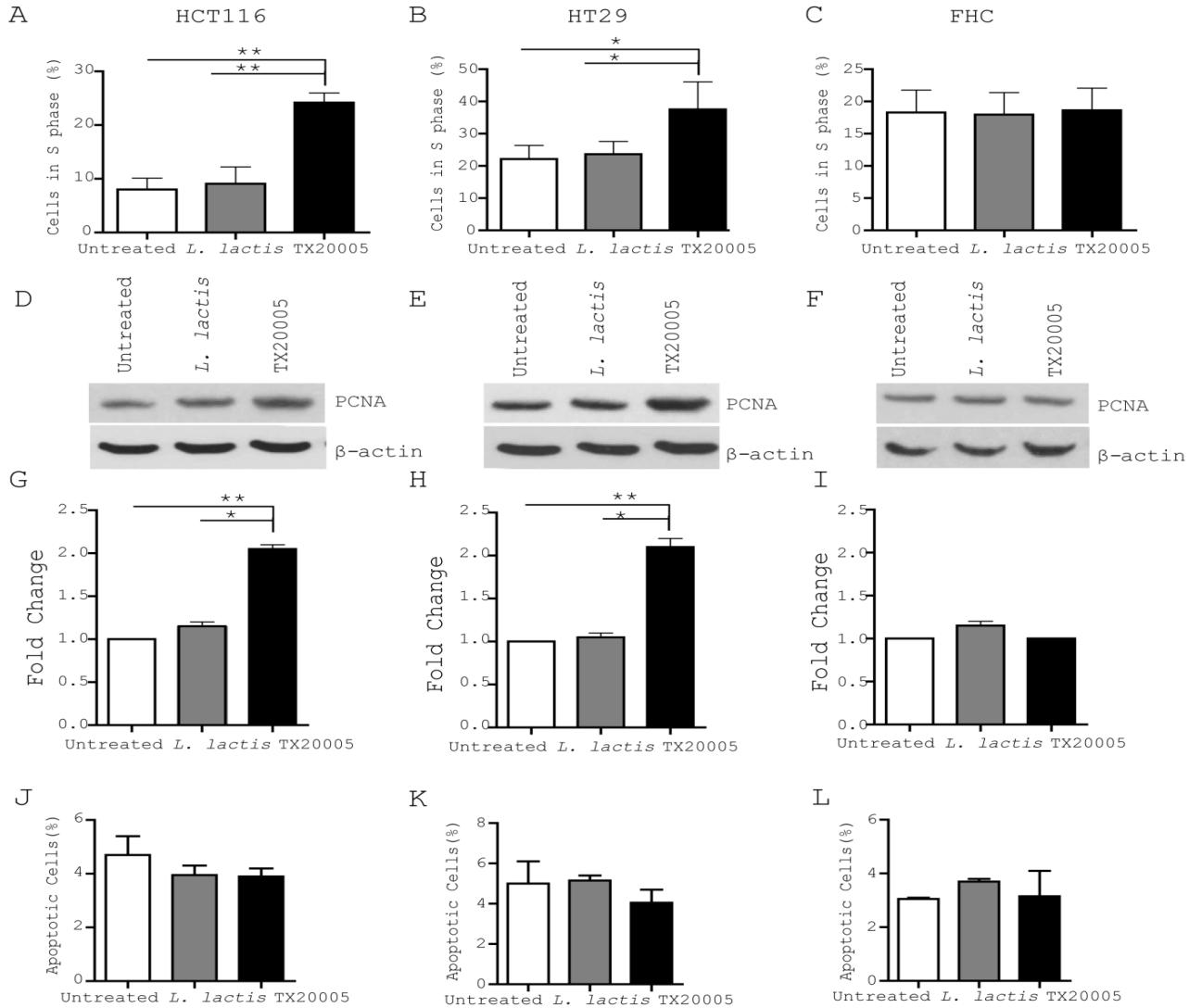
The increased viable cell numbers after co-culture with *Sg* could be due to increased proliferation, reduced apoptosis, or both. We therefore examined the effect of *Sg* on cell proliferation and apoptosis. Cells co-cultured with *Sg* or *L. lactis* were labeled with bromodeoxyuridine (BrdU) and analyzed by flow cytometry. Co-culture with *Sg* TX20005 resulted in ~1.6



**Figure 1: *Sg* stimulates cell proliferation in responsive colon cancer cell lines.** Human colon cancer cell lines HCT116 (A), HT29 (B), LoVo (C), SW480 (D), and SW1116 (E), human lung cancer cell line A549 (F), human kidney epithelial cell line HEK293 (G), and normal human colon epithelial cell lines CCD841CoN (H) and FHC (I) were tested. Cells were seeded into the wells of 6-well plates at  $1 \times 10^4$  cells per well and incubated for 12 hours. Stationary phase bacteria were washed with sterile phosphate buffered saline, pH 7.4 (PBS) and resuspended in the appropriate cell culture media. Bacterial suspension or media only were then added to the wells at  $1 \times 10^2$  cfu/well, and incubated for 24 or 48 hours. Cells were stained with trypan blue and counted in an automated cell counter. Data are presented as the mean  $\pm$  SEM. Each experiment was done with duplicate wells and repeated at least three times. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ,  $t$  test. Experiment performed by Ritesh Kumar, Ph.D.

- 2 and ~ 0.6 – 0.7 fold increase in the percentage of S phase cells in HCT116 and HT29 cells, respectively, compared to *L. lactis* treated cells or cells only control (Fig. 2a-2b). No significant changes in the percentage of S phase cells were observed in FHC cells following treatment with TX20005, as compared to untreated or *L. lactis*-treated FHC cells (Fig. 2c). We further determined the level of proliferating cell nuclear antigen (PCNA), a marker for cell proliferation [87], in cells treated with *Sg*, *L. lactis* or cells only. The results showed that HCT116 and HT29 cells treated with TX20005 had significantly higher levels of PCNA compared to cells treated with *L. lactis* or cells only control (Fig. 2d-2i). No difference was observed in PCNA levels in FHC cells between the different treatment groups, as expected. These results indicate that *Sg* promotes cell proliferation in responsive cells.

We next examined the effect of *Sg* on cell apoptosis in HCT116, HT29, and FHC cells co-cultured with TX20005, *L. lactis*, or media only. The cells were stained with anti-Annexin V antibodies and propidium iodide followed by flow cytometry analysis. No significant difference was observed in the percentage of apoptotic cells between the different treatment groups in any of the cell lines (Fig. 2j-2l). To further confirm this, we compared the level of cleaved caspase

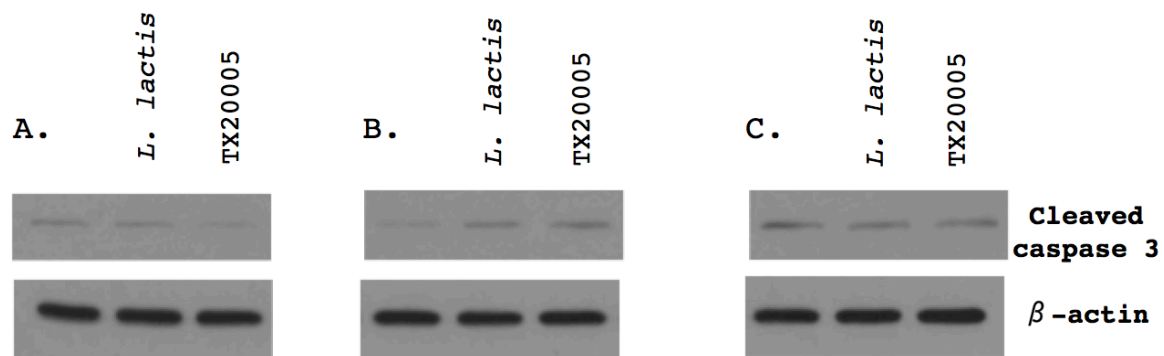


**Figure 2: *Sg* promotes cell proliferation but does not affect apoptosis.** HCT116, HT29, or FHC cells ( $\sim 1 \times 10^5$ /well) were incubated with *L. lactis* or TX20005 ( $\sim 1 \times 10^5$ /well) or media only for 12 hours. Cells were pulsed with 10  $\mu$ M BrdU for 30 mins, incubated with anti-BrdU antibodies and secondary antibodies, and analyzed by flow cytometry (**A** – **C**). The level of PCNA was determined by western blot assays using total cell lysates from cells co-cultured with TX20005, *L. lactis* or media only. Representative images are shown (**D** – **F**). Band intensity was quantified using Image J, normalized to  $\beta$ -actin, and combined from at least three independent experiments (**G** – **I**). Apoptotic cells were detected by staining with PI and anti-Annexin V antibodies and secondary antibodies, followed by flow cytometry (**j** – **l**). Each experiment was done with duplicate wells and was repeated at least three times. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ,  $t$  test. Experiment performed by Ritesh Kumar, Ph.D.

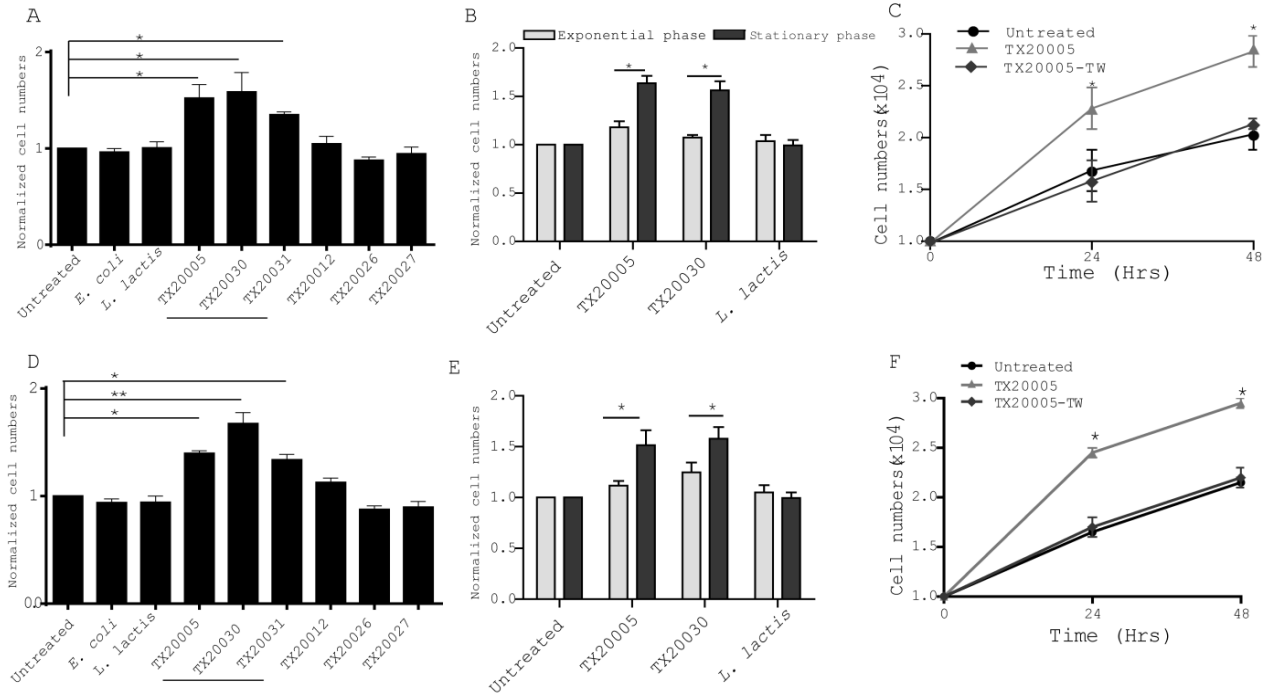
3 and observed no difference between the different treatment groups in any of the cell lines tested (Fig. 3). Taken together, these results indicate that *Sg* does not affect cell apoptosis, but promotes colon cancer cell proliferation in a cell context-dependent manner.

**2) The proliferation-promoting effect of *Sg* was *Sg*-specific and depends on bacterial growth phase and direct contact between bacteria and responsive cells.** We next examined the effect of an expanded panel of bacterial strains on HT29 and HCT116. The panel included *Sg* strains TX20005, TX20030 and TX20031, and strains of closely related species within the SBSEC - *S. infantarius* (TX20012), *S. macedonicus* (TX20026), and *S. pasteurianus* (TX20027). *E. coli* strain XL-1 Blue and *L. lactis* were included as negative control bacteria. All three *Sg* strains significantly increased HT29 (Fig. 4a) and HCT116 (Fig. 4d) cell numbers whereas none of the other bacterial strains had any effect.

In the co-culture experiments described above, the bacteria added to the wells were from stationary phase cultures. We examined the effect of exponential phase cultures of TX20005, TX20030 and *L. lactis* on HT29 and HCT116 cells. In contrast to stationary phase bacteria, exponential phase TX20005 or TX20030 did not cause any significant increase in HT29 (Fig. 4b) or HCT116 (Fig. 4e) cell numbers compared to



**Figure 3. Detection of cleaved caspase 3 in cells treated with *S. gallolyticus*.** Approximately  $1 \times 10^5$  cells were incubated with media only, *L. lactis* or TX20005 ( $\sim 10^5$  cfu) for 12 hrs in a 6 well plate. Whole cell lysates were prepared as described in the Methods and Materials section and analyzed by western blot assays. (A) HCT116; (B) HT29; (C) FHC. The experiment was repeated three times and representative images are shown. Experiment performed by Ritesh Kumar, Ph.D.

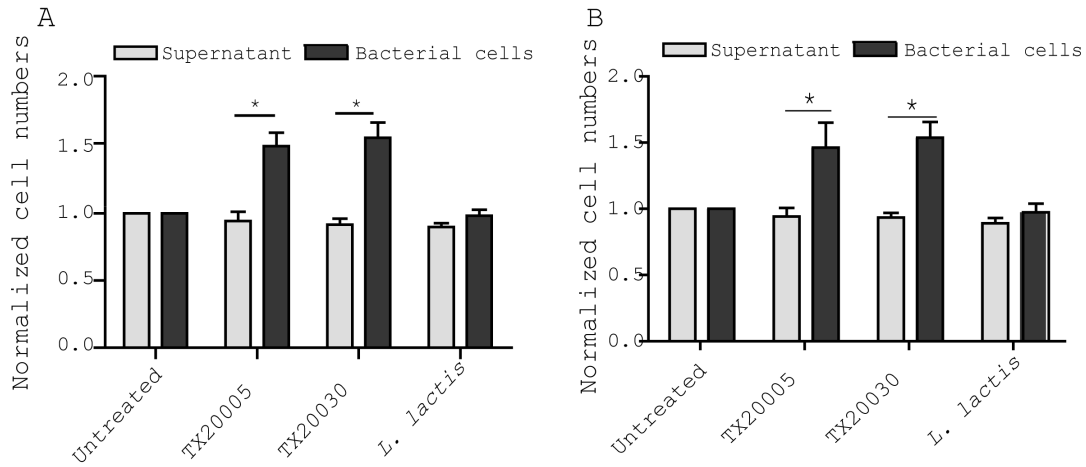


**Figure 4: Promotion of cell proliferation requires *Sg*-specific factors and depends on bacterial growth phase and direct contact with CRC cells.** **A** and **D**. Species closely related to *Sg* do not promote cell proliferation. Stationary phase bacteria were added to HT29 (**A**) and HCT116 (**D**) cells, co-cultured for 24 hours and viable cell numbers enumerated. TX20005, TX20030 and TX20031, *Sg*; TX20012, *S. infantarius*; TX20026, *S. macedonicus*; TX20027, *S. pasteurianus*. **B** and **E**. Promotion of cell proliferation requires stationary but not exponential phase *Sg*. TX20005 bacteria harvested at exponential or stationary phase of growth were added to HT29 (**B**) and HCT116 (**E**) cells and co-cultured with for 24 hours. Viable cell numbers were enumerated. **E** and **F**. Promotion of cell proliferation requires direct contact between *Sg* and responsive cells. Stationary phase TX20005 bacteria were added to transwell inserts (0.4  $\mu$ m pore) (TX20005-TW) or directly to cells and co-cultured with HT29 (**E**) and HCT116 (**F**) cells for 24 and 48 hours. Data are presented as the mean  $\pm$  SEM. Each experiment was done with duplicate wells and was repeated at least three times. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ,  $t$  test. Experiment performed by Ritesh Kumar, Ph.D.

the controls, suggesting that the ability of *Sg* to promote cell proliferation is growth phase-dependent.

We next examined whether secreted bacterial factors or bacterial metabolites in the culture supernatant were sufficient to promote colon cancer cell growth. Supernatants from stationary phase cultures of TX20005, TX20030 and *E. coli* were collected and filtered to remove any residual bacteria. HT29 and HCT116 cells were cultured in media only or media supplemented with the culture supernatants. The results showed that culture supernatants were insufficient to promote cell proliferation (Fig. 5). To distinguish between the possibilities that the proliferation-promoting effect of *Sg* required bacterial-attached factors, and that the factors/metabolites in the culture supernatants were unstable and required a continuous presence of live bacteria in the culture, we used a transwell system in which bacteria were cultured in inserts with permeable membranes of 0.4  $\mu\text{m}$  pore size. This pore size allows the passage of secreted bacterial factors and metabolites but not bacteria. Culturing bacteria in transwells resulted in a complete loss of the proliferation-promoting effect of TX20005 on both HT29 and HCT116 cells (Fig. 4c, Fig. 4f). Taken together, these results suggest that the proliferation-promoting effect of *Sg*

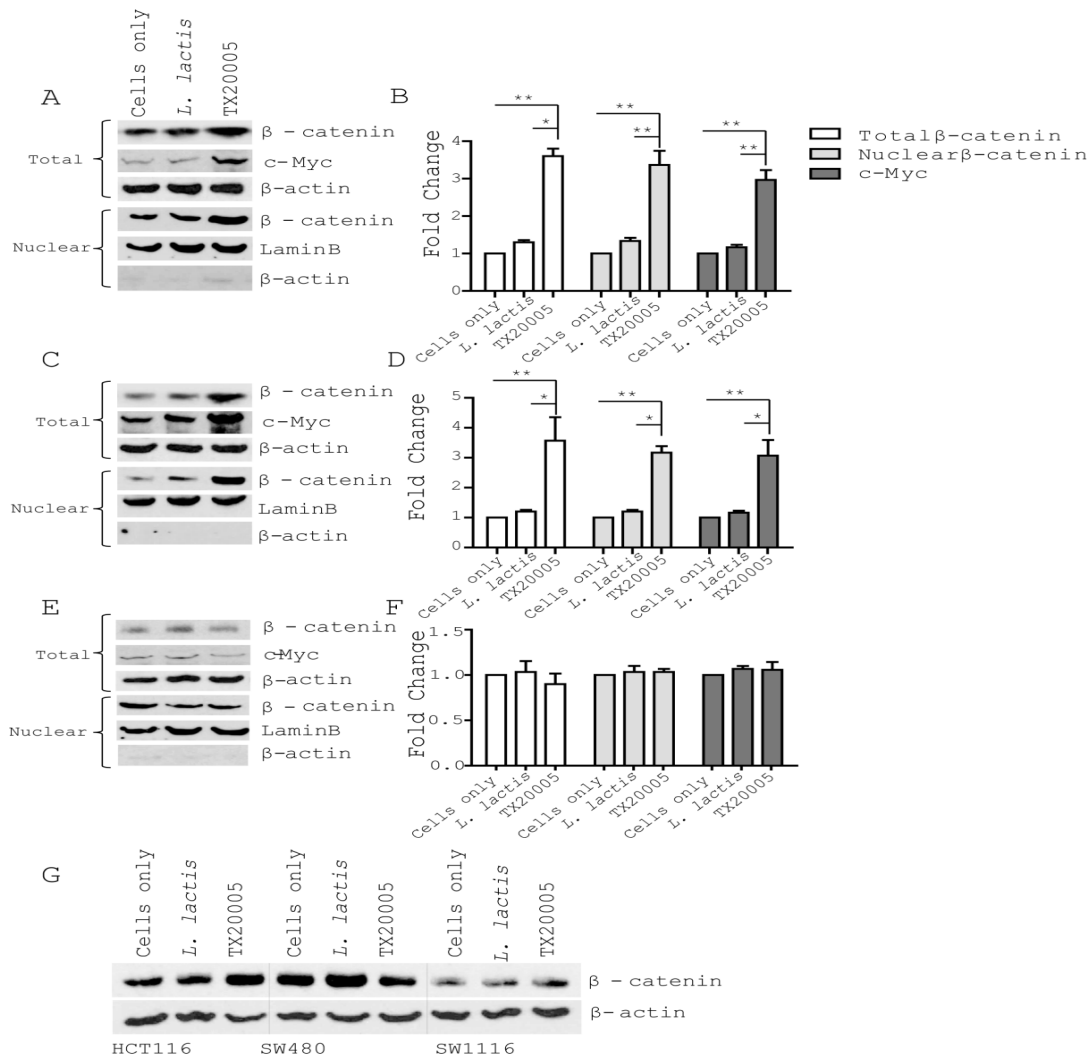




**Figure 5. *S. gallolyticus* culture supernatant had no effect on cell proliferation.**  $1 \times 10^4$  HT29 (A) and HCT116 (B) cells were co-cultured with bacterial supernatant or  $1 \times 10^2$  bacterial cells collected from stationary phase culture. Cells were then incubated for 24 hours and viable cells enumerated. Cell numbers are normalized to the untreated samples at 24 hours. Data is presented as the mean  $\pm$  SEM. Each experiment was performed with duplicate wells and repeated at least three times. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ,  $t$  test. Experiment performed by Ritesh Kumar, Ph.D.

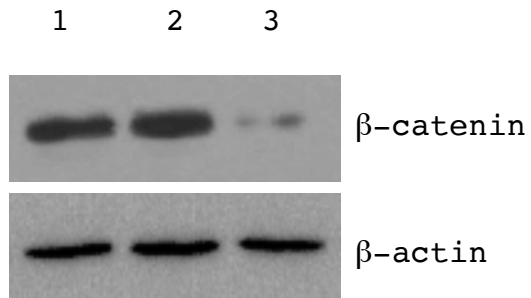
is dependent on *Sg*-specific factors, bacterial growth phase and direct contact between bacteria and responsive cells.

**3) *Sg* promotes cell proliferation in a  $\beta$ -catenin dependent manner.** The Wnt/ $\beta$ -catenin signaling pathway regulates cell fate and proliferation and is a critical pathway in colon tumorigenesis [88-90]. We investigated the effect of *Sg* on  $\beta$ -catenin in responsive and unresponsive cells. For HCT116 and HT29 cells, co-culture with TX20005 led to a significantly elevated level of total  $\beta$ -catenin compared to cells co-cultured with *L. lactis* or no bacteria (Fig. 6a-6d). In contrast, no increase in  $\beta$ -catenin level was observed in unresponsive FHC, SW480 and SW1116 cells following *Sg* treatment (Fig. 6e-6g). Upon activation,  $\beta$ -catenin is translocated into the nuclei and triggers the enhanced expression of downstream targets, such as c-Myc[91]. We then examined the level of nuclear  $\beta$ -catenin. The results showed that HCT116 and HT29 cells co-cultured with TX20005 had significantly increased nuclear  $\beta$ -catenin compared to cells co-cultured with *L. lactis* or cells only (Fig. 6a-6d). No change in nuclear  $\beta$ -catenin was observed in FHC cells under the same experimental conditions (Fig. 6e and 6f). In accordance with this observation, the level of c-Myc in HCT116 and HT29 was also significantly increased following treatment by TX20005 compared to that in the control groups (Fig. 6a-

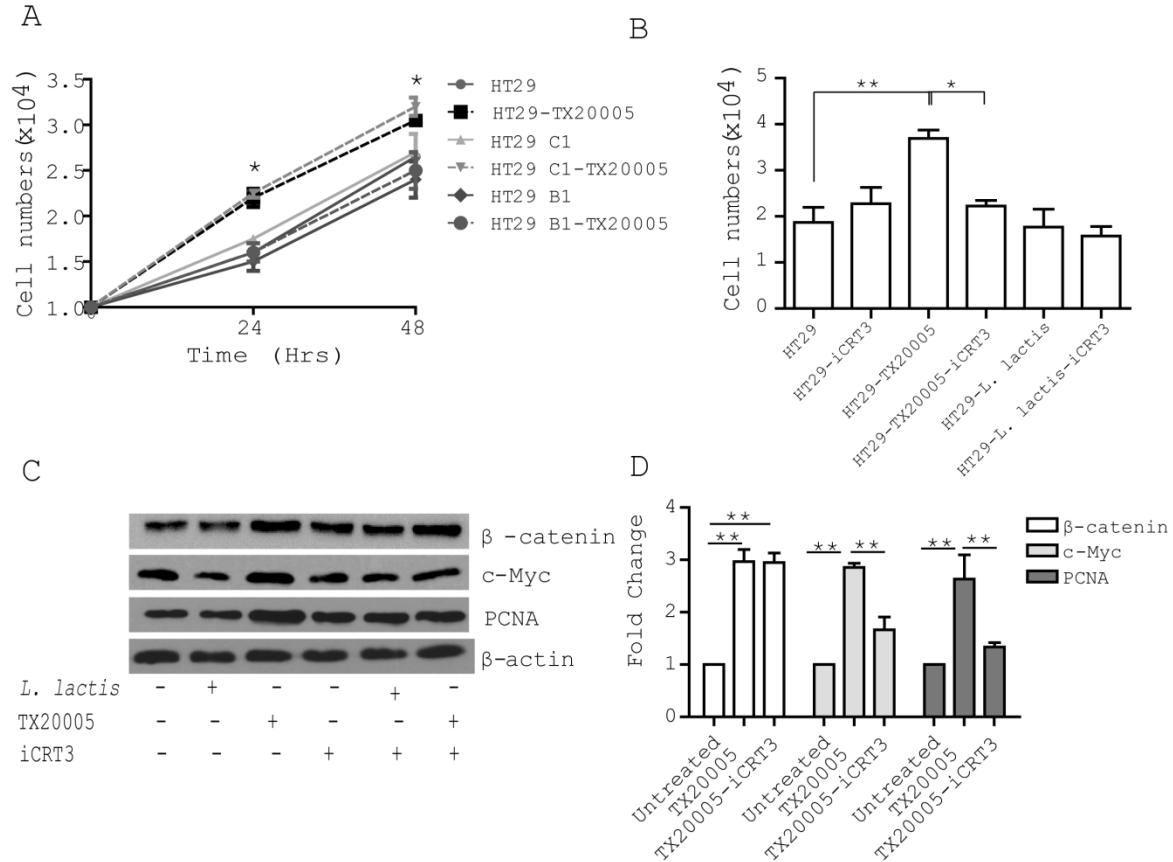


**Figure 6: *Sg* increases the level of β-catenin and c-Myc in HCT116 and HT29 cells.** Approximately  $1 \times 10^5$  cells/well were incubated with bacteria ( $\sim 10^5$  cfu/well) or media only for 12 hours in a 6 well plate. Whole cell or nuclear lysates were extracted and analyzed by western blot assays using specific antibodies. Representative images are shown (A, C, E and G). Band intensity was quantified using Image J, normalized to β-actin or lamin B first and then normalized to cells only control. Results combined from at least 3 experiments are shown (B, D and F). **A** and **B**, HCT116; **C** and **D**, HT29; **E** and **F**, FHC. **G**. *Sg* does not increase β-catenin level in SW1116 or SW480 cells. Experiment performed by Ritesh Kumar, Ph.D.

6d). No difference in the level of c-Myc was observed in FHC cells, as expected (Fig. 6e and 6f). Taken together, these results suggest that treatment of responsive cells with *Sg* results in up-regulation of  $\beta$ -catenin and its oncogenic downstream targets. To determine the role of  $\beta$ -catenin in *Sg*-mediated cell proliferation,  $\beta$ -catenin stable knockdown cells were generated using specific shRNA. Knockdown was confirmed using western blot assays (Fig. 7). In co-culture experiments,  $\beta$ -catenin knockdown completely abolished the effect of *Sg* on cell proliferation, whereas HT29 cells transfected with a control shRNA showed a similar increase in cell numbers as untransfected cells (Fig.8a). To further confirm this, we used a  $\beta$ -catenin responsive transcription (CRT) inhibitor iCRT3, which disrupts  $\beta$ -catenin-TCF4 interaction[92]. In the presence of iCRT3, TX20005 treatment of HT29 cells did not increase cell proliferation compared to the control groups (Fig. 8b). We next examined the effect of TX20005 on the level of c-Myc and PCNA in the presence of iCRT3. Treatment of HT29 cells with iCRT3 significantly reduced the effect of TX20005 on c-Myc and PCNA expression (Fig. 8c-8d). Taken together, these results indicate that promotion of cell proliferation by *Sg* is through up-regulation of  $\beta$ -catenin dependent signaling.



**Figure 7:**  $\beta$ -catenin expression in untransfected HT29 cells (lane 1), HT29 cells transfected with control shRNA (lane 2), and HT29 cells transfected with a  $\beta$ -catenin specific shRNA, as assessed by immunoblotting. Experiment performed by Ritesh Kumar, Ph.D.



**Figure 8: *Sg* promotes cell proliferation in a  $\beta$ -catenin dependent manner.**

**A.** Knockdown of  $\beta$ -catenin abolished the effect of *Sg*. Untransfected HT29 cells,  $\beta$ -catenin stable knockdown HT29 cells (HT29-B1) or HT29 cells transfected with a control shRNA (HT29-C1) were seeded into the wells of 6-well plates at  $\sim 1 \times 10^4$  cells/well and incubated for 12 hours. Stationary phase bacteria were added to the wells at  $\sim 1 \times 10^2$  cfu/well, and incubated for 24 or 48 hours. Cells were stained with trypan blue and viable cells counted in an automated cell counter. **B - D.** Inhibition of  $\beta$ -catenin transcriptional activity by iCRT3 renders cells unresponsive to *Sg*. Stationary phase TX20005 or *L. lactis* bacteria resuspended in the appropriate cell culture media were added to the wells at  $\sim 1 \times 10^2$  cfu/well in the presence or absence of iCRT3, incubated for 24 hours and viable cells enumerated (**B**). Total cell lysates were prepared and subject to western blot assays to compare  $\beta$ -catenin, c-Myc and PCNA protein levels. Representative images are shown (**C**). Band intensity was quantified using Image J, normalized to  $\beta$ -actin first and then to the cells only control (**D**). Data in panels a, b and d are presented as the mean  $\pm$  SEM. Each experiment was done with duplicate wells and was repeated at least three times. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ,  $t$  test. Experiment performed by Ritesh Kumar, Ph.D.

These *in vitro* data using cultured human colon cancer cells indicate that *Sg* promotes colon cancer cell proliferation by activating  $\beta$ -catenin signaling, and the promotion requires stationary bacteria and direct contact between *Sg* and colon cancer cells. The Wnt/ $\beta$ -catenin signaling pathway is the most critical pathway in colon tumorigenesis [88-90]. The finding that *Sg* upregulates  $\beta$ -catenin is therefore highly pertinent. The key question now is whether *Sg* promotes tumorigenesis *in vivo*.

In this study, the effect of *Sg* on tumor growth in a xenograft model was examined. In addition, an azoxymethane (AOM)-induced mouse model of CRC was used to examine the effect of *Sg* on colon tumorigenesis. The results showed that *Sg* treatment resulted in larger tumors in the xenograft model. Furthermore, in the AOM model, mice treated with *Sg* had significantly more macroscopic tumors, higher tumor burden, higher average dysplasia grade, and increased cell proliferation and  $\beta$ -catenin level in colon crypts compared to control mice. These results shed light on a tumor-promoting role of *Sg* and have important implications with respect to microbial contributions to CRC as well as clinical practices to combat CRC.

## **EXPERIMENTAL PROCEDURE:**

**Bacterial strains and culture conditions.** *S. bovis* group strains (*Sg*, *S. pasteurianus*, *S. infantarius*, *S. macedonicus*, provided by Barbara E. Murray, University of Texas Medical School, Houston, TX) [85], *Lactococcus lactis* MG1363 (provided by Timothy J. Foster, Trinity College Dublin, Ireland), and *E. coli* XL-1 Blue were grown at 37°C in brain-heart infusion (BHI) broth with shaking or on BHI agar (Difco Laboratories, Sparks, MD).

**Cell lines and growth conditions.** Human colon cancer cell lines HCT116 and HT29 were cultured in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO, USA) supplemented with 10% fetal bovine serum (FBS) (GIBCO, USA).

**Adherence assay.** This was performed following a procedure described previously with slight modifications [93]. Cells were seeded onto the wells of 24-well tissue culture plates at  $10^6$  cells/well. Bacteria from a stationary phase or exponential phase culture were washed twice in PBS, resuspended in DMEM supplemented with 10% FBS, and added to the wells at a multiplicity of infection (MOI) of 10. The plates were incubated in a humidified incubation chamber at 37°C with 5% CO<sub>2</sub> for 1 hour. Each well was washed three times with sterile PBS to remove unbound bacteria. To determine the number of



associated bacteria, cells were lysed with sterile PBS containing 0.025% Triton X-100 and dilution plated. All experiments were performed in triplicate and repeated at least three times. Adherence was expressed as a percentage of total bacteria added.

**Animal experiments.** Animal studies were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at the Texas A&M Health Science Center, Institute of Biosciences and Technology. Mice were fed with standard ProLab IsoPro RMH3000 (LabDiet). (1) **Xenograft model.** HCT116 cells ( $1 \times 10^6$ ) were incubated with TX20005 or *L. lactis* (MOI = 1) for 12 hours. The cells were immediately washed, trypsinized and mixed with Matrigel (Corning, MA) according to the manufacture's instructions and subcutaneously injected (100  $\mu$ l) into the dorsal flap of 5-week-old nude mice (Jackson Laboratory, Bar Harbor, ME). Three hours after the injection, mice were administered a broad-spectrum antibiotic imipenem (MSD) by intraperitoneal (i.p.) injection (150 mg/kg body weight). Tumor diameters were measured with a digital caliper, and tumor volume calculated using the formula: Volume =  $(d1 \times d1 \times d2)/2$ , with d1 being the larger dimension[94]. (2) **AOM-induced mouse model of CRC.** Eight-week old female A/J mice (Jackson Laboratory, Bar Harbor, ME) were treated with AOM (10 mg/kg body weight) by i.p. injection once a week for 2 or 4

weeks. Mice were then given ampicillin (1g/L) in drinking water for one week and switched to antibiotic-free water 24 hours prior to bacterial inoculation. Mice were orally gavaged with saline, TX20005 or *L. lactis* using a feeding needle ( $\sim 1 \times 10^8$  cfu/mouse) at a frequency of three times per week for 24 weeks, or once a week for 12 weeks and were euthanized one week after the final gavage. One hour before sacrifice, mice received an i.p. injection of BrdU at 100 mg/kg body weight. Colons were removed by cutting from the rectal to the cecal end and opened longitudinally for visual evaluation. Tumor number was recorded and tumor size measured using a digital caliper. Tumor burden was calculated as the sum of all the tumor volumes of one mouse. Visual evaluation was carried out by two blinded observers.

**Histology and immunohistochemistry.** At necropsy, colons from 3 randomly selected mice from each group were "Swiss rolled" from the rectal to the cecal end, fixed in Methcarn (60% methanol, 30% chloroform, and 10% glacial acetic acid), paraffin embedded, and cut into 5 $\mu$ m sections across. Every 10 sections were stained with hematoxylin/eosin (H&E) and histological evaluation performed by a pathologist in a blinded fashion. Pathological scores were given using the following scale [95]: 0, no dysplasia; 1, mild dysplasia characterized by aberrant crypt foci (ACF), +0.5 for small

gastrointestinal neoplasia (GIN) or multiple ACF; 2, moderate dysplasia with GIN, +0.5 for multiple occurrences or small adenoma; 3, severe or high grade dysplasia restricted to mucosa; 3.5, adenocarcinoma (involvement through muscularis mucosa); 4, adenocarcinoma (through submucosa and into or through the muscularis propria). Inflammation was scored using the following scoring matrix [96]: 0, normal; 1 -  $\leq 1$  multifocal mononuclear cell infiltrates in lamina propria accompanied by minimal epithelial hyperplasia and slight to no depletion of mucous from goblet cells; 2, involves more of intestine or more frequent, occasional small epithelial erosions, no submucosa involvement; 3, moderate inflammation plus submucosa neutrophils, crypt abscesses, ulcers; 4, most of colon; transmural; crowding of epithelial cells with elongated crypts, ulcers plus crypt abscesses [96].

Proliferating crypt cells were detected by staining every 10 sections with anti-BrdU antibodies and counting BrdU-positive cells. A total of ~100 crypts were counted per mouse and the percentage of BrdU+ cells vs. total crypt epithelial cells counted was calculated. Apoptosis was determined by performing TUNEL assay on every 10 sections. Crypts were counted in the same manner as for BrdU+ cells. Sections were also stained for  $\beta$ -catenin. A Leica DM2000 LED microscope was used for imaging. Paraffin embedding, sectioning,

histochemistry and immunohistochemistry were performed by the Histology Core, Gulf Coast Digestive Diseases Center, Baylor College of Medicine, Houston, TX.

**Detection of *Sg* by qPCR.** Fecal pellets were collected from mice at the end of 12-week gavage with TX20005. DNA was extracted using QIAamp Fast DNA Stool Mini Kit (Qiagen) following manufacturer's instructions. PCR primers were designed using *Sg*-specific sequences (forward primer – 5' TGACGTACGATTGATATCATCAAC 3', reverse primer – 5'CGCTTAACACATTTTGTAGCTAATACG 3'). qPCR was performed using Fast Plus EvaGreen qPCR Master Mix (Biotium) in a Viia 7 Real Time PCR System (Applied Biosystems) with the following cycling condition: 95°C for 10 minutes followed by 40 cycles at 95°C for 30 seconds and 60°C for 1 minute.  $\Delta$ CT was normalized to the results from qPCR reactions using universal 16S rRNA primers.

**Immunofluorescence detection of *Sg* in the mouse colon.** Rabbit serum was raised against formalin killed TX20005 (Rockland Immunochemicals). The antiserum and pre-bleed serum were tested against TX20005, *S. infantarius* (TX20012), *S. macedonicus* (TX20026), *S. pasterianus* (TX20027), *E. coli* XL-1 Blue, and *L. lactis* MG 1363, to determine the specificity of the antibodies. The antiserum specifically recognized *Sg* not

other bacterial strains under the experimental conditions (Fig. S8). Methcarn-fixed paraffin embedded colon sections (5  $\mu$ m) from mice treated twice with AOM and 24 weeks of oral gavage with bacteria were used to detect *Sg* using an optimized procedure. Briefly, sections were deparaffined with xylene and rehydrated in an ethanol gradient. The slides were incubated in a citrate buffer at 95°C for 15 min, cooled to room temperature (RT), rinsed with PBS and incubated in blocking buffer (PBS containing 1% Saponin and 20% BSA) for 30 min. The slides were then incubated with rabbit anti-*Sg* serum (1:250 dilution) at 4°C overnight, washed with PBS, and incubated with donkey-anti-rabbit Alexa 594 (1:500 dilution in PBS) for 1 hr at RT. The slides were washed again, stained with DAPI, mounted and examined in a DeltaVision Elite microscope.

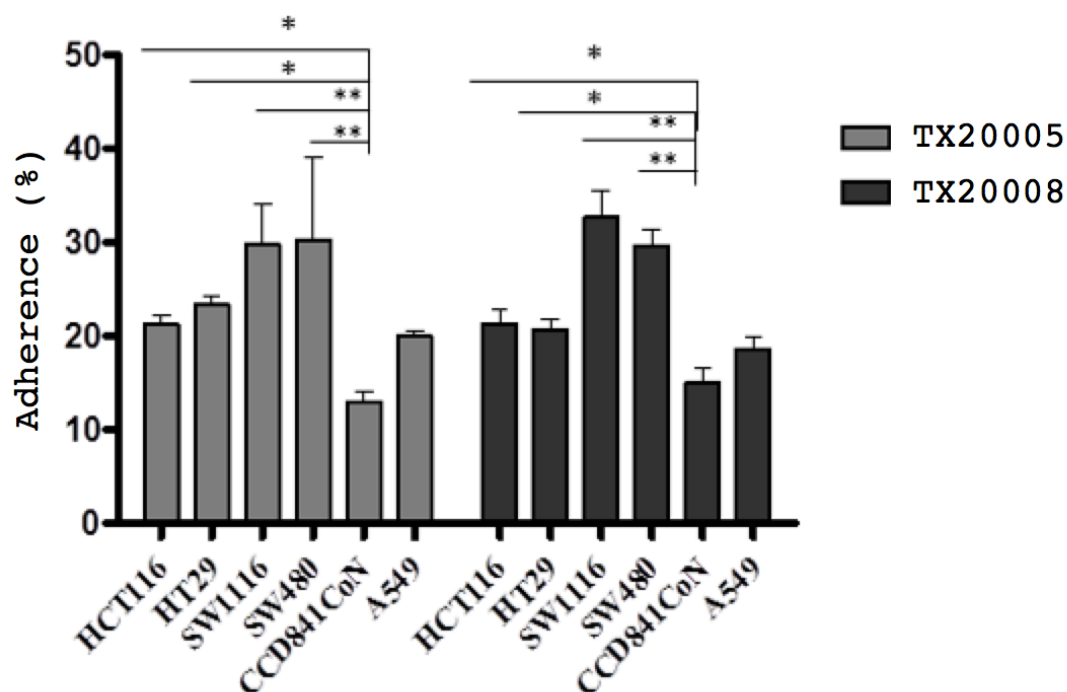
**Statistical analyses.** Pairwise comparison was done using Student's *t*-test. Pearson correlation analysis was performed to determine any correlation between *Sg* burden and tumor number and burden, respectively. Analyses were carried out using the Graphpad Prism 6 software.

## **RESULTS:**

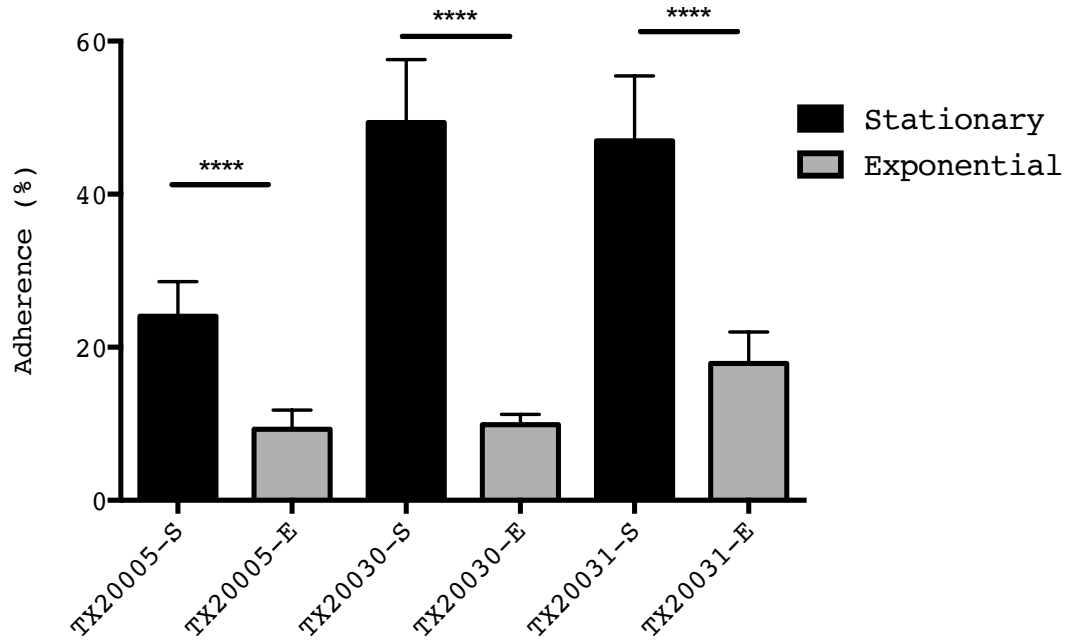
***Sg* adheres to both responsive and unresponsive cells.** Studies from our lab showed that *Sg* was able to promote the proliferation of HT29 and HCT116 cells, but not lung cancer

cell line A549 or normal colon epithelial cell line CCD 841. Since direct contact between bacteria and cancer cells is required to promote cell proliferation, we investigated the ability of *Sg* to adhere to responsive and unresponsive cell lines. The results showed that both TX20005 and TX20030 adhered to HCT116, HT29, and A549 cells at a similar level (~20% of the initial inoculum) and adhered slightly higher to SW1116 and SW480 (~30% of the initial inoculum). Adherence to CCD 841 CoN colonic epithelial cells was significantly lower than to the cancer cell lines (~15% of the initial inoculum) (Fig. 9). Together these results showed that the effects of *Sg* on cell proliferation were not in direct concordance with the ability of the bacteria to adhere to the cultured cell lines.

Exponential and stationary phase bacteria were also evaluated for their adherence to both HCT116 and HT29 cell lines. Stationary phase *Sg* strains adhered significantly more to these cell lines than exponential phase *Sg* strains (Fig. 10). These results show a similar effect as seen with exponential and stationary phase bacteria in our cell proliferation co-culture experiments, with stationary phase bacteria playing the important role in promoting cell proliferation. These results indicate the importance of stationary phase bacteria in both adherence and cell proliferation.



**Figure 9. Adherence of *S. gallolyticus* to different cell lines.** Stationary phase culture TX20005 and TX20030 were washed twice in PBS, resuspended in DMEM supplemented with 10% FBS, and added to the wells containing different cells at a multiplicity of infection (MOI) of 10, as described in the Methods and Materials section. The plates were incubated in a humidified incubation chamber at 37°C with 5% CO<sub>2</sub> for 1 hr. Each well was washed three times with PBS to remove unbound bacteria. To determine the number of adhered bacteria, cells were lysed with sterile PBS containing 0.025% Triton X-100 and dilution plated. All experiments were performed in triplicate wells and repeated at least three times. Adherence was expressed as the percentage of adhered bacteria vs. total bacteria added. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ;  $t$  test. Experiment performed by Ritesh Kumar, Ph.D.

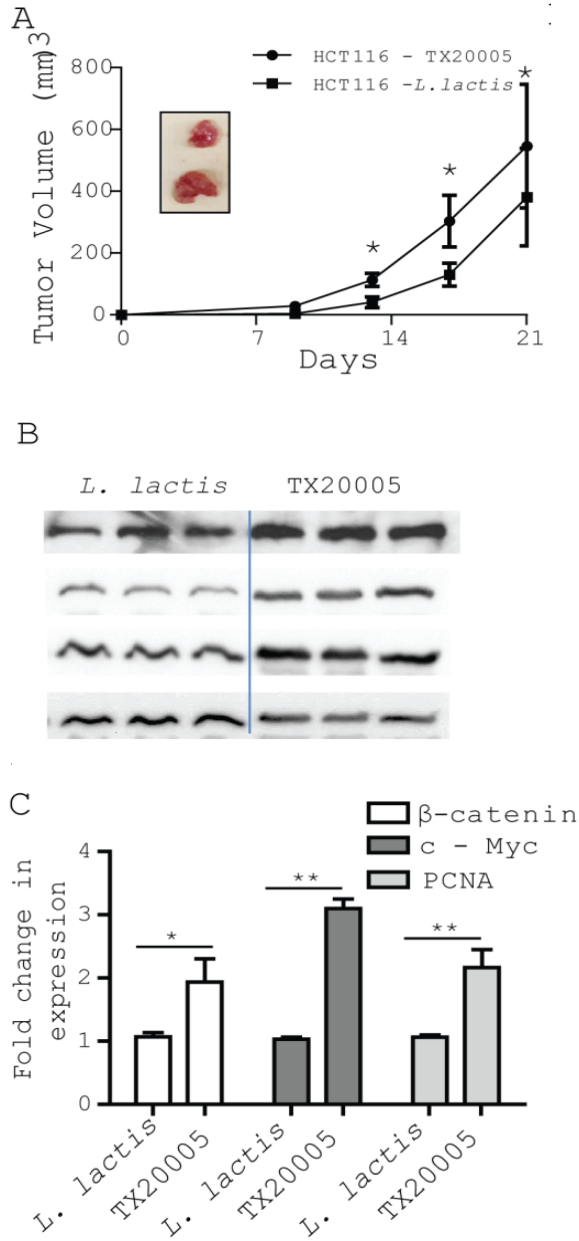


**Figure 10. Adherence of *S. gallolyticus* and closely related strains to HCT116 cells.** Stationary phase and exponential phase culture of TX20005, TX20030, and TX20031 were washed twice in PBS, resuspended in DMEM supplemented with 10% FBS, and added to the wells containing HCT116 cells at a multiplicity of infection (MOI) of 10, as described in the Methods and Materials section. The plates were incubated in a humidified incubation chamber at 37°C with 5% CO<sub>2</sub> for 1 hr. Each well was washed three times with PBS to remove unbound bacteria. To determine the number of adhered bacteria, cells were lysed with sterile PBS containing 0.025% Triton X-100 and dilution plated. All experiments were performed in triplicate wells and repeated at least three times. Adherence was expressed as the percentage of adhered bacteria vs. total bacteria added. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ;  $t$  test.



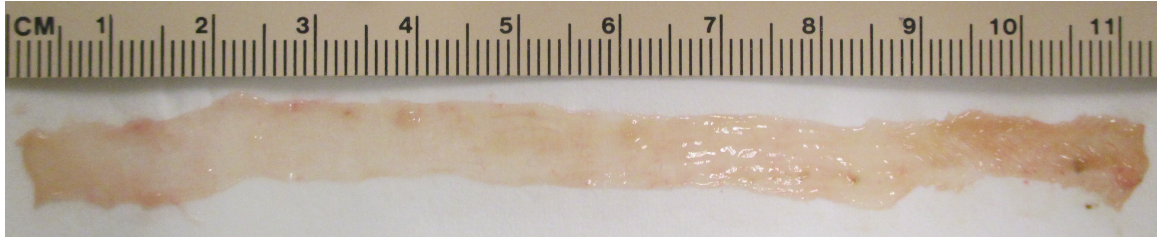
***Sg* promotes tumor growth in a xenograft model.** HCT116 cells treated with TX20005 or *L. lactis* were injected into nude mice and tumor growth was monitored (Fig. 11). Starting from day 13, TX20005-treated cells formed significantly larger tumors than *L. lactis*-treated cells. Expression of  $\beta$ -catenin, c-Myc and PCNA was analyzed in tumors obtained at day 21 (Fig.11b-11c). A significant increase in the levels of  $\beta$ -catenin, c-Myc and PCNA were observed in tumors from TX20005-treated cells compared to those from *L. lactis*-treated cells. These results indicate that TX20005 treatment promoted tumor growth in the xenograft model.

***Sg* promotes colon tumor development in an AOM-induced mouse model of CRC.** To further evaluate the role of *Sg* in tumor development, we used an AOM-induced mouse model of CRC. This model is commonly used to represent sporadic CRC. Mice were treated with 2 doses of AOM followed by antibiotic treatment for a week and then orally gavaged with TX20005, *L. lactis* or saline for 24 weeks. Colons were harvested for visual examination for macroscopic tumors (Fig. 12). Overall, most of the tumors were found in the distal portion of the colon. We observed that *Sg*-treated mice had more tumors per mouse compared to the saline control ( $p = 0.03$ ) and *L. lactis*-treated mice although the difference with the latter group was not statistically significant ( $p = 0.08$ ) (Fig. 13a). In

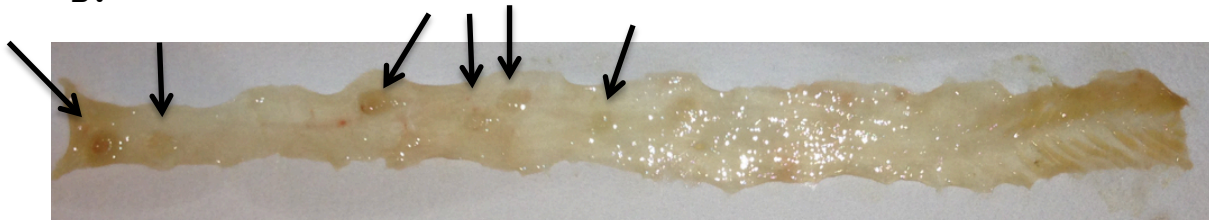


**Figure 11: *Sg* treatment promotes tumor growth in a xenograft model. A.** *Sg*-treated cells developed larger tumors in nude mice.  $\sim 1 \times 10^5$  HCT116 cells were treated with TX20005 or *L. lactis*, mixed with Matrigel and injected into the dorsal flap of nude mice ( $n=5/\text{group}$ ) as described in the Methods and Materials section. Tumor size was measured during the indicated time period with a digital caliper. **B-C.** *Sg*-treated xenografts had higher levels of  $\beta$ -catenin, c-Myc and PCNA compared to *L. lactis*-treated ones. Three tumors were randomly selected from each group and collected on day 21. Protein extracts were analyzed by western blot assays (**B**). Protein level was normalized to  $\beta$ -actin first and then to *L. lactis*-treated controls (**C**). Data are presented as the mean  $\pm$  SEM. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ,  $t$  test.

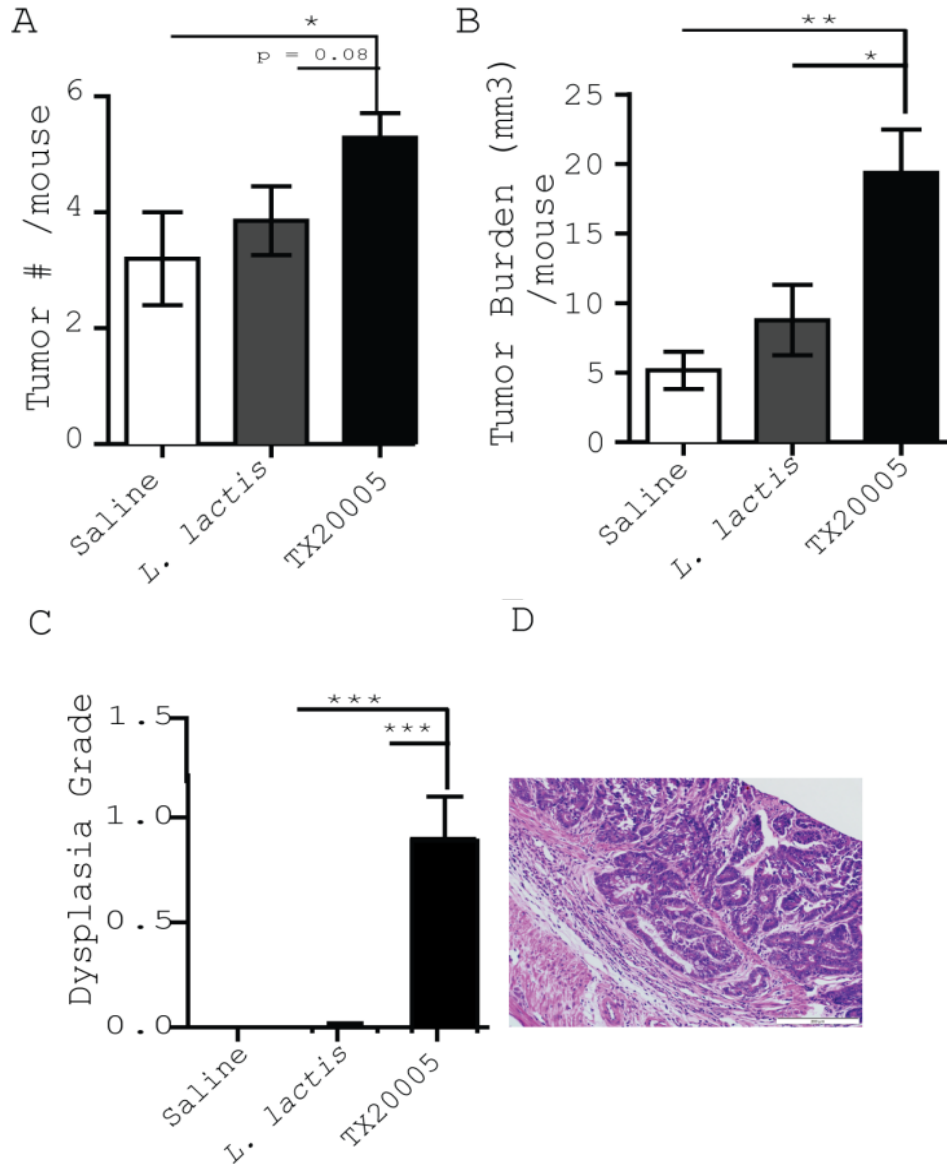
A.



B.



**Figure 12.** At necropsy, mouse colons were removed and cut from the distal to the proximal end in a longitudinal direction. Tumors were visually evaluated for tumor number and size. Representative images from saline treated mice (A) and TX20005 treated mice (B) are shown.

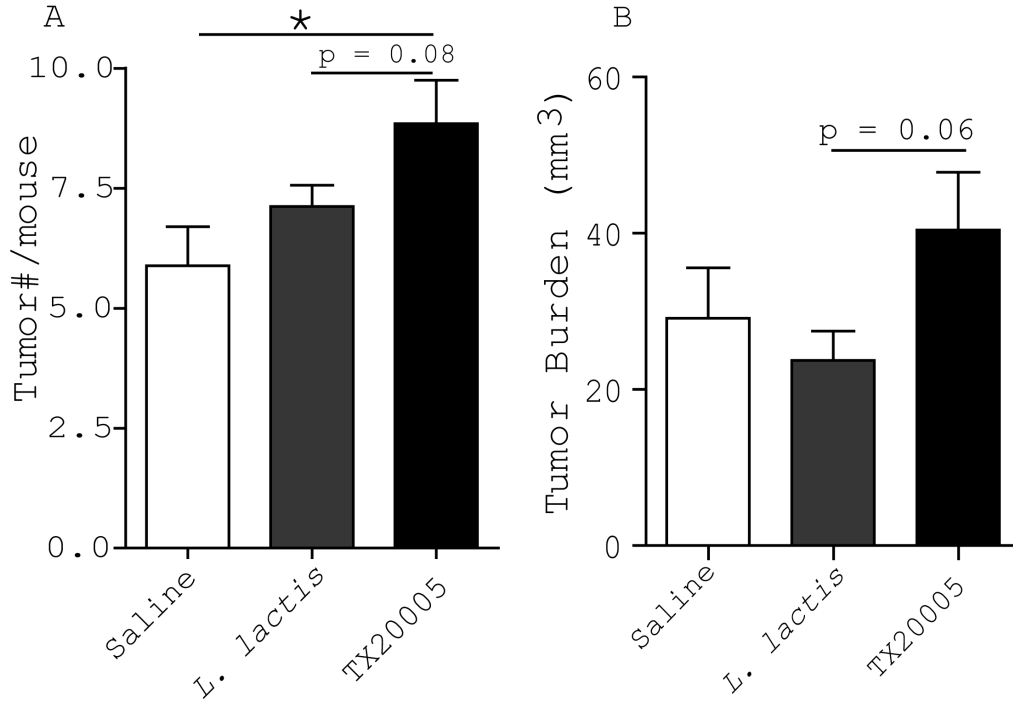


**Figure 13: *Sg* promotes colon tumor development in an AOM-induced mouse model of CRC. A-D.** A/J mice were administered 2 weekly i.p. injections of AOM, followed by treatment with Amp (1g/L) in drinking water for 1 week and then oral gavage of bacteria or saline for 24 weeks. Colons were visually examined for the number of tumors (**A**) and tumor burden (**B**) was calculated as described in the Methods and Materials section. H&E stained colon sections were evaluated for dysplasia (**C**) according to the scale described in the Methods and Materials section. An image from the TX20005-treated group with a dysplasia grade of 3.5 is shown.  $n = 5$  for saline,  $n = 7$  for *L. lactis* and TX20005, respectively. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ;  $t$  test.

addition, tumor burden in *Sg*-treated mice was significantly higher compared to both the saline and *L. lactis* control groups (Fig. 13b).

H&E stained colon sections were evaluated. Colons from *Sg*-treated mice showed a significantly higher average dysplasia grade compared to those from *L. lactis*-treated or saline control mice (Fig. 13c). Adenocarcinomas were observed in *Sg*-treated mice but not in the control groups (Fig. 13d).

We further tested the effect of *Sg* on tumor development using a different shorter procedure, in which mice were treated with four doses of AOM and gavaged with bacteria for 12 weeks. Similar to the results from the first longer procedure, a significant increase in tumor numbers was observed in *Sg*-treated mice compared to the saline control (Fig. 14a). When compared to the *L. lactis* group, mice gavaged with TX20005 also had more tumors; however, the difference was not statistically significant ( $p = 0.08$ ). Tumor burden also displayed a similar trend as that observed in the longer procedure, in which *Sg*-treated mice had a higher average tumor burden than the other two groups (Fig. 14b). In the shorter procedure, however, the difference was not statistically significant, perhaps due to reduced duration, less bacterial gavage or more AOM injections in this second procedure. Overall, results from the two procedures show a consistent

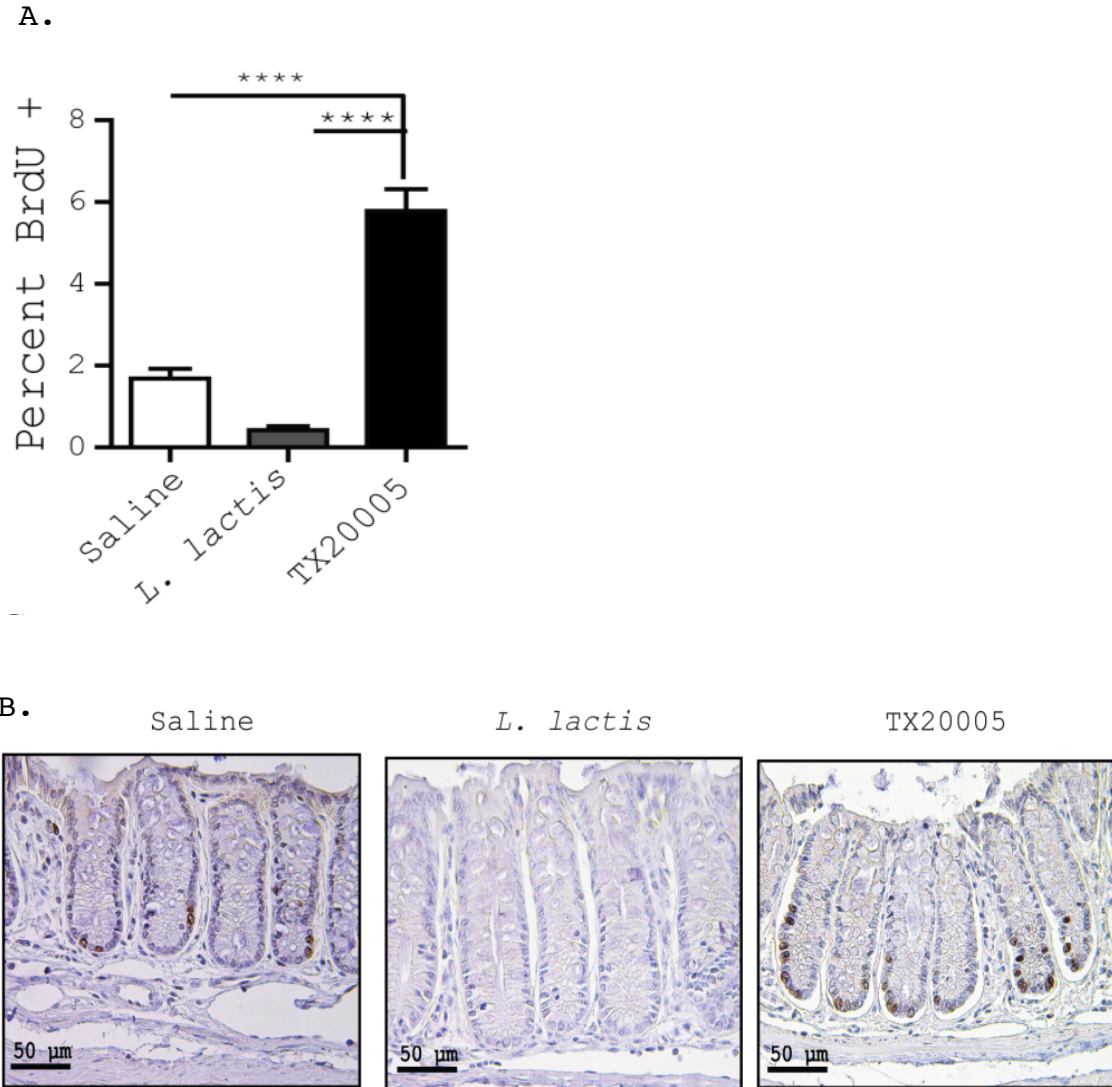


**Figure 14. *S. gallolyticus* promotes colon tumor development in an AOM-induced mouse model of CRC.** A/J mice were administered with 4 weekly i.p. injections of AOM, followed by treatment with Amp (1g/L) in drinking water for 1 week and oral gavage of *L. lactis* (n = 17), TX20005 (n = 19) or saline (n = 17) for 12 weeks. Colons were visually examined to determine tumor number (**A**). Tumor size was measured and tumor burden (**B**) was calculated as described in the Methods and Materials section. Data shown is mean ± SEM. \*,  $p < 0.05$ , t test.

trend towards *Sg* acting as a promotional agent for tumor development in the mouse colon.

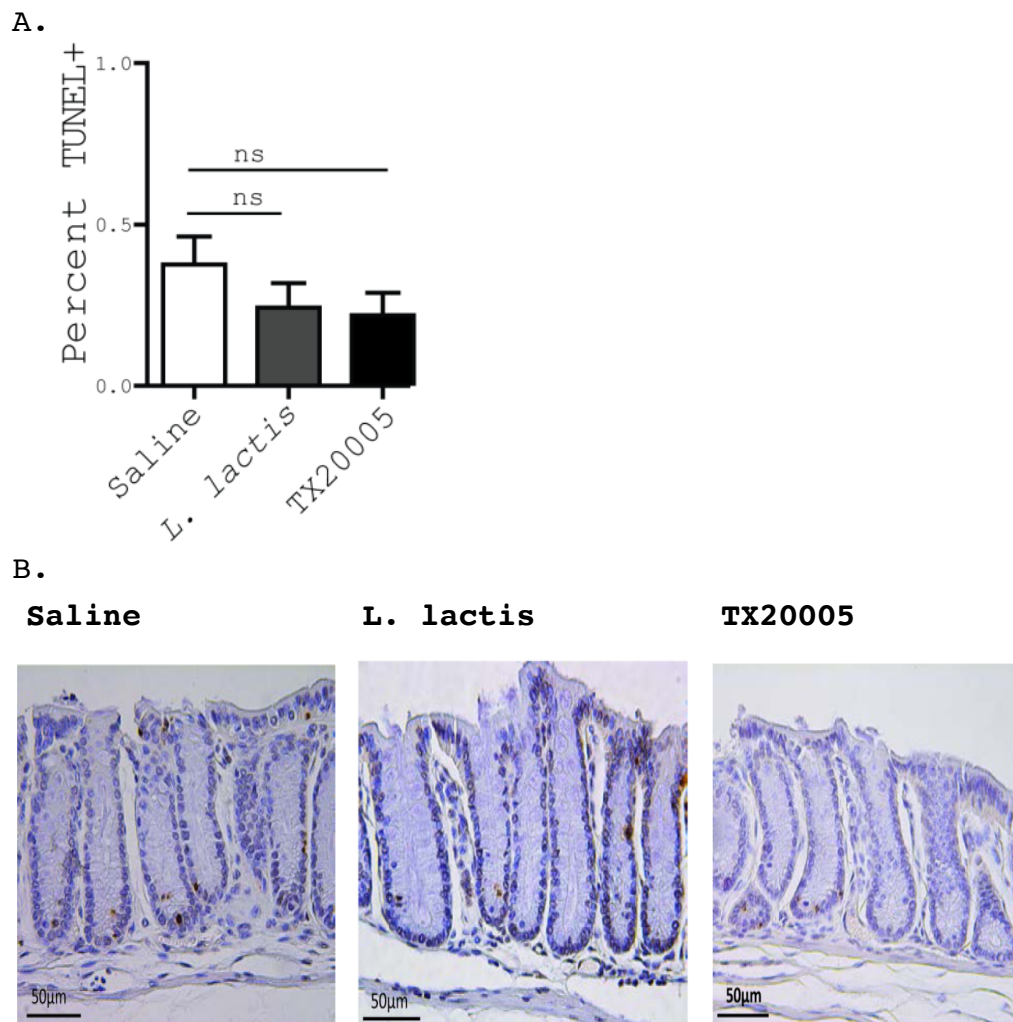
***Sg* promotes colonic crypt cell proliferation in vivo.** We next examined cell proliferation and apoptosis in mouse colonic crypt cells by determining the percentage of proliferating cells or apoptotic cells per crypt. Mouse colons were sectioned consecutively and every 10<sup>th</sup> section was counted for cells that stained positive for BrdU (proliferation marker) or cells that stained positive for TUNEL (apoptotic marker). Approximately 100 crypts were counted per mouse. *Sg*-treated animals had a significantly higher percentage of proliferating cells (BrdU<sup>+</sup>) in the colonic crypts compared to *L. lactis*- or saline-treated control groups (Fig. 15 a-b). In contrast, we did not observe any significant difference in the percentage of apoptotic cells between the different treatment groups as determined by TUNEL assays (Fig. 16 a-b). In addition, *Sg*-treated mice had higher levels of  $\beta$ -catenin in the colon epithelium as compared to *L. lactis*-treated or saline controls (Fig. 17a). These results are consistent with the observations from our *in vitro* cell culture assays.

***Sg* does not induce strong inflammatory responses.** A panel of cytokines (TNF $\alpha$ , IL-6, COX-2, IL-1 $\beta$ , IL-10, IL-17, and IL-23) were selected for their role in inflammation and evaluated in the normal and tumor tissues of mice. There was no

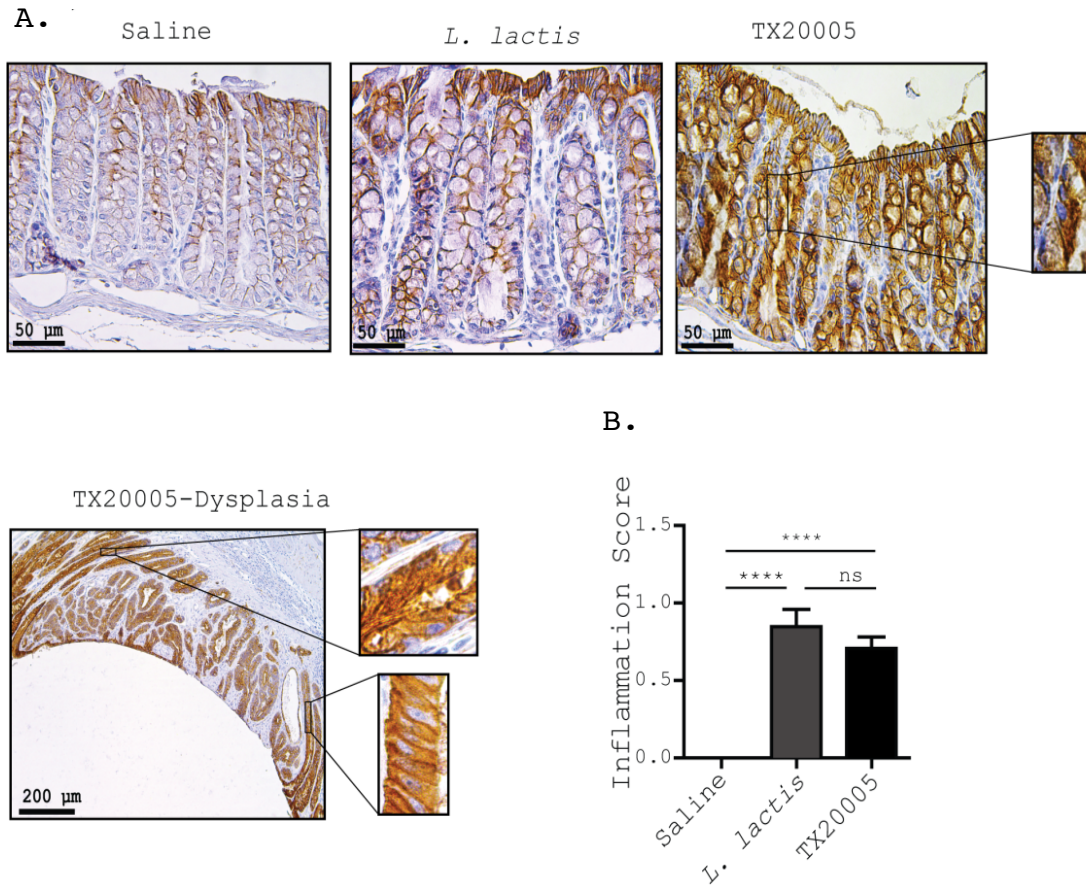


**Figure 15: *Sg* promotes colon tumor development in an AOM-induced mouse model of CRC.** A/J mice were administered 2 weekly i.p. injections of AOM, followed by treatment with Amp (1g/L) in drinking water for 1 week and then oral gavage of bacteria or saline for 24 weeks. Proliferating cells were determined by staining colon sections for BrdU incorporation (**A** and **B**).  $n = 5$  for saline,  $n = 7$  for *L. lactis* and TX20005, respectively. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ;  $t$  test.





**Figure 16. TUNEL assay of colon sections from mice treated with AOM and bacteria.** A/J mice were administered with 2 weekly i.p. injections of AOM, followed by treatment with Amp (1g/L) in drinking water for 1 week and oral gavage of saline, *L. lactis*, or TX20005 for 24 weeks. Methcarn-fixed colon sections (5 µm) were subject to TUNEL assays to detect apoptotic cells (**A** and **B**). (n = 3/group). \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; *t* test.

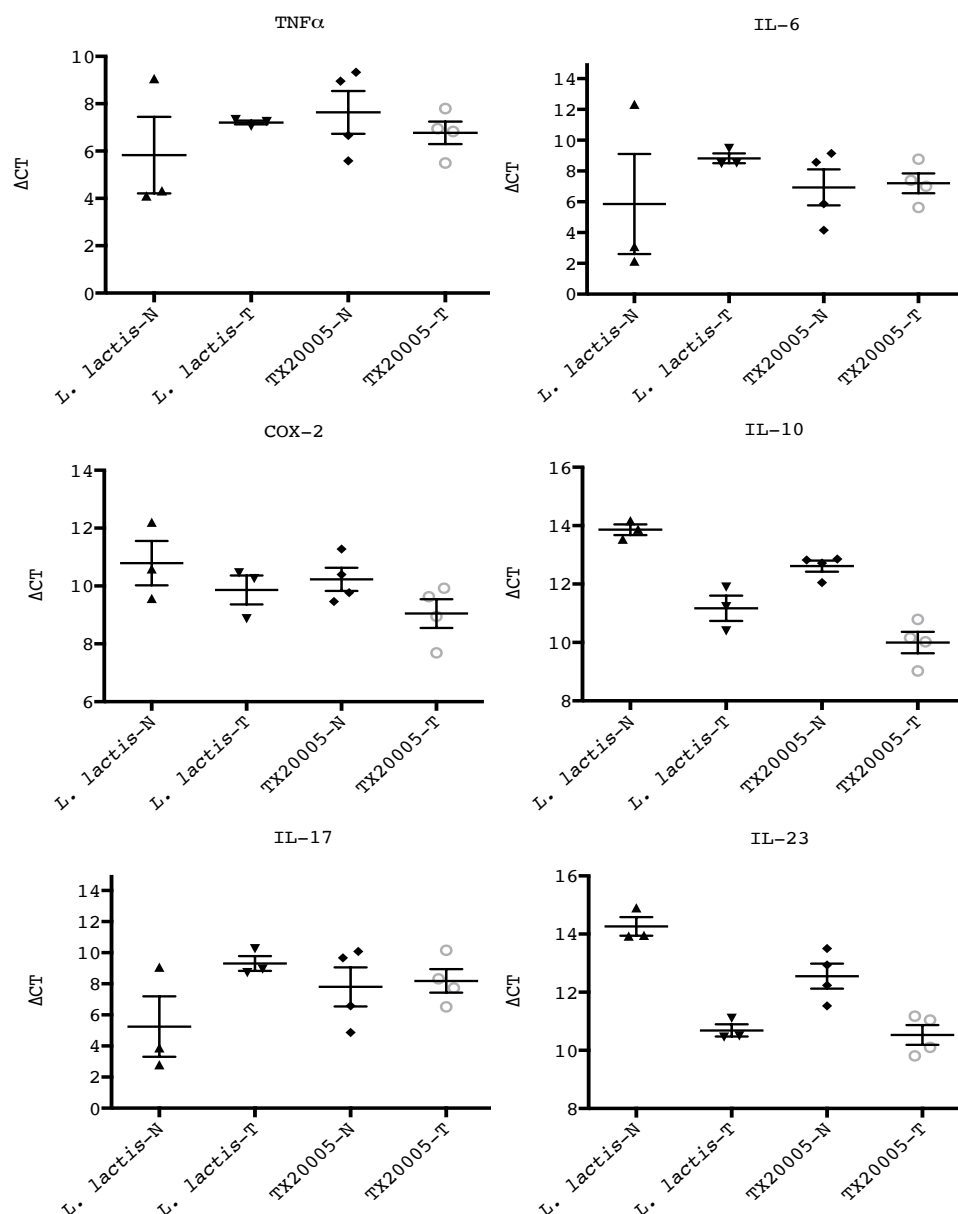


**Figure 17. *Sg* increases B-catenin levels, but does not promote inflammation in a mouse model.** Colon sections were also stained for  $\beta$ -catenin (**A**) H&E stained colon sections were also evaluated for inflammation according to the scale described in the Methods and Materials section and average inflammation score for each treatment group is shown (**B**).  $n = 5$  for saline,  $n = 7$  for *L. lactis* and TX20005, respectively. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ;  $t$  test.

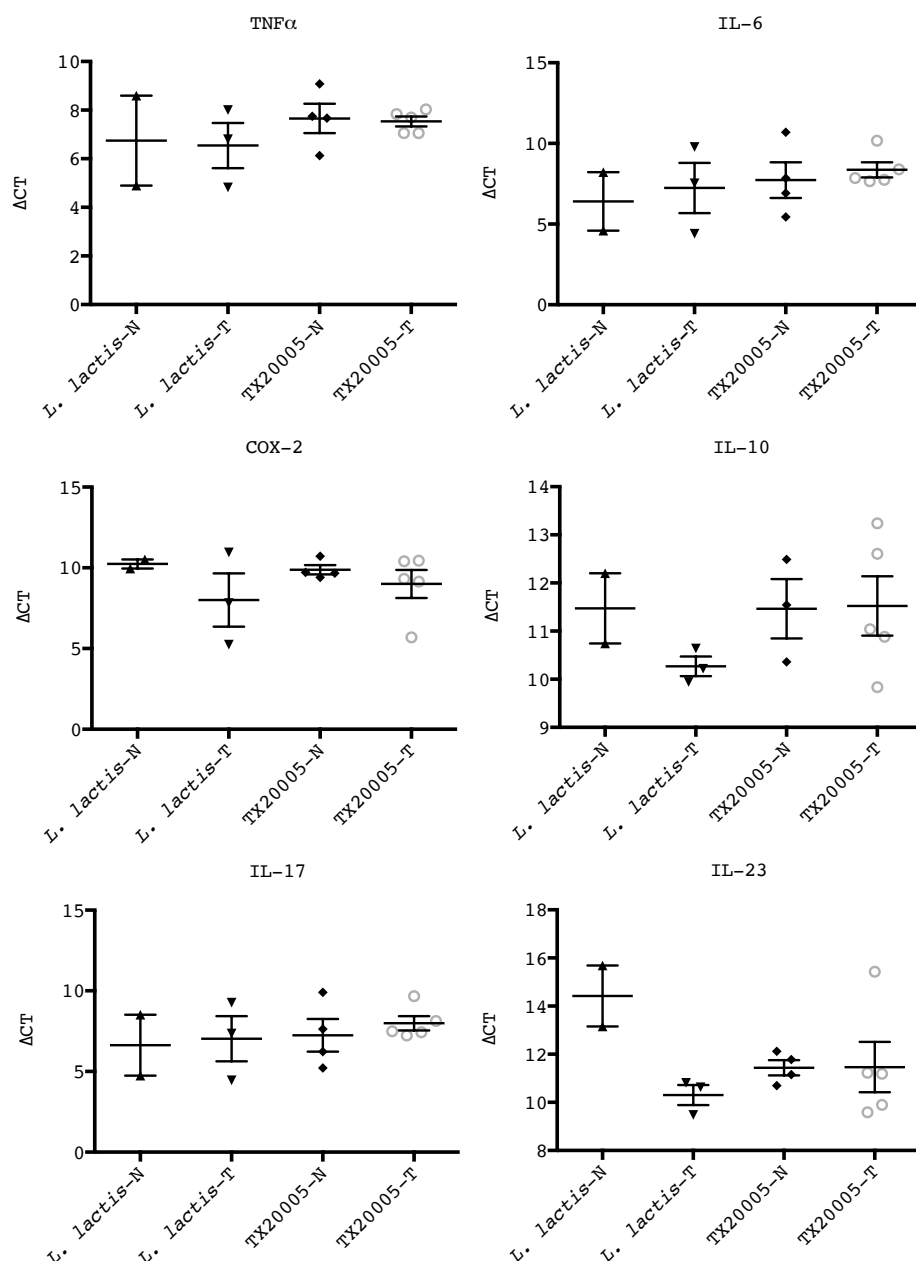
significant change in these cytokines between *Sg*- and *L. lactis*- treated groups in either the 2 AOM (Fig. 18) or 4 AOM group (Fig. 19), indicating that *Sg* does not induce a strong inflammatory response.

Inflammation in the colon of mice was also scored by evaluating the H&E stained slides. Both *Sg*- and *L. lactis*-treated groups displayed significantly higher average inflammation scores compared to the saline group. However, overall inflammation in these groups was mild, with few areas of lymphocyte infiltration and minimal epithelial hyperplasia. There was no apparent difference between the *Sg*- and *L. lactis*-treated groups (Fig. 17b), suggesting that in terms of inducing inflammation *Sg* and *L. lactis* have a similar effect.

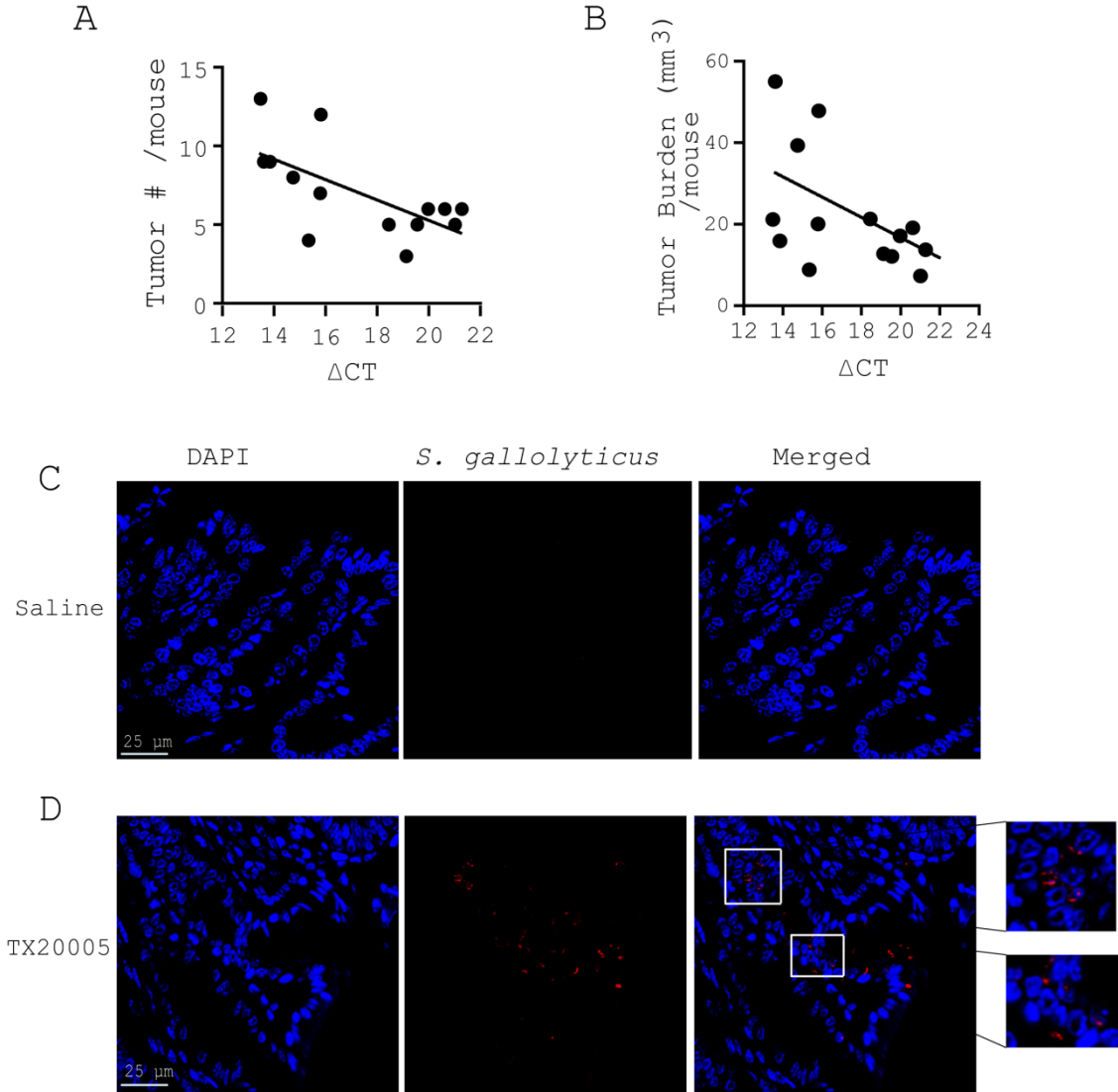
***Sg* abundance in the colon correlates with tumor multiplicity and tumor burden.** To determine whether the abundance of TX20005 in the colon correlates with tumor number or burden in the mice, we collected fecal material from mice at the end of the 12-week gavage experiment. Relative abundance of *Sg* was determined by qPCR using *Sg* specific primers. The development of the qPCR procedure is described in more detail in Chapter 3. We observed statistically significant correlations between the relative abundance of TX20005, tumor number (Fig. 20a, Pearson's  $r = -0.6548$ ,  $p =$



**Figure 18. Sg does not induce strong inflammatory responses.** At necropsy, tumor and adjacent normal tissues were collected from mice in the two AOM treatment group and immediately stored in liquid nitrogen. Tissues were then processed for RNA using the All-Prep DNA/RNA/Protein Mini kit (Qiagen) and converted to cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche). PCR primers to TNF $\alpha$ , IL-6, COX-2, IL-10, IL-17, and IL-23 were used in a qPCR reaction using SYBR Green qPCR Master Mix (Roche) in a Viia 7 Real Time PCR System (Applied Biosystems). The following cycle conditions were used: 95°C for 10 minutes followed by 40 cycles at 95°C for 30 seconds and 60°C for 1 minute.  $\Delta CT$  was normalized to the results from the qPCR reactions using  $\beta$ -actin primers.



**Figure 19. *Sg* does not induce strong inflammatory responses.** At necropsy, tumor and adjacent normal tissues were collected from mice in the four AOM treatment group and immediately stored in liquid nitrogen. Tissues were then processed for RNA using the All-Prep DNA/RNA/Protein Mini kit (Qiagen) and converted to cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche). PCR primers to TNFα, IL-6, COX-2, IL-10, IL-17, and IL-23 were used in a qPCR reaction using SYBR Green qPCR Master Mix (Roche) in a Viia 7 Real Time PCR System (Applied Biosystems). The following cycle conditions were used: 95°C for 10 minutes followed by 40 cycles at 95°C for 30 seconds and 60°C for 1 minute. ΔCT was normalized to the results from the qPCR reactions using β-actin primers.

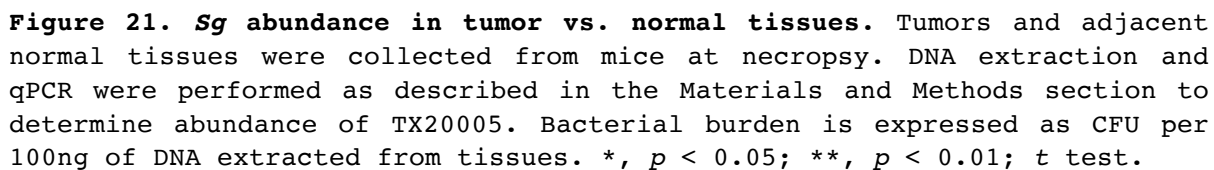


**Figure 20. Correlation of bacterial burden with tumor number and burden.** **A** and **B**. Fecal pellets were collected from mice at the end of the 12-week oral gavage of TX20005 (14 mice). DNA was extracted and analyzed by qPCR to determine the relative abundance of TX20005 as described in the Methods and Materials section. Pearson correlation analysis was performed between  $-\Delta$ CT and tumor number (**A**) and burden (**B**), respectively. **C** and **D**. Detection of *Sg* in colon tumor tissues. Methcarn-fixed paraffin embedded colon sections (5  $\mu$ m) from mice treated twice with AOM and 24 weeks of oral gavage with saline (**C**) or TX20005 (**D**) were incubated with anti-TX20005 antiserum and secondary antibodies as described in the Methods and Materials section. Nuclei were stained with DAPI.

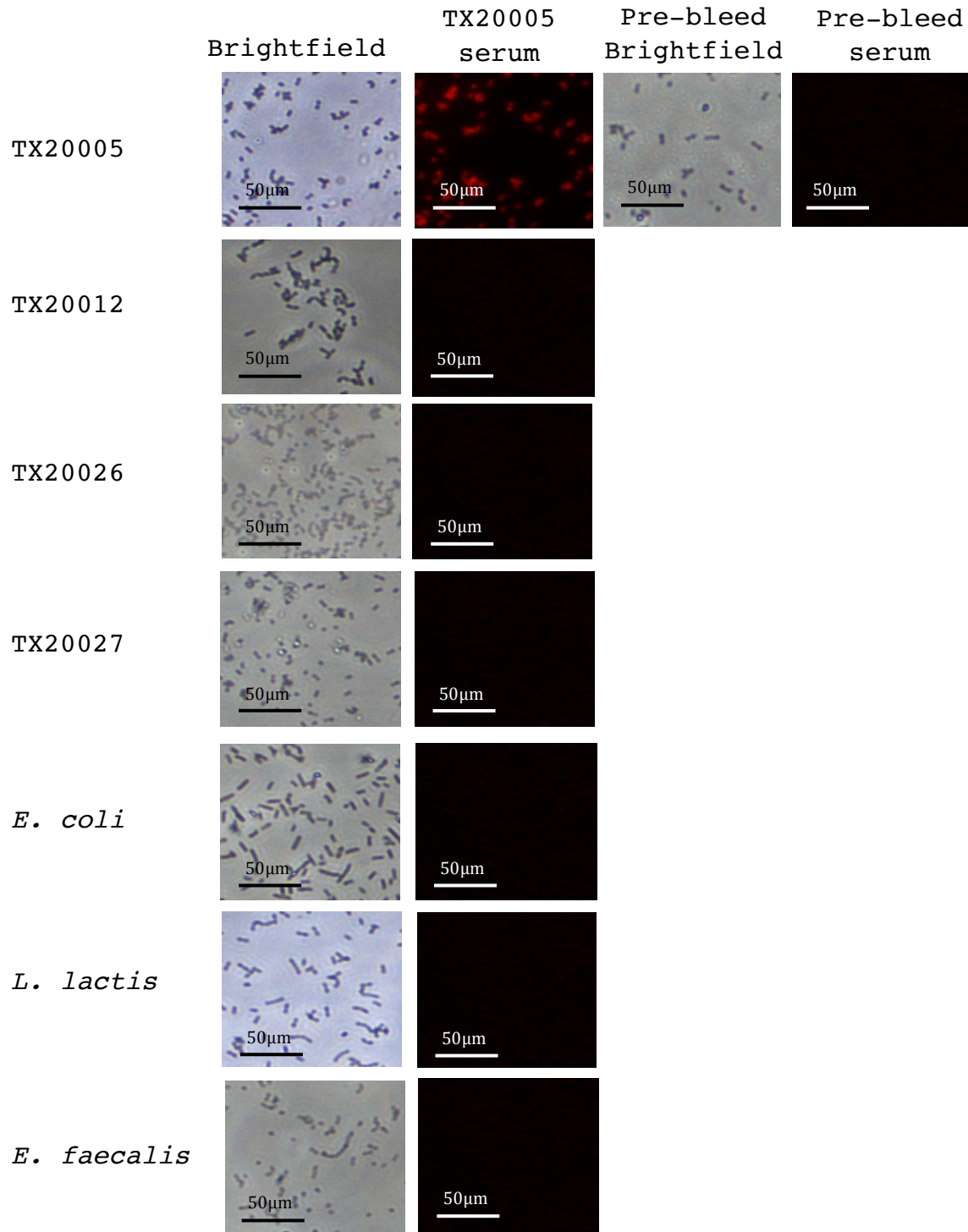
0.0111), and tumor burden (Fig. 20b, Pearson's  $r = -0.5404$ ,  $p = 0.0460$ ), respectively, suggesting a dose effect of *Sg*.

***Sg* abundance in tumor vs. normal tissues.** To evaluate the association of *Sg* with tumor vs. normal tissues in TX20005 treated mice, we collected tumor and adjacent normal tissues from mouse colons. Relative abundance in these tissues was determined by qPCR. We observed a statistically significant difference between bacterial burden in tumor tissues in comparison to normal tissues (Fig. 21). This suggests a preferential association of *Sg* with tumor tissues.

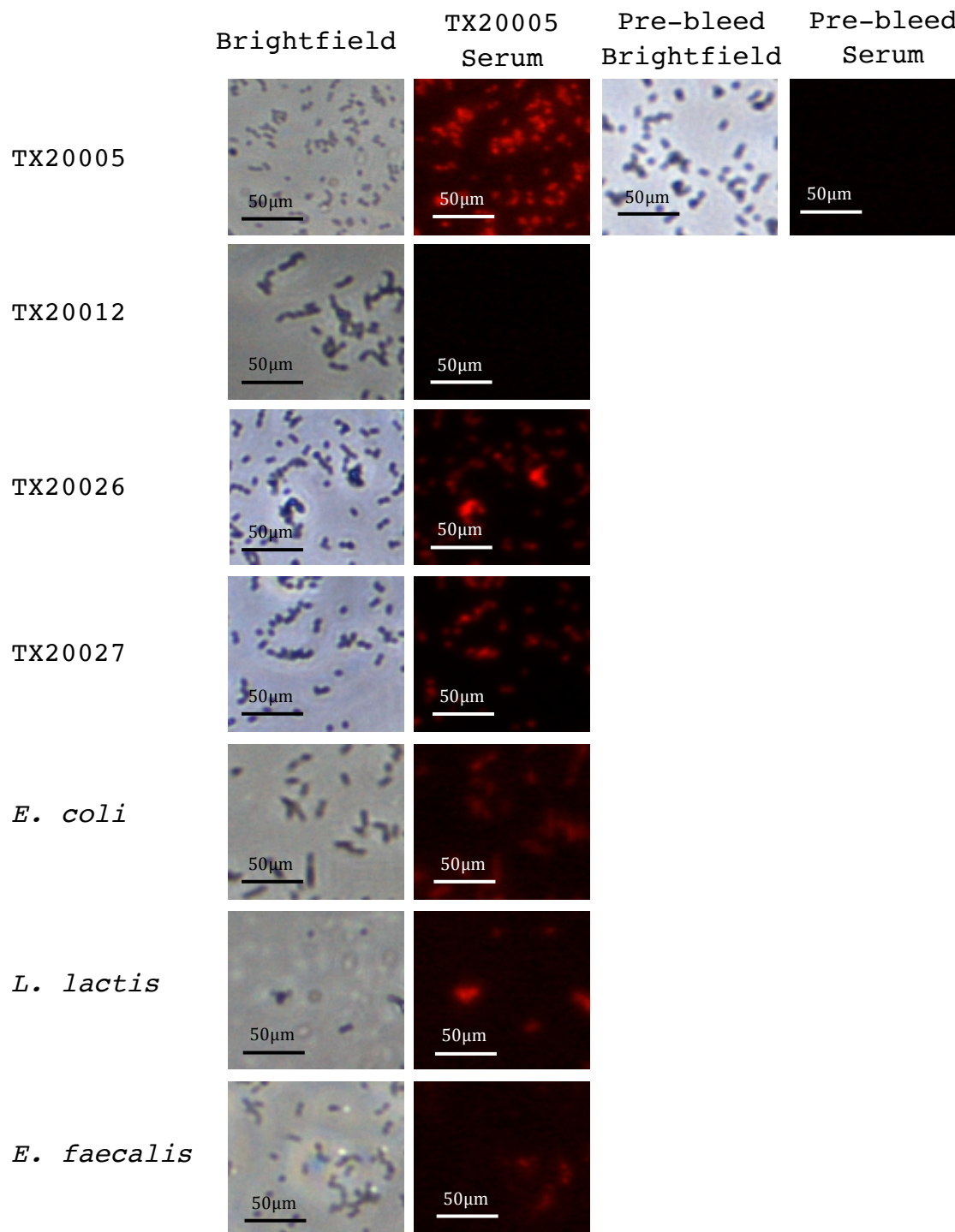
**Immunofluorescence detection of *Sg* within tumor tissues in the mouse colon.** Rabbit serum against formalin-killed TX20005 was raised. The specificity of the antiserum for two rabbits was tested against a panel of different bacteria. I tested strains of closely related species in SBSEC including *S. infantarius*, *S. macedonicus*, and *S. pasteurianus*. I also included *Enterococcus faecalis* strain V583, *E. coli* XL1-Blue and *L. lactis*. Antiserum was specific against *Sg* in the first rabbit (Fig. 22), however, the antiserum from the second rabbit showed non-specific binding to control strains (Fig. 23). Antiserum from the rabbit producing *Sg* specific antibodies was used in subsequent experiments.







**Figure 22. Specificity of rabbit 1 anti-Sg serum.** Bacteria were attached to poly-L-lysine coated coverslips, fixed with 2% paraformaldehyde, blocked with PBS containing 5% goat serum, and incubated with rabbit 1 anti-TX20005 serum (1:250) or pre-bleed serum (1:250), followed by donkey anti-rabbit Alexa Fluor (1:1000).



**Figure 23. Specificity of rabbit 2 anti-*Sg* serum.** Bacteria were attached to poly-L-lysine coated coverslips, fixed with 2% paraformaldehyde, blocked with PBS containing 5% goat serum, and incubated with rabbit 2 anti-TX20005 serum (1:250) or pre-bleed serum (1:250), followed by donkey anti-rabbit Alexa Fluor (1:1000).

Antiserum was then used to stain methcarn-fixed mouse colon sections. In colon sections, we observed positive staining in *Sg*-treated mice but not in the saline control group (Fig. 20c and 20d), further indicating that the antiserum was specific. *Sg* bacteria were found within tumor tissues. The presence of *Sg* around normal-looking crypts was observed only occasionally, suggesting a preferential association of *Sg* with tumor tissues.

#### **DISCUSSION:**

CRC is the second to third most common cancer and a leading cause of cancer death in the world. Annually, over a million people are diagnosed with CRC and ~700,000 die due to CRC [30]. In recent years, the role of microbial agents in CRC development has gained increasing recognition, raising hope that we may be able to exploit microbes to improve CRC diagnosis, prevention and treatment. To achieve this goal, a clear understanding of how precisely microbes exert their influence on tumor development is important. *Sg* has long been known to display a strong association with CRC, and yet virtually nothing was known about the nature of this association or the molecular mechanism underlying the association. The results described in this study provide exciting new insights into a tumor-promoting role of *Sg* that

is dependent on cell context, specific bacterial factors, direct contact with colon cancer cells, and  $\beta$ -catenin.

In cell-based assays carried out in our lab, *Sg* increased cell proliferation without altering apoptosis, whereas bacterial species closely related to *Sg* failed to recapitulate these effects *in vitro*, suggesting *Sg*-specific factors are involved. This finding is consistent with previous clinical observations that among the closely related species in the *S. bovis* group, *Sg* displays a particularly strong association with CRC. The results also show that promotion of cell proliferation by *Sg* depends on bacterial growth phase and direct contact between the bacteria and responsive colon cancer cells, whereas secreted bacterial factors or soluble metabolites had no apparent effect on cell proliferation as demonstrated by the results from bacterial culture supernatants and transwell assays. These results suggest that the *Sg* factor(s) responsible for mediating the promotion of cell proliferation is surface associated and up-regulated in bacterial stationary phase and that promotion of cell proliferation is likely mediated by interactions between the *Sg* surface factor and specific cell surface receptor(s). Further studies are needed to characterize the nature of these cell surface interactions.

Several lines of evidence indicating that *Sg* promotes cell proliferation through  $\beta$ -catenin. *Sg*-treated responsive cells had significantly increased levels of total and nuclear  $\beta$ -catenin, c-Myc and PCNA compared to *L. lactis*-treated cells or cells only control. *Sg* did not increase  $\beta$ -catenin level in unresponsive cells. Furthermore, knockdown of  $\beta$ -catenin in responsive cells by shRNA or inhibition of its transcriptional activity by a specific inhibitor abolished *Sg*'s effect on cell proliferation, c-Myc and PCNA. The Wnt/ $\beta$ -catenin signaling pathway regulates cell proliferation and cell fate. Dysregulation of this pathway plays a central role in the development of CRC [97-101]. It is highly pertinent, therefore, that *Sg* also targets this critical pathway in the etiology of CRC. Studies on other tumor-promoting bacteria indicate that diverse strategies are used to influence  $\beta$ -catenin signaling. For example, *Fusobacterium nucleatum* modulates  $\beta$ -catenin signaling by binding to E-cadherin through its FadA adhesin [50]. *Bacteroides fragilis* secretes a zinc-dependent metalloprotease toxin that cleaves E-cadherin, leading to nuclear translocation of  $\beta$ -catenin, increased c-Myc expression and cell proliferation [102]. *Helicobacter pylori*, which is an important cause for gastric cancer, activates  $\beta$ -catenin signaling in multiple ways including affecting the

expression of Wnt ligands [36], activating Wnt receptors [35], suppressing GSK3 $\beta$  [34, 37], interfering with  $\beta$ -catenin/TCF4 complex by down regulating the gastric tumor suppressor *Runx3* [103-105], and interacting with E-cadherin to disrupt the E-cadherin/ $\beta$ -catenin complex [33]. In addition, there have been numerous studies in recent years linking microRNA (miRNA) dysregulation to CRC (recent reviews [106-110]). Evidence indicates that microbes (*e.g.*, *H. pylori*, *Citrobacter rodentium*, and human papillomavirus (HPV)) can regulate  $\beta$ -catenin signaling and cell proliferation by affecting certain miRNAs [111-113].

In the case of *Sg*, all five of the colon cancer cell lines we tested contain mutations in the Wnt/ $\beta$ -catenin signaling pathway; HT29, LoVo, SW480 and SW1116 have mutations in APC whereas HCT116 contains a mutated version of  $\beta$ -catenin that results in increased protein stability [114, 115]. *Sg* further increases  $\beta$ -catenin level in HT29, HCT116, and LoVo, but not in SW480 and SW1116 cells. It is possible that *Sg* up-regulates  $\beta$ -catenin at a more upstream level or by affecting factors outside the canonical Wnt/ $\beta$ -catenin signaling pathway. The fact that TX20005 adheres to unresponsive colon cancer cells as well as, or even better than, responsive cells suggests that the differential effects of *Sg* on responsive and

unresponsive cells are not due to any difference in the amount of bacteria adhering to these cells. Rather, whether or how the signal is transduced from the cell surface where *Sg* is attached is likely to be responsible for the difference. It is also possible that *Sg* adheres to different receptors on responsive and unresponsive cells. Overall, our results suggest that the effect of *Sg* depends on specific cell context. This implies that not everyone colonized by *Sg* may be equally affected; some individuals with certain genetic or epigenetic makeup may be more susceptible to the tumor-promoting effect of *Sg* than others. Identifying host factors that render cells responsive to *Sg* will be important.

The results from mouse models suggest that *Sg* promotes tumor development. *Sg*-treated cells developed larger tumors in the mouse xenograft model than cells treated with control bacteria. Higher levels of  $\beta$ -catenin, c-Myc and PCNA were also observed in *Sg*-treated xenografts compared to *L. lactis*-treated ones. In the AOM model, mice treated with *Sg* had more tumors and higher tumor burden compared to *L. lactis* or saline-treated mice. This was confirmed using two different experimental procedures. In addition, *Sg*-treated mice had a higher percentage of proliferating cells and stronger  $\beta$ -catenin staining in colonic crypts compared to the control groups. Apoptosis in colon epithelial cells of *Sg*-treated mice

was similar to that in *L. lactis*-treated mice. These findings are consistent with the results from cell culture assays. Furthermore, a significant correlation was observed between *Sg* bacterial burden in the mouse colon and tumor number and burden, respectively, suggesting a dose effect. Finally, *Sg* bacteria were detected within tumor tissues; consistent with the *in vitro* finding that direct contact between *Sg* and cancer cells is important. The observation that *Sg* and *L. lactis* induced similar levels of inflammatory responses suggests that *Sg*-induced immune responses may not play a major role in *Sg*-mediated tumor promotion. However, this does not exclude the possibility that *Sg* may induce specific types of immune reactions that favor tumor development. In addition, the role of microbiota in *Sg*-mediated tumor promotion remains unclear. The results here suggest a direct effect of *Sg* on colon epithelial cells. However, whether *Sg* functions in concert with other microbial agents in the gut or elicits specific responses when mixed with certain other microbes is unknown. Further studies are needed to clarify these issues. Overall, the results presented here support a model in which an increase in colon epithelial cell proliferation through up-regulation of  $\beta$ -catenin by *Sg* is an important mechanism for *Sg*-mediated tumor promotion.



In summary, this is the first report demonstrating a tumor-promoting role of *Sg*, an organism that has long been known to have a strong association with CRC. The findings here have important clinical implications. Going forward, identifying the *Sg* factor(s) responsible for promoting cell proliferation and tumor development, and host factors that render cells responsive to *Sg*, will be important for understanding how *Sg* functions as a tumor-promoting agent and for developing optimized strategies to fight CRC by taking both bacterial and host factors into consideration.

### **Chapter 3: Prevalence of *Sg* in human patient samples.**

#### **INTRODUCTION:**

Within the *S. bovis* group *Sg* has the strongest association with CRC (~7 fold higher risk compared to infections caused by other *S. bovis* species) [10], suggesting an *Sg*-specific mechanism in the *Sg*-CRC association. It has been reported that 25 to 80% of patients with *S. bovis* bacteremia and 18 to 62% of patients with *S. bovis* endocarditis have CRC [11, 69, 116-120]. This wide range of association frequencies is most likely due to the lack of differentiation of *Sg* from other species within the *S. bovis* group, especially in earlier studies. In addition, a recent study followed patients with endocarditis due to *Sg* or *Enterococcus spp.* for an average of ~5.5 years and found that a significantly higher percentage of *Sg* endocarditis patients developed a new colonic neoplasm during the follow-up period, compared to enterococcal endocarditis patients (45.2% vs. 21%,  $p = 0.01$ ) [121].

A few studies have investigated the association between patients with *S. bovis* endocarditis and the different stages of CRC. Hoen *et al.* reported that 46.9% of patients with *S. bovis* endocarditis had adenomas, while 9.4% of patients had carcinomas, indicating an association with early colonic lesions [122]. A study by Abdulamir *et al.* found that *Sg*

selectively associates with the most aggressive polyps in the large intestine, and villous or tubulovillous adenomas [8]. Since colon cancer progresses from adenomas to carcinomas through genetic mutations, the association between *Sg* and adenomatous polyps seems to have significance and could indicate a promoter role of *S. bovis* in polyp progression.

Compared to the well-documented association between *Sg* infection and CRC, the prevalence of *Sg* in CRC patients is relatively poorly defined. While previous studies approximate that 2.5–15% of the normal population is colonized with *Sg*, there is limited data documenting the prevalence of *Sg* in CRC patients [123, 124]. A recent study by Abdulmir *et al.* evaluated the presence of *Sg* in tumor tissues of CRC patients with or without a history of bacteremia within the last 2 years through the use of conventional PCR and *in situ* hybridization (ISH) [66]. Their group detected *Sg* in 48.7% (PCR) and 46.1% (ISH) of patients with a history of bacteremia and 32.7% (PCR) and 28.8% (ISH) of patients without a history of bacteremia. This was significantly higher than the 4% (PCR) and 2% (ISH) of healthy patients that were positive for *Sg*. They also used absolute quantitative PCR to calculate the copy number of *Sg* DNA in these tissues. They found that patients with a history of bacteremia were more highly colonized than patients without a history of bacteremia and both CRC subsets

were significantly more colonized than normal control patients. This suggests that some CRC patients are “silently” infected with *Sg*, without concomitant *Sg* bacteremia or endocarditis. Additionally, a recent prospective study on 203 colonoscopy patients found a clear relationship between positivity for *S. bovis* in the colonic suction fluid and presence of malignant tumors and large polyps in the colon [65]. Specifically, all 17 malignant tumors diagnosed in this cohort were *S. bovis* positive. Due to this strong clinical association, it is recommended that patients with *Sg* infections undergo colonic evaluation.

In this study, we surveyed resected tumor and matched normal colon tissues from CRC patients and analyzed for the presence of *Sg*. We demonstrated that *Sg* is present in the majority of CRC patients and is more abundant in tumor tissues than in normal tissues from CRC patients. In addition, we evaluated the presence of another *S. bovis*, non-*Sg* strain and were only able to detect this strain in a small proportion of patients, indicating the specific association of *Sg* with CRC.

#### **EXPERIMENTAL PROCEDURE:**

**Patient characteristics.** Patient samples were provided by Dr. Scott Kopetz, Department of Medical Gastrointestinal Oncology, University of Texas M. D. Anderson Cancer Center,

Houston, TX. Patient identifiers (privileged health information - PHI) were anonymized. Summary patient information was provided by members of the Kopetz lab and can be seen in Table 2.

**Bacterial strains and culture conditions.** *S. bovis* group strains (*Sg*, *S. pasteurianus*, *S. infantarius*, *S. macedonicus*, provided by Barbara E. Murray, University of Texas Medical School, Houston, TX) [85], *Lactococcus lactis* MG1363 (provided by Timothy J. Foster, Trinity College Dublin, Ireland), and *E. coli* XL-1 Blue were grown at 37°C in brain-heart infusion (BHI) broth with shaking or on BHI agar (Difco Laboratories, Sparks, MD).

**Identification of *S. gallolyticus* by qPCR.** The genome sequences of three *Sg* strains (NC\_015215.1, NC\_013798.1 and NC\_017576.1) were compared to those of closely related species in the *S. bovis* group (*S. pasteurianus*, *S. infantarius* and *S. macedonicus*) to identify *Sg* unique sequences, which were then used to design *Sg*-specific primers (forward primer - 5' TGACGTACGATTGATATCATCAAC 3', reverse primer - 5'CGCTTAACACATTTTGTAGCTAATACG 3'). The primers were tested on a panel of *Sg* and non-*Sg* strains using conventional PCR to determine their specificity. One pair of primers that correctly amplified from all the *Sg* strains and none of the

**Table 2:** Patient characteristics.

Characteristic	
Age at surgery, mean (SEM)	62.5
Sex	
Male	88
Female	58
Tumor Stage	
II	59
III	53
IV	5

non-*Sg* strains were chosen. qPCR was performed using Fast Plus EvaGreen qPCR Master Mix (Biotium) in a Viia 7 Real Time PCR System (Applied Biosystems) with the following cycling condition: 95°C for 10 minutes followed by 40 cycles at 95°C for 30 seconds and 60°C for 1 minute.  $\Delta$ CT was normalized to the results from qPCR reactions using universal 16S rRNA primers. *S. pasteurianus* (*Sp*) specific primers (forward primer-ATGGATAGTCATAGAATTGA reverse primer-GGACAATGCCCTCATCTAGC) were developed following the same strategy as described for *Sg* primers. The primers were tested on a panel of *Sp* and non-*Sp* strains and *Sp* specific primers were chosen.

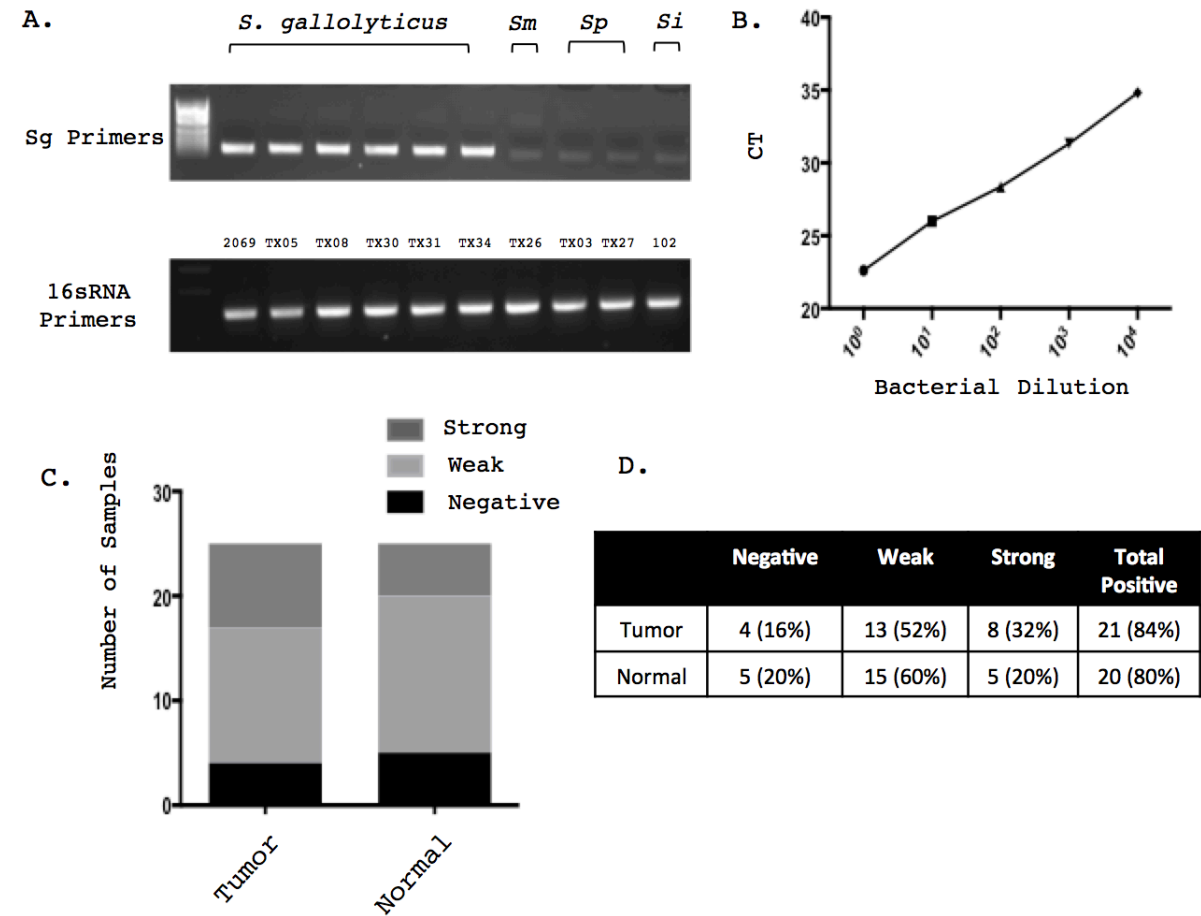
## **RESULTS:**

**Establishing a protocol for specific detection of *Sg* in colon tissues.** *Sg*-specific primers targeting *Sg*-unique sequences were designed and tested against a panel of 6 *Sg* strains and 4 strains of closely related species (*S. infantarius*, *S. macedonicus*, and *S. pasteurianus*, previously belonging to the same *S. bovis* group as *Sg*). The primers correctly identified all 6 *Sg* strains and none of the 4 non-*Sg* strains (Fig. 24a). The primers were then used in qPCR reactions to detect *Sg* in mouse colon tissues spiked with serially diluted *Sg* to establish a standard linear range (Fig.

24b). Mouse colon tissues without *Sg* were used as a control. This procedure was then used on ~25 pairs of colon tumor and matched normal tissues from CRC patients (Fig. 24c-d). The positive samples were further analyzed for melting temperature ( $T_m$ ) to determine the presence of spurious amplification. Of the samples with the correct  $T_m$ , PCR products were purified using a Qiagen gel extraction kit and subject to DNA sequencing. The results showed that all of the products contained the correct *Sg* sequence.

**Detection of *S. gallolyticus* subsp. *pasterianus* in colon tumor and normal tissues from CRC patients.** *S. gallolyticus* subsp. *pasterianus* (previously *S. bovis* biotype II/2) was closely related to *Sg*, however patients with endocarditis due to *Sp* did not have a strong association with CRC [13, 20, 125-129]. *Sp*-specific primers were designed and tested for their specificity by PCR. The results showed that the primers correctly identified *Sp* but not *Sg* (Fig. 25a). The primers were then used in qPCR reactions to detect *Sp* in mouse colon tissues spiked with serially diluted bacteria to establish a standard linear range (Fig. 25b). The results showed that *Sp* were detected in ~8% of tumor and 12% of normal tissues. These percentages were much lower than those of *Sg*, consistent with previous observations (Fig. 25c-d).



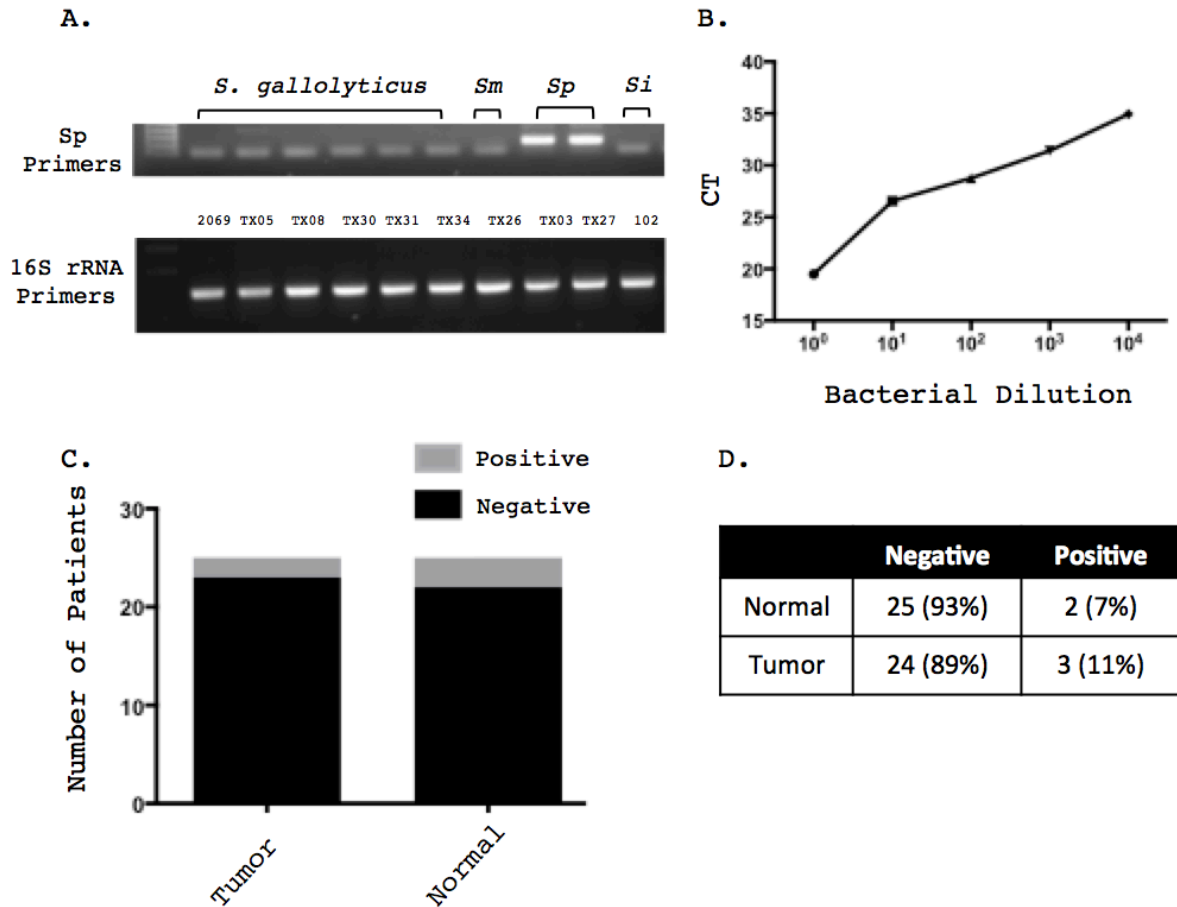


**Figure 24. Establishing a protocol for specific detection of *Sg* in colon tissues.** *Sg*, *Sm*, *Sp*, *Si*, and 16sRNA primers were used to determine *Sg* primer specificity by PCR. PCR conditions are as follows: annealing temperature 60°C, 30 cycles (A). DNA was extracted from serially diluted *Sg* spiked mouse tissues and qPCR was performed to generate a standard curve as described in Materials and Methods (B). Quantification of 25 matched tumor and normal samples with no CT, low CT, or high CT. Cutoff for high CT (strong) was more than 5 CTs from the mean and cutoff for low CT (weak) was within 5 CTs of the mean (C-D).

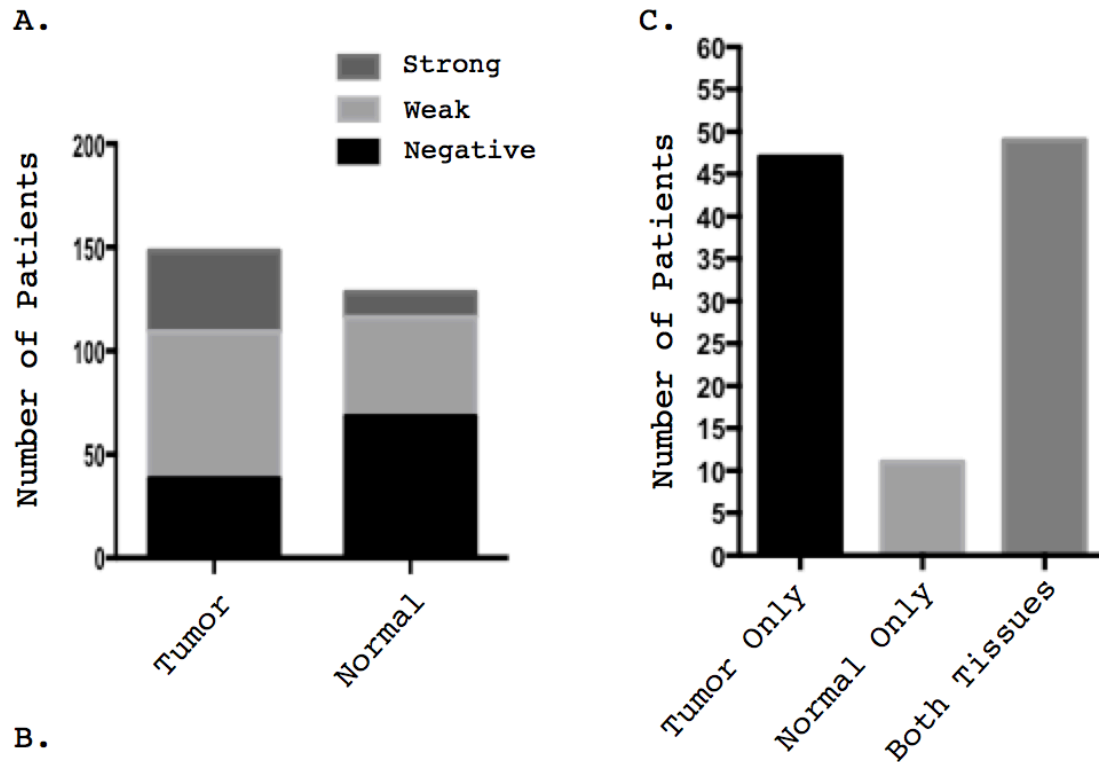
*Sg* is present in the majority of CRC patients and preferentially associates with tumor tissues. I further analyzed an additional 121 tumors and 101 adjacent normal tissues from CRC patients. Overall, we found that ~74% of tumor tissues and ~47% of the normal tissues were positive for *Sg* ( $p < 0.0001$ , tumor vs. normal, Fisher's exact test), suggesting *Sg* is present in the majority of CRC patients and preferentially associates with tumor tissues (Fig. 26a, c). We further divided the positive samples into those with relatively high or low abundance of *Sg*. The high abundance is arbitrarily defined as with a 5 CT cutoff from the mean. More tumor tissues were highly enriched with *Sg* (26%) than normal tissues (9%) indicating a higher bacterial abundance in the tumor tissues (Fig. 26a-b).

## **DISCUSSION:**

McCoy and Mason first reported an association of *Sg* with CRC in 1951 and this association has since been verified by numerous case reports and case series. However, most reports are retrospective and only evaluate CRC prevalence in patients with an *Sg* infection. On the other hand, few studies have investigated the prevalence of *Sg* infections within the CRC population. In this study, our results demonstrate that *Sg* is found at a high prevalence within the CRC population and that



**Figure 25. Detection of *S. gallolyticus* subsp. *pasterianus* in colon tumor and normal tissues from CRC patients.** *Sg*, *Sm*, *Sp*, *Si*, and 16sRNA primers were used to determine *Sp* primer specificity by PCR. PCR conditions are as follows: annealing temperature 60°C, 30 cycles (A). DNA was extracted from serially diluted *Sp* spiked mouse tissues and qPCR was performed to generate a standard curve as described in Materials and Methods (B). Quantification of 27 matched tumor and normal samples that are positive or negative for *Sp* (C-D).



	Negative	Weak	Strong	Total Positive
Tumor	38 (26%)	71 (48%)	39 (26%)	110 (74%)
Normal	68 (53%)	48 (38%)	12 (9%)	60 (47%)

Fisher's exact test,  $p < 0.0001$

**Figure 26. *Sg* is present in the majority of CRC patients and preferentially associates with tumor tissues.** An additional 121 tumor tissues and 101 matched normal tissues were evaluated for the presence of *Sg* using primers and the qPCR method described previously. Quantification of these samples was determined based on no CT, low CT, or high CT. Cutoff for high CT (strong) was more than 5 CTs from the mean and cutoff for low CT (weak) was within 5 CTs of the mean (A-B). The number of patients with *Sg* present in only the tumor tissue, normal tissue, or both tissues was calculated (C).

*Sg* has a preferential association with tumor tissues vs. matched normal tissues.

In this study we developed a method for detecting *Sg* in patient samples. Previously, *Sg* was identified through biochemical analysis and conventional PCR [66]. Here, we have developed a sensitive and specific qPCR technique for detecting *Sg* that can be performed in a high-throughput manner. This technique allows for the detection of *Sg* and a determination of abundance. From this analysis, ~74% of CRC patients samples were positive for *Sg*, which is higher than the results reported by Adulamir *et al.* (32.7% in patients without a history of bacteremia). This may be explained by our study's use of qPCR, a more sensitive technique than conventional PCR. Additionally, *Sg* has a preferential association with tumor tissues. In comparison, a closely related non-*Sg* strain was almost completely absent from these patient samples. These results corroborate previous reports in the literature, demonstrating a high prevalence of *Sg* in CRC cases-- showing a significantly higher prevalence of *Sg* over closely related strains.

## **Chapter 4: *Sg* adherence correlates with cell proliferation.**

### **INTRODUCTION:**

*Sg* is estimated to colonize 2-15% of the normal population [123, 124]. However, an approximately 5-fold increase of *Sg* is seen in patients with CRC [77] and approximately 60% of patients diagnosed with an *Sg* infection were found to have a polyp or CRC [79]. Until recently it was unknown whether *Sg* was a cause or a consequence of CRC, but our group has demonstrated the tumor promoting abilities of *Sg* both *in vitro* and *in vivo*, (described in Chapter 2) further strengthening the importance of *Sg*.

Currently there is limited information concerning *Sg* adhesins and cell receptors responsible for *Sg*-host interactions. We have shown that *Sg* can adhere to CRC cell lines, but the mechanism of interaction has yet to be elucidated. Recently, a study was published that defined the role of Pil3B in mediating adherence to CRC cell lines and colonizing the mouse gut [77]. However, other factors are involved as demonstrated by the ability of pil3 deficient *Sg* to bind CRC cells and colonize the gut, albeit to a lesser degree.

Studies from our laboratory showed that the ability of *Sg* to promote colon cancer cell proliferation is strain

dependent, i.e., some *Sg* strains were able to promote cell proliferation whereas others were not (Fig. 4a, d). Here I characterized the ability of two of these strains to adhere to colon cancer cells and to colonize the mouse colon. The results showed that the strain that was able to promote proliferation also adhered more efficiently to CRC cell lines and more efficiently colonized the mouse gut. Additionally, the adherent *Sg* strain correlated with increased tumor burden in a xenograft mouse model.

#### **EXPERIMENTAL PROCEDURE:**

**Bacterial strains and culture conditions.** *S. bovis* group strains (*Sg*, *S. pasteurianus*, *S. infantarius*, *S. macedonicus*) were grown at 37°C in brain-heart infusion (BHI) broth with shaking or on BHI agar (Difco Laboratories, Sparks, MD).

**Cell lines and growth conditions.** Human colon cancer cell line HCT116 was cultured in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO, USA) supplemented with 10% fetal bovine serum (FBS) (GIBCO, USA).

**Adherence assay.** This was performed following a procedure described previously with slight modifications [93]. Cells were seeded onto the wells of 24-well tissue culture plates at  $10^6$  cells/well. Bacteria from a stationary phase culture were washed twice in PBS, resuspended in DMEM supplemented with 10%

FBS, and added to the wells at a multiplicity of infection (MOI) of 10. The plates were incubated in a humidified incubation chamber at 37°C with 5% CO<sub>2</sub> for 1 hour. Each well was washed three times with sterile PBS to remove unbound bacteria. To determine the number of associated bacteria, cells were lysed with sterile PBS containing 0.025% Triton X-100 and dilution plated. A  $\beta$ 1 integrin antibody was purchased from Developmental Studies Hybridoma Bank and used at a concentration of 1.25  $\mu$ g/ml or 2.5  $\mu$ g/ml for adherence blocking experiments. All experiments were performed in triplicate and repeated at least three times. Adherence was expressed as a percentage of total bacteria added.

**Animal experiments.** Animal studies were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at the Texas A&M Health Science Center, Institute of Biosciences and Technology. Mice were fed with standard ProLab IsoPro RMH3000 (LabDiet). (1) **Colonization model.** Eight-week old female A/J or C57bl/6 mice (Jackson Laboratory, Bar Harbor, ME) were orally gavaged with TX20005 or TX20008 using a feeding needle ( $\sim 1 \times 10^9$  cfu/mouse) and then euthanized after 3 or 7 days. Colons were removed and frozen at -20°C until DNA extraction was performed. (2) **Xenograft model.** HCT116 cells ( $1 \times 10^6$ ) were incubated with TX20005 or TX20008 (MOI = 1) for 12 hours. The cells were



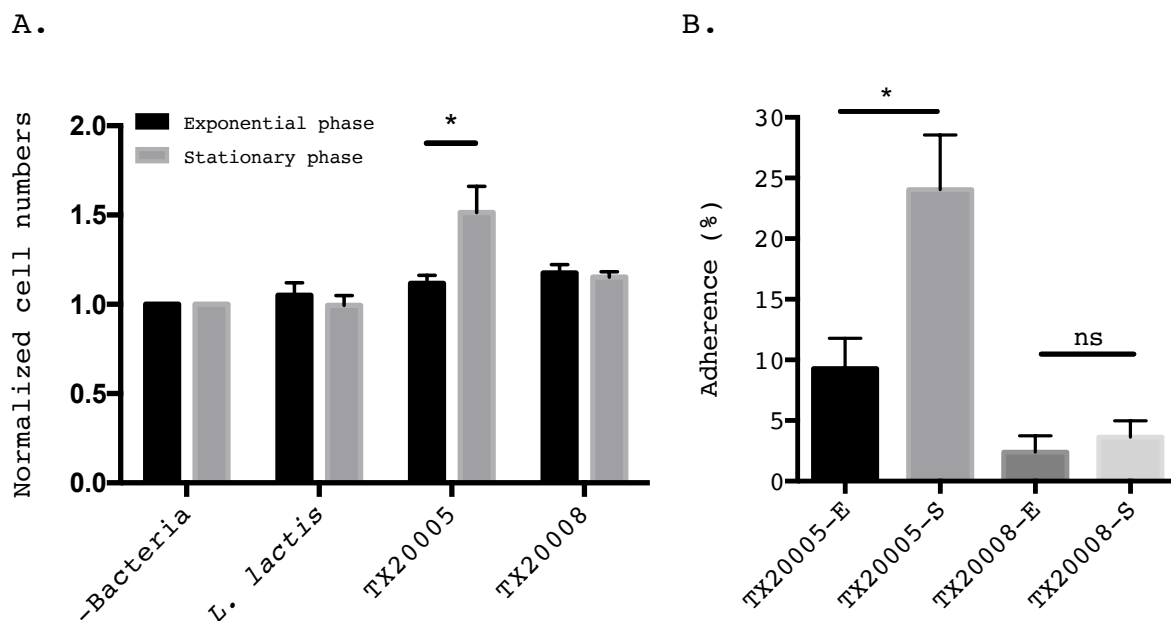
immediately washed, trypsinized and mixed with Matrigel (Corning, MA) according to the manufacture's instructions and subcutaneously injected (100  $\mu$ l) into the dorsal flap of 5-week-old nude mice (Jackson Laboratory, Bar Harbor, ME). Three hours after the injection, mice were administered a broad-spectrum antibiotic imipenem (MSD) by intraperitoneal (i.p.) injection (150 mg/kg body weight). Tumor diameters were measured with a digital caliper, and tumor volume calculated using the formula: Volume = (d1xd1xd2)/2, with d1 being the larger dimension[94].

**Detection of *Sg* by qPCR.** Fecal pellets were collected from mice at days 3 and 7. DNA was extracted using QIAamp Fast DNA Stool Mini Kit (Qiagen) following manufacturer's instructions. DNA was extracted from frozen colons at days 3 and 7 according to standard protocol. PCR primers were designed using *Sg*-specific sequences (forward primer – 5' TGACGTACGATTGATATCATCAAC 3', reverse primer – 5'CGCTTAACACATTTTGTAGCTAATACG 3'). qPCR was performed using Fast Plus EvaGreen qPCR Master Mix (Biotium) in a Viia 7 Real Time PCR System (Applied Biosystems) with the following cycling condition: 95°C for 10 minutes followed by 40 cycles at 95°C for 30 seconds and 60°C for 1 minute.  $\Delta$ CT was normalized to the results from qPCR reactions using universal 16S rRNA primers.

## RESULTS:

**The ability of *Sg* to adhere to cell lines correlates with its ability to promote cell proliferation.** Unpublished data from our lab showed that TX20005 has the ability to promote cell proliferation in HCT116 cells, whereas TX20008 does not (Fig. 27a). We also demonstrated that the proliferation-promoting effect of *Sg* requires stationary phase bacteria, and direct contact between bacteria and cells. Due to these requirements, I next examined the ability of TX20005 and TX20008 to adhere to HCT116 cells. I determined that TX20005 adhered significantly better to these cells than TX20008, suggesting a correlation between the ability to adhere to cell lines and the ability to promote cell proliferation (Fig. 27b).

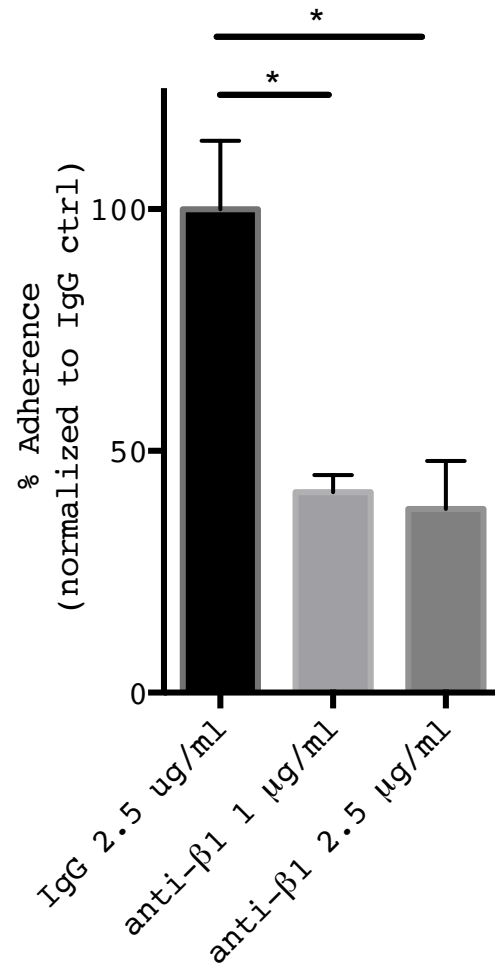
**Involvement of  $\beta 1$  integrin in mediating *Sg* adherence to colon cancer cells.** In unpublished data we have shown that *Sg* promotes cell proliferation of HCT116 cells in a  $\beta$ -catenin dependent manner. These findings involving  $\beta$ -catenin were a pivotal step in pursuing whether *Sg* infection was a cause or a consequence of CRC, and appeared to bolster causal evidence. Since the  $\beta 1$  integrin can regulate the Wnt/ $\beta$ -catenin signaling pathway, this led us to investigate a possible role of  $\beta 1$  integrin as a receptor for mediating *Sg* adherence.



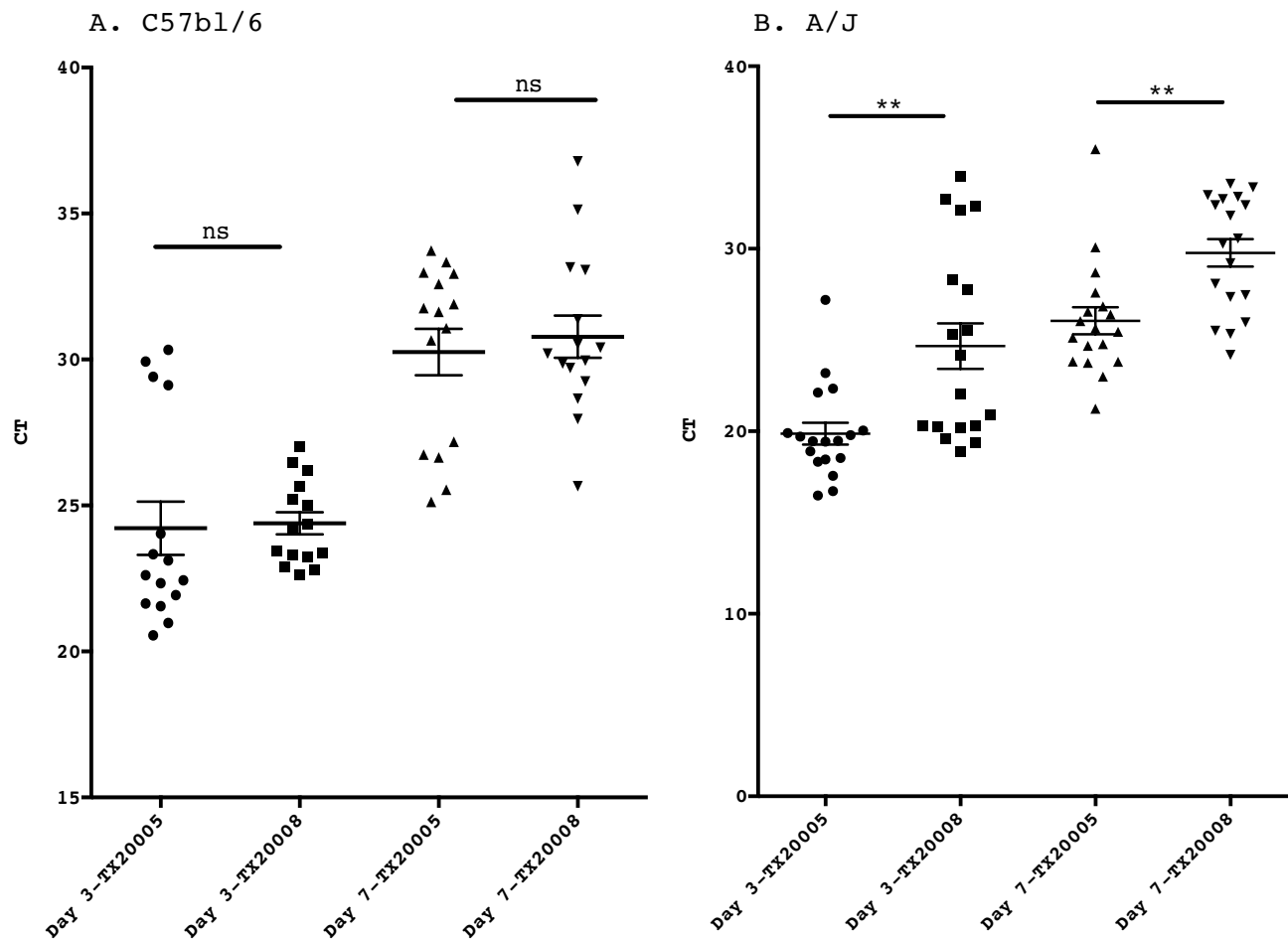
**Figure 27. *Sg*'s ability to adhere to cell lines correlates with its ability to promote cell proliferation.** Proliferation (A) and adherence (B) were determined for TX20005 and TX20008 treated HCT116 cells as described previously. Briefly, for proliferation,  $1 \times 10^4$  HCT116 cells were seeded in plates and incubated for 12 hours. Cells were then co-cultured with  $1 \times 10^2$  TX20005, TX20008, or no bacteria for 24 hours. Cells counts were performed using an automated cell counter. For adherence, TX20005 was added to wells containing HCT116 cells at a multiplicity of infection (MOI) of 10, as previously described. The plates were incubated for 1 hr. To determine the number of adhered bacteria, cells were lysed with sterile PBS containing 0.025% Triton X-100 and dilution plated. Adherence was expressed as the percentage of adhered bacteria vs. total bacteria added. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; t test.

Adherence assays were performed in the presence of a  $\beta 1$  integrin-blocking antibody to determine if *Sg* TX20005 adheres to colon cancer cells via the integrin. In comparison to controls, the blocking antibody significantly reduced *Sg* TX20005 adherence to HCT116 cells (Fig. 28). These results suggest involvement of  $\beta 1$  integrin in *Sg* adherence to CRC cells. Further studies are needed to confirm the involvement of  $\beta 1$  integrin in *Sg* adherence to colon cancer cells and to identify the  $\alpha$  subunit of the integrin.

***Sg* TX20005 is more efficient at colonizing A/J mice colons than *Sg* TX20008.** Next, we evaluated the ability of TX20005 and TX20008 to colonize the mouse colon. This was performed in both C57bl/6 and A/J mice and analyzed for bacterial burden in colon tissues. For colon tissues, I first evaluated *Sg* burden in the proximal and distal colons by sectioning the colon into two parts. However, we found no difference in *Sg* burden between the proximal and distal colons and subsequently combined proximal and distal colons for analysis. At 3 days post inoculation, there was no significant difference in bacterial burden between mice gavaged with TX20005 and TX20008. Similar results were seen at day 7 (Fig. 29a). However, in A/J mice, there was a significant difference between TX20005 and TX20008 abundance at both days 3 and 7.



**Figure 28. Involvement of  $\beta 1$  integrin in mediating *Sg* adherence to colon cancer cells.** HCT116 cells were pre-treated with  $\beta 1$  integrin-blocking antibody (Developmental Studies Hybridoma Bank) at a concentration of 1.25  $\mu\text{g/ml}$  or 2.5  $\mu\text{g/ml}$  or an IgG control (Cell Signaling Technologies) at a concentration of 2.5  $\mu\text{g/ml}$  for 30 minutes in a humidified chamber at 37°C with 5%  $\text{CO}_2$ , then washed three times with PBS to remove unbound antibody. TX20005 was added to wells at an MOI of 10 and incubated for 1 hr at 37°C. Cells were then washed three times to remove non-adherent bacteria and plated. Adherence was expressed as the percentage of adhered bacteria vs. total bacteria added and normalized to the IgG control. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ;  $t$  test.

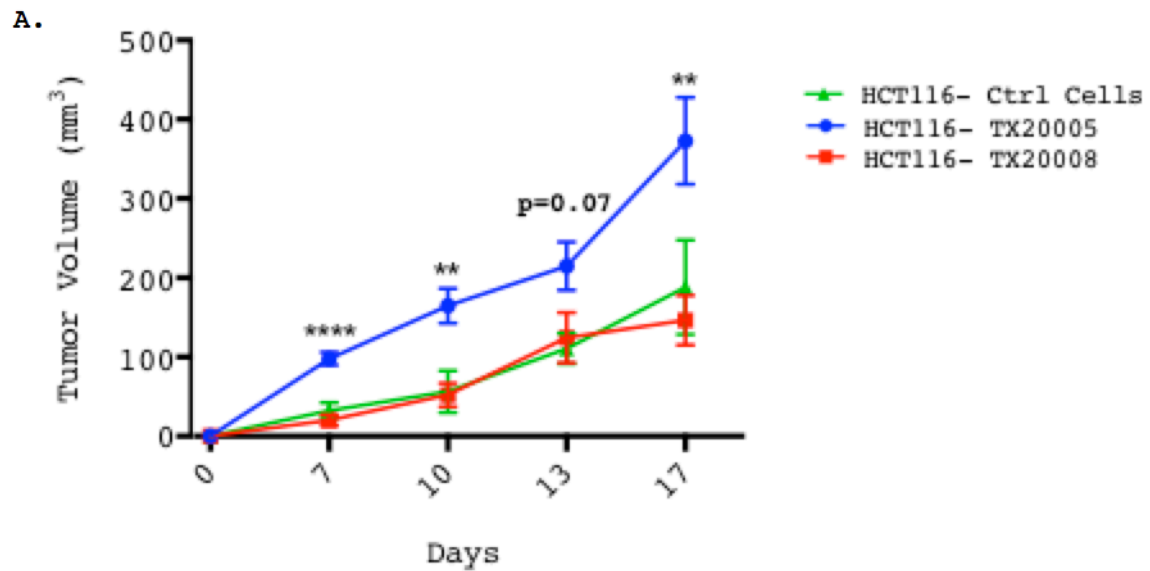


**Figure 29. Colonization of mice by *Sg*.** (A) C57bl/6 and (B) A/J mice were treated with Ampicillin (1g/L) in drinking water for 1 week and then orally gavaged with TX20005 at a dose of  $1 \times 10^9$  CFU/mouse. At days 3 and 7 post-gavage, mice were sacrificed and colons were collected. DNA was extracted and qPCR was performed as described in Materials and Methods for detection of TX20005. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ;  $t$  test.

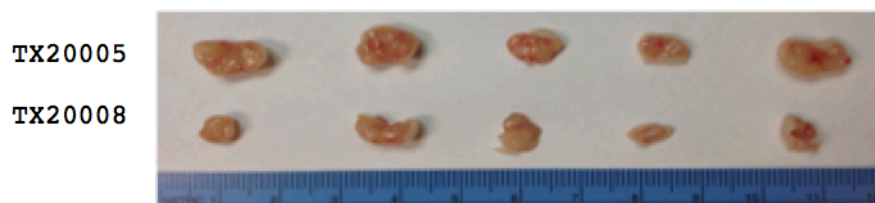
This may suggest that the host genetic background plays a role in *Sg* colonization.

***Sg* TX20008 does not promote tumor growth in a xenograft model.** To further evaluate the correlation between adherent strains and the ability to promote proliferation we evaluated the effects of TX20005 and TX20008 in a xenograft model. Cells were co-cultured with TX20005 or TX20008 for 12 hours at an MOI of 1 and then injected subcutaneously into the dorsal flap of nude mice. Mice were monitored for 17 days and tumor size was measured every 3-4 days. At day 7, mice injected with cells co-cultured with TX20005 had significantly larger tumors and this trend continued through day 17 (Fig. 30).

**Investigation of the potential of TX20008 to promote tumor growth in the AOM-model.** To further determine the tumor promoting effects of TX20008 in vivo, we examined its effect in the AOM mouse model. Mice were given 4 weekly injections of AOM, followed by one week of antibiotic treatment and 24 weeks of bacterial oral gavage. Unfortunately, mice treated with TX20008 did not survive. After the second bacterial gavage 3 out of 5 mice died unexpectedly. With only two TX20008 mice remaining the experiment was ended, due to the lack of statistical value from two mice. This experiment was repeated and again, 8 out of 10 mice died shortly after gavages were



B.



**Figure 30. TX20005 promotes tumor growth in a xenograft model.**  $\sim 1 \times 10^5$  HCT116 cells were treated with TX20005 or TX20008, mixed with Matrigel and injected into the dorsal flap of nude mice (n=5/group) as described previously. Tumor size was measured during the indicated time period with a digital caliper (A). Mice were euthanized when tumors exceeded 15mm and tumors were collected for final measurements (B).



administered. The cause of lethality by TX20008 is currently unknown.

## **DISCUSSION:**

CRC is one of the leading causes of cancer death, with over a million people diagnosed annually [30]. Recently, the role of microbial agents in cancer development has gained significant interest, with many bacteria being linked to its development [28, 63, 130]. Many examples from the literature have further increased this interest such as *H. pylori* in the development of gastric cancer [6, 130] and *F. nucleatum* in the promotion of colon cancer [46, 48, 131]. Understanding more details about the roles of these bacteria in cancer development will lead to greater prevention, earlier diagnoses, and improved treatment strategies.

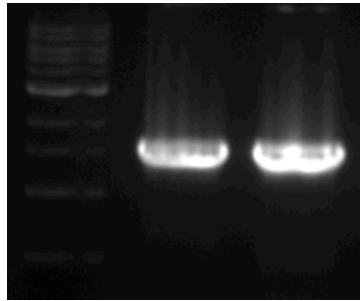
Since the 1950s *Sg* has been associated with CRC, but it has not been determined whether it is a cause or a consequence of CRC. However, our group recently has shown that both *in vitro* and *in vivo* *Sg* leads to increased cell proliferation and tumor development through a currently unknown mechanism. We also found that direct contact between CRC cells and *Sg* was required, which led us to investigate the ability of *Sg* to adhere to CRC cells. *In vitro* we found that while some of our *Sg* strains were able to promote proliferation, others were

not. Strains that were capable of promoting proliferation were also capable of adhering to CRC cell lines significantly better. These results are consistent with our finding that direct contact between *Sg* and colon cancer cells is required for the bacteria to stimulate proliferation. The results also suggest that there is polymorphism among *Sg* strains with respect to their ability to adhere to colon cancer cells and to promote cell proliferation. Furthermore, it is possible that those *Sg* strains defective in cell adherence and promotion of cell proliferation are also defective in promoting tumor development in vivo. Thus, further studies to identify the bacterial adhesins responsible for this interaction and how those vary among different strains are needed. With respect to host receptors mediating *Sg* adherence, the result suggests a role for  $\beta 1$  integrin in the interaction. Kwok *et al.* in 2007 reported that *H. pylori* interacted with and activated host cells by binding to integrin  $\alpha 5\beta 1$  [132]. We were able to show through an adherence assay that when we treated CRC cells with a  $\beta 1$  integrin blocking antibody we significantly decreased the ability of *Sg* to bind to cells, indicating a possible role of this integrin in the *Sg*-CRC cell interaction. Further studies such as gene knockdown or inhibitory or competitive ligands, are required to confirm  $\beta 1$  integrin involvement.

In mice, a bacterial colonization model, showed that *Sg* colonized the mouse gut; however, strain-to-strain variation was observed. TX20005 was better able to colonize A/J mice at both day 3 and day 7 post-infection than TX20008, as evidenced by an increased bacterial burden. Currently, the reasons for differences in strain colonization patterns are unknown, but one possibility is a differential gene expression or heterogeneous bacterial expression of surface adhesins. Previously, a study was published which underlined the significance of Pil3 in bacterial adherence to CRC cells and in bacterial colonization of the mouse distal colon [77]. When Pil3 was deleted decreased levels of bacterial binding and colonization were observed. On the other hand, over expression of Pil3 increased gut colonization. I have verified the presence of the *pil3* locus by conventional PCR in both TX20005 and TX20008 (Fig. 31). However, further analysis is needed to verify the sequences of these loci. Subsequently, it will be important to determine differences in Pil3B expression levels between these two strains.

Sillanpaa *et al.* also noted a strong diversity among *Sg* strains and binding to ECM proteins (such as collagen I, collagen IV, collagen V, fibrinogen, and fibronectin), strongly suggesting differences in surface components of *Sg* strains [81]. Further studies should be performed to identify

TX05 TX08



**Figure 31. Presence of Pil3 locus in TX20005 and TX20008.** Genomic DNA was extracted from TX20005 and TX20008 using a standard phenol/chloroform extraction method. Conventional PCR was performed using Pil3B primers constructed by Martins et al (Forward- GCAGTACATATGCAAACAGTTGACTCAGGT , Reverse- CCAAAGGATCCTCATGAAGGCAATTCTGCACC ). Standard PCR conditions were used, with an annealing temperature of 60°C and 30 cycles.

*Sg* surface components and evaluate their cognate host cell binding partners in order to explore colonization variation among *Sg* strains.

The results from the xenograft model suggest that *Sg* strains that are more adherent and more efficiently colonize mice also lead to increased tumor development. This reiterates the idea that direct bacteria-host cell contact is required for cell proliferation both in vitro and in vivo. Another area of interest is to evaluate whether *Sg* is a "hit and run" bacteria or whether its presence is required throughout tumorigenesis. In the literature *H. pylori* has been shown to play an important role in the early stages of tumor progression, but is no longer required for tumorigenesis in later stages [133]. Since the ability of *Sg* to colonize mice appears to play an important role in tumorigenesis it will be interesting to see if *Sg*'s presence is required at various stages of CRC development.

In summary, the data indicate variations among different *Sg* strains with respect to the ability to adhere to host cells, to promote cell proliferation and influences on CRC development. Going forward, it is important to distinguish the bacterial factors responsible for these processes, determine their surface expression patterns, and identify the mechanisms of these interactions among different clinical isolates.

## Chapter 5:

### DISCUSSION:

***Sg* promotes tumor development.** More than 60 years after initial reports emerged linking *Sg* to CRC, the increasing importance of these bacteria remains a focus of studies investigating host-microbe interactions. In general, evidence for the importance of microbes in colon tumorigenesis has been mounting over the last several decades. While we now better understand the role that some of these microbes play in tumor promotion/development, the roles of other microbes remain unclear. If we can better understand the host-microbe interaction, this will allow us to improve upon current cancer diagnostics and treatments by incorporating this understanding into clinical strategies.

Although the association of *Sg* endocarditis/ bacteremia with CRC has been well documented, the nature of this association has remained unknown. It has been unclear whether *Sg* played a role in promoting tumorigenesis or whether it was an opportunistic pathogen that merely took advantage of the favorable tumor environment. Few experiments have been performed evaluating the effects of *Sg* on cell proliferation and tumor promotion until now, yet these studies hold great

importance in further evaluating and understanding the relationship between *Sg* and CRC.

From our *in vitro* work we were able to demonstrate that *Sg* is able to promote cell proliferation, but not apoptosis. This finding is important because proliferation without apoptosis is indicative of tumor growth. Interestingly, the closely related, non-*Sg* strains did not demonstrate the same proliferative effects, strongly indicating that *Sg* possesses unique properties that allow for these changes. Moreover, these results are consistent with what has been reported in the literature where the strong association with CRC was specific to *Sg* and not other closely related strains such as *Sp* and *Si*. Our results also indicate the importance of bacterial growth phase and direct bacteria-cell contact in *Sg*-stimulated cell proliferation. As evidenced by our transwell assay, secreted bacterial factors or soluble metabolites are not sufficient to increase proliferation. This data suggests that there are specific surface components expressed by *Sg* that mediate an increase in cell proliferation and that these components are expressed on bacteria that are growing in the stationary phase. It is also possible that direct bacteria-cell contact is required for the secretion of cytosol-localized factors. These factors may then mediate cell proliferation. The specific bacterial factor(s) and host

receptor(s) are currently unknown, and identification of these factors requires further studies.

In the literature it is well demonstrated that a dysregulation of the  $\beta$ -catenin pathway plays an important role in CRC development, as it is critical in regulating cell fate and proliferation [98-102]. In our studies, we demonstrated *Sg* promotion of host cell proliferation through this pathway. First, CRC cells treated with *Sg* had significantly increased levels of nuclear  $\beta$ -catenin, c-Myc, and PCNA compared to an *L. lactis* control. Additionally, when the  $\beta$ -catenin pathway was inhibited by shRNA or a specific  $\beta$ -catenin inhibitor the effect of *Sg* on cell proliferation, c-Myc, and PCNA was abolished. There are many other examples in the literature of tumor promoting bacteria and their ability to modulate  $\beta$ -catenin activity. For example, *H. pylori*, which is one of the most well understood bacteria in relation to its tumor promoting abilities activates  $\beta$ -catenin signaling. It is able to affect the expression of Wnt ligands [36], activate Wnt receptors [35], suppress GSK3 $\beta$  [34, 37], interfere with the  $\beta$ -catenin/TCF4 complex [103-105], and disrupt the E-cadherin/ $\beta$ -catenin complex [33]. *F. nucleatum* is another bacteria that has been recently linked to CRC and is shown to affect  $\beta$ -catenin signaling by binding E-cadherin through its FadA



adhesin [50]. Additionally, *S. typhi* has been shown to secrete AvrA and activate  $\beta$ -catenin signaling [51].

Our laboratory investigated proliferative effects of *Sg* on five colon cancer cell lines: HT29, HCT116, LoVo, SW480, and SW1116. Our results showed *Sg* promoted cell proliferation in HT29, HCT116, and LoVo cells, all of which we term responsive cell lines. The other two, SW480 and SW1116, were unresponsive to *Sg* with respect to cell proliferation. Notably, these cell lines all contained mutations in the Wnt/ $\beta$ -catenin signaling pathway, with HT29, LoVo, SW480, and SW1116 having mutations in APC and HCT116 having a mutation in  $\beta$ -catenin that results in increased stability. We showed that *Sg* was able to increase  $\beta$ -catenin levels in HT29, HCT116, and LoVo cells, but not the other two cell lines. This could suggest that *Sg* activates  $\beta$ -catenin further upstream or regulates factors outside of the canonical Wnt/  $\beta$ -catenin signaling pathway. Our adherence assays demonstrated that *Sg* can adhere to both responsive and unresponsive cells in similar numbers, and in some cases, can adhere better to the unresponsive cells lines. This indicates that the effects of *Sg* on these CRC cells may not be due strictly to adherence ability, but rather how *Sg* impacts signal transduction within these cells. It is also possible that *Sg* may bind different receptors in each of these distinct cell lines, therefore

resulting in different cellular responses, especially regarding  $\beta$ -catenin signaling. This could explain the differences we are seeing in cell proliferation. Overall, this data suggests cell context plays an important role in determining the effect of *Sg* on the cell. In a clinical application, this would imply that *Sg* may not effect all individuals equally, but that specific genetic or epigenetic backgrounds are necessary to predispose certain individuals to *Sg*-induced tumorigenesis. Therefore, it will be important to identify host cell receptors that are necessary for individuals to be affected by colonization of *Sg*.

To further investigate the association of *Sg* with CRC, both xenograft and AOM mouse models were used. Results from both of these studies suggest an ability of *Sg* to promote tumor development. In the xenograft model, *Sg* treated cells were able to form larger tumors and resulted in higher levels of  $\beta$ -catenin, c-Myc, and PCNA. Mice in the AOM model had more tumors and an increased tumor burden when treated with *Sg* in comparison to the *L. lactis* control. Furthermore, specific examination of colonic crypts from the test groups demonstrated both a higher percentage of proliferating cells and increased  $\beta$ -catenin staining. In contrast, we saw no differences in staining for apoptotic cells between *Sg* treated mice and our saline or *L. lactis* controls. These results are

consistent with our *in vitro* analyses. We also found a correlation between *Sg* abundance in the mouse colon and tumor number and burden, suggesting bacterial abundance is important in tumor development. Additionally, *Sg* bacteria were detected within tumor tissues and appeared to be more abundant within these tumor tissues than surrounding normal tissues. This reiterates the idea that direct contact is important between *Sg* and cancer cells.

Inflammation is known to contribute to CRC development. However, in our mouse experiments, *Sg* and *L. lactis* also induced similar levels of inflammatory responses in the mouse colon, as evaluated with IHC. This was further confirmed by cytokine profiling. This suggests that immune responses to *Sg* may not play an important role in *Sg*-mediated promotion of tumor development, but does not rule out other specific effects of *Sg* in inducing immune reactions that favor tumor development. It will also be interesting to examine the role of the gut microbiota and their relationship with *Sg*. While it appears *Sg* has a direct affect on tumor promotion, it is unknown if *Sg* may work in concert with other microbes to have this effect or if a certain microbiome makes individuals more susceptible to *Sg*-mediated tumorigeneis. Overall, our data supports a mechanism by which *Sg* increases cell proliferation through  $\beta$ -catenin up-regulation. Further studies are needed to

determine *Sg* ligands capable of binding to host cell receptors resulting in host cellular proliferative responses and whether or not the interaction of *Sg* with host cells is able to promote tumorigenesis.

**Prevalence of *Sg* in CRC patients.** Due to the known association of *Sg* endocarditis/ bacteremia with CRC in patients and our current data suggesting the ability of *Sg* to promote tumor development it is important to evaluate the prevalence of *Sg* in the CRC population. If *Sg* is highly present in CRC patients, this could provide the basis for a useful screening method to determine which patients are at risk for CRC and possibly lead to earlier detection. Currently, only patients with *Sg* endocarditis or bacteremia undergo further colonic evaluation, but those that are “silently” infected by *Sg* are still at risk. In addition, our finding that *Sg* promotes colon tumor development raises the possibility that *Sg* may be targeted as a part of the treatment to improve patient outcomes.

In our analysis of tumor and matched normal tissue samples from patients we found that significantly more tumor samples (74%) were positive for *Sg* than the matched normal samples (47%) and this falls within the range of previously reported studies. Our study looked specifically at *Sg*, without combined contributions of other closely related, non-*Sg*

strains. Additionally, 26% of the tumor samples were highly enriched for *Sg*, whereas only 9% of the normal samples were highly enriched, reiterating the importance of direct contact between *Sg* and cancer cells. This aligns with the findings from our *in vitro* and *in vivo* work. It will be interesting to evaluate additional samples from healthy patients with no pathologies to determine the prevalence of *Sg* in these patients. In comparison, *Sp*, a closely related strain, was detected in only a few samples. This data is similar to previous findings in the literature with *Sg* being the predominant *S. bovis* biotype associated with CRC. Additionally, we will evaluate the impact of *Sg*-positivity on global methylation as a well-described field effect for a subset of colorectal cancer.

Overall, this data suggests a strong association of *Sg* with CRC. *Sg* is more readily abundant in the tumor tissues of these patients in comparison to matched normal tissues and is more prevalent than a closely related *Sp* strain.

**Polymorphism in *Sg* strains.** Currently, we know that a direct interaction between *Sg* and host cells is important in promoting cell proliferation. As a consequence, we evaluated the ability of several *Sg* strains to adhere to CRC cell lines. From this we found that several of these strains were able to adhere to CRC lines more efficiently than others and the

strains that adhered were able to promote cell proliferation, whereas those that did not were not able to. We further evaluated two *Sg* strains in both C57BL/6 and A/J mice. *Sg* strain TX20005 is adherent and TX20008 is non- or poorly adherent to *in vitro* cultured colon cancer cell lines. We determined colonization at three or seven days after initial *Sg* inoculation. The *Sg* strain that was able to adhere to cell lines also more efficiently colonized the colons of A/J mice compared to the strain that adhered poorly to *in vitro* cultured cells. We did not see this difference in C57bl/6 mice, which suggests the host genetic background may make a difference in *Sg* colonization. Additionally, these results suggest that there is variation among *Sg* strains in terms of their ability to interact with colon cells and to stimulate cell proliferation. The specific *Sg* factors that mediate cell adherence and cell proliferation are currently unknown. In the literature, it has been suggested that Pil3 plays an important role in *Sg*'s ability to adhere to cells and colonize the distal mouse colon. It will be interesting to evaluate both the presence of Pil3 and expression levels between our adherent and non-adherent strains. Further studies are required to identify the bacterial and host factors involved in this interaction.

We attempted to evaluate the ability of TX20008 to promote tumor development in our AOM mouse model. However, TX20008 was unexpectedly virulent in this model and killed mice before any colon tumors had the time to develop. We then resorted to the xenograft model. When nude mice were injected with cells treated with TX20005 we saw a significant increase in tumor size in comparison to TX20008 treated cells. Thus, it is important for an *Sg* strain to adhere to CRC cells in order to promote cell proliferation and to be able to colonize mouse tissues in order to promote tumor development.

**Implications to CRC prevention, diagnosis and treatment.**

Individuals over the age of 60 are at the highest risk for CRC, and currently, colonoscopy is the standard screening method for CRC [134]. Although this method is effective at detecting CRC, the compliance is low. It is estimated that approximately 23 million people are not up to date on their screening [30]. The five-year survival rate for patients with stage I colon cancer is 92%, but decreases significantly for stage IV to 11% (Table 3). Therefore, more non-invasive screening methods are needed that are both reliable and affordable for detecting cancers early.

Several studies have evaluated the seroprevalence of *Sg* in CRC patients. One study suggested measuring IgG antibody titers against *Sg* [8]. *Sg* is more abundant in fecal samples

**Table 3:** Five-year survival rate for different stages of colon cancer.

Colon cancer survival rate, by stage	
Stage I	92%
Stage IIA	87%
Stage IIB	63%
Stage IIIA	89%
Stage IIIB	69%
Stage IIIC	53%
Stage IV	11%



from CRC patients [119, 124, 135] and it is speculated that alterations at the site of the lesion allows *Sg* entry to the blood stream [136], inducing an antibody response. Also, *Sg* has been shown to highly colonize tumor lesions [83, 137] and this colonization deeply within the tumor tissue increases the likelihood of a systemic response including the production of IgG antibodies [66]. Other studies have suggested using ELISA-based assays to specifically detect pilus components, in combination with other detectable *Sg* antigens, may be used to determine at risk groups [68]. Ultimately, knowing the relationship between *Sg* and CRC will help in further validating these ideas and developing better detection methods for CRC. The finding that *Sg* promotes CRC development also raises the possibility that *Sg* should be included in clinical strategies to treat CRC. Many questions remain in this regard. For example, should CRC patients be routinely screened for the presence of *Sg*? Should *Sg*-positive patients be treated with antibiotics to eliminate *Sg*? On the other hand, it is unclear whether *Sg*'s effect can last after its elimination from the gastrointestinal tract. Finally, should vaccination against *Sg* be considered? These questions require additional carefully planned and well-controlled studies.

**Conclusion and future directions.** In summary, the results presented here suggest a model in which *Sg* mediates tumor

promotion through the up-regulation of  $\beta$ -catenin, which leads to increased cell proliferation. This is the first report providing evidence for *Sg*'s role in CRC. Furthermore, we provided additional evidence for the strong association between *Sg* and CRC in patients. Previously, studies primarily focused on *Sg* patients with endocarditis or bacteremia and the co-currence of CRC, but here we evaluated these patients from the CRC perspective to determine the prevalence within these patients. Finally, our results suggest that specific *Sg* factors mediate adherence to colon cells and promote cell proliferation. These factors are also likely to be important for promoting tumor development. Future studies will address identifying both bacterial ligands and host receptors responsible for the tumor promoting affects seen by *Sg* and determining the mechanism involved. These studies provide valuable insight into this *Sg*-CRC association and demonstrate the need for better diagnostic tests in detecting CRC. If we can utilize this knowledge and understand precisely how microbes interact with and affect the gut this will allow us to exploit these interactions for our benefit.

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