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MODULATION OF COLORECTAL CANCER BY THE PROBIOTIC ORGANISM *LACTOBACILLUS REUTERI*

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Abstract

Probiotics are beneficial to gastrointestinal health and have anti-inflammatory properties. An experiment was conducted to determine if *L. reuteri* could protect mice from azoxymethane-induced colorectal cancer (AOM-CRC). A/J mice were randomly assigned to controls, pre-treatment, or post-treatment groups. The mice in the groups were injected with 10mg/kg body weight of AOM and treated with *L. reuteri*; given only AOM; given only *L. reuteri*; or given neither AOM nor *L. reuteri*. At the end of a 26-week latency period, the mice were euthanized, dissected, and colons examined for tumors. *L. reuteri* did not protect animals against tumor formation. However, *L. reuteri* treatment had a significant effect on tumor number when the mice were segregated by gender ($p = 0.014$). There was no significant effect of regimen on tumor number ($p = 0.667$) or tumor size ($p = 0.197$). Ultimately, *L. reuteri* exhibited probiotic properties as a potential prophylactic treatment for colitis.

Keywords: Colorectal Cancer, Azoxymethane, *Lactobacillus reuteri*

Introduction

According to the American Cancer Society (2014), colorectal cancer (CRC) remains the 3rd most frequently diagnosed cancer in the United States, behind lung cancer (the leading cause of cancer-related deaths for both genders, and behind prostate cancer (men) and breast cancer (women). An emerging area of active research is the connection between the commensal flora in the gastrointestinal tract, host genetics, and cancer biology/pathogenesis. Twenty percent of CRC is caused by hereditary factors (Fearnhead et al., 2002). Another 20% of CRC can be traced to chronic inflammation caused by parasites, harmful bacteria and viral infections (Venning et al., 2013). The remaining determinants linked to CRC are related to environmental factors and lifestyle choices (Chan et al., 2010). Diet is a major factor that can modulate the health of the gastrointestinal tract and can alter the gut microbial composition of an organism (De Filippo et al., 2010). Probiotics are naturally found in the gut of animals but in varying amounts depending on the organism. Consuming additional probiotics could enhance the diversity of the commensal flora and the health of the gut (Barbara et al., 2005), and could potentially prove chemotherapeutic for organisms with CRC. The objective of this study was to determine if *Lactobacillus reuteri* (*L. reuteri*), a common inhabitant of the gastrointestinal tracts of most mammals, could modulate the severity of CRC in mice injected with the colon-specific, chemical carcinogen, azoxymethane (AOM).

Literature Review

CRC is the third most commonly diagnosed cancer in the United States. In 2014,136,830 people were diagnosed with CRC, and of these, 50,310 were expected to die (American Cancer Society, 2014). The colon is made up of different segments that include the ascending colon, transverse colon, descending colon, sigmoid colon, and the rectum. The most proximal segment is the ascending colon and the more distal segments are the descending and sigmoid colon (American Cancer Society, 2014). The majority of CRC cases are not caused by single genetic determinants, but are considered sporadic cancers (Frank et al., 2007). Of all sporadic colonic tumors, 75% are found in the descending colon (Li and Lai, 2009); however, CRC that is found in the more proximal segments of the colon has a better prognosis (Loupakis et al., 2015).

The risk factors for CRC include age, gender, physical activity, heredity, and diet. The older a person is, the greater the likelihood that he/she will develop cancer of the colon. Men develop CRC at a higher rate than women (American Cancer Society, 2014; 2011). The incorporation of more physical activity into one's daily life reduces one's chances of developing CRC (Tárraga López et al., 2014). Eighty percent of all colon cancers are not genetically linked (Frank et al, 2007), and most of these tumors are caused by small aberrations in the DNA paired with one of the aforementioned risk factors (Bissahoyo et al., 2005). Diet is another very important factor in influencing the development of CRC (Parnaud et al., 1997). Increased fruit and vegetable intake lessens the chances of developing colon cancer (Koushik et al., 2007) and increased consumption of meat cooked at high temperatures increases the risk of developing CRC (Kim, 2013).

Studies involving germfree mice revealed that bacteria are co-factors in the development of both inflammatory bowel disease (IBD) and colon tumors, inducing inflammation and tumor formation in certain genetic mouse models of CRC due to peroxidative stress (Chu et al., 2004). The inflammation which leads to CRC is reportedly caused by inappropriate responses to gastrointestinal (GI) bacteria (Sellon et al., 1998). This inflammation is implicated in ulcerative colitis-related CRC, as this form of IBD increases cancer risk 20-30% (Rufo and Bousvaros, 2006). Microflora can regulate epithelial cell signaling for immune reactions. (Chu et al., 2004) and skew an immune response in a way that either potentiates or protects animals from disease. Lactobacilli have been found to induce gene transcription in epithelial cells (Zoetendal et al, 2002); induce secretion of antibodies like IgA (Zoetendal et al., 2002), and usurp the host epithelial cell machinery to provide for their own needs (Mahida, 2004). Bacteria can even regulate the production of cytokines and chemokines like Nf-kappa-B and IL-8 (Mahida, 2004), as well as IL-1, IL-6, IL-10, IL-12, and TNF-alpha (Karlsson et al., 2004; Raz et al., 2007).

The intestinal microflora can determine the pre-immune antibody repertoire in rats and modulate the development of the gut-associated lymphoid tissue (GALT) via stress responses (Rhee et al., 2004). Additionally, bacteria contribute to the regulation of intestinal angiogenesis and the induction of oral tolerance. GI commensal organisms live in a symbiotic association with the lumen or mucosal layers of the GI tract, though a few species, such as the *Lactobacilli*, live attached to the epithelium. Some of these organisms are involved in promoting GI and immune system homeostasis, as well as tissue and immune system development (Guarner, 2006). Lack of proper interactions between these bacteria and their host could disrupt this delicate ecosystem and lead to disease (Falk et. al., 1998). However, certain species of probiotic organisms have

been shown to offer protection for diseases like CRC (Fukui et al., 2001) and IBD (Shultz et al., 2002).

Probiotics are believed to confer anti-carcinogenic properties like decreased inflammation, enhanced immune function, and anti-tumorigenic activity by a number of mechanisms, including inhibiting growth of colonic cells, increasing the production of conjugated linoleic acids, increasing concentrations of beneficial bacteria, reducing colorectal proliferation and the capacity of fecal water to induce necrosis, reducing the levels of pathogenic micro-organisms, binding to potential food carcinogens, improving epithelial barrier function, and reducing bacterial enzymes which hydrolyse pre-carcinogenic compounds, such as beta-glucuronidase (Lee et al., 2007; Ewaschuk et al., 2006; Geier et al., 2006; Rafter et al., 2007). Lorea-Baroja et al., (2007) found that consumption of a probiotic yogurt, supplemented with *L. reuteri* and other organisms, led to anti-inflammatory effects in the host. *L. reuteri* and *L. fermentum* were found to have immunomodulatory effects in a trinitrobenzenesulfonic acid model of rat colitis (Peran et al., 2007). Still, not enough intervention studies have been conducted to evaluate the efficacy of probiotics in GI diseases like IBD and CRC (Bergonzelli et al., 2005).

IL-$10^{-/-}$ animals develop spontaneous colitis under specific-pathogen-free (SPF) conditions but remain disease-free if kept germfree (Schultz et al., 2002; Ruiz et al., 2006). This is due to aggressive T lymphocyte responses to the enteric flora (Cong et al., 1998). The microflora has also been implicated as the causal factor in other models of colitis (Chu et al., 2004; Esworthy et al., 2003). So the microflora is key in the development of colitis, but among the microflora certain members were found to protect mice against the disease (Shultz et al., 2002). Colitis in 129SvEv IL-$10^{-/-}$ animals could be prevented by pre-treating the animals with *L. plantarum 299V* (Schultz et al., 2002).

In a comparative study of mice with and without colitis (Pena et al., 2004), it was found that IL-$10^{-/-}$ mice that developed colitis were largely colonized by species of *L. johnsonii*, *L. reuteri*, *L. vaginalis* and *L. paracasei* were found in the mice that did not develop colitis and *L. reuteri* was found to have an immuno-inhibitory effect on TNF-alpha production, which could be related to the lack of colitis in the animals tested. *L. reuteri* was later found to reduce *H. hepaticus*-induced IBD in this same mouse model (Pena et al., 2005). Casas and Dobrogosz (2000) found that *L. reuteri* is the only Lactobacillus species that inhabits the gastrointestinal tract of all vertebrates and mammals. They also reported that there is host-specificity to how the *L. reuteri* strains function as probiotics. *L. reuteri* was demonstrated to protect against viral, fungal, and protozoan infections as well as to improve GI biology and morphology. The broad spectrum activity of *L. reuteri* led us to believe it might prove therapeutic against experimental CRC and/or IBD.

Based on the above, the study sought to determine whether the probiotic organism *L. reuteri* would abrogate carcinogenesis in AOM-injected A/J mice (AOM-CRC), which show a high penetrance and multiplicity for experimental colon cancer in response to AOM. Consequently, the authors assessed the chemotherapeutic properties of *L. reuteri* in a mouse model of CRC disease.

Materials and Methods

Bacterial Strains

Pure cultures of *L. reuteri* isolates 4000 and 4020 were provided by Walter Dobrogosz and Norris Carbal (North Carolina State University, Raleigh, NC and BioGaia, Inc. Raleigh, NC 27617). *L. reuteri* strains were grown in stationary Man, Rogosa, and Sharpe (MRS) broth (Remel Products, Lenexa, KS 66215), under anaerobic conditions at 27 °C.

Mice

A/J mice (Jackson Labs, Bar Harbor, ME, 04609) were housed under specific pathogen free (SPF) conditions in the Department of Lab Animal Medicine (DLAM) at the University of North Carolina at Chapel Hill (UNC-CH). The animals were maintained on standard sterilized chow and water. Animals were introduced into the study at 8-16 weeks old. A total of 34 animals were used in the study, with 12 of those being female animals. The mice were distributed across 13 cages.

Experimental Colon Cancer

Probiotics are known to be beneficial to gastrointestinal health and when added to the diet can be used as a preventive measure for GI-related illnesses. In order to investigate whether *L. reuteri* could protect mice from azoxymethane-induced colorectal cancer (AOM-CRC), AOM-susceptible mice were given probiotic supplementation and tumor incidence, location, and multiplicity were measured using the AOM-CRC model. The AOM-CRC model was chosen, because it is the model that most closely recapitulates sporadic human colon cancer (Chen and Huang, 2009).

A/J mice that were more than 2 months old were randomly assigned to control, pre-treatment, or post-treatment groups. The pre-treatment, post-treatment, and probiotic-only control groups contained six mice each. The nothing-added control and the *L. reuteri* control group contained 4 mice each. Pre-Treatment groups were given 10^8/*L. reuteri* colony-forming units (CFU) per mouse/day by adding the organisms to water bottles. Water bottles were changed every 2-3 days to preserve consistent *L. reuteri* numbers across cages. Two weeks later, all mice earmarked for treatment were injected intraperitoneally once a week for four weeks with 10mg/kg body weight of AOM (Sigma-Aldrich Corp. St. Louis, MO) suspended in 1X Phosphate Buffered Saline (PBS).

At 13 weeks, the animals in the post-treatment group were started on the *L. reuteri* probiotic regimen. The AOM control animals were given the probiotic (*L. reuteri*) but were not injected with AOM. *L. reuteri* control animals were injected with AOM but not given the probiotic (*L. reuteri*). Negative control animals were neither injected with the AOM nor treated with the *L. reuteri*. At the end of a 26-week latency period, all mice were euthanized by isoflourine overdose and dissected. Using the procedure previously described by Bissahoyo et al., (2005), colons were removed, flushed with PBS, splayed open, and scored for tumors. Colons were then rolled from the distal to proximal end, and preserved in 10% Neutral Buffered Formalin. Processed samples were dehydrated in 70% ethanol then paraffin embedded. Tissue blocks were sectioned (6 µm) and stained with hemotoxylin and eosin for histopathological analysis.

Statistical Analysis

It was hypothesized that the anti-inflammatory properties of *L. reuteri* would reduce the incidence of colorectal cancer in A/J mice. Data were subjected to ANOVA (StatView). Values are presented as the mean of n experiments $\pm$ standard deviation. All values with a p value < 0.05 were considered significant. Treatment, gender, cage, cage density, mouse age, treatment regimen, weight, and colon length were determined and assessed for their role in modulating tumor size, and tumor location.

Results and Discussion

All bacterial species were successfully cultured and used to associate the mice (data not shown). Bacterial identities were verified by gram-staining and growth in differential media. The mice which did not receive AOM-injections did not develop any detectable tumors (Table 1).

The mice treated with *L. reuteri* alone did not evidence any increase in tumor incidence compared to other animals in other treatment groups (data not shown) but did reduce inflammation in this experimental model (data not shown). Surprisingly, *L. reuteri* supplementation did not significantly alter morbidity in AOM-treated animals as measured by tumor number (Figure 1), mortality (Figure 2), tumor penetrance (Figure 3), or tumor multiplicity (Figure 4), although there was a significant effect of gender on tumor penetrance and multiplicity (Figure 3, Figure 4), especially in animals which did not receive any probiotic. Total tumor numbers ($p = 0.834$), tumor size ($p = 0.237$) and multiplicity ($p = 0.237$) were not significantly different for *L. reuteri*-treated animals compared to AOM-only treated animals (data not shown).

There was a significant effect of gender on tumor number, with females having a higher tumor burden than males ($p = 0.014$; Figure 1). This could be due to slight differences in the weights of females vs. male mice or it could be due to hormonal differences between the mice. The females were nulliparous and were prevented from mating, but there may have still been gender-specific physiological differences in the mice that confounded the data collected. However, gender did not affect tumor size ($p = 0.920$) nor tumor location ($p = 0.211$; data not shown). Age was not a significant factor in tumor number ($p = 0.528$) nor was tumor size ($p = 0.070$), but age exerted a significant effect on tumor location ($p = 0.003$), with younger mice having more proximal tumors compared to older animals (data not shown). The relevance of this finding is not clear and additional studies need to be conducted to gain more insight into how this affects cancer prognosis. However, distal tumors are associated with worse prognosis (Gervaz et al., 2001) compared to proximal tumors. There was no effect of regimen (no treatment vs. pre-treatment, vs. post-treatment) on tumor number ($p = 0.667$, data not shown) or tumor size ($p = 0.197$; data not shown). This may indicate that the dosage used in this study might need to be increased in order to see a significant effect of the probiotic supplementation.

Table 1. Animals used in the AOM-CRC *L. reuteri* Study

	*Treat.	Gender	Cage	Weight (g)	Tumors (mm)	Size (mm)	Location (mon)	Density (mm)	Age	Colon L.	Regimen
1.	R	F	D001	27.600	5.000	2.200	36.000	3.000	3.000	115.000	PRE
2.	R	F	D001	22.300	2.000	3.750	22.500	3.000	3.000	80.000	PRE
3.	R	F	D001	25.400	4.000	3.130	18.750	3.000	3.000	90.000	PRE
4.	C	F	D002	20.800	2.000	2.500	22.500	1.000	3.000	95.000	CON
5.	R	M	D003	32.100	1.000	1.500	0.000	3.000	3.000	90.000	PRE
6.	R	M	D003	32.100	2.000	3.000	10.000	3.000	3.000	105.000	PRE
7.	R	M	D003	26.400	0.000	0.000	.	3.000	3.000	80.000	PRE
8.	C	M	D004	25.100	0.000	0.000	.	5.000	3.000	60.000	CON
9.	C	M	D004	25.400	0.000	0.000	.	5.000	3.000	95.000	CON
10.	C	M	D004	23.400	0.000	0.000	.	5.000	3.000	85.000	CON
11.	C	M	D004	24.600	1.000	2.000	25.000	5.000	3.000	100.000	CON
12.	C	M	D005	20.300	2.000	3.000	15.000	2.000	3.000	95.000	PRE
13.	R	M	D005	24.400	1.000	3.500	15.000	2.000	3.000	80.000	PRE
14.	R	M	D006	25.000	2.000	2.500	37.500	5.000	4.000	105.000	POST
15.	R	M	D006	25.100	0.000	0.000	.	5.000	4.000	95.000	POST
16.	R	M	D006	32.800	0.000	0.000	.	5.000	4.000	90.000	POST
17.	R	M	D006	29.900	0.000	0.000	.	5.000	4.000	90.000	POST
18.	R	M	D006	27.500	0.000	0.000	.	5.000	4.000	100.000	POST
19.	C	M	D007	28.800	0.000	0.000	.	2.000	4.000	105.000	CON
20.	C	F	D008	25.200	0.000	0.000	.	1.000	4.000	115.000	CON
21.	R	F	D11	22.300	2.000	1.500	37.500	5.000	4.000	95.000	POST
22.	R	F	D11	22.800	1.000	2.000	30.000	5.000	4.000	105.000	POST
23.	R	F	D11	21.600	3.000	2.830	30.000	5.000	4.000	115.000	POST
24.	C	M	D12	27.500	0.000	0.000	.	1.000	3.000	105.000	CON
25.	R	M	B571	27.800	2.000	2.500	10.000	4.000	3.000	85.000	PRE
26.	R	M	B571	26.800	2.000	3.250	30.000	4.000	3.000	105.000	PRE
27.	R	M	B571	27.300	0.000	0.000	.	4.000	3.000	100.000	PRE
28.	C	M	B579	26.400	0.000	0.000	.	3.000	3.000	110.000	CON
29.	C	M	B579	27.000	0.000	0.000	.	3.000	3.000	80.000	CON
30.	C	M	B579	24.500	1.000	3.000	15.000	3.000	3.000	110.000	CON
31.	C	F	B580	18.900	7.000	2.790	17.900	4.000	3.000	85.000	CON
32.	C	F	B580	25.900	4.000	3.380	22.500	4.000	3.000	50.000	CON
33.	C	F	B580	21.600	3.000	3.130	13.300	4.000	3.000	90.000	CON
34.	C	F	B580	23.500	2.000	2.750	7.500	4.000	3.000	85.000	CON

***** *Treat.=treatment (R=L. reuteri; C=Control) , Gender, Cage, Weight in grams, # of Tumors, Size in mm, Location (mm from distal end), Density (number of animals/cage), Age (in months), Colon L=colon length in mm, Regimen*

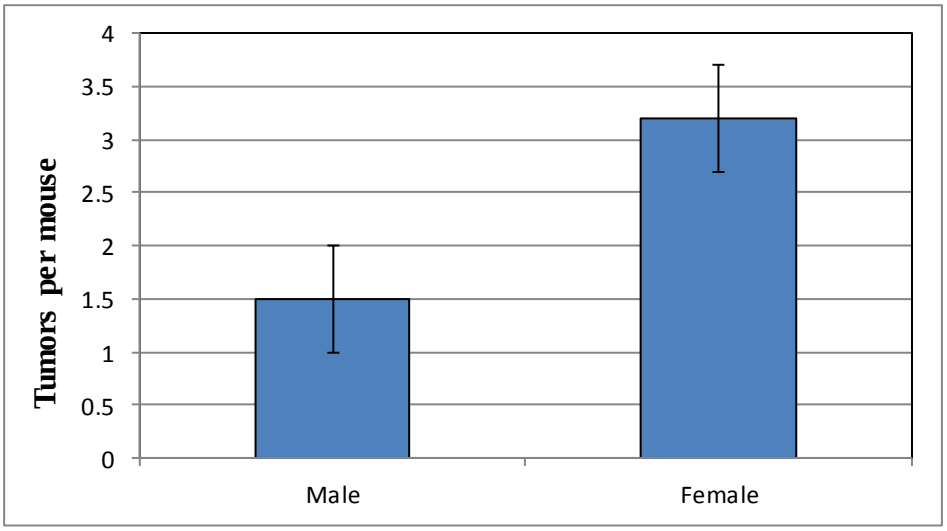

Figure 1. Effect of Gender Supplementation on Tumor Number

When compared to the control mice with normal mucosa (Figure 5), mice given AOM injections developed adenomas with high grade dysplasia (Figure 6), and gastrointestinal intra-epithelial neoplasia with high grade dysplasia (Figure 7). Mice treated with *L. reuteri*-only did not develop any tumors. These data confirmed that AOM causes colon-specific tumor in the A/J mouse strain after the **26-week** latency. The development of dysplastic lesions (both low and high grade) as well as frank tumors, suggests that the AOM injection protocol models the step-wise cancer progression seen in human CRC.

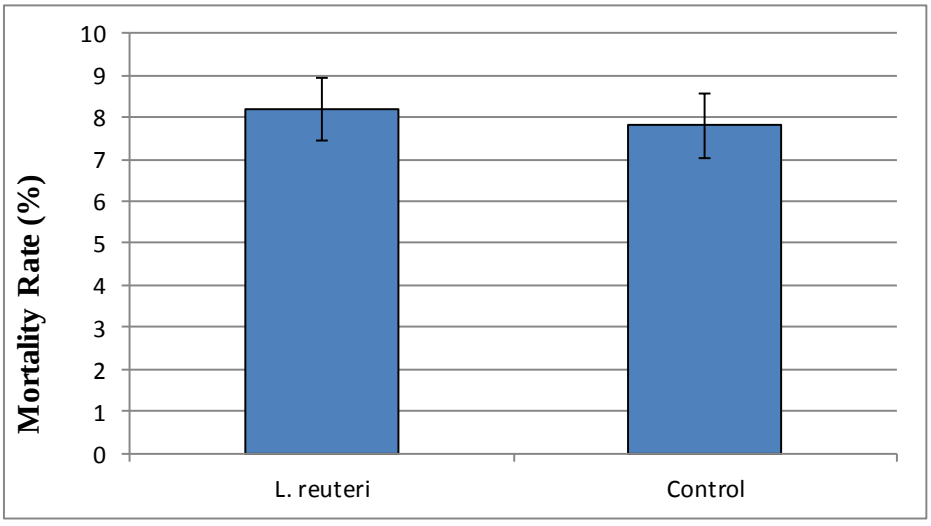

Figure 2. Effect of *L. reuteri* supplementation on Mortality Rate, by treatment

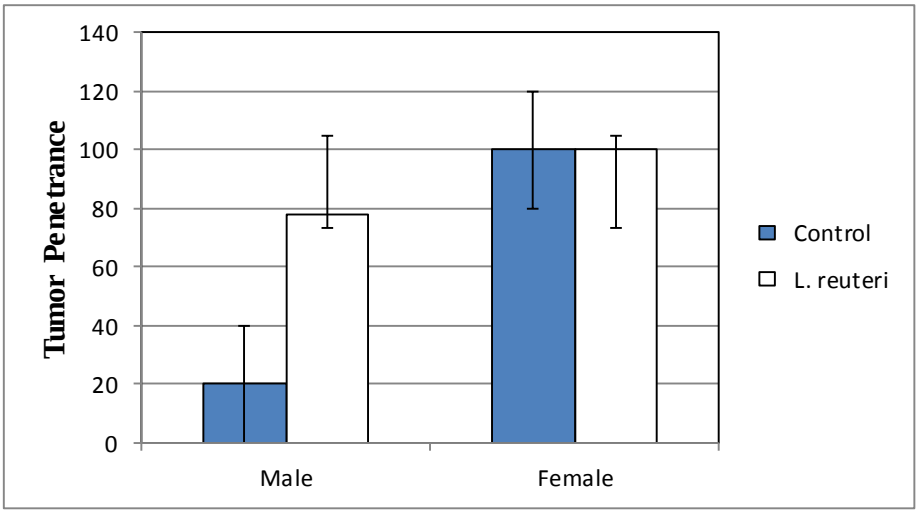

Figure 3. Effect of *L. reuteri* supplementation on Tumor Penetrance, by Gender, by Treatment

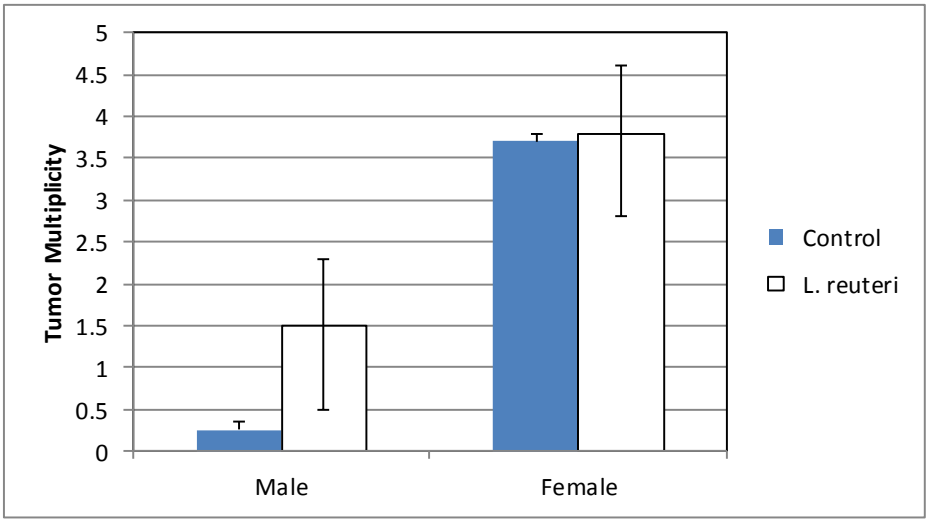

Figure 4. Effect of *L. reuteri* supplementation on Tumor Multiplicity, by Gender, by Treatment

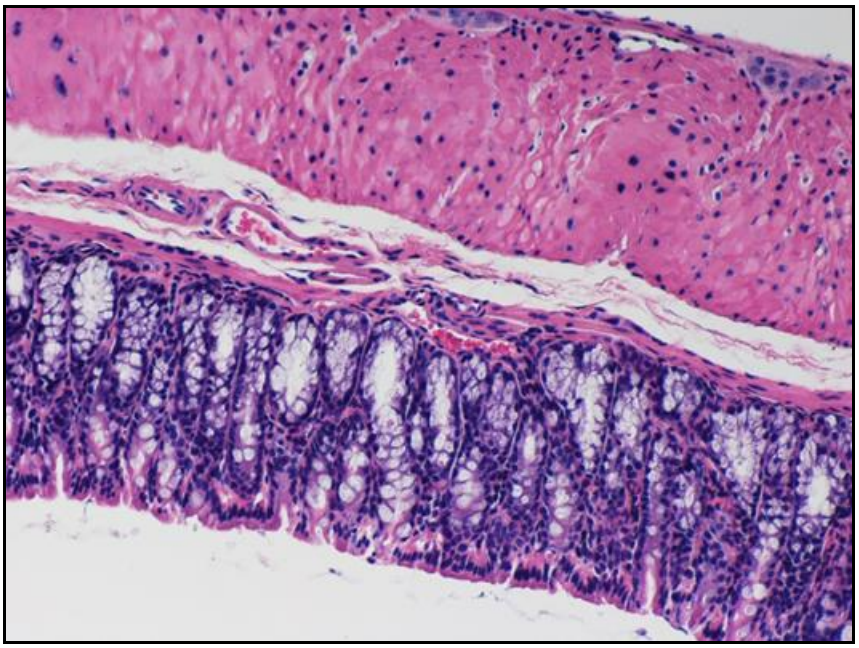

Figure 5. Normal mucosa, submucosa and muscularis of colon

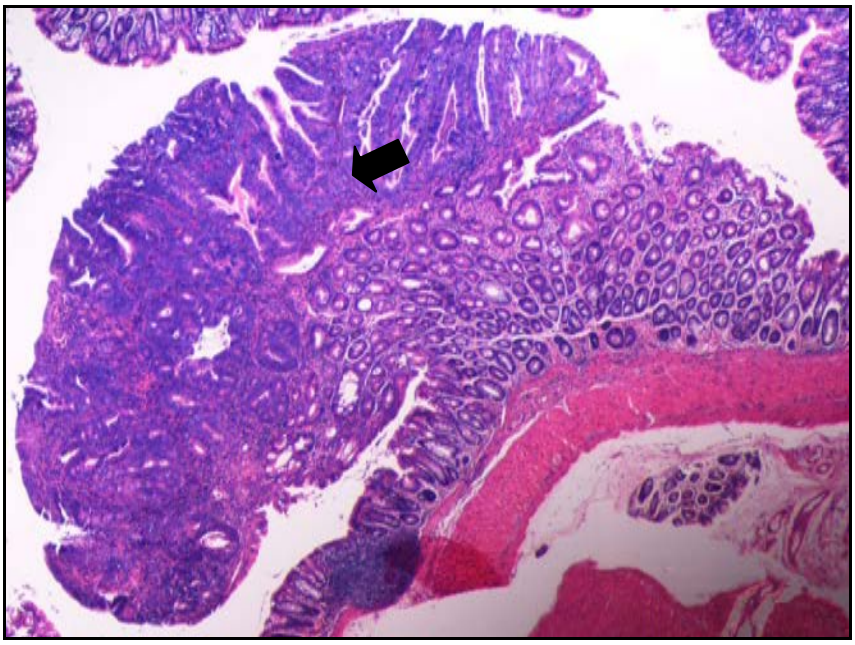

Figure 6. Adenoma of colon, papillary, with high grade dysplasia, group 129 SvEv ASF, 100x

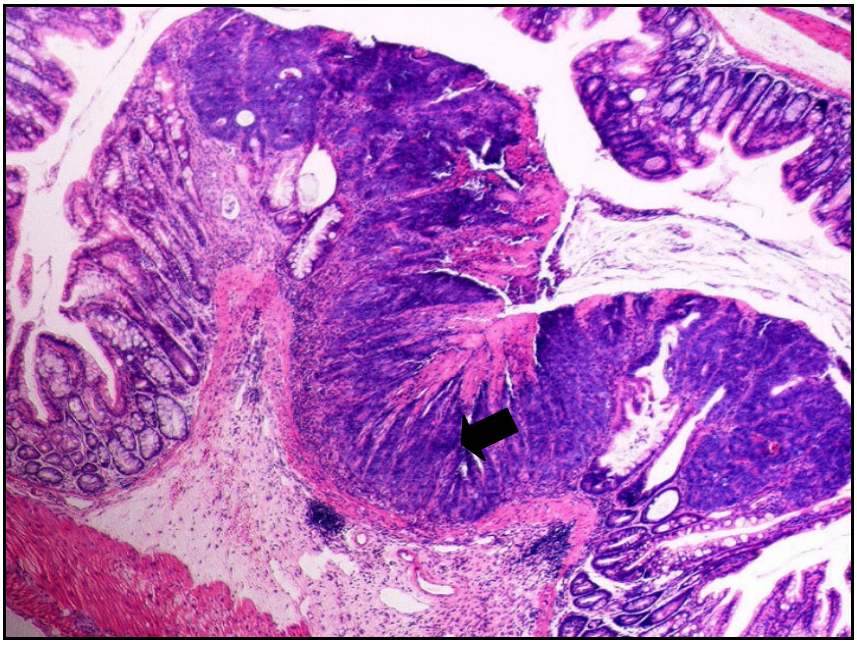

Figure 7. Gastrointestinal intraepithelial neoplasia, colon, with high grade dysplasia, group 129 SvEv GF (100x)

Conclusion

It was determined that *L. reuteri* was not effective in reducing colon cancer severity in our mouse model, but that gender had a significant effect on AOM-induced colorectal cancers. Age had a significant effect on tumor location, with older age being associated with worse prognosis. Additional studies regarding dosage and routes of administration should be conducted. It may be that a higher dosage of the *L. reuteri* might have proven more effective. It may also be important to start the administration of *L. reuteri* during development as a strategy (Ex. beginning the supplementation at a neonatal stage). A future goal of the researchers is to test the efficacy of other lactobacillus strains in this and other experimental models of CRC. The potential of *L. reuteri* for use as probiotic therapy in GI diseases warrants further investigation.

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