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Use of miRNA inhibitors to investigate probiotic regulation of E-cadherin expression

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Abstract

Studies have repeatedly focused on the positive effects of probiotic bacteria on mammalian gastrointestinal health and have confirmed the ability of probiotics, like lactobacilli, to decisively influence the intestinal microbiota balance and the gut immune system. The four species of lactobacilli used in this study, *L. fermentum*, *L. acidophilus*, *L. gasseri*, and *L. rhamnosus*, are known to stimulate the integrity of the intestinal barrier by promoting the expression of the adherence junction protein E-cadherin. Another major influence on E-cadherin concentrations is the presence of the transcription repressor SNAIL. This study aims to further investigate the mechanisms by which these species of lactobacilli and the SNAIL transcription factors exert regulation on E-cadherin.

The presence of SNAIL transcription factors down-regulate the expression of E-cadherin and this is associated with various carcinomas. If it is found that the lactobacilli and the SNAIL factors regulate E-cadherin expression by similar mechanisms (these specific miRNA), potential lactobacilli therapies could be utilized in cancer treatments. This study did not reveal direct relationships between specific lactobacilli and SNAIL factors analyzed. Further trials should be done with additional combinations. Still, previous claims about the regulatory functions of the SNAIL transcription factors and lactobacilli were confirmed.

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Introduction

Probiotic bacteria have drawn increasing interest in recent years as nutritional supplements. The consumption of probiotics as live cultures in dairy products and in dietary supplement pills has become a common practice to improve health. Probiotics are understood to be "live microorganisms that, which when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2001). They are non-pathogenic and benefit the health of the gut by improving the microbial balance (Zyrek et al., 2006). In addition to enhancing gut barrier function by refining the gut barrier integrity, probiotics stimulate the immune system, ease dietary intolerance, and reduce cancer risk as well as allergy symptoms and the risk of cardiovascular disease (O'Toole & Cooney, 2008). While probiotics have been observed to have a wide range of positive effects on human health, traditionally they are most often used in the treatment of bowel disorders and inflammatory diseases, including *Colitis ulcerosa* and Chrohn's disease (Nissle, 1959).

Lactobacilli are a common type of probiotic and four species of *Lactobacilli* are utilized in this study: *Lactobacillus rhamnosus, Lactobacillus acidophilus, Lactobacillus gasseri, and Lactobacillus fermentum*. All four species have been extensively studied and their probiotic properties are well described (Lyra et al., 2010; O'Toole & Cooney, 2008; Ventura et al., 2009; West et al., 2011; Zyrek et al., 2006). Zyrek et al. (2006) and Hummel et al. (2011) performed experiments to examine the biochemical means by which probiotic bacteria improve the gut barrier function. In the presence of *L. acidophilus, L. gasseri, and L. fermentum*, E-cadherin mRNA was upregulated. E-

cadherins are junctional molecules that are most concentrated in adherence junctions. The apical junction complex is composed of adherence junctions and tight junctions, and the adherence junctions are essential to the assembly of tight junctions. Tight junctions limit diffusion of molecules between the absorptive apical surface and the basolateral surface of intestinal epithelial cells. Therefore the cell can maintain two separate activities on opposite sides to establish polarity: absorption at one face and release into circulation at the other face. The tight junctions also create a tight seal between intestinal epithelial cells that acts as a selectively permeable barrier of diffusion in the paracellular space and play a role in cell differentiation (Zyrek et al., 2006). E-cadherins are especially abundant in epithelial cells, like intestinal epithelial cells in the gastrointestinal tract (Takeichi, 1990).

The complete signal transduction pathway by which the presence of probiotic bacteria influences the regulation of E-cadherins is only partially understood. Various transcription factors that contribute to the regulation of E-cadherin expression have been identified and include the SNAIL transcription factors, SNAI1 and SNAI2 (Reinhold et al., 2010). Both SNAIL transcription factors function as repressors of E-cadherin transcription by binding to the E-cadherin promoter region (Batlle et al., 2000 and Guaita et al., 2002). The SNAIL transcription factors are abundantly expressed by fibroblasts and some epithelial tumor cells that lack adequate amounts of E-cadherin and their expression has repeatedly been associated with the development of carcinomas (Batlle et al., 2000; Guaita et al., 2002; Liu et al., 2012; Roy et al., 2005). As illustrated in Figure A., it is hypothesized that SNAIL expression is naturally suppressed

by microRNA molecules (miRNA's). miRNA's are noncoding and relatively short RNA's that range from 21 to 23 nucleotides long. miRNA's often post transcriptionally regulate specific mRNA's (Bissels et al., 2009). When miRNA molecules block SNAIL production, E-cadherin expression occurs. Liu et al. (2012) performed an experiment in which overexpression of a miRNA (hsa-miR-9) induced the down-regulation of transcription factor SNAIL 1 as well as the up-regulation in E-cadherin expression. MicroRNA inhibitors can be introduced experimentally and block the action of the targeted miRNA. As illustrated in Figure A., SNAI1 and SNAI2 miRNA inhibitors block the action of the miRNA's and relieve the suppression of SNAI1 and SNAI2 transcription factors, thus allowing them to repress E-cadherin transcription.

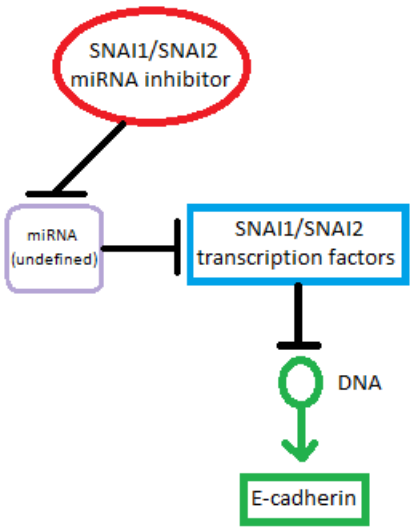

Figure A. Transcriptional effect of SNAIL miRNA inhibitors*: Diagram of action taken by SNAIL miRNA inhibitors to repress the miRNA that suppress the SNAIL transcription factors. Expressed SNAIL factors bind to the promoter region of the E-cadherin gene and repress transcription of E-cadherin mRNA.*

In this study, human T84 colon cells were cultured and co-incubated with one of the probiotics, *L. acidophilus, L. gasseri, L. rhamnosus, or L. fermentum,* and one of the

SNAIL miRNA inhibitors. For each experiment controls included just T84 cells, T84 cells co-incubated with the probiotic, and T84 cells that were incubated with the probiotic and then an unspecific miRNA. Experimental tests included co-incubation with a bacteria followed by a miRNA inhibitor and a miRNA inhibitor followed by a bacteria. As stated above, the SNAI1 and SNAI2 miRNA inhibitors prevent the expression of the miRNA's that inhibit the SNAIL factors and thus work to remove the repression of the SNAIL factors. These factors are then free to repress the transcription of E-cadherin. Additionally, E-cadherin regulation of analyzed lactobacilli is differently affected by the two miRNA inhibitors/transcription factors. We hypothesized that, amongst other mechanisms, probiotics induce the expression of E-cadherin by suppressing either SNAI1 or SNAI2 expression. Since SNAIL miRNA inhibitors are known to relieve SNAIL suppression, we tested whether probiotics would override the effect of the miRNA inhibitors. Such results would suggest that the miRNA inhibitors and the probiotics act via the same mechanism and that the lactobacilli up-regulate the expression of E-cadherin in part by inducing the expression of the miRNA's that inhibit SNAIL transcription factor expression.

The signaling mechanism by which the bacteria act on the gastrointestinal barrier is chiefly unknown (Ventura et al., 2009), and this study further investigates the mechanisms by which probiotic bacteria promote gut barrier function. The knowledge gained from a more complete understanding of probiotic function can lead to a better utilization of the bacteria as a medical treatment of diseases that disrupt the integrity of the gut barrier. Additionally, the utilization of specific miRNA inhibitors provides further

insight into their function within cell signaling and could open avenues to their use as therapeutic agents in various diseases. The connection between the beneficial effects of lactobacilli and the down-regulation of SNAIL transcription factors within epithelial cells could open avenues for lactobacilli to be used as a therapeutic agent in treating carcinomas known to be associated with the expression of SNAIL factors. Further understanding of the miRNA's that repress SNAIL expression could also lead to the direct employment of these miRNA's as a potential treatment of carcinomas as well.

Materials and Methods

Cell Culture

T84 human intestinal epithelial cell line (ECACC, Salisbury, UK) was grown in an incubator at 37 °C in a flask filled with 16 mL of medium that consisted of the following proportions: 90% DMEM Ham's F12 medium, 9% FCS (fetal calf serum), and 1% antibiotics (Penicillin/Streptomycin 100 µg/mL) (PAALaboratories, Cöolbe, Germany). The medium was changed every other day. Cells were counted and seeded onto 24-well plates to prepare for co-incubation experiments: medium was removed from the 75 cm^2 flask containing the T84 cell line, 1 mL of trypsin was added and flask was shaken to release cells from flask, 8 mL of medium was added, removed, and centrifuged at 800 xg for 4 minutes, pellet was re-suspended in 2 mL of medium, and then 10 µL of cells were placed onto cell counting plate. The amount needed to obtain $0.8x10^5$ cells in each well was calculated. Cells were grown overnight and medium was changed to infection medium (medium minus the antibiotics) prior to co-incubation.

Bacterial Culture

L. fermentum (PZ1162), *L. gasseri* (PZ1160), *L. rhamnosus* (PZ1121), and *L. acidophilus* (PZ1041) (PZ=Pharmazentrale GmbH, Herdecke, Germany) were stored at -80 °C. Bacteria was cultured and prepared for experiments: 1 centrifuge tube (1.5 mL) of frozen lactobacilli was added to 10 mL MRS broth and 2.5 mL of 25% Na_2HPO_4 buffer (to neutralize the pH value to 7). Culture was incubated at 37 °C overnight (without shaking) prior to usage.

Co-incubation and miRNA inhibitor Transfection experiments

For each experiment with one species of lactobacilli and one SNAIL miRNA inhibitor, 5 wells were utilized: (1) T84 cells only, (2) T84 and lactobacilli, (3) T84 co-incubated with lactobacilli and then transfected with SNAIL miRNA inhibitor, (4) T84 transfected with SNAIL miRNA inhibitor and then co-incubated with lactobacilli, and finally (5) T84 cells first co-incubated with lactobacilli and then transfected with an unspecific miRNA inhibitor. Cells were incubated with an MOI (multiplicity of infection) of 1.5 (1.5 bacteria per epithelial cell). This determined amount of lactobacilli (2 µL) was added to wells (3) and (5) and incubated at 37 °C for 2 hours. All wells were then washed 2 times with PBS (with Ca^{2+} and Mg^{2+}) and then filled with 450 µL of serum-free Opti-MEM-I® medium (Invitrogen, Darmstadt, Germany). X-tremeGene siRNA Transfection Reagent (Roche, Mannheim, Germany) was used for transfection experiments according to protocol. The two mixtures of 40 µL of Opti-MEM plus 10 µL of transfection reagent and 50 µL of Opti-MEM plus 7.5 µL SNAIL miRNA inhibitor or 1.5 µL of unspecific miRNA were combined and incubated for 20 minutes. Wells (3), (4), and (5) were transfected with corresponding transfection mixtures and incubated at 37 °C for 5 hours. Cells washed again 2 times with PBS (with Ca^{2+} and Mg^{2+}) and then medium was replaced by infection medium in wells (2) and (4) and regular medium in wells (1), (3), and (5) again to be left overnight at 37°C (not shaking). The next day wells (2) and (4) are co-incubated with 1.5 MOI of lactobacilli (2 µL).

For the second half of experiments, jetPRIME™ Transfection Reagent (pegLab, Erlangen, Germany) was used. Because protocol for the jetPRIME™ reagent called for a different concentration of cells within the well-plate, amount of SNAIL miRNA inhibitors and jetPRIME™ buffer and transfection reagent were changed accordingly. 4.12 µL of the SNAIL miRNA inhibitor was added to 100 µL of the buffer and pipetted up and down. Upon addition of 6 µL of the transfection reagent, the mix was incubated at room temperature for 10-15 minutes and then added to the cells. Additionally, the jetPRIME™ protocol called for an incubation period of 24 hours with the transfection reagent. In fear that the cells might die, the same length of transfection time as in the X-tremeGene protocol (5 hours) was followed. All other steps were completed as previously stated. Lactobacilli and SNAIL transcription factor experimental combinations included those stated in Table 1.

X-tremeGene siRNA Transfection Reagent	
L. fermentum	SNAI1 miRNA inhibitor
jetPRIME™ Transfection Reagent	
L. acidophilus	SNAI1 miRNA inhibitor
L. gasseri	SNAI2 miRNA inhibitor
L. rhamnosus	SNAI2 miRNA inhibitor

Table 1. Co-incubation and Transfection experiment contents: *Table illustrates the combinations of probiotic bacteria and SNAIL transcription factors that were probed. Additionally, the type of transfection reagent used in the experiment is shown.*

RNA Extraction

Upon completion of experiments, total RNA was extracted from cells using the RNeasy MiniKit (Qiagen, Hilden, Germany). After removing medium, cells were washed 2 times with PBS (with Ca^{2+} and Mg^{2+}) then 350 µL of RLT-Buffer Lysis was added. The contents were then transferred to 1.5 mL centrifuge tubes and vortexed. 350 µL of 70% EtOH was added to each tube and contents were pipetted up and down and then transferred to RNeasy columns which were set inside 2 mL tubes. Columns were centrifuged for 15 seconds at ≥8000 xg and flow-through in the 2 ml tube was discarded. 700 µL of Buffer RWI was added. The RNeasy column was again centrifuged for 15 seconds at ≥8000 xg, and flow through was discarded. 500 µL of Buffer RPE was added to the RNeasy column, centrifuged at ≥8000 xg for 15 seconds, and flow through was discarded. One last addition of 500 µL of Buffer RPE was added to the RNeasy column and then centrifuged for 2 minutes at ≥8000 xg. The RNeasy column was removed and placed in a new 2 mL tube and centrifuged at full speed for 1 minute. Finally the RNeasy column was placed in a new 1.5 mL centrifuge tube, 20 µL of RNase-free water was added, and it was centrifuged for 1 minute at 8000 xg. Addition of water and centrifugation was repeated one more time. Upon completion, the RNA was ready for cDNA preparation or was stored at -80 °C.

cDNA Preparation

cDNA preparation was completed by using the Transcriptor Reverse Transcriptase Kit (Roche, Mannheim, Germany). First, a mixture of 0.13 µL of OligodT and 7.87 µL of H_2O

was added to the RNA containing centrifuge tubes and incubated for 10 minutes at 65 °C for denaturation. Then, 4 µL of RT-Buffer, 0.5 µL of RNase Inhibitor, 2 µL of dNTP-Mix, and 0.5 µL of reverse transcriptase was added to each of the tubes. The mix was then incubated for 30 minutes at 55 °C to allow the cDNA to be made and then for 5 minutes at 85 °C to inactivate the enzyme. cDNA was stored at -20 °C.

Quantitative Real-Time PCR

Variation in E-cadherin gene expression was analyzed and recorded by qualitative RT-PCR via the LightCycler system (Roche, Mannheim, Germany) and LightCyler software Version 3.5 (Roche, Mannheim, Germany). First, each cDNA sample was diluted with distilled water to 1:10, 1:100, and 1:1000. E-cadherin, as well as HPRT I, forward and reverse primers were designed by the Universal Probe Library Assay Design Center (Roche, Mannheim, Germany) and were manufactured by Eurofins MWG GmbH (Ebersberg, Germany). HPRT I (hypoxanthine phosphoribosyl-transferase I) was used as a reference. Primers were diluted with water to 1:10. The Roche Mix was then prepared by diluting the Syber green with water 1:2. The LightCyler® Capillaries (Roche, Mannheim, Germany), as well as the primers, cDNA, and Roche Mix, were all kept on ice during preparation. 4 µL of Roche Mix was added to each of the 32 capillaries. Then 4 µL of cDNA dilution and finally 2 µL of primer was added to each capillary according to Table 2.

Capillary	RT-PCR Capillary Contents
1.	HPRT_T84 1:10
2.	HPRT_T84 1:100
3.	HPRT_T84 1:1000
4.	E-cad_T84 1:10
5.	E-cad_T84 1:100
6.	E-cad_T84 1:1000
7.	HPRT only (negative control)
8.	HPRT_T84_*Lactobacilli* 1:10
9.	HPRT_T84_*Lactobacilli* 1:100
10.	HPRT_T84_*Lactobacilli* 1:1000
11.	E-cad_T84_*Lactobacilli* 1:10
12.	E-cad_T84_*Lactobacilli* 1:100
13.	E-cad_T84_*Lactobacilli* 1:1000
14.	E-cad only (negative control)
15.	HPRT_T84_*Lactobacilli*+SNAIL miRNA inhibitor 1:10
16.	HPRT_T84_*Lactobacilli*+SNAIL miRNA inhibitor 1:100
17.	HPRT_T84_*Lactobacilli*+SNAIL miRNA inhibitor 1:1000
18.	E-cad_T84_*Lactobacilli*+SNAIL miRNA inhibitor 1:10
19.	E-cad_T84_*Lactobacilli*+SNAIL miRNA inhibitor 1:100
20.	E-cad_T84_*Lactobacilli*+SNAIL miRNA inhibitor 1:1000
21.	HPRT_T84_SNAIL miRNA inhibitor+*Lactobacilli* 1:10
22.	HPRT_T84_SNAIL miRNA inhibitor+*Lactobacilli* 1:100
23.	HPRT_T84_SNAIL miRNA inhibitor+*Lactobacilli* 1:1000
24.	E-cad_T84_SNAIL miRNA inhibitor+*Lactobacilli* 1:10
25.	E-cad_T84_SNAIL miRNA inhibitor+*Lactobacilli* 1:100
26.	E-cad_T84_SNAIL miRNA inhibitor+*Lactobacilli* 1:1000
27.	HPRT_T84_*Lactobacilli*+unspecific miRNA inhibitor 1:10
28.	HPRT_T84_*Lactobacilli*+unspecific miRNA inhibitor 1:100
29.	HPRT_T84_*Lactobacilli*+unspecific miRNA inhibitor 1:1000
30.	E-cad_T84_*Lactobacilli*+unspecific miRNA inhibitor 1:10
31.	E-cad_T84_*Lactobacilli*+unspecific miRNA inhibitor 1:100
32.	E-cad_T84_*Lactobacilli*+unspecific miRNA inhibitor 1:1000

Table 1Table 2. RT-PCR Capillary Contents: *RT-PCR Capillary number and corresponding contents to be added for each experimental trial.*

Results

Procedures were undertaken with *L. fermentum* (1162) and SNAI1 miRNA inhibitor, *L. acidophilus* (1041) and SNAI1 miRNA inhibitor, *L. gasseri* (1160) and SNAI2 miRNA inhibitor, and *L. rhamnosus* (1121) and SNAI2 miRNA inhibitor (refer to Figures 1. – 4., respectively). Controls included: unchanged T84 colon cells, T84 cells co-incubated with bacteria, and T84 cells co-incubated with bacteria and then transfected with an unspecific miRNA inhibitor that has no effect on the adherence junction proteins. An up regulation of the second control with the addition of the bacteria as compared to the first control with only the T84 cells assures proper co-incubation was achieved. The third control, with the addition of an unspecific miRNA inhibitor, serves as a negative control for the transfections. Introduction of the SNAIL miRNA inhibitors produces a down-regulation of E-cadherin mRNA.

As seen in Figure 1., amounts of E-cadherin mRNA detected by quantitative RT-PCR were up-regulated by the presence of *L. fermentum*. The concentrations of E-cadherin mRNA after co-incubation with the lactobacilli and unspecific miRNA inhibitor were not definitively lower than the T84 + *L. fermentum* sample and were up-regulated compared to the T84 cells alone. The effect of the lactobacilli on the cells was neutralized by transfection of the SNAI1 miRNA inhibitor. Experiments conducted with *L. fermentum* co-incubation and SNAI1 miRNA inhibitor transfection successfully demonstrated proper co-incubation and were in line with the expected results for controls.

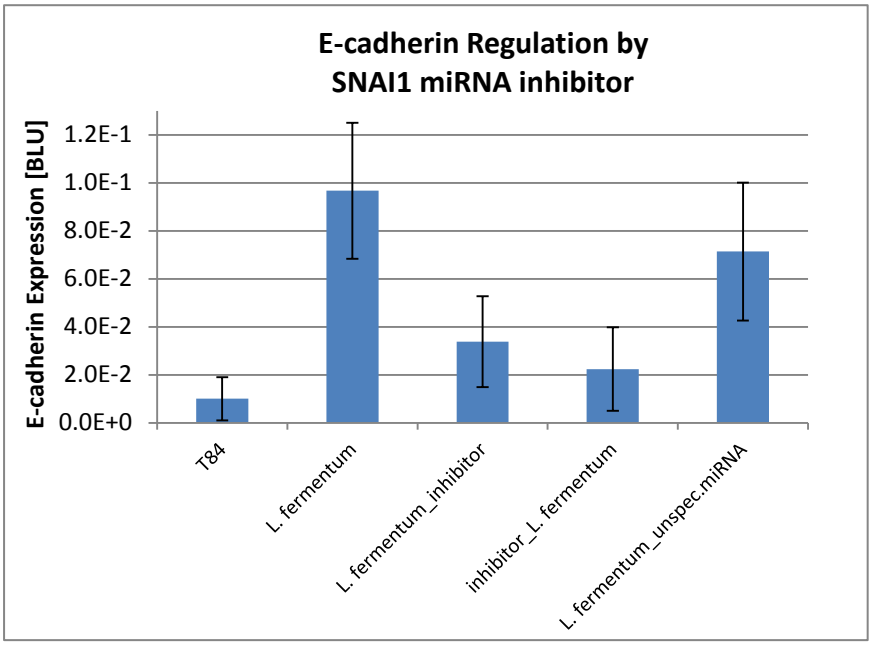

Figure 1. E-cadherin Regulation by SNAI1 miRNA inhibitor and L. fermentum: *Analysis of E-cadherin regulation in T84 Cells co-incubated with Lactobacillus fermentum (1162) and transfected with the SNAI1 miRNA inhibitor.*

Experimentation with *L. acidophilus* and the SNAI1 miRNA inhibitor produced a similar pattern of results as with *L. fermentum*, but impact on up-regulation was less (Figure 2.). Slight up-regulation of E-cadherin could be speculated after co-incubation with *L. acidophilus*. The post bacterial incubation transfection of the unspecific miRNA inhibitor produced concentrations of E-cadherin that were fairly similar to the levels present in the T84 cells without any exposure to bacteria. Without a clear up-regulation of E-cadherin that was unhindered by the presence of a negative control miRNA inhibitor, this data cannot be utilized as a proper control for the experiment. There was a down-regulation of E-cadherin mRNA concentrations after transfection with the SNAI1 miRNA inhibitor.

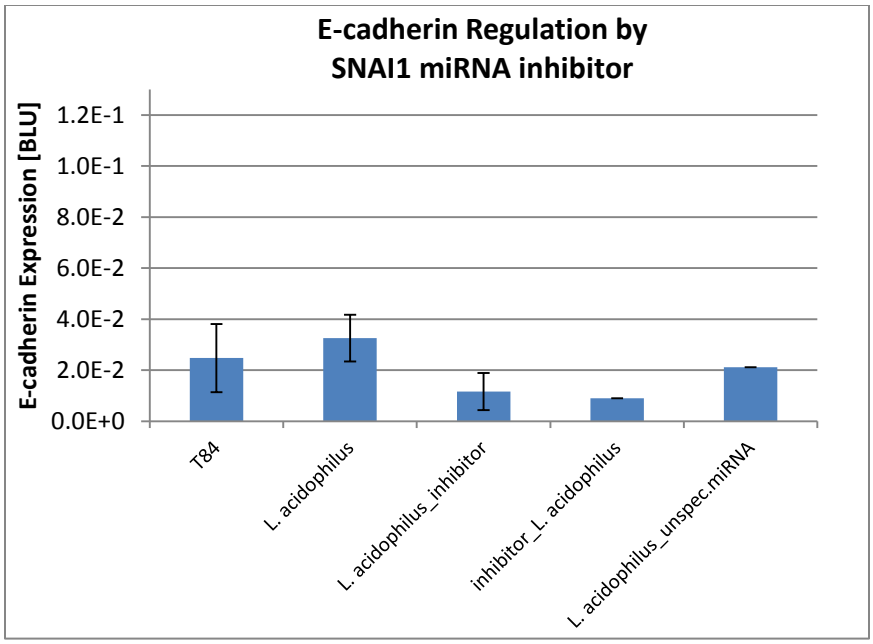

Figure 2. E-cadherin Regulation by SNAI1 miRNA inhibitor and L. acidophilus: *Analysis of E-cadherin regulation in T84 Cells co-incubated with Lactobacillus acidophilus (1041) and transfected with the SNAI1 miRNA inhibitor.*

The co-incubation and transfection experiments conducted with *L. gasseri* and the SNAI2 miRNA inhibitor were not as definitive (Figure 3.). Slight up-regulation of E-cadherin mRNA concentrations could be noted in the *L. gasseri* co-incubated T84 cells as compare to the unchanged T84 cell sample. Neither a concretely elevated level of E-cadherin mRNA concentrations after transfection of the unspecific miRNA inhibitor, nor a definitive down-regulation of E-cadherin mRNA concentrations for transfection of the SNAI2 miRNA inhibitor after incubation of the bacteria could be observed. Furthermore, there appeared to be no certain up-regulation when T84 cells were exposed to the lactobacilli after the miRNA inhibitor transfection.

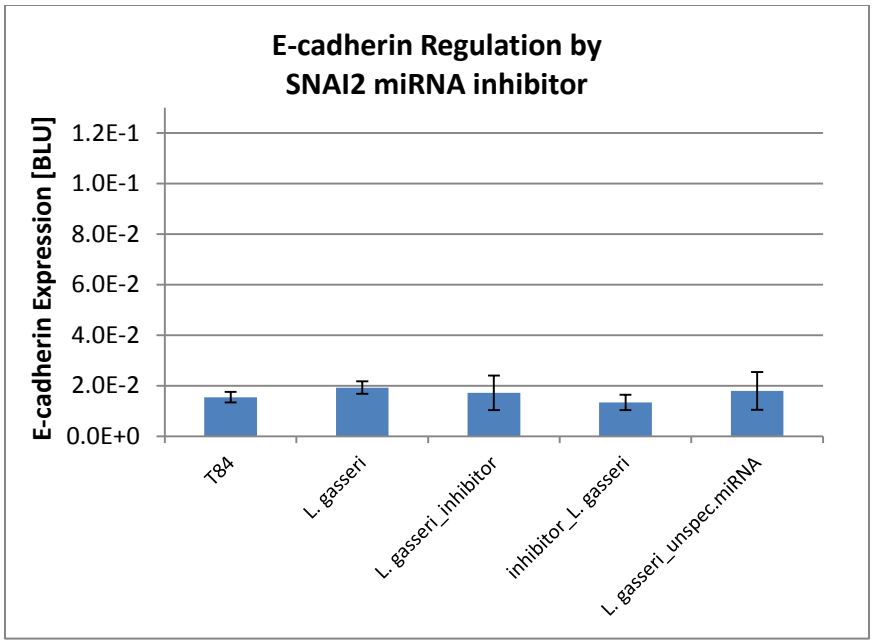

Figure 3. E-cadherin Regulation by SNAI2 miRNA inhibitor and L. gasseri*: Analysis of E-cadherin regulation in T84 Cells co-incubated with Lactobacillus gasseri (1160) and transfected with the SNAI1 miRNA inhibitor.*

Figure 4. displays data for the experiment with *L. rhamnosus* and the SNAI2 miRNA inhibitor. While it could not be definitely argued, a very slight rise in E-cadherin concentrations is seen within the T84 cells upon co-incubation with the *L. rhamnosus*. Although the projected up-regulation of the third control that was transfected with an unspecific miRNA inhibitor after lactobacilli exposure could not be conclusively supported, E-cadherin mRNA levels were clearly down-regulated in the third sample in response to transfection with the SNAI2 miRNA inhibitor after co-incubation with the lactobacilli (which suggests some relationship). The negative regulatory effects produced in E-cadherin mRNA levels by the presence of the SNAI2 miRNA inhibitor were not neutralized by later co-incubation of *L. rhamnosus* in the fourth sample.

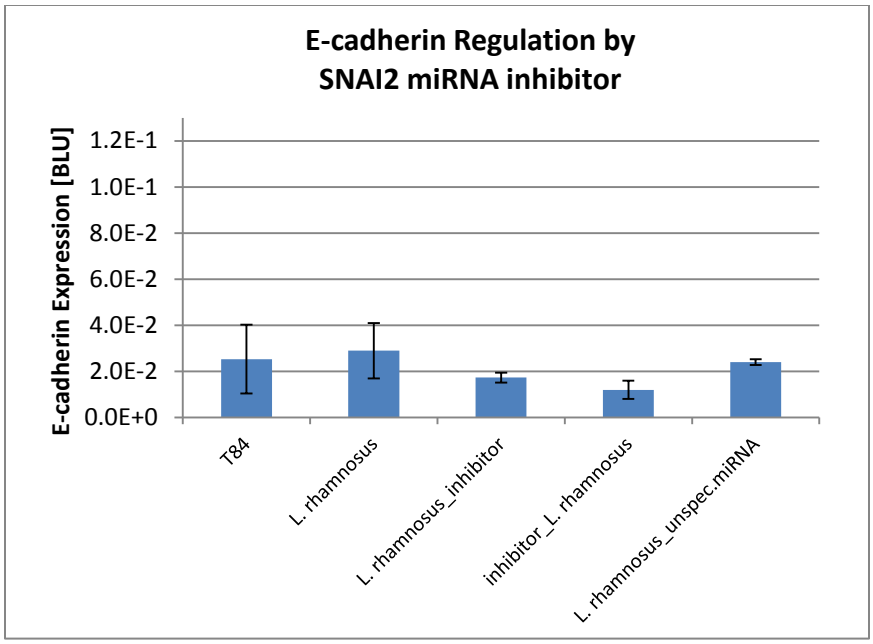

Figure 4. E-cadherin Regulation by SNAI2 miRNA inhibitor and L. rhamnosus: *Analysis of E-cadherin regulation in T84 Cells co-incubated with Lactobacillus rhamnosus (1121) and transfected with the SNAI2 miRNA inhibitor.*

Discussion

In an effort to characterize the cell signaling induction of adherence junction complexes of T84 colon cells by the presence of lactobacilli, miRNA studies were performed with the miRNA inhibitors of SNAIL transcriptional factors (SNAI1 and SNAI2). The SNAIL transcription factors are known to be repressors of E-cadherin expression (Batlle et al., 2000 and Guaita et al., 2002). Four species of lactobacilli were used in this study: *L. fermentun, L. acidophilus, L. gasseri,* and *L. rhamnosus.* The first three species of lactobacilli are known to confer positive benefits on the T84 cells by up-regulation of E-cadherin protein (Zyrek et al., 2006 and Hummel et al., 2011), and the later, *L. rhamnosus*, has not been confirmed to directly influence the adherence junctions by positive regulation of E-cadherin proteins. Still, increases in transepithelial resistance (TER) upon co-incubation of T84 cells with *L. rhamnosus* confirmed that this species of bacteria also positively affects the gut barrier integrity (Hummel et. al, 2011). Therefore, an affirmative and reproducible up-regulation of E-cadherin expression after exposure to *L. fermentum, L. acidophilus,* and *L. gasseri* was expected, but the ability of *L. rhamnosus* to induce expression was uncertain.

In order to determine if probiotic regulation and SNAIL transcriptional regulation of E-cadherin occurred through similar mechanisms, miRNA studies were utilized. This study aimed to test whether or not regulatory effects of these lactobacilli acted on the same miRNA's that influence the functionality of the SNAIL transcription factors. If this was the case, the lactobacilli could override the regulatory effects that miRNA inhibitors

have on the SNAIL transcription factors. Alternatively, the lactobacilli could activate transcription independently of SNAIL factors.

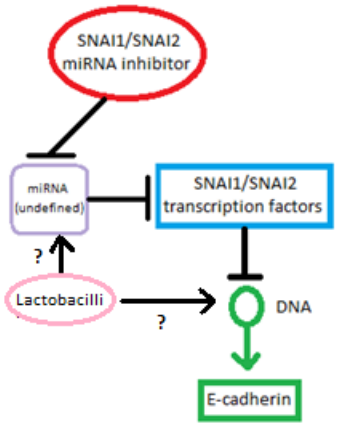

Figure B. Transcriptional effect of SNAIL miRNA inhibitors + Potential Lactobacilli effect: *Diagram of action taken by SNAIL miRNA inhibitors to repress the miRNA that suppress the SNAIL transcription factors. Expressed SNAIL factors bind to the promoter region of the E-cadherin gene and repress transcription of E-cadherin mRNA. Diagram also includes the hypothesized influence of lactobacilli on the regulation of E-cadherin by the potential positive influence on the miRNA's that inhibit the SNAIL transcription factors or an alternative activation of transcription independent of the SNAIL factors.*

For this study, we only investigated the potential relationship between *L. fermentum* and SNAI1, *L. acidophilus* and SNAI1, *L. gasseri* and SNAI2, and *L. rhamnosus* and SNAI2, due to time and supply constraints. Further trials should be performed with the other four combinations (*L. fermentum* and SNAI2, *L. acidophilus* and SNAI2, *L. gasseri* and SNAI1, and *L. rhamnosus* and SNAI1) to fully articulate the potential connection between the two mechanisms of regulating E-cadherin expression.

The first experiment with *L. fermentum* and SNAI1 miRNA inhibitor (Figure 1.) was the most reproducible one out of the four completed. Previous understandings of the effect of *L. fermentum* were upheld as the experiment showed a clear up-regulation of E-cadherin mRNA after co-incubation with the bacteria. Additionally, the known function

of the SNAI1 miRNA inhibitor was confirmed, as well as the function of the SNAI1 transcription factor in its ability to repress the transcription of E-cadherin. While the experiment was set up to test whether or not the negative effect of the SNAIL transcription factors would be overridden by the lactobacilli, results did not produce expected results. Still, the change in E-cadherin mRNA concentrations upon exposure to both the bacteria and the miRNA inhibitor could be suggestive of some type of relationship between the mechanisms by which the SNAIL transcription factors and the probiotics effect the expression of E-cadherin. While this may be a possibility, we cannot confirm that *L. fermentum* has a regulatory effect on the miRNA that represses the SNAI1 transcription factor and they could be explained to act through independent, alternative mechanisms as well.

The first experiment with *L. fermentum* and the SNAI1 miRNA inhibitor was the only one that was completed with the routinely used transfection reagent (X-tremeGene), while the others were performed with a substitute (jetPRIME™). Concentrations were amended for the trials with the second transfection reagent. As seen in the results of the later three experiments, results were not as definite and error bars were higher than in the first experiment with *L. fermentum* and SNA1 miRNA inhibitor. The qRT-PCR procedure performed is a sensitive method, and the undesirable size of the error bars can also be due to lack of familiarity of the researcher with the experimental techniques, especially in preparing dilutions. This error could be lowered with greater precision through practice.

The second experiment was carried out with *L. acidophilus* and the SNAI1 miRNA inhibitor (Figure 2.). Again, the expected effects of *L. acidophilus* and the SNAI1 miRNA inhibitor were reconfirmed by this experiment. The results also suggest a relationship between the pathways, but the up-regulation of E-cadherin was not restored upon co-incubation of *L. acidophilus* after transfection with the SNAI1 miRNA inhibitor. This data also does not define a clear relationship between *L. acidophilus* and the SNAI1 transcription factor regulation of E-cadherin concentrations.

The third experiment included the probiotic *L. gasseri* and the SNAI2 miRNA inhibitor (Figure 3.). While results were not as definitive as in experiments one and two, there was still a quantifiable up-regulation detected by the RT-PCR in E-cadherin after exposure to *L. gasseri* and a down-regulation with influence of the SNAI2 miRNA inhibitor. Again, we could not confirm that the lactobacilli and the SNAIL transcription factor regulate E-cadherin expression by a similar mechanism, considering that the effects of *L. gasseri* did not clearly impede the SNAI2 transcription factor's suppression of E-cadherin transcription.

Considering that *L. rhamnosus* is not known to positively affect the gut-barrier function by means of up-regulating E-cadherin (Hummel et. al, 2011), the ambiguity presented by the error bars in the first two samples of Figure 4. does not make this experiment a wash. On the other hand, this experiment does reconfirm the understanding of the SNAI2 transcription factor to impose a negative effect on the transcription of E-cadherin. Once again, no evident results linked the mechanisms by which this species of lactobacilli regulated E-cadherin expression and the mechanisms that are utilized by the

SNAI2 transcription to regulate this junction protein. Testing the other SNAIL factor, the SNAI1 miRNA inhibitor, with *L. rhamnosus* would be a very interesting study and if results showed an up-regulation in the miRNA inhibitor then probiotic treatment, then there would be more reason to believe that this probiotic does in fact have an effect on E-cadherin expression.

Liu et al. (2012) performed a study that directly identified an association between manipulating the concentration of a hsa-miR-9 and the upregulation of E-cadherin and correlating down-regulation of SNAI1. This study, as well as others, link higher concentrations of SNAIL transcription factors with the occurance of various carcinomas (Batlle et. al, 2000; Guaita et al., 2002; Roy et al., 2005). Further research into the specific funtion and identity of possible miRNA's that regulate SNAIL transcription factors (or other factors) can provide insight to new potential cancer treatments. miRNA's are naturally present in cells and act on the regulation of cell functions in many ways. Manipulation of these naturally occuring nucleotides could provide a therapy that is not as harmful as conventional chemotherapy approaches.

Furthermore, the means by which regulation of these miRNA's is achieved is a key factor of this possible therapy. There is a link between the regulatory function of the probiotic lactobacilli and the SNAIL transcription factors on the expression of E-cadherin. This influence on E-cadherin concentrations is a vital factor to the barrier function and consequently the health of the epithelial cells of the gastrointestinal barrier. Probiotics are used today as a non-invasive, natural supplement to improve the health of the gut and as therapy for various gastrointestinal disorders. It has been shown that probiotics

have the ability to reduce the risk of cancer (O'Toole et. al, 2008). If it can be seen that these same probiotics have regulatory effects on SNAIL transcription factors, there is a possibility for probiotics to be further utilized in cancer therapy. These experiments showed some connection between the effect of these transcription factors and probiotics. While some of the results were ambiguous, they still support the existence of a relationship. Further research is needed to investigate whether or not the lactobacilli influence up-regulation of E-cadherin via an overlapping pathway with the SNAIL transcription factors.

In addition to the need for further experiments, additional trials of each experiment, and increased precision of techniques, there are some other components of these trials that may be considered in further research in order to ensure verifiable and reproducible results. Monitoring the effect of the miRNA inhibitor directly and what its influence on the T84 cells alone may be a task worthy of consideration. Additionally, it is always important to assure that the fewest variables possible exist between samples. To insure less deviation, one might consider devising a plan that would limit the variation of time after co-incubation prior to RNA extraction. While consistency of the transfection times was maintained, the time after the co-incubation of the lactobacilli in the third and fifth sample is much longer than that of the second and fourth. With differentiated lengths of time proceeding the co-incubation period of the lactobacilli, it can be questioned whether or not the positive effect of E-cadherin was sustained or had diminished over time.

Further understanding the ways by which SNAIL transcription factors are regulated would also help strengthen the conclusions aimed at by this study. While it is known that SNAI1 and SNAI2 repressors act by binding to the 5'-CACCTG-3' sequence of the E-cadherin promoter region, the E-Box (Batlle et al., 2000 and Guaita et al., 2002), the complete regulation by the SNAIL factors is largely unknown. Once bound to the E-Box region, there is the question of how the SNAIL factor is released in order for transcription to proceed. This may be where the miRNA's are utilized. Whether or not the miRNA inhibition of the SNAIL transcription factors is vital pre and/or post-binding to the E-cadherin gene is a question worth further investigation. Liu et al. (2012) gave us reason to believe that the concentration of these miRNA's (such as has-miR-9) is vital to SNAIL functionality, but further insight to how these miRNA act on the SNAIL transcription factors and if probiotics, like lactobacilli, have any effect on them is essential to the possibility of the use of miRNA's, and possibly probiotics, as a potential therapeutic control of SNAIL factors in cancerous cells.

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