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Consumption of a natural high intensity sweetener enhances activity and expression of rabbit intestinal Na^+/glucose cotransporter 1 (SGLT1) and improves colibacillosis-induced enteric disorders.

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Running title: Natural sweetener improves enteric disorders.

ABSTRACT

Absorption of glucose, via intestinal Na^+/glucose cotransporter 1 (SGLT1), activates salt and water absorption and is an effective route for treating *Escherichia coli* (*E. coli*)-induced diarrhea. Activity and expression of SGLT1 is regulated by sensing of sugars and artificial/natural sweeteners by the intestinal sweet receptor, T1R2-T1R3 expressed in enteroendocrine cells. Diarrhea, caused by the bacterial pathogen *E-coli* is the most common post-weaning clinical feature in rabbits, leading to mortality. We demonstrate here, that in rabbits with experimentally *E-coli* - induced diarrhea, inclusion of a supplement containing stevia leaf extract (SL) in the feed improves clinical signs of disease. We show that the rabbit intestine expresses T1R2-T1R3. Furthermore, intake of SL enhances activity and expression of SGLT1, and the capacity to absorb glucose. Thus, a natural plant extract sweetener can act as an effective feed additive for lessening the negative impact of enteric disease in animals.

Key words: Colibacillosis; Oral rehydration; Rabbit; SGLT1; Intestinal T1R2-T1R3

INTRODUCTION

Na^+/glucose co-transporter 1, SGLT1, is the major route for absorption of glucose across the intestinal brush border membrane. Absorption of glucose via SGLT1 activates electrolyte and water absorption. In humans, this strategy has been used in oral rehydration therapy, which is the safest and most effective remedy for treating life-threatening diarrhea induced by agents such as *Vibrio cholerae* and *Escherichia coli*.[1,2] The condition is caused by toxic peptides produced by bacteria stimulating the conversion of guanosine 5′-triphosphate (GTP) to cyclic guanosine 5′-monophosphate (cGMP) by the enzyme guanylate cyclase. Increased intracellular cGMP inhibits intestinal fluid uptake, resulting in net fluid secretion and thus diarrhea.

The gut epithelium can sense sugars and artificial sweeteners via the sweet receptor comprising of Taste family 1 Receptor 2 (T1R2) and 3 (T1R3) expressed on the luminal membrane of enteroendocrine cells (EEC).[3,4] This results in secretion of gut hormones, glucagon-like peptide 1 (GLP-1), glucagon- like peptide 2 (GLP-2) and glucose-dependent insulinotropic peptide (GIP) from EEC.[5,6] GLP-2 upregulates SGLT1 activity and expression[7,8] in neighboring absorptive enterocytes via a neuro-paracrine pathway.[6,9] GLP-2 also increases villus height and intestinal barrier function[10,11], thereby promoting gut health. These effects have also been reported in piglets;[12] calves and ruminants.[13]

The sweet taste receptor is similarly activated by natural, high-intensity sweeteners, such as stevia[14] leading to increased expression and activity of SGLT1, providing the capacity for enhanced glucose (electrolyte and water) absorption.

Rabbits are raised for a variety of commercial reasons. Their meat, wool and fur are valuable commodities, as is their nitrogen-rich manure and high protein milk. They are also very popular as household pets. Diarrhea is the most common post-weaning clinical feature in rabbits, leading to significant rates of mortality. With current trends aimed at decreasing the

use of antibiotics, feed additives that can improve rabbit health and performance in the face of disease is highly desirable. This is especially relevant in Europe, where antibiotic use in animal feed is already banned, and the use of natural alternatives, for disease prevention, is encouraged. Furthermore, in Europe the use of artificial sweeteners, used routinely in farm animal nutrition,[12,13] is prohibited as supplements in rabbit feed. It is not known if a natural high-intensity sweetener such as stevia leaf extract (**SL**), which can be used in rabbit feed, will elicit similar effects as seen with artificial sweeteners in farm animals, assisting to prevent and ameliorate enteric diseases in rabbits.

Here we show that when rabbits are challenged with colibacillosis, inclusion of a supplement containing SL in rabbit feed leads to significant reduction in diarrhea and bloat, improved health, and decreased mortality. Furthermore, we demonstrate that rabbit intestine expresses the intestinal sweet receptor T1R2-T1R3, and that inclusion of SL in the feed results in upregulation of SGLT1 activity, protein and mRNA abundance in the small intestine. Thus, a better understanding of the molecular mechanism underlying intestinal nutrient absorption provides a rational strategy for using a natural feed additive for alleviating enteric disorders and promoting health and well-being of animals.

MATERIALS AND METHODS

Chemicals

SL supplement (containing 25% stevia leaf extract and 2% *capsicum oleoresin* (for concentration see below); TakTik Rabbit), was from Pancosma, Geneva, Switzerland. Zymo Total RNA isolation kit with on-column DNase 1 digestion, was from Cambridge Bioscience, Cambridge, UK. dT_{20} primers and superscript III reverse transcriptase was from Life Technologies, Paisley, UK and QIAquick PCR purification kit from Qiagen, Crawley, West Sussex, UK. Consensus primers for mammalian T1R2 and T1R3 were purchased from Eurogentec, Seraing, Belgium. Q5 Hot Start High-Fidelity DNA Polymerase was purchased from New England Biolabs, Hitchin, Herts, UK, and pGEM-T Easy vector from Promega, Southampton, UK. SYBR green JumpStart *Taq* ReadyMix, dithiothreitol, benzamidine, phenolmethylsulfonyl fluoride, Bio-Max Light Chemiluminescence Film, β-actin antibody (clone AC-15), D.P.X. neutral mounting medium, donkey serum, 10% neutral buffered formalin and Mayer's Haemalum (3.3 mmol/L Mayer's Haemalum-haematoxylin, 1 mmol/L sodium iodate, 0.42 mmol/L potassium alum) were purchased from Sigma-Aldrich, Poole, Dorset, UK. Bio-Rad protein assay solution and polyvinylidene difluoride (PVDF) membrane were from Bio-Rad Laboratories Ltd. Hemel Hempstead, UK. The antibody to SGLT1 was raised in rabbits (custom synthesis) to a recombinant peptide corresponding to amino acids 554-640 of rabbit SGLT1 protein. Horseradish peroxidase-linked secondary antibodies were purchased from DAKO Ltd, Cambridge, UK. Immobilon Western Chemiluminescent HRP Substrate and cellulose acetate/nitrate filter were purchased from Millipore, Hertfordshire, UK and [U-^{14}C]-D-glucose (10.6 GBq/mmol) from Perkin Elmer, Seer Green, Bucks, UK. Scintillation fluid (Optiphase HiSafe 3), was purchased from Fisher Scientific, UK and Eosin Y solution (1 % [w/v] eosin aqueous) was from HD Supplies, Buckingham, Bucks, UK. Chromogranin A antibody was from Abcam, Cambridge, UK. Antibodies to T1R2 and T1R3

are from Santa Cruz Biotechnology, INC., Heidelberg, Germany, and IgG Cy3- FITC-conjugated secondary antibodies from Stratech Scientific, Newmarket, UK. 4′,6-diaminido-2-phenylindole (DAPI) was purchased from Vector Laboratories, Peterborough, UK.

Phase 1:

Animals, Treatments and Experimental condition. The animal experiment was conducted at the Talhouet Research Center (Saint Nolff, France). All animal procedures were approved by the Ethical Committee for Animal Experimentation of NEOVIA and by the Ministry of Higher Education, of Research and Innovation, France (experimental reference # 03835.03). Animal numbers were determined based on power calculations conducted using data from previous experiments performed in the same facility wherein colibacillosis challenge was used. Thirty-six day old Souche Hyplus PS59 rabbits (http://www.hypharm.fr; n = 300) were weaned, blocked by sex, litter origin and body weight, and assigned to one of four dietary treatments (n = 75/trt): un-supplemented diet or a diet supplemented with 50, 75, or 100 ppm of SL, containing a maximum of 3.3, 4.9 and 6.5 µM capsaicin). Animals were housed in cages (5 rabbits per cage) with *ad libitum* access to feed and water. Rabbit feed was formulated for a typical fattening ration containing 15.5% crude protein and ME of 22.9 kcal/100 g feed. All feed was free of antibiotics and medications, including coccidiostats. The room was maintained at 19°C and was illuminated between 0700 and 1700 each day. All animals were monitored daily.

On day 44 of age (day 0 of infection), all rabbits were orally inoculated with 5 x 10^6 CFU/mL of *E. Coli* O103 LY265 inoculum (INRA, Nouzilly, France; dose determined in preliminary experiments and validated in several separate experiments).

Measurements. Feed intake was measured daily per cage by weighing of refusals. Live weights of individual rabbits were measured on days -2, 5, 12, 19 and 26 post infection (corresponding to 42, 49, 56, 63, 70 d of age), and average daily gain (ADG) was calculated from individual body weights. Feed efficiency (G:F; gain/feed) was calculated per cage. Morbidities [visual signs of diarrhea and discoloration of feces, bloat (swollen stomach, dull fur, low energy), and mobility] were assessed daily by two technicians trained by a veterinarian. Morbidities were not quantified but were simply noted as present or absent based on subjective visual observation by both technicians. The same technicians performed the scoring throughout the study to avoid variation due to observer. Mortalities were also recorded daily; dead animals were removed from cages upon detection and visible clinical signs were noted. At the peak of mortality during clinical disease, a random selection of rabbits (n = 10) was necropsied to verify colibacillosis as the cause of death (via *E. coli* serotyping of intestinal content).

The experiment ended on day 26 post infection (when animals were 70 d of age) and all remaining animals were euthanized by a trained technician. Average body weight at 70 d of age was multiplied by the number of animals alive to estimate production weight per treatment.

Statistical Analysis. Data were analyzed by ANOVA using the SAS Mixed Procedure with a Dunnett's adjustment for multiple comparisons and orthogonal contrasts to test for linearity. Treatment and time were fixed effects whereas sex and cage were treated as random effects. Statistical significance was set at $p < 0.05$.

Phase 2:

154 The experiments in Phase 2 were undertaken to understand the molecular mechanisms
155 underlying the intestinal response of rabbits to SL.

156 **Animals, dietary trial, gut tissue sampling.** The animal experiment was conducted at the
157 Talhouet Research Center (Saint Nolff, France). All animal procedures were approved by the
158 Ethical Committee for Animal Experimentation of NEOVIA and by the Ministry of Higher
159 Education, of Research and Innovation, France (experimental reference # 03835.03). Animal
160 numbers were determined using gut responses and variation associated with supplementation
161 with artificial sweetener reported in published articles.[6,12,13] Forty-two 60-day-old Souche
162 Hyplus PS59 rabbits (http://www.hypharm.fr) were blocked by sex and body weight and
163 assigned to one of two dietary treatments starting on day 61 of age (n = 21 rabbits/treatment):
164 un-supplemented diet or a diet supplemented with 75 ppm SL (dose chosen based on
165 responses observed in Phase 1). Animals were housed in cages (5 rabbits per cage) with *ad*
166 *libitum* access to feed and water. Rabbit feed was formulated for a typical fattening ration
167 containing 15.5% crude protein and ME of 22.9 kcal/100 g feed. All feed was free of
168 antibiotics and medications, including coccidiostats. The room was maintained at 19°C and
169 was illuminated between 0700 and 1700 each day. After the 9-day treatment period (the
170 period of 9 days was selected to cover the gut epithelial cell turnover that takes 4-5 days in
171 the majority of species and was extended to 9 days due to travel delays for the researcher
172 from the UK travelling to France for harvesting intestinal tissues) at 70 days of age (same
173 slaughter age as phase 1), all rabbits were weighed and euthanized by intra-cardiac injection
174 of Euthasol® after sedation starting at 9am. Intestinal tissues were removed, duodenal: 10 cm
175 distal to the pyloric caeca, ileal: 10 cm proximal from the ileocecal valve, jejunal; at the mid-
176 point between the pyloric caeca and ileocecal valve. Tissue samples collected from 10
177 rabbits/treatment (blocked by sex and body weight at slaughter), rinsed in ice cold saline and
178 either placed into cryovials or wrapped in aluminum foil and frozen immediately in liquid

nitrogen or pinned to dental plastic and fixed in 10% neutral buffered formalin at 4°C. Fixed tissues were transferred to 20% sucrose in PBS after 24 h and stored at 4°C. Frozen tissues were stored at -80°C before shipping to the UK on dry ice, whilst fixed samples were shipped to the UK on wet ice, for subsequent analysis.

Cloning of rabbit T1R2 and T1R3. Total RNA was isolated from rabbit intestinal tissues using the Zymo Total RNA isolation kit with on-column DNase 1 digestion. RNA was quantified by UV spectrophotometry (assuming an OD_{260} value of 1 = 40 μg/mL) and integrity determined by agarose gel electrophoresis. Complementary DNA (cDNA) was prepared using oligo dT_{20} primers and superscript III reverse transcriptase, purified using QIAquick PCR purification kit and quantified by UV spectrophotometry (assuming an OD_{260} value of 1 = 33 μg/mL). Consensus primers for mammalian T1R2 and T1R3 are listed in Table 1. Each PCR reaction mix contained 0.5 μmol/L of each forward and reverse primer, 0.5 U of Q5 Hot Start High-Fidelity DNA Polymerase, and 25 ng template cDNA in a final volume of 25 μl. PCR cycling was carried out as follows: initial denaturation at 98°C for 1 min, 25 cycles of denaturation at 98°C for 10 s, annealing for 10 s and extension at 72°C for 30 s, followed by a final extension step at 72°C for 2 min. PCR amplicons were gel purified using 1% agarose gels, cloned into pGEM-T Easy vector and custom sequenced (Eurofins-MWG, Ebersberg, Germany). Sequence alignments and amino acid translations were performed using commercial software (Vector NTI, Life Technologies).

The radial phylogram shown in Figure 7, depicting the phylogenetic relationship of rabbit T1R3 to various mammalian homologs, was constructed by neighbor-joining analysis[15] of distance matrices generated using the PROTDIST program (Jones-Taylor-Thornton similarity model),[16] as part of the phylogenetic inference package, PHYLIP.[17]

204 **Table 1.** Primers used for PCR and qPCR.

Primer name	Accession No.	sequence	Tm / °C
RbACTB S	NM_001101683	5'-CCTTCTACAACGAGCTGCGAG-3'	51.4
RbACTB AS	NM_001101683	5'-GCCCTCGTAGATGGGTACTG-3'	49.9
RbPOLR2A S	XM_017348893.1	5'-ACGCTGCTCTTCAACATCCA-3'	60
RbPOLR2A AS	XM_017348893.1	5'-CCAGCGTAGTGGAAGGTGTT-3'	60
RbB2M S	XM_008269078.2	CTAGTCTTGTTCCCCTGCCT	58.9
RbB2M AS	XM_008269078.2	ATCAATCTGGGGCGGATGAAA	60
RbT1R2 S	XM_017346518	5'-TCTGGAACGTCAGCTTCACC-3'	52.5
RbT1R2 AS	XM_017346518	5'-GTGCTTCAGCATGGGGTAGT-3'	51.6
RbT1R3 S		5'-GCAAGTTCTTCAGCTTCTTCCT-3'	51.5
RbT1R3 AS		5'-TACATGTTCTCCAGGAGCTGC-3'	51.9
RbSGLT1 S	NM_001101692	5'-TGTCAAGGCTGGCTGTATCC-3'	51.4
RbSGLT1 AS	NM_001101692	5'-CTCCTCTGGTTCCACGCAA-3'	51.3

205

206 **Quantitative PCR.** Relative mRNA expression in the intestine was determined by
207 quantitative real-time PCR (qPCR). cDNA was prepared from total RNA as described above
208 and diluted to 5 ng/μL. Primers to rabbit SGLT1, β-actin (ACTB), RNA polymerase II
209 (POLR2A), and β-2-microglobulin (B2M) were designed using Primer-BLAST[18] and
210 purchased from Eurogentec (see Table 1). Each qPCR reaction consisted of 25 ng cDNA
211 template, 1 X SYBR green JumpStart *Taq* ReadyMix and 900 nmol/L of each primer in a
212 total volume of 25 μL. The PCR cycling consisted of initial denaturation at 95°C for 2 min
213 followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. Assays were performed in
214 triplicate using a RotorGene 3000 (Qiagen) with relative abundance calculated using RG-
215 3000 comparative quantification software (Qiagen). Abundance of SGLT1 mRNA was
216 normalized to the genomic mean of ACTB, POLR2A and B2M housekeeping genes, the
217 expression of which did not change throughout the study. qPCR assays without the RT step
218 were routinely employed as negative controls and showed no amplification. Melt curve

analysis showed no primer dimer formation in the assays. PCR amplicons were cloned into pGEM-T easy vectors and sequenced to confirm veracity.

Preparation of Brush Border Membrane Vesicles. Brush border membrane vesicles (BBMV) were isolated from different regions of rabbit small intestinal tissues based on the procedure described by Shirazi-Beechey et al.,[19] with modifications outlined by Rowell-Schäfer et al.[20] and Dyer et al.[21] All steps were carried out at 4°C. Tissues were thawed in a buffer solution (100 mmol/L mannitol, 2 mmol/L HEPES/Tris pH 7.1 with protease inhibitors, 0.5 mmol/L dithiothreitol, 0.2 mmol/L benzamidine, and 0.2 mmol/L phenolmethylsulfonyl fluoride), cut into small pieces and vibrated for 1.5 min at speed 5 using a FUNDAMIX vibro-mixer (DrM, Dr Mueller AG, Maennedorf, Switzerland), in order to free intestinal epithelial cells. The filtrate was then homogenized using a Polytron (Ystral, Reading, Berkshire, UK) for 20 s. Next, $MgCl_2$ was added to a final concentration of 10 mmol/L and the solution stirred on ice for 20 min. The suspension was then centrifuged for 10 min at 3,000 x *g* (SS34 rotor, Sorvall, UK) and the resulting supernatant was spun for 30 min at 30,000 x *g*. The pellet was suspended in buffer (100 mmol/L mannitol, 0.1 mmol/L $MgSO_4$, and 20 mmol/L HEPES/Tris pH 7.1) and homogenized with 10 strokes of a Potter Elvehjem Teflon hand-held homogenizer before centrifuging for 30 min at 30,000 x *g*. The final pellet was re-suspended in an isotonic buffer solution (300 mmol/L mannitol, 0.1 mmol/L $MgSO_4$, and 20 mmol/L HEPES/Tris pH 7.4) and homogenized by passing through a 27-gauge needle several times. The protein concentration in the BBMV was estimated by its ability to bind Coomassie blue according to the Bio-Rad assay technique. Porcine γ-globulin was used as the standard. Inclusion of protease inhibitors in the buffers is essential for avoiding SGLT1 protein degradation.

In preparation for western blot analysis, aliquots of freshly prepared BBMV were diluted with sample buffer (62.5 mmol/L Tris/HCl pH 6.8, 10% [v/v] glycerol, 2% [w/v] SDS, 0.05% [v/v] β-mercaptoethanol, 0.05% [w/v] bromophenol blue) and stored at -20°C until use. The remaining BBMV were divided into aliquots and stored in liquid nitrogen or used immediately for glucose uptake studies.

Western Blotting. The abundance of SGLT1 and β-actin proteins in the BBMV isolated from rabbit small intestine was determined by western blotting as described previously.[12,21] Protein components of BBMV (20 μg) were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis on 8% (w/v) polyacrylamide mini gels, containing 0.1% (w/v) SDS, and electrotransferred to polyvinylidene difluoride (PVDF) membrane. The PVDF membranes were blocked for 1h at RT in PBS containing 0.5% (w/v) non-fat dried milk, and 0.05% (v/v) Tween-20 (PBS-TM). Incubation for 1 hour with the SGLT1 antibody diluted 1:1,000 in PBS-TM then followed.

Immuno-reactive bands were detected by incubation for 1 h with affinity purified horseradish peroxidase-linked anti-rabbit secondary antibody diluted 1:2,000 in PBS-TM, and visualized using Immobilon Western Chemiluminescent HRP Substrate and Bio-Max Light Chemiluminescence Film. The intensity of the immunoreactive bands was quantified using scanning densitometry (Total Lab, Newcastle-upon-Tyne, UK).

The PVDF membranes were stripped by 3 x 10 min washes in 137 mmol/L NaCl, 20 mmol/L glycine/HCl (pH 2.5) and then re-probed with a monoclonal antibody to β-actin used as a loading control. Blocking solution consisted of 0.1% (v/v) Triton X-100 and 0.1 mmol/L EDTA in PBS (PBS-TE) and 5% (w/v) skimmed milk powder. PBS-TE was used for the incubation and washing buffers. Horseradish peroxidase-linked anti-mouse secondary antibody diluted 1:2,000 in PBS-TE was used, and visualized as above.

Measurement of Na^+-dependent glucose uptake. Na^+-dependent glucose uptake into rabbit intestinal BBMV was measured as described.[12,19] The uptake of D-glucose was initiated by the addition of 100 µL of incubation medium (100 mmol/L NaSCN [or KSCN], 100 mmol/L mannitol, 20 mmol/L HEPES/Tris [pH 7.4], 0.1 mmol/L $MgSO_4$, 0.02% [w/v] NaN_3 and 0.1 mmol/L [U-^{14}C]-D-glucose [10.6 GBq/mmol,]) to BBMV (100 µg protein) at 37°C. The reaction was stopped after 3 s by the addition of 1 mL of ice-cold stop buffer (150 mmol/L KCl 20 mmol/L HEPES/Tris [pH 7.4], 0.1 mmol/L $MgSO_4$, 0.02% [w/v] NaN_3 and 0.1 mmol/L phlorizin). Aliquots (0.9 mL) of the reaction mixture were removed and filtered under vacuum through a 0.22 µm pore cellulose acetate/nitrate filter. The filter was washed with 5 x 1 mL of ice-cold stop buffer, placed in a vial containing 4 mL of scintillation fluid and the radioactivity retained on the filter measured using a Tri-Carb 2910TR Liquid Scintillation Analyzer (PerkinElmer, Bucks, UK). All uptakes were measured in duplicate.

Morphometry. Rabbit small intestinal tissue was fixed and cryo-protected before embedded in OCT (Fisher Scientific, UK), frozen at -20°C and then kept at -80°C until use. Tissue blocks were sectioned (10 µm) on a cryostat (Leica, CM 1900UV-1-1, Milton Keynes, Buckinghamshire, UK) and thaw-mounted onto polylysine-coated slides. Morphometric analysis was performed as described previously.[12] The sections were exposed to tap water for 1 min, transferred to Mayer's Haemalum for 1 min and washed gently with running tap water for 5 min. They were stained with eosin Y solution for 30 s and subsequently dehydrated by stepwise washing in 70% ethanol (v/v) for 2 x 1-min, absolute ethanol for 2 x 1-min, and xylene for 3 x 1-min, before mounting with D.P.X. neutral mounting medium.
Digital images were captured with an Eclipse E400 microscope and DXM 1200 digital camera (Nikon, Kingston upon Thames, Surrey, UK), analyzed using ImageJ software

(Wayne Rasband, US National Institutes of Health, Bethesda, MD) and calibrated using a 100 μm gradient slide. The crypt depth and the villus height were measured as the average distance from crypt base to crypt-villus junction and villus base to villus tip, respectively. The villus height and the crypt depth measurements were taken from an average of sixteen well oriented crypt-villus units. A minimum of three images were captured per section with a minimum of 8 sections prepared per animal, with each section being 5 sections apart within the block. All images were captured under the same conditions with care taken to ensure that the same villus was not counted twice.

Immunohistochemistry. Immunohistochemistry was performed as previously described.[22] Tissue sections (10 μm thick, on polylysine coated slides) were washed five times for 5 minutes each in PBS. Slides were then incubated for 1 hour in blocking solution (10% (v/v) donkey serum in PBS) at room temperature in a humidified chamber. Subsequently, sections were incubated overnight at 4°C with primary polyclonal antibodies. The antibody to Chromogranin A (1:100), T1R2 (1:200) and T1R3 (1:200). The T1R2 antibody was raised against a peptide corresponding to residues 426-570 of mouse T1R2 that shares 66% homology with rabbit T1R2, and T1R3 to a peptide corresponding to the c-terminus of human T1R3. Cloned rabbit T1R3 shares 69% homology to human T1R3. After incubation of sections with primary antibodies, slides were washed five times for 5 minutes each in PBS and subsequently stained for 1 hour at room temperature using a 1:500 dilution of Cyanine 3 (Cy3)- or Fluorescein isothiocyanate (FITC)-conjugated anti-goat, anti-rabbit and anti-mouse IgG secondary antibodies. The composition of the buffer containing antibodies (primary or secondary) was 2.5% (v/v) donkey serum, 0.25% (w/v) NaN_3, and 0.2% (v/v) Triton X-100 in PBS. Finally, slides were washed five times for 5 minutes each in PBS and then mounted with Vectashield Hard Set Mounting Medium with (DAPI). Immunofluorescent labeling of Chromogranin A,

T1R2 and T1R3 proteins was visualized using an epifluorescence microscope (Nikon, Kingston-Upon-Thames, UK), and images were captured with a digital camera (model C4742-96-12G04, Hamamatsu Photonics, Welwyn Garden City, UK). Omission of primary antibodies was routinely used as the control.

Statistical Analysis. All parameters were tested for normality by the Shapiro–Wilk test. For comparison of SGLT1 expression in intestinal tissues and measurements of crypt-depth/villus height in intestinal tissues a Student's two-tailed t-test was used to determine statistical significance (GraphPad Prism 5, GraphPad Software Inc., La Jolla, CA). The level of statistical significance was set at $p < 0.05$.

330 **RESULTS**

331 **Phase 1 studies**

332 **Assessment of rabbit performance.** Rabbit daily weight gain, feed intake, and gain to feed
333 ratios are presented in Figure 1. *E-coli* inoculation markedly decreased daily weight gain ($p <$
334 0.001; Figure 1A) across all dietary treatments until surviving animals had recovered by day
335 19 post infection (63 days of age). There was no effect of dietary supplementation on daily
336 weight gain ($p > 0.70$; Figure 1A). Feed intake was not affected by inoculation Figure 1B).
337 There was a trend for an effect of dietary treatment ($p < 0.10$) such that the animals
338 supplemented with 75 ppm SL consumed more feed in the middle of the clinical phase of
339 disease (day 12 post infection; 56 days of age; Figure 1B). Inoculation markedly decreased
340 efficiency of growth ($p < 0.001$; Figure 1C) and this was not influenced by dietary treatment
341 ($p > 0.50$). None of the groups recovered to pre-inoculation feed efficiency for the duration of
342 the study.

343

344 **Mortality and morbidities.** Rabbit rates of mortality are presented in Figure 2. The
345 colibacillosis challenge had a strong effect and elicited a marked increase in mortality rate (p
346 < 0.001; Figure 2A), with peak levels reached on day 19 post infection (63 d of age). There
347 was no effect of dietary treatment on percent rate of mortality ($p > 0.30$); however, the 75
348 ppm SL group had nearly half the mortality rate of the control group. Cumulative death was
349 increased by inoculation ($p < 0.001$; Figure 2B), and the number of dead animals tended to
350 decrease with doses of 50 and 75 ppm ($p < 0.20$; Figure 2B). The average body weight on
351 day 26 post infection (70 d of age) was multiplied by the number of animals alive on that day
352 to give production weights of 112.5, 119.2, 121.2, and 111.9 g for animals supplemented with
353 0, 50, 75, and 100 ppm SL, respectively.

Morbidities (diarrhea and bloat) are presented in Figure 3. The majority of morbidities observed were diarrhea, and this was increased with inoculation ($p < 0.001$; Figure 3A). All animals reached pre-inoculation levels by day 26 post infection (70 d of age) and there was no effect of dietary treatment on percent morbidities ($p > 0.40$; Figure 3A). The cumulative number of morbidities also increased with inoculation ($p < 0.001$; Figure 3B) and there was a significant treatment effect characterized by fewer morbid animals in the 50 and 75 ppm SL groups, compared to control animals ($p < 0.01$; Figure 3B).

Phase 2 studies

Rabbit SGLT1 expression and activity is enhanced by feed supplementation with the natural high-intensity sweetener, stevia leaf. SGLT1 expression and activity was determined along the length of the small intestine in rabbits, fed a diet supplemented with SL and the same diet without SL (control diet). Irrespective of diets, levels of SGLT1 mRNA, protein and function were highest in the duodenum>jejunum>ileum. There was a 1.4- ($p < 0.05$) and 1.3-fold ($p < 0.001$) increase in SGLT1 mRNA abundance in the duodenum and jejunum of rabbits fed a diet supplemented with SL compared to control diet (Figure 4A). SGLT1 protein abundance measured in BBMV increased by 1.6- ($p < 0.001$) and 1.6-fold ($p < 0.001$) in the duodenum and jejunum of rabbits fed a diet supplemented with SL compared to control (Figure 4B). This was matched by 1.8- ($p < 0.050$) and 1.7-fold ($p < 0.050$) increase in the initial rates of D-glucose transport into BBMV in the duodenum and jejunum of rabbits fed a diet supplemented with SL compared to the control diet (Figure 4C). No increases in either SGLT1 mRNA, protein abundance, or the initial rate of D-glucose transport into BBMV were observed in the ileum of rabbits fed a diet supplemented with SL, compared to control diet (Figure 4). There was a 1.4- ($p < 0.0010$) and 1.3-fold ($p < 0.0010$) increase in villus height in the duodenum and jejunum of SL fed rabbits compared to controls

(Figure 5). There was no difference in the average villus heights of control and SL fed rabbits in the ileum.

Expression of T1R2 and T1R3 in the rabbit intestine. For rabbit T1R2, PCR primers were designed against the predicted mRNA sequence available on the National Center for Biotechnology Information (NCBI) non-redundant nucleotide database. PCR amplicons using rabbit jejunal cDNA and the designed T1R2 primers resulted in a 152 bp amplicon, which was found to be a 100% match to the predicted NCBI sequence, revealing that rabbit intestine expresses T1R2 (Figure 6). An alignment of the full-length rabbit T1R2 mRNA sequence showed 55.3% homology with cow, pig, human, mouse and rat T1R2.

As no sequence information on rabbit T1R3 was available from the current release of the rabbit genome (NCBI OryCun2.0 Annotation Release 102), it was necessary to clone rabbit T1R3 to obtain mRNA sequence data and verify its expression in the rabbit intestine. PCR amplification using rabbit jejunal cDNA and consensus mammalian T1R3 primers resulted in a 1221 bp fragment that was screened against the National Center for Biotechnology Information (NCBI) non-redundant nucleotide database, via BlastN,[23] identifying the amplified sequence as being homologous to T1R3 in many other mammalian species. The mRNA fragment was subsequently translated to produce a sequence of 407 amino acids (corresponding to residues 116-515 of human T1R3). Phylogenetic analysis was performed to construct a radial phylogram depicting the relationship of rabbit T1R3 to homologs in various other mammalian species for which sequence information was available (Figure 7). The NCBI accession number for the mRNA sequence of rabbit T1R3 is MK182098.

Immunofluorescence detection for the sweet receptor subunits, T1R2 and T1R3, as well as the classical marker for enteroendocrine cells, chromogranin A, was performed on frozen tissue sections of rabbit duodenum and jejunum. As shown in figure 8, T1R2 and T1R3 were co-expressed in the same cell (Figure 8A). Furthermore, both T1R2 and T1R3 were co-expressed with chromogranin A, confirming receptor subunits expression in the enteroendocrine cell (Figure 8B).

DISCUSSION

Feeding of low-level antibiotics has been a routine procedure for controlling enteric pathogens, preventing disease, improving health and growth, in particular in post weaning-animals.[24] However, increasing antibiotic resistance and rising consumer concern over prophylactic antibiotic use in animal production has led to a concerted search for effective alternatives. In humans, oral rehydration therapy, which relies on absorption of glucose via SGLT1, activating electrolyte and water absorption is a safe and effective method for the treatment of *E-coli*- and *Vibrio cholerae*- induced diarrhea.[1] The discovery that sensing of sugars and sweeteners by the gut-expressed sweet receptor T1R2-T1R3 enhances the expression and activity of SGLT1,[4] has allowed the design of novel strategies for animal nutrition that use artificial sweeteners to combat diarrheal and enteric diseases.[25]

E. coli- induced diarrhea is endemic in rabbits and results in high rates of morbidity and mortality. In the EU, artificial sweeteners are not permitted to be used in rabbit feed.

We hypothesized that rabbit intestine expresses the intestinal sweet receptor T1R2-T1R3, and that a natural high intensity sweetener (stevia) activates the receptor leading to SGLT1 upregulation improving E-coli–induced enteric disorders. The supplement used in this study contained a small amount (2%) of *capsicum oleoresin* (~ 4.9 μM capsaicin) shown to influence immunity. However, using heterologous expression of rabbit T1R2-T1R3 we have

428 determined that capsaicin does not activate rabbit T1R2-T1R3. In contrast, stevia leaf extract
429 (SL) activates the receptor in a dose-dependent manner (unpublished data, paper in
430 preparation).
431 Work in the laboratory of Tavakkkolizadeh and colleagues[26,27] have questioned the role of
432 vagal afferent fibres in SGLT1 regulation by vagotomy and de-afferentation with 1 mg
433 capsacin applied per animal. They have concluded that vagal de-afferention abolishes SGLT1
434 upregulation in response to increased luminal glucose. They have further proposed that the
435 specific involvement of vagal afferent fibres and enteric nervous system in glucose-sensing
436 initiated regulatory pathway controlling SGLT1 expression remains unclear.[27]
437 Bates, Sharkey and Meddings 1998,[28] have shown that guinea pigs treated with vehicle, and
438 thus having intact vagal afferent were able to increase the ability to enhance intestinal glucose
439 transport when switched from a low- to a high-carbohydrate containg diet. In contrast,
440 animals that received 32.8 mM solution of capsaicin demonstrated no adaptation to
441 alterations in dietary composition.[28] Interestingly Nassar et al. 1995,[29] have shown that
442 capsaicin (160 and 800 μM) reduces significantly intestinal alanine absorption when perfused
443 either intraluminally or applied topically to the vagus nerve, concluding the involvement of
444 vagal capsaicin sensitive primary afferent fibres in this inhibitory mechanism.[29] Thus, it
445 appears that vagal de-afferentiation may have a generalized effect on inhibiting a range of
446 intestinal nutrient absorptive processes.
447 The results of studies carried out by Strearns et al., 2010 and Bates et al., 1998 are in contrast
448 to this study. In our study, rabbits fed diets that included stevia and capcaicin (maximum
449 capsaicin concentration, 4.9 μM) were able to upregulate glucose transporter
450 expression/activity compared to those fed the same diet without capsaicin.
451 We have shown recently that electric field stimulation of an isolated segment of the intestine
452 results in 2-3 fold increase in SGLT1 upregulation.[6] This increase is abolished in presence of

453 the nerve blocking agent tetrotodoxin, indicating the involvement of enteric nervous system
454 in the regulatory pathway. We used this strategy because sensing of glucose or artificial
455 sweeteners via T1R2-T1R3, expressed in enteroendocine cells, stimulates GLP-2 release, and
456 GLP-2 via binding to its receptor (GLP-2R) present in enteic neurons induces an action
457 potential.[30] We showed that electric field stimulation of enteric neurons, induces a neural
458 response leading to secretion of specific neropeptides that upregualte SGLT1 expression in
459 the neighbouring absorptive enterocyte, by enhancing half-life of SGLT1 mRNA and thus
460 increased in SGLT1 protein abundance[6]. Our studies strongly supports the involvement of
461 enteric neurons in a glucose-sensing initiated pathway regulating SGLT1 expression.[6]
462 Addititional studies are required to address if there is a specific involvement of vagus nerve
463 in SGLT1 regulatory pathway.
464 In this study we determined the effect of supplementation of feed with an additive containing
465 SL on rabbit intestinal SGLT1 expression, as sweeteners are known to enhance Na^+-
466 dependent glucose absorption in other mammalian species.[4,12,13] Since the regulatory pathway
467 controlling SGLT1 expression/function is initiated by activation of the gut-expressed sweet
468 receptor T1R2-T1R3, we aimed to identify if these receptor subunits were expressed in the
469 rabbit intestine. The gene for rabbit T1R2 has previously been identified from the rabbit
470 genome sequence, located on chromosome 13 (NCBI OryCun2.0 Annotation Release 102);
471 however, no information was available for rabbit T1R3. To determine expression of T1R2
472 and T1R3 in rabbit intestine, a PCR based strategy was used to demonstrate that rabbit
473 intestine does indeed express both receptor subunits T1R2 and T1R3 at mRNA level.
474 Moreover, by immunohistochemistry we showed that T1R2, T1R3 proteins are co-expressed
475 in the same intestinal enteroendocrine cell. Furthermore, SGLT1 mRNA, protein abundance
476 and glucose transport function were increased ~2-fold by dietary inclusion of SL, providing a
477 higher capacity for the rabbit intestine to absorb glucose, electrolyte and water. There was

also 1.4-fold increase in villus height in rabbits consuming SL, likely due to GLP-2 action.[13] In these dietary studies, we maintained rabbits on diets with and without SL for 9 days. We have shown SGLT1 upregulation, with a similar increase in magnitude after 1 day or 5 day in response to increased dietary carbohydrates or sweeteners[4,6], indicating that increase in SGLT1 expression occurs in existing absorptive enterocytes.[6,7] However, since this was the first time that we were assessing potential SGLT1 upregulation in response to inclusion of a natural sweetener in the feed of rabbits, we selected a 5-day dietary trial in order to cover intestinal epithelial cell turnover that takes 4-5 days in the majority of species. This period was extended to 9 days because of the researcher travel delays from the UK to France for harvesting rabbit intestinal tissues.

We also assessed the effect of supplementation of rabbit feed with SL on relieving *E. coli*-induced enteric disorders and observed that inclusion of SL in the feed decreases morbidity associated with disease. Although we did not observe a linear dose response, there was a clear trend for improved morbidities at the two lower doses. Such hormetic responses to plant-based supplements are common; very low doses are beneficial whereas higher doses are non-specific and detrimental[31]. To our knowledge this is the first report evaluating the effect of a natural artificial sweetener on rabbit health and performance, and the associated molecular mechanisms.

The *E. coli* challenge elicited a marked impact on performance, characterized by blunted feed intake, decreased daily gain, and efficiency of growth. These are classical signs of infection that not only lead to stressed animals but have a devastating economic impact in rabbit production. The use of artificial sweetener to prevent decreased performance during stress has been explored previously. For example, Sterk et al.[32] reported that supplementation of weanling piglets with artificial sweetener prevented the decrease in feed intake around weaning. Similar observations have been made for receiving feedlot cattle with respect to

503 both feed intake[33] and daily weight gain.[34] Whilst in this study the inclusion of SL in the feed
504 had some impact on feed intake, the major effect is at the gut level where activation of gut-
505 expressed T1R2-T1R3 by sweeteners results in the secretion of GLP-2, a gut hormone that
506 can alter appetite,[35] and also the intestinal uptake of glucose leading to improved efficiency
507 of growth.[6] It has indeed been shown that artificial sweeteners directly introduced into the
508 lumen of the intestine, bypassing the oral cavity, lead to an increase in expression of SGLT1
509 and higher rates of intestinal glucose absorption.[27]
510 It was noteworthy that the *E. coli* challenge in this study was quite severe, with mortality rate
511 peaking at nearly 60% during the clinical trials in some groups. Despite the severity of the
512 disease challenge, the supplement showed a trend for a positive effect on cumulative
513 morbidities at the lower doses. This observation was consistent with those we made during
514 our preliminary experiments to establish the optimal timing and dose of inoculation (data not
515 shown). Previous work using an artificial high-intensity sweetener has revealed similar
516 effects during enteric disease challenge,[36] but this is the first report on the impact of a natural
517 high-intensity sweetener for prevention of clinical signs of enteritis in rabbits. The positive
518 impact of the supplement on morbidities, combined with the molecular responses we
519 observed, are consistent with an increase in GLP-2 secretion which is known to be essential
520 for gut repair after injury,[25] and also an enhancement in nutrient absorption.[6] In this scenario,
521 the increased glucose, electrolyte and water absorption at the intestinal level likely decreased
522 the clinical signs of disease associated with diarrhea, and the enteric lesions caused by the
523 pathogen were likely reduced or repaired in supplemented animals due to GLP-2 effect.
524

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528 **Author contributions**

529 ⊥A.W.M. and M.A.A-R. contributed equally to this work.

530 S.P.S-B. is responsible for conception of studies described in phase-2, with A.W.M.

531 designing and carrying out experiments on isolating rabbit intestinal BBMV, western

532 blotting, glucose transport function, morphometric analyses and determining intestinal

533 expression of T1R2. M.A.A-R. performed immunohistochemistry. K.D. cloned and

534 sequenced rabbit T1R3. S.P.S-B., A.W.M. and K.D. analyzed and interpreted the data. E.G.

535 carried out feed trial studies described in phase 1 and -2 of the study. C.I. assisted with the

536 experimental design, and D.M.B. conceptualized the project and the potential application of

537 the supplement in rabbits. E.H.W. designed, directed, discussed phase-1 and -2 animal studies

538 and commented on the paper. S.P.S-B. wrote the paper.

539

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646 **Figure 1.** Growth performance of rabbits supplemented with 0, 50, 75, or 100 ppm of an
647 additive containing natural high-intensity sweetener (SL) and inoculated with *Escherichia*
648 *coli* on day 44 of age (red arrow). Data presented are LS Means, with SEM indicated on each
649 panel. A, daily weight gain; B, daily feed intake; C, gain:feed.

650 **Figure 2.** Mortality of rabbits supplemented with 0, 50, 75, or 100 ppm of an additive
651 containing natural high-intensity sweetener (SL) and inoculated with *Escherichia coli* on day
652 44 of age (red arrow). A, percent mortality over time. B, total number of dead animals over
653 time. Mortality was calculated by dividing the number of new deaths recorded at each time
654 point by the number of animals alive at the previous time point, and then multiplying by 100.

655 **Figure 3.** Morbidity (diarrhea, abnormal feces, and/or bloat) of rabbits supplemented with 0,
656 50, 75, or 100 ppm of an additive containing natural high-intensity sweetener (SL) and
657 inoculated with *Escherichia coli* on day 44 of age (red arrow). A, percent morbidity over
658 time. B, cumulative morbidity over time. * = $p < 0.05$; ** = $p < 0.01$

659 **Figure 4**. **Expression and activity of SGLT1 in the intestine of control rabbits and in**
660 **rabbits maintained on the same diet supplemented with SL.** Brush border membrane
661 vesicles (BBMV) and RNA were isolated from small intestinal tissues of rabbits fed either a
662 control diet (C) or a diet supplemented with an additive containing natural high-intensity
663 sweetener (SL). A: Level of SGLT1 mRNA abundance normalized to β-actin, RNA
664 polymerase II and β-2-microglobulin mRNA. B: Expression of SGLT1 and β-actin proteins
665 in BBMV isolated from the small intestine assessed by western blotting (*left panel*).
666 Densitometric analysis of SGLT1 protein abundance normalized to β-actin (*right panel*). C:
667 Initial rates of Na^+-dependent [$U^{14}C$]-D-glucose uptake into BBMV. Data were generated in
668 triplicate. Results are shown as mean ± SEM; n = 7 animals. Statistically significant results

669 determined using a Student's two-tailed t-test where * = $p < 0.050$; ** = $p < 0.010$; *** = p
670 <0.001.

671 **Figure 5. Morphometric analysis of the rabbit intestine**. A) Representative light
672 micrographs of small intestinal tissues of control and natural high-intensity sweetener (SL)
673 fed rabbits. Images were obtained at 4X magnification. B) Morphometric analyses of villus
674 height and crypt depths are shown as histograms, in (μm) ± SEM. Control (□), SL-fed (■); n
675 = 5 animals. Statistically significant results were determined using Student's two-tailed t-test
676 where ** = $p < 0.010$.

677 **Figure 6.** Alignment of rabbit T1R2 mRNA sequence with the corresponding region of cow,
678 pig, human, mouse and rat T1R2 (numbers in parentheses relate to initiating nucleotide).

679 **Figure 7**. Radial phylogram, derived from amino acid sequences, depicting the phylogenetic
680 relationship of rabbit T1R3 to various mammalian homologs. The scale bar represents the
681 number of substitutions per amino acid position.

682 **Figure 8**. **Co-expression of T1R2 and T1R3 in rabbit small intestine**. A) A representative
683 image shows expression of T1R2 (green), T1R3 (red) and merged image (yellow) in serial
684 sections of rabbit small intestine as determined by double immunohistochemistry. B) A
685 typical image showing expression of T1R2 or T1R3 (green), the enteroendocrine marker,
686 chromogranin A (ChA, red) and merged image (yellow). Specificity of primary antibodies for
687 T1R2 and T1R3 have been validated in mice.[6] Omission of primary antibodies for T1R2 or
688 T1R3 showed no non-specific immunoreactivity with secondary antibodies. (-T1R2 control
689 & -T1R3 control). All images were taken under 400X magnification, scale bar represents 20
690 μm.

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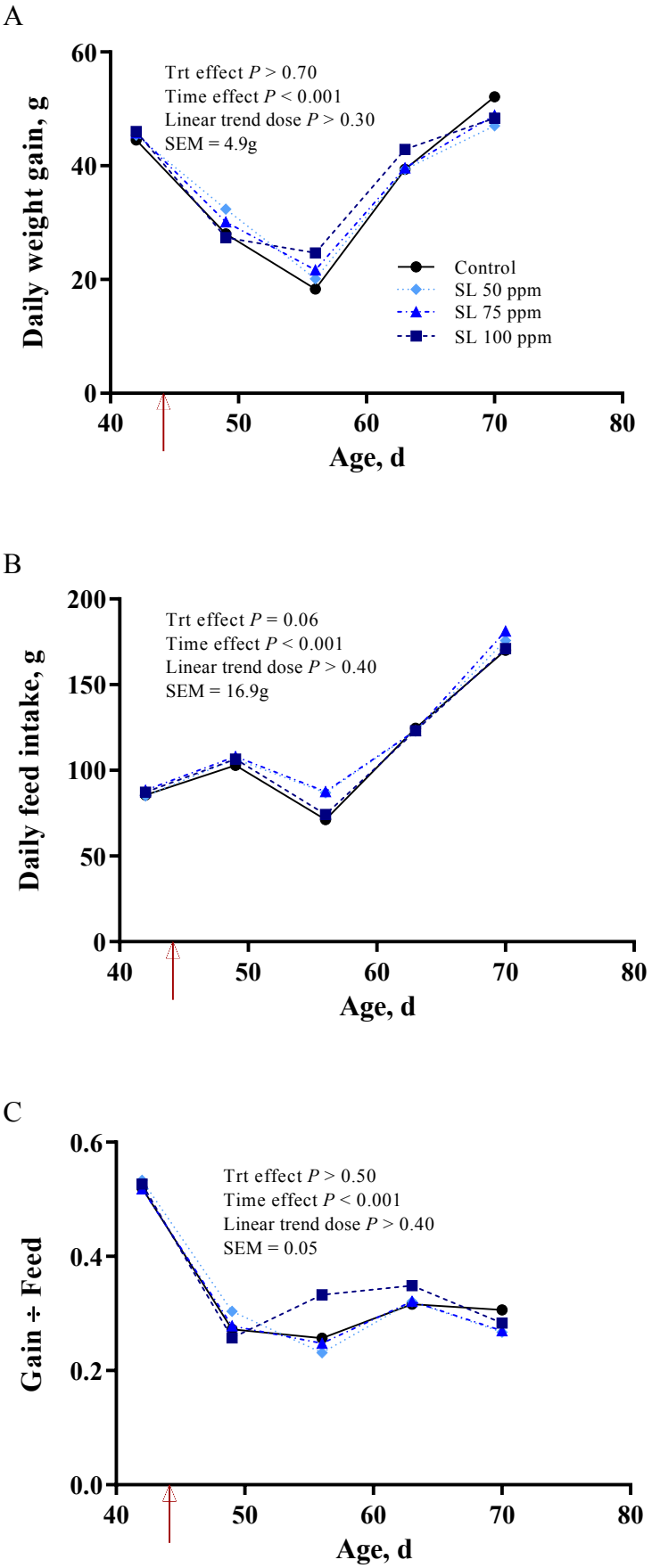
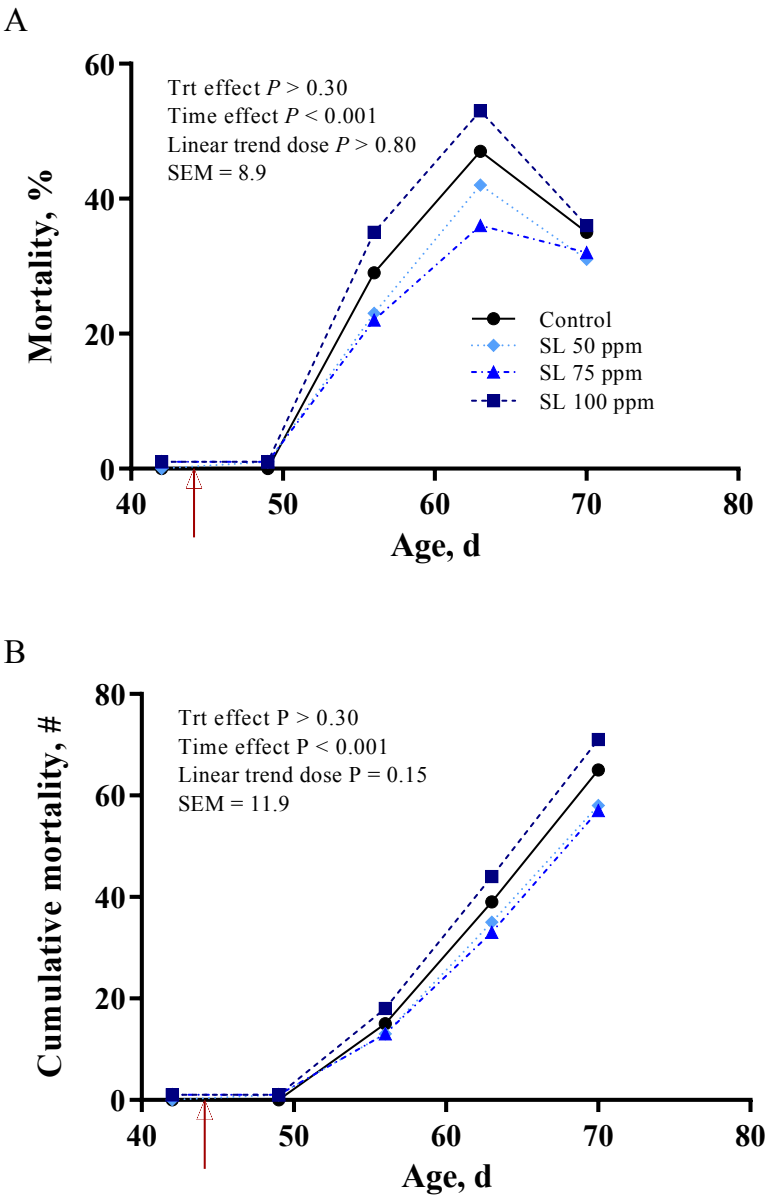

A
Daily weight gain, g
Trt effect P > 0.70
Time effect P < 0.001
Linear trend dose P > 0.30
SEM = 4.9g
Control
SL 50 ppm
SL 75 ppm
SL 100 ppm
Age, d
B
Daily feed intake, g
Trt effect P = 0.06
Time effect P < 0.001
Linear trend dose P > 0.40
SEM = 16.9g
Age, d
C
Gain ÷ Feed
Trt effect P > 0.50
Time effect P < 0.001
Linear trend dose P > 0.40
SEM = 0.05
Age, d

693 **Figure 1**

694 **Figure 2**



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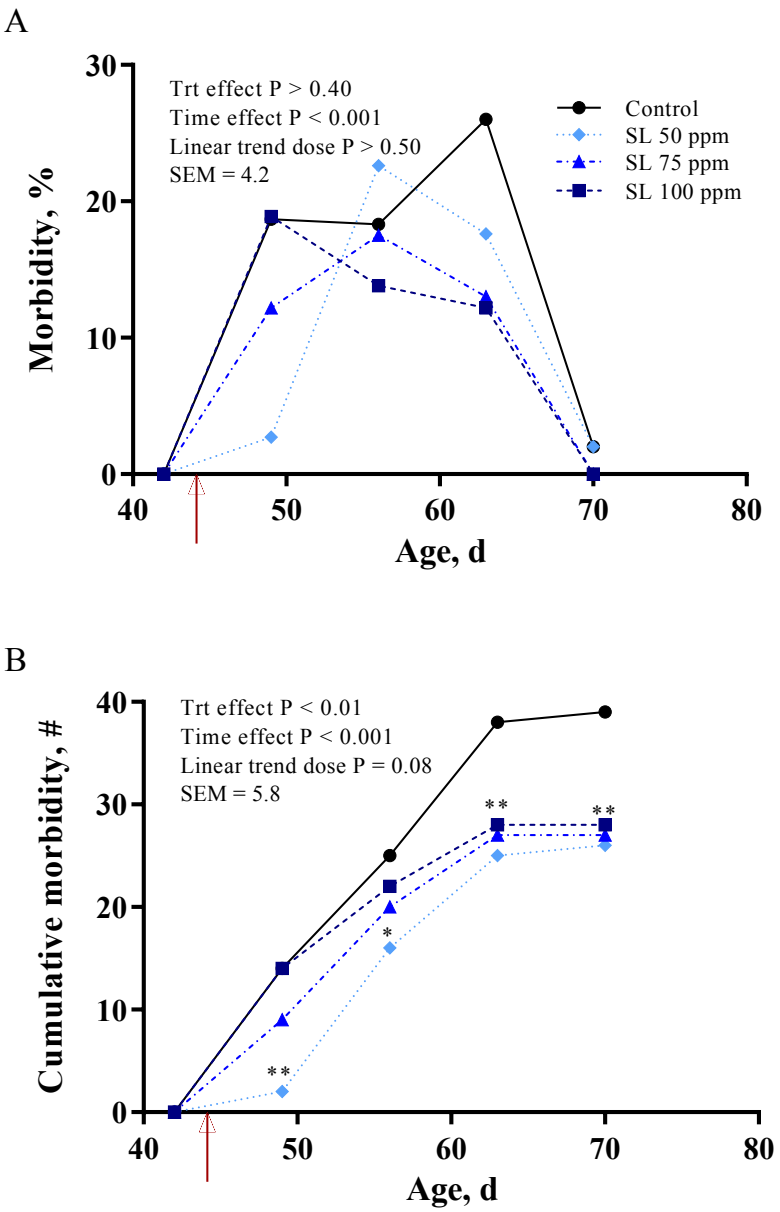
697 **Figure 3**



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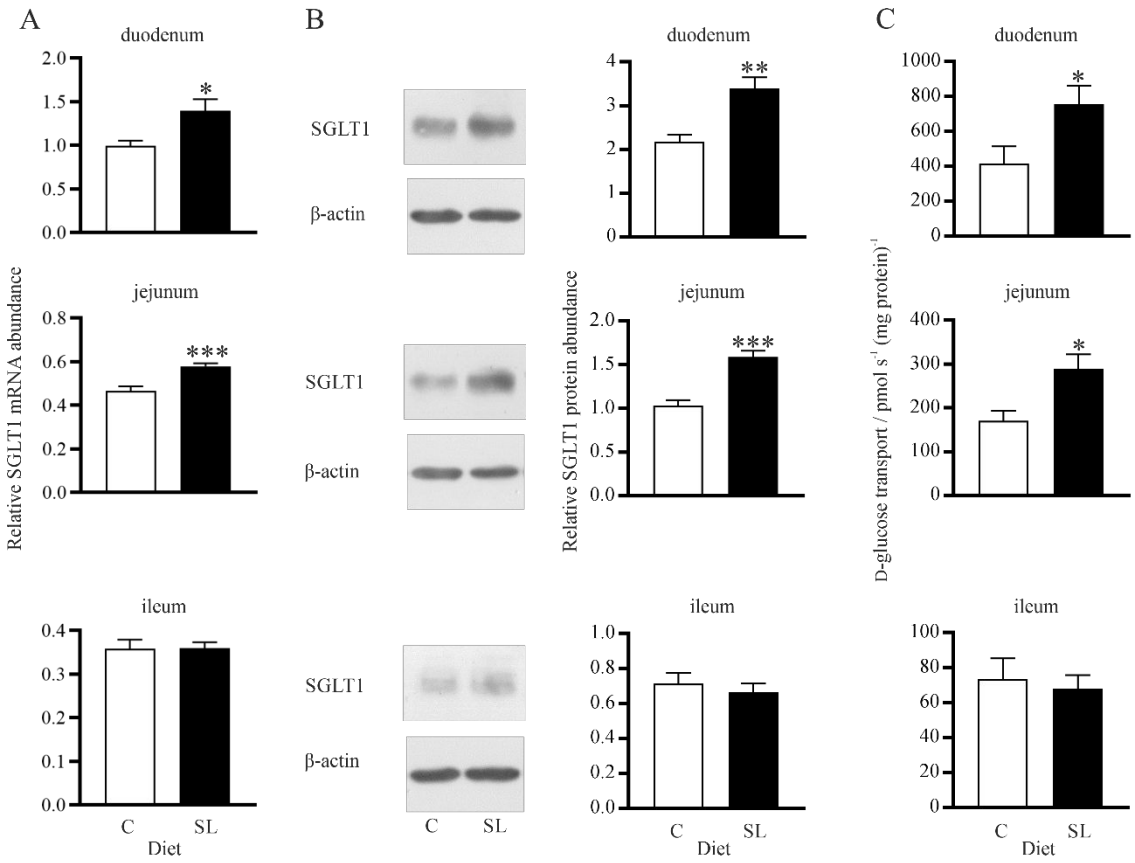
700 **Figure 4**



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704 **Figure 5**

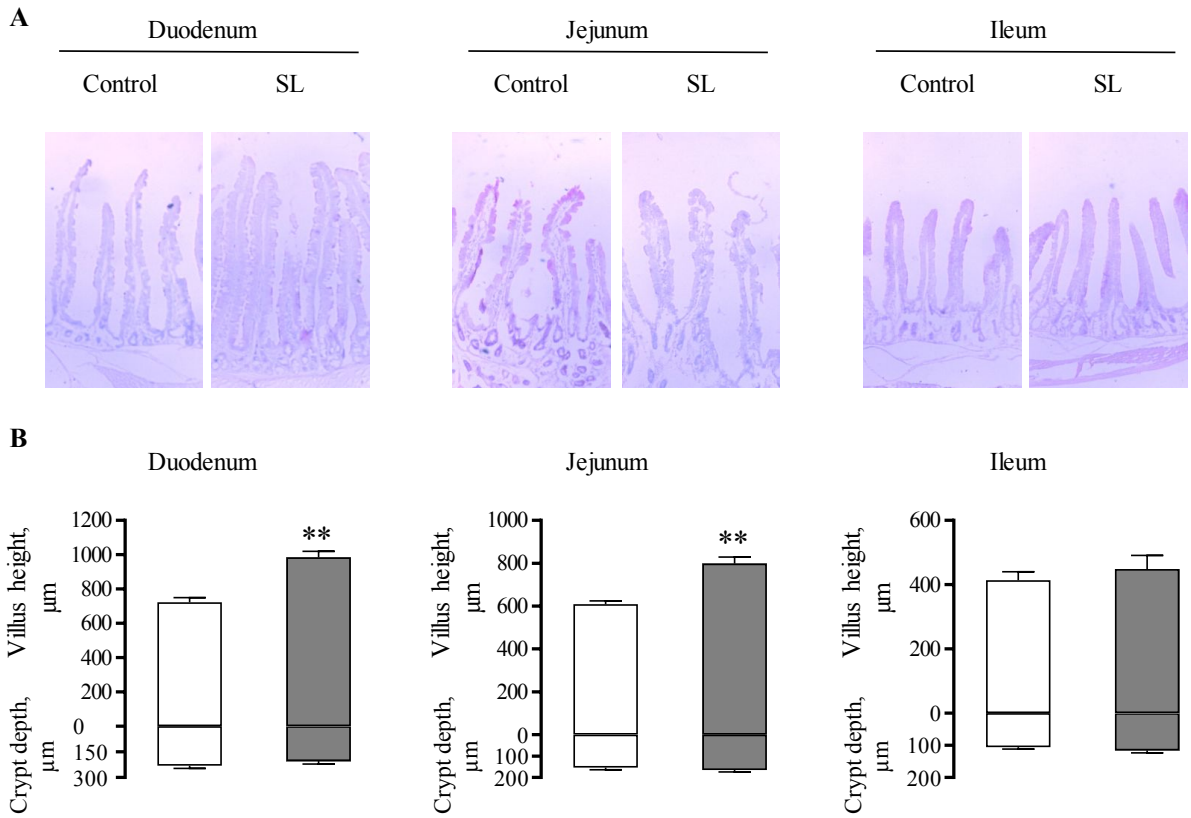

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Figure 6

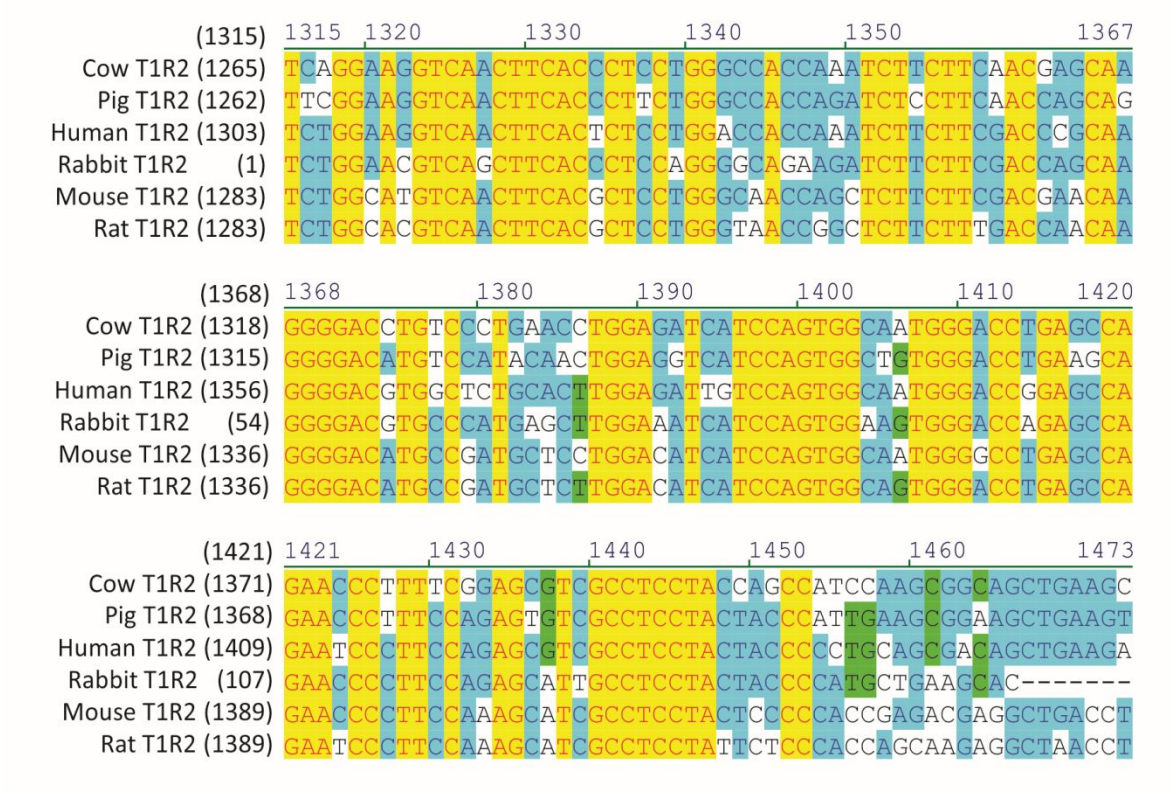
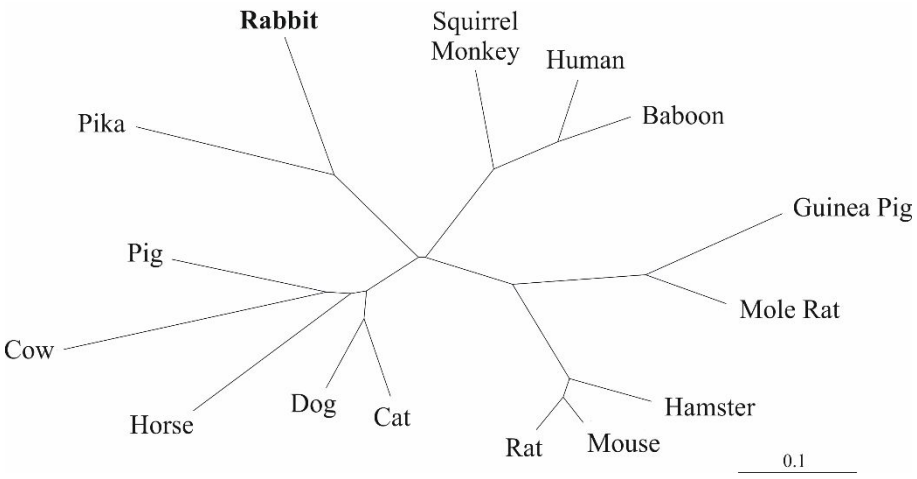
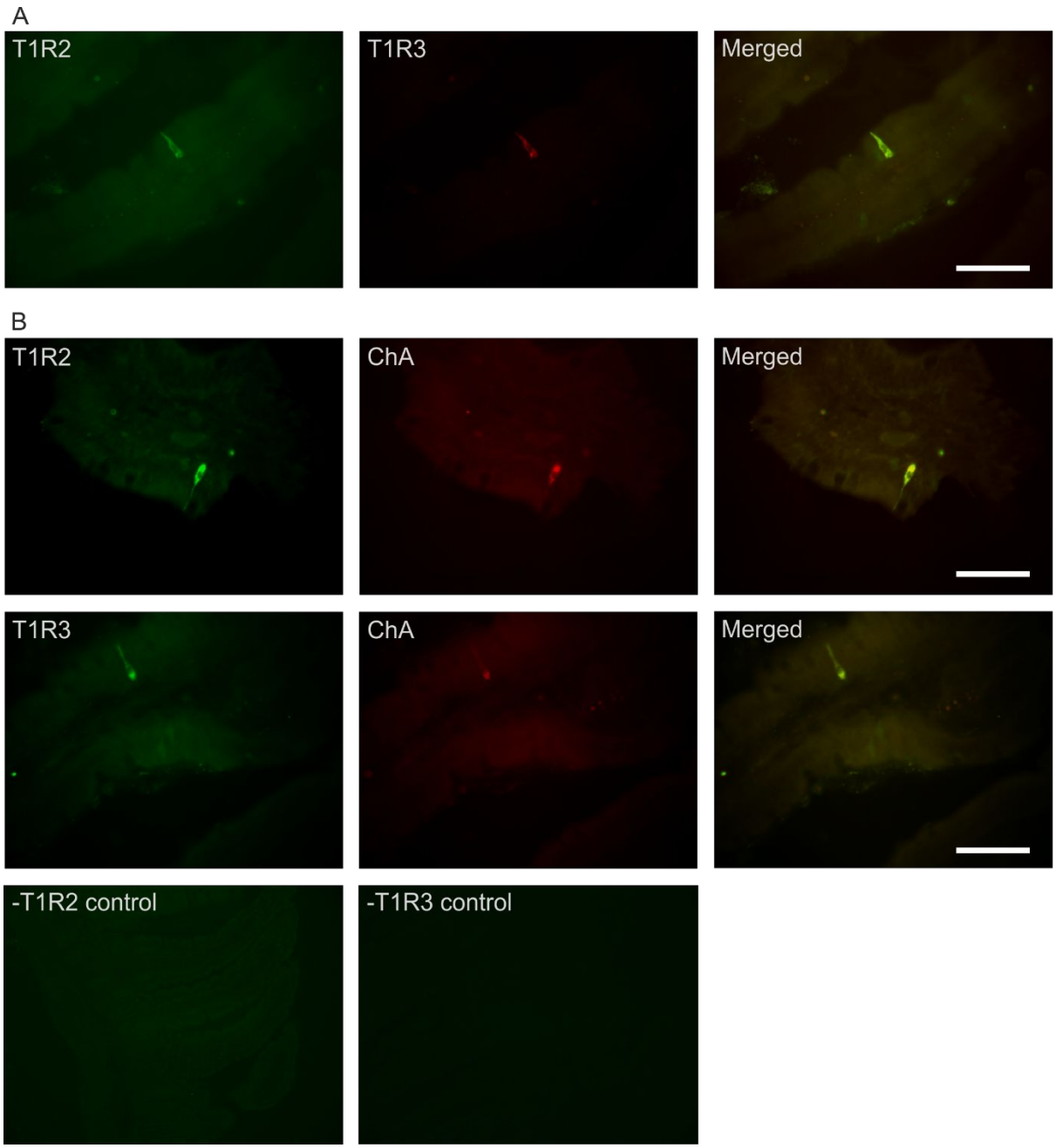


Figure 7



712 **Figure 8**



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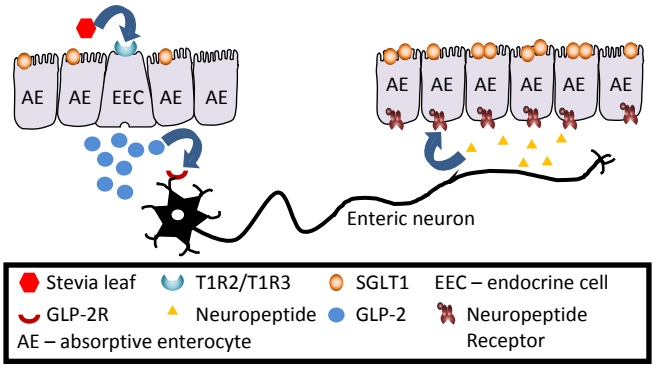
715 TOC


AE
AE
EEC
AE
AE
AE
AE
AE
AE
AE
AE
Enteric neuron
Stevia leaf
T1R2/T1R3
SGLT1
EEC – endocrine cell
GLP-2R
Neuropeptide
GLP-2
Neuropeptide Receptor
AE – absorptive enterocyte

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