The effect of altered trace aminergic signalling and estrogen on intestinal inflammation, within an IBS context

by

Lesha Pretorius

Dissertation presented for the degree of Doctor of Philosophy in Physiological Sciences in the Faculty of Science at Stellenbosch University

Supervisor: Prof. Carine Smith

Co-supervisor: Dr. Anton Du Preez Van Staden

December 2022

The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF

Declaration

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third-party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

This dissertation includes three original papers published in peer-reviewed journals and two unpublished publications. The development and writing of the papers (published and unpublished) were the principal responsibility of myself, and for each of the cases where this is not the case, a declaration is included in the dissertation indicating the nature and extent of the contributions of co-authors.

Declaration with signature in possession of candidate and supervisor.

Abstract

Irritable bowel syndrome (IBS) is a widespread (≈10% global prevalence) female predominant functional gastrointestinal (GI) disorder. While it is known that IBS is underpinned by relative microbial dysbiosis and chronic microinflammation, current therapeutic strategies often only provide transient symptomatic relief (with relative neglect of inflammation) and are thus unsatisfactory in many cases. As such, the development of targeted therapeutics to alleviate GI inflammation and consequential symptomologies are required. We suggest that the trace aminergic system, which connects several IBS risk factors (sex, dysbiosis, diet, inflammation and anxiety), may be a pretermitted regulatory system that could be manipulated as a therapeutic target. In addition, existing data supports an interpretation of sex dependence in trace aminergic signalling. As such, fluctuations of female reproductive hormones, such as 17β-estradiol (E2), may alter subsequent signalling cascades. Therefore, this thesis aimed to investigate the GI modulatory effects of selected trace amines (TAs), with consideration of the context of female predominance.

To elucidate mechanisms at play, a multidisciplinary approach was necessitated. As such, multiple model systems were utilised, including both *in vitro* (microbial cultures and human tissue cultures) and *in vivo* (zebrafish larval) models. In this regard, microbial (probiotic and commensal strains) culturing techniques, coupled with the development of a novel multianalyte mass spectrometry methodology, allowed for the accurate assessment of microbial TA generation. Indeed, data generated in these studies highlighted firstly, the dependence of probiotic secretome profile on host hormonal status, and secondly, that specific rooibos supplementation strategies may be able to negate E2-induced alterations in secretome TA profiles, both of which have important implications in TA-associated symptom management in females with GI disorders.

Data generated *in vitro* in HT-29 colon adenocarcinoma cells and *in vivo* in zebrafish larvae, in which the effects of increased TA load were assessed, demonstrated potential differences in the mechanisms of actions between TYR and AGM in particular. In this regard, extensive occludin redistribution was observed following TYR-exposure, which was associated with increased reactive oxygen species and pro-inflammatory cytokine levels, as well as tight junction disruption – an outcome prevented by E2 treatment. In contrast, AGM administration promoted the colocalization of ZO-1 and occludin to promote tight junction integrity but was also associated with risk of pro-oxidant damage when AGM metabolism was insufficient.

In conclusion, this dissertation contributes significantly to our understanding of the role of TAs in GI physiology, consistently illustrating (across *in vitro* and *in vivo* models), that while some TAs may promote disease symptomology, others may have therapeutic benefit when responsibly administered. From a therapeutics standpoint, data presented here crucially highlights the importance of dosage and administration optimisation to achieve benefit and minimize adverse side effects when targeting TA signalling in the context of functional GI disease. In addition, potential mechanistic insights by which E2 - *or rather the transient cyclic lack thereof* - is associated with trace aminergic signalling, was elucidated.

Opsomming

Prikkelbare derm-sindroom (IBS) is ‘n wydverspreide (≈10% global voorkoms) vroulik-oorheersde funksionele gastroïntestinale (GI) versteuring. Alhoewel dit bekend is dat IBS gekenmerk word deur onderliggende relatiewe mikrobiese disbiose en chroniese mikro-inflammasie, bied huidige terapeutiese strategieë dikwels net verbygaande simptomatiese verligting (met relatiewe verwaarlosing van inflammasie) en is dus in baie gevalle onbevredigend. Die ontwikkeling van geteikende terapeutika om GI-ontsteking en gevolglike simptome te verlig, is dus nodig. Ons stel voor dat die spoor-aminergiese stelsel, wat verskeie IBS-risikofaktore (seks, disbiose, diëet, inflammasie en angs) verbind, ‘n voorafbepaalde regulatoriese stelsel kan wees wat as terapeutiese teiken gemanipuleer kan word. Daarbenewens ondersteun bestaande data ‘n interpretasie van geslagsafhanklikheid in spoor-aminergiese seinoordrag. As sodanig kan wisseling in vlakke van vroulike voortplantingshormone, soos 17β-estradiol (E2), daaropvolgende seinoordrag verander. Daarom het hierdie tesis gemik om die GI modulerende effekte van geselekteerde spoor-amiene (TAs) te ondersoek, met inagneming van die vroulike konteks.

Om betrokke meganismes toe te lig, was ‘n multidissiplinêre benadering genoodsaak. Dus is veelvuldige modelstelsels gebruik, insluitend beide *in vitro* (mikrobiese kulture en menslike selkulture) en *in vivo* (sebravislarwe) modelle. In hierdie verband het (probiotiese en kommensale) mikrobiese kweektegnieke, tesame met die ontwikkeling van ‘n nuwe multi-analiet massaspektrometrie metodologie, die akkurate assessering van mikrobiese TA profiele moontlik gemaak. Inderdaad, data wat in hierdie studies gegenereer is, het eerstens aangedui dat probiotiese sekretoomprofiel van gasheer se hormonale status afhanklik is. Tweedens beklemtoon data dat spesifieke rooibos aanvullings moontlik E2-geïnduseerde veranderinge in sekretoom TA-profiele kan verhoed, wat albei belangrike implikasies vir TA-geassosieerde simptoombestuur by vroue met GI-afwykings inhou.

Data gegenereer *in vitro* in HT-29 kolon adenokarsinoomselle en *in vivo* in sebravislarwes, waarin die effekte van verhoogde TA-lading ge-evalueer is, het potensiële verskille in die meganismes van aksies tussen veral ρ-tiramien (TYR) en agmatien (AGM) getoon. In hierdie verband is omvattende okludien herverspreiding waargeneem na TYR-blootstelling, wat geassosieer is met verhoogde reaktiewe suurstofspesies en pro-inflammatoriese sitokienvlakke, sowel as stywe aansluiting proteïen ontwrigting – ‘n uitkoms wat deur E2 behandeling voorkom is. Daarteenoor het AGM-toediening die ko-lokalisering van ZO-1 en

okludien bevorder om stywe aansluitingsintegriteit te bevorder, maar was ook geassosieer met risiko van pro-oksidantskade wanneer AGM metabolisme onvoldoende was.

Ten slotte, dra hierdie proefskrif aansienlik by tot ons begrip van die rol van TAs in GI fisiologie, wat konsekwent illustreer (in beide *in vitro* en *in vivo* modelle) dat hoewel sommige TAs siekte simptomologie kan bevorder, ander terapeutiese voordeel kan hê wanneer dit verantwoordelik toegedien word. Vanuit ‘n terapeutiese oogpunt beklemtoon data wat hier aangebied word, die belang van dosis- en toedieningsoptimering om voordeel te bewerkstellig en nadelige effekte te verminder wanneer TA-seinoordrag in die konteks van funksionele GI-siekte geteiken word. Daarbenewens is potensiële meganistiese insigte verkry, waaronder die rol van E2 – *of eerder die tydelike sikliese vermindering daarvan* – in spoor-aminergiese seinoordrag.

Dedication

This dissertation is dedicated to my father, Dr Gerard Pretorius. Dankie dat pappa jou passie vir wetenskap met my gedeel het.

“Whatever you do, do it heartily, as unto the Lord, and not unto men”

– Colossians 3:23

Acknowledgements

To Prof Carine Smith, thank you for believing in me. Your supervision has been invaluable, and something that I feel very lucky to have experienced. I am very grateful for all the opportunities you have afforded me and can truly say I have had one of the most versatile educations.

To Du Preez, Hannes, Tracy and Tash, thank you for all of your guidance and assistance in the lab. You have all taught me the value of collaboration and I am sincerely thankful for all the technical support.

To my parents, thank you for all the unconditional love and support. You have always motivated me to persevere through thick and thin, and it is such a privilege to achieve this milestone with you both by my side. Thank you to the rest of my family for all the encouragement, you have been the best support system I could ask for.

To Sheray, thank you for being the most inspirational person I know. You have taught me the value of hard work, and I will forever be grateful. Thank you for always having my best interest at heart, your support has meant the world to me over the past few years.

To my dearest friends, Maria, Simei, Vicci and Ane, thank you for taking the time and putting in the effort to listen, support and encourage me every step of the way. You have all given me so much perspective and always kept me motivated.

To the members of the Smith Group, thank you for always celebrating the little wins, and for all the practical, technical, and emotional support. Your comradery has made all the difference!

All figures were created with https://biorender.com/.

Table of Contents

List of Figures

p<0.0001. Asterisk's (*) represent TA dose effect under the same E2 condition, while hashtags (#) represent effect of E2.

Supplementary Figure 3.1: Effect of 1 nM 17β-estradiol on the growth of **(A)** *Lactobacillus plantarum*, **(B)** *Enterococcus mundtii*, **(C)** *Saccharomyces boulardii*, **(D)** *Lactobacillus reuteri*, and **(E)** *Lactobacillus rhamnosus* over a 16-hour period. Data is expressed as a mean ± SD.

Supplementary Figure 3.2: Effect of low (0.1 nM) levels of 17β-estradiol (E2) treatment on the percentage of relative area of the imaged HT-29 cell monolayer that stained positive for tight junction proteins following selected TA-exposure: **(A, E & I)** β-phenylethylamine (PEA), **(B, F & J)** tryptamine (TRP), **(C, G & K)** ρ-tyramine (TYR), and **(D, H & L)** agmatine (AGM). **(A to D)** Effect on colocalization of occludin and ZO-1, **(E to H)** effect on total occludin, and **(I to L)** effect on total ZO-1 signal. The data is represented as mean ± SEM. Statistical analysis: 2-way ANOVA's with Šídák's multiple comparisons tests: * = *p*<0.05, ** = *p*<0.01, *** = *p*<0.001, **** = *p*<0.0001. Asterisk's (*) represent TA dose effect under the same E2 condition, while hashtags (#) represent effect of E2.

Figure 4.1: Changes in the concentration of fifteen major phenolic constituents between three rooibos samples: green rooibos ethanol extract (GRE), green rooibos aqueous extract (GR) and fermented rooibos aqueous extract (FR). Abbreviations: PPAG: phenylpropenoic acid glucoside

Figure 4.2: Effect of rooibos on the growth of (A) *Lactobacillus plantarum*, (B) *Enterococcus mundtii*, (C) *Saccharomyces boulardii*, and (D) *Lactobacillus reuteri*. Data is expressed as a mean ± SD, n=3. Statistical analysis: 2-way ANOVA with Dunnett's multiple comparison tests; * = *p*<0.05, ** = *p*<0.01, *** = *p*<0.001, **** = *p*<0.0001, compared to control media conditions. Abbreviations: GREM: green rooibos ethanol extract enriched media, GRM: aqueous green rooibos enriched media, FRM: aqueous fermented rooibos enriched media, OD: optical density.

Figure 4.3: Changes in the relative distribution (% of total) of trace amines in the secretome of *Lactobacillus plantarum* following rooibos and estradiol (E2) treatment. Rooibos treatments were as follows: (A & B) control media, (C & D) green rooibos extract media (GRE), (E & F) green rooibos media (GR), and (G & H) fermented rooibos media (FR). E2 treatments were as follows: (A, C, E, G) control (no E2), and (B, D, F, H) 1 nM E2 supplementation. The area of each pie chart is indicative of the fold change (total TA content) expressed relative to control (A). Abbreviations: TYR: ρ-tyramine, PEA: β-phenethylamine, TRP: tryptamine, AGM: agmatine, PUT: putrescine.

Figure 4.4: The effect of rooibos (GRE, GR and FR) and estradiol (E2) treatment on secretion of (A) ρ-tyramine (TYR), (B) β-phenethylamine (PEA), (C) tryptamine (TRP), (D) agmatine (AGM), and (E) putrescine (PUT) from *Lactobacillus plantarum*. The data are represented as the mean concentration of quantified trace amine (n=3) ± SEM. Statistical analysis: 2-way ANOVA with Tukey's multiple comparison tests; * = *p*<0.05, ** = *p*<0.01, *** = *p*<0.001, **** = *p*<0.0001, Ψ = main ANOVA effect of E2, Ω = main ANOVA effect of rooibos. Asterisk's (*) represent TA dose effect under the same E2 condition, while hashtags (#) represent effect of E2. Abbreviations: GRE: ethanol extract of green rooibos, GR: aqueous extract of green rooibos, FR: aqueous extract of fermented rooibos.

Figure 4.5: Changes in the relative distribution (% of total) of trace amines in the secretome of *Enterococcus mundtii* following rooibos and estradiol (E2) treatment. Rooibos treatments were as follows: (A & B) control media, (C & D) green rooibos extract media (GRE), (E & F) green rooibos media (GR), and (G & H) fermented rooibos media (FR). E2 treatments were as follows: (A, C, E, G) control (no E2), and (B, D, F, H) 1 nM E2 supplementation. The area of each pie chart is indicative of the fold change (total TA content) compared to control (A). Abbreviations: TYR: ρ-tyramine, PEA: β-phenethylamine, TRP: tryptamine, AGM: agmatine, PUT: putrescine.

Figure 4.6: The effect of rooibos (GR, GRE and FR) and estradiol (E2) treatment on secretion of (A) ρ-tyramine (TYR), (B) β-phenethylamine (PEA), (C) tryptamine (TRP), (D) agmatine (AGM), and (E) putrescine (PUT) from *Enterococcus mundtii*. The data are represented as the mean concentration of quantified trace amine (n=3) ± SEM. Statistical analysis: 2-way ANOVA with Tukey's multiple comparison tests; * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001, Ψ = main ANOVA effect of E2, Ω = main ANOVA effect of rooibos. Asterisk's (*) represent TA dose effect under the same E2 condition, while hashtags (#) represent effect of E2. Abbreviations: GRE: ethanol extract of green rooibos, GR: aqueous extract of green rooibos, FR: aqueous extract of fermented rooibos.

Figure 4.7: Changes in the relative distribution (% of total) of trace amines in the secretome of *Lactobacillus reuteri* following rooibos and estradiol (E2) treatment. Rooibos treatments were as follows: (A & B) control media, (C & D) green rooibos extract media (GRE), (E & F) green rooibos media (GR), and (G & H) fermented rooibos media (FR). E2 treatments were as follows: (A, C, E, G) control (no E2), and (B, D, F, H) 1 nM E2 supplementation. The area of each pie chart is indicative of the fold change (total TA content) compared to control (A). Abbreviations: TYR: ρ-tyramine, PEA: β-phenethylamine, TRP: tryptamine, AGM: agmatine, PUT: putrescine.

Figure 4.8: The effect of rooibos (GR, GRE and FR) and estradiol (E2) treatment on secretion of (A) ρ-tyramine (TYR), (B) β-phenethylamine (PEA), (C) tryptamine (TRP), (D) agmatine (AGM), and (E) putrescine (PUT) from *Lactobacillus reuteri*. The data are represented as the mean concentration of quantified trace amine (n=3) ± SEM. Statistical analysis: 2-way ANOVA with Tukey's multiple comparison tests; * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001, Ψ = main ANOVA effect of E2, Ω = main ANOVA effect of rooibos. Asterisk's (*) represent TA dose effect under the same E2 condition, while hashtags (#) represent effect of E2. Abbreviations: GRE: ethanol extract of green rooibos, GR: aqueous extract of green rooibos, FR: aqueous extract of fermented rooibos.

Figure 4.9: Changes in the relative distribution (% of total) of trace amines in the secretome of *Saccharomyces boulardii* following rooibos and estradiol (E2) treatment. Rooibos treatments were as follows: (A & B) control media, (C & D) green rooibos extract media (GRE), (E & F) green rooibos media (GR), and (G & H) fermented rooibos media (FR). E2 treatments were as follows: (A, C, E, G) control (no E2), and (B, D, F, H) 1 nM E2 supplementation. The area of each pie chart is indicative of the fold change (total TA content) compared to control (A). Abbreviations: TYR: ρ-tyramine, PEA: β-phenethylamine, TRP: tryptamine, AGM: agmatine, PUT: putrescine.

Figure 4.10: The effect of rooibos (GR, GRE and FR) and estradiol (E2) treatment on secretion of (A) ρ-tyramine (TYR), (B) β-phenethylamine (PEA), (C) tryptamine (TRP), (D) agmatine (AGM), and (E) putrescine (PUT) from *Saccharomyces boulardii*. The data are represented as the mean concentration of quantified trace amine (n=3) ± SEM. Statistical analysis: 2-way ANOVA with Tukey's multiple comparison tests; * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001, Ψ = main ANOVA effect of E2, Ω = main ANOVA effect of rooibos. Asterisk's (*) represent TA dose effect under the same E2 condition, while hashtags (#) represent effect of E2. Abbreviations: GRE: ethanol extract of green rooibos, GR: aqueous extract of green rooibos, FR: aqueous extract of fermented rooibos.

Supplementary Figure 4.1: Chromatograms of different rooibos preparations following q-TOF analyses targeting phenolic compounds.

Figure 5.1: Effects of green rooibos extract (GRE) and 17β-estradiol (E2) on the percentage of relative area of imaged HT-29 cell monolayer that stained positive for tight junction proteins occludin (A to D) and ZO-1 (E to H) following selected TA-exposure (200 ng/mL): (A, E) β-phenethylamine (PEA), (B, F) tryptamine (TRP), (C, G) ρ-tyramine (TYR), and (D, H) agmatine (AGM). Data are represented at mean

(% of total imaged area) ± SEM. Statistical analysis: 2-way ANOVAs with Tukey's multiple comparison tests: * = p <0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001.

Figure 5.2: Representative fluorescent micrographs showing the effects of trace amines (TA), green rooibos extract (GRE), 17β-estradiol (E2) and combined treatments on tight junction proteins in HT-29 cell monolayers. Green signal = ZO-1, red signal = occludin, yellow signal = colocalized ZO-1 and occludin signal, and blue signal = Hoechst. All fluorescent images are maximum intensity projections of acquired z-stacks, imaged using the 60 x oil immersion objective. Scale bar = 10 μm. Abbreviations: PEA: β-phenethylamine, TRP: tryptamine, TYR: ρ-tyramine, AGM: agmatine

Figure 5.3: Effects of green rooibos extract (GRE) and 17β-estradiol (E2) on the concentration of prostaglandin E2 (PGE2) secreted by HT-29 cell monolayers following selected TA-exposure (200 ng/mL): (A) β-phenethylamine (PEA), (B) tryptamine (TRP), (C) ρ-tyramine (TYR), and (D) agmatine (AGM). Data are represented at mean ± SD, n=3. Statistical analysis: 2-way ANOVAs with Tukey's multiple comparison tests: * = p <0.05, ** = p <0.01, *** = p < 0.001.

Figure 5.4: Effects of green rooibos extract (GRE) and estradiol (E2) on the concentration of interleukin-8 (IL-8) secreted by HT-29 cell monolayers following selected TA-exposure (200 ng/mL): (A) β-phenethylamine (PEA), (B) tryptamine (TRP), (C) ρ-tyramine (TYR), and (D) agmatine (AGM). The data are represented at mean ± SD, n=3. Statistical analysis: 2-way ANOVAs with Tukey's multiple comparison tests: * = p <0.05, ** = p <0.01, *** = p < 0.001, **** = p < 0.0001.

Supplementary Figure 5.1: Changes in the relative distribution (% of total) of fifteen major phenolic constituents due to differential rooibos processing: (A) green rooibos ethanol extract (GRE), (B) green rooibos, and (C) fermented rooibos. Abbreviations: PPAG: phenylpropenoic acid glucoside.

Supplementary Figure 5.2: WST-1 results showing the effect of varying green rooibos extract (GRE) doses on HT-29 colon cell viability. Data are represented as mean % of control ± SD, n=3. Statistical analysis: One-way ANOVA with Tukey's multiple comparison test, *** = p<0.001, **** = p<0.0001.

Figure 6.1: Schematic illustrating longitudinal sections through larvae that enabled the correct orientation and assessment of the desired – mid-intestinal – gut region.

Figure 6.2: Basal locomotion of zebrafish larvae (7 dpf) following selected trace amine (TA) exposure in the absence or presence of an inflammatory stimulus (TNBS; 25 μg/mL, 2-day exposure). Total distance travelled (mm) over a 10-minute period was captured utilising Ethovision software following 24 hr selected TA treatments: β-phenethylamine (PEA), tryptamine (TRP), ρ-tyramine (TYR), and agmatine (AGM). Data are represented as individual scatter dot plots with mean ± SEM, n=12. Statistical analyses: 2-way ANOVA with Tukey's multiple comparison tests; */$^{\#}$ = p<0.05, ***/$^{\#\#\#}$ = p<0.001, **** = p<0.0001. Hashtags (#) represent differences within TNBS treatment groups, while asterisks (*) represent differences between TNBS treatment groups.

Figure 6.3: Length of neutral red stained mid-intestine (μm) in zebrafish larvae (7 dpf) following exposure to selected trace amines (TAs): β-phenethylamine (PEA), tryptamine (TRP), ρ-tyramine (TYR), and agmatine (AGM), in the absence or presence of TNBS (25 μg/mL, 2-day exposure). Data are expressed as mean ± SD, n=9. In addition, TNBS treatment with subsequent prednisolone co-administration is represented as mean (white dotted line) ± SD (greyed area) as the standard internal rescue control. Statistical analyses: 2-way ANOVA with Tukey's multiple comparison tests; */$^{\#}$ = p<0.05, **/$^{\#\#}$ = p<0.01, ****/$^{\#\#\#\#}$ = p<0.0001. Hashtags (#) represent TA effects within TNBS treatment groups, while asterisks (*) represent effects between TNBS groups for the same TA treatment.

Figure 6.4: Representative fluorescent micrographs showing the effects of selected trace amines (TAs), TNBS, 17β-estradiol (E2), prednisolone, and combined treatments on tight junction proteins in zebrafish larvae (7 dpf) mid-intestines. Green signal = ZO-1, red signal = occludin, blue signal = Hoechst. All micrographs were imaged using a 60x objective; scale bar = 10 μm. Abbreviations: PEA: β-phenethylamine, TRP: tryptamine, TYR: ρ-tyramine, AGM: agmatine.

Figure 6.5: Mid-intestinal immunofluorescent tight junction protein signal in stained zebrafish larvae (7 dpf) treated with 25 μM β-phenethylamine (PEA) or tryptamine (TRP) or ρ-tyramine (TYR) or agmatine (AGM), in the absence or presence of TNBS and 17β-estradiol (E2). In addition, the effects of TNBS treatment (yellow bar), and TNBS and prednisolone (Pred) co-administration are represented (white bar). In (A) the effect on ZO-1 intensity is reported while in (B) the effect on occludin intensity is reported. Data are expressed as mean fluorescent intensity ± SEM, n=6. Statistical analyses: One-way ANOVA with Tukey's multiple comparison tests; */#/@ = p<0.05, **/##/@@ = p<0.01, ### = p<0.001, ****/#####/@@@@ = p<0.0001. Asterisks (*) represent differences compared to the control group, hashtags (#) represent differences compared to the TNBS only group, asperands (@) represent differences compared to the AGM only group.

Figure 6.6: Hydrogen peroxide (H_2O_2) levels in pooled zebrafish larvae (7 dpf; n=25 per pooled sample). In (A) the effect of TNBS (25 μg/mL, 2-day exposure) in combination with prednisolone (P: 25 μg/mL, 1-day exposure), 17β-estradiol (E2: 20 nM, 2-day exposure) and tamoxifen (TAM: 2.5 μM, 2-day exposure) are displayed. In (B) H_2O_2 outcome in response to trace amine (TA) dose responses (25 μM, 50 μM, and 100/200 μM) are displayed. Additionally, the basal (control) H_2O_2 level is depicted (black dotted line). Data are expressed as mean concentration per total protein as % of control ± SD, n=6. Statistical analyses: One-way ANOVA with Tukey's multiple comparison tests; * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001. Asterisks (*) represent differences compared to the control group (dotted line). Abbreviations: β-phenethylamine (PEA), (C) tryptamine (TRP), (D) ρ-tyramine (TYR), and (E) agmatine (AGM).

Figure 6.7: Hydrogen peroxide (H_2O_2) levels in pooled zebrafish larvae (7 dpf; n=25 per pooled sample) treated with 25 μM (left column) or 100 μM (right column) of selected trace amines: (A&B) β-phenethylamine (PEA), (C&D) tryptamine (TRP), (E&F) ρ-tyramine (TYR), and (G&H) agmatine (AGM), in the absence (-) or presence (+) of TNBS (25 μg/mL, 2-day exposure), 17β-estradiol (E2: 20 nM, 2-day exposure) and tamoxifen (TAM: 2.5 μM, 2-day exposure). In addition, the mean ± SD of TNBS only condition is represented by the dotted black line and greyed box respectively, located next to the y-axis. Data are expressed as mean concentration per total protein as % of control ± SD, n=6. Statistical analyses: One-way ANOVA with Tukey's multiple comparison tests; * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001.

Figure 6.8: Prostaglandin E2 (PGE2) levels in pooled zebrafish larvae (7 dpf; n=25 per pooled sample). In (A) the effect of TNBS (25 μg/mL, 2-day exposure) in combination with prednisolone (P: 25 μg/mL, 1-day exposure), 17β-estradiol (E2: 20 nM, 2-day exposure) and tamoxifen (TAM: 2.5 μM, 2-day exposure) are displayed. In (B) H_2O_2 outcome in response to trace amine (TA) dose responses (25 μM, 50 μM, and 100/200 μM) are displayed. Additionally, the basal (control) PGE2 level is depicted (black dotted line). Data are expressed as mean concentration per total protein as % of control ± SD, n=3. Statistical analyses: One-way ANOVA with Tukey's multiple comparison tests; * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001. Asterisks (*) represent differences compared to the control group (dotted line). Abbreviations: β-phenethylamine (PEA), (C) tryptamine (TRP), (D) ρ-tyramine (TYR), and (E) agmatine (AGM).

Figure 6.9: Prostaglandin E2 (PGE2) levels in pooled zebrafish larvae (7 dpf; n=25 per pooled sample) treated with 25 μM (left column) and 100 μM (right column) of selected trace amines: (A&B) β-phenethylamine (PEA), (C&D) tryptamine (TRP), (E&F) ρ-tyramine (TYR), and (G&H) agmatine (AGM), in the absence (-) or presence (+) of TNBS (25 μg/mL, 2-day exposure), 17β-estradiol (E2: 20 nM, 2-day exposure) and tamoxifen (TAM: 2.5 μM, 2-day exposure). In addition, the mean ± SD of TNBS only condition is represented by the dotted black line and greyed box respectively, located next to the y-axis. Data are expressed as mean concentration per total protein ± SD, n=3. Statistical analyses: One-way ANOVA with Tukey's multiple comparison tests; * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$.

Supplementary Figure 6.1: Representative micrographs of neutral red stained zebrafish larvae.

List of Tables

List of Abbreviations

α2-ADR	α2-Adrenergic receptor
5-HT	5-Hydroxytryptamine (serotonin)
AADC	Aromatic L-amino acid decarboxylase
AGM	Agmatine
BBB	Blood brain barrier
CAF	Central analytical facility
COX	Cyclooxygenase
CVD	Cardiovascular disease
E2	17β-Estradiol
E3	Embryo medium
EC	Enterochromaffin
ELISA	Enzyme-linked immunosorbent assay
ENS	Enteric nervous system
ER	Estrogen receptor
FA	Formic acid
FBS	Fetal bovine serum
FGID	Functional gastrointestinal disorder
FR	Fermented rooibos
FRM	Aqueous fermented rooibos enriched media
GI	Gastrointestinal
GIT	Gastrointestinal tract
GLP-1	Glucagon-like peptide-1
GPCR	G protein-coupled receptor

GPER (GPR30)	G protein-coupled estrogen receptor 1
GR	Green (unfermented) rooibos
GRE	Green rooibos extract (EtOH extract)
GREM	Green rooibos ethanol extract enriched media
GRM	Aqueous green rooibos enriched media
HPA	Hypothalamic pituitary axis
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IS	Internal standard
LAB	Lactic acid bacteria
LB	Luria Bertani
LC	Liquid chromatography
MAO	Monoamine oxidase
MAOI	Monoamine oxidase inhibitor
MDA	Malondialdehyde
MP	Mobile phase
MRM	Multiple reaction monitoring
MRS	De Man, Rogosa and Sharpe
MS	Mass spectrometry
NE	Norepinephrine
NO	Nitric oxide
O/N	Overnight
OD	Optical density
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PEA	β-Phenethylamine
PEA-d4	Phenethylamine-d4
PFA	Paraformaldehyde
PGE2	Prostaglandin E2
PMN	Polymorphonuclear
PPAG	Phenylpropenoic acid glucoside
PUT	Putrescine
PUT-d8	Putrescine-d8
PYY	Peptide tyrosine tyrosine
qTOF	Quadrupole time-of-flight
RCT	Randomized control trial
SD	Standard deviation
SEM	Standard error of mean
SERT	Serotonin reuptake transporter
SIBO	Small intestinal bacterial overgrowth
SPE	Solid phase extraction
T_1AM	3-Iodothyronamine
TA	Trace amine
TAAR	Trace amine-associated receptor
TAM	Tamoxifen
TJP	Tight junction protein
TMA	Trimethylamine
TMAO	Trimethylamine *N*oxide

TNBS	2,4,6-trinitrobenzene-sulfonic acid
TRP	Tryptamine
TYR	ρ-Tyramine
UPLC	Ultra-performance liquid chromatography
WCX	Weak cation exchanger
YPD	Yeast extract peptone dextrose
ZO-1	Zona occludens-1

Chapter 1

Introduction

Irritable bowel syndrome (IBS) is a highly predominant functional gastrointestinal disorder (FGID) (Oswiecimska et al., 2017, Sperber et al., 2021). Currently, IBS is estimated to affect around 1 in 10 individuals, relating to a global prevalence of 10 % (Endo et al., 2015, Black and Ford, 2020). Importantly, IBS is distinctly different from inflammatory bowel diseases (IBD). In this regard, FGIDs such as IBS, have no obvious structural gastrointestinal (GI) alterations, and as such diagnosis and subsequent classification is based on clinical symptomology (Rome IV Criteria). Despite the fact that IBS is not a life-threatening condition, suboptimal treatment, lack of highly predictive diagnostic biomarkers, and the complexity and heterogeneity of symptomology makes management difficult and unsatisfactory in many cases (Moayyedi et al., 2017). Therefore, it is evident that a more thorough understanding of the pathophysiology underpinning IBS is required to improve the therapeutic strategies and armamentarium currently being implemented.

In addition, and of particular importance to this thesis, epidemiological studies have consistently shown female predominance for IBS in patient populations. Generally, female predominance in this context is reported as a ratio of 2:1, however, this ratio can increase to up to 5:1 in tertiary care settings (Mulak et al., 2014). In fact, gonadal maturation often coincides with IBS symptom onset (Heitkemper and Jarrett, 2008), and GI symptom fluctuation and exaggeration (flare ups) are often reported during pre-menses/menses and pre-menopausal phases (Kane et al., 1998, Whitehead et al., 1990, Heitkemper et al., 2002), implicating – at least to some degree – female reproductive hormones as confounding factors in the aetiology of IBS.

Given the lack of effective therapies in this context, as well as a suboptimal understanding of the regulatory mechanisms or maladaptation which are associated with or underpin IBS pathophysiology (Chong et al., 2019), this thesis aimed to investigate the actions of the trace aminergic system, as an overlooked regulatory role player. Trace amines (TAs) are a class of biogenic amines that are produced endogenously at very low concentrations in neuronal tissues. In the GI context, however, high levels of TAs can originate from microbial sources i.e. microbial transformation of L-amino acids in the gut, or from direct consumption of certain microbially fermented foods. Given that the gut microbiome is attributed several regulatory roles, not only for maintenance of the GI environment but also systemic host functioning (Yue et al., 2020, Agus et al., 2021, Chen et al., 2021), relative gut dysbiosis (which is a recognized

risk factor for IBS (Wang et al., 2020) and importantly the ensuing functional dysbiosis (i.e. changes in microbial secretomes), may contribute to altered TA levels, potentially implicating the gut microbiota in subsequent disease progression and symptomology. In the IBS context specifically, the trace aminergic system links together several risk factors, such as sex (Liberles & Buck, 2006), diet (Barbieri et al, 2019), dysbiosis (Ponnusamy et al., 2011, Jacobs et al., 2016, Santoru et al., 2017), inflammation (Babusyte et al., 2013, Islam et al., 2017), and anxiety, potentiating its association with other regulatory systems such as the inflammatory or redox systems. Indeed, we endeavored not only to investigate the role of the trace aminergic system in the context of aberrant functioning related to potential pathology (as hypothesized), but also in the context of a 'return to homeostasis' (i.e. what is 'normal' and how should the system be targeted to return to 'normal'). Moreover, the physiological relevance of the trace aminergic system in GI conditions, as well as potential therapeutic targets, are discussed.

For the purpose of this dissertation the specific effects of four selected TAs - β-phenethylamine (PEA), tryptamine (TRP), ρ-tyramine (TYR) and agmatine (AGM) - were primarily investigated. These TAs were selected due to their known capacity for generation (from L-amino acid precursors – which are in rich supply in the gut) and secretion by representative GI microbes (Luqman et al., 2018, Nakamura et al., 2019, Pugin et al., 2017). In addition, these TAs have known peripheral roles in the gastrointestinal tract (GIT) and have been subsequently identified as potential biomarkers of dysbiosis/GI disorders (Jacobs et al., 2016, Ponnusamy et al., 2011, Santoru et al., 2018). Importantly, these TAs also seem to play roles in the regulation of inflammatory and redox functioning (Babusyte et al., 2013, Milosevic et al., 2022, Islam et al., 2017).

Seeing that IBS is a complex, multifactorial disease, it was clear that a multidisciplinary approach would be required to not only better understand the aetiology of IBS, but also to make advancements in terms of therapeutic strategies. Indeed, a significant strength of this thesis is the multi-model approach utilized, which consisted of *in vitro* investigations in microbial (commensal and probiotic) and tissue (HT-29 colon adenocarcinoma) cultures, as well as *in vivo* experimentation in a zebrafish larval model of TNBS-induced GI inflammation.

This thesis is presented in a hybrid format. The work presented in Chapters 2 (literature review), 3 and 4 (original data) has been published, while manuscripts of further original data (as presented in Chapters 5 and 6) are currently under review. Specifically, an in-depth literature review deliberating the proposed role of TAs and their associated receptor in IBS, as well as the putative links to sex will be presented in Chapter 2. Original data will be presented

in the subsequent four chapters. Chapter 3 presents data from *in vitro* microbial, and HT-29 cell cultures, demonstrating the effects of TAs on gut epithelial tight junction protein (TJP) and inflammatory status, as well as the effects of 17β-estradiol (E2) on the microbial TA production and downstream effects of TAs. Chapter 4 will present data on the characterisation of three different rooibos preparations, as well as their respective effects on microbial growth and secretomes TA profiles, compared to E2. Chapter 5 will present data that describes the specific differences of E2 and rooibos to prevent specific TA-induced TJP damage, as well as inflammatory profile outcomes. In addition, the potential synergistic actions of rooibos and AGM as therapeutic strategies are proposed. Finally, Chapter 6 will present data that describes the *in vivo* effects of TAs in a zebrafish larval model of GI inflammation in the presence and absence of E2 and tamoxifen (TAM), to elucidate mechanisms and major role players in the content of GI inflammation. This model in particular, allowed for investigation into potential targets for future therapeutic investigations. These chapters will be followed by a synthesis in Chapter 7 and a full reference list in Chapter 8.

Chapter 2

Literature Review

The trace aminergic system: a gender-sensitive therapeutic target for IBS?

This chapter has been published in the Journal of Biomedical Sciences (Impact Factor: 8.41).

Citation reference: Pretorius, L., Smith, C. (2020) The trace aminergic system: a gender-sensitive therapeutic target for IBS? *J Biomed Sci.* 27:95. https://doi.org/10.1186/s12929-020-00688-1

2.1 Abstract

Due to a lack of specific or sensitive biomarkers, drug discovery advances have been limited for individuals suffering from irritable bowel syndrome (IBS). While current therapies provide symptomatic relief, inflammation itself is relatively neglected, despite the presence of chronic immune activation and innate immune system dysfunction. Moreover, considering the microgenderome concept, gender is a significant aetiological risk factor. We believe that we have pinpointed a “missing link” that connects gender, dysbiosis, diet, and inflammation in the context of IBS, which may be manipulated as therapeutic target. The trace aminergic system is conveniently positioned at the interface of the gut microbiome, dietary nutrients and by-products, and mucosal immunity. Almost all leukocyte populations express trace amine associated receptors and significant amounts of trace amines originate from both food and the gut microbiota. Additionally, although IBS-specific data are sparse, existing data supports an interpretation in favour of a gender dependence in trace aminergic signalling. As such, trace aminergic signalling may be altered by fluctuations of especially female reproductive hormones. Utilizing a multidisciplinary approach, this review discusses potential mechanisms of actions, which include hyperreactivity of the immune system and aberrant serotonin signalling, and links outcomes to the symptomology clinically prevalent in IBS. Taken together, it is feasible that the additional level of regulation by the trace aminergic system in IBS has been overlooked, until now. As such, we suggest that components of the trace aminergic system be considered targets for future therapeutic action, with the specific focus of reducing oxidative stress and inflammation.

2.2 Introduction

Irritable bowel disease (IBS) is a functional gastrointestinal disorder, which is prevalent in more than 10% of the global population (Endo et al., 2015). Although IBS is easily recognised (recurrent abdominal pain associated with change in stool consistency and frequency, as is the case in inflammatory bowel disorder (IBD) (Bennet et al., 2015), but in the absence of

structural abnormalities (Lacy and Patel, 2017)), there is still a lack of any sensitive or specific biomarkers (Rodino-Janeiro et al., 2018), limiting advancement in terms of drug discovery for treatment of this debilitating condition.

It is important to note that epidemiological studies have consistently shown female predominance for IBS in patient populations, with ratios of up to 5:1 in tertiary care settings (Mulak et al., 2014). Furthermore, many women with IBS report gastrointestinal (GI) symptom fluctuation and exaggeration (flares) during pre-menses and pre-menopausal phases (Kane et al., 1998, Whitehead et al., 1990, Heitkemper et al., 2002). Although, some of these symptoms seem to be common among non-IBS or normally asymptomatic women too (Mulak et al., 2014, Bharadwaj et al., 2015b), however, the fact that IBS symptom onset often coincides with gonadal maturation, again implicates hormone levels as confounder in IBS aetiology (Heitkemper and Jarrett, 2008).

In terms of current IBS therapy, treatment strategies range from microbiota-based therapies (probiotics, prebiotics, synbiotics, non-absorbable antibiotics and faecal microbiota transplants) to opioid receptor agonist/antagnoists, and dietary interventions, most of which provide symptomatic relief (Chong et al., 2019). Of specific interest to this review, present-day strategies seem focused on addressing clinically evident symptoms only, with relative neglect of inflammation, despite the fact that chronic immune activation and innate immune system dysfunction is implicated in IBS pathogenesis (Lazaridis and Germanidis, 2018). The importance of considering inflammation in IBS, is underlined by several factors. Firstly, a recent review concluded that gender-differences in inflammation – specifically the fact that prognosis in chronic inflammatory conditions are worse in females, in line with the female predominance in IBS – cannot be fully accounted for by hormonal differences between genders (Casimir et al., 2018). Secondly, psychosocial stress is the most generally recognized risk factor for both development and relapse of IBS (Vannucchi and Evangelista, 2018). Here again, females are more at risk, with a significant female predominance reported for anxiety and depression-associated disorders (Altemus et al., 2014). Taken together, it is clear that gender is a significant role player in IBS risk, but that hormone differences alone are probably not the only predictor of outcome.

We believe that we have identified a “missing link” that ties together gender, diet, inflammation and anxiety in the context of IBS, which may be exploited as a therapeutic target. The trace aminergic system was first described in non-human mammals, as having a “sexual cue” function (Liberles and Buck, 2006). Most trace amine associated receptors associate closely with olfactory neurons, suggesting a pheromone-type function. In line with this, significant

gender differences were reported for trace amine levels (Liberles and Buck, 2006). Furthermore – and specifically relevant to the review topic and IBS – trace amine synthesis has been reported in human neurons, where it is thought to modulate neuronal signalling (Pei et al., 2016, Miller, 2011, Berry, 2004), and high levels of trace amines have been found in specific foods, as well as bacterial secretomes (Moracanin et al., 2015, Ruiz-Capillas and Herrero, 2019, Barbieri et al., 2019, Lorencová et al., 2012). Taken together, it is therefore possible that the trace aminergic system may be an additional level of control/maladaptation in IBS that has been largely overlooked until now. It is also clear that in order to make advances in terms of therapeutic strategies, or even better understanding of disease aetiology, a multi-disciplinary approach is required.

This review therefore aims to provide an integrated and holistic picture of IBS aetiology, including a critical assessment of current methodologies employed in this context where relevant. Drawing from different disciplines in science, we then provide a comprehensive review of the literature on the trace aminergic system, in support of our hypothesis that this system may be targeted therapeutically in the context of IBS.

2.3 The complexity that is IBS

Given the difficulty of IBS management, it would be foolish to underestimate the complexity of the disease aetiology. For the purpose of the current discussion, in the next few sections, we provide an overview of only the most relevant processes at play.

2.3.1 Oxidative stress and inflammation

Inflammation and oxidative stress go hand in hand, especially in chronic inflammatory disorders, where the poorer prognosis in females has been specifically linked to greater oxidative damage resulting from inflammation (Akira and Takeda, 2004, Flak et al., 2013). While the susceptibility of cells to oxidative stress is largely variable between individuals and specific tissue types, a review by Jones *et al*., (2012) explains that the GI tract (GIT) is a particularly high reactive oxygen species environment. Furthermore, in cancer literature, the presence of estrogen receptors (ERs) are commonly known to render cells more sensitive to oxidative stress via diminished antioxidant activities (Mobley and Brueggemeier, 2004, Betts et al., 2017). Similar results have been observed in IBS patients. For example, in a study including 36 IBS patients, plasma activities of xanthine oxidase and adenosine deaminase, and plasma concentrations of malondialdehyde (MDA) and nitric oxide, were significantly higher in patients than controls, while superoxide dismutase, catalase and glutathione

peroxidase activities were significantly lower (Mete et al., 2013). These results suggest that altered oxidant-antioxidant responses are prevalent in patients with IBS. While both males and females formed part of the study, no analysis in gender differences was performed. Furthermore, increased oxidative stress-related markers (elevated MDA, decreased total antioxidant capacity) were reported in 90 IBS patients more recently (Choghakhori et al., 2017b), with a tendency for female patients to have a worse redox profile (Choghakhori et al., 2017a). Together, these data suggest oxidative damage to be a major contributor to female predominance of IBS.

Of course, plasma redox status is not necessarily an accurate indication of the status at tissue level. Although clinical evidence of oxidative stress within the intestinal wall is lacking, studies in rodent models of IBS have reported evidence suggestive of oxidative stress in the intestinal wall as well. For example, the total antioxidant capacity (measured by FRAP) of large intestine homogenates of rats (IBS induced by restraint-stress) was significantly reduced compared to controls (Zamani et al., 2005, Mozaffari et al., 2011). Moreover, Mozaffari *et al*., (2011) reported significant increases in myeloperoxidase activity and lipid peroxidation in the same homogenates. These reductions in antioxidant capacity reportedly correlated with gastrointestinal symptomology as well. While similar studies in humans are lacking, it has been reported that neutrophil counts in colonic biopsies of patients with IBS are significantly increased compared to control (Chadwick et al., 2002), which may in turn result in increased myeloperoxidase levels, for example, in the colon tissue. Additionally, a very recent study utilising confocal laser endomicroscopy, reported that patients with IBS have a six-fold higher prevalence of colorectal mucosa micro-inflammation than healthy controls (Robles-Medranda et al., 2020). Considering that inflammation and oxidative stress are linked, often occurring in tandem, it is likely that local effects of oxidative damage/stress are implicated in IBS-related gastrointestinal symptomology.

As is the case for many chronic inflammatory diseases, it is difficult to know whether oxidative stress or inflammation manifests first. However, given the self-perpetuating cycle of oxidative stress and inflammation, it is not unexpected that a pro-inflammatory phenotype (increased TNFα and IL-17, decreased IL-10) is prevalent in IBS (Lazaridis and Germanidis, 2018). Also, in the context of inflammation, a female bias has been reported, with females exhibiting higher inflammatory capacity and generally having poorer prognosis in chronic inflammatory disease (Casimir et al., 2018).

Inflammation is, however, not just an outcome of oxidative stress, but is interconnected with other significant role players in IBS, as demonstrated in the following sections.

2.3.2 Serotonin dysregulation

Serotonin - or 5-hydroxytryptamine (5-HT) - is a well-known neurotransmitter and neuro-hormone, which modulates several GI functions, such as motility, visceral sensitivity, immune function and blood flow (Kim and Camilleri, 2000). Additionally, due to its prominent role in the gut-brain axis, perturbations in 5-HT signalling have also been implicated in the pathophysiology of IBS (Camilleri, 2009, Chong et al., 2019, Crowell, 2004, Gershon, 2013, Gershon and Tack, 2007, Padhy et al., 2015, Pata et al., 2004). Mucosal serotonergic enterochromaffin (EC) cells are sensory transducers that respond to luminal stimuli by secreting 5-HT into the intestinal wall to stimulate the primary afferent nerve fibers of the enteric nervous system (Makker et al., 2015, Bellono et al., 2017). Although relatively sparse (less than 1% of intestinal epithelia), EC cells produce more than 90% of the body's 5-HT and have been suggested to affect a variety of physiological and pathophysiological states (Gershon, 2013, Mawe and Hoffman, 2013).

Indeed, in IBS, evidence of dysregulated serotonergic signalling has been established. The most reproducible results indicate that patients with diarrhoea-predominant IBS have higher blood levels of 5-HT (Bearcroft et al., 1998), while patients with constipation-predominant IBS have lower blood levels of 5-HT (Dunlop et al., 2005, Atkinson et al., 2006) compared to healthy controls.

Also here, a gender-dependence is evident: 17β-estradiol (E2) regulates the concentration of 5-HT *via* two mechanisms. Firstly, E2 increases synthesis of tryptophan hydroxylase (Bethea et al., 2000a, Bethea et al., 2000b), which is the rate-limiting factor in the conversion of tryptophan to 5-HT, thereby increasing the concentration of 5-HT (Blum et al., 1996, Sze et al., 2000). Secondly, E2 inhibits gene expression of the serotonin reuptake transporter (SERT), and also acts as a SERT inhibitor, consequently promoting the actions of 5-HT by increasing its availability in synapses and interstitial spaces (Pecins-Thompson et al., 1998, Ofir et al., 2003). Beyond increasing concentration and availability of 5-HT, E2 also modulates the actions of 5-HT. This is because the activation of E2 receptors affects the state and distribution of 5-HT receptors. For example, higher levels of E2 in the presence of progesterone (Prog), upregulates ERβ - resulting in upregulation of the 5-HT_{2A} receptor (Ostlund et al., 2003) (Kugaya et al., 2003, Moses-Kolko, 2003) - and downregulates ERα (Cheng et al., 2005) - resulting in a decreased NFκB-associated activation of 5-HT_{1A} receptors (Wissink et al., 2001). Therefore, during the reproductive phase of a female lifespan (higher E2 and Prog levels), E2 causes an increase in the density and binding of the 5-HT_{2A} receptor.

It is notable that the 5-HT_{2A} receptor gene is expressed in the brain and the gut (Raote et al., 2007), and has been reported as the main 5-HT receptor in the perception of pain (Tokunaga et al., 1998), which may contribute to female bias in pain processing, specifically in an IBS context (Meleine and Matricon, 2014). Interestingly, a study by Pata *et al*. (2004) implicated 5-HT_{2A} receptor gene polymorphisms as a genetic component of IBS pathophysiology. Specifically, a high incidence of homozygous C allele of the 102T/C polymorphism (also reported in patients with depression and anxiety) and homozygote A allele of the −1438 G/A promoter region was reported in patients with IBS. Moreover, the patients with T/T genotype had a significantly higher visual analogue score (determines severity of chronic abdominal pain) than patients with other genotypes, suggesting that the T/T genotype potentates pain perception, although it is not unique to IBS. It remains to be elucidated if a gender bias exists for this type of mutation. Nevertheless, in line with this data, abdominal pain is a hallmark of IBS and is often a result of colonic distension and visceral hypersensitivity (Crouzet et al., 2013). Of further relevance, it has been reported that $5HT_{2A}$ −/− mice had smaller enterocytes, fewer paneth cells, and thinner muscle layers, compared to 5-HT_{2A} +/+ littermates (Florica-Howells et al., 2002). However, since this receptor does not seem to affect colonic transit time, IBS treatments targeting 5-HT receptors have classically focused on 5-HT_3 (facilitates enteric to central nervous system signalling and promotes gut motility), 5-HT_4 (augments peristalsis and intestinal secretion), 5-HT_{1B} (initiates peristalsis) receptors (Fayyaz and Lackner, 2008) and even 5-HT_{1A}R – for which decreased activity has been linked to exacerbated symptoms of depression (Gorinski et al., 2019), a known co-morbidity in IBS. The role of $5HT_{2A}$ receptors in the context of IBS-related pain remains to be fully elucidated.

2.3.3 Altered colonic ion secretion

While colonic ion secretion is critically important in maintaining GI motility, there is no concrete evidence that patients with IBS (regardless of the subtype) suffer from primary secretory diathesis (Camilleri, 2015). Moreover, it is reasonable to suggest that different IBS subtypes would be characterised by different secretory ion profiles, resulting in either constipation (more common in females) or diarrhoea (more common in males). In terms of a reproductive hormone effect, E2 is a known modulator of ion-secretion, also independent of its effect on 5-HT signalling. Both ERα and ERβ have been detected in distal colonic crypts (Thomas et al., 1993), where E2 was shown to inhibit epithelial chloride ion secretion in female rats (Condliffe et al., 2001), resulting in significant water and salt retention during high estrogen states (O'Mahony and Harvey, 2008). Of interest, the gender bias for the anti-secretory action of estrogen was attributed to gender specificity of ion transporter protein expression profiles (Condliffe et al., 2001). Furthermore, E2 reduced currents mediated by the KCNQ1:KCNE3

potassium channel in an Ussing chamber model (Alzamora et al., 2011). Similarly, more recent data shows that E2 links to intracellular calcium, cystic fibrosis transmembrane conductance regulator and Cl^-/HCO_3^- secretion (Yang et al., 2017). Seeing that E2 inhibits colonic chloride ion secretion (consequentially reducing water movement to the lumen), it makes sense that females with IBS generally present with reduced colonic transit/GI motility and constipation, symptoms which alter drastically during menses.

2.3.4 Gut dysbiosis

Up to now, gender-association has been a continuous thread through all factors contributing to IBS aetiology. However, although it is clear in other disease contexts, e.g. auto-immune disease, that a gender bias indeed also exist in terms of gut microbial content and/or function (Rizzetto et al., 2018), this association is less clear in IBS, due to a relative lack of research in this context.

Nevertheless, the gut microbiota is widely regarded as a regulatory system that actively mediates numerous physiological functions as part of its symbiotic relationship with its host, *via* generation of metabolites to affect both nearby and distant organs (Marcobal et al., 2013, Wikoff et al., 2009), including the brain (Matsumoto et al., 2013). This ability to predict clinical phenotype was even recently suggested to be superior to the predictive power of genetics (Rothschild et al., 2018). Indeed, altered bacterial composition, the so-called dysbiosis, is associated with a spectrum of diseases, including neuropathology and inflammatory conditions (Distrutti et al., 2016). For example, germ-free animals demonstrate delayed gastric emptying and intestinal transit, reduced migrating motor complex cycling and propagation, and reduced GABA and VAP-33 gene expression, when compared with animals raised in a normal laboratory environment (Barbara et al., 2016). Thus, it is not surprising that some form of compositional dysbiosis (altered microbial alpha and/or beta diversity) has been implicated as an etiological factor in the development of various gastrointestinal disorders, including IBS (Neish, 2009, Park et al., 2011, Quigley, 2009, Klem et al., 2017), where it is thought to drive persistent low-grade inflammation and chronic gut dysfunction (Collins et al., 2009). Globally, data on IBS patients suggest reductions in microbial diversity, altered proportions of specific bacterial groups, shifts between mucosal and luminal bacterial abundance (Carroll et al., 2010), and a higher degree of temporal instability of microbiota (Distrutti et al., 2016), when compared to healthy individuals.

However, there are several shortcomings of exploring compositional dysbiosis in the context of IBS. Firstly, the compositional alterations reported in the literature are not specific to IBS - similar changes in microbial diversity are found in numerous diseases and conditions (Collins

et al., 2009, Distrutti et al., 2016). This raises questions regarding their specificity as potential disease biomarkers. Similarly, some authors have criticized whether a relative microbial imbalance that is assessed in a cross-sectional approach, would accurately represent a disease or reflect whether this imbalance occurs secondary to disease-related behaviours (poor lifestyle choices), as this requires analysis of consecutive samples with simultaneous disease variation (Enck and Mazurak, 2018). Secondly, many of these studies have produced conflicting results within the IBS population (Dlugosz et al., 2015) for various reasons commonly ascribed to methodology or study design (Lozupone et al., 2013, Mavrangelos et al., 2017, Pozuelo et al., 2015, Tap et al., 2017). Thirdly, an improvement in the fairly basic genus-species analysis most often employed (Rodino-Janeiro et al., 2018) is required before significant advances in knowledge gain on the topic are made. This is corroborated by probiotic-focused studies that have emphasized strain-specificity in bacterial function (Gharbi et al., 2019), let alone species specificity. Fourthly, the profile of a 'normal' or healthy microbiota is unknown. In our opinion, and those of several other authors, it is impossible to define a healthy microbiome due to the high degree of inter-individual variation (Costello et al., 2009, Dave et al., 2012), cultural habitual dietary habits and other population-specific factors. This adds to the complexity of selecting suitable controls for gut microbiome studies. Accordingly, extreme care to choose controls similar not only in age and gender, but also cultural background and nutritional habits, is vital to the reliability of any study (but may at the same time also limit its broader relevance). Lastly, efforts to standardize testing, with the introduction of the 'dysbiosis index' (Casen et al., 2015) has thus far been unsuccessful, since the predictive value of the index was reportedly low and non-specific (Enck and Mazurak, 2018). Given the fact that compositional analysis does not seem to be sufficiently sophisticated yet to lead to therapeutic advancement in medicine, another option is to rather consider functional effects of the total microbiome secretome (i.e. the secretory products of all microbes present in the gut) on its host.

Indeed, another grouping of researchers tends towards analysing *functional* dysbiosis, or the alterations of the microbial secretomes, in diseased states (Roager and Licht, 2018, De Preter and Verbeke, 2013, Yu et al., 2018, Yu et al., 2019, Zhu et al., 2019, Jeffery et al., 2020). This is achieved through mass spectrometry or nuclear magnetic resonance techniques, and allows researchers to associate the absence or presence of certain microbial-associated molecules to disease symptomology (Vernocchi et al., 2016), which may provide an avenue for discovery of disease-specific biomarkers or novel targets for therapeutic treatment (Andrews et al., 2020). Given the fact that at least some microbial secretory products are competitive survival tactics, the relative absence of a "competing species" may alter secretome content (and thus effect on host cells) significantly, but linearity of secretome

change to bacterial predominance cannot be assumed, lending further support for favouring analysis of functional dysbiosis.

In the context of microbiome-associated functional effects, recent studies seem to highlight metabolites derived from microbial transformation of dietary components as having significant effects on several physiological processes (Zhang and Davies, 2016, Koh et al., 2016, Koppel et al., 2017, Rowland et al., 2018). One of the first studies to explore microbial metabolism in the IBS context reported that these patients had increased production of hydrogen gas (H_2) (fasted breath test) (Kumar et al., 2010b). The authors suggest that the difference in H_2 production may be associated with small intestinal bacterial overgrowth (SIBO), since SIBO is common among IBS patients (Ghoshal et al., 2010) and is associated with higher levels of H_2 production in fasted states (Kerlin and Wong, 1988). Additionally, altered proportions of specific bacterial species, such as decreased *Lactobacilli* in IBS patients, may alter the amount/distribution of the by-products of microbial metabolism (H_2). Indeed, *Lactobacilli* are less gas producing than some other bacteria, such as *Clostridia* and *Enterobacteriaceae* (Nobaek et al., 2000, O'Mahony et al., 2005). This is confirmed by another study in which colonization by *Clostridium* spp. was associated with excess gas production, abdominal discomfort and bloating among IBS patients (Salonen et al., 2010). As such, the relative dysbiosis of IBS patients may result in higher levels of excreted H_2 as a functional consequence. Indeed, altered microbial fermentation of carbohydrates results in the excessive production of H_2 and methane gases, the elimination of which is essential to maintain efficient fermentation in the gut.

While H_2 only represents a single by-product of microbial metabolism, these findings demonstrate the potential of microbial-derived metabolites to alter host functioning. The most obvious example of this lies in the relatively successful use of probiotics as a treatment option for individuals with IBS. Probiotics are “live strains of strictly selected microorganisms which, when administered in adequate amounts, confer a health benefit on the host” (Hill et al., 2014). Theoretically, probiotics, primarily those containing *Lactobacillus* and *Bifidobacterium* spp., should beneficially modulate the gut microbiota through production of antimicrobial proteins, which should reduce pathogenic bacteria and interfere with epithelial adhesion (Didari et al., 2015, Ford et al., 2014, Mayer et al., 2014, Simren et al., 2013), among several other mechanisms of action (Distrutti et al., 2016). In a meta-analysis including 35 RCTs, probiotics were shown to have beneficial effects with regard to abdominal pain, bloating and flatulence scores in IBS patients (Ford et al., 2014). Additionally, authors described superiority of multispecies probiotics to single species probiotics but found no specific combination of multispecies probiotics predominant to another. As such, clarification with regards to which

combinations of species are effective in treating specific IBS subtypes, and optimum treatment dosages and durations are still required (Lee and Bak, 2011). Thus, not enough is known for compounding of probiotics into treatment formulations, or to accurately prescribe probiotic strategies.

An integrated presentation of IBS-associated pathology is also presented visually in Figure 2.1. Taken together, the female bias towards exacerbation of IBS symptomology is clear, as well as its prominent links to the actions of microbial-derived metabolites, or as we suggest, the trace aminergic system.We believe that trace amines (which are, at least in part, by-products of microbial metabolism) may provide the molecular link to explain the association between gut microbiome dysbiosis, IBS, inflammation and central nervous system conditions such as depression and anxiety (both high incidence co-morbidities in IBS).

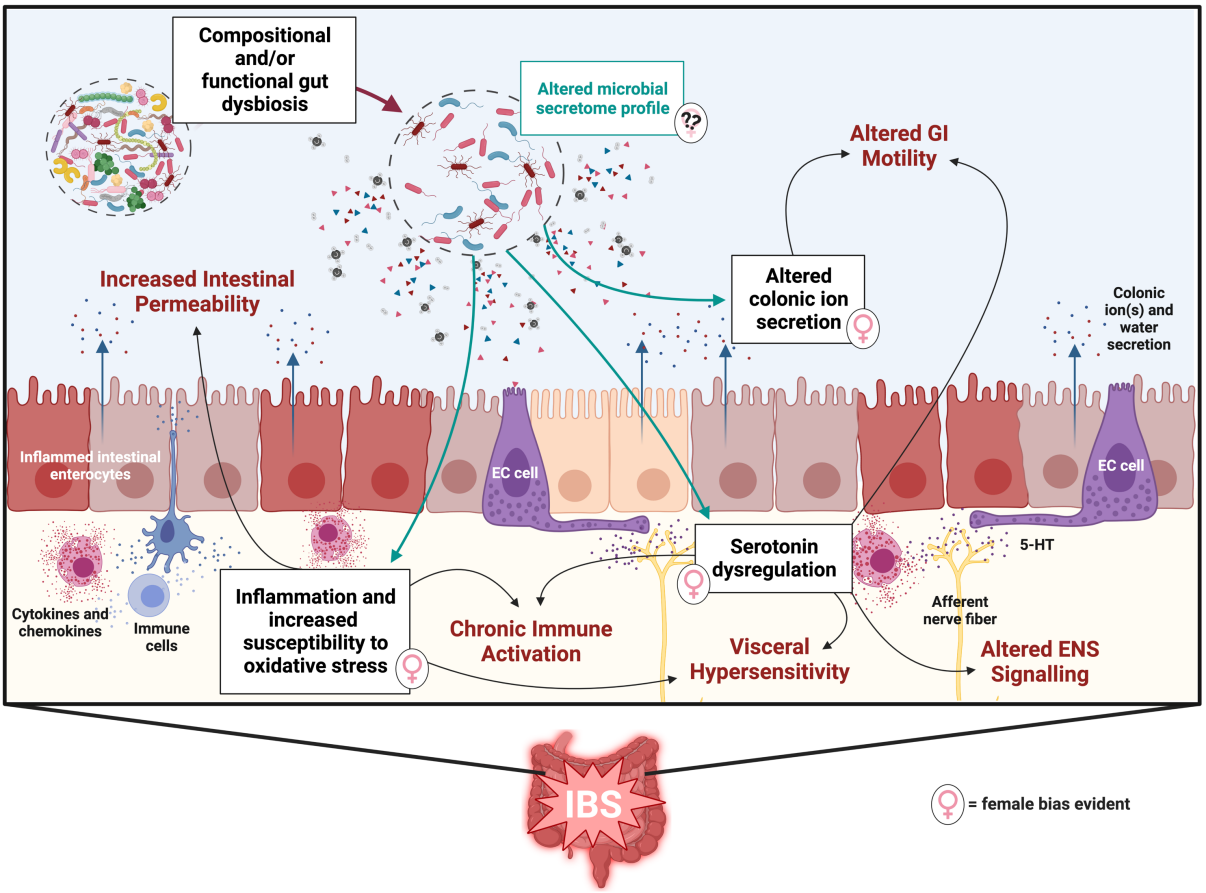


Figure 2.1: A simplified visualisation of IBS-associated pathology. Abbreviations: GI; gastrointestinal, EC; enterochromaffin, 5-HT; serotonin, ENS; enteric nervous system

2.4 The trace aminergic system

Trace amines (TA) are a class of biogenic amines produced endogenously in humans, but also present in bacterial secretomes and certain foods. Common TA include β-phenylethylamine (PEA), tryptamine (TRP) and ρ-tyramine (TYR), which are derived from their respective amino acid precursors L-phenylalanine, L-tryptophan, and L-tyrosine. Synthesis of TA primarily occurs through the enzymatic action of aromatic L-amino acid decarboxylases (AADC) (Berry, 2004). While endogenous synthesis of TA is often reported to be neuronal, AADC are also present in non-neuronal tissues, including the epithelium of the GI tract (Lauweryns and Van Ranst, 1998, Vieira-Coelho and Soares-da-Silva, 1993). Additional sources of TA include those derived from food and microbes.

Trace amine-associated receptor (TAAR) 1 is the most thoroughly studied of the receptors in humans and has both central (acts as a rheostat of dopaminergic, glutamatergic, and serotonergic neurotransmission) and peripheral (regulates nutrient-induced hormone secretion and immune responses) roles (Gainetdinov et al., 2018). In the next few sections, we review different aspects of the trace aminergic system, as they relate to IBS.

2.4.1 TA “toxicity” risk

Interestingly, foods containing high levels of TA, such as cheese, wine, sausages and other fermented foods - all commonly linked to exacerbated symptoms in IBS - are foodstuffs largely dependent on bacterial fermentation (Joosten and Northolt, 1989, Moracanin et al., 2015, Moses-Kolko, 2003). Lactic acid bacteria (LAB) are considered the primary biogenic amine producers in fermented foods. Indeed, various *Lactobacillus* spp. starter cultures have been studied with the aim of mitigating the potential health risks (headaches, heart palpitations, vomiting and diarrhoea) associated with excessive biogenic amines levels (Aymerich et al., 2006). Interestingly, the authors of this study reported that TYR and PEA were produced by 14.4% and 12.4% of LAB isolates (fermented sausages) respectively, all belonging to *L. curvatus* species. As such, the authors recommended that *L. sakie* be used as the predominant LAB in preparation of these sausages in the future. In fact, the inability of a strain to synthesize biogenic amines is included in the selective criterion for malolactic starter cultures (Torriani et al., 2010). It is, however, important to highlight that the biogenic amine producing ability is a strain-specific characteristic, as variability in aminobiogenetic potential between different strains belonging to the same species is evident (Barbieri et al., 2019). Regardless, the link between TA levels and fermentation is clear. Indeed, the expression (transcriptional induction) and/or activation (catalytic modulation) of LAB amino acid

decarboxylation systems is reportedly an adaptive response to energy depletion, and is considered a strategy that counteracts acid stress (Pessione, 2012), since decarboxylase activity can lead to membrane energization and increased environmental pH. Moreover, the strain dependent (rather than species specific) presence of decarboxylases genes involved in biogenic amine production eludes to horizontal gene transfer between strains as an adaptive mechanism of survival in specific environments (such as the GIT) (Coton and Coton, 2009, Lucas et al., 2005, Marcobal et al., 2006a). Therefore, these decarboxylation mechanisms represent an important ecological tool, which can confer a competitive advantage in acid or nutritional stress conditions (Romano et al., 2014, Pereira et al., 2009, Perez et al., 2015, Fernandez de Palencia et al., 2011).

Of the potential health risks related to biogenic amines, the most severe symptoms are said to be caused by histamine and TYR (Barbieri et al., 2019). Interestingly, a study investigating the self-reported food intolerance of 197 IBS patients, reported that 84% of these individuals recounted symptoms related to at least one of the surveyed foodstuffs, of which, 58% experienced GI symptoms from foods rich in biogenic amines (wine, beer, salami and cheese) (Bohn et al., 2013). Additionally, histamine-containing food were also considered as causes of IBS-related symptoms. The resultant symptoms are reportedly induced *via* chemo-stimulation of gut or immune cell receptors (Gibson et al., 2015). Of note, the authors also reported that females reported more food items causing symptoms than males (Bohn et al., 2013), although no potential explanation was provided. This study, along with several others, emphasises the high perceived food intolerance among IBS patients (Dainese et al., 1999, Simren et al., 2001, Monsbakken et al., 2006). Histamine (a weak TAAR1 agonist) is directly involved in inflammation, while TYR intoxication facilitates the “cheese reaction”. This reaction, most commonly described in the context of cheese consumption, is the result of a food-drug interaction, where the food can be any TYR rich food and the drug usually a non-selective monoamine oxidase inhibitor (MAOI). Mechanistically, TYR increases sympathetic responses by indirectly acting as a sympathomimetic (displaces stored norepinephrine (NE)), thereby increasing the levels of circulating NE. The use of MAOI exacerbates this action by inhibiting the metabolism of both TYR and NE. As such, symptoms of the “cheese reaction” include dietary-induced migraine, nausea, vomiting, increased cardiac output, respiratory difficulties and elevated blood glucose levels (Marcobal et al., 2012). This has had important implications in patients using MAOI, in which not even low levels of biogenic amines can be metabolised effectively (Ruiz-Capillas, 2004). MOAI have been prescribed to patients with chronic anxiety to improve 5-HT signaling, and intriguingly, 54 to 94% of treatment-seeking IBS patients will have a co-morbid psychiatric disorder (Drossman et al., 2003, Roy-Byrne et al., 2008) of which, anxiety and depression are the most common. The resultant biogenic amine sensitivity

that these patients experienced led to the development of new generation MAOI, so-called reversible MAO-A inhibitors (McCabe-Sellers et al., 2006).

As mentioned, histamine is indeed a weak TAAR1 agonist (Zucchi et al., 2006), and various reports suggest between 5 to >50 μM are required for the activation of TAAR1 (similar potency as TRP, NE and synephrine) (Borowsky et al., 2001, Wolinsky et al., 2007, Lindemann et al., 2005). Even though patients with IBS have reportedly elevated levels of histamine in mucosal supernatants/biopsies, of up to 50 ng/mL*mg (Grabauskas et al., 2020, Buhner et al., 2009), it is unlikely that these endogenous levels result in TAAR1 activation. However, exogenous or dietary histamine consumption could contribute significantly to the levels of histamine in the gut, contributing to potentially detrimental effects – although most likely independent of TAAR1 activation. For example, certain fish and varieties of cheeses contain up to 2000 mg/kg of histamine, and the ingestion of 75 mg of histamine is reported to cause symptoms of intoxication in healthy individuals (Wöhrl et al., 2004, Rauscher-Gabernig et al., 2009). A recent study by del Rio *et al*., (2017) also reported that co-treatment (on HT-29's) with TYR and histamine was associated with stronger (or synergistic) cytotoxic effects *in vitro* than treatment with either TYR or histamine alone, an effect achieved in the absence of TAAR1. These results indicate that histamine increases the cytotoxicity of TYR at concentrations prevalent in some foods (levels generally considered safe for consumption) (Del Rio et al., 2017).

While the symptoms of the "cheese reaction" and histamine intoxication are not specific to IBS, other biogenic amines may similarly trigger IBS-specific symptoms by promoting visceral hypersensitivity *via* the action of bioactive mediators and/or luminal distention (Cuomo et al., 2014, Balemans et al., 2017, Wouters et al., 2016). As such, other biogenic amines (PEA, putrescine, cadaverine, agmatine and spermidine) can also cause toxicity, but in cases where multiple biogenic amines are present, they are said to potentiate the effects of histamine and TYR by inhibiting their metabolizing enzymes (Pegg, 2013). Taken together, the toxicity of any biogenic amine will depend on the type of amine, the amount of amine, the individual host sensitivity or allergy, and the consumption of MOA inhibitory drugs (or ethanol), which inhibits or reduces the aminooxidase enzymatic systems responsible for the detoxification of exogenous amines (Sathyanarayana Rao and Yeragani, 2009).

While these health risks are well-researched with regard to food safety and regulation (Ruiz-Capillas and Herrero, 2019), it is concerning that many LAB are commonly used as probiotics. Indeed, another study has raised concern that some *Lactobacillus rhamnosus* strains often used in probiotics may produce biogenic amines (Lorencová et al., 2012). Moreover, not only

LAB predominant probiotics should be considered, but probiotics with *Enterococcus*, *Streptococcus*, and *Lactococcus* species may also potentially produce biogenic amines (Barbieri et al., 2019). In fact, decarboxylase activity is often expressed independently of cell viability, since these enzymes maintain activity after cell lysis, even in harsh environmental conditions (Gardini et al., 2012, La Gioia et al., 2011, Rossi et al., 2011, Moreno-Arribas and Lonvaud-Funel, 1999). As such, it may be premature to advocate probiotic treatment as a blanket supplementation strategy for therapeutic relief of IBS patients, and at least some individualisation is required to increase efficacy and mitigate risks of adverse outcomes. Beyond the importance of investigating the decarboxylation activity of probiotic or functional cultures before their use, in the context of gastrointestinal disease and symptomology, it may also be important to elucidate negative effects (if any), that chronic exposure to low levels of these biogenic amines could cause.

2.4.2 Microbial-derived TA modulate host functioning

It is proposed that through the production of bioactive metabolites, such as biogenic amines, the gut microbiota may increase an individual's susceptibility to GI inflammation *via* modification of intestinal epithelial function and mucosal immune activity (Christian and Berry, 2018, Wlodarska et al., 2015). For example, several intestinal microbes synthesize various amino acid decarboxylases, which means that they have the capability to sequester amino acids, convert them into TA, and thereby alter the distribution of metabolites, such as calcium, 5-HT, trimethylamine *N*-oxide (TMAO) and immune cell mediators in the host, as part of the symbiotic relationship between the gut microbiome and host. However, changes in TA metabolism have already been correlated to both inflammation of the bowel (Wilson et al., 2015) and decreased microbiome complexity (dysbiosis) (Santoru et al., 2017), which suggests that in pathological states, this altered TA metabolism may have functional consequences, that may manifest as or promote disease symptomology. Given the strong links of IBS with depression, and that microbial-derived biogenic amines are similar in structure to monoamine neurotransmitters, TA/TAAR1 should be considered as potential biomarkers and/or therapeutic targets. To motivate this point, this section will discuss (i) the significant presence of TA-producing microbes in the gut, (ii) the optimal conditions of a dysbiotic gut for the generation of microbial-derived TA and, finally (iii) an example of functional modulation by trimethylamine (TMA) and TMAO.

Firstly, in intestinal metagenomes of healthy individuals, tryptophan decarboxylase homologs were found to be present in 9% to 17% of individuals, suggesting that microbe derived TRP may be more prevalent in the gut than previously thought (Williams et al., 2014). Indeed, the gut microbiome of IBS patients is often dominated by Firmicutes (Jeffery et al., 2012), the

phylum from which the majority of the tryptophan decarboxylases derive. In fact, the gut microbiota features a myriad of metabolizing enzymes, such as various decarboxylases, dehalogenases, and amine oxidases, which may facilitate the formation of other TAs (octopamine and synephrine), as well as functionally active TA-metabolites. While no studies have investigated the percentage of TA producing bacteria in IBS populations compared to healthy individuals, these results suggest that the TA-production capacity of the gut microbiome is significant and has been largely overlooked or underestimated.

Secondly, according to two independent *in vitro* studies on known microbial producers of TA (*Lactobacillus brevis* CECT 4669, *Enterococcus faecium* BIFI-58 and *E. faecium* EF37), various physiochemical factors influence microbial synthesis of TA (Gardini et al., 2001, Marcobal et al., 2006b). These factors include incubation temperature and time, environmental pH, pyridoxal-5-phosphate supplementation, sodium chloride concentration and most importantly, amino acid substrate availability, most of which are optimal within the human GIT. Of note, some of these factors may be altered in pathological states, towards promoting microbial TA production. For example, luminal pH is reportedly altered in individuals with dysbiosis, and this may contribute to mucosal inflammation and enterocyte dysfunction (Blachier et al., 2017). With regard to IBS, Ringel-Kulka *et al*., (2015) reported that colonic intraluminal pH levels were significantly lower in IBS patients (all disease subtypes) when compared to controls. Similar findings have also been reported in patients with IBD (Nugent et al., 2001), with one study reporting low colonic luminal pH values (pH 5.3 patients *vs* pH 6.8 controls), which were associated with active disease states (Sasaki et al., 1997). Interestingly, a pH range of 4 to 5.5 is reported to increase amino acid decarboxylase activities and thus enhance TA production (Gardini et al., 2001, Marcobal et al., 2006b). This microbial response is a well-documented adaption to pH/acid stress (as already briefly discussed in section 3.1), and suggests that more efficient TA production may occur in individuals with dysbiosis.

Finally, TMA is a selective agonist of TAAR5 (Liberles and Buck, 2006, Wallrabenstein et al., 2013, Zhang et al., 2013), and there is no known mammalian pathway for its synthesis. As such, the production of TMA seems to be exclusive to the metabolism of choline, betaine, and carnitine by microbes (Craciun and Balskus, 2012, Kalnins et al., 2015, Zhu et al., 2014), with the administration of antibiotics to mice, reducing the levels of urinary TMA (Yap et al., 2008, Frohlich et al., 2016). Interestingly, raised levels of TMA have been reported to result from dysbiosis at various mucosal sites, such as intestines, in both mice and human models (Fennema et al., 2016, Zhang and Davies, 2016). While increased levels of TMAO are generally associated with extra-intestinal diseases (CVD), TMAO may cause intestinal

inflammation and oxidative stress (Chan et al., 2019). Considering that TAAR5 expression has been reported for several leukocyte populations (Babusyte et al., 2013), particularly B lymphocytes, it is clear that increased levels of either TMA or TMAO could be involved in initiating or perpetuating intestinal inflammation, as is common in IBS. Moreover, altered levels of TMA/TMAO can reportedly alter the growth and secretion of metabolites of several intestinal bacteria (Hoyles et al., 2018). As such, TMA/TMAO not only affects host functioning, but can alter the luminal environment too, perpetuating dysbiosis.

2.4.3 Mechanism of action of TA in the gut

Perhaps due to a relative lack of cross-disciplinary communication in this context, despite the knowledge of their existence, or perhaps as a result of the bias in favour of compositional, rather than functional assessment of the gut microbiome, data on the specific actions of TA in the human gut are still relatively limited. However, some insights into the function of TA in the context of IBS may be derived by considering their extra-intestinal effects.

2.4.3.1 Direct cellular effects of TA

Direct cytotoxic effects of PEA, TRP and TYR on MonoMac-6 and HEK293 cell lines was investigated (MTT assay) (Luqman et al., 2018). Data showed that 62.5 μg/mL of each TA independently reduced MonoMac-6 viability by 20%, and 125 μg/mL of TRP reduced MonoMac-6 viability by 80%. While the HEK293 cells were more resistant to the cytotoxic effects of the TA, 500 μg/mL of TRP also reduced viability by more than 80%. From this study, the most cytotoxic TA seems to be TRP, while TYR had the least cytotoxic effects. However, the lack of an *in vivo* context limits the interpretations which can be made by these data. Moreover, the lack of reported absolute concentrations of endogenous TAs in the gut/intestinal mucosa of humans questions the physiological relevance of these findings. To “bridge” this gap in literature, we propose the consideration of, firstly, the contribution of exogenous TA consumption (in food) to levels in the gut. For example, it has been previously reported that dietary concentrations of PEA and TYR indeed stimulate the gut, altering intestinal blood flow in an *ex vivo* model (Broadley et al., 2009). Secondly, we considered that the contribution of TAs derived from major TA-producing microbes *in vitro*, suggests that the TA range selected for the generation of the WST-1 data is feasible. For example, *Staphylococcus pseudintermedius* ED99 cultured in media containing 2 mg/mL of L-tryptophan, L-phenylalanine and L-tyrosine produces 231 ± 10 μg/mL TRP, 557 ± 8 μg/mL of PEA and 360 ± 9 μg/mL TYR *in vitro* (Luqman et al., 2018). These data suggest that in the gut, in the presence of potentially numerous TA-producing microbes, significant concentrations of TAs may be present. Thus, while the endogenous levels of TAs are not known, the exogenous

(and potentially endogenous) contribution may be significant enough to warrant physiological relevance.

Moreover, the presence or production of TA is reported to enhance the ability of *Staphylococcus* and *Enterococcus* spp. to adhere to intestinal epithelium, promoting consequential internalization and enterocyte cytokine secretion (Fernandez de Palencia et al., 2011, Luqman et al., 2018), potentiating colonization as a potential adaptive advantage for these species. Indeed, TA bound to the α2-adrenergic receptor induced cytoskeletal reorganisation, which facilitated host cell colonization to boost adherence of both TA-producing and non-TA-producing bacteria (Luqman et al., 2018). Interestingly, the addition of 10mM tyrosine (which resulted in formation of ± 140nmol TYR) significantly improved bacterial adherence to colon epithelium by 3-fold, while direct supplementation with 140μM of TYR did not affect adherence (Fernandez de Palencia et al., 2011). The authors speculated that the activation of the TYR biosynthetic pathway, rather than the production of TYR, could be involved in the enhancement of microbial adhesion. Nevertheless, this data illustrates the complex mechanisms at play to facilitate TA effects. Taken together, these results suggest that dose specificity is an important consideration. What is not known, is the range in which microbes potentially benefit from TA (producing or produced), while conferring host cytotoxicity, and what implications this could have for IBS.

In terms of enteroendocrine function, PEA was reported to stimulate gastrin secretion from stomach G cells in a rat model (Dial et al., 1991) – which in the IBS context is linked to ulceration and dyspepsia (El-Salhy et al., 2014). This again points to a direct detrimental effect of TA in the IBS context.

With regard to colonic ion secretion, TRP in particular was reported to promote colonic ion secretion (Williams et al., 2014), however the nature of this ion secretion and potential preference to a specific ion(s) were not reported and thus, require further investigation before interpretation of the significance of this finding is possible. Nevertheless, this data potentially suggests that TRP-mediated signalling might affect GI transit. Furthermore, from the known effects already outlined for E2, we can postulate that in general E2 and TAs, such as TRP, have opposing effects on colonic ion secretion and GI motility. This could explain an exacerbation of symptomology during menses, when ion secretion and thus GI motility shifts from one side of the spectrum to the other in female patients, an effect that would be heightened in the presence of a high TA (or at least TRP) load. The potential of manipulating TRP levels to achieve optimal GI transit in IBS, warrants TA profiling in IBS.

2.4.3.2 Modulation of oxidative stress and inflammation

Intracellular accumulation of Ca^{2+} is commonly associated with oxidative stress, damage, and inflammation in various chronic conditions. Of relevance in this context, binding of TA to human TAAR1 results in the influx of Ca^{2+} as a result of activated TAAR1 coupling to $G\alpha_s$ and G_q proteins (Navarro et al., 2006). Upon stimulation of these G-proteins, intracellular messengers such as cAMP and IP_3 accumulate and activate downstream proteins such as PKB and PKC, which mobilize intracellular Ca^{2+} stores, as well as promote extracellular Ca^{2+} influx (Borowsky et al., 2001, Bunzow et al., 2001, Lindemann et al., 2005, Bradaia et al., 2009). Excessive Ca^{2+} influx may lead to endoplasmic reticulum stress and mitochondrial dysfunction, rendering a cell with an unfavourable redox profile, and thus several regulatory mechanisms intricately control intracellular Ca^{2+} levels. However, in overabundance of TA, regulatory mechanisms may be overwhelmed. In line with this, histamine – a known mediator of inflammation and known to be increased in IBS – has also been reported to increase the intracellular Ca^{2+} response in an EC cell line (P-STS) (Pfanzagl et al., 2019). Apart from the resultant direct oxidative stress, histamine and TA may also exacerbate aberrant 5-HT signalling linked to IBS symptomology (altered GI motility, visceral hypersensitivity, and immune activation). Interestingly, TA may also indirectly cause increased susceptibility to oxidative stress through their modulation of serotonin, as $5\text{-}HT_{2A}$ receptor activity was found to increase intracellular calcium *via* the mitogen-activated protein kinase pathway (Watts, 1998). This may cause an intracellular Ca^{2+} burden within the surrounding intestinal tissue, resulting in symptoms such as abdominal pain. As such, it may be important to consider targeting TA availability or modulation of TAAR1 expression in an effort to curb Ca^{2+}-associated visceral hypersensitivity in IBS patients with severe abdominal pain.

Another common trace amine, 3-iodothyronamine (T_1AM), may also contribute to changes in Ca^{2+} homeostasis and alter the pro- and antioxidant balance in the intestine and surrounding tissue by interacting with various receptors (such as α2-adrenergic receptor) (Zucchi et al., 2014, Hoefig et al., 2016). For example, T_1AM reportedly increased the amount of hydrogen peroxide released by rat liver mitochondria (Venditti et al., 2011). Interestingly, Chiellini *et al*., (2012) reported that exogenous T_1AM (and its metabolites) primarily undergo biliary and urinary excretion, and subsequent reports have suggested the presence of significant endogenous levels of T_1AM in stomach and intestine, at least in mice (Hoefig et al., 2015, Chiellini et al., 2012), suggesting that the pro-oxidative effects of T_1AM may not be limited to the liver. While the precise biosynthesis of T_1AM in humans remains to be confirmed, Hoefig *et al*., (2015) has demonstrated that the intestine expresses the enzymatic machinery (intestinal deiodinases and ornithine decarboxylase) required for T_1AM biosynthesis from thyroxine, while other authors highlight the potential of the gut microbiota to generate T_1AM

(Saba et al., 2010, Glossmann and Lutz, 2017). In terms of relevance to IBS, interactions of T_1AM with histaminergic circuitries has been proposed (Zucchi et al., 2014), warranting investigation in the context of both inflammation and oxidative stress related symptomology.

In terms of inflammation a relatively recent metabolomics study indicated elevated faecal PEA levels in patients with Crohn disease (Jacobs et al., 2016), suggesting a pro-inflammatory effect for trace aminergic signalling. Similarly, abundance of *Faecalibacterium prausnitzii* - a species that has a reported role in mitigating inflammation in the colon (Miquel et al., 2015, Lopez-Siles et al., 2017) - also correlated inversely with PEA levels in IBD patients (Santoru et al., 2017). Since significant consumption of phenylalanine is associated with growth of *F. prausnitzii* (Heinken et al., 2014), its absence in dysbiotic conditions may increase the availability of phenylalanine to TA-producing microbes, resulting in elevated PEA levels. This potential mechanism should be evaluated more comprehensively in IBS-specific models.

Furthermore, PEA and TYR are chemotactic for polymorphonuclear (PMN) cells (Babusyte et al., 2013), major role players in inflammation and in particular, secondary damage to host tissue during inflammation. Considering that low levels of TA are normally present in the GIT – the interface between mucosal immunity and microbes – it is possible that in dysbiosis, the ensuing immune activation and altered microbial behaviour may promote or exacerbate intestinal inflammation. Within the context of IBS, and specifically female predominance, it is necessary for future experiments to elucidate the contribution of TA to chronic intestinal inflammation, and whether or not female reproductive hormones affect this in any way.

2.4.4 The role of TAARs

Most TAAR-related research to date was performed in microbiology contexts. Although some neurophysiology investigations have reported on TAAR, this niche is largely unexplored. Generally, the available literature seem to suggest that TA and TAAR are not exclusively “paired”, with TA able to act as ligand for several other receptors. Furthermore, TAARs – in particular TAAR1 – seems to be a dose-dependent factor in TA-associated effects, as its presence have been linked to opposite effects than described for TA.

For example, in contrast to the over secretion of gastrin linked to TA, TAAR1 activation by a selective small molecule agonist was associated with elevated plasma levels of peptide tyrosine tyrosine (PYY) and glucagon-like peptide-1 (GLP-1) (Raab et al., 2016), which may be protective, as decreased levels of secreted PYY and GLP-1 from L-cells are implicated in IBS pathogenesis and symptomology (El-Salhy et al., 2020, O'Malley, 2019). Similarly, in terms of modulation of leukocyte responses specifically, TAAR1 is differentially expressed in

several leukocyte populations, such as PMN, B and T lymphocytes, monocytes, and natural killer cells (Babusyte et al., 2013, Nelson et al., 2007, Sriram et al., 2016, D'Andrea et al., 2003), thus TAAR1 activation may regulate leukocyte differentiation and activation. Indeed, expression of both TAAR1 mRNA and protein components are upregulated in primary human lymphocytes after activation with PMA and PHA (Wasik et al., 2012, Nelson et al., 2007). However, one would expect in the context of IBS, that chronic (rather than acute) activation may downregulate TAAR expression overtime. As such, the importance of conducting *in/ex vivo* testing should be emphasized, and certainly warrants future investigations. Furthermore, T helper lymphocyte differentiation toward Th2 phenotype may be regulated by the activation of leukocyte TAAR1 and TAAR2 (Babusyte et al., 2013). However, in the same study, TAAR1 and TAAR2 activation were also reported to mediate IL-4 secretion from T lymphocytes and immunoglobulin E (IgE) secretion from B lymphocytes (Babusyte et al., 2013). Of relevance to the allergy-like symptoms prevalent in IBS, both IL-4 and IgE mediate allergic inflammatory responses (Tan et al., 1992, Paul and Zhu, 2010), inducing mast cells to release histamine upon IgE binding, and are implicated as central role players in IBS (Barbara et al., 2006, Lee and Lee, 2016). This seemingly dichotomous role for TAAR in the activation of immune cells remain to be further elucidated.

In terms of its effect on serotonergic signalling, highest predominance of TAAR1 is described in neuronal aminergic pathways (Berry, 2016, Revel et al., 2011). Of specific relevance, in a rodent knockout model, the absence of TAAR1 was associated with increased aminergic (dopaminergic and serotonergic) signalling (Pei et al., 2016), again suggesting a dampening effect for TAAR1. In terms of applicability to this review, IBS-associated 5-HT dysregulation – which is implicated in altered GI motility (Lundgren, 1998, Turvill et al., 2000) and visceral hypersensitivity (Mayer, 2011) – is already therapeutically targeted by 5-HT_3R antagonists (Gershon and Tack, 2007, Mawe et al., 2006). Assuming that a higher TA load results in reduced TAAR1 expression (specifically in EC cells) in chronic conditions, a more accurately targeted approach might include modulation of trace aminergic signalling, thereby eliminating the cause of 5-HT dysregulation.

It is important to note, however, that TAAR signalling is additionally complicated by three factors. Firstly, genetic variations in the form of TAAR polymorphisms, as well as their clinical relevance, cannot be excluded (Christian and Berry, 2018), since function-altering polymorphisms of TAAR1 (Shi et al., 2016) and TAAR2 (Bly, 2005) have already been reported. Secondly, TAAR expression is often recorded in acute, *in vitro* models. According to basic ligand/receptor relations, receptor expression is generally downregulated when ligands are overexpressed chronically, however, this notion is complicated by the very location and

nature of TAARs. It is generally accepted that TAAR1 is primarily located intracellularly (Pei et al., 2016). How this unique behaviour translates to altered receptor expression is still unknown. Lastly, TAAR reportedly undergoes heterodimerization, which may result in biased signalling outputs (Berry et al., 2017). Indeed, it has been reported that this heterodimerization modulates the signalling capacities of GPCRs, thereby altering their sensitivity for ligands (Rutigliano et al., 2017, Dinter et al., 2015). Putative candidates include adrenergic, serotoninergic, dopaminergic and glutaminergic receptors, and as such, signal modulation by receptor pairs is largely underestimated, especially considering the vast expression of GPCRs in any one cell (Regard et al., 2008). Interestingly, GPCR distribution and expression is dependent on several factors, including gender and disease condition {Afzal, 2020 #190;Bychkov, 2011 #188;Leysen, 2021 #187;Miller, 2017 #189}. To add to the complexity, intermediate receptor pairings have also been suggested (Smith and Milligan, 2010), further complicating pharmacological and drug discovery studies. As such, Berry *et al*., (2016) suggested targeting GPCR dimers, which should mitigate undesired side effects and increase ligand selectivity.

These data suggest that TAAR1 may have a modulatory (down-regulatory) role in trace aminergic signalling, but that this effect may be dependent on nature of receptors with which heterodimers are formed upon ligand binding. Given the clear role for TA in IBS aetiology and symptomology, elucidation of the potential of TAAR as therapeutic target is high priority.

The importance of the trace aminergic system as a multipronged role player, which effects many sites, is visually represented in Figure 2.2. This includes a summary of the known functions of TA in the GIT, and the plausible links to IBS symptomology.

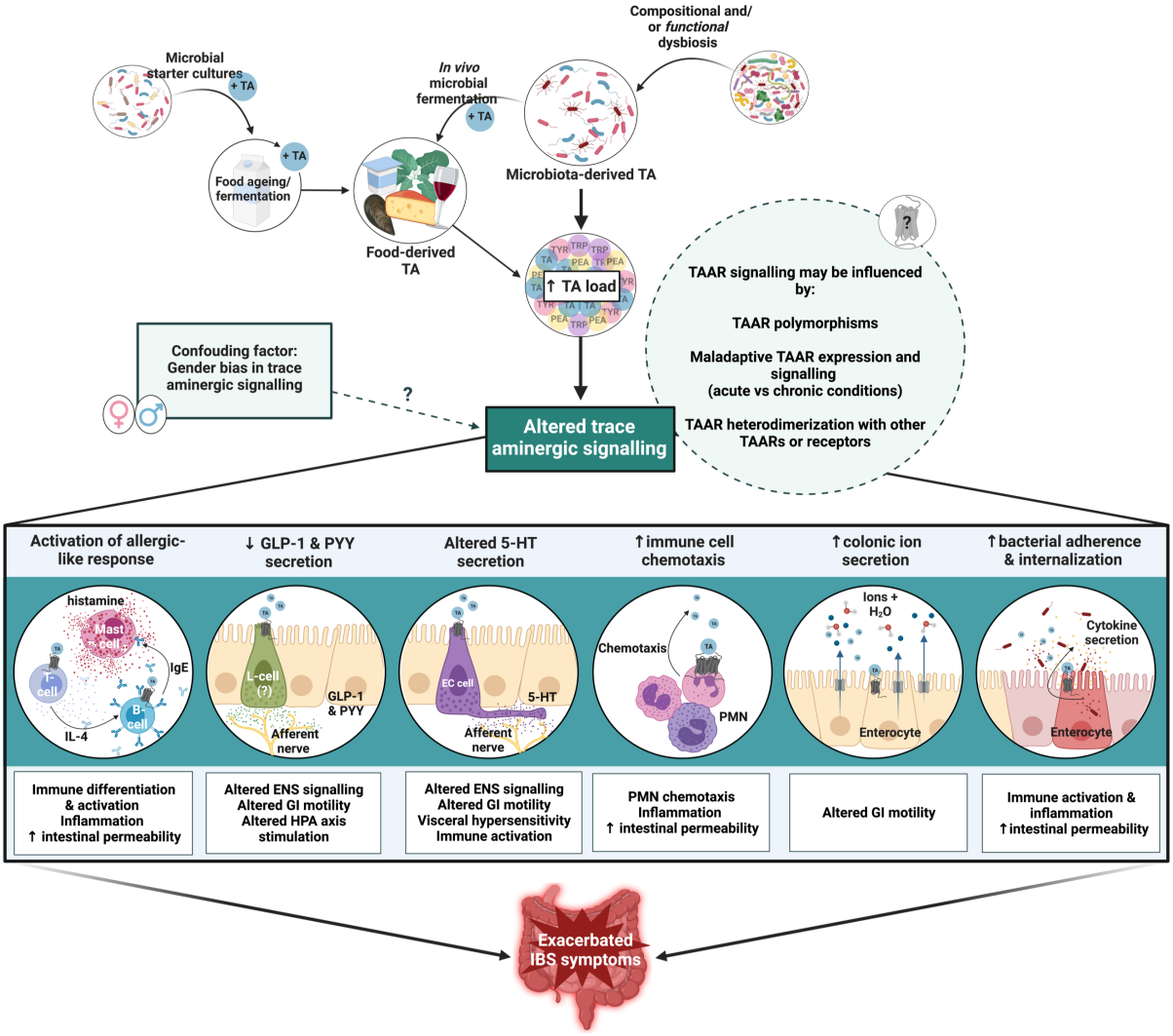


Figure 2.2: Altered trace aminergic homeostasis as a potential aetiological factor in IBS pathogenesis. The predominant risk factor promoting altered trace aminergic signalling in the gastrointestinal tract is functional microbial dysbiosis, which varies trace amine load. The altered signalling is gender dependent, and results in functional consequences, which manifest as clinical IBS symptoms. Abbreviations: TA; trace amine, TAAR; trace amine associated receptor, IgE; immunoglobulin E, IL-4; interleukin-4, GLP-1; glucagon-like peptide-1, PYY; peptide tyrosine tyrosine, HPA; hypothalamic pituitary axis, 5-HT; serotonin; ENS; enteric nervous system, GI; gastrointestinal; PMN; polymorphonuclear cells.

2.4.5 Is trace aminergic signalling gender dependent?

Turning attention back now to the female predominance in IBS, it is important to consider whether trace aminergic signalling also shows gender-dependence, as this may impact on not only drug discovery, but also patient management. Indeed, with the emergence of the concept “microgenderome”, researchers have shown that the microbiome is both shaped by reproductive hormones and that the microbes in turn are able to regulate levels of these hormones (Flak et al., 2013). An example of this is prevalent when one considers the more specific “estrobolome” (Kwa et al., 2016, Plottel and Blaser, 2011), which collectively encompasses intestinal microbes (or rather their gene repertoire) capable of producing

estrogen metabolizing enzymes (such as β-glucuronidase). In healthy individuals the actions of the estrobolome increases intestinal reabsorption of estrogens, while in dysbiotic conditions this is reduced (Baker et al., 2017). Similarly, the microgenderome can also modulate 5-HT signaling (as discussed earlier) and interestingly again do so via estrogens. As such, the effect that altered trace aminergic signalling could have on 5-HT release and action, could be predetermined by lifelong exposure to and priming by E2.

Furthermore, TAAR signalling is differentially activated by distinct TA profiles in different genders. Indeed, in a study by Liberles and Buck, (2006) murine TAAR5 was reported to respond strongly to extremely diluted urine from male mice, but not female mice or prepubescent males. Notably, three ligands identified for murine TAARs (mTAARs) are natural components of mouse urine: PEA, isoamylamine and TMA, which act as ligands for mTAAR4, mTAAR3 and mTAAR5 respectively, of which isoamylamine and TMA are enriched in male *vs* female mouse urine (Gavaghan McKee et al., 2006, Nishimura et al., 1989). Furthermore, isoamylamine in male urine is reported to act as a pheromone, fast-tracking the onset of puberty in female mice (Nishimura et al., 1989). Thus, by utilizing mTAAR5, mice could theoretically determine the gender and sexual status of other mice, which suggest that at least some mTAARs detect social cues (Halpern, 2003, Novotny, 2003) that may stimulate certain behaviours or physiological responses. While this study has not been replicated in humans, we are of the opinion that altered trace aminergic signalling (as reported in modern chronic diseases) may be the result of altered gender dynamics. In modern society, with the rise of female emancipation and modern hygiene practices, both females and males may have inadvertently altered their 'social cues'. This of course remains to be confirmed, as this may have far-reaching implications for disease preventative strategies in IBS-high risk populations. Nevertheless, the little available data suggest that amount and distribution of at least some TA within a host are gender (or even sexual status) specific, which may have implications for diseases in which onset parallels the onset of puberty, such as IBS.

Another link between gender and altered TA homeostasis is chronic psychological stress. According to several studies, elevated PEA levels in urine correlated with increased stress and stress response, in both humans and rodents (Paulos and Tessel, 1982, Snoody et al., 1985, Grimsby et al., 1997, Dlugos et al., 2012). Typically, it was thought that women are more vulnerable to life stress (Seeman, 1997, Young and Korszun, 2010), and are more prone to depression, anxiety and somatization than men (Altemus, 2006, Blanchard et al., 2001, Corney and Stanton, 1990). Women also seem to present with exaggerated IBS symptoms when stressed (Heitkemper and Jarrett, 1992). In addition, gender-related differences in the prevalence of depression becomes apparent after menarche and continued until peri-

menopause (Halbreich and Kahn, 2001), which parallels IBS symptom peak onset. These studies, along with several more recent studies, report evidence for sexual dimorphism in stress response in the context of IBS (Videlock et al., 2016, Kennedy et al., 2014b, Kano et al., 2017). However, the link between chronic stress and increased urinary PEA levels is less clear, although limited research to date does again point to an estrogen link in this context. Interestingly, in a study investigating the relationship of urinary PEA levels and personality traits (MMPI) in healthy individuals, reported that males had lower PEA levels (Moises et al., 1985), a finding that had previously also been reported (Philips, 1987). Although concrete mechanisms and IBS specific data is lacking, current data supports an interpretation in favour of a gender dependence in trace aminergic signalling. As such, fluctuations of especially female reproductive hormones may alter trace aminergic signalling. Given the comprehensive body of literature that already exist on female reproductive hormone replacement therapy, it may be possible to expand on the manipulation of hormone levels for therapeutic effect in IBS. This option warrants further research in this context.

2.5 Where to from here?

From the literature reviewed here, both female reproductive hormones (especially E2) and TA potentially modulate EC cell functioning. While no studies have directly explored the role of an altered trace aminergic system in patients with IBS, it is alluded to. The fact that TAARs are present in almost all leukocyte populations, and the supply of significant amounts of their ligands (TA) originate from food and gut microbiota, suggests that the trace aminergic system is conveniently positioned at the interface of diet/nutrition, gut microbiome, and mucosal immunity, all of which are implicated as aetiological factors in IBS pathogenesis. In terms of proposed mechanisms, dyshomeostasis of the trace aminergic system may result in altered colonic ion secretion, hyperreactivity of the immune system and fluctuations of 5-HT levels causing aberrant 5-HT signalling. While disruption of trace aminergic homeostasis may occur due to TAAR polymorphisms or increased TA supply due to changes in diet, functional microbial dysbiosis seems to be the predominant risk factor. Aberrant trace aminergic functioning can result in altered leukocyte differentiation, activation, and chemotaxis, all while microbes more efficiently adhere to and infiltrate intestinal epithelium. The ensuing pro-inflammatory state of the gut could manifest in the symptomology clinically prevalent in IBS. As such, we suggest that microbial-derived TA (and the functional consequences perpetuated by the trace aminergic system) should be considered aetiological factors in the pathogenesis of IBS. Furthermore, since an altered trace aminergic system results in fluctuations of intestinal 5-HT, which is already targeted for modulation by current medications for IBS, then

it is feasible to suggest that TAARs be considered targets for future therapeutic action, with the specific focus of reducing oxidative stress and inflammation.

2.6 Conclusions

In conclusion, the microgenderome concept may explain, at least in part, the gender bias observed in many chronic inflammatory conditions. The notion of host-intrinsic factors, which are reinforced and manipulated by commensal bacteria, could underpin the relationship between an altered trace aminergic homeostasis and female predominance in IBS. In order to elucidate the nature of relationship between the trace aminergic system and reproductive hormones, specifically E2, and their influence on IBS, areas of overlap, such as modulation of serotonin and ion secretion and susceptibility to oxidative stress and inflammation requires further investigation.

2.7 Hypothesis statement

Given the female predominance of IBS, and that the trace aminergic system links several IBS risk factors (sex, diet, inflammation etc.), I firstly hypothesise that E2 may alter the functional capacity of (commensal and probiotic) gut microbes, in the context of TA secretion. Secondly, I hypothesise that there is an E2-associated interaction that determines whether increased abundance of TA will result in a negative outcome or not, in the context of intestinal inflammation. Thirdly, given the very low endogenous levels of TAs, I further hypothesize that TA metabolites may have a significant effect on TA risk profile.

Chapter 3

Alterations to microbial secretomes by estrogen may contribute to sex bias in irritable bowel syndrome

This chapter has been published in the Journal of Inflammopharmacology (Impact Factor: 5.093).

Citation reference: Pretorius, L., Van Staden, AD., Van der Merwe, JJ., Henning, N., Smith, C. (2022) Alterations to microbial secretomes by estrogen may contribute to sex bias in irritable bowel syndrome. *Inflammopharmacology*. 30:267-281. https://doi.org/10.1007/s10787-021-00906-8

3.1 Abstract

Introduction:
Irritable bowel syndrome (IBS) is a female predominant functional gastrointestinal disorder, underpinned by microbial dysbiosis and microinflammation. We suggest that changes in trace amine (TA) load and metabolism may link together diet, inflammation and sex in this context.
Methods:
The effect of E2 treatment on microbial growth and TA generation was assessed using liquid chromatography and tandem mass spectrometry methodology. To investigate the effects of TAs on the gut, WST-1, prostaglandin E2 and tight junction protein dynamics were investigated in TA treated (HT-29) colon epithelial monolayer cultures.
Results:
Differential E2-dependent alterations of the TA production capabilities of microbes were observed. Significantly, E2 treatment resulted in a 50% increase in tryptamine secretion from a probiotic microbe ($p<0.0001$). Moreover, *in vitro* experiments indicated that TA treatment exerted type-specific effects in the gut, e.g. reducing mitochondrial functionality, even at low doses of tryptamine ($p<0.0001$) and ρ-tyramine ($p<0.001$). Additionally, prostaglandin E2 levels were significantly increased following ρ-tyramine and agmatine treatment ($p<0.05$). In terms of functionality, all investigated TAs resulted in occludin redistribution and loss of zona occludens-1 and occludin co-localization.
Conclusion:
Increases in the gastrointestinal TA load may contribute to a relatively pro-inflammatory outcome in the intestine, along with tight junction protein disruption. Additionally, fluctuating levels of endogenous E2 may modulate microbially-derived TA levels, potentially explaining exaggerated gastrointestinal symptomology in females during low E2 phases. Thus, current data warrants subsequent investigations in appropriate *in vivo* models to fully elucidate the role of the trace aminergic system in the sex bias observed in IBS.

3.2 Introduction

The use of clinical symptoms to diagnose functional gastrointestinal disorders (FGIDs) - due to the lack of sensitive or specific biomarkers - is limiting advancement in terms of drug discovery for treatment of affected individuals. Irritable bowel syndrome (IBS) is the most common FGID and is prevalent in more than 10% of the global population (Endo et al., 2015). Furthermore, according to epidemiological studies, and of particular interest to this study, IBS has consistently shown female predominance (Mulak et al., 2014, Canavan et al., 2014a, Chatila et al., 2017, Wilkins et al., 2012). Indeed, IBS symptom onset often coincides with gonadal maturation (Heitkemper and Jarrett, 2008), implicating female hormone levels as aetiological confounder, despite the fact that the relative contribution of menstrual cycle to IBS-like gastrointestinal symptomology remains unclear. Notably, some researchers suggest that there exists an estrogen-dependent maladaptive intestinal epithelial response to environmental factors (Alonso et al., 2008, Alonso et al., 2012), suggesting that female susceptibility to IBS may involve an estrogen driven maladaptation of the gut barrier to stressors, rather than phase-dependent changes.

In terms of pathophysiology, it remains ambiguous which factors trigger or augment IBS in different individuals (Chong et al., 2019). However, recent evidence highlights the influence of biological sex, diet and food intolerance, gastrointestinal infections, relative gut dysbiosis, low-grade mucosal inflammation and innate immune system dysfunction, as well as psychosocial factors, on IBS onset and progression (Chong et al., 2019, Gu et al., 2019, Lazaridis and Germanidis, 2018). Notably, psychosocial stress is the most generally recognized risk factor for both development and relapse of IBS (Vannucchi and Evangelista, 2018), suggesting a significant departure from normalcy in the gut-brain axis in these patients (Kennedy et al., 2014a). When these factors act in tandem with genetic (Ek et al., 2015, D'Amato, 2013) and/or epigenetic (Mahurkar et al., 2016) predisposition, they may promote gut-related alterations such as the increased intestinal permeability reported in IBS populations (Piche et al., 2009, Zhou et al., 2009, Lee et al., 2013, Mujagic et al., 2014, Hanning et al., 2021), which – even when not clinically evident – potentiates activation of systemic and neural immune and endocrine responses. The abnormal intestinal and microbial secretory and sensorimotor outputs that ensue - gastrointestinal dysmotility and visceral hypersensitivity - are associated with IBS symptom duration and severity (Enck et al., 2016, Ford et al., 2017, Ohman and Simren, 2010).

Importantly, although chronic immune activation and innate immune system dysfunction is implicated in IBS pathogenesis (Lazaridis and Germanidis, 2018), IBS is generally regarded

a non-inflammatory syndrome. The persistent micro-inflammation of IBS is therefore largely overlooked in treatment strategies. While it would be optimistic to implicate inflammation as a central aetiological factor in a syndrome with the complexity of IBS, unresolved inflammation – even at low level – will certainly impact negatively on therapeutic outcome. The importance of considering the micro-inflammation in IBS (and especially in females), is underlined by the fact that sex-differences in inflammation – specifically that in chronic inflammatory conditions prognosis is often worse in females, is in line with the female predominance in IBS – cannot be fully attributed to hormonal differences between sexes (Casimir et al., 2018). Furthermore, psychosocial stress is associated with a pro-inflammatory phenotype – here again a significant female predominance exists (Altemus et al., 2014). In this context, we have suggested that the trace aminergic system may link together diet, inflammation, and sex, and could potentially be exploited as a therapeutic target (Pretorius and Smith, 2020). Indeed, significant sex differences have been reported for trace amine (TA) levels (Liberles and Buck, 2006) and high levels of TAs have been measured in specific foods and bacterial secretomes, including those of probiotic nature (Barbieri et al., 2019, Lorencová et al., 2012, Moracanin et al., 2015, Ruiz-Capillas and Herrero, 2019).

As ligands, TAs are present at their highest concentrations at the interface between mucosal immunity and microbes. In addition, trace amine-associated receptors are also conveniently situated along the gastrointestinal tract (Gwilt et al., 2020) and are also expressed by most leukocytes (Babusyte et al., 2013). In the IBS context specifically, foods with high trace amine context (e.g., fermented foods) are associated with symptom flare-up. For example, a study by Bohn *et al.,* (2013) investigating the self-reported food intolerance of 197 IBS patients, reported that 84% of these individuals' recounted symptoms related to at least one of the surveyed foodstuffs, of which, 58% experienced GI symptoms from foods rich in biogenic amines (wine, beer, salami and cheese). Of note, the authors also reported that females reported more food items causing symptoms than males (Bohn et al., 2013). Moreover, other biogenic amines may similarly trigger IBS-specific symptoms by promoting visceral hypersensitivity *via* the action of bioactive mediators and/or luminal distention (Cuomo et al., 2014).Furthermore, probiotics – which are commonly prescribed for the management of IBS – secrete trace amines, which may explain at least in part, its effect on gut health (Barbieri et al., 2019, Lorencová et al., 2012). Taken together, these studies support our theory that altered TA metabolism may have functional consequences, that may promote disease symptomology. It is therefore feasible that the trace aminergic system may be an additional level of control/maladaptation in IBS that has been largely overlooked until now.

However, the mechanism of actions of TAs in the gut are relatively under-researched. According to several *in vitro* studies, TAs reportedly (i) stimulate bacterial adherence to gut epithelial cells, promoting consequential internalization and enterocyte cytokine secretion (Fernandez et al., 2007, Luqman et al., 2018), (ii) promote colonic ion secretion (Williams et al., 2014), thereby altering gastrointestinal motility, (iii) promote serotonin secretion and signalling (Roshchina, 2010, Yano et al., 2015), the perturbations of which are already implicated in the pathophysiology of IBS (Gershon, 2013), and finally (iv) are chemotactic for polymorphonuclear cells (Babusyte et al., 2013), which are major role players in inflammation and in particular, secondary damage to host tissue during inflammation. Taken together, despite the knowledge of their existence, data on the specific actions of TA in the human gut – and the potential role of estrogen - are still relatively limited. Therefore, this study aimed to elucidate the effects of 17β-estradiol (E2) on the TA production of several commensal and probiotic microbes, as well as to investigate the effects of TA load, and combined TA and E2 treatment on the inflammatory and functional profile of the gut, in the context of IBS and its female predominance, in order to define and/or refine potential therapeutic targets.

3.3 Methods and materials

3.3.1 Microbial culture

3.3.1.1 Maintenance

Bacterial cultures were grown in 51 g/L De Man, Rogosa and Sharpe (MRS) broth (Sigma-Aldrich, 69966) with 0.1% Tween 80 at 37°C, while yeast stocks were grown in 50 g/L yeast extract peptone dextrose (YPD) broth (Sigma-Aldrich, Y1375) at 30°C. Frozen bacterial and yeast inoculates were made up to a final concentration of 40% glycerol in broth and stored at - 80°C. Additionally, amplification and subsequent sequencing of bacterial 16S rDNA and yeast ITS regions were performed prior to experimentation, to confirm identity (refer to supplementary material).

3.3.1.2 Microbial secretome trace amine content

To assess the potential of (clinically established) probiotic bacteria *Lactobacillus plantarum* 423, *Enterococcus mundtii* ST4SA and *Saccharomyces boulardii* 17*,* as well as commensal bacteria *L. reuteri*, and *L. rhamnosus,* to generate and secrete selected Tas, β-phenylethylamine (PEA), tryptamine (TRP), ρ-tyramine (TYR) and agmatine (AGM), a protocol adapted from Luqman *et al.*, (2018) was used. Briefly, 10 mL inoculates of all microbes were cultured under conventional conditions for 24 hrs. Thereafter, each culture was pelleted, washed twice in 1x PBS (pH 7.2), and resuspended in 1 mL of minimal media solution (0.2 M

TRIS HCl, 10% $^{m}/_{v}$ glucose and 1 mg/mL of L-phenylalanine (Sigma, P17008), L-tryptophan (Sigma, T0254), L-tyrosine (Sigma, T3754), and L-arginine (Sigma, A5131)) at pH 7.5. Additionally, microbes were also treated with low (0.1 nM) and high (1 nM) concentrations of E2 (Sigma, E2758). The densely resuspended cultures were incubated at 37°C for another 24 hours, before the supernatants were collected and stored in aliquots for mass spectrometry (MS) analysis.

Sample preparation and extraction: To quantify the levels of TAs in the bacterial and yeast conditioned supernatants, a novel liquid chromatography (LC)-MS method, adapted from D'Andrea *et al*., (2019), was developed. A solid phase extraction (SPE) protocol utilizing Supelclean™ LC-WCX SPE Tubes with 100 mg bed wt. and 1 mL volume (Supelco, 505595) to extract the TAs from microbial conditioned media was optimized to improve eluent purity, analyte sensitivity and maximally reduce the lower limits of quantification. Briefly, WCX SPE cartridges were conditioned with 1 mL absolute methanol (MeOH), followed by equilibration with 1 mL 50 mM ammonium acetate pH 5.5. The samples, quality controls, blanks and calibrators were buffered (1:1) with 50 mM ammonium acetate containing a final concentration of 100 ng/mL of internal standard (IS), phenethylamine-d4 (Toronto Research Chemicals, P321336). Following equilibration, 1 mL of buffered sample was loaded into a cartridge. Cartridges were then washed twice with 500 μL 25% MeOH in H_2O before eluting with 1 mL acetonitrile containing 5% formic acid (FA). The eluent was evaporated to dryness under vacuum utilising a Genevac miVac Duo Sample Concentrator at 40°C (-OH function). Samples were reconstituted in 500 μL H_2O with 0.1% FA, and 2 μL was injected for LC-MS/MS analysis.

LC-MS/MS: The method was developed on the SHIMADZU 8040 LC-MS system. Mobile phase (MP) A was H_2O with 0.1% FA and MP B was 100% MeOH with 0.1% FA. The samples/controls/blanks/calibrators were injected onto an Agilent Poroshell 120 EC-C18 column (2.7μm, 4.6 x 100 mm) at a flow rate of 0.4 mL/min. A gradient elution method was used, starting with 40% B for 30 seconds, increasing %B to 60% to 4 min and holding it at 60% B until 4.5 min, after which the %B was reduced to 40% by 5 min and 40% B held up to 5.5 minutes. The total run time was 5.5 min. For the detection of AGM, 5 mM ammonium formate was added to each MP, and the flow rate was reduced to 0.35 mL/min. Additionally, the run time was extended to 7 min and the elution gradient was again used, starting with 10% B for 30 seconds, then increasing B to 95% by 4 min and holding it at 95% until 5 min, before reducing B to 10% by 6 min and holding it at 10% up to 7 min. The acquisition was set in positive electrospray ionisation mode with multiple reaction monitoring (MRM) with electrospray ionization using Argon as the collision-induced dissociation gas, at conditions

presented in Table 3.1. Instrument control, acquisition and the analysis of data was provided by LabSolutions Version 5.97 software (Shimadzu Corporation). For TA quantification, calibration curves were established with commercially available TAs (Toronto Research Chemicals, P321335, T894600 & T898500; Sigma, A7127). Integrated data was exported to Microsoft Excel for further analysis.

Table 3.1: Multiple reaction monitoring conditions for LC-MS/MS.

Analyte	Elution time (min)	Precursor ion (m/z)	Product ions (m/z)	Collision energy
PEA	4,280	122,1000 $[M + H]^+$	105,0500 (Q) 77,0000 51,0000	14 29 44
TRP	4,511	161,1078 $[M + H]^+$	144,1000 (Q) 117,0500 115,000	13 27 35
TYR	3,154	138,1000 $[M + H]^+$	121,0500 (Q) 77,000 91,000	21 28 15
AGM	4,234	130,9500 $[M + H]^+$	72,0500 (Q) 59,9500 113,9000	15 14 17
PEA-d4 (IS)	4,281	126,2000 $[M + H]^+$	109,0500 (Q) 79,1000 51,9000	15 28 46

Abbreviations: IS: internal standard, Q: quantifier ion.

3.3.2 Human colon epithelium cell culture

3.3.2.1 Trace amine exposure

Colon adenocarcinoma (HT-29) cells, kindly donated by Dr Tanya Davis, were cultured in 5 mM D-galactose supplemented glucose free RMPI (Gibco, 11879020) culture medium to facilitate differentiation. The culture media was additionally supplemented with 10% heat inactivated, gamma irradiated fetal bovine serum (FBS) (Biowest, S181Y-500) and 1% PenStrep. Cells were sub-cultured with 1x trypsin and maintained in a humidified incubator at 5% CO_2 at 37°C. When not actively cultured, the cells were cryopreserved in culture media containing 10% DMSO at $2x10^6$ cells/mL. All experiments were performed between passage 10 to 14. All experiments were done in triplicate and repeated at least three times.

For TA exposure, $4x10^4$ cells/well were seeded in a 96-well microtiter plate in 5 mM D-galactose supplemented glucose free RMPI (Gibco, 11879020) and were incubated at 37°C in 5% CO_2. The cells were treated with various concentrations of PEA, TRP, TYR and AGM, in the presence or absence of 0.1 nM E2 (total reaction volume of 100 μL per well) for 24 hrs. At the end of the incubation period, mitochondrial reductive capacities were assessed as indirect indicator of TA cytotoxicity. Briefly, 5 μL of WST-1 reagent (Abcam, ab155902) was

added to each well. After an additional 90 min incubation (37°C in 5% CO_2), the absorbance of each well was determined at 450 nm, using a plate reader (BIO-TEK, EL800). Data were collected utilizing the KC Junior Software before exporting to Microsoft Excel for further analysis.

In addition, culture supernatant prostaglandin E2 (PGE2) concentrations were assayed using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (E-EL-0034, Elabscience), following the manufacturer's guidelines.

3.3.2.2 Tight junction protein immunofluorescent staining

To assess the effects of TA treatment on tight junction protein (TJP) integrity, Zona occludens-1 (ZO-1), a cytoskeletal linker protein, and occludin, an integral membrane protein, were fluorescently stained in treated HT-29 cell monolayers. Briefly, HT-29 cells were seeded at $1.5x10^5$ cells/well (24-well plate) onto sterilized 12 mm round glass coverslips, which were previously coated with ECL cell attachment matrix (Merck, 08-110). After 9 days of culturing, with media changes every second day, the cell monolayers were treated with PEA, TRP, TYR and AGM or media vehicle (for control) in the presence or absence of 0.1 nM E2 for 24 hours. Thereafter, the cell monolayers were washed once with PBS before being fixed (ice cold 4% PFA and 50% MeOH solution) for 15 min at - 20°C. The cell monolayer was washed again, once with PBS and then once with S-PBS (0.1% saponin in PBS) prior to blocking (20% FBS and 5% donkey serum in S-PBS) for 1 hr at room temperature. Overnight primary antibody incubations at 4°C followed, utilising the primary antibodies: 1:250 mouse anti-ZO-1 (Invitrogen, 33-9100) and 1:250 rabbit anti-occludin (Novus Biologicals, NBP1-87402) in blocking buffer. Next, the cell monolayers were washed 3x with S-PBS, prior to incubation with secondary antibodies: 1:250 Alexa Fluor 488 donkey anti-mouse (Invitrogen, A-21202) and Alexa Fluor 594 donkey anti-rabbit (Invitrogen, A-21207) in blocking buffer for 1 hr at room temperature. Finally, the cell monolayers were incubated with Hoechst (ThermoFisher Scientific, 33342) for 20 min before undergoing 4x washes with PBS. The glass coverslips were then mounted onto microscope slides with Dako Fluorescent Mounting media (S3023). Fluorescently stained cell monolayers were imaged with a Carl Zeiss Confocal LSM 780 Elyra PS1 using the 60x oil-immersion objective. Respective Z-stack (10 slices) images were captured, and maximum intensity projections were analysed for co-localisation of ZO-1 and occludin utilizing Zeiss ZEN imaging software.

3.3.3 Statistical analyses

Statistical analyses of all data were completed by utilizing GraphPad Prism Version 9.1.2 (www.graphpad.com, San Diego, CA). TA quantification and WST-1 results are represented as mean ± standard deviation (SD), while PGE2 and TJP status results are represented as mean ± standard error of mean (SEM). Statistical analyses included: (i) One-way ANOVA with Bonferroni post-hoc test for TA quantification data, (ii) 2-way ANOVA with Bonferroni post-hoc test for WST-1 data, and (iii) 2-way ANOVA's with Šídák's multiple comparisons tests for PGE2 and TJP data. A p-value of < 0.05 was considered statistically significant.

3.4 Results

3.4.1 E2 alters the TA production capabilities of microbes without acutely effecting their growth

With the exception of *L. plantarum,* which exhibited no E2-dependent changes in TA production capability (Fig. 3.1. A), all probiotic organisms assessed showed significant, yet differential responses to E2 treatment. For example, E2-exposures were associated with a dose-dependent increase in PEA, TRP and TYR production by *E. mundtii* (Fig. 3.1. B), while the effects of high doses of E2 on *S. boulardii* resulted in the drastic decrease in PEA and TYR levels (Fig. 3.1. C). Notably, *E. mundtii* was the only microbe that produced quantifiable amounts of TRP, and interestingly, a single high dose of E2 treatment significantly increased this concentration compared to both control conditions ($p<0.0001$, 50% increase), and a single low dose of E2 treatment ($p<0.001$, 37% increase). Similar trends were observed for the production of PEA ($p<0.01$) and TYR ($p<0.01$) too (Fig. 3.1. B). Moreover, from the results depicted in Figure 3.1. C, a single high dose of E2 treatment significantly reduced the concentration of PEA produced by *S. boulardii* ($p<0.05$, 91% decrease). Of particular interest is the differential response of *S. boulardii* to low *vs* high E2 in terms of TYR production capabilities. In this case, a single low dose of E2 treatment increased the amount of TYR produced ($p<0.001$, 69% increase compared to no treatment), while a single high dose of E2 treatment nearly abolished production completely ($p<0.001$, 71% decrease compared to no treatment).

Moving on to commensal microbes, the production of PEA by *L. reuteri* did not seem to be altered by E2 treatment, but an effect of E2 was observed for *L. rhamnosus*, with a tendency for high dose E2 treatment to increase PEA production ($p=0.056$) when compared to no E2 treatment (Fig. 3.1. E). In terms of TYR production, statistically significant differences were apparent for *L. reuteri* – high dose E2 treatment reduced TYR levels when compared to no

(p<0.01, 30% decrease) and low-E2 (p<0.01) groups (Fig. 3.1. D) – and for *L. rhamnosus* – high dose E2 treatment reduced the amount of TYR produced (p<0.01, 6% decrease) compared to no E2 treatment (Fig. 3.1. E). Notably, no statistically significant E2-dependent alterations in AGM production capabilities were reported for any assessed organism. Of importance, previous growth curve experiments confirmed that microbial growth, measured by optical density, were not significantly altered by hormone treatment (refer to supplementary material), eliminating the contribution of hormone-dependent growth changes as a confounding variable in TA production.

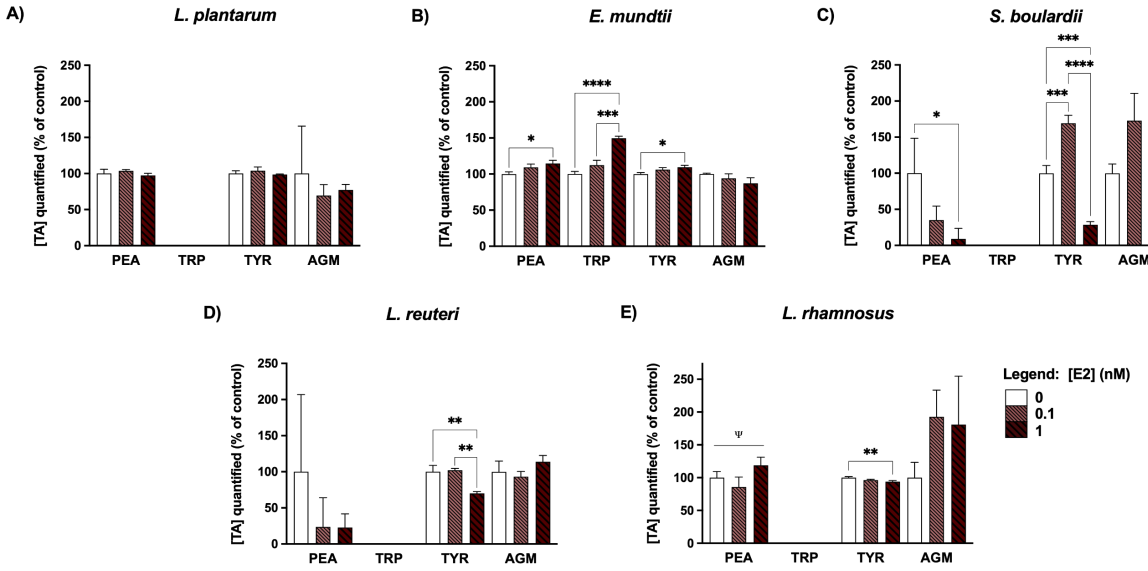


Figure 3.1: The effect of low (0.1 nM) and high (1 nM) concentrations of 17β-estradiol (E2) on **(A)** *Lactobacillus plantarum,* **(B)** *Enterococcus mundtii,* **(C)** *Saccharomyces boulardii,* **(D)** *L. reuteri*, and **(E)** *L. rhamnosus* to generate and secrete β-phenylethylamine (PEA), tryptamine (TRP), ρ-tyramine (TYR) and agmatine (AGM). The data is represented as the mean concentration of quantified trace amine (as a percentage of the 0 nM E2 group) ± SD. Statistical analysis: One-way ANOVA with Bonferroni post-hoc tests; * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001, ψ = main ANOVA effect.

3.4.2 Cytotoxic effects of TAs on HT-29 cells are differentially buffered by E2

The WST-1 results showing the effect of a range of TA doses on the percentage of metabolically active HT-29 cells in the presence of low (0.1 nM) E2 are presented in Figure 3.2. Cell viabilities, as an indirect outcome of metabolic activity, were reduced significantly by all TAs at higher concentrations (AGM<PEA<TYR<TRP, under control conditions), indicated by statistically significant main ANOVA effects for TA dose. Specifically, the onset of significant loss of metabolically active cells under control conditions were recorded at doses ≥ 200 μg/mL PEA and AGM (p<0.001 & p<0.01 respectively), 25 μg/mL TYR (p<0.01) and 18.75 μg/mL TRP (p<0.05). With the addition of E2 treatment, the onset of significant loss of metabolically

active cells were recorded at doses ≥ 800 μg/mL AGM and TYR ($p<0.001$ & $p<0.0001$ respectively), 200 μg/mL PEA ($p<0.0001$) and 50 μg/mL TRP ($p<0.001$). With reference to Figure 3.2. A, the onset of significant HT-29s cytotoxicity at a dose of 200 μg/mL PEA were not altered by E2 treatment. Nevertheless, compared to control conditions, E2-exposed cells had significantly reduced viable cells at this concentration ($p<0.05$, 10% less). In contrast, low doses of E2 treatment delayed the onset of significant cytotoxicity as a result of TRP treatment, from 25 to 50 μg/mL. Indeed, in Figure 3.2. B, the ability of E2 to buffer against TRP-induced cytotoxicity were evident at 25 μg/mL ($p<0.001$), 50 μg/mL ($p<0.01$), and 100 μg/mL ($p<0.0001$) doses. Similarly, with regards to the effects of TYR, the administration of E2 delayed the onset of significant cytotoxicity drastically, from 25 μg/mL under control conditions, to 800 μg/mL in the presence of E2. These effects can be observed in Figure 3.2. C, where E2 treatment significantly increased the number of viable cells following 100 μg/mL ($p<0.05$, 12% increase), 200 μg/mL ($p<0.0001$, 18% increase) and 800 μg/mL ($p<0.01$, 14% increase) TYR treatment. And finally, in terms of AGM, although no significant differences between control and E2 groups were observed (Fig. 3.2. D), the onset of AGM-induced cytotoxicity was delayed in HT-29 cells in the presence of E2, from 200 μg/mL to 800 μg/mL.

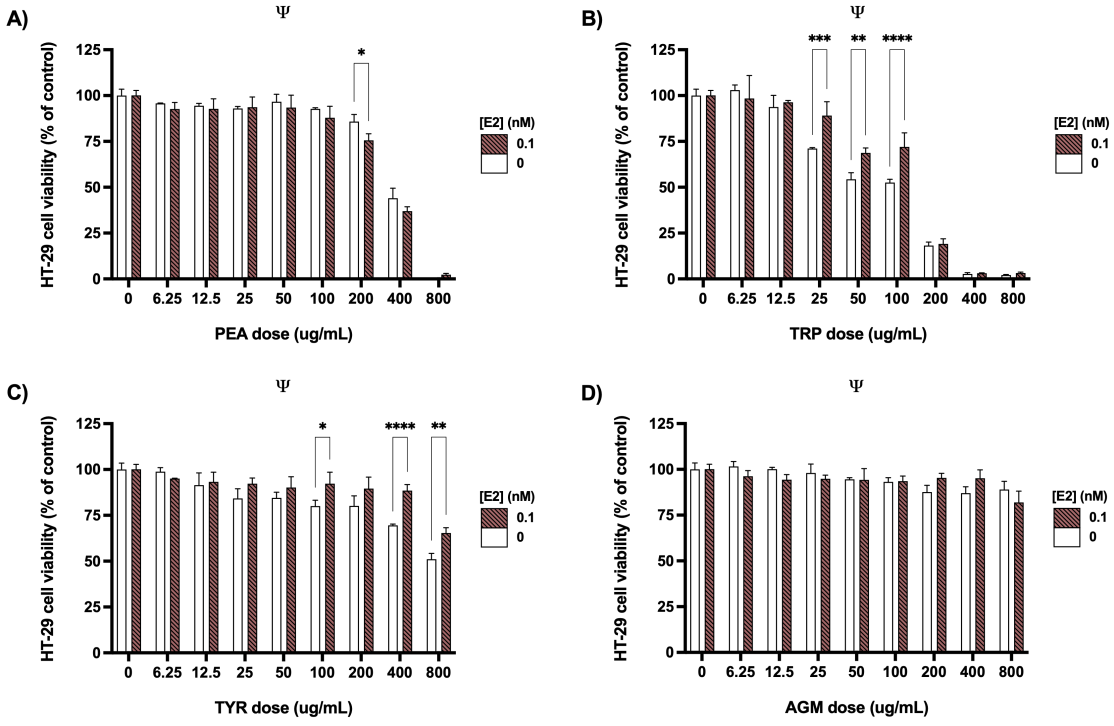


Figure 3.2: WST-1 results showing the effect of varying concentrations of trace amines **(A)** β-phenylethylamine (PEA), **(B)** tryptamine (TRP), **(C)** ρ-tyramine (TYR), and **(D)** agmatine (AGM) on the percentage of metabolically active HT-29 cells in the absence or presence of low (0.1 nM) levels of 17β-estradiol (E2). The data is represented as mean % of control ± SD. Statistical analysis: 2-way ANOVA with Bonferroni post-hoc tests; * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$, ψ = main ANOVA effect for TA dose.

Additionally, low- and high-TA doses were inferred from these results and used for subsequent experimentation. Importantly, low doses were defined by a non-significant change (less than 10%) to cellular viability, while high doses were defined by a dose that resulted in the significant loss of metabolically active cells, with a loss of between 10 to 20% of viable cells. Notably, given the pronounced sensitivity of HT-29s to TRP-mediated cytotoxicity, the high-TRP dose employed (18.75 μg/mL) was reasonably lower than the high doses for PEA, TYR, and AGM (200 μg/mL).

3.4.3 TA-induced changes to the inflammatory profile of HT-29 cells are differentially altered by E2

In Figure 3.3 the effects of 24-hour exposure of low and high doses of TAs on secreted PGE2 levels are reported. Interestingly, the results of PEA, TRP and TYR seem to follow the same dose-dependent trajectory (inverted U) – although the effects of TYR are more pronounced in this regard. Indeed, low doses of PEA ($p<0.05$) resulted in a significant decrease in PGE2 levels compared to control (Fig. 3.3. A), and while the effects of low dose TRP and TYR are not significant, treatment similarly resulted in a 13 pg/mL (37% reduction) and 10 pg/mL (30% reduction) decrease in PGE2 levels (Fig 3.3. B & C), respectively. At higher doses, recovery to baseline levels following PEA and TRP treatments were apparent, while high dose TYR treatment resulted in a significant increase in PGE2 levels compared to baseline ($p<0.05$). In terms of AGM, treatment with this TA resulted in a dose-dependent increase in PGE2 levels, with 200 ug/mL AGM increasing PGE2 secretions by 26 pg/mL ($p<0.05$, 74% increase, Fig. 3.3. D).

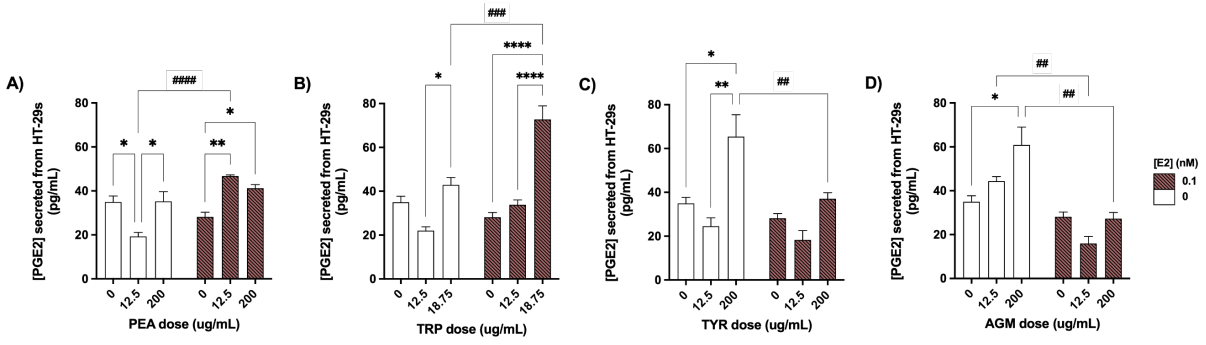


Figure 3.3: Effect of low (0.1 nM) levels of 17β-estradiol (E2) treatment on the concentration of Prostaglandin E2 (PGE2) secreted by HT-29 cells following selected TA-exposure: **(A)** β-phenylethylamine (PEA), **(B)** tryptamine (TRP), **(C)** ρ-tyramine (TYR), and **(D)** agmatine (AGM). The data is represented as mean ± SEM. Statistical analysis: 2-way ANOVA with Šídák's multiple comparisons; * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$. Asterisk's (*) represent TA dose effect under the same E2 condition, while hashtags (#) represent effect of E2.

In terms of the effect of E2 in this context, importantly, baseline levels of PGE2 were not significantly altered by E2. However, differential E2-dependent changes were again observed for all TAs. In this regard, E2 treatment promoted PGE2 secretion from cells treated with 12.5. ug/mL PEA ($p<0.0001$) and 18.75 ug/mL TRP ($p<0.001$) when compared to control conditions. In contrast, the additions of E2 were associated with reduced PGE2 secretion from cells treated with 200 ug/mL TYR ($p<0.01$) and both low and high doses of AGM ($p<0.01$ for both).

3.4.4 TA-induced changes to the functional profile of HT-29 cells are not altered by E2, despite baseline differences

In terms of effects of TA-exposure on TJPs, representative fluorescent images (Fig. 3.4. A) visually demonstrate occludin redistribution in response to TA treatment. Indeed, visual comparison of control and TA-treated micrographs clearly represent the irregular distribution of occludin from membrane-located junction sites to intracellular localizations. AGM treatment, specifically at higher doses, resulted in the loss of monolayer integrity altogether. With regard to ZO-1, representative micrographs visually demonstrate ZO-1 signal intensity reduction following TA-exposure (Fig. 3.4. B). Lastly, in Figure 3.4. C, the effects of TA treatment on the colocalization between occludin and ZO-1 are displayed. These representative micrographs visually demonstrate that low dose TA treatment results in a decrease in the colocalization signal intensity along the cell periphery, compared to control conditions. In addition, at high TA doses, observable loss of cellular structure and morphology are associated with an increase in the intracellular localization of colocalized signal, as well as clumping of colocalized ZO-1 and occludin.

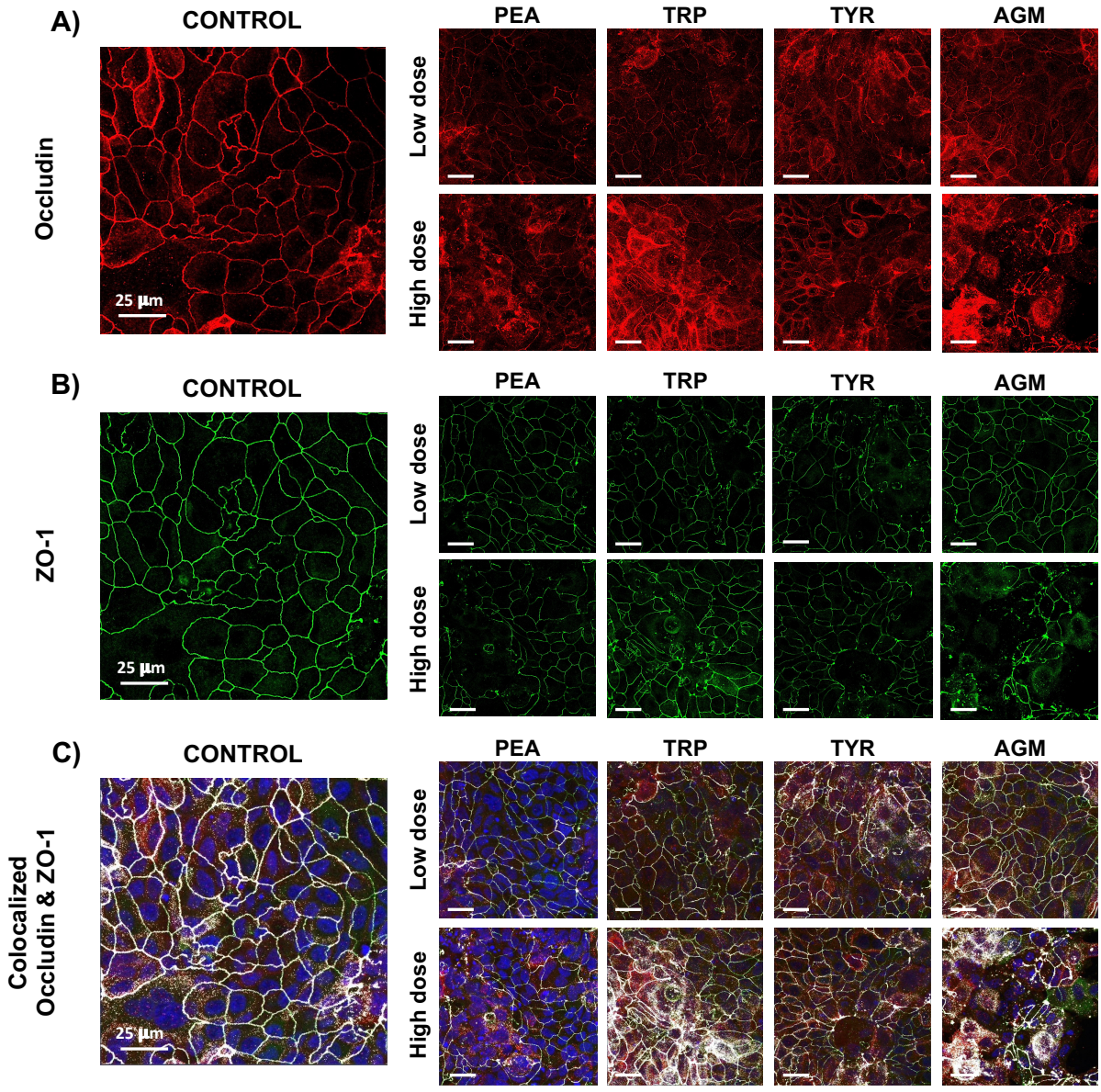


Figure 3.4: Representative fluorescent images showing the effects of TA treatment on **(A)** total occludin status (red signal) **(B)** total ZO-1 status (green signal), and **(C)** colocalization between ZO-1 and occludin (white signal) in HT-29 cell monolayers. Control represents a media and vehicle only treated HT-29 cell monolayer. Blue staining = Hoechst. All fluorescent images are maximum intensity projections of acquired z-stacks, imaged using the 60x oil immersion objective. Scale bar = 25 µm.

Furthermore, in terms of the quantitative effect of TA exposure on TJPs, results depicting the relative area (%) of stained HT-29 cell monolayers for uncolocalised occludin and ZO-1 are reported here (Fig. 3.5.). The assessment of these parameters, as opposed to changes in total levels (data in supplementary material), facilitated the evaluation of changes in the cellular distribution of these proteins in response to TA treatment. Seeing that many TJPs have dual roles dependent on cellular localization, our assessment is more informative of the functional consequences associated with dysregulated localization. Regarding occludin status, two distinctive responses were observed. The first groups together the effects of PEA and TRP,

that similarly exhibited a dose-dependent tendency to increase 'uncolocalised' (i.e. not in association with ZO-1) occludin signal (Fig. 3.5. A & B). Indeed, high doses of PEA and TRP significantly increased the percentage of 'uncolocalised' occludin compared to control ($p<0.01$ & $p<0.001$ respectively) and low dose conditions ($p<0.01$ & $p<0.05$ respectively). The second groups together the effects of TYR and AGM, which both significantly increased the percentage of 'uncolocalised' occludin signal at low doses ($p<0.05$ & $p<0.01$ respectively) but seemed to return towards basal levels at higher doses (Fig. 3.5. C & D). In terms of an E2 effect, in the absence of TA treatment, E2 treatment resulted in a significant increase in the percentage of 'uncolocalised' occludin. Despite these baseline differences, no significant or E2-dependent changes were observed in response to PEA, TYR or AGM treatment, and only at high concentrations of TRP (Fig. 3.5. B), were there a significant difference between control and E2 groups, with the E2 group showing significantly reduced 'uncolocalised' occludin signal ($p<0.001$).

In Figure 3.5. (E to G) the effects of TA treatment on ZO-1 status are displayed. Again, two distinctive responses with identical TA groupings were observed. Firstly, low doses of both PEA and TRP treatment resulted in a significant decrease in 'uncolocalised' ZO-1 signal compared to control ($p<0.01$ & $p<0.001$ respectively). Interestingly, at high doses, there are a partial recovery in these levels (Fig. 3.5. E & F). Secondly, although no statistical significances were recorded, TYR and AGM exposures seem to tend towards a dose-dependent decreased in the percentage of 'uncolocalised' ZO-1 (Fig. 3.5. C & D). Moreover, with regard to the effect of E2, in the absence of TA treatment, E2 treatment resulted in a significant decrease in the percentage of 'uncolocalised' ZO-1. Similar to the effects on occludin status, in spite of these baseline differences, no significant changes between control and E2 groups were reported for either PEA, TYR or AGM treatment, and only at high concentrations of TRP, were a significant decrease in the percentage of 'uncolocalised' ZO-1 signal as a result of E2 treatment ($p<0.01$).

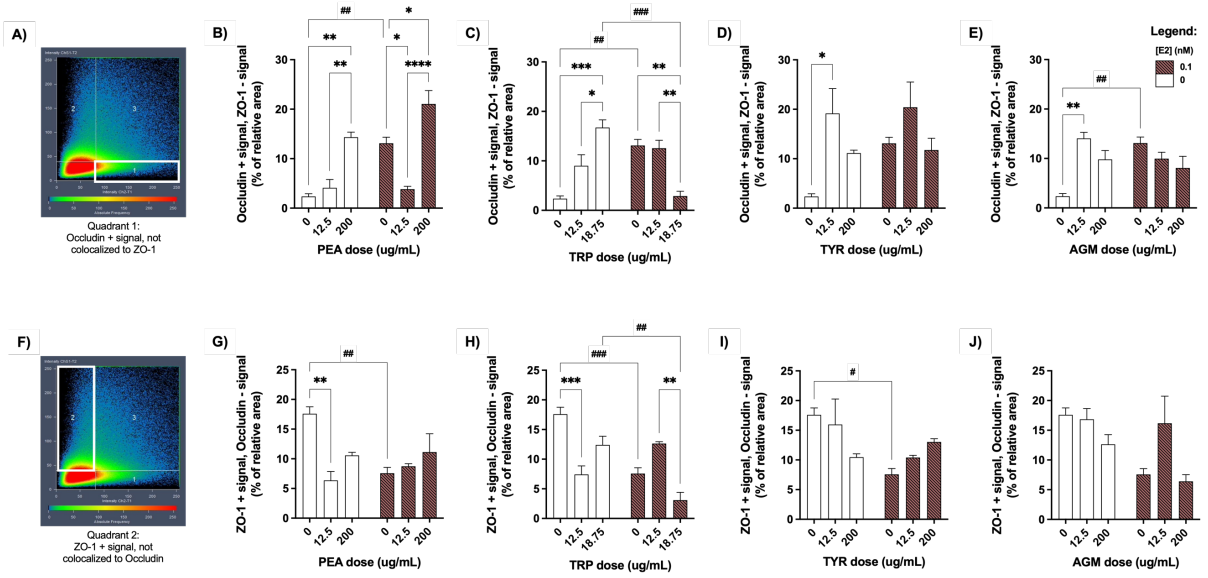


Figure 3.5: Effect of low (0.1 nM) levels of 17β-estradiol (E2) treatment on the percentage of relative area of the imaged HT-29 cell monolayer that stained positive for tight junction proteins following selected TA-exposure: **(C&G)** β-phenylethylamine (PEA), **(D&H)** tryptamine (TRP), **(E&J)** ρ-tyramine (TYR), and **(F&I)** agmatine (AGM). **(C to F)** Effect on occludin, but not ZO-1 positive signal – indicated by quadrant 1 in **(A)**, demarcated by the white box. **(G to I)** Effect on ZO-1, but not occludin positive signal – indicated by quadrant 2 in **(B)**), demarcated by the white box. **(A&B)** Representative scatter plot generated by colocalization analyses. The data is represented as mean ± SEM. Statistical analysis: 2-way ANOVA's with Šídák's multiple comparisons tests: * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001. Asterisk's (*) represent TA dose effect under the same E2 condition, while hashtags (#) represent effect of E2.

3.5 Discussion

Current data contributes several novel findings which contribute to elucidation of factors which underpin female predominance in IBS. Firstly, while it is well known that estrogens are implicated in host-microbial communication (Baker et al., 2017, Ervin et al., 2019), current literature does not conclusively expound on the effect of reproductive hormones on gut microbes in healthy or dysbiotic states (Thackray, 2019), nor has this relationship been investigated in probiotic microbes often used to manage IBS. In terms of direct effects, current data illustrates that the growth of several microbes, including the probiotic varieties *L. plantarum, E. mundtii* and *S. boulardii* – commonly prescribed to IBS patients (Horvath et al., 2011, Kim et al., 2005, McFarland, 2010) – and commensal varieties *L. reuteri* and *L. rhamnosus*, was not altered significantly by varying concentrations of E2. This lack of direct dependency of microbial growth curves on E2 in monoculture however should not be interpreted as an unaffected compositional microbiome. Rather, it is necessary to also consider potential E2-dependent functional changes – i.e. differences in secretome profile –

in order to elucidate potential competition between gut microbes which indeed may influence microbiome composition.

In terms of interpretation of E2-associated changes in the secretome, the unaltered growth curves do confirm that changes in microbial growth rate was not a confounding variable in *in vitro* experiments presented here. Turning attention to the analysis of microbial secretome in the current study, the specific focus was on potential differences in TA profile content, as an indicator of functional dysbiosis. E2-dependent alterations in microbial TA production was indeed a novel illustration, in line with our theory of TA involvement in the female bias seen in IBS (Pretorius and Smith, 2020). The nature of these responses to E2 were clearly different between the various microbes assessed – and even between probiotic varieties. Furthermore, within the same organism, high vs low doses of E2 could have opposite effects on TA production. Together, this data highlights that the mechanisms by which E2 affects TA production is indeed complex; clearly more comprehensive investigation is required to fully elucidate role players and mechanisms involved. In the context of female bias of IBS, and considering reproductively mature, pre-menopausal women undergo cyclic hormonal fluctuations and gonadal maturation which often coincides with IBS symptom onset (Heitkemper, 2008), current data suggest that different microbes may potentially secrete different levels of microbial-derived metabolites (such as TAs), depending on the host phase of menstrual cycle. Interestingly, from the probiotic strains assessed, *L. plantarum* was the only assessed microbe to show no E2-dependent changes in TA production capability *in vitro*, and while it is premature to suggest that this strain may therefore represent a sex-neutral probiotic option, these findings warrant investigation into sex specific – or even menstrual phase specific – probiotic options based on secretomes alterations in response to hormone levels. Additionally, the dependence of probiotic secretome profile - and per extension their exerted effect on their host – on host hormonal status, may explain some reports on inefficiency of probiotics to ‘treat’ IBS (Lee and Bak, 2011). Current data highlights the need for more comprehensive investigation into the effects of probiotics to adequately inform on optimal consumer profiles, as well as efficacy and safety of specific probiotics in specific disease contexts. A limitation of the current study – which was aimed at elucidating specific responses of individual microbes to estrogen – is that the interplay of different microbes in combination could not be evaluated. The current study should thus be followed up with a more complex study design which can also account for microbial interaction.

In addition, as estradiol may also condition gut epithelial tissue itself, we transitioned into an *in vitro* colon cell model in order to investigate the physiological implications of varying TA levels, in the context of estradiol. Using a TA concentration range in line with the levels of TAs

produced by microbes *in vitro* (Luqman et al., 2018), as well as TA concentrations in or transiting through the human gut (Pugin et al., 2017), we illustrated that TA treatments in the μg/mL range are a significant stressor – of which TRP seem most cytotoxic – that disrupts mitochondrial functioning and results in the loss of metabolically active cells. In addition, we report varying degrees of E2-dependent buffering of these cytotoxic effects for some, but not all TAs, which suggests that, at least for females, the effects of fluctuating E2 levels may alter the response of the gut barrier to stressors. This is also in line with anecdotal evidence of exaggerated IBS symptomology during menses, when E2 levels are at their lowest (Bharadwaj et al., 2015a, Heitkemper et al., 2003, Jane et al., 2011). Although speculation at this point, since HT-29 cells do express both ERβ and GPER (GPR30) (Arai et al., 2000, Bustos et al., 2017, Campbell-Thompson et al., 2001, Chatzinikolaou et al., 2007, Gilad et al., 2005, Gilligan et al., 2017, Looijer-van Langen et al., 2011), this may suggest that trace amines may exert their undesired effect via binding to the estrogen receptor (ER) and that high enough levels of estrogen may competitively bind ER to inhibit/limit the effect of TA load. Receptor binding studies may shed more light on this potential mechanism. Furthermore, the lack of an *in vivo* context to more accurately simulate this complex interplay between various role players, limits the interpretations which can be made from these data and as such, warrants more robust investigations.

Nevertheless, the demonstrated effects of TA load on the inflammatory and tight junction protein profile of HT-29 cells provide insight into their possible mechanisms of actions in the gut and specifically in the context of IBS. In this context, low doses of PEA, TRP and TYR (although not significant for the latter) resulted in a decrease in PGE2 levels when compared to baseline, while high doses of TYR in particular, resulted in a significant increase in PGE2 levels, suggestive of a relatively more pro-inflammatory outcome at high TA load. A potential mechanism of action linking TA to PG production, involves the enzymatic decomposition of TA via monoamine oxidase (MAO) (Bortolato et al., 2008, Lindemann and Hoener, 2005), which results in the formation of hydrogen peroxide as a by-product. The subsequent increase in hydrogen peroxide stimulates endoperoxide synthesis, essentially increasing cyclooxygenase (COX) activity and PG synthesis downstream (Seregi and Hertting, 1984). As such, future experimentation investigating PG synthesis in response to TA-exposure, should also assess MAO activity/levels. Moreover, AGM treatment resulted in a PGE2 profile different from those of PEA, TYR and TRP, namely a dose-dependent increase in PGE2 levels, suggesting that a different mechanism of action may exist for this TA. We suggest that the effects of AGM may result from its direct actions within HT-29 cells, as opposed to metabolites generated via degradation. Indeed, Mayeur *et al.,* (2005) reported that AGM accumulates rapidly in HT-29 cells, due to the presence of agmatine-specific transporters in this cell line.

Additionally, AGM catabolism via agmatinase was negligible (only 2% of accumulated AGM was catabolized) in these cells (Mayeur et al., 2005). Interestingly, AGM reportedly modulates nitric oxide (NO) synthesis, with differential inhibition and activation of NO synthase isoforms reported in literature (Demady et al., 2001, Gadkari et al., 2013). Although speculative, it is plausible that an increase in AGM accumulation could have increased NO synthesis in our study. It is well known that a resultant increase in NO activates COX enzymes to produce increased amounts of PGs (Salvemini et al., 2013), potentially explaining the dose-dependent increase in PGE2 following AGM treatment. This differential and potentially more direct effect/action of AGM is not surprising, seeing that PEA, TYR and TRP are all primary amines, while AGM is a tertiary amine. Nevertheless, most of AGM's functions are tissue and concentration specific and require more thorough investigations in the gut.

In terms of TJP, defects of the intestinal epithelial barrier – with associated TJP alterations – are observed in a significant proportion of patients with IBS (Piche, 2014). This increased intestinal permeability reportedly promotes and perpetuates low grade inflammation, contributing to IBS symptomology, specifically abdominal pain (Piche, 2014). Data presented here, illustrates TAs to alter TJP status *in vitro* to varying degrees, with AGM-exposure being the most detrimental to monolayer integrity. Indeed, excessive AGM intake has recently been cautioned for its potentially detrimental effects on intestinal integrity (Oliphant and Allen-Vercoe, 2019). This effect may be attributed to AGMs ability to reduce the synthesis and promote the degradation of other polyamines (Mayeur et al., 2005). Normally, polyamines improve the integrity of gastrointestinal epithelium by increasing TJP expression (Guo et al., 2005), promoting intestinal restitution and increasing mucus secretion (Rao et al., 2012). Therefore, AGM-mediated degradation of polyamines could have significant undesired effects on barrier integrity. Indeed, in terms of occludin, two distinctly different responses to TA-exposure were observed. While PEA and TRP similarly displayed a dose-dependent tendency to increase 'uncolocalised' occludin protein expression, both TYR and AGM significantly increased the percentage of 'uncolocalised' occludin expression only at low doses, an effect that was blunted at higher doses. Moreover, representative fluorescent images demonstrated occludin redistribution in response to TA treatment. Interestingly, occludin redistribution stimulates caspase-3 transcription and thus apoptosis (Kuo et al., 2019). This mechanism of action correlates with the results from the PEA and TRP responses to the corresponding WST-1 data, i.e. a dose-dependent increase in redistributed occludin should promote cell death. However, this effect of occludin was not evident after TYR or AGM treatment. A potential explanation for this inconsistency can be inferred from the PGE2 results. To reiterate, at both high doses of TYR and AGM, PGE2 levels were increased significantly. According to literature, an inflammatory stimulus inhibits an apoptotic stimulus by promoting occludin degradation

(Kuo et al., 2019), which may account for the lack of upregulated expression of 'uncolocalised' occluding in response to TYR and AGM. Furthermore, the decrease in 'uncolocalised' occluding expression at high doses of both TYR and AGM could be as a result of cell death and corresponding occludin loss at this stage.

Moreover, in terms of the combined TA and E2 results, significant findings suggest that when E2 is combined with TRP specifically, a decrease in 'uncololocalised' occludin expression ensues, which is opposite to the independent effects of both TRP and E2. Keeping in mind that in the absence of E2, increased occludin expression was correlated to significant loss of metabolically active cells, this "downregulation" (that occurs only in combination treatment), may explain the delay in the onset of cell death in TRP treated cells. Interestingly, the outcomes of combination of E2 and TA treatment are not universal. In fact, our results suggest the different TAs interact differently with E2, and the nature of the interaction not only alters TJP status, but also cell sensitivity/vulnerability to stressors. Whether this represents E2-dependent actions of TAs or rather E2-dependent priming of cellular sensitivity and tight junction status to stressors (or even a combination), remains to be elucidated.

On face value, however, the occludin results seem to contradict literature pertaining to occludin expression in IBS. In fact, increased proteasome-mediated *degradation* of occludin in patients with IBS has been reported (Coeffier et al., 2010), with lower levels of occludin expression positively correlating to clinical IBS outcomes (symptom duration and abdominal pain intensity) (Bertiaux-Vandaele et al., 2011). Accordingly, to Kuo *et al.,* (2019) in chronic inflammatory conditions, the resultant downregulation of occludin – and consequentially reduced caspase-3 expression – has been described as an anti-apoptotic process, which aims to maintain mucosal homeostasis, and may explain the body of literature that reports lower occludin levels in IBS patients. In terms of the potential effects of TAs in this context, our results demonstrate individual TA responses (high dose TYR and AGM), which increased PGE2 levels and decreased 'uncolocalised' occludin signal *in vitro*. As such, the implementation of appropriate *in vivo* models is required to elucidate the consequences that chronic exposure to elevated TA levels could have, and if those effects translate to adaptive occludin diminishment in robust models of gastrointestinal inflammation.

Furthermore, in terms of ZO-1, similar responses were observed to all TAs employed. 'Uncolocalised' ZO-1 expression decreased significantly at low doses of TA (although only reaching statistical significance for PEA and TRP). Interestingly, at high doses, there is a partial recovery in these levels. These results are in line with IBS literature, where a reduction in ZO-1 protein levels in colonic mucosal biopsies of patients with IBS (n=50), compared to

controls (n=30), has been reported (Bertiaux-Vandaele et al., 2011). These findings could therefore suggest that chronic exposure to low doses of TAs (that importantly do not result in cell death), could contribute to the reported low ZO-1 expression in IBS. Again, appropriate *in vivo* investigations are required to confirm this. In addition, current data report a baseline E2 TJP phenotype, which is characterised by an increase in 'uncolocalised' occludin and decreases in 'uncolocalised' ZO-1 and colocalisation between occludin and ZO-1. In a study that set out to assess the effects of ovarian hormones and hormonal cycling on paracellular permeability in intestinal epithelium, authors reported that menstrual cycle phase was associated with colonic permeability in female rats, with increasing permeability correlating to a decrease in or lower concentrations of E2 (Braniste et al., 2009). Interestingly, utilising an *in vitro* colon model in the same study, Braniste *et al.,* (2009) reported that E2 increased occludin protein expression, but had no effect on ZO-1. This E2-dependent increase in occludin expression has also been reported in cervical epithelial cells (Zeng et al., 2004) and rabbit esophageal tissues (Honda et al., 2016). More recently, the analysis of primary human gut tissues by Zhou *et al.,* (2017) indicated that ZO-1 expression was decreased in females compared to males, independently of ERβ expression. Relatedly, the same authors reported that *in vitro* E2 treatment decreased ZO-1 promoter activity, mRNA, and protein expression in colon cells (Zhou et al., 2017). As such, our results seem consistent with literature. In the context of IBS, recent literature reports that colonic mucosa ZO-1 mRNA expression was lower only among females IBS patients when compared to controls (Lee et al., 2020c). The authors suggested that this finding may indicate gender differences in IBS pathophysiology and could partially explain female vulnerability to IBS.

Finally, TA treatment resulted in a decrease in the colocalization of occludin and ZO-1 along the cell periphery, although the extent of this response varied between TAs. According to several studies in many different tissues, the reduced colocalisation between occludin and ZO-1 is related to tight junction disruption and increased epithelial permeability measured by transepithelial/trans-endothelial electrical resistance (Alonso et al., 2008, Peerapen and Thongboonkerd, 2011, Petecchia et al., 2012, Price et al., 2014). In addition, at high TA doses, observable loss of cellular structure and morphology were associated with an increase in the intracellular localization of colocalized signal, as well as clumping of colocalized ZO-1 and occludin. This is again in line with literature. Bertiaux-Vandaele *et al.,* (2011) reported irregular (and even cytosolic) distribution of ZO-1, occludin, and claudin-1 in colonic biopsies from patients with IBS, compared to the typical reticular pattern observed in control biopsies. While the precise mechanisms of actions TA-associated tight junction disruption are unclear, we suggest that TA binding to the α2-adrenergic receptor (expressed in HT-29 cells), may have

stimulated actin re-organization that resulted in ZO-1 disassembly and subsequent degradation/loss. Indeed, findings from a receptor inhibition experiment by Luqman *et al.,* (2018) suggested that TAs (PEA, TRP and TYR) promote α2-adrenergic receptor-mediated actin re-organisation, which facilitated the effects of TAs to boost adherence to and stimulate subsequent internalization of intestinal microbes into HT-29 cells. Taken together the TJP results reported in our study reiterates the complexity of TA-TJP interplay in a multi-microbe populated environment, necessitating investigation in whole organism models to fully elucidate the role of TA load in the context of sex bias in IBS.

3.6 Conclusion

To conclude, current data suggest that microbially-derived TA profile can be altered by fluctuating E2 levels, and that this altered TA content in the gut may compromise gut health by promoting a relatively more pro-inflammatory cellular phenotype and decreasing gut barrier integrity via breakdown of epithelial TJP function. Data suggest that TAs may exert their observed effects via α-adrenergic receptors and ER, but more focussed research is required to fully elucidate ligand-receptor interactions at play. Taken together, current data illustrate a clear role for estrogen in TA-mediated outcomes on gut health, which may have implications in female susceptibility to IBS. Current data demonstrates the complexities of E2 and TA interactions and limitations of cellular models, highlighting the requirement for further research employing suitable *in vivo* models.

3.7 Supplementary materials

Supplementary data relating to microbial secretome analyses

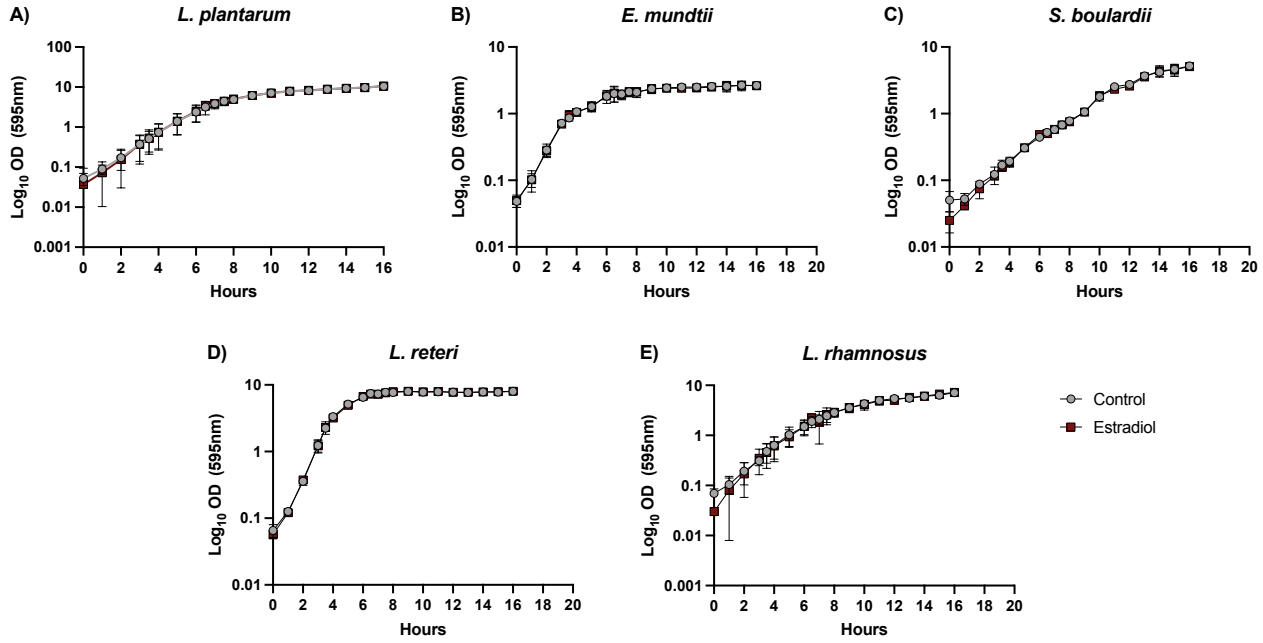
Microbial culture

Microbial sequencing: To confirm the authenticity of the bacterial and yeast strains, DNA from each cultured organism was isolated using *Quick*-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, D6005) and the partial bacterial 16S rDNA (F8 5'-CACGGATCCAGACTTTGATYMTGGCTCAG-3' and R1512 5'-GTGAAGCTTACGGYTAGCTTGTTACGACTT-3') region and yeast D1D2 (F63 5'-GCATATCAATAAGCGGAGGAAAAG-3' and LR3 5'-GGTCCGTGTTTCAAGACGG-3') region was amplified with polymerase chain reactions (PCR). Next, the PCR products were purified with GeneJET PCR Purification Kit (Thermo Scientific, K0701) prior to cloning with CloneJET PCR Cloning Kit (Thermo Scientific, K1231). The cloned pJET vectors were transformed into chemically competent DH5α *Escherichia coli*. Clones were subsequently plated onto Luria Bertani agar (1.2 % $^{m}/_{v}$) supplemented with 100 µg/mL ampicillin. Overnight colonies were used to inoculate 5 mL LB broth supplemented with 100 µg/mL ampicillin and incubated with aeration at 37°C overnight. Plasmid DNA was extracted from overnight cultures with the use of the PureYield™ Plasmid Miniprep System (Promega, A1222). Plasmids digested to confirm PCR product inserts and plasmid DNA samples were sent for DNA sequencing using primers provided in the CloneJET PCR cloning kit (CAF, Stellenbosch University) for analyses.

Microbial growth curves: The growth of the following microbes was assessed over a 16 hr period: (i) bacteria, including the probiotic species *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST4SA, as well as commensal *L. reuteri* and *L. rhamnosus* species, which were generously donated by Prof Leon Dicks (Department of Microbiology, Stellenbosch University), and (ii) a yeast, *Saccharomyces boulardii*, which was isolated from a commercial probiotic capsule (Inteflora 250). Briefly, at time 0 hrs, each organism was diluted to an optical density (OD) of 0.05, measured with the SmartSpec™ Plus Spectrophotometer (BIO RAD, 170-2525) at a wavelength of 595 nm. All cultures (200 mL) were incubated at 37°C (to simulate body temperature) under aerobic conditions for the duration of the growth curve. Hormone treatment of 1 nM 17β-Estradiol (E2) (Sigma, E2758) was added at time points 0 hrs, 3.5 hrs and 7 hrs to replicate a mean E2 concentration, normally circulating during the follicular phase of the menstrual cycle (Albert et al., 2015). Each hour, for 16 hrs, as well as at 30 min before and after hormone treatments, 1 mL of

resuspended broth mixture was deposited into a clean plastic cuvette and the OD was determined as for time 0 hrs.

Effect of E2 on microbial growth

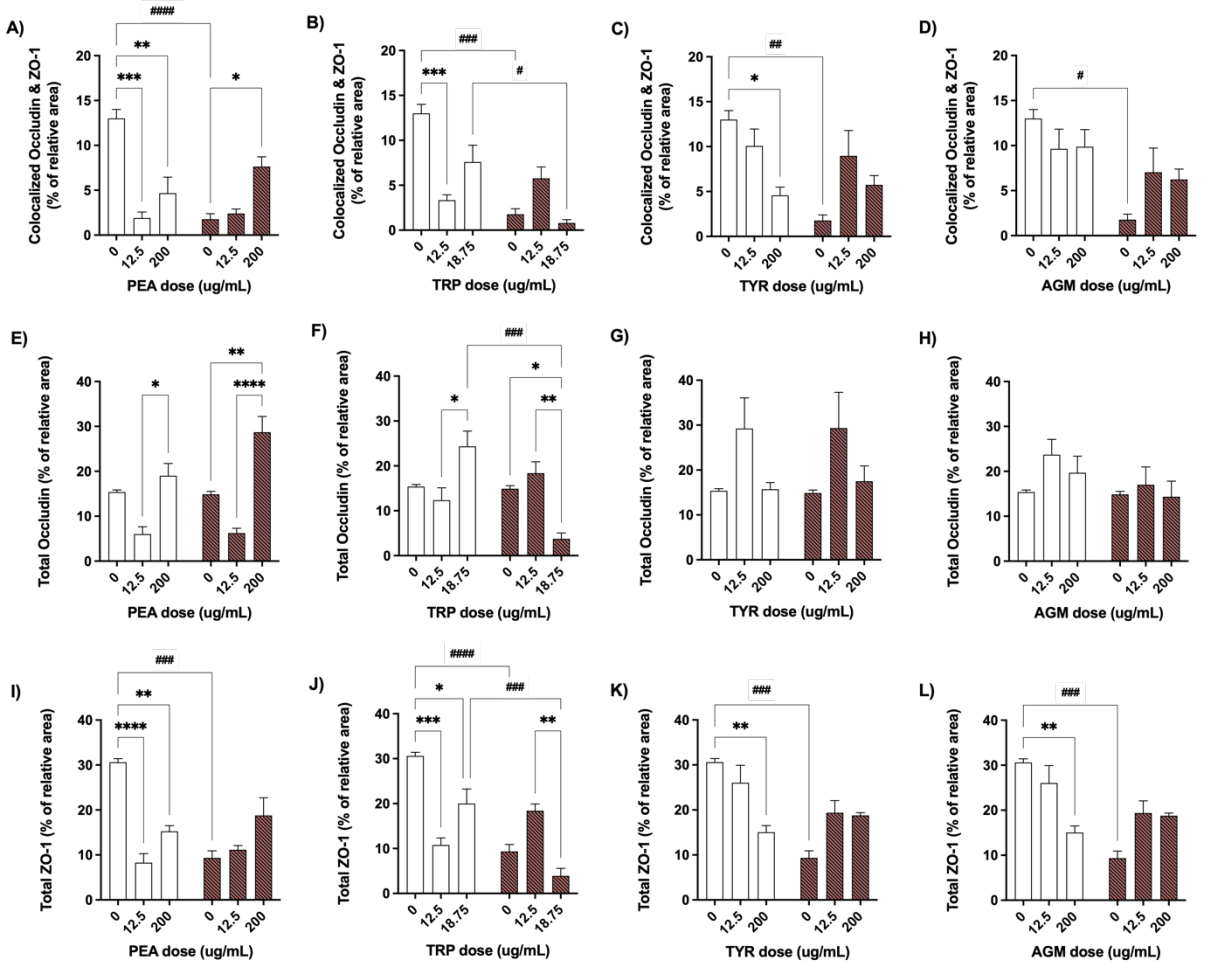


Supplementary Figure 3.1: Effect of 1 nM 17β-estradiol on the growth of **(A)** *Lactobacillus plantarum*, **(B)** *Enterococcus mundtii*, **(C)** *Saccharomyces boulardii*, **(D)** *Lactobacillus reuteri*, and **(E)** *Lactobacillus rhamnosus* over a 16-hour period. Data is expressed as a mean ± SD.

Supplementary data relating to immunofluorescent staining of tight junction proteins

Tight junction protein immunofluorescent staining: Tight junction protein staining was performed as described in the main text. Additional information reported below includes quantitative data for total fluorescent occludin and ZO-1 signal ('uncolocalised' data is reported in the main text since changes in the cellular distribution of these proteins in response to TA treatment was of importance to this study), as well as the colocalization between these two tight junction proteins following colocalization analyses.

TA-induced changes to the functional profile of HT-29 cells are not altered by E2, despite baseline differences



Supplementary Figure 3.2: Effect of low (0.1 nM) levels of 17β-estradiol (E2) treatment on the percentage of relative area of the imaged HT-29 cell monolayer that stained positive for tight junction proteins following selected TA-exposure: **(A, E & I)** β-phenylethylamine (PEA), **(B, F & J)** tryptamine (TRP), **(C, G & K)** ρ-tyramine (TYR), and **(D, H & L)** agmatine (AGM). **(A to D)** Effect on colocalization of occludin and ZO-1, **(E to H)** effect on total occludin, and **(I to L)** effect on total ZO-1 signal. The data is represented as mean ± SEM. Statistical analysis: 2-way ANOVA's with Šídák's multiple comparisons tests: * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001. Asterisk's (*) represent TA dose effect under the same E2 condition, while hashtags (#) represent effect of E2.

Chapter 4

Rooibos (*Aspalathus linearis*) alters secretomes trace amine profile of probiotic and commensal microbes *in vitro*.

This chapter has been published in the Journal of Ethnopharmacology (Impact Factor: 5.195).

Citation reference: Pretorius, L., Van Staden, AD., Kellermann, TA., Henning, N., Smith, C. (2022) Rooibos (*Aspalathus linearis*) alters secretomes trace amine profile of probiotic and commensal microbes *in vitro*. *J Ethnopharmacol.* 297:115548.
https://doi.org/10.1016/j.jep.2022.115548

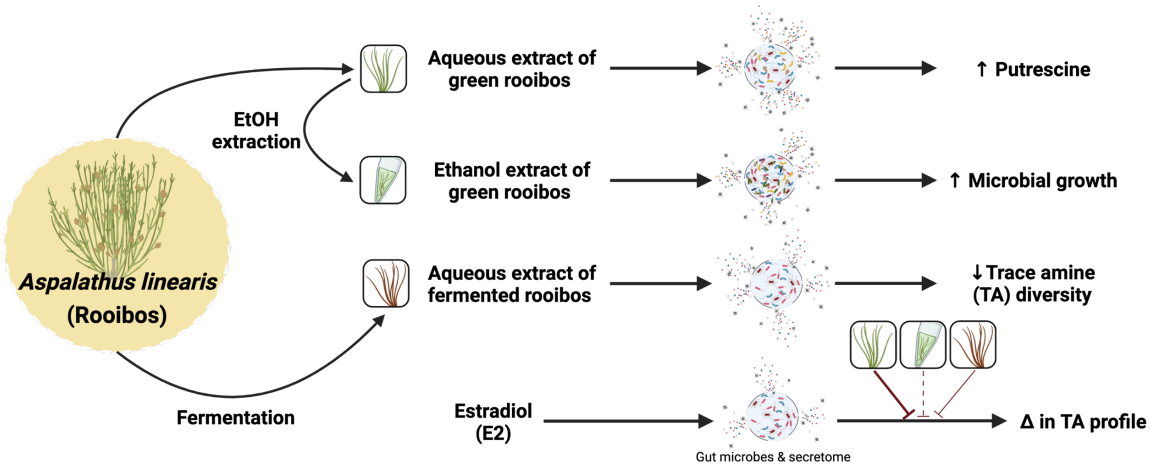
4.1 Abstract

Ethnopharmacology relevance
Aspalathus linearis (Burm.f.) R. Dahlgren (rooibos) tea is anecdotally renowned for its calming effect in the context of gastrointestinal discomfort, but little scientific support is available to elucidate potential mechanisms of action. Enhancement of dietary polyphenol content to improve gut health *via* prebiotic-like modulation of the gut microbiota has gained significant research interest. Given the known high polyphenol content of rooibos, rooibos tea may potentially exert a prebiotic effect in the gut to facilitate an improvement in chronic inflammatory gastrointestinal conditions.
Aim of the study
This study aimed to determine the prebiotic or health-modulating potential of rooibos tea in terms of its effect on gut microbial growth and secretome trace amine composition, as well as to determine how differential rooibos processing alters this activity.
Methods
Three rooibos preparations (green and fermented leave aqueous extracts, as well as a green leaf ethanol extract) were compared in terms of their phenolic composition (qTOF-LC/MS). Moreover, the effect of rooibos exposure on growth and secretome trace amine levels of probiotic and commensal microbes were assessed (LC/MS). In addition, given the known female bias prevalent for many gastrointestinal disorders, experiments were conducted in the absence and presence of estradiol.
Results
Polyphenolic composition of rooibos was drastically reduced by fermentation. Aqueous extracts of both green and fermented rooibos improved microbial growth, although fermented rooibos had the most pronounced effect ($p<0.01$). In terms of secretome trace amine profile, both aqueous extracts of rooibos seemed to facilitate increased putrescine secretion ($p<0.0001$) and decreased tryptamine production ($p<0.0001$). Estradiol seemed to suppress trace amine secretion by bacteria (*Lactobacillus plantarum, Lactobacillus reuteri* and *Enterococcus mundtii*) but increased it in yeast (*Saccharomyces boulardii)*.

Conclusion

Rooibos altered gut probiotic and commensal microbial growth and secretome trace amine profiles *in vitro*, suggesting it has potential to modulate gut microbial composition and functionality as a prebiotic. Current data suggest that these effects are highly dependent on raw material processing. Finally, rooibos may be able to prevent estradiol-induced alterations in trace amine profile, which may have important implications for patient management in female-predominant gastrointestinal disorders.

Graphical abstract



4.2 Introduction

Aspalathus linearis (Burm.f.) R. Dahlgren (rooibos), a well-known antioxidant herbal tea, has gained popularity for its potential health promoting properties – which is mainly ascribed to its diverse polyphenol content. Indeed, rooibos contains numerous polyphenols, including dihydrochalcones (aspalathin & nothofagin), flavones (luteolin, chryoeriol, (iso)-orientin & (iso)-vitexin), flavanones ((R)- and (S)-eriodictyol-6-C-glucoside, and (R)- and (S)-eriodictyol-8-C-glucoside), flavonols (quercetin, rutin & hyperoside), lignans and hydroxycinnamic derivatives (Malongane et al., 2018, Stander et al., 2017, Walters et al., 2017), for which various health benefits have been described (Smith and Swart, 2018). Rooibos tea is commercially distributed in two forms, unfermented or green, and fermented – the latter of which has a substantially reduced polyphenol content (Stalmach et al., 2009) as a result of the fermentation process.

Traditionally, among many other uses, rooibos tea is consumed to aid digestive problems/stomach cramps and nervous tension (Mahomoodally, 2013, Viljoen et al., 2022). In this regard, the antispasmodic actions of rooibos tea are reportedly mediated through the activation of K^+ ion channels (Gilani et al., 2006), warranting the use of rooibos tea in hyperactive gastrointestinal disorders, however, very little research is available in this context.

More specifically, rooibos has been attributed moderate phytoestrogenicity (Shimamura et al., 2006). This warrants investigation of rooibos supplementation in female predominant gastrointestinal disorders, such as irritable bowel syndrome (IBS).

In this context, it is well-known that the gut microbiome plays a fundamental role in both maintenance of health, as well as in the pathogenesis of various diseases (Lavelle and Sokol, 2020, Lobionda et al., 2019, Valdes, 2018, Zheng et al., 2020). As such, an individualised approach toward nutrition and medication – as potential substrates for microbes – is becoming increasingly important in modulating the gut microbiome (Milutinovic et al., 2021). Indeed, despite the complexity and variability of this interplay, recent research clearly shows that the interaction between the gut microbiota and undigested foods can yield various metabolites that could promote or jeopardise human health (Koh and Backhed, 2020), seeing that signals from microbial metabolites influence immune maturation and homeostasis, host energy metabolism and maintenance of mucosal integrity (Lavelle and Sokol, 2020), to name a few. Many of these dietary substrates are prebiotics, which are selectively utilized by host colonic microbes conferring health benefits, by promoting the growth and the activity of these beneficial bacterial strains. Until recently, the prebiotic concept was limited to select non-digestible carbohydrates. However, phytochemicals, particularly polyphenols, also reportedly exert potentially prebiotic effects (Rodriguez-Daza et al., 2021). Thus, enhancement of dietary polyphenol content to modulate the gut microbiome has potentiality to improve health and prevent disease.

In terms of specific candidate prebiotics, polyphenols are a varied class of secondary plant metabolites, which are found in most healthy diets (present in vegetables, fruits, spices, and medicinal plants). Owing to their chemical structure, they are poorly absorbed (<5 to 10 % of the ingested polyphenols are resorbed in the small intestine) and thus reach the colon, where they are bio-transformed by the resident microbes (Ozdal et al., 2016). As reviewed by Rodriguez-Diaz et al., (2021), the direct beneficial effect of polyphenols on the gut microbiota relies on two main modes of action, namely the simultaneous antimicrobial effect on potential pathogens, and the stimulation of beneficial microbes. Specifically, polyphenols can stimulate several keystone bacterial species such as *Bacteroides thetaiotaomicrom*, *Faecalibacterium prausnitzii*, *Akkermansia muciniphila*, and Bifidobacteria and Lactobacilli spp. (Anhê et al., 2015, Gonzalez-Sarrias et al., 2018, Rodriguez-Daza et al., 2020, Roopchand et al., 2015) to have a positive impact on gut health (Catalkaya et al., 2020).

The use of tea to modulate the gut microbiota is not a new notion. In fact, a review by Liu et al. (2020), suggested that teas or tea constituents (tea liquids, tea extracts, polyphenols,

polysaccharides and teasaponin) of *Camellia sinensis* (green tea, oolong tea, Fuzhuan brick tea and Pu-erh tea) possess a prebiotic-like effect which can alleviate gastrointestinal dysbiosis. The authors further suggested the potential of strategically selected tea extracts to be developed into functional foods. In line with this, we proposed that rooibos and its polyphenol constituents could similarly regulate the gut microbiota. In support of this, a study in vervet monkeys suggested that green rooibos and its major bio-active phenolic compound, aspalathin, enhanced the relative abundance of health promoting butyrate-producing bacteria such as *Faecalibacterium prausnitzii*, *Prevotella stercorea* and *P. copri* (Mangwana, 2020).

This study aimed to add to this literature by determining the prebiotic or health-modulating potential of rooibos tea in terms of its effect on gut microbial growth and secretome composition. Specifically, probiotic strains *Lactobacillus plantarum*, *Enterococcus mundtii* and *Saccharomyces boulardii*, as well as a commensal strain of *Lactobacillus reuteri*, were cultured in the absence or presence of three different rooibos preparations (aqueous extracts from unfermented and fermented leaves, as well as an ethanol extract from unfermented leaves), and their growth and secretome trace amine (TA) content assessed. TA content was decided on as a marker of functional capacity since microbial conversion of dietary substrates (amino acids) into small bioactive molecules (TAs) represents a potential regulatory mechanism by which gut microbes alter intestinal physiology (Bhattarai et al., 2018, Han et al., 2021, Lee et al., 2020b, Neis et al., 2015, Santoru et al., 2017). In addition, the different rooibos preparations (aqueous extract of raw green rooibos (GR), aqueous extract of raw fermented rooibos (FR), and ethanol extract of green rooibos (GRE)) were compared in terms of their phenolic composition. Lastly, given the exacerbated symptomology associated with cyclic fluctuations of estradiol (E2) in female predominant gastrointestinal disorders – such as IBS (Heitkemper and Jarrett, 2008, Mulak et al., 2014) – and our previous report of estrogen-mediated changes in secretome TA levels (Pretorius et al., 2022b), the effect of rooibos to counter E2 effects were investigated.

4.3 Methods and materials

4.3.1 List of reagents

Reference and deuterated standards purchased from Toronto Research Chemicals (Canada) include β-phenethylamine (PEA; P321335, CAS 64-04-0), tryptamine (TRP; T894600, CAS 61-54-1), ρ-tyramine (TYR; T898493, CAS 60-19-5), phenethylamine-d4 (PEA-d4; P321336, CAS 87620-08-4) and putrescine-d8 (PUT-d8; D416027, CAS 709608-92-4). In addition, reference standards for agmatine (AGM; A7127, CAS 2482-00-0) and putrescine (PUT;

D13208, CAS 110-60-1) were purchased from Merck/Sigma, as well as estradiol (E2; E2758, CAS 50-28-2), L-phenylalanine (P17008, CAS 63-91-2), L-tryptophan (T0254, CAS 73-22-3), L-tyrosine (T3754, CAS 60-18-4), L-arginine (A5131, CAS 1119-34-2), DMSO (276855, CAS 67-68-5), TRIS base (T1503, CAS 77-86-1), D-(+)-glucose (G7528, CAS 50-99-7), de Man, Rogosa & Sharpe broth; (MRS; 69966), M17 broth (56156) and Yeast peptone glucose broth (YPD; Y1375). For LC/MS analyses Methanol 215 (MeOH; H409L, CAS 67-56-1) and acetonitrile (H048L, CAS 75-05-8) were purchased from ROMIL pure chemistry (Cambridge), while the ammonium acetate (A114-50, CAS 631-61-8), ammonium formate (All550, CAS 540-69-2), and formic acid (FA; A117-50, CAS 64-18-6) utilised were purchased from Fisher chemical (USA).

4.3.2 Rooibos preparations

Both unfermented (green) and fermented rooibos (or *Aspalathus linearis* (Burm.f.) R. Dahlgren (official name according to http://www.theplantlist.org (access date: 07/06/2022)) were assessed. Unfermented and fermented leaves from the same raw plant source material, as well as an ethanol extraction from the unfermented leaves, were kindly donated by Mr Roy van Brummelen (Van Brummelen Consultants, Pretoria, South Africa). Water extracts of green (GR) and fermented (FR) rooibos were obtained by steeping raw intact tea leaves in boiling water for 10 min (hot plate with a stirrer bar), before vacuum filtration (Whatman filter paper) and centrifugation at 10 000 rpm were utilised to remove any sediment. The resultant tea supernatant was concentrated and filter sterilized (0.22 μm pore size) to create working stocks. The final experimental treatment concentration was equivalent to 8-fold the concentration of rooibos tea normally consumed (which is prepared by steeping 2.5 g of tea leaves per 200 mL boiling water for a few minutes). To obtain this 8-fold concentration aqueous extracts were prepared at 10% $^{m}/_{v}$ and GRE was prepared at 100 μg/mL (reconstituted in DMSO and diluted in water), which we have previously determined to also equate to an 8-fold concentration of tea in terms of polyphenol content (Lopez et al., 2022). The decision to utilise a single concentration of each rooibos preparation was based on previously generated data by our group and others. Firstly, from human supplementation studies with a chronic administration design, it has been concluded that less than 6 cups of rooibos per day is not enough to result in significant antioxidant benefit - although it was able to significantly reduce cortisol synthesis in humans and rodents (Schloms et al., 2014),suggesting mild anti-inflammatory outcome. This is in line with literature suggesting the use of at least 6-8 cups of tea as daily supplement (Breiter et al., 2011, Marnewick et al., 2011, Rodgers et al., 2016). Secondly, the dose chosen here, which equates to 8 cups of tea in terms of polyphenol content, has been shown to exert sufficient antioxidant capacity to protect

human neuronal cells from H_2O_2-associated oxidative damage in cell culture models (Lopez et al., 2022), which by nature has a more acute design and is more similar to the design of the current study. Thirdly, dose-responses performed in HT-29 colon cells (Supp. Fig 5.2) – the cell type commonly used for research focused on gut heath - confirmed optimal cell viability at our selected dose, supporting our decision.

4.3.3 Quantification of phenolic constituents

We employed the method described by Stander et al. (2017) to quantify fifteen major phenolic constituents commonly found in rooibos tea. Briefly, tea samples were extracted with 50% MeOH and 1% FA. A Waters Synapt G2 Quadrupole time-of-flight (q-TOF) mass spectrometer (MS) connected to a Waters Acquity ultra-performance liquid chromatograph (UPLC) was used for high-resolution UPLC-MS analysis (CAF, Stellenbosch University). Chromatographic separation was achieved on a Waters HSS T3 column (1.7 µm, 2.1 × 100 mm), and the column temperature was maintained at 55 °C. For all samples an injection volume of 2 µL was used and run using a binary mobile phase gradient which consisted of (A) 0.1% FA in H_2O and (B) 0.1% FA in acetonitrile. The flow rate was set to 0.3 mL/min throughout the set 29 min run time with the following separation conditions: the gradient started at 0% solvent B for 1 min and increased to 28% B over 22 mins in a linear way. It was then increased to 40% solvent B until 22.5 min, followed by an increase to 100% solvent B until 23 min. Solvent B was held at 100% until 24.5 min, before being reduced to 0% solvent B to re-equilibrate to initial conditions for the final 4.5 min. Electrospray ionization was used in negative mode with a cone voltage of 15 V. Constituents were identified according to their accurate mass, MS/MS fragments, UV maxima and retention times as previously described (Stander et al., 2017) and quantified relative to rutin reference standards.

4.3.4 Microbial growth curves

To assess the effect of the different rooibos preparations on microbial growth, bacterial cultures (*L. plantarum* 423, *L. reuteri*, and *E. mundtii* ST4SA) were inoculated from frozen 40% glycerol stocks into MRS broth (51 g/L) and incubated at 37°C overnight (O/N), before being streaked out onto MRS agar (MRS with 1.5% $^{w}/_{v}$ agar) and incubated at 37°C until visible colonies formed (24 to 48 h). MRS broth was inoculated with a single colony of the respective bacteria and incubated at 37°C (biological repeat = different colony) and this was used for subsequent experiments. For the growth curves, rooibos preparations were added to filter sterilized M17 media (42 g/L, supplemented with 0.5% glucose) to a final 1/3 ratio (control: tea replaced with ultrapure milli-Q water). The various rooibos supplemented media preparations were inoculated at 1% (v/v) with respective O/N bacterial cultures. Inoculated preparations

(150 uL/well) were pipetted into 96-well microtiter plate, and at respective time points the total volume of wells were removed and used to determine optical density (OD) at 595 nm. Samples were diluted as needed to keep values below 1.0.

Similarly, *S. boulardii* 17 was streaked out from frozen 40% glycerol stocks into YPD broth (Yeast extract 10 g/L, peptone 20 g/L and 20 g/L glucose) and incubated at 30°C O/N, before being streaked out onto YPD agar (YPD with 1.5% $^{w}/_{v}$ agar) and incubated at 30°C until visible colonies formed (24 to 48 h). Separate colonies were used to inoculate YPD broth and incubated at 30°C for 48 h. These cultures were used to inoculate tea media preparations as above with YPD (instead of M17) used as the base media. The inoculated preparations were used to fill the wells of a 96-well microtiter plate (200 uL/well). OD readings were recorded as above.

4.3.5 Microbial secretome analyses

As mentioned previously, TA content was chosen as a marker of functional capacity since these microbially-derived bioactive molecules regulate intestinal physiology. In line with our theory of TA involvement in IBS and its female bias (Pretorius and Smith, 2020), we have decided to assess the effect of different rooibos preparations on β-phenethylamine (PEA), tryptamine (TRP), ρ-tyramine (TYR), agmatine (AGM) and putrescine (PUT) generation (from amino acid precursors) and secretion by representative gastrointestinal microbes. These TAs were specifically selected since they have known roles in the gastrointestinal tract (GIT), and/or have been implicated in dysbiosis or gastrointestinal disorders and are readily formed from precursor L-amino acids which are in rich supply in the gut.

Conditioned media: Briefly, *L. plantarum, L. reuteri, E. mundtii,* and *S. boulardii* were cultured to stationary phase (overnight) under optimal conditions. Each culture was pelleted and washed once in phosphate buffered saline (PBS; pH 7.2), before being resuspended in an equal volume of pH 7.4 minimal media solution (0.2 M TRIS HCl, 5% $^{m}/_{v}$ glucose, and 1 mg/mL amino acid mix: L-phenylalanine, L-tryptophan, L-tyrosine, and L-arginine), and supplemented with either 10% $^{m}/_{v}$ GR, 10% $^{m}/_{v}$ FR, 100 ug/mL GRE or no rooibos (vehicle control). Additionally, all experiments were conducted in the absence or presence of 1 nM E2 in the minimal media phase. Resuspended cultures were incubated at 37 °C for 24 h in 12-well plates, before supernatants were collected and stored in aliquots at -80 °C for batch analysis.

Sample preparation and extraction: To quantify TA levels in microbial conditioned media a protocol from D'Andrea et al. (2019) was adapted. Briefly, Supelclean™ LC-WCX SPE cartridges (Supelco, 505595) were conditioned with 1 mL absolute MeOH, followed by 2 equilibrations with 1 mL 50 mM ammonium acetate pH 5. The samples, quality controls, blanks

and calibrators were buffered (200 μL: 800 μL) with 50 mM ammonium acetate pH 5. Loaded samples (with the exception of the double blank) were spiked with 20 μL internal standard mix containing 1.25 μg/mL phenethylamine-d4 and 10 μg/mL putrescine-d8. Cartridges were washed with 1 mL 5% MeOH in H_2O, 1 mL 10% MeOH in H_2O and finally 1 mL 20% MeOH in H_2O, before eluting twice with 1 mL acetonitrile containing 5% FA. The eluent was evaporated to dryness under vacuum utilising the Genevac miVac Duo Sample Concentrator at 40°C. Samples were reconstituted in 500 μL MeOH:H_2O with 0.1% FA, and analysed on the LC-MS/MS in 96-well plates.

LC-MS/MS analysis: The method was developed on the SHIMADZU 8040 LC-MS system. For chromatographic separation samples were injected onto an Agilent Poroshell 120 EC-C18 column (2.7 μm, 3.0 x 100 mm) at a flow rate of 0.4 mL/min. The column oven temperature was maintained at 30°C. For all samples an injection volume of 5 μL was used and run using a binary mobile phase gradient which consisted of (A) 0.1% FA and 5 mM ammonium formate in Milli-Q water (Synergy® polished) and (B) 0.1% FA and 5 mM ammonium formate in 95% Methanol and 5% Synergy® polished Milli-Q. Mobile phases were controlled to elute in a gradient as follows: hold mobile phase B at 5% for 30 sec, increase mobile phase B to 95% until 4 min, hold mobile phase B at 95% until 5 min, followed by reduction to 5% at 6 min. The system was re-equilibrated at 5% mobile phase B until 9 min. The acquisition was set in positive electrospray ionisation mode with multiple reaction monitoring (MRM) using Argon as the collision-induced dissociation gas (see Table 4.1 for MRM conditions). Instrument control, acquisition and the analysis of data was provided by LabSolutions Version 5.97 software (Shimadzu Corporation). For TA quantification, calibration curves were established with commercially available TAs. Integrated data were exported to Microsoft Excel for further analysis.

4.3.6 Statistical analyses

All microbial experiments were conducted in triplicate and repeated at least three times. Triplicate values were averaged to yield a final n=3 for all data points presented. Statistical analyses of all data were completed utilizing GraphPad Prism Version 9.1.2 (www.graphpad.com, San Diego, CA). Microbial growth curve data are represented as mean ± standard deviation (SD) over time, while TA quantifications from secretomes are represented as both relative distribution (mean % of total TA output per organism), as well as absolute (mean ± standard error of mean (SEM) concentration. Statistical analyses included 2-way ANOVAs with Tukey's multiple comparison tests for both the microbial growth curve

data, as well as for the TA quantification from secretome data set. A p-value of < 0.05 was considered statistically significant.

Table 4.1: Multiple reaction monitoring (MRM) conditions using Argon as collision-induced dissociation gas, for detection of trace amines in the positive electrospray ionisation mode on LC-MS/MS.

Analyte	Retention time (min)	MRM transition
ρ-Tyramine (TYR)	2.850	138,1000 → 121,0500 138,1000 → 77,000* 138,1000 → 91,000
β-Phenylethylamine (PEA)	3.720	122,1000 → 105,0500* 122,1000 → 77,0000 122,1000 → 51,0000
Tryptamine (TRP)	3.961	161,1078 → 144,1000* 161,1078 → 117,0500 161,1078 → 115,0000
Agmatine (AGM)	1.204	130,9500 → 72,0500* 130,9500 → 59,9500 130,9500 → 113,9000
Putrescine (PUT)	1.130	89,0000 → 72,1500*
Phenylethylamine-d4 (PEA-D4)	3.711	126,2000 → 109,0500* 126,2000 → 79,1000 126,2000 → 51,9000
Putrescine-d8 (PUT-D8)	1.134	97,1500 → 80,1000*

* MRM transition used for quantification

4.4 Results

4.4.1 Effect of fermentation and extraction on the composition of rooibos tea

Initial q-TOF data (Supplementary Table 4.1 to 4.6) highlighted that GR had a more diverse polyphenol content than both FR and GRE preparations, such as the presence of epicatechin for example. However, due to the lack of rooibos-specific constituents in available MSP spectral libraries, a more targeted approach was employed to quantify fifteen major phenolic constituents found in rooibos (chromatograms of samples available in supplementary material). From these results, GR had the highest aspalathin and nothofagin content, as expected (Fig. 4.1). Fermentation most notably lowered the levels of rutin, aspalathin and nothofagin, along with slight reductions in the levels of isoorientin, vicenin-2, vitexin, orientin, isovitexin, phenylpropenoic acid glucoside (PPAG) and quercetin-3-O-robinobioside, when compared to both the original levels found in GR and those found in the GRE (Fig. 4.1). However, FR had the highest levels of all eriodyctiol derivatives and isomers, increasing its total flavanone content compared to the other tea preparations. This is in line with literature reporting that oxidation of aspalathin during fermentation increases both dihydro-isoorientin ((R)- and (S)-eriodictyol-6-C-glucoside) and dihydro-orientin ((R)- and (S)-eriodictyol-8-C-

glucoside) (Krafczyk and Glomb, 2008). Moreover, extraction of GR to formulate the GRE particularly concentrated orientin and PPAG constituents, while also increasing the levels of iso-orientin, vicenin-2, luteolin-7-O-glucoside, and quercetin-3-O-robinobioside to higher levels than found in GR alone. In terms of approximate total polyphenol content of extracted tea samples, GR and GRE faired similarly with 460 μg/mL and 469 μg/mL respectively, while fermentation halved this, to a total of 224 μg/mL for FR.

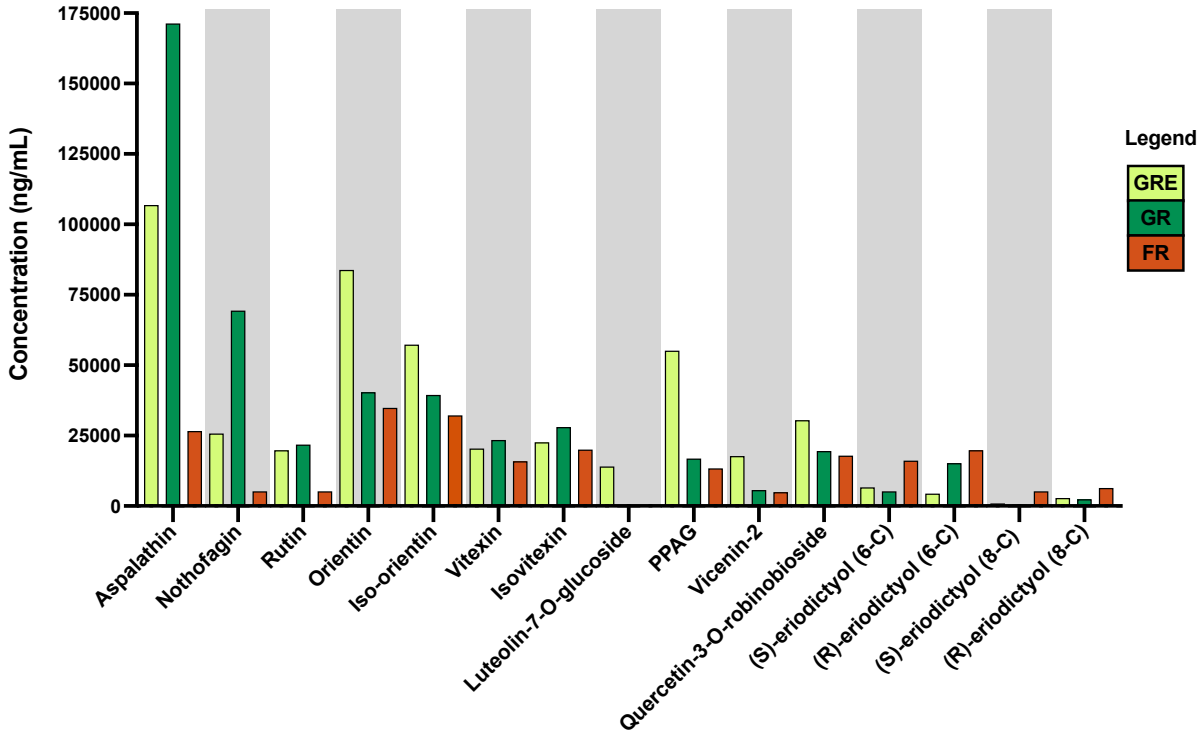


Figure 4.1: Changes in the concentration of fifteen major phenolic constituents between three rooibos samples: green rooibos ethanol extract (GRE), green rooibos aqueous extract (GR) and fermented rooibos aqueous extract (FR). Abbreviations: PPAG: phenylpropenoic acid glucoside

4.4.2 Effect of rooibos tea on microbial growth is dependent on its processing

According to growth curve data (Fig. 4.2), GR and FR improved the growth of all assessed microbes, while GRE has no lasting net effect on bacterial growth yet seemed to reduce the growth of yeast significantly compared to control ($p<0.001$). Interestingly, FR improved growth most significantly across the board for bacterial cultures at the final time point of assessment (A: $p<0.01$, B & D: $p<0.001$), while in the case of *S. boulardii*, both GR and FR improve growth equally well (Fig. 4.2 C, $p<0.0001$ for both).

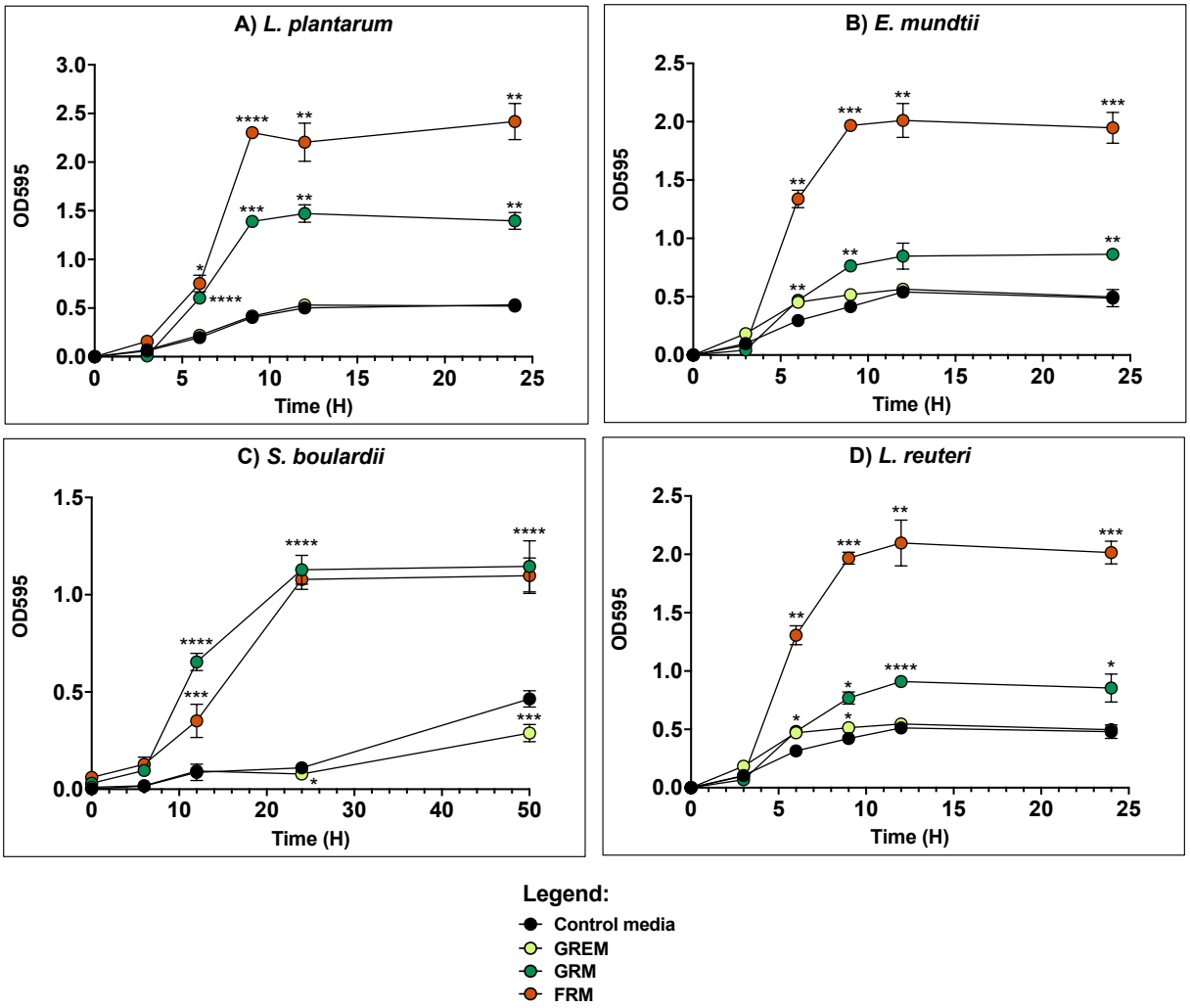


Figure 4.2: Effect of rooibos on the growth of (A) *Lactobacillus plantarum*, (B) *Enterococcus mundtii*, (C) *Saccharomyces boulardii*, and (D) *Lactobacillus reuteri*. Data is expressed as a mean ± SD, n=3. Statistical analysis: 2-way ANOVA with Dunnett's multiple comparison tests; * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$, compared to control media conditions. Abbreviations: GREM: green rooibos ethanol extract enriched media, GRM: aqueous green rooibos enriched media, FRM: aqueous fermented rooibos enriched media, OD: optical density.

4.4.3 Effect of rooibos on microbial secretome trace amine profile

Alike to the growth curve data, TA secretion patterns were hugely dependent on preparation of the rooibos treatments. In Figures 4.3, 4.5, 4.7 and 4.9, the relative distribution of TAs in microbial secretomes under different conditions are presented. To aid with interpretation, the area of pie charts are indicative of total TA levels measured *for a particular microbe*, to allow for simultaneous visualisation and comparison of both absolute production and relative distribution of TAs. Under control conditions these total levels are 186 828 ± 778 ng/mL for *E. mundtii* – as the most predominant TA producer assessed - as well as 14 553 ± 7188 ng/mL for *L. reuteri*, 165 ± 53 ng/mL for *S. boulardii* and 56 ± 12 ng/mL for *L. plantarum*. In addition, absolute concentrations of TA detected are also presented graphically in Figures 4.4, 4.6, 4.8

and 4.10 to allow for within-microbe, TA-specific analysis of treatment (E2 and rooibos) effects.

In the case of *L. plantarum*, under control conditions (Fig. 4.3A), TYR made up the highest proportion (66.49 %) of the total TAs secreted by the microbes, followed by TRP, AGM and PEA. Treatment with GRE (Fig. 4.3C) reduced TA diversity and slightly lowered total absolute production due to significantly reduced TRP production ($p<0.0001$) and the loss of PEA and AGM when compared to control (Fig. 4.4). However, both aqueous extracts had a significant effect on both absolute TA production and TA profile (main ANOVA effect for rooibos, $p<0.0001$). Firstly, GR treatment (Fig. 4.3E) altered the relative distribution of the secreted TAs, with 71.85 % of the total contribution consisting of PUT, while only 21.52 % was from TYR. Treatment with GR also increased total secretome TA content most significantly (10.28-fold) when compared to control. This increase was associated with a remarkable increase in PUT levels ($p<0.0001$), although significant increases in TYR, PEA and AGM were also observed ($p<0.0001$ for all), while TRP production decreased significantly compared to control ($p<0.05$) (Fig. 4.4). Similarly, treatment with FR (Fig. 4.3G) altered relative distribution of TAs to approximately even contributions from TYR (48.5%) and PUT (44.5%) and also increased (2-fold) the total TA output when compared to control, but less so than seen in GR. Most significant were increases in TYR ($p<0.01$), AGM ($p<0.05$) and PUT ($p<0.0001$) levels (Fig. 4.4), which as for GR, occurred at the cost of TRP, which decreased significantly when compared to control ($p<0.0001$). Regarding additional E2 treatment (Fig. 4.3B, D, H & F), specific effects of E2 on TA distribution were only observable under control conditions, where TYR, PEA and AGM production were lost in the presence of E2. In contrast, under rooibos-supplemented conditions, no significant effect of E2 on TA distribution was evident. However, E2 treatment did generally lower the total TA levels secreted when compared to their respective media controls (Fig. 4.4), which is supported statistically by main ANOVA effects for E2 regarding PEA, TRP and AGM production ($p<0.0001$ for all) by *L. plantarum*.

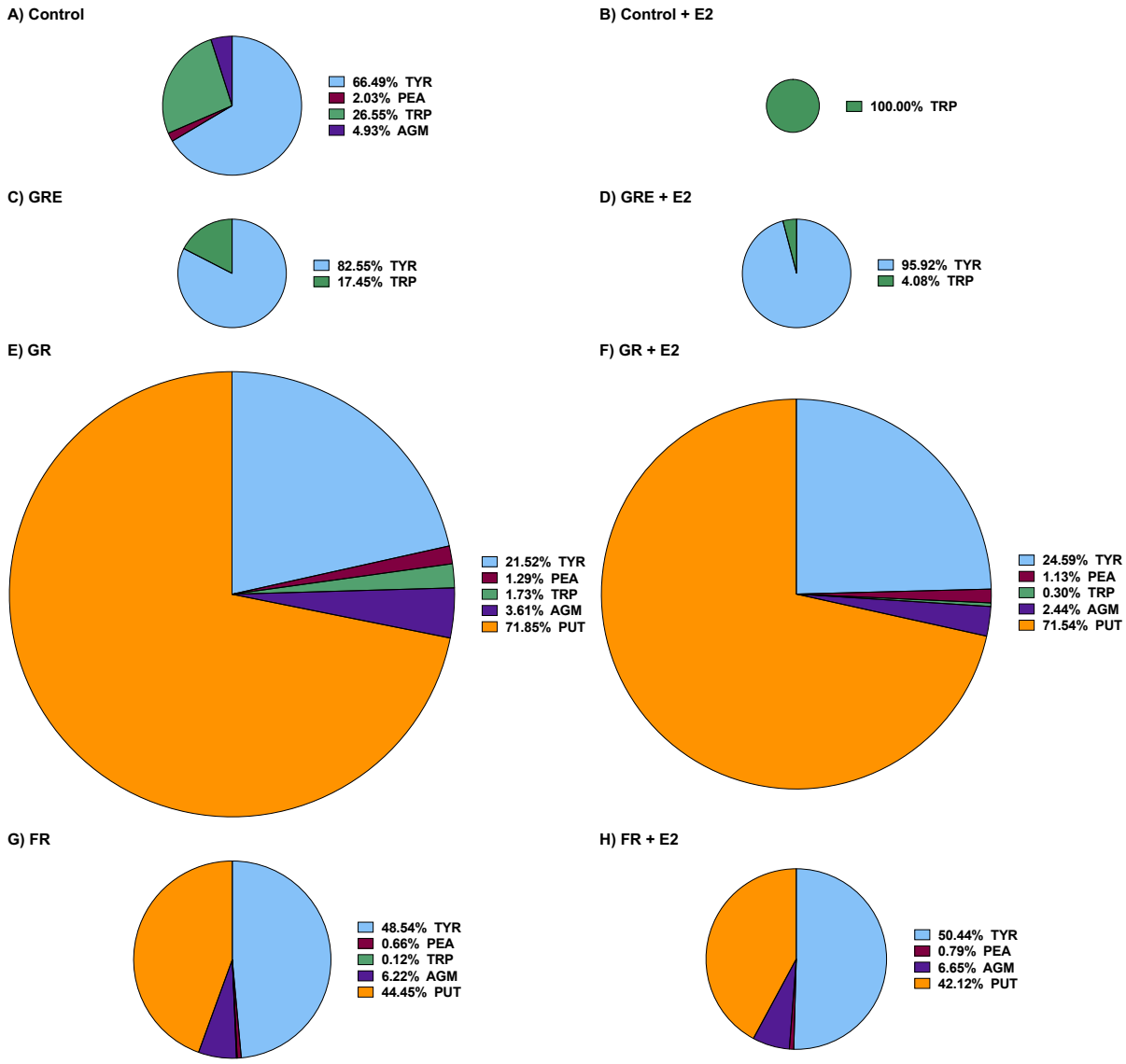


Figure 4.3: Changes in the relative distribution (% of total) of trace amines in the secretome of *Lactobacillus plantarum* following rooibos and estradiol (E2) treatment. Rooibos treatments were as follows: (A & B) control media, (C & D) green rooibos extract media (GRE), (E & F) green rooibos media (GR), and (G & H) fermented rooibos media (FR). E2 treatments were as follows: (A, C, E, G) control (no E2), and (B, D, F, H) 1 nM E2 supplementation. The area of each pie chart is indicative of the fold change (total TA content) expressed relative to control (A). Abbreviations: TYR: ρ-tyramine, PEA: β-phenethylamine, TRP: tryptamine, AGM: agmatine, PUT: putrescine.

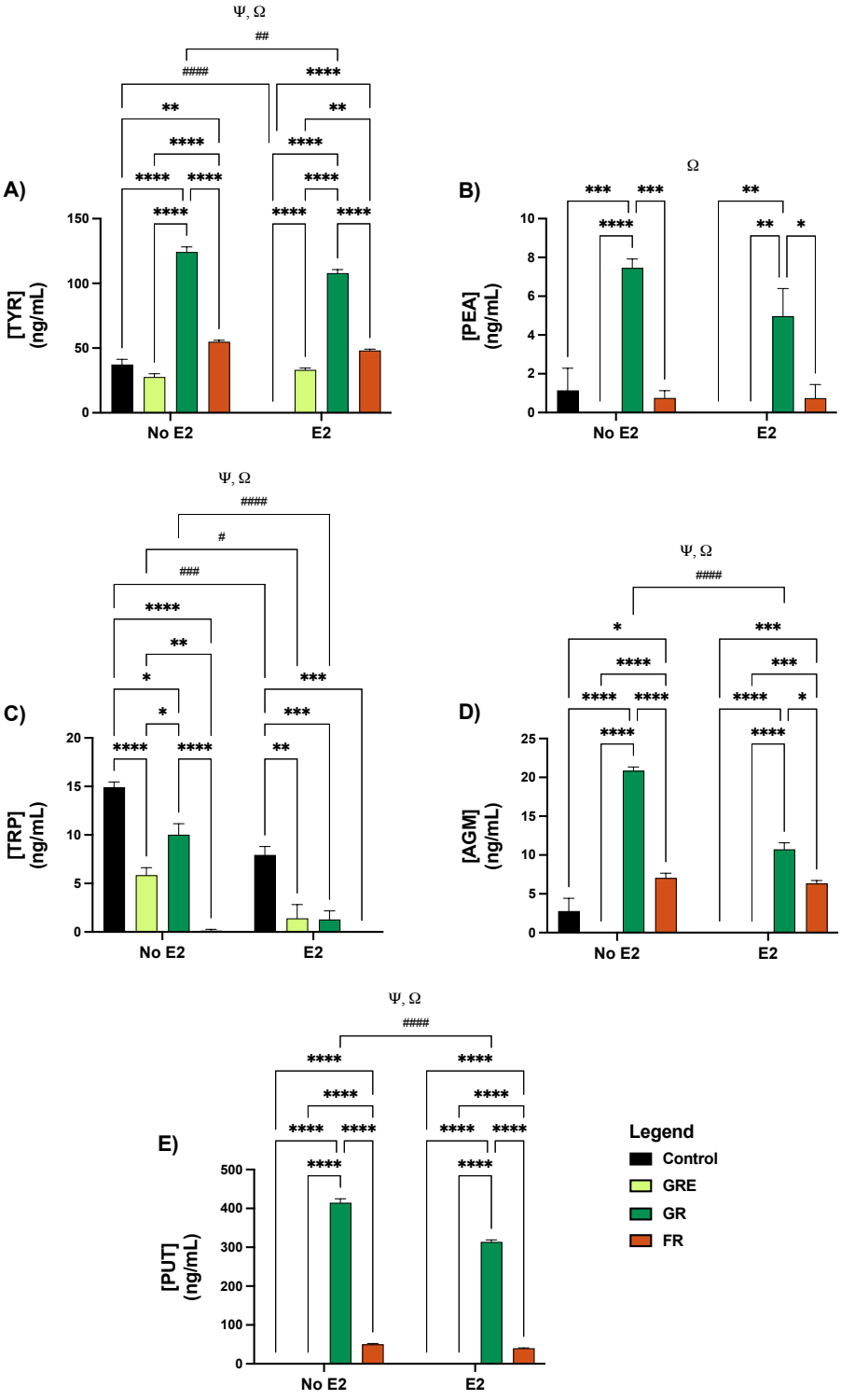


Figure 4.4: The effect of rooibos (GRE, GR and FR) and estradiol (E2) treatment on secretion of (A) ρ-tyramine (TYR), (B) β-phenethylamine (PEA), (C) tryptamine (TRP), (D) agmatine (AGM), and (E) putrescine (PUT) from *Lactobacillus plantarum*. The data are represented as the mean concentration of quantified trace amine (n=3) ± SEM. Statistical analysis: 2-way ANOVA with Tukey's multiple comparison tests; * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$, Ψ = main ANOVA effect of E2, Ω = main ANOVA effect of rooibos. Asterisk's (*) represent TA dose effect under the same E2 condition, while hashtags (#) represent effect of E2. Abbreviations: GRE: ethanol extract of green rooibos, GR: aqueous extract of green rooibos, FR: aqueous extract of fermented rooibos.

In contrast, total TA production by *E. mundtii* was only slightly modulated by rooibos treatments (Fig. 4.5), which may imply a reduced sensitivity towards modulation by rooibos. For this microbe, control conditions resulted in the most TA diversity and highest total TA secretion, with TYR making up 89.34 % of the total TA output. Notably, *E. mundtii* was the only microbe (of those assessed) to produce all selected TAs under control conditions. In the absence of added E2, treatment with GRE resulted in the loss of AGM and PUT ($p<0.0001$ for both) as well as significant absolute decreases in TYR ($p<0.05$) and TRP ($p<0.0001$) levels (Fig. 4.5C & Fig. 4.6). GR treatment reduced the secretion of TRP, AGM and PUT significantly ($p<0.0001$ for all) but seemed to maintain TA diversity – albeit at lower levels compared to control (Fig. 4.5E & Fig. 4.6). FR treatment was similarly associated with a decreased production of all TAs assessed ($p<0.0001$ for TRP, AGM and PUT), with the exception of TYR and PEA, which did not show significant decreases from control (Fig. 4.5G & Fig. 4.6). In most cases (except GRE), E2 treatment (Fig. 4.5) reduced total TA content (main effect of E2, $p<0.05$ for TYR & PEA, $p<0.0001$ for TRP, AGM & PUT, Fig. 4.6), and altered TA diversity (except GR) compared to respective media controls. In terms of magnitude of change, however, these findings suggest that *E. mundtii* TA production may be least sensitive to effects of E2, and rooibos of the microbes assessed.

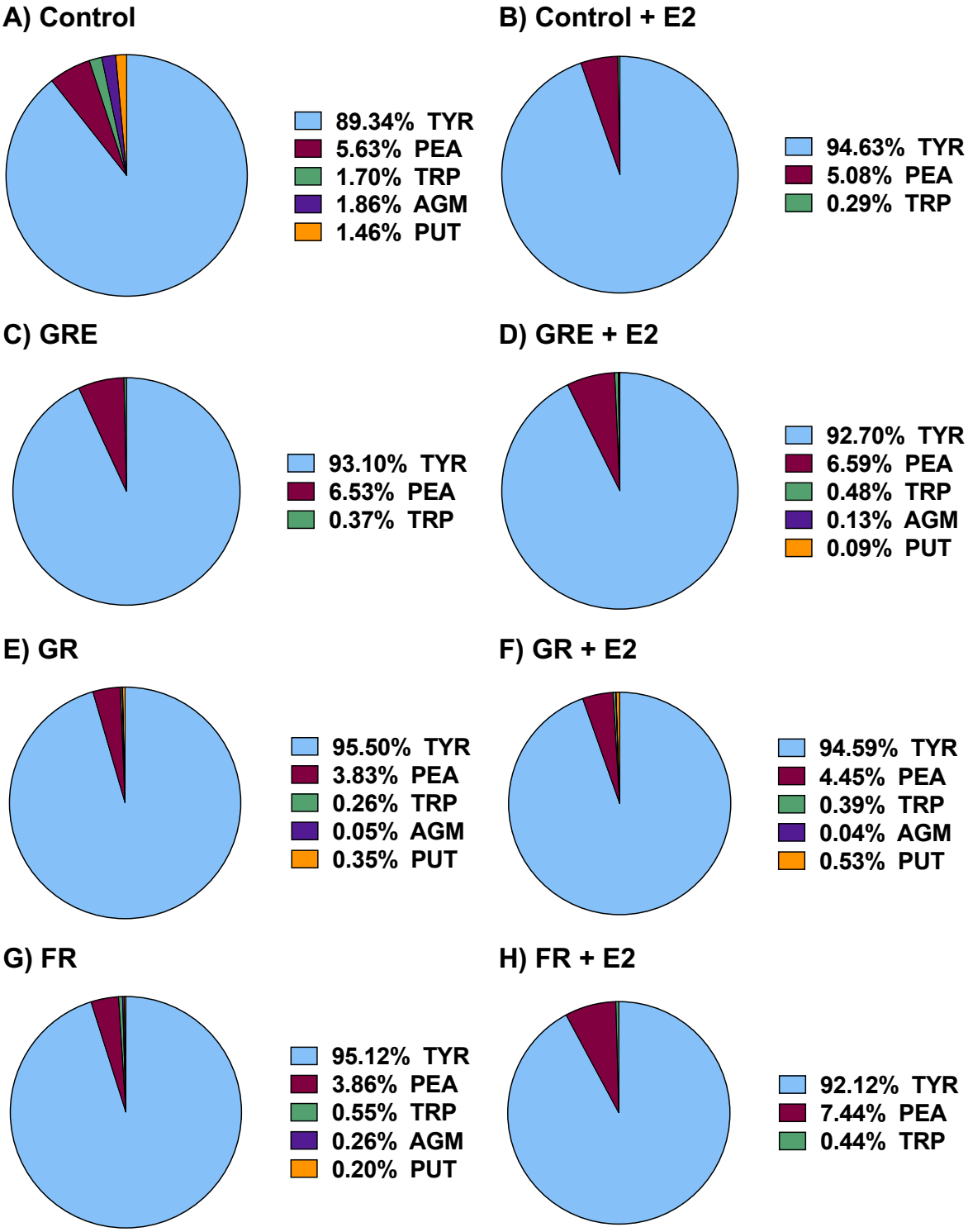


Figure 4.5: Changes in the relative distribution (% of total) of trace amines in the secretome of *Enterococcus mundtii* following rooibos and estradiol (E2) treatment. Rooibos treatments were as follows: (A & B) control media, (C & D) green rooibos extract media (GRE), (E & F) green rooibos media (GR), and (G & H) fermented rooibos media (FR). E2 treatments were as follows: (A, C, E, G) control (no E2), and (B, D, F, H) 1 nM E2 supplementation. The area of each pie chart is indicative of the fold change (total TA content) compared to control (A). Abbreviations: TYR: ρ-tyramine, PEA: β-phenethylamine, TRP: tryptamine, AGM: agmatine, PUT: putrescine.

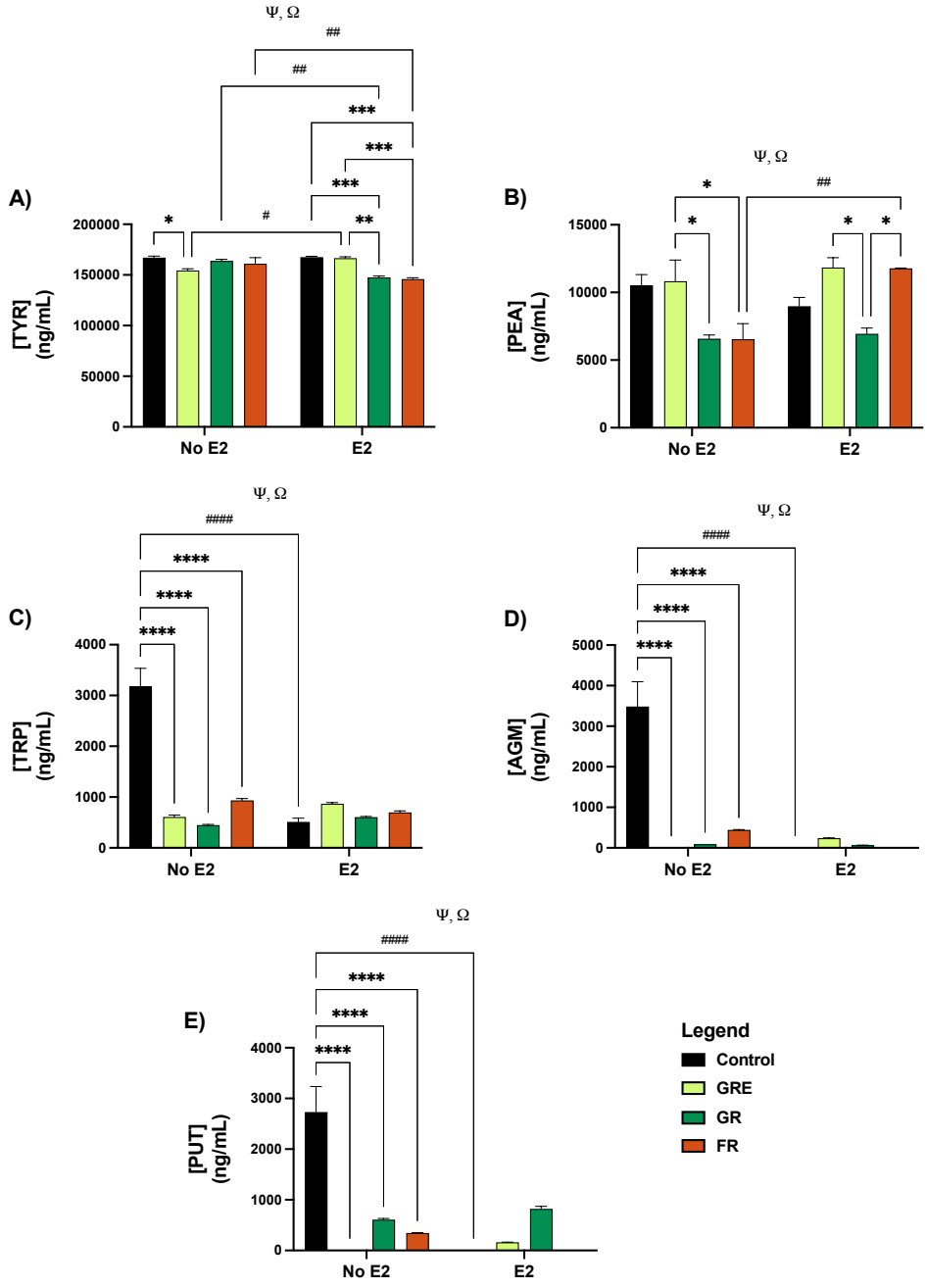


Figure 4.6: The effect of rooibos (GR, GRE and FR) and estradiol (E2) treatment on secretion of (A) ρ-tyramine (TYR), (B) β-phenethylamine (PEA), (C) tryptamine (TRP), (D) agmatine (AGM), and (E) putrescine (PUT) from *Enterococcus mundtii*. The data are represented as the mean concentration of quantified trace amine (n=3) ± SEM. Statistical analysis: 2-way ANOVA with Tukey's multiple comparison tests; * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$, Ψ = main ANOVA effect of E2, Ω = main ANOVA effect of rooibos. Asterisk's (*) represent TA dose effect under the same E2 condition, while hashtags (#) represent effect of E2. Abbreviations: GRE: ethanol extract of green rooibos, GR: aqueous extract of green rooibos, FR: aqueous extract of fermented rooibos.

Moving on to the response of *L. reuteri*, as for the other bacteria assessed, TYR again made up the highest proportion of the total TAs secreted (98.82%) under control conditions, followed

by relatively small amounts of TRP and PEA only (Fig. 4.7A). Treatment with GRE did not affect the relative TA distribution or total production. (Fig. 4.7C). In contrast, treatment with GR decreased the total TA output by 3.5-fold (Fig. 4.7E), due to less TYR (ns) and decreased in TRP ($p<0.0001$) secretion (Fig. 4.8C). Despite this decrease, GR treatment significantly increased AGM ($p<0.001$) and PUT ($p<0.0001$) secretion when compared to control (Fig. 4.8). FR treatment similarly resulted in a decrease in the total TA output (2.7-fold compared to control, Fig. 4.7G), again due to significant loss of TRP ($p<0.0001$) (Fig. 4.8C). In terms of E2 treatment (Fig. 4.7B, D, H & F), for all groups, TA diversity and relative distributions seemed unaffected, although, as for other bacteria, significantly reduced total TA levels were observed (3- to 20-fold; ANOVA main effect of E2, $p<0.01$ for TYR and $p<0.05$ for TRP, Fig. 4.8).

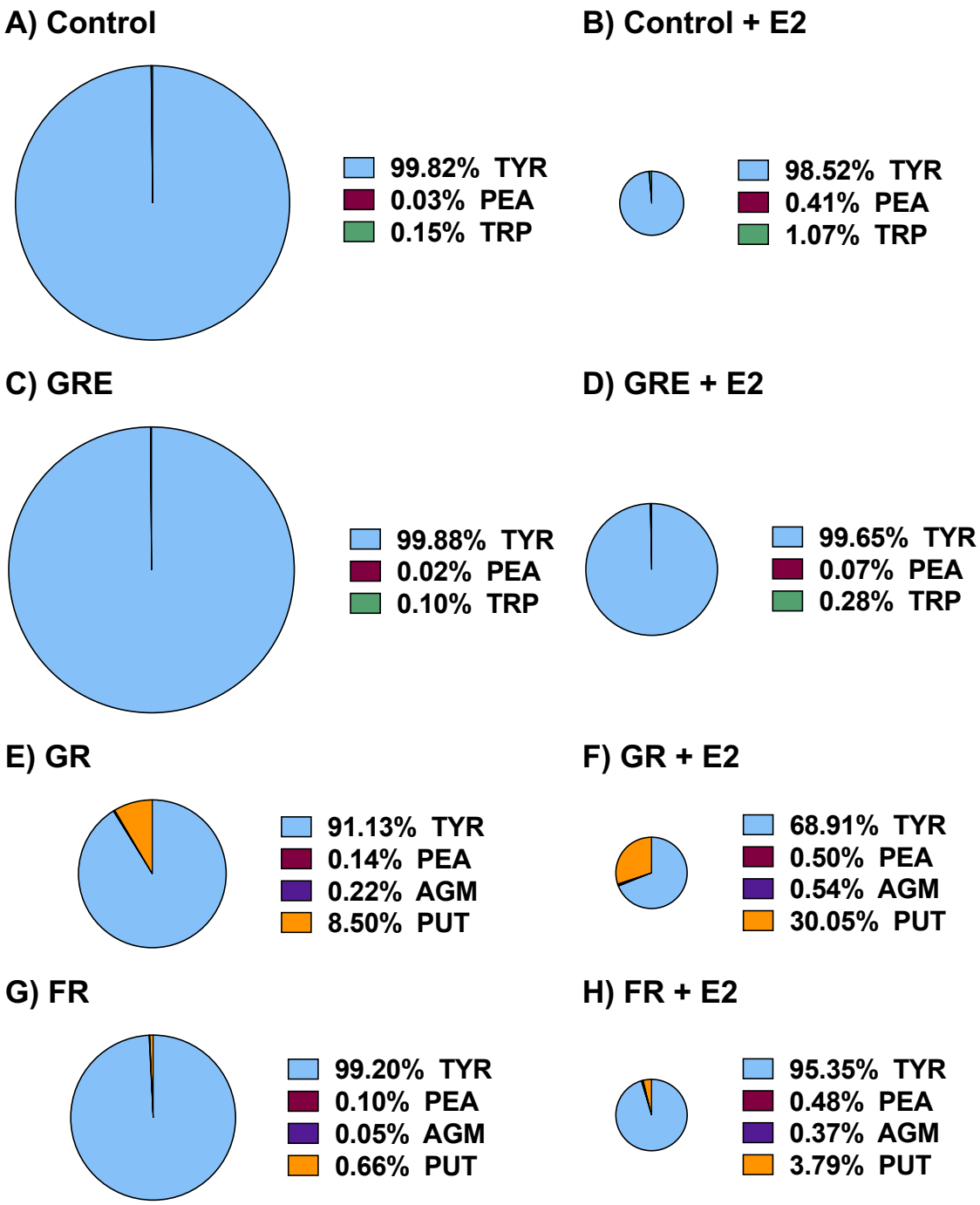


Figure 4.7: Changes in the relative distribution (% of total) of trace amines in the secretome of *Lactobacillus reuteri* following rooibos and estradiol (E2) treatment. Rooibos treatments were as follows: (A & B) control media, (C & D) green rooibos extract media (GRE), (E & F) green rooibos media (GR), and (G & H) fermented rooibos media (FR). E2 treatments were as follows: (A, C, E, G) control (no E2), and (B, D, F, H) 1 nM E2 supplementation. The area of each pie chart is indicative of the fold change (total TA content) compared to control (A). Abbreviations: TYR: ρ-tyramine, PEA: β-phenethylamine, TRP: tryptamine, AGM: agmatine, PUT: putrescine.

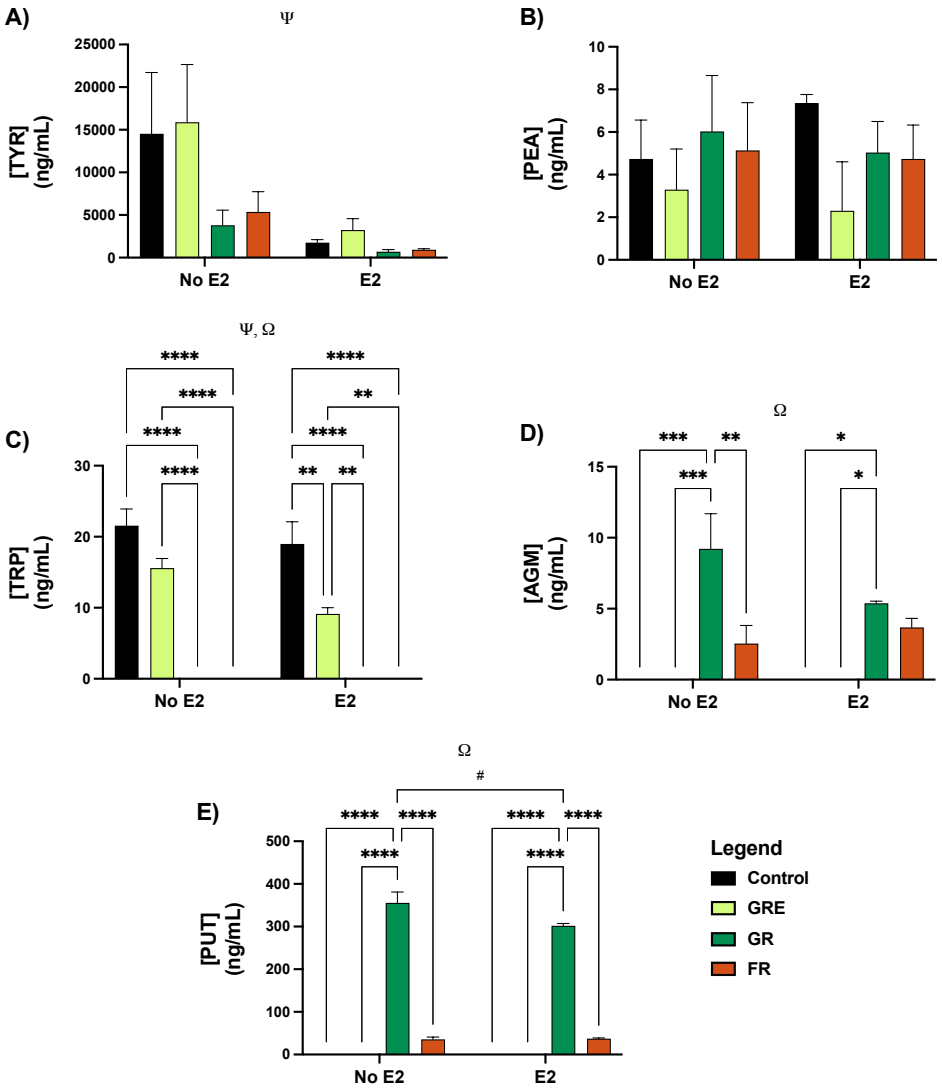


Figure 4.8: The effect of rooibos (GR, GRE and FR) and estradiol (E2) treatment on secretion of (A) ρ-tyramine (TYR), (B) β-phenethylamine (PEA), (C) tryptamine (TRP), (D) agmatine (AGM), and (E) putrescine (PUT) from *Lactobacillus reuteri*. The data are represented as the mean concentration of quantified trace amine (n=3) ± SEM. Statistical analysis: 2-way ANOVA with Tukey's multiple comparison tests; * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$, Ψ = main ANOVA effect of E2, Ω = main ANOVA effect of rooibos. Asterisk's (*) represent TA dose effect under the same E2 condition, while hashtags (#) represent effect of E2. Abbreviations: GRE: ethanol extract of green rooibos, GR: aqueous extract of green rooibos, FR: aqueous extract of fermented rooibos.

Finally, *S. boulardii* – as the only yeast assessed – under control conditions PUT made up the majority (65.02 %) of the total amount of TAs secreted, followed by AGM, TRP and PEA (Fig. 4.9A). Interestingly, in contrast to its prominence in the secretome of all bacteria assessed in the current study, no TYR was produced under control conditions. However, treatment with GRE significantly increased TYR levels (61.34 % of total, $p<0.0001$), thereby also increasing total TA content 1.9-fold compared to control (Fig. 4.9C & Fig. 4.10A). Despite this increase, levels of PEA ($p<0.0001$) and TRP ($p<0.01$) decreased significantly compared to the control

(Fig. 4.10B & C). Similarly, GR treatment increased the total TA content (3.76-fold compared to control, Fig. 4.9E), again with major increases in TYR (24.03 % of total, $p<0.01$), as well as PUT (73.49 % of total, $p<0.0001$). Interestingly, secretion of PEA ($p<0.01$), TRP, and AGM ($p<0.0001$ for both) were reduced significantly despite the increase in total TA content (Fig. 4.10). In contrast, FR treatment was not associated with a significant effect on total TA secretion when compared to control (Fig. 4.9G), although it did alter the relative distribution of TAs, decreasing TRP, PEA and AGM output considerably ($p<0.0001$ for all) (Fig. 4.10). In terms of E2 treatment, this yeast again showed a different response than the generally decreased TA production by bacteria in the presence of E2, as illustrated in the current study. Intriguingly, under control and FR media conditions (Fig. 4.9B & H), E2 treatment was associated with increased total TA outputs, while under GRE and GR media conditions E2 treatment was linked to decreased total TA content (Fig. 4.9D & F). Indeed, main ANOVA effects for E2 were observed for PEA, TRP and AGM ($p<0.0001$ for all) production from *S. boulardii*. In terms of relative distribution of TAs, with the exception of control media conditions, E2 supplementation did not seem to have a significant effect. Under control conditions, however, the addition of E2 stimulated TYR production at the diminishment of PEA and TRP ($p<0.0001$ for both) (Fig. 4.10).

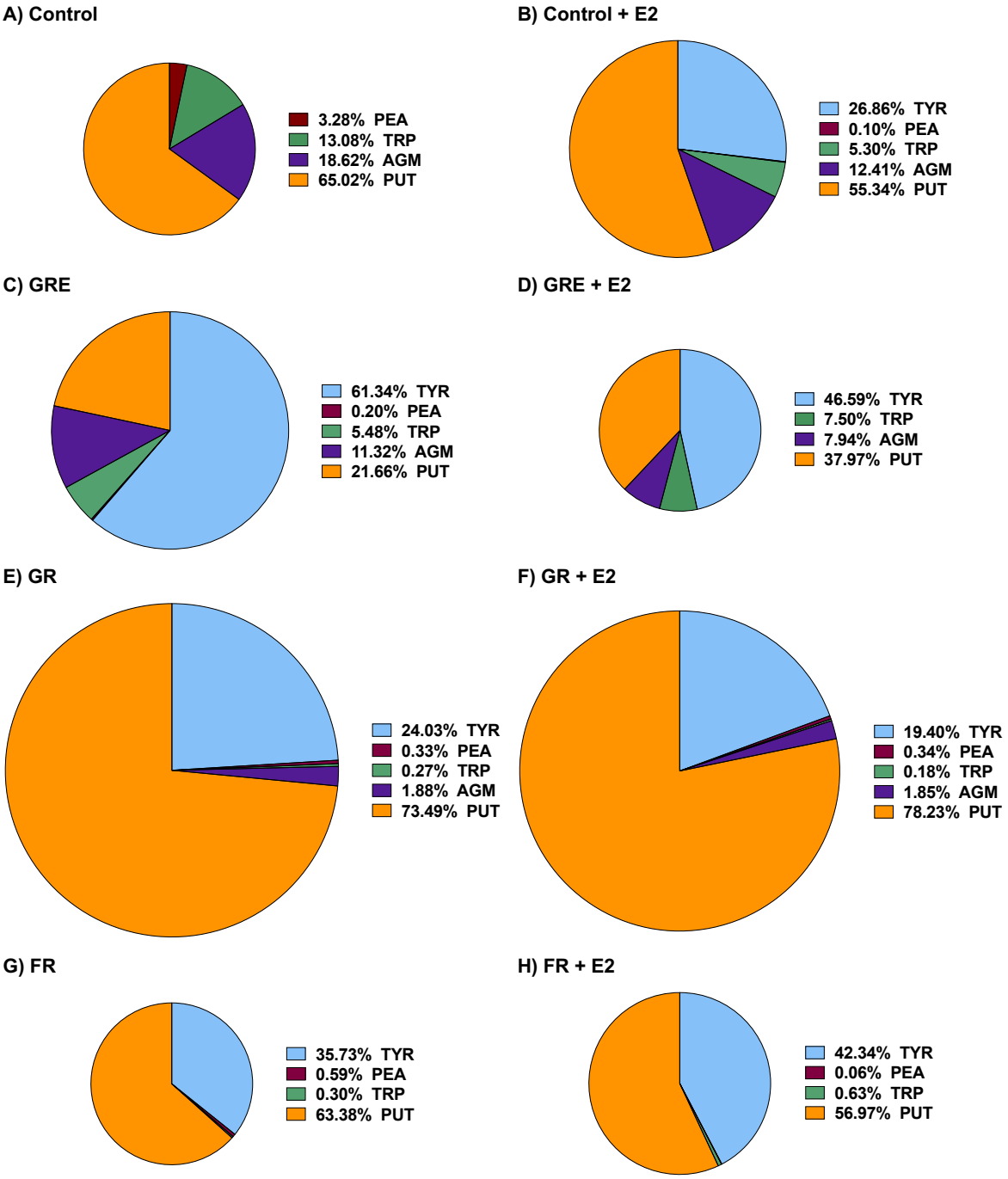


Figure 4.9: Changes in the relative distribution (% of total) of trace amines in the secretome of *Saccharomyces boulardii* following rooibos and estradiol (E2) treatment. Rooibos treatments were as follows: (A & B) control media, (C & D) green rooibos extract media (GRE), (E & F) green rooibos media (GR), and (G & H) fermented rooibos media (FR). E2 treatments were as follows: (A, C, E, G) control (no E2), and (B, D, F, H) 1 nM E2 supplementation. The area of each pie chart is indicative of the fold change (total TA content) compared to control (A). Abbreviations: TYR: ρ-tyramine, PEA: β-phenethylamine, TRP: tryptamine, AGM: agmatine, PUT: putrescine.

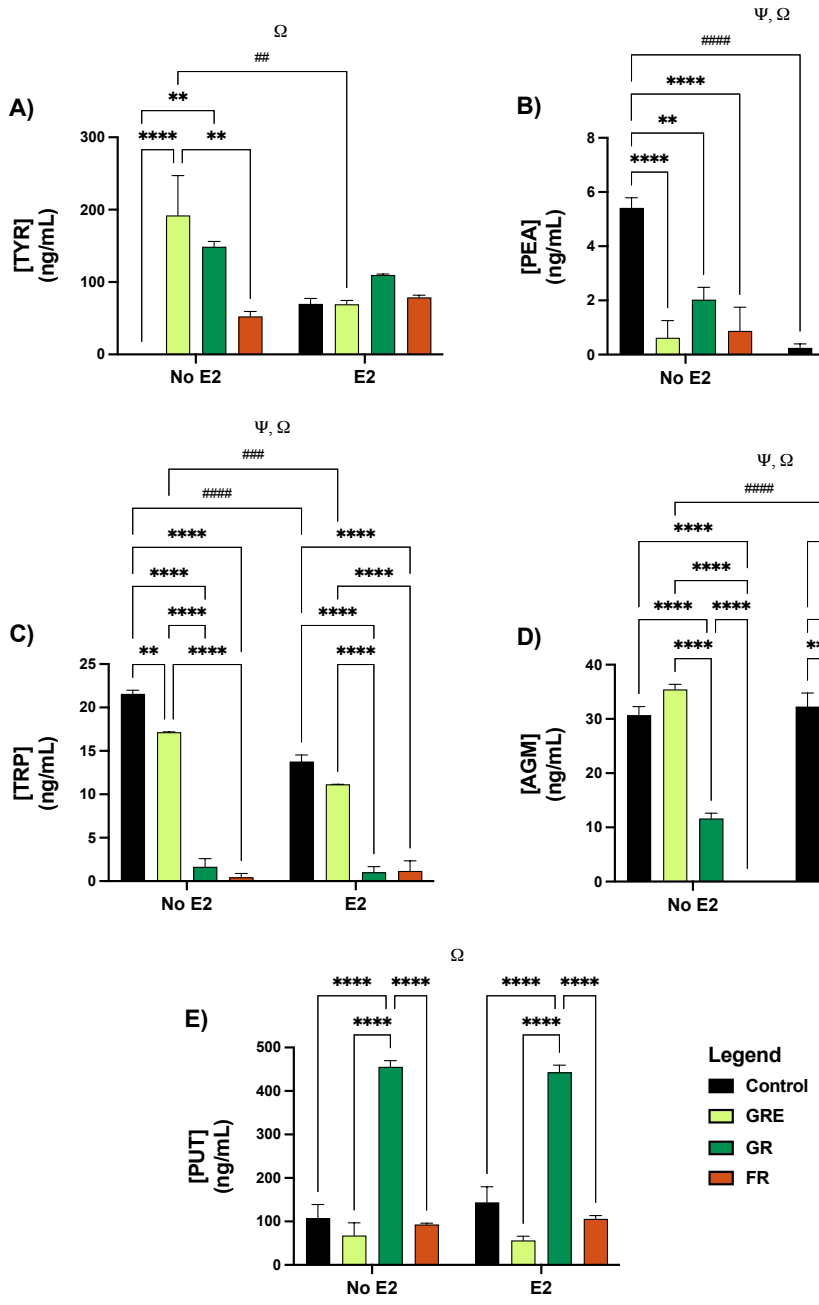


Figure 4.10: The effect of rooibos (GR, GRE and FR) and estradiol (E2) treatment on secretion of (A) ρ-tyramine (TYR), (B) β-phenethylamine (PEA), (C) tryptamine (TRP), (D) agmatine (AGM), and (E) putrescine (PUT) from *Saccharomyces boulardii*. The data are represented as the mean concentration of quantified trace amine (n=3) ± SEM. Statistical analysis: 2-way ANOVA with Tukey's multiple comparison tests; * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001, Ψ = main ANOVA effect of E2, Ω = main ANOVA effect of rooibos. Asterisk's (*) represent TA dose effect under the same E2 condition, while hashtags (#) represent effect of E2. Abbreviations: GRE: ethanol extract of green rooibos, GR: aqueous extract of green rooibos, FR: aqueous extract of fermented rooibos.

Taken together, treatment with both aqueous extracts of rooibos generally seemed to facilitate an increase in microbial PUT production and decrease in TRP production. It is important to note that these effects were not direct effects of changes in growth patterns (growth curves

performed in these media preparations showed no differences, data not shown). The effect on PUT was greater in magnitude after treatment with GR than FR, suggesting some loss of this effect after rooibos fermentation. This effect of rooibos was also likely lost in the ethanol extraction process, as this outcome was not observed in response to GRE treatment. In terms of a potential additional effect of E2, total TA secretion in bacteria was relatively suppressed in the presence of E2. In contrast, in the yeast, *S. boulardii*, TA secretion was increased in the presence of E2, while its response to E2 across different rooibos treatment conditions was highly variable. The fact that this variability in the response to E2 was only evident in this microbe, highlights the complexity of microbe, and E2 and treatment constituent interplay in determining TA profile in the gut microbial secretome. Lastly, in terms of specific TA responses to E2, TRP was produced at the highest concentrations under control conditions and in the absence of E2 by all microbes assessed here, which may indicate a similar effect of rooibos and E2 to reduce TRP levels.

4.5 Discussion

The current study presents novel findings on the effects of rooibos tea on probiotic and commensal microbial growth and their TA secretion capacities, which may, at least in part, inform on the potential mechanisms of action by which the anecdotal effects of rooibos to beneficially modulate gastrointestinal dysfunction/discomfort occur. In addition, by using different rooibos treatments prepared from the same raw plant material, we were able to present comparative data to demonstrate potential effects of fermentation and/or harsher extraction processing on the potential modulatory capacity of rooibos in the context of gut health.

Firstly, current data illustrates that aqueous extracts of both green and fermented rooibos, but not an ethanol extract of green rooibos, promoted the growth of these microbes in monoculture *in vitro*. The distinct difference between FR and GR's capacity to promote growth may be attributed to the fact that the process of fermentation increases the availability of simple rather than complex energy sources for microbial metabolism and subsequent growth. In the same line of thinking, we and many others have shown that variable processing of rooibos (fermentation or extraction) alters the concentrations of major phenolic constituents (Joubert and de Beer, 2011). The different effect of GR and FR *vs.* GRE on microbial growth suggests that total polyphenol content *per se* may not be the main driver for this difference, but rather perhaps specific constituents that are maintained only in aqueous preparation of raw tea material (i.e. preparation according to the indigenous knowledge practices). Indeed, initial q-TOF data indicated that only the two aqueous extracts (FR and GR) potentially contain

chlorogenic acid (4-caffeoylquinic acid), which in a recent study was highlighted as a bioactive compound in coffee that promoted *in vitro* probiotic bacterial growth (Sales et al., 2020). This expands on reports from several *in vitro* studies demonstrating the direct favourable impact of polyphenols on beneficial bacteria regarding their growth in pure culture (reviewed by Rodriguez-Daza et al. (2021)).

The limited extent to which rooibos changed secretome TA content have important implications for use of the tea as daily health supplement. The beneficial effects of rooibos are likely at least in part the result of synergistic actions between constituents, as previously reported for rooibos and many other plant extracts, (Bennett et al., 2018, Qin et al., 2018, Smith and Swart, 2018). Thus, the very targeted effect of rooibos to reduce tryptamine levels in favour of putrescine highlights a potential mechanism for the beneficial effect of isolated rooibos constituents recently reported in gastrointestinal health (Cheng et al., 2020). At the same time, such a targeted effect is unlikely to carry risk of causing dysbiosis itself on chronic consumption. Thus, although regular consumption of rooibos tea is acknowledged as a functional food in terms of disease prevention (Smith and Swart, 2018, Abdul and Marnewick, 2021), there may also be scope for an individualised or disease-specific approach to rooibos tea processing and supplementation *via* these elucidated targets.

The current study design (microbial monoculture) limits our interpretation in terms of net TA profile outcome in the gut – and the potential of rooibos to modulate this profile. Nevertheless, current data allows for interpretations into the potential prebiotic effect of rooibos on particular microbes. This knowledge may inform on the microbes most sensitive to manipulation (of their TA secretory profile) by rooibos consumption or rooibos constituent supplementation, to promote gut health of their human hosts. Firstly, TYR formation *via* decarboxylation of tyrosine is a well-known characteristic of a variety of lactic acid bacteria including lactobacillus and enterococcus species (Marcobal et al., 2012, Pugin et al., 2017). In fact, TYR biosynthesis is transcriptionally induced at low pH as an adaptation to acid-stress response (Linares et al., 2009, Perez et al., 2015, Wolken et al., 2006), which improves the fitness of these species in acidic environments, such as the caecum portion of the GIT (Fernandez de Palencia et al., 2011, Pessione, 2012). As such, it was unsurprising that TYR made up the highest proportion of our analysed TA output under control conditions for all 3 bacteria assessed in our study. In terms of the implications of TYR for human health, elevated TYR levels in the gut have been linked to both local and systemic effects (Millichap and Yee, 2003, Yano et al., 2015), such as increasing the synthesis and secretion of serotonin (5-HT) from gut epithelial enterochromaffin cells into circulation *in vitro* (RIN14B chromaffin cells) and *in vivo* (germ free mice) (Yano et al., 2015). In addition, TYR has been shown to stimulate ileal contractions and neuropeptide

Y release *ex vivo* (Broadley et al., 2009). Authors also reported that TYR relaxed mesenteric vasculature but constricted peripheral vasculature (aorta and coronary arteries), suggesting that post-prandially, TYR may aid/promote digestion *via* stimulation of the gut and improved gastrointestinal circulation. As such, it has been suggested that some beneficial effects of probiotic lactobacillus and enterococcus species could be attributed to increased gut activity *via* their generation of TAs (Broadley et al., 2009). However, these beneficial effects facilitated by TYR seems highly dose dependent. For example, an increase in 5-HT release may manifest not only as anxiety (Marcinkiewcz et al., 2016) – a common co-morbidity in gastrointestinal disease (Dubinsky et al., 2021, Fond et al., 2014, Mikocka-Walus et al., 2016, Wu, 2012) – but may also negatively impact digestion, causing increased mucus production and preventing colonic NaCl reuptake, which may lead to diarrhoea, or altered smooth muscle contractility, which may result in either diarrhoea or constipation (Ormsbee and Fondacaro, 1985). Indeed, in intestinal cell culture models, relatively high doses of TYR have been reported to be cytotoxic (Del Rio et al., 2017, Linares et al., 2016) and genotoxic (Del Rio et al., 2018), to modulate inflammatory cytokine/chemokine release (Fernandez de Palencia et al., 2011, Pretorius et al., 2022b) and to alter tight junction protein expression (Pretorius et al., 2022b). Moreover, in a cross-sectional study evaluating the gut microbiome in an Italian cohort of patients with inflammatory bowel disease, TYR was found to be significantly elevated compared to healthy controls (Santoru et al., 2017). Interestingly, for all assessed bacterial cultures in this study, GRE treatment either decreased or left TYR secretion unchanged compared to control conditions, which may indicate a potential beneficial effect of GRE treatment in certain disease contexts where elevated levels of TYR may be associated with disease outcome/symptomology. Additionally, from the growth curve results, we can expect this action to not be at the expense of microbial growth and composition per extension. Given the high incidence of GIT disorders globally and the huge impact it has on both the economy (*via* absence from work) and quality of life (Sperber et al., 2021, Tack et al., 2019), this potential benefit to be gained from rooibos warrants more comprehensive investigation *in vivo.*

In addition to TYR, PUT was also illustrated as a significant role player to be considered in the current context. In contrast to the high TYR secreted by the three bacteria assessed in the current study, PUT was the most abundant TA in the secretome of the probiotic yeast, *S. boulardii*. This is in line with literature illustrating in another strain of *S. boulardii,* the synthesis and secretion of measurable amounts of polyamines (PUT, spermine and spermidine) (Moré and Vandenplas, 2018). Indeed, it is the secretion of these polyamines, which enhances the expression of intestinal digestive enzymes, promotes nutrient uptake transporters and has a cumulative trophic action on the gut, that may in part account for the efficacy of *S. boulardii* as a probiotic (Kelesidis and Pothoulakis, 2012, Li et al., 2021b, Pais et al., 2020). As such,

the potential of GR to specifically increase PUT secretion from *S. boulardii* and potentially stimulate polyamine metabolism in the gut may be a beneficial mechanism of action to further investigate in the context of inflammatory bowel conditions.

In terms of pronounced treatment effects, with the exception of *E. mundtii* – which was the microbe least affected by tea treatment in general – both FR and GR supplementation increased PUT output. Regarding the implications of increased PUT in the gut, the described effects of PUT in literature are inconsistent, due to differences in models, doses, and treatment durations. Nevertheless, dietary supplementation with 0.2 % PUT was reported to mitigate mucosal atrophy, improve anti-inflammatory function and intestinal integrity and to decrease the incidence of diarrhoea in weaning piglets (Liu et al., 2019). In support of these findings, Nakamura et al., (2021), reported that commensal bacterium-derived PUT was absorbed and utilized by the host to increase the abundance of anti-inflammatory macrophages in the colonic lamina propria (similar reprogramming to that of IL-4 stimulation). Additionally, these bacterial mediators ameliorated colitis-induced symptomology in mice. Furthermore, the benefit from increased PUT production may also be indirectly achieved by reducing the levels of agmatine (AGM), for which we have recently illustrated negative outcome in terms of tight junction functionality and inflammatory status (increased prostaglandin E2) in HT-29 gut epithelial cells in culture (Pretorius et al., 2022b). Indeed, in support of this interpretation, PUT production from AGM metabolism by commensal gut bacteria has previously been reported (Nakamura et al., 2019). In contrast to these beneficial findings, PUT was reported to disrupt tight junction integrity in *ex vivo* mouse colons (Grosheva et al., 2020). In the same study, 200 mg/kg PUT administration (10-day duration) was also shown to exacerbate colonic inflammation and increased gut permeability in a mouse colitis model. Moreover, in colonic cell culture models, higher doses of PUT were reported to have dose-dependent cytotoxic effects, although to a lesser extent than TYR (Del Rio et al., 2019). Taken together, the implications of increased PUT levels in the gut depends on its physiological availability/dose and metabolism in the gut. Therefore, to interpret the potential effect of rooibos to increase PUT in the gut context, more thorough investigations should broaden investigation into downstream metabolites in appropriate *in vivo* models.

Moreover, GR and FR – and to a lesser extent GRE too – treatments were also associated with decreased TRP across the board. Although TRP is present in the microbial secretomes at much lower concentrations than TYR or PUT, it may be of relevance to the current context given that TRP is also implicated in a 5-HT-dependent pathway affecting gut function (Modoux et al., 2021). Moreover microbially-derived TRP may be more prevalent in the human gut than previously thought (Williams et al., 2014), especially in patient populations whose gut

microbiome are dominated by Firmicutes, such as patients with IBS (Jeffery et al., 2012). The balance between microbial tryptophan synthesis and its metabolism (into TRP) determines local gastrointestinal and circulating tryptophan availability for the host. Altered tryptophan metabolism could have profound effects on the gut microbial composition and metabolism, as well as the host immune system (Gao et al., 2018). Reported gastrointestinal effects of TRP specifically include its well documented effects to increase intestinal motility (Bhattarai et al., 2018, Roager and Licht, 2018, Williams et al., 2014, Wlodarska et al., 2015). Specifically, Williams et al. (2014) reported that TRP (at doses comparable to other microbially-derived metabolites) promoted colonic ion secretion, suggesting that TRP-mediated signalling could affect the transit of luminal contents. Also, Bhattarai et al. (2018) found that TRP accelerated gastrointestinal transit *via* activation of 5-HT_4R and stimulated anion-dependent fluid secretion in the proximal colon in a humanized mouse model (Bhattarai et al., 2018). This influence of TRP on intestinal transit time makes it another interesting candidate for investigation in IBS, which often manifests as chronic diarrhoea or constipation. Moreover, activation of the same receptor (5-HT_4R) stimulated mucus release from goblet cells, which was beneficial in preventing barrier disruption and decreasing disease activity in only female mice following induction of colitis (Bhattarai et al., 2020), highlighting sex-specific effects. Like other tryptophan metabolites, TRP can also act as a ligand for aryl hydrocarbon receptor which regulates intestinal immunity (Islam et al., 2017). Finally, TRP has also been reported to induce the release of 5-HT (Takaki et al., 1985), which may have additional implications of gastrointestinal motility (Gershon, 2013). Given the many similarities in effects of TRP and TYR, it may be relevant to investigate the potential for synergy between the two TAs, as a cumulative effect of the two TAs may exacerbate their individual effects on gut health. Nevertheless, although more research is clearly required in this context to more comprehensively elucidate the effects of TRP in the context of other tryptophan derivatives not assessed in the current study, rooibos treatment is again illustrated as having potentially modulatory effects of the gut.

Finally, considering the female bias in conditions such as IBS and Crohn's disease (Greuter et al., 2020, Kim and Kim, 2018), as well as in the common co-morbidity anxiety (Dubinsky et al., 2021, Hu et al., 2021), and the similarly central role of 5-HT-dependent mechanisms in symptomology of these conditions (Gershon, 2013, Jorandli et al., 2020), it is of interest to include the potential confounding/modulatory effect of female reproductive hormones in the current context. Although there were some exceptions, it is of relevance that E2 in general seemed to have a dampening effect on TA signalling. This fact alone suggests that females in particular may benefit from supplementation with the appropriate probiotic, or indeed a prebiotic such as rooibos. Overall, the effect of E2 on microbial TA production was relatively

insignificant in the presence of rooibos supplementation, which may be favourable in terms of designing a rooibos prebiotic supplementation strategy, as potential differences in TA load resulting from E2 fluctuations (i.e. menstrual cycles) will not be a significant confounder.

In terms of limitations, we acknowledge that the use of microbial monocultures – although purposely chosen according to our specific aims – is not an accurate simulation of *in vivo* events, where changes in environment (e.g. pH) and presence of competing microbes and additional ingested nutrients, will likely also play important modulatory roles in terms of microbial TA secretion. In addition, the effect of secreted TAs, either locally or systemically, can only realistically be evaluated in an *in vivo* model. Nevertheless, current data suggest that the use of rooibos tea as a prebiotic in the context of various gastrointestinal diseases is probable for two main reasons. Firstly, both aqueous extracts of rooibos tea promoted the growth of known probiotic organisms. Hence, one potential mechanism of beneficial effect could be related to favourable modulation of the gut microbiota composition and/or activity, which has been demonstrated for other high phenolic content nutraceuticals (Ankolekar et al., 2011, Milutinovic et al., 2021). Secondly, rooibos modulated the levels of microbially-derived TYR, PUT and TRP, which are all TAs that have significant effects in the GIT as discussed. In specific disease-contexts, these effects of rooibos may suggest beneficial outcome in terms of symptomology.

4.6 Conclusion

We have demonstrated that polyphenol content of rooibos tea extracts are highly dependent on method of preparation, with both harsher solvents and prior fermentation reducing the concentration and/or variety of polyphenols. Fermented rooibos promoted probiotic microbial growth most prominently. This suggests than habitual consumption of rooibos in this form may play a role in maintenance of gut microbiota populations. Green (unfermented) rooibos most significantly modulated microbial secretome TA content. While the aqueous green rooibos extract significantly improved microbial production of PUT – a gut protective TA – the ethanol extract did not. In contrast, the ethanol extract of green rooibos seemed to reduce secretome TA diversity most significantly, which may potentially facilitate its development into a disease-specific phytomedicine. In addition, we have shown that rooibos extracts have the potential to largely prevent estradiol-associated fluctuations in TA profile and thus potentially symptomology of female predominant gastrointestinal conditions such as IBS and Crohn's disease. Given the limitations of our model (isolated microbial culture) and the extreme complexity of chronic inflammatory disorders such as IBS, our results – although positive – remain to be interrogated in a physiologically relevant, *in vivo* model. Nevertheless, our data

comprehensively describe consistently beneficial effects of rooibos on microbial TA secretion profile. This suggests that rooibos may possess a prebiotic-like effect at the level of the gut. These positive data warrants mechanistic studies on whole extracts as well as selected active components, in the context of gastrointestinal health.

4.7 Supplementary materials

q-TOF analyses

Liquid Chromatography-Quadruple Time-of-Flight Tandem Mass Spectrometry (LC-MS/MS):
Modified tea preparations (1 mg/mL GR and FR, 300 ug/mL GRE) were analysed on a liquid chromatography-quadrupole time-of-flight tandem MS instrument (Shimadzu LCMS-9030 qTOF, Shimadzu Laboratory, Auckland Park Kingsway Campus). Chromatographic separation was performed on a Shim-pack Velox PFPP column (2.7 μm, 2.1 x 100 mm). The column temperature was maintained at 40°C. For all samples an injection volume of 10 µL was used and run using a binary mobile phase gradient which consisted of (A) 0.1% FA in Milli-Q water (HPLC grade) and (B) Methanol (HPLC grade). The flow rate was set to 0.4 mL/min throughout the set 55 min run. The chromatographic effluents were further analysed utilizing the qTOF high-definition mass spectrometer. Data-independent acquisition was performed with a precursor ion range of *m/z* 100 to 1000, an isolation window width of 20 *m/z*, and a product ion range of *m/z* 100 to 1000 in both positive and negative electrospray ionisation modes.

Data processing and metabolite annotation:
Raw data obtained from the Shimadzu LCMS-9030 qTOF were converted to .mzML open access format and processed using MS-DIAL (version 4.80). Potential metabolite constituents were identified by matching the generated MS/MS spectra against freely available MSP libraries (Downloaded from: http://prime.psc.riken.jp/compms/msdial/main.html#MSP) for natural compounds (Fiehn/Vaniya natural product library, GNPS and ReSpect were used). Reference matches were exported to MS-FINDER (version 3.52) and compound annotation analyses (formula and structure) were performed. These data sets were exported to Microsoft Excel for further processing.

Supplementary results

Supplementary Table 4.1: Potential metabolites present in all tea samples following q-TOF analyses.

Metabolite	Title	Green rooibos				Green rooibos extract				Fermented rooibos				Formula	Theoretical mass	Ontology
		MS 1 count	MS/MS count	Precursor M/Z	Precursor type	MS 1 count	MS/MS count	Precursor M/Z	Precursor type	MS 1 count	MS/MS count	Precursor M/Z	Precursor type			
1	Quercetin	1091, 115 1195, 119	39, 39 30, 30	303.0491 303.0492	[M+H]+	967, 106 1072, 121	22, 22 22, 22	303.049 303.0498	[M+H]+	997	24	303.0494	[M+H]+	C15H10O7	302.04265266	Flavonols
2	Avocadene 1-acetate	2056	16	327.2523	[M-H]-	2046	16	327.2529	[M-H]-	1369	27	327.2527	[M-H]-	C19H36O4	328.259360024	Fatty Acyls (Long-chain fatty alcohols)
3	Melibiose Sucrose	188	89	365.1049	[M+Na]+	161	67	365.1047	[M+Na]+	146	28	365.1048	[M+Na]+	C12H22O11	342.116211524	Fatty Acyls (Fatty acyl glycosides of mono- and disaccharides) Disaccharide
4	Octocrylene	4369	105	362.2108	[M+H]+	4394	109	362.2105	[M+H]+	4288	99	362.211	[M+H]+	C24H27NO2	361.204179104	Diphenylmethanes
5	Citroside A	497 466	9 1	409.182 409.1822	[M+Na]+	488 492	5 9	409.1825 409.1823	[M+Na]+	466	6	409.1826	[M+Na]+	C19H30O8	386.192567598 386.19406792 386.193035962	Terpene glycosides
6	2-acetoxy-4-pentadecylbenzoic acid (Acetylsalicylic acid derivative)	5041	149	413.2655	[M+Na]+	5068	159	413.2656	[M+Na]+	4930	126	413.2656	[M+Na]+	C24H38O4	390.277009696	Benzoic acid esters
7	3-Genistein-8-C-glucoside Isovitexin (Apigenin-6-C-glucoside) Vitexin (Apigenin 8-C-glucoside)	387, 287, 340 310, 306 427 90, 74, 828 81, 118, 93	32, 19, 15 14, 21 53 67, 38, 67 41, 76, 41	431.0969 431.0968 431.0971 433.1121 433.1122	[M-H]- [M+H]+	387, 287 306 427, 427 1002, 98 716, 828 791	32, 19 21 53, 53 23, 23 38, 67 41	431.0969 431.0968 431.0971 433.1119 433.1121 433.1122	[M-H]- [M+H]+	340, 387, 287 306 1074 716 791, 1074, 894	15, 32, 19 21 24 38 41, 76, 41	431.0969 431.0968 455.094 433.1121 433.1122	[M-H]- [M+H]+	C21H20O10	432.1038305 432.104614882 432.10564684	Isoflavone (Genistein) Flavone (Isovitexin and Vitexin)
8	Quercitrin (Quercetin O-glycoside) Orientin (Luteolin-8-C-glucoside) Isoorientin (Luteolin-6-C-glucoside)	456 340, 456 408 491 942, 1072, 104 1092, 113	44 8, 72 10 200 154, 169, 169 13, 13	493.0971 447.0919 447.0918 447.0922 449.1071 449.1069	[M+FA-H]- [M-H]- [M+H]+	319, 353 333 92 786, 843, 92 1106, 115	45, 8 33 15 79, 65, 65 16, 16	447.0917 447.0918 471.0886 449.1069 449.1071	[M-H]- [M+Na]+ [M+H]+	323 348 326 740, 847, 90 90	6 31, 71, 66, 74, 74 27	447.0911 447.0919 447.092 449.1069 471.0888	[M-H]- [M+H]+ [M+Na]+	C21H20O11	448.09805936 448.099061138 448.099213484 448.099396752 448.099529502 448.10056146	Flavonols (Quercitrin) Flavones (Orientin and Isoorientin)
9	Diosmetol 7-glucoside	1311 139	14 14	463.1221	[M+H]+	1372 146	20 20	463.1229	[M+H]+	137	11	463.1233	[M+H]+	C22H22O11	462.114395184 462.116211524	Flavones
10	Peonidin-3-glucoside	1311	14	463.1221	[M]+	1372	20	463.1229	[M]+	1279	11	463.1233	[M]+	C22H23O11	463.123004598 463.124036556	Anthocyanidins
11	Isoquercitrin (Quercetin 3-glucoside) Hyperoside (Quercetin 3-galactoside)	351 374 1127 1116	11 13 24 18	463.0864 463.0867 465.1013 465.1015	[M-H]- [M+H]+	317	4	463.0865	[M-H]-	325 325	4 10	463.0866 463.0865	[M-H]-	C21H20O12	464.09297398 464.09365974 464.094444122	Flavonols
12	Diosmin (diosmetin 7-O-rutinoside)	405 137	2 9	607.1646 609.1794	[M-H]- [M+H]+	449	2	607.1637	[M-H]-	137	8	609.1808	[M+H]+	C28H32O15	608.17114341 608.171618224 608.172056408 608.174120324	Flavones
13	Rutin (Quercetin 3-rutinoside) Quercetin 3-O-robinobioside Panasenoside	385 433 1127 1181, 94, 120 119 115	30 24 15 11, 4, 11 23 38	609.1443 609.1442 611.1594 611.16 633.1411 633.1415	[M-H]- [M+H]+ [M+Na]+	322 337 1067 96 967, 106 106 117	5 9 10 4 6, 6 7 10	609.144 609.1444 611.1591 611.1595 611.1599 633.1409 633.1418	[M-H]- [M+H]+ [M+Na]+	313 320 102 102 99	9 9 3 11 7	609.1442 609.1443 611.1603 633.1415 633.1429	[M-H]- [M+H]+ [M+Na]+	C27H30O16	610.15101553 610.151320964 610.151504232 610.151884558 610.152036904 610.152061376 610.152125684 610.152352922 610.15338488	Flavone/flavonol glycosides (Quercetin 3-rutinoside/Quercetin 3-O-robinobioside) Panasenoside (Flavonoid glycosides)
14	Delphinidin-3-O-(6"-O-alpha-rhamnopyranosyl-beta-glucopyranoside) Cyanidin-3, 5-di-O-glucoside	433 385	24 30	609.1442 609.1443	[M-2H]-	313 320	9 9	609.1442 609.1443	[M-2H]-	337 896	9 4	609.1444 611.1595	[M-2H]- [M]+	C27H30O16	611.159329264 611.15970959 611.160177954	Anthocyanidins

Supplementary Table 4.2: Potential metabolites present in green rooibos and green rooibos extract following q-TOF analyses.

Metabolite	Title	Green rooibos				Green rooibos extract				Formula	Theoretical mass	Ontology
		MS 1 count	MS/MS count	Precursor M/Z	Precursor type	MS 1 count	MS/MS count	Precursor M/Z	Precursor type			
15	Syringin	61	27	395.1304	[M+Na]+	54	16	395.1303	[M+Na]+	C17H24O9	372.142032348	Phenolic glycosides
16	5a,6a-Epoxy-7E-megastigmene-3b,9e-diol 9-glucoside	569	6	411.1981	[M+Na]+	535	6	411.1982	[M+Na]+	C19H32O8	388.209717984	Fatty Acyls (Fatty acyl glycosides of mono- and disaccharides) Disaccharide
17	Isolariciresinol 9'-O-beta-D-glucoside	103	11	545.1985	[M+Na]+	89	10	545.1989	[M+Na]+	C26H34O11	522.210111908	Lignan glycosides

Supplementary Table 4.3: Potential metabolites present in green and fermented rooibos following q-TOF analyses.

Metabolite	Title	Green rooibos				Fermented rooibos				Formula	Theoretical mass	Ontology
		MS 1 count	MS/MS count	Precursor M/Z	Precursor type	MS 1 count	MS/MS count	Precursor M/Z	Precursor type			
18	Adenosine	808 85	39 39	268.1036	[M+H]+	604 69	15 15	268.1033	[M+H]+	C10H13N5O4	267.096753896	Purine nucleosides
19	Cocamidopropyl Betaine methyl (2S)-6-amino-2-(dodecanoylamino)hexanoate	2402 60	51 6	343.2948 365.1195	[M+H]+ [M+Na]+	2331	53	343.295	[M+H]+	C19H38N2O3 C14H22N4O3	342.129967342 342.288243076	Fatty acid amide (Cocamidopropyl Betaine) N-acyl-alpha amino acids and derivatives?
20	4-Caffeoylquinic acid?	523, 68	7, 7	355.1012	[M+H]+	194	7	353.0861	[M-H]-	C16H18O9	354.092580056 354.094050198	(Cinnamate ester)
21	Apiin (Apigenin-7-apioglucoside) Isovitexin 2"-O-arabinoside Schaftoside	294 102 95 88	10 18 44 47	563.1388 565.1539 565.1542 565.1543	[M-H]- [M+H]+	86 89	9 12	565.155 565.1547	[M+H]+	C26H28O14	564.146024928 564.146958264 564.147905576	Flavones

Supplementary Table 4.4: Potential metabolites only present in green rooibos following q-TOF analyses.

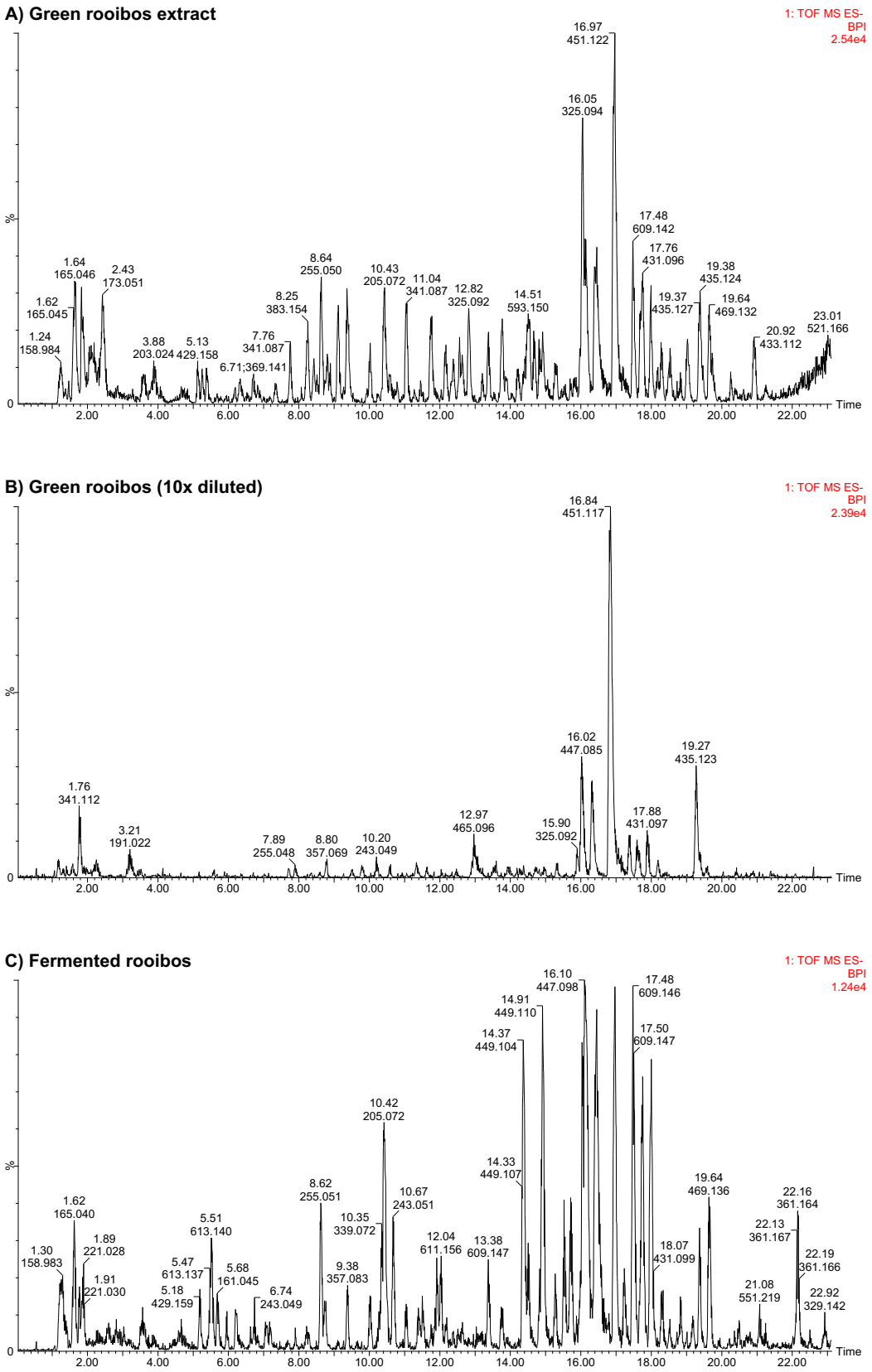
Metabolite	Title	Green rooibos				Formula	Theoretical mass	Ontology
		MS 1 count	MS/MS count	Precursor M/Z	Precursor type			
22	Adenine	126 85	20 13	136.0611 136.0612	[M+H]+	C5H5N5	135.05449516	6-aminopurines
23	Trigonelline Anthranilic acid	1440	28	138.0543	[M+H]+	C7H7NO2	137.047678464	Alkaloids and derivatives (Trigonelline) Aminobenzoic acids
24	Citric acid	621	6	191.0189	[M-H]-	C6H8O7	192.027002596	Tricarboxylic acids and derivatives
25	L-Tryptophan	63	40	205.0965	[M+H]+	C11H12N2O2	204.089877624	Indolyl carboxylic acids and derivatives
26	Cordycepin (3'-deoxyadenosine)	70	18	252.1082	[M+H]+	C10H13N5O3	251.100501884	Purine nucleosides
27	Guanosine	393	7	282.0833	[M-H]-	C10H13N5O5	283.090331124	Purine nucleosides
28	Epicatechin	439 462, 61	15 26, 26	291.0854 291.0855	[M+H]+	C15H14O6	290.079038168	Flavanols
29	trans-o-Coumaric acid 2-glucoside	263 66 77 63	5 21 14 17	325.0917 344.1332 344.133 344.1334	[M-H]- [M+NH4]+	C15H18O8	326.099135578 326.100167536	Hydroxycinnamic acids
30	3-p-coumaroylquinic acid	223 241 499	5 6 7	337.0913 337.0917 339.1063	[M-H]- [M+H]+	C16H18O8	338.099135578	Cinnamate ester
31	Guanosine cyclic monophosphate	141	25	346.0537	[M+H]+	C10H12N5O7P	345.046096962	Cyclic nucleotide
32	Icariside F2	492	2	425.1411	[M+Na]+	C18H26O10	402.151432324	Glycosyloxyflavone
33	Phenylethyl primeveroside	498, 54 447, 64 507	10, 10 3, 3 9	439.1557 439.1564 439.1568	[M+Na]+	C19H28O10 C13H29N4O9P	416.166366448 416.167215138 416.168247096	O-glycosyl compounds
34	Betavulgarin 2'-glucoside	103 118	9 185	475.1203 475.1209	[M+H]+	C23H22O11	474.113057792 474.113709424	Isoflavone
35	Maltotriose	188	96	527.1572	[M+Na]+	C18H32O16	504.167870236	Trisaccharide
36	Kaempferol-3-O-rutinoside (Nicotiflorin) Kaempferol-3-O-glucoside-7-O-rhamnoside (Astragalin 7-rhamnoside) Vicenin 2 (Isovitexin derivative)	365 293 364 372 751 130	2 16 4 3 66 4	593.1482 593.1494 593.1498 593.1504 595.1647 595.1662	[M-H]- [M+H]+	C27H30O15	594.155557654 594.15665392 594.156969938 594.157305552 594.15847026	Flavanols/Flavones
37	Kaempferol-3-O-(6'''-trans-p-coumaroyl-2''-glucosyl)rhamnoside	99 55 89 110	7 2 3 3	763.1821 763.1824 763.1825 763.1831	[M+Na]+	C36H36O17	740.192839578 740.193382834 740.193807228	Cinnamate ester & Glycosyloxyflavone

Supplementary Table 4.5: Potential metabolites only present in green rooibos extract following q-TOF analyses.

Metabolite	Title	Green rooibos extract				Formula	Theoretical mass	Ontology
		MS 1 count	MS/MS count	Precursor M/Z	Precursor type			
38	Salicylic acid	190	1	137.0234	[M-H]-	C7H6O3	138.12	Benzoic acid
39	Oxythiamine disulfide monosulfoxide	76	1	603.1667	[M+Na]+	C24H32N6O7S2	580.177389364	Thiazoles

Supplementary Table 4.6: Potential metabolites only present in fermented rooibos following q-TOF analyses.

Metabolite	Title	Fermented rooibos				Formula	Theoretical mass	Ontology
		MS 1 count	MS/MS count	Precursor M/Z	Precursor type			
41	Gentisic acid (2,5-Dihydroxybenzoic acid) Pyrocatechuic acid (2,3-Dihydroxybenzoic acid)	207	2	153.0183	[M-H]-	C7H6O4	154.12	Hydrobenzoic acids
42	Diosmetin	602	2	299.0546	[M-H]-	C16H12O6	300.062356146	Flavone
43	Astilbin	555, 602	45, 50	451.1225	[M+H]+	C21H22O11	450.116211524	Flavanone
44	3b,6a-Dihydroxy-alpha-ionol 9-[apiosyl-(1->6)-glucoside] 7,8-Dihydrovomifoliol 9-[apiosyl-(1->6)-glucoside]	485 588	4 4	543.2413 543.2416	[M+Na]+	C24H40O12	520.25197672	Fatty Acyls (Fatty acyl glycosides of mono- and disaccharides)



Supplementary Figure 4.1: Chromatograms of different rooibos preparations following q-TOF analyses targeting phenolic compounds.

Chapter 5

Aspalathus linearis (rooibos) and agmatine may act synergistically to beneficially modulate intestinal tight junction integrity and inflammatory profile

This chapter has been published in a special issue; “Recent Advances in Natural Products Discoveries for Antioxidants: Pharmacology and Mechanisms” in the Journal of Pharmaceuticals (Impact Factor: 5.215).

Citation reference: Pretorius, L., Smith, C. (2022) *Aspalathus linearis* (Rooibos) and Agmatine May Act Synergistically to Beneficially Modulate Intestinal Tight Junction Integrity and Inflammatory Profile. *Pharmaceuticals*, 15:1097-1113.

5.1 Abstract

Introduction:
In order to promote gastrointestinal health, significant increases in the prevalence of gastrointestinal disorders should be paralleled by similar surges in therapeutics research. Nutraceutical interventions may play a significant role in patient management. The current study aimed to determine the potential of *Aspalathus linearis* (rooibos) to prevent gastrointestinal dysregulation resulting from high dose trace amine (TA) exposure. Considering the substantial female bias in functional gastrointestinal disorders, and the suggested phytoestrogenicity of rooibos, the study design allowed for a comparison between effects of an ethanol extract of green rooibos and 17β-estradiol (E2).
Methods:
The effect of rooibos and E2 pre-treatment on tight junction protein (TJP); occludin and ZO-1) dynamics were investigated in TA treated (HT-29) colon epithelial monolayers. In addition, prostaglandin E2 (PGE2) and cytokine analyses were performed on culture supernatants.
Results:
High levels of ρ-tyramine (TYR) and agmatine (AGM), but not β-phenethylamine (PEA) or tryptamine (TRP), resulted in PGE2 hypersecretion, increased TJP secretion and (dissimilarly) disrupted TJP cellular distribution profile. Modulating benefits of rooibos and E2 were TA-specific. Rooibos pre-treatment generally reduced IL-8 secretion across all TA conditions and prevented PGE2 hypersecretion after exposure to both TYR and AGM, but was only able to normalise TJP levels and distribution profile in AGM-exposed cells. In contrast, E2 pre-treatment prevented only TYR-associated PGE2 hypersecretion and TJP dysregulation.
Conclusion:
Together, data suggest that antioxidant and anti-inflammatory effects of rooibos, rather than phytoestrogenicity, affected benefits illustrated for rooibos.

5.2 Introduction

Functional gastrointestinal disorders (FGIDs) are highly prevalent (approximately 40% of the global population) conditions that considerably reduce quality of life, placing enormous economic burdens on healthcare systems worldwide (Canavan et al., 2014b, Tack et al., 2019, Sperber et al., 2021). Of importance, many FGIDs have reported female predominance (Avramidou et al., 2018, Chatila et al., 2017, Heitkemper and Jarrett, 2008, Mulak et al., 2014), suggesting a potential estrogen-driven vulnerability to gastrointestinal disturbances. Current therapeutic strategies (including drug and biological treatments) are not yet addressing this aspect and have been associated with adverse systemic effects such as adverse cardiovascular events (Tack et al., 2012, Wilkins et al., 2012), which may exacerbate patient discomfort. As such, the use of functional foods/nutraceuticals is gaining research interest to promote gastrointestinal health while minimising adverse effects (Elmaliklis et al., 2019, Luvian-Morales et al., 2021, Lyu et al., 2017, Mijan and Lim, 2018, Shimizu, 2012, Wan et al., 2019, Yanni and Kourkoutas, 2021).

Of particular interest to the current study context, rooibos herbal tea (brewed from unfermented or fermented *Aspalathus linearis*), is a widely consumed traditional South African tisane that has already been suggested as a functional food (Abdul and Marnewick, 2021, Smith and Swart, 2018) due to its substantial and unique blend of bioactive polyphenols which have been linked to potent antioxidant actions (Bond and Derbyshire, 2020, Hoosen, 2019, Villaño et al., 2010). In terms of promoting gut health specifically, scant literature suggests beneficial actions (anti-inflammatory, anti-nociceptive, anti-spasmodic) of several bioactive constituents of rooibos, such as iso-orientin (Cheng et al., 2020, Küpeli et al., 2004), quercetin (Lin et al., 2019, Qin et al., 2019, Shi et al., 2020, Sun et al., 2020, Zou et al., 2016), chrysoeriol, orientin, vitexin and rutin (Gilani et al., 2006) in various *in vitro* and *in vivo* models, which could potentially reduce FGIDs symptomology. We have recently expanded on this body of literature by demonstrating that rooibos – in a manner very dependent on extraction/processing – beneficially modulated the secretory profile of gut microbes (Pretorius et al., 2022a). In the same study, 17β-estradiol (E2)-associated changes in microbial secretory function were largely negated by rooibos, suggesting that rooibos supplementation may stabilize gut signalling profile in females, which may aid patient management. Related to this, a moderate phytoestrogenicity has been reported for rooibos (Monsees and Opuwari, 2017, Shimamura et al., 2006, Sirotkin, 2021), which makes it an interesting candidate nutraceutical for investigation in female predominant disorders, such as irritable bowel syndrome.

According to Wan et al (2019), two crucial factors determine gut health, namely the gut microbiota and the intestinal epithelial barrier. Indeed, in line with our current hypothesis of trace aminergic involvement in exaggeration of gastrointestinal symptomology (Pretorius and Smith, 2020), we have recently demonstrated the deleterious effects of high trace amine (TA) load on intestinal epithelial tight junction proteins (TJPs) and inflammatory status (Pretorius et al., 2022b). In this regard, we propose that the known poor absorption and low systemic bioavailability of rooibos and some of its polyphenolic constituents (Breiter et al., 2011, Kreuz et al., 2008, Stalmach et al., 2009) may not be a significant limitation if beneficial modulation of rooibos occurs directly at an intestinal epithelial level. In support of this notion, a recent review suggested that dietary polyphenols can be metabolised by gut microbes, which could exert beneficial effects toward the gut epithelium, as well as systemically (Luca et al., 2020). In terms of intestinal barrier integrity and intestinal inflammation, protective effects of flavonoids have been described in several studies utilising both *in vitro* and *in vivo* models (De Santis et al., 2015, Gil-Cardoso et al., 2016, Mijan and Lim, 2018, Uyanga et al., 2021), suggesting nutraceutical interventions may ameliorate gastrointestinal dysfunction and inflammation, which underpins many gastrointestinal conditions. However, to the best of our knowledge, no studies have investigated the effects of rooibos on intestinal cells.

As such, to determine the potential of rooibos to promote gut health, this study aimed to investigate the use of a selected (refer to supplemental material) rooibos preparation (ethanol extract of green rooibos; GRE) as pre-treatment to protect against TA-induced TJP disruption, with concurrent inflammatory status assessments in colon adenocarcinoma (HT-29) cells. To enable probing potential phytoestrogenicity of rooibos, a parallel experiment using E2 pre-treatment instead of rooibos was also included.

5.3 Methods and materials

5.3.1 Rooibos preparation

An ethanol extract from unfermented (green) rooibos leaves (GRE) was kindly donated by Mr Roy van Brummelen (Van Brummelen Consultants, Pretoria, South Africa). A profile of the major constituents of the extract is presented in the supplementary material (Supp. Fig. 1). This extract was reconstituted in DMSO and diluted in cell culture media to final experimental concentration of 100 μg/mL.

5.3.2 Cell culture maintenance

Colon adenocarcinoma (HT-29) cells were kindly donated by Dr Tanya Davis. For general maintenance cells were cultured in 5 mM D-galactose supplemented glucose free RPMI (Gibco, 11879-020) culture medium to facilitate differentiation. Culture media was additionally supplemented with 10% heat inactivated, gamma irradiated fetal bovine serum (FBS) (Biowest, S181Y-500) and 1% PenStrep. Cells were sub-cultured with 1x trypsin and maintained in a humidified incubator at 5% CO_2 at 37°C. All experiments were done in triplicate and repeated at least three times. In addition, to optimize GRE dosage cell viability utilizing the WST-1 assay was performed (Supp. Fig. 2).

5.3.3 Tight junction protein immunofluorescent staining

In order to investigate the ability of GRE to mediate gut health, HT-29 cells were exposed to TA doses previously demonstrated to detrimentally affect TJP profile (Pretorius et al., 2022b). Briefly, HT-29 cells were seeded at $1.5x10^5$ cells/well (24-well plate) onto sterilized 12 mm round glass coverslips, which were previously coated with ECL cell attachment matrix (Merck, 08-110) and refreshed every other day. After 8 days of culturing, cell monolayers were pre-treated with either 100 μg/mL GRE, 1 nM E2, or a media vehicle (control). After 24 hrs of pre-treatment, the cell monolayers were treated with fresh media containing 200 μg/mL TA (β-phenethylamine (PEA), or tryptamine (TRP), or ρ-tyramine (TYR), or agmatine (AGM)) or media vehicle (control) in the presence or absence of GRE (100 μg/mL) or E2 (1 nM) for an additional 24 hrs. The supernatants were collected for additional analyses, after which the cell monolayers were washed once with PBS. Monolayers were fixed (ice cold 4% PFA and 50% MeOH solution) for 15 min at - 20°C, washed again with S-PBS (0.1% saponin in PBS), and blocked (20% FBS and 5% donkey serum in S-PBS) for 1 hr at room temperature. Overnight primary antibody incubations at 4°C followed, utilising the primary antibodies: 1:250 mouse anti-ZO-1 (Invitrogen, 33-9100) and 1:250 rabbit anti-occludin (Novus Biologicals, NBP1-87402) in blocking buffer. Next, the monolayers were washed 3x with S-PBS, prior to incubation with secondary antibodies: 1:250 Alexa Fluor 488 donkey anti-mouse (Invitrogen, A-21202) and Alexa Fluor 594 donkey anti-rabbit (Invitrogen, A-21207) in blocking buffer for 1 hr at room temperature. Finally, the monolayers were incubated with Hoechst (ThermoFisher Scientific, 33342) for 20 min before undergoing 4x washes with PBS. The glass coverslips were then mounted onto microscope slides with Dako Fluorescent Mounting media (Diagnostech, S3023). Fluorescently stained cell monolayers were imaged with the Carl Zeiss Confocal LSM 780 Elyra PS1 using the 60x oil-immersion objective. Respective Z-stack (10 slices) images were captured, and maximum intensity projections were analysed for co-localisation of ZO-1 and occludin utilizing Zeiss ZEN imaging software.

5.3.4 Supernatant analyses

Cell culture supernatant prostaglandin E2 (PGE2) concentrations were assayed using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (E-EL-0034, Elabscience), following the manufacturer's guideline protocol. In addition, supernatants were analysed for IFN-γ, IL-1β, IL-6, IL-8, IL-10 and TNFα using a Human Magnetic Luminex Screening Assay (LXSAHM-06, R&D Systems), following the manufacturer's guideline protocol.

5.3.5 Statistical analyses

All experiments were conducted in triplicate and repeated at least three times. Triplicate values were averaged to yield a final n=3 for all data points presented. Statistical analyses of all data were completed utilizing GraphPad Prism Version 9.1.2 (www.graphpad.com, San Diego, CA). TJP data are represented qualitatively as representative images, as well as quantitatively as mean $\pm$ standard error of mean (SEM), while PGE2 and cytokine data are represented as means $\pm$ standard deviation (SD). Statistical analyses entailed 2-way ANOVAs with Tukey's multiple comparison tests for TJP, PGE2 and cytokine data. A p-value of < 0.05 was considered statistically significant.

5.4 Results

5.4.1 Rooibos and estradiol have differential protective effects on tight junction integrity following trace amine exposure

TJP (occludin and zona occludens-1 (ZO-1)) levels and distribution profiles were assessed in HT-29 colon adenocarcinoma monolayers as an indication of gut barrier epithelial integrity. These data are represented both quantitatively (Fig. 5.1) and qualitatively (Fig. 5.2). In terms of conditioning of cells with either E2 or GRE, in the absence of TA-exposure, both treatments maintained – if not enhanced – normal cellular morphology, although no changes in total ZO-1 expression was evident (Fig. 5.1). More specifically, E2 seemed to have a beneficial modulatory effect on cell size and shape, resulting in a more uniformly sized cell monolayer (Fig. 5.2F), while GRE treatment reduced occludin signal significantly (Fig. 5.2K) compared to control conditions ($p<0.05$; Fig. 5.1).

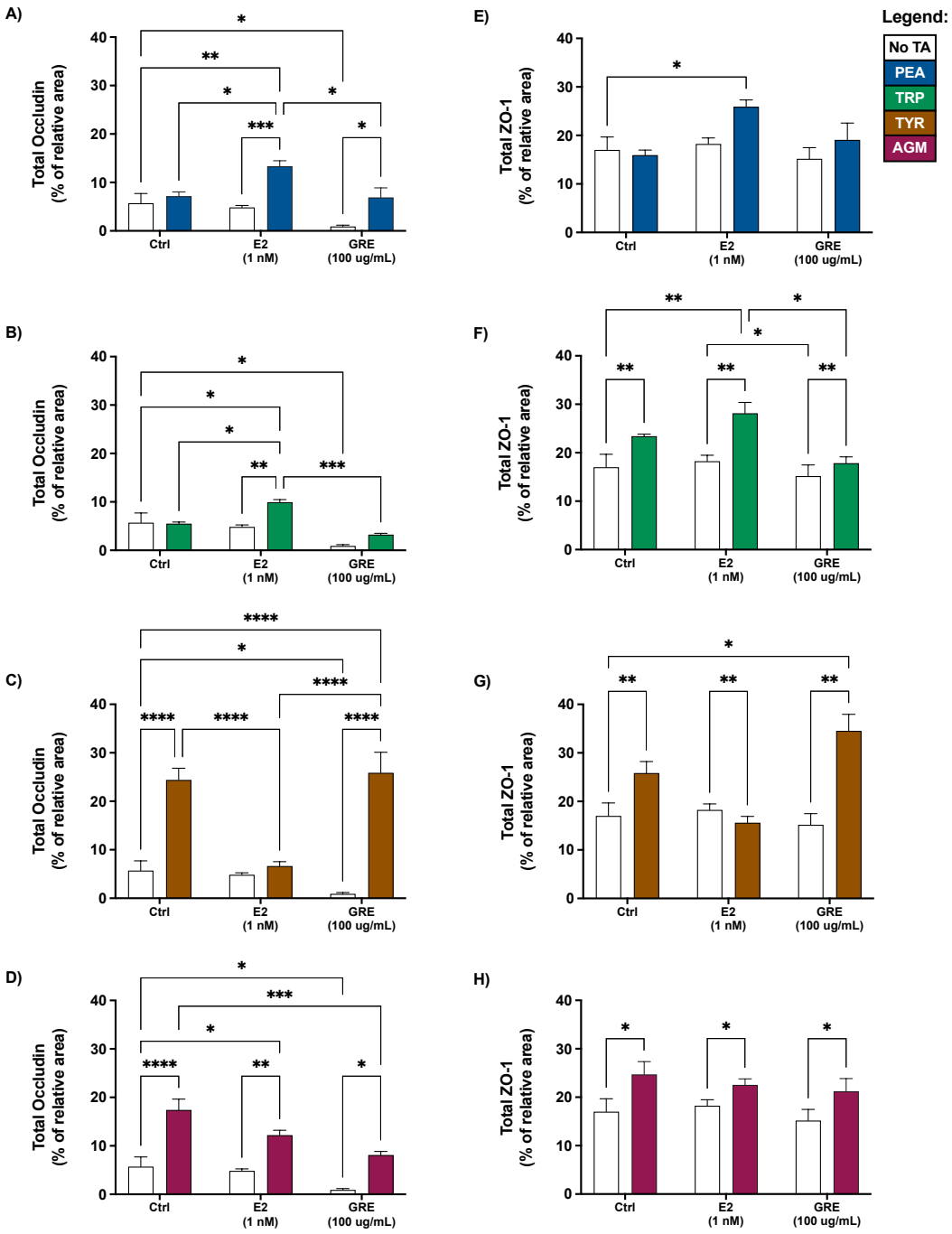


Figure 5.1: Effects of green rooibos extract (GRE) and 17β-estradiol (E2) on the percentage of relative area of imaged HT-29 cell monolayer that stained positive for tight junction proteins occludin (A to D) and ZO-1 (E to H) following selected TA-exposure (200 ng/mL): (A, E) β-phenethylamine (PEA), (B, F) tryptamine (TRP), (C, G) ρ-tyramine (TYR), and (D, H) agmatine (AGM). Data are represented at mean (% of total imaged area) ± SEM. Statistical analysis: 2-way ANOVAs with Tukey's multiple comparison tests: * = p <0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001.

In the presence of TAs and without pre-treatment (control), TJPs were differentially disrupted (clustered or dispersed and irregularly localised) and cellular morphology was altered to varying degrees by all TA included. Exposure to PEA and TRP seemed the least detrimental,

while AGM exposure induced the most significant damage to monolayer integrity (Fig. 5.2E). Although PEA treatment did not alter TJP levels significantly (Fig. 5.1A, E), qualitatively a mild intracellular redistribution of occludin, as well as larger areas without ZO-1 expression, was observed (Fig. 5.2B). TRP treatment only altered cellular morphology slightly (Fig. 5.2C), despite significantly increased total ZO-1 expression levels (Fig. 5.1F; $p<0.01$ *vs* control). In contrast, TYR treatment significantly increased occludin (Fig. 5.1C; $p<0.01$) most of all TAs assessed. This ≈ 20 % increase was associated with significant internalisation and clustering of occludin (Fig. 5.2D). TYR treatment also significantly increased ZO-1 expression (Fig. 5.1G; $p<0.0001$), which appeared less organised than control. AGM treatment prominently increased total occludin (Fig. 5.1D; $p<0.05$) and ZO-1 expression levels (Fig. 5.1H; $p<0.05$). Notably, AGM treatment clustered TJPs intensely at membrane junctions in areas of intact monolayer – evidenced by the intense yellow signal in some, but not all cells (Fig. 5.2E) – but also resulted in interspersed areas where TJP disruption is evident. These different outcomes for TYR and AGM in terms of TJP profile, highlights the importance of assessing both expression levels (Fig. 5.1) and cellular distribution profiles (Fig. 5.2) of TJPs.

Moreover, the effects of E2 pre-treatment on the cellular response to TA-exposure was variable. In the context of PEA and TRP treatment, the presence of E2 significantly increased occludin (Fig. 5.1A, B) and to a lesser extent ZO-1 expression (Fig. 5.1E, F). These results are reflected in the respective representative images which depict increased occludin clustering and internalization (Fig. 5.2G) and higher levels of ZO-1 signal (Fig 5.2H). Markedly, following TYR treatment, E2 pre-treated monolayers had normalised occludin and ZO-1 levels (Fig. 5.1C, G). Indeed, the E2 and TYR group had significantly lower occludin than both other TYR groups ($p<0.0001$ for both), which translated to regular TJP distribution and localization (Fig. 5.2I), resembling a control profile. In terms of AGM, no obvious E2 effect was apparent.

Turning attention to rooibos, despite the significant dampening effect of GRE pre-treatment on occludin levels in the absence of TAs, GRE pre-treatment was unable to prevent the occludin response to TA exposure, with the exception of the response to AGM exposure, which was significantly inhibited (Fig. 5.1D). In this context, cellular morphology was generally maintained, and TJP distribution was visually similar to control cells in the absence of TA treatment (Fig. 5.2O). Regarding PEA, TRP and TYR treatment, GRE pre-treatment had no effect on absolute TJP levels, however, cellular morphology in the combination TYR group appeared somewhat modulated (Fig. 5.2N). Taken together, both E2 and GRE pre-treatment had very specific effects, which suggest complex, yet different mechanisms of actions.

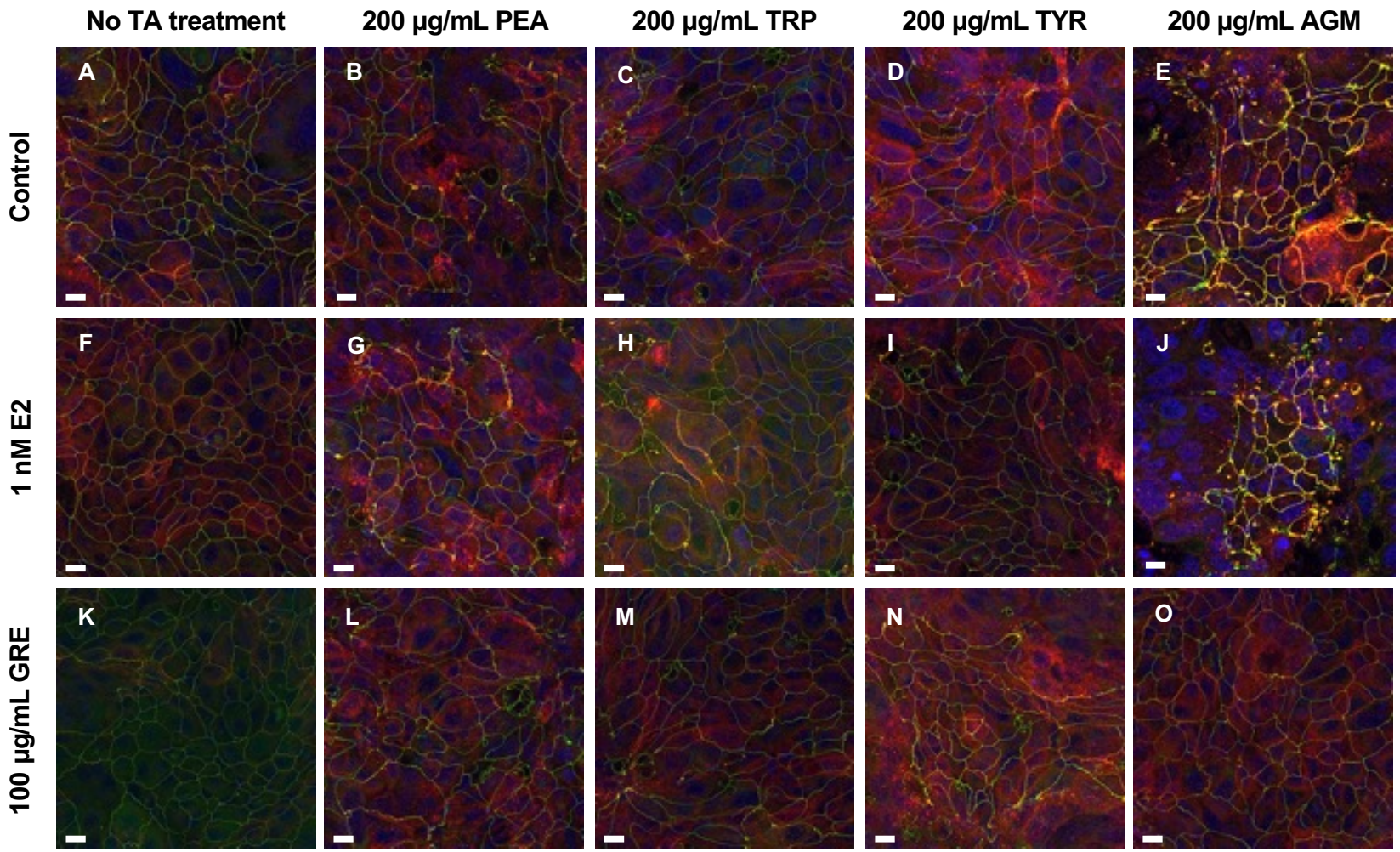


Figure 5.2: Representative fluorescent micrographs showing the effects of trace amines (TA), green rooibos extract (GRE), 17β-estradiol (E2) and combined treatments on tight junction proteins in HT-29 cell monolayers. Green signal = ZO-1, red signal = occludin, yellow signal = colocalized ZO-1 and occludin signal, and blue signal = Hoechst. All fluorescent images are maximum intensity projections of acquired z-stacks, imaged using the 60 x oil immersion objective. Scale bar = 10 µm. Abbreviations: PEA: β-phenethylamine, TRP: tryptamine, TYR: ρ-tyramine, AGM: agmatine.

5.3.2 Rooibos and estradiol differentially modulate prostaglandin E2 secretion following trace amine-exposure

In terms of assessment of inflammatory profile, prostaglandin E2 (PGE2) and inflammatory cytokine levels were determined. In the absence of TA-exposure, neither E2 or GRE pre-treatment affected PGE2 secretion by HT-29 cells (Fig. 5.3). Similar to the TJP data, PEA and TRP exposure did not result in increased PGE2 secretion, with no apparent effect of either E2 or GRE pre-treatment in this context (Fig. 5.3A, B). In contrast, TYR-exposure under control conditions, increased PGE2 levels significantly ($p<0.01$ *vs* control, Fig. 5.3C). Both E2 and GRE prevented this increase, maintaining PGE2 at baseline levels. However, while this modulatory effect on TYR-induced PGE2 secretion was mediated by both E2 and GRE, from representative micrographs of these cell monolayers, only the effects from E2 seemed to translate into a more beneficial outcome in terms of maintenance of TJP integrity and cellular morphology (Fig. 5.2I). Lastly, in response to AGM exposure, a significant but mild PGE2 response was only evident in the presence of E2, while GRE significantly reduced PGE2 response *vs* the no pre-treatment AGM-exposed condition (Fig. 5.3D; $p<0.05$).

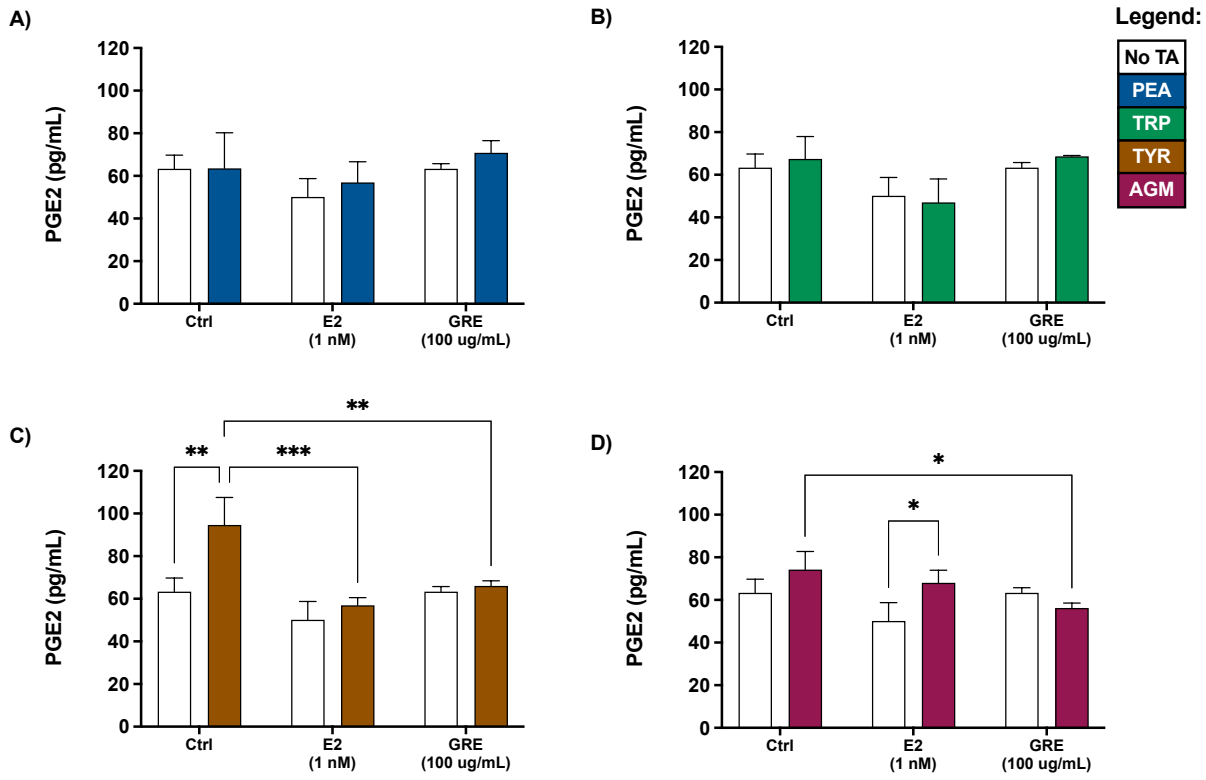


Figure 5.3: Effects of green rooibos extract (GRE) and 17β-estradiol (E2) on the concentration of prostaglandin E2 (PGE2) secreted by HT-29 cell monolayers following selected TA-exposure (200 ng/mL): (A) β-phenethylamine (PEA), (B) tryptamine (TRP), (C) ρ-tyramine (TYR), and (D) agmatine (AGM). Data are represented at mean ± SD, n=3. Statistical analysis: 2-way ANOVAs with Tukey's multiple comparison tests: * = p <0.05, ** = p <0.01, *** = p < 0.001.

5.4.3 Rooibos, but not estradiol, differentially modulates IL-8 secretion following trace amine-exposure

In terms of cytokine secretion, the overall cellular cytokine response was quite low, with only IL-8 secreted at detectable levels (Fig. 5.4). All TAs employed seemed to reduce the IL-8 response, although not reaching statistical significance for TRP. In the absence of TA treatment, GRE pre-treatment reduced the IL-8, while in the presence of PEA and TYR, TAs enhanced this effect. In contrast, E2 pre-treatment did not seem to have an effect on IL-8 release, suggesting that E2 and GRE are independent role players in this context.

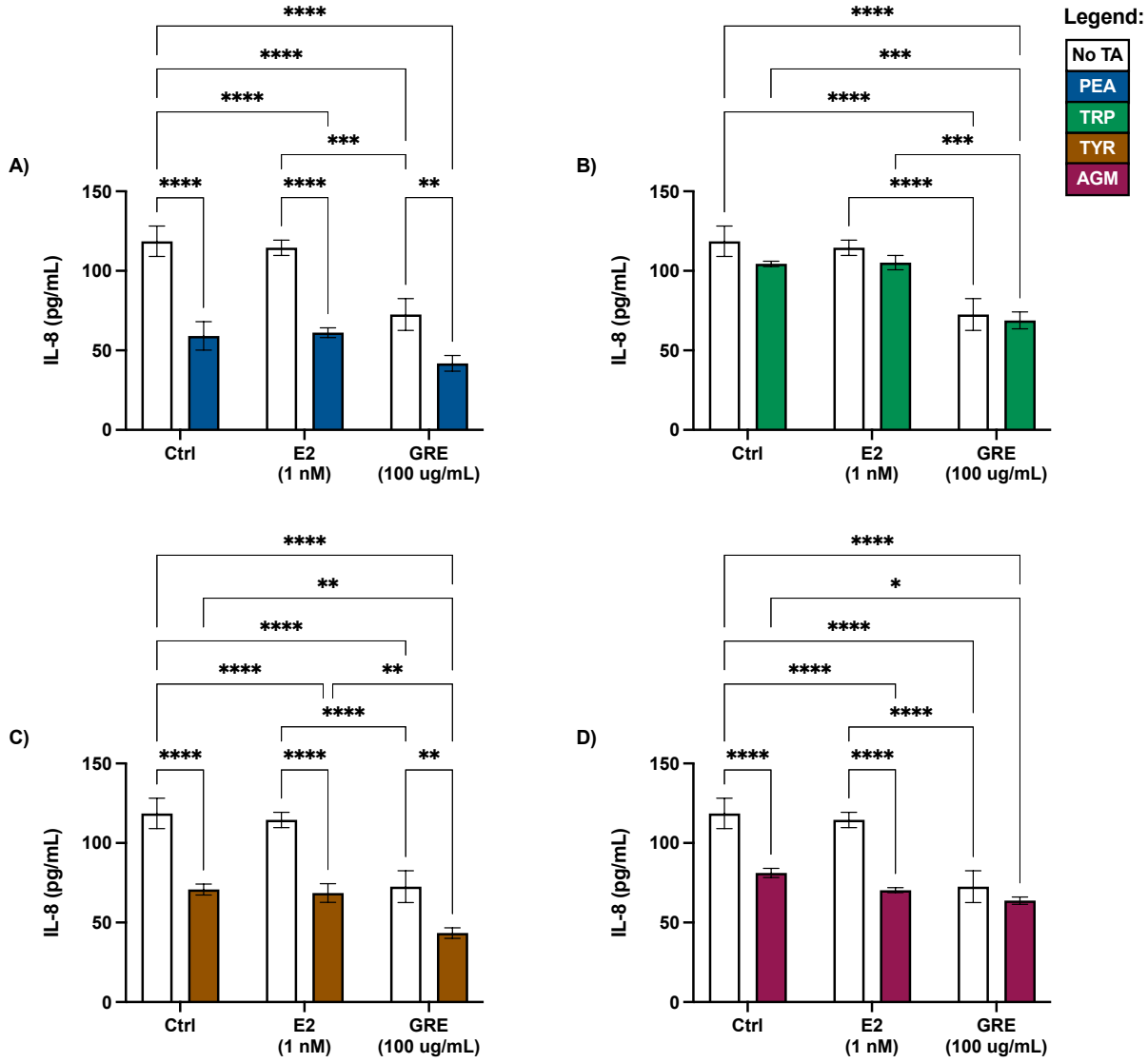


Figure 5.4: Effects of green rooibos extract (GRE) and estradiol (E2) on the concentration of interleukin-8 (IL-8) secreted by HT-29 cell monolayers following selected TA-exposure (200 ng/mL): (A) β-phenethylamine (PEA), (B) tryptamine (TRP), (C) ρ-tyramine (TYR), and (D) agmatine (AGM). The data are represented at mean ± SD, n=3. Statistical analysis: 2-way ANOVAs with Tukey's multiple comparison tests: * = p <0.05, ** = p <0.01, *** = p < 0.001, **** = p < 0.0001.

5.5 Discussion

Gastrointestinal epithelia are exposed to numerous exogenous (dietary, microbial and medicinal) metabolites/compounds and endogenous stimuli (inflammatory, hormonal and neural), which can influence gastrointestinal homeostasis. For the epithelial cells to both absorb necessary nutrients as well as prevent the entry of potentially harmful microbes or dietary antigens, dynamic regulation of intestinal barrier permeability is crucial. In this context, polyphenol-rich nutraceuticals have seen a dramatic increase in their use as dietary supplements. This is likely due to their widely reported protection against oxidative damage and inflammation, and beneficial modulation of intestinal barrier integrity (Bernardi et al., 2020, De Santis et al., 2015, Gil-Cardoso et al., 2016, Mijan and Lim, 2018, Samodien et al., 2021, Uyanga et al., 2021), which supports a role for them in management of chronic inflammatory conditions associated with increased intestinal permeability.

Current data yielded the following main novel findings: i) pre-treatment of HT-29 cells with E2 or GRE both improved cellular ultrastructure, albeit differently ii) exposure to TAs generally suppressed the inflammatory response in terms of inflammatory cytokine (IL-8) secretion, although TYR (and to a lesser degree AGM) elicited a significant PGE2 response, which was associated with increased total levels of and altered TJP distribution, iii) E2 pre-treatment prevented the PGE2 response to TYR only, normalising TJP levels and distribution, while iv) GRE prevented TA-induced PGE2 and IL-8 secretion in both TYR and AGM, but was only able to normalise TJP levels and distribution profile for AGM.

Current data illustrate that pre-treatment with both E2 and GRE improved HT-29 monolayer morphology, which adds to literature reporting known beneficial modulation of tight junction barriers in this context. In the context of E2, this outcome was potentially mediated by estrogen receptor (ER)-β and/or G protein-coupled ER 1 (GPER) interactions – both of which have been reported in HT-29 cells (Bustos et al., 2017, Jacenik et al., 2019, Wada-Hiraike et al., 2006). This interpretation is in line with literature describing destabilization of cellular integrity and cytoarchitecture as a feature of ERβ-/- mice (Wada-Hiraike et al., 2006), suggesting that activation of ERβ by E2 promoted uniform cellular architecture. This notion also aligns with exaggerated gastrointestinal symptomology during menses, when E2 levels are at their lowest (Kim and Kim, 2018). In the context of rooibos, however, literature regarding its effects on TJPs are sparse and usually in the context of blood-brain-barrier (BBB) assessments. To our knowledge, the results demonstrated in this study are the first to inform on the potential of rooibos to beneficially modulate colonic TJPs following TA (AGM specifically) exposure.

According to literature, long-term consumption of fermented rooibos tea was found to protect against BBB disruption and brain oedema following ischemic injury in rats (Akinrinmade et al., 2017). Similarly, Fisher et al. (2020) reported protective effects of an aspalathin rich rooibos tincture in an *in vitro* BBB model. Notably this study utilised bEnd5 cells, which do not express the TJP occludin (Fisher et al., 2020). In our study, an ethanol extract of green rooibos decreased total occludin signal significantly under baseline conditions (absence of TA treatment), which in the context of the report from Fisher et al (2020), suggests more than one potential mechanism of action for rooibos-related beneficial modulation of tight junction barriers. Importantly, this downregulation of total absolute occludin expression levels did not compromise cellular integrity or morphology. A potential explanation for this finding is related to rooibos uptake and transport in the gut. In this regard, it has been strongly suggested that aspalathin – the major constituent of GRE used – is potentially transported paracellularly (Bowles et al., 2017), motivating that the decrease in occludin observed may be due to an enhancement of paracellular transport in this context, although this requires more thorough investigations to elucidate the actual mechanism at play.

In terms of TA-induced effects on inflammatory status, although the levels of PGE2 – as a prominent prostanoid – seemed to associate with changes in TJP status induced by TA-exposure (specifically TYR and AGM) in this study, cytokine analyses revealed a relatively suppressed IL-8 response following TA treatment across the board. This seeming contradiction to the increased PGE2 levels is in line with literature though. For example, according to Kelly et al (2015), a feed-forward cycle between gut barrier dysfunction and inflammatory processes exists. In this context, authors suggested that an increase in gut permeability, which is generally associated with TJP disruption, could precede mucosal inflammation to stimulate an inflammatory response (Kelly et al., 2015). Also, increases in PGE2 generally precede increases in IL-8 levels (Srivastava et al., 2012). Once produced from membrane phospholipids, PGE2 is secreted, either *via* passive diffusion or active transport to exert para- or autocrine effects – which may explain the higher PGE2 levels observed.

Current data implicates TYR especially in PGE2 stimulation. This has specific relevance to gastrointestinal disorders, as elevated levels of faecal TYR were identified as a differential biomarker in patients with inflammatory bowel disease in a cross-sectional study (Santoru et al., 2017) and TYR has demonstrated cellular cytotoxicity at high doses (Del Rio et al., 2018, Pretorius et al., 2022b). Furthermore, we and others have demonstrated that TYR is an abundant microbial metabolite (Luqman et al., 2018, Pugin et al., 2017, Pretorius et al., 2022a). Of particular relevance, TYR formation *via* L-tyrosine decarboxylation is a well-known

characteristic of lactic acid bacteria (LAB) (Barbieri et al., 2019), which are commonly consumed as probiotic supplements. Considering TYR as a microbial metabolite, as well as the report by Luqman et al (2018) that demonstrated promotion of bacterial adherence and subsequent internalization (HT-29s) in the presence of TYR, a low or delayed cytokine response may be in line with microbial adaptations to evade host immune systems (Reddick and Alto, 2014). Furthermore, in terms of additional mechanisms that can potentially explain the low cytokine response, despite the generally robust PGE2 response, we hypothesise that occludin-related congestion of the golgi complex (Yu et al., 2015) – *via* caveolin-1-dependent mechanisms – may have caused delayed cytokine secretion and may potentially explain the discrepancy between the PGE2 and IL-8 results. In support of this interpretation, our representative micrographs depicted prominent occludin redistribution following TA treatment in general, with most extensive internalization in the presence of TYR. Importantly, caveolin proteins associate with the golgi complex to facilitate transport from the plasma membrane into the cell (de Almeida, 2017, Feng et al., 2013). As such, endocytosis of occludin in this context is likely mediated by caveolin-1 (Marchiando et al., 2010), which forms caveolae (plasma membrane invaginations), and are reportedly essential for immune-mediated TJP regulation. Furthermore, in general caveolin-1 has been reported to limit the inflammatory response (de Almeida, 2017). In support of this LPS and other microbial products have been shown to activate caveolin, which may assist their (microbial) entry into cells/across barriers when there is compromised TJP integrity (Lei et al., 2005, de Almeida, 2017). Certainly, some pathogens exploit caveolae as a route of internalisation that would allow their survival, since it avoids the lysosomal pathway (Machado et al., 2012). In fact, caveolin-1 expression increased the susceptibility of M-cells to Salmonella infection (Lim et al., 2009), implicating caveolin-1 in the gateway of microbial pathogen internalization. While this mechanism of action was not confirmed in the current study, it may – at least in part – explain the inflammatory profile reported here. As such, it would be interesting to assess potential links between caveolin-1, occludin internalisation and TYR in future studies.

Despite the relatively similar effects of TYR and AGM – in line with our previous work (Pretorius et al., 2022b) – to alter both PGE and IL-8 levels and increase total TJP concentrations, an important differentiating factor in these effects are related to the distribution of occludin in particular. In the case of TYR, as mentioned previously, extensive occludin internalisation (i.e. loss of colocalization with ZO-1) was observed, while exposure to AGM promoted ZO-1 and occludin colocalization in areas of intact monolayer, but with apparent loss of TJP expression in other areas. In the current study, the inclusion of pre-treatment conditions (E2 and GRE) serves to illustrate potential differences in mechanisms of actions between TYR and AGM. For example, in the context of E2 pre-treatment, E2 prevented TYR-

induced PGE2 increases, normalising TJP levels and cellular distribution, particularly that of occludin. While the precise mechanism of action by which TYR disrupts TJPs is unknown, we suggest – at least in part – that hydrogen peroxide, as a by-product of oxidation of TYR via monoamine oxidase (MAO), may induce oxidative stress and damage that could disrupt junctional proteins and integrity. Indeed, the intestine is predominated (<80%) by MAO-A isoform (Tipton, 2018), by which TYR and other TAs (but not PEA, which is highly selective for MAO-B) are deaminated. In addition, a study investigating the effect of MAO substrates on endogenous prostaglandin synthesis in rat brain homogenates reported that the presence of tyrosine (the precursor L-amino acid to TYR) caused a 2-fold increase in cyclooxygenase activity, increasing PGE2 levels significantly (Seregi and Hertting, 1984), suggesting that the hydrogen peroxide formed during amine degradation stimulated prostaglandin synthesis. While it should be considered that TYR-related internalization of occludin may be mediated by more complex mechanisms, it has been reported that PGE2 may disrupt intestinal epithelial barrier function, and specifically redistribute occludin towards intracellular locations *in vitro* (Rodriguez-Lagunas et al., 2010). Regarding the preventative E2 effect in this context, well described anti-inflammatory effects of estrogen or ERβ agonists have been described in models of chronic intestinal inflammation (Harnish et al., 2004, Harris, 2007, Jacenik et al., 2019, Looijer-van Langen et al., 2011). Specifically, E2 is reported to mitigate oxidative damage caused by hydrogen peroxide in several tissue types (Farruggio et al., 2019, Giddabasappa et al., 2010, Khan et al., 2021, Ruan et al., 2014), including in HT-29s, where E2 treatment prevented oxidative damage of the mucus layer, and reduced apoptosis and permeability following hydrogen peroxide challenge (Diebel et al., 2015). Taken together, if E2 pre-treatment quenched TYR-related hydrogen peroxide, the subsequent increase in PGE2 and occludin redistribution could have been alleviated, as seen in current data. The fact that the (known antioxidant) rooibos pre-treatment was also able to reduce the PGE2 response – although not the normalisation of TJP profile in the presence of TYR, supports a role for antioxidants in this context, but also suggests that more than one mechanism is at play. The relative importance of a direct antioxidant response of E2 (as described above) vs. other mechanisms – such as potential ERβ and/or GPER interactions – in the context of TYR-associated risk remains to be elucidated. Regardless, in terms of the practical implications of the current data, the fact that the presence of E2 seems to negate TYR-induced epithelial disruptions, suggests that female patients with elevated gut TYR levels could potentially have significantly exaggerated gastrointestinal symptomology during menses. This fact, as well as the high proportion of TYR secreted by LAB, as already mentioned, also cautions against prescription of these bacteria as probiotic supplements in FGID.

Moving on to address the different outcome to AGM exposure, the effects of rooibos suggest that redox balance is hugely important in optimising effects of AGM, which in the literature is mostly ascribed beneficial roles, albeit at much lower levels. Taking a closer look at the effects of AGM, literature generally suggests a neuroprotective effect (Xu et al., 2019), particularly in the context of BBB stabilization and AGM-related reductions in BBB permeability (Ahn et al., 2015). In the current study, representative micrographs importantly reflected increases in ZO-1 and occludin colocalization along the cell membrane, which we suggest may depict an promotion of TJP integrity, which would be in line with the mentioned beneficial effects of AGM on the BBB.

Despite this favourable assessment of AGM function on TJPs, the interspersed areas lacking TJP suggest breakdown of cellular membrane integrity – a less desired outcome. However, given the fact that rooibos normalised this profile, we would like to argue that this negative outcome is an artefact of the cellular model used, for two reasons. Firstly, it was unlikely that AGM was metabolised into its downstream polyamine metabolites (putrescine, spermidine and spermine), as would normally happen *in vivo*. Indeed, exogenous AGM accumulation inside HT-29s, with minimal subsequent catabolism - ascribed to AGM-associated downregulation of ornithine decarboxylase activity and expression - has been reported (Mayeur et al., 2005). Thus, at high doses, AGM may have directly resulted in epithelial cell apoptosis. This interpretation is in line with reports of AGM enhancing the release of pro-apoptotic pro-oxidant factors, such as cytochrome c, to potentially induce apoptosis *via* selective permeabilization of the outer mitochondrial membrane (Martinis et al., 2020).

Secondly, the relative absence of AGM-associated polyamines in the cellular model, had further significance. These polyamines – which are non-enzymatic antioxidants (Mironczuk-Chodakowska et al., 2018, Rider et al., 2007) - have been reported to improve gastrointestinal epithelial integrity and restoration by enhancing TJP expression (Guo et al., 2005, Rao et al., 2012). This protective effect is attributed to the stabilization of lipids in the cell membrane by polyamines, particularly spermine (Fujisawa and Kadoma, 2005). Thus, while AGM has beneficial effects on TJP colocalization, by preventing its own metabolism into antioxidant polyamines, increased availability of AGM may facilitate oxidative damage to surrounding cells, which is in line with current data, as well as literature cautioning against excessive AGM intake (Oliphant and Allen-Vercoe, 2019). Current data clearly illustrates the importance of dose regulation of AGM, as there seems to be a fine line between beneficial and detrimental effects.

In the context of AGM, an important role for rooibos is demonstrated. Firstly, Dludla et al (2020) recently reported that a specific rooibos formulation (combination aspalathin and PPAG – both present in notable amounts in the GRE utilised here) exerted anti-apoptotic characteristics specifically (Dludla et al., 2020). Secondly, the high polyphenol (i.e. also non-enzymatic antioxidant) content of GRE was likely mitigating the relative lack of polyamine-related antioxidant activity here. In terms of specific constituents of the GRE responsible for this benefit, one candidate would be quercetin, as the most ubiquitous polyphenolic flavonoid known to prevent against oxidative damage to DNA oligonucleotides by reactive oxygen species (Moussa et al., 2019) – and which was concentrated in our GRE extract (Supp. Fig. 5.1).

5.6 Conclusion

Taken together, current data expands on the literature implicating TYR as a major trigger in gastrointestinal disorders by suggesting that manipulation of E2 (or its receptors) may provide therapeutic effect. Furthermore, our data suggest that combination treatment with AGM and GRE may have substantial benefit in the context of intestinal inflammation and barrier disruption. These benefits of GRE seem to be directly linked to its antioxidant and – to a lesser extent – its anti-inflammatory characteristics, rather than to a phytoestrogenic effect. These data warrants further treatment development in a robust *in vivo* model, so that limitations of cell culture models may be overcome.

5.7 Supplementary materials

Quantification of phenolic constituents

We employed the method described by Stander et al. (2017) to quantify fifteen major phenolic constituents commonly found in rooibos tea. Briefly, tea samples were extracted with 50% MeOH and 1% FA. A Waters Synapt G2 Quadrupole time-of-flight (q-TOF) mass spectrometer (MS) connected to a Waters Acquity ultra-performance liquid chromatograph (UPLC) was used for high-resolution UPLC-MS analysis (CAF, Stellenbosch University). Chromatographic separation was achieved on a Waters HSS T3 column (1.7 µm, 2.1 × 100 mm), and the column temperature was maintained at 55 °C. For all samples an injection volume of 2 µL was used and run using a binary mobile phase gradient which consisted of (A) 0.1% FA in H_2O and (B) 0.1% FA in Acetonitrile. The flow rate was set to 0.3 mL/min throughout the set 29 min run time with the following separation conditions: the gradient started at 0% solvent B for 1 min and increased to 28% B over 22 mins in a linear way. It was then increased to 40% solvent B until 22.5 min, followed by an increase to 100% solvent B until 23 min. Solvent B was held at 100% until 24.5 min, before being reduced to 0% solvent B to re-equilibrate to initial conditions for the final 4.5 min. Electrospray ionization was used in negative mode with a cone voltage of 15 V. Constituents were identified according to their accurate mass, MS/MS fragments, UV maxima and retention times as previously described (Stander et al., 2017) and quantified relative to rutin reference standards.

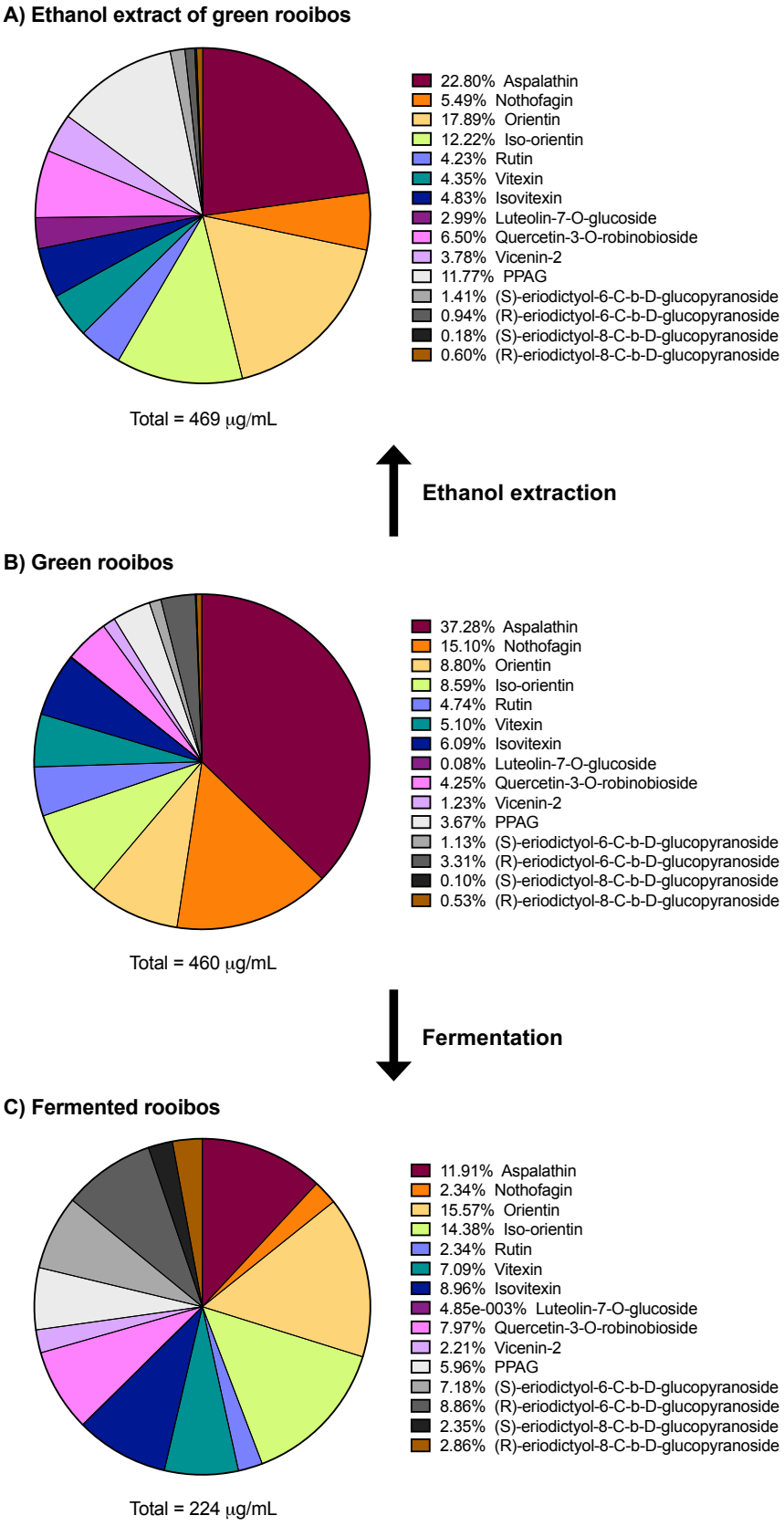
Choice of rooibos formulation

The decision to utilise a single rooibos preparation in this study (i.e. GRE) was manifold. Firstly, it is well-known that various factors influence the polyphenolic content, and subsequent antioxidant capacity, of rooibos. Some of these factors include geographical variety variations (Stander et al., 2017), season and quality grade (Joubert et al., 2012), processing (Pretorius et al., 2022a) and tea preparation i.e. brewing method (Damiani et al., 2019). As mentioned in the introduction, we have previously characterised 15 major polyphenolic constituents in green rooibos (GR), fermented rooibos and GRE (used in this study). Secondly, from these results (Supplementary Figure 1) we observed that the process of ethanol extraction concentrated constituents with well documented antioxidant activities. Indeed, orientin (An et al., 2012, Nayak and Uma, 2006, Praveena et al., 2014), iso-orientin (Cheel et al., 2005, Sarıkahya et al., 2011, Yuan et al., 2018), luteolin-7-O-glucoside (Park and Song, 2019, Rehfeldt et al., 2022, Song and Park, 2014), quercetin-3-O-robinobioside (Novaes et al., 2019, Xu et al., 2019, Zheng et al., 2017), and vicenin-2 (Duan et al., 2019, Lee et al., 2020a, Li et al., 2021a) were all present in higher levels in GRE compared to the original GR stock, and have well-documented anti-oxidant activities. Thirdly, the specific antioxidant activity of GRE utilised

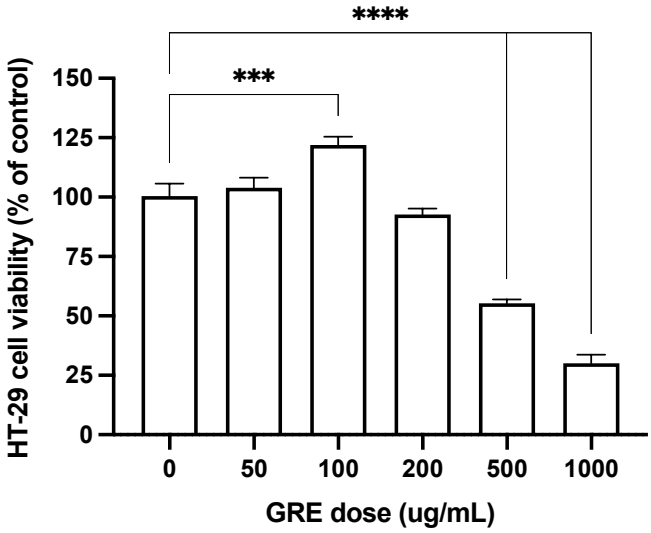
here has previously been determined (Lopez et al., 2022), which could be linked to outcome measures in this study. Fourthly, GRE also had higher phenylpropenoic acid glucoside (PPAG) levels than GR stock, and has been reported to confer protection downstream of oxidative stress induction (Himpe et al., 2016), presumably via anti-apoptotic mechanisms (Dludla et al., 2020). Taken together, we suggest that there is scope for specific rooibos constituent compounding (selected bioactive compound combinations and distributions) to promote synergistic actions to obtain optimal therapeutic benefit. As such, we selected to utilise GRE in this study, as it represented the rooibos formulation with the most abundant distribution of a wide variety of polyphenolic constituents with known antioxidant and anti-apoptotic activities.

Choice of rooibos dose

Due to the substantial polyphenolic content (Iswaldi et al., 2011, Stander et al., 2017) and documented antioxidant activity of GRE (Lopez et al., 2022, Marnewick, 2014), it was important to establish a feasible dose to investigate in cell culture. Previous work executed on GRE by our group utilized a final experimental concentration of 100 μg/mL *in vitro*, which was determined to equate to an 8-fold concentration of rooibos tea in terms of polyphenol content (Lopez et al., 2022). To determine the optimal dose of GRE for this current study, HT-29 cell viability was assessed in response to a range of GRE (0 to 1000 μg/mL). For this purpose, a WST-1 assay was used to assess the activity of mitochondrial enzymes (succinate-tetrazolium reductase system) in treated cells. Briefly, HT-29 cells were seeded in a 96-well microtiter plate with $4x10^4$ cells/well in phenol free media RMPI (Gibco, 11835-030) and were incubated at 37°C in 5% CO_2 after treatments. After a 24 hr incubation, 5 μL of WST-1 reagent (Abcam, ab155902) was added to each well and left to incubate for 90 min before the absorbance of each well was read on a plate reader (BIO-TEK, EL800) at 450 nm. Data were collected utilizing the KC Junior Software before exporting to Microsoft Excel for further analysis.



Supplementary Figure 5.1: Changes in the relative distribution (% of total) of fifteen major phenolic constituents due to differential rooibos processing: (A) green rooibos ethanol extract (GRE), (B) green rooibos, and (C) fermented rooibos. Abbreviations: PPAG: phenylpropenoic acid glucoside.



Supplementary Figure 5.2: WST-1 results showing the effect of varying green rooibos extract (GRE) doses on HT-29 colon cell viability. Data are represented as mean % of control ± SD, n=3. Statistical analysis: One-way ANOVA with Tukey's multiple comparison test, *** = $p<0.001$, **** = $p<0.0001$.

Results from the WST-1 assay suggested that 100 ug/mL of GRE had a beneficial effect of HT-29 cell viability ($p<0.001$), and as such was selected as the dose that was utilised in subsequent experimentation. Furthermore, these results suggest that an over-dose of GRE (>200 μg/mL), as with all antioxidants (Joubert et al., 2005), can have detrimental effects on cell mitochondrial functionality and survival.

Chapter 6

Tyramine-induced gastrointestinal dysregulation is attenuated via estradiol associated mechanisms in a zebrafish larval model

This chapter has been submitted to the Journal of Biomedical Sciences (Impact Factor: 8.41).

Title: Tyramine-induced gastrointestinal dysregulation is attenuated via estradiol associated mechanisms in a zebrafish larval model

Authors: Pretorius, L., & Smith, C.

6.1 Abstract

Introduction:
Development of targeted therapeutics to alleviate gastrointestinal (GI) inflammation and its debilitating consequences (i.e. GI symptomology) are required. In this context, the trace aminergic system may link together sex, diet and inflammation. Utilising a zebrafish larval model of GI inflammation (TNBS-exposure), the current study aimed to investigate mechanisms by which excess amounts of trace amines (TAs) may influence GI health. In addition, we probed the potential role of 17β-estradiol (E2) and its receptors, given the known female-predominance of many GI disorders.
Methods:
To assess GI functionality and integrity following TA administration, live imaging techniques (neutral red staining) and post-mortem immunofluorescent staining of tight junction proteins (occludin and ZO-1) were analysed respectively. In addition, behavioural assays, as an indication of overall wellbeing, as well as whole-body H_2O_2 and prostaglandin E2 assays were performed to inform on oxidative and inflammatory status.
Results:
Excess β-phenethylamine (PEA), tryptamine (TRP) and ρ-tyramine (TYR) resulted in adverse GI and systemic effects. In this regard, clear beneficial effects of E2 to modulate the effects of PEA, TRP and TYR was evident. Moreover, agmatine (AGM) displayed potential protective effects on GI epithelium and whole-body oxidative status, however, potential to induce systemic inflammation suggests the importance of dosage and administration optimisation.
Conclusions:
Considering both local and systemic analyses performed in the current study, TYR seems like the most prominent TA to have damaging GI effects, feasibly exacerbating GI inflammation. In this context, the relative lack of E2 (i.e. during menses) may provide mechanistic insights into the reported female-predominance of GI disorders. Moreover, an effective therapeutic in this context may be required to maintain GI TA load despite fluctuating E2 levels.

6.2 Introduction

Over the last few decades substantial increases in the incidence of gastrointestinal (GI) disorders associated with GI inflammation have been observed. Indeed, 6.8 million cases of inflammatory bowel disease (IBD) were reported globally in 2017 (Alatab et al., 2020) and more than 40% of individuals around the world experience functional GI disorders (FGIDs) to some degree (Sperber et al., 2021). Albeit to variable extents, most GI disorders are underpinned by altered mucosal and innate immune function (Gersemann et al., 2012, Lazaridis and Germanidis, 2018). A better understanding of intestinal immunity is vital in the search for targeted therapeutics with which to alleviate symptomology and improve quality of life of affected individuals. We have identified two complexities in this context. Firstly, consistent epidemiological evidence of female predominance related to specific GI disorders, such as irritable bowel syndrome (IBS) and Crohn's disease (Greuter et al., 2020, Kim and Kim, 2018) exist, which cannot be fully attributed to absolute hormonal differences between sexes (Bharadwaj et al., 2015b, Casimir et al., 2018, Mulak et al., 2014). Secondly, altered gut trace aminergic signalling and its pertinent links to sex, diet, and inflammation, has been implicated in the context of GI disorders (Gwilt et al., 2020, Christian and Berry, 2018, Pretorius and Smith, 2020). Indeed, elevated levels of faecal ρ-tyramine (TYR), β-phenethylamine (PEA) and tryptamine (TRP) has been reported in IBD (Santoru et al., 2018), Crohn's disease (Jacobs et al., 2016) and IBS (Ponnusamy et al., 2011) respectively.

Despite the potential implications for trace amines (TAs) as role players in intestinal inflammation and homeostasis – illustrated by abovementioned metabolomics studies – limited knowledge on gut-specific actions of TAs from robust models exists. Nevertheless, literature seems to suggest that TRP and TYR have the most potential to modulate GI motility (Bhattarai et al., 2020, Bhattarai et al., 2018, Williams et al., 2014, Takaki et al., 1985, Yano et al., 2015), while all TAs assessed in the current study (PEA, TRP, TYR and agmatine; AGM) seem to be directly or indirectly implicated in the regulation or stimulation of intestinal inflammatory processes (Babusyte et al., 2013, Islam et al., 2017, Rooks and Garrett, 2016). Indeed, our group has previously demonstrated the *in vitro* effects of TYR in particular to disrupt tight junction proteins (TJPs) and promote inflammatory prostanoid release (Pretorius et al., 2022b) (Pretorius and Smith, manuscript under review). However, limitations associated with *in vitro* experimental settings – specifically related to subsequent TA metabolism – limit interpretive power, highlighting the need for *in vivo* investigations to accurately model roles of TAs and their metabolites in the presence of endogenous regulatory systems. Moreover, considering that TAs are microbially derived metabolites that seem to confer some adaptive/advantageous effects to enhance microbial survival and subsequent host infiltration

(Fernandez de Palencia et al., 2011, Luqman et al., 2018, Marcobal et al., 2012), and that TAs are in high supply in certain foods which are associated with a high-perceived food intolerance among patients with IBS (Barbieri et al., 2019, Bohn et al., 2013), it is also important to elucidate negative effects (if any) to both acute high dose and chronic low dose exposures. In addition, considering the female predominance associated with many GI disorders, as well as our previous work demonstrating 17β-estradiol (E2)-associated modulation of microbial TA secretion (Pretorius et al., 2022b, Pretorius et al., 2022a), as well as E2-mediated protection against TYR-induced TJP damage (Pretorius and Smith, manuscript under review), the *in vivo* actions of TAs in the presence and absence of E2 warrants investigation.

In this regard, the use of zebrafish (*Danio rerio*) larvae to study GI disease and inflammation (Brugman, 2016, Chuang et al., 2019, Hanyang et al., 2017, Oehlers et al., 2013) has become increasingly popular. Zebrafish have comparable GI systems to humans, with highly analogous immune systems (as a key mediator between gut microbes and host) as well (Flores et al., 2020). Within this concept, their adaptive immune system is only functional four to six weeks post fertilization (Novoa and Figueras, 2011), thus the effects of the innate immune system can be investigated relatively unconfounded at early larval stages. This is of particular interest considering the innate immune system dysfunction prevalent in IBS and IBD (Kamada and Rogler, 2016, Lazaridis and Germanidis, 2018). In addition, established methods exist for introducing hormones such as E2 into zebrafish larvae (Souder and Gorelick, 2017), allowing development of a model in which female predominance can be explored.

Taken together, data on the *in vivo* actions of TAs in the gut, as well as the potential modulatory role of E2 in high dose TA signalling, are insufficient. Given our recent data illustrating the importance of endogenous role players in redox balance (Pretorius and Smith, manuscript under review), which affects the effects exerted by TAs, an *in vivo* model is likely the only way in which mechanisms and role players may be accurately elucidated. As such, this study employed a zebrafish larval model of GI inflammation to investigate *in vivo* mechanisms by which high doses of TA may influence intestinal health, while probing the potential confounding role of E2 and its receptors.

6.3 Methods

6.3.1 Animal husbandry & ethical considerations

Wild-type zebrafish (*Danio rerio*) embryos were obtained from the Zebrafish Research Unit (Division of Clinical Pharmacology, Department Medicine, Stellenbosch University). Zebrafish embryos and larvae were maintained in embryo medium (E3; 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2 \bullet 2H_2O$, 0.33 mM $MgSO_4 \bullet 7\ H_2O$, $1.3x10^{-5}$ % $^{w}/_{v}$ methylene blue in autoclaved RO water) at 28°C and 40-60% humidity, with a 14:10 light:dark cycle, and refreshed daily for the duration of the protocol. All experimental protocols were approved by the Stellenbosch University Animal Research Ethics Committee (Ref# ACU-2021-21677).

6.3.2 Induction of gastrointestinal inflammation by TNBS- and TA-exposure

GI inflammation was induced by 25 μg/mL 2,4,6-trinitrobenzene-sulfonic acid (TNBS; ThermoFisher, 28997) via immersion (TNBS was added to E3 used to refresh daily), from 5 days post-fertilisation (dpf), until 7 dpf (2-day duration). As a standard treatment control, 25 μg/mL prednisolone (Sigma, P6004) (reconstituted in DMSO and diluted in E3) administered via immersion for 24 hrs was utilised. (Thus, co-administration of TNBS and prednisolone occurred for the final 24 hrs of induction (6 to 7 dpf)). In addition, either 20nM 17β-estradiol (E2; Sigma, E2758) or the general estrogen receptor (ER) antagonist 2.5 μM tamoxifen (TAM; Sigma, T5648), were supplemented directly into E3 for 48 hrs (i.e. for the entire duration of TNBS-exposure).

In terms of TA exposure, 25 μM, 50 μM or 100/200 μM of β-phenethylamine (PEA; Toronto Research Chemicals, P321335) or tryptamine (TRP; Toronto Research Chemicals, T894600) or ρ-tyramine (TYR; Toronto Research Chemicals, T898493) or agmatine (AGM; Sigma, A7127) or a vehicle control (control) were supplemented in E3 for 24 hrs, from 6 to 7 dpf for all experiments.

6.3.3 Behavioural assay

As a general measure of overall wellbeing, basal activity levels of zebrafish larvae were tracked and recorded using Daniovision larval activity tracking equipment and Ethovision software (Noldus, Wageningen, Netherlands). Activity tracking of all larvae was conducted on 7 dpf. A smoothing profile of 0.2 mm (minimum distance moved) and frame rate of 25/s were applied.

6.3.4 Neutral red staining & live imaging

Neutral red staining was performed at endpoint by incubating larvae in 2 μg/mL neutral red (Sigma, 72210) in fresh E3 for 5 hrs. After 5 hrs excess neutral red was washed away by replacing E3. Live larvae were then anesthetised with 0.016 mg/mL ethyl 3-aminobenzoate methanesulfonate salt (Tricaine; Sigma, A5040) in E3 for ~ 5 min. Once larvae were sedated, they were transferred to a microscope slide in a drop of tricaine supplemented E3, manipulated to the correct orientation and imaged on a stereomicroscope (WPI, Florida, USA) at 2x magnification for representative images and 3x for quantification purposes. Once the images were captured, the larvae were placed in fresh E3 to reverse the effects of the anaesthetic. Images were exported to ImageJ for analysis.

6.3.5 Tight junction protein visualisation

Wholemount immunofluorescent staining

Larvae at 7 dpf were euthanised (tricaine overdose) and fixed in 4% paraformaldehyde overnight at 4°C. After fixation, larvae were washed twice with 1x PBS (pH= 7.4) before undergoing permeabilization in ice cold acetone for 7 min at -20°C. Larvae were washed twice again in PBS before incubation in blocking buffer (5% donkey serum, 20% fetal bovine serum (FBS) and 0.2% Triton-X in PBS) for 3 hrs at room temperature. Thereafter, overnight primary antibody incubations at 4 °C followed, utilising 1:100 mouse anti-ZO-1 (Invitrogen, 33-9100) and 1:250 rabbit anti-occludin (Novus Biologicals, NBP1-87402) in antibody diluent (1% donkey serum and 4% FBS in PBS with 0.1% Tween 20 (PBS-T)). Next, larvae were washed 3x 5 min in PBS-T, prior to incubation with secondary antibodies: 1:250 Alexa Fluor 488 donkey anti-mouse (Invitrogen, A-21202) and Alexa Fluor 594 donkey anti-rabbit (Invitrogen, A-21207) in antibody diluent overnight at 4 °C. Finally, the larvae were incubated with Hoechst (ThermoFisher Scientific, 33342) for 20 min at room temperature. In preparation for embedding, larvae were washed 3x 5 min with PBS-T and 1x 5 min with PBS.

JB-4 resin embedding and sectioning

Stained whole larvae were embedded using a JB-4 embedding kit (Sigma, EM0100) according to a protocol published by Sullivan-Brown et al. (2011), with minor modifications. Infiltration with infiltration solution proceeded directly after staining was completed in a three-stage process: (1) 1x 1 min at room temperature, (2) 1x 1 hr at room temperature, and finally (3) 1x overnight at 4 °C. After infiltration, larvae were embedded in freshly made embedding solution in polyethylene molding cup trays and embedding stubs (Polysciences, 11643A & 23197), overnight at 4 °C. Embedded samples were sectioned longitudinally (5 μm) using a standard rotary microtome fit with a tungsten blade. Sections were mounted onto microscope slides with Dako fluorescent mounting media (Diagnostech, S3023) and visualised by brightfield and

fluorescence microscopy using a Nikon® ECLIPSE Ti2 inverted microscope and images were captured using NIS-Elements D v 5.30.02 software. The immunofluorescent intensities were quantified for each treatment group, with a minimum of 6 samples per group, using colour threshold and area measurements with ImageJ v 2.1.0 software. For TJP quantification, micrographs captured using 10x objective were utilised, while representative micrographs were captured using the 60x objective.

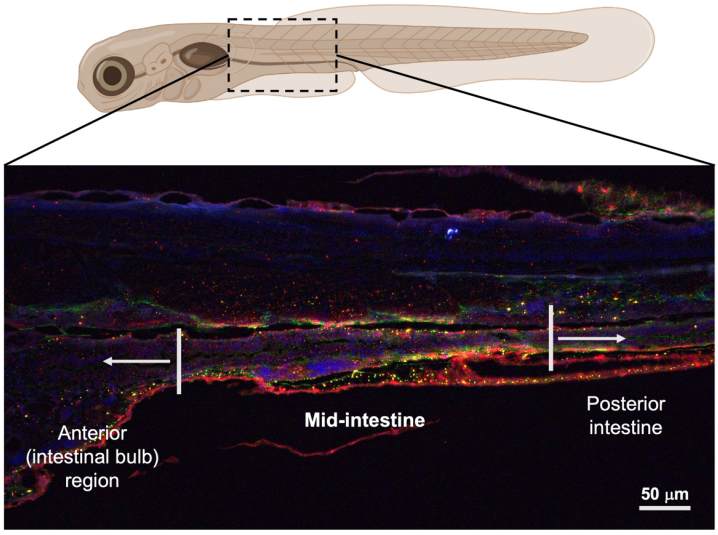


Figure 6.1: Schematic illustrating longitudinal sections through larvae that enabled the correct orientation and assessment of the desired – mid-intestinal – gut region.

6.3.6 Hydrogen peroxide assay

Hydrogen peroxide (H_2O_2) concentrations in whole zebrafish larvae were determined using a commercial H_2O_2 colorimetric assay kit (Elabscience, E-BC-K102-S) according to manufacturer's instructions with minor modifications. Briefly, after exposure of zebrafish larvae to TA treatments for 24 hrs, 25 zebrafish larvae (7 dpf) per sample were pooled and homogenized in 250 µL ice cold PBS (pH 7.4). Homogenates were centrifuged at 10000 x*g* and 4 °C for 10 min and supernatants were collected for the assay. In a 96-well microtiter plates, 100 µL/well of the buffer solution (reagent 1) was incubated at 37 °C for 10 min. After incubation, 20 µL/well of H_2O_2 standard or sample was added, followed by 100 µL/well of ammonium molybate (reagent 2). Immediately after, absorbance was read at 415 nm on a microplate reader (Victor Nivo Multimode Plate Reader, PerkinElmer). The assay was performed in triplicate, on samples from six different experiments and absorbance corrected for total protein content per sample (determined *via* Bradford assay).

6.3.7 Prostaglandin E2

PGE2 concentrations in whole zebrafish larvae were determined using a commercial PGE2 ELISA (Elabscience, E-EL-0034) following the manufacturer's guideline protocol. Briefly, after exposure of zebrafish larvae to TA treatments for 24 hrs, 25 zebrafish larvae (7 dpf) per sample were pooled and homogenized in 250 µL ice cold PBS (pH 7.4). Homogenates were centrifuged at 10000 x*g* and 4 °C for 10 min and supernatants were collected for the assay. The assay was performed in triplicate, on samples from three different experiments and absorbance corrected for total protein content per sample (determined *via* Bradford assay).

6.3.8 Statistical analyses

Visualization and statistical analyses of all data were completed utilizing GraphPad Prism version 9.4.0 (www.graphpad.com, San Diego, CA). Data from the behavioural assay (n=12 per group) was presented as individual scatter dot plots with mean ± standard error of mean (SEM) and was analyzed by 2-way ANOVAs with Tukey's multiple comparison tests. Data from the neutral red staining experiment (n=9 per group) were analyzed with 2-way ANOVA with Tukey's multiple comparison tests and represented as mean ± standard deviation (SD). Data depicting TJPs (ZO-1 and occludin; n=6 per group) were analyzed with a one-way ANOVA with Tukey's multiple comparison tests and represented as mean fluorescent intensity ± SEM. Finally, whole body H_2O_2 (n=6 pooled samples per group) and PGE2 (n=3 pooled samples per group) data were analyzed with one-way ANOVAs with Tukey's multiple comparison tests and represented as mean concentration per total protein (determined *via* Bradford assay) as % of control ± SD. A p-value of <0.05 was considered statistically significant.

6.4 Results

6.4.1 Differential effect of trace amines to modulate general swimming activity

TNBS administration significantly reduced larval locomotion compared to control levels (Figure 6.2; $p<0.0001$). Moreover, TNBS exposure reduced the variability within treatment groups, resulting in a more clustered response, indicating a similar sensitivity of the larvae to TNBS. With prednisolone co-administration, a significant rescue effect was observed ($p<0.05$ *vs* control TNBS), and a restoration to normal distribution/variability was evident. In the absence of TNBS, both PEA and AGM treatment did not significantly alter activity levels, although treatment did increase the variability within each respective treatment group. In contrast, TRP and TYR treatment significantly decreased locomotion ($p<0.001$ and $p<0.05$ respectively). Moreover, TRP treatment – similarly to TNBS treatment – elicited a response with clustered

distribution around the mean (i.e. reduced inter-group variability) suggesting that of the TAs assessed, the larval activity were most sensitive to TRP and/or its metabolites. In the presence of TNBS, none of these TAs (PEA, TRP and TYR) significantly exacerbated or countered the effects of TNBS. In contrast, AGM treatment partially rescued this effect, restoring normal inter-group distribution.

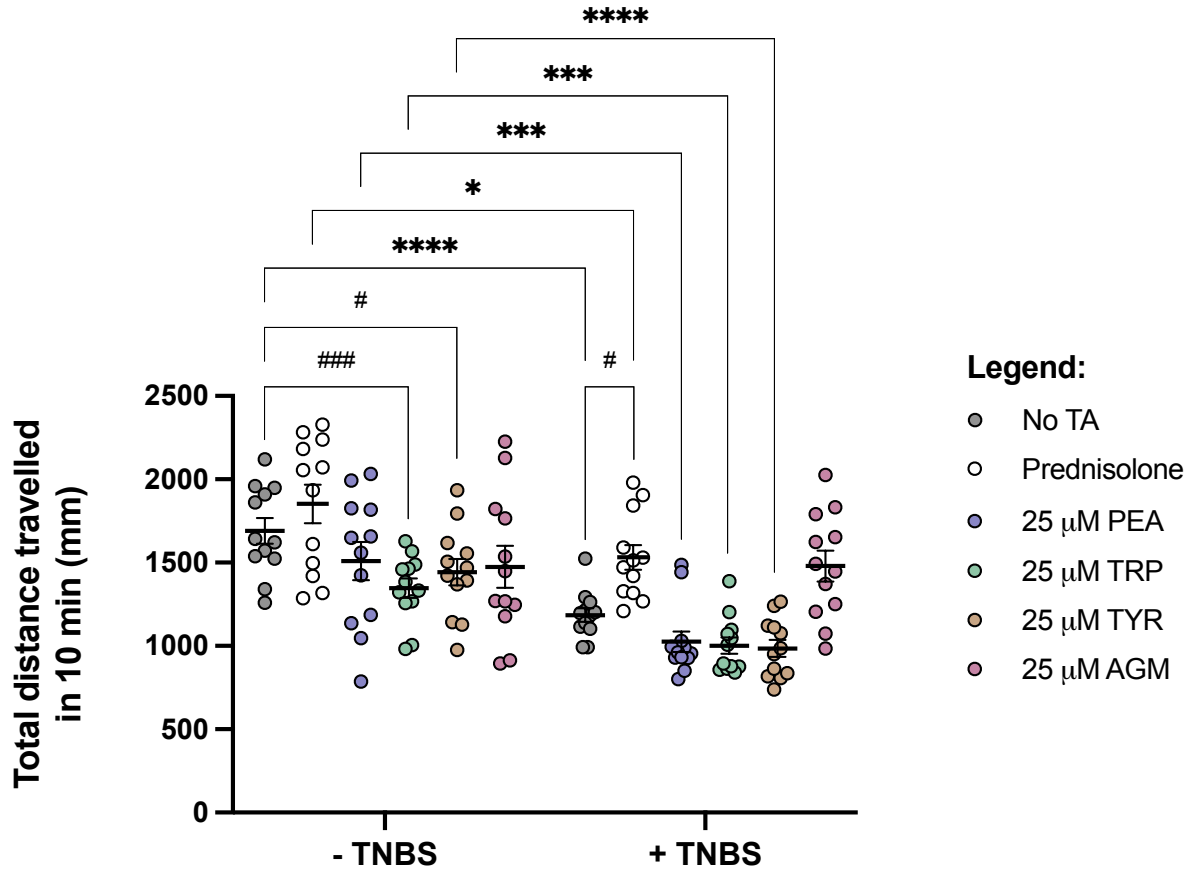


Figure 6.2: Basal locomotion of zebrafish larvae (7 dpf) following selected trace amine (TA) exposure in the absence or presence of an inflammatory stimulus (TNBS; 25 μg/mL, 2-day exposure). Total distance travelled (mm) over a 10-minute period was captured utilising Ethovision software following 24 hr selected TA treatments: β-phenethylamine (PEA), tryptamine (TRP), ρ-tyramine (TYR), and agmatine (AGM). Data are represented as individual scatter dot plots with mean ± SEM, n=12. Statistical analyses: 2-way ANOVA with Tukey's multiple comparison tests; */# = p<0.05, ***/### = p<0.001, **** = p<0.0001. Hashtags (#) represent differences within TNBS treatment groups, while asterisks (*) represent differences between TNBS treatment groups.

6.4.2 Differential effect of trace amines to modulate gastrointestinal endocytic capacity

Neutral red staining of the mid-intestine serves as an indication of GI endocytic functional capacity, represented quantitatively in Figure 6.3 and qualitatively (representative micrographs) in the supplementary material (Supp. Fig. 6.1). In our model of GI inflammation, TNBS-exposure significantly reduced the average length of the stained mid-intestine from 363 μm to 281 μm ($p<0.0001$), while the co-administration of prednisolone (329.7 ± 30.81 μm) rescued this effect significantly compared to the TNBS group ($p<0.01$). In the absence of TNBS treatment, significant reductions in stained mid-intestinal length were observed following PEA ($p<0.01$) and TYR ($p<0.01$) treatments, while the TRP treatment group was not different from control. In contrast, the presence of AGM increased staining significantly ($p<0.05$). In the presence of TNBS, however, both PEA and TRP treatment abolished staining completely ($p<0.0001$ for both), suggesting a cumulative effect to reduce GI endocytic functionality. In the case of TYR, the presence of TNBS did not significantly exacerbate the effect of TYR. In the presence of AGM, although TNBS significantly reduced staining ($p<0.0001$ vs. AGM -TNBS), levels did not differ from either control, suggesting that the protective effects of AGM to promote staining at least partially mitigated the effects of TNBS in this context.

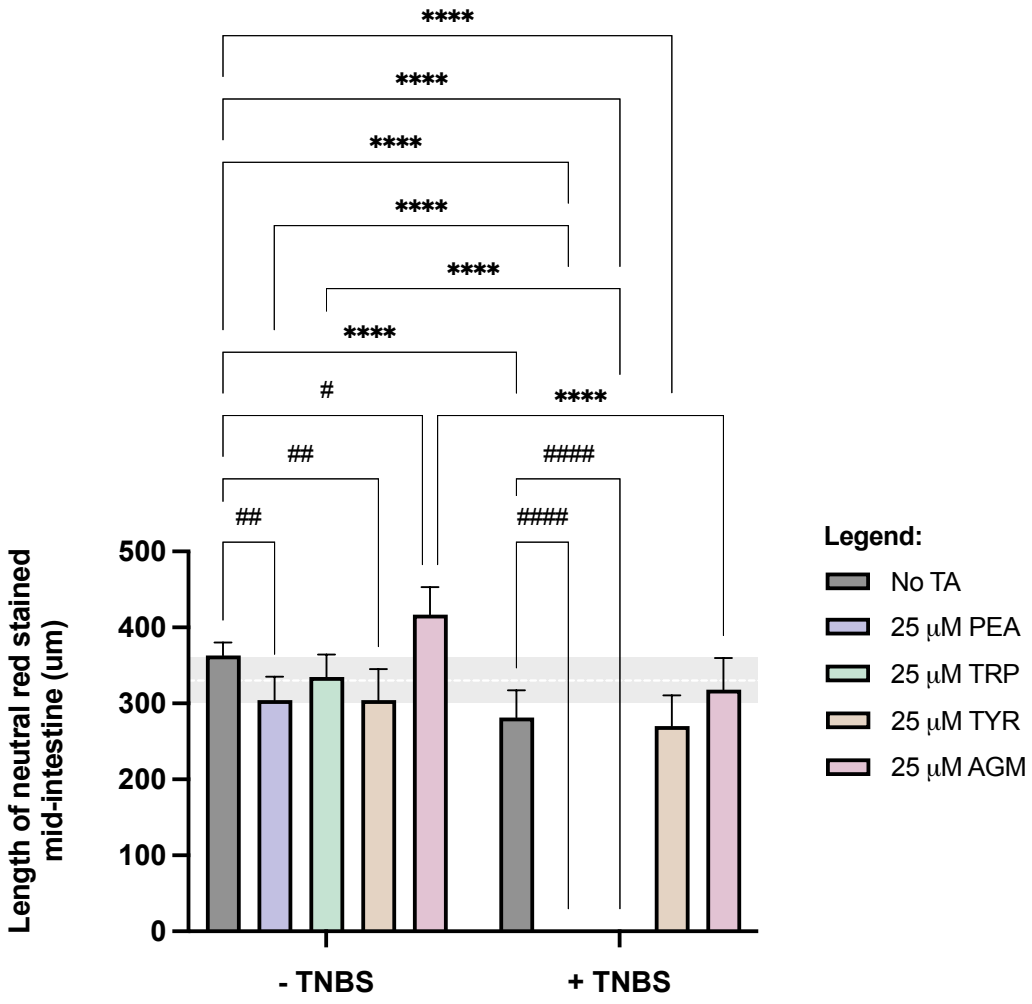


Figure 6.3: Length of neutral red stained mid-intestine (μm) in zebrafish larvae (7 dpf) following exposure to selected trace amines (TAs): β-phenethylamine (PEA), tryptamine (TRP), ρ-tyramine (TYR), and agmatine (AGM), in the absence or presence of TNBS (25 μg/mL, 2-day exposure). Data are expressed as mean ± SD, n=9. In addition, TNBS treatment with subsequent prednisolone co-administration is represented as mean (white dotted line) ± SD (greyed area) as the standard internal rescue control. Statistical analyses: 2-way ANOVA with Tukey's multiple comparison tests; */$^{\#}$ = p<0.05, **/$^{\#\#}$ = p<0.01, ****/$^{\#\#\#\#}$ = p<0.0001. Hashtags (#) represent TA effects within TNBS treatment groups, while asterisks (*) represent effects between TNBS groups for the same TA treatment.

6.4.3 Differential effects of trace amines to modulate gastrointestinal tight junction proteins

Zona occludens-1 (ZO-1) – a cytoskeletal linker protein – and occludin – an integral membrane protein – form part of tight junction protein complexes and were assessed to determine the modulatory effects of TAs on TJP integrity. In this regard, representative micrographs are displayed in Figure 6.4 and the corresponding quantitative data are represented in Figure 6.5. Qualitatively, in the absence of TA treatment, the deleterious effect of TNBS-exposure was clearly observed (Fig. 6.4F), with reduced ZO-1 staining being the most prominent visual difference. This was confirmed by image analysis – TNBS treatment significantly reduced both

ZO-1 (Fig. 6.5A; $p<0.05$) and occludin (Fig. 6.5B; $p<0.01$) expression. This effect of TNBS was limited by the presence of both E2 (Fig. 6.4K) and prednisolone (Fig. 6.4P) to similar extents. Statistically, prednisolone partially rescued both ZO-1 and occludin levels (Fig. 6.5). Indeed, ZO-1 staining was visually prominent compared to the TNBS group (Fig. 6.4F), and the cellular architecture demarcated by this staining was more uniform in shape and size.

Continuing to TA treatments, under control conditions, PEA treatment visually disrupted cellular morphology, and seemed to slightly decrease general signal intensity (Fig. 6.4B). Interestingly, TRP treatment seemed to somewhat maintain cellular morphology, however, epithelial cell size seemed larger, potentially indicating subtle edema (Fig. 6.4C). While TYR treatment most notably reduced ZO-1 signal intensity, there seemed to be an elevated presence of intracellular occludin in this group (Fig. 6.4D). However, despite these visual indications, PEA, TRP and TYR treatments did not statistically alter absolute levels for either ZO-1 or occludin (Fig. 6.5) compared to control conditions. In contrast, AGM treatment seemed to increase both occludin and ZO-1 signal intensity, with increased ZO-1 clustering evident (Fig. 6.4E). Statistically, AGM treatment significantly increased total occludin ($p<0.0001$), but not ZO-1 intensity when compared to control.

In the presence of TNBS, each respective TA treatment group seemed to fair worse (Fig. 6.4F-J), with decreases in total signal compared to their respective TA control groups. Interestingly, compared to the TNBS control group, none of the TAs assessed altered ZO-1 intensity significantly, but PEA, TRP and TYR treatments significantly increased occludin intensity ($p<0.05$ for all), thereby mitigating the effect of TNBS on occludin. Importantly, this effect was not observed for AGM, which in the presence of TNBS had significantly lower ZO-1 ($p<0.05$) and occludin intensities ($p<0.0001$) compared to AGM treatment in the absence of an inflammatory stimulus (Fig. 6.5), but not lower than control, which suggests AGM-associated maintenance of tight junction protein status in an inflammatory GI context.

In the presence of E2, both PEA and TRP groups, as well as TYR to a lesser extent, showed improvement of gastrointestinal morphologies (Fig. 6.4L, M & N). Linking these observations to quantitative results, PEA, TRP and TYR groups had normalised ZO-1 levels, which were significantly increased when compared to the TNBS control (PEA and TYR: $p<0.05$, TRP: $p<0.07$), and similarly, occludin levels were also significantly improved (PEA: $p<0.001$, TRP: $p<0.0001$, TYR: $p<0.01$). These findings suggest a significant effect of E2 to beneficially protect against TNBS-induced damage *via* potentially TA-related signalling/mechanisms. In this context, AGM seemed to be the exception, as outcome seemed independent of E2 (Fig. 6.4O).

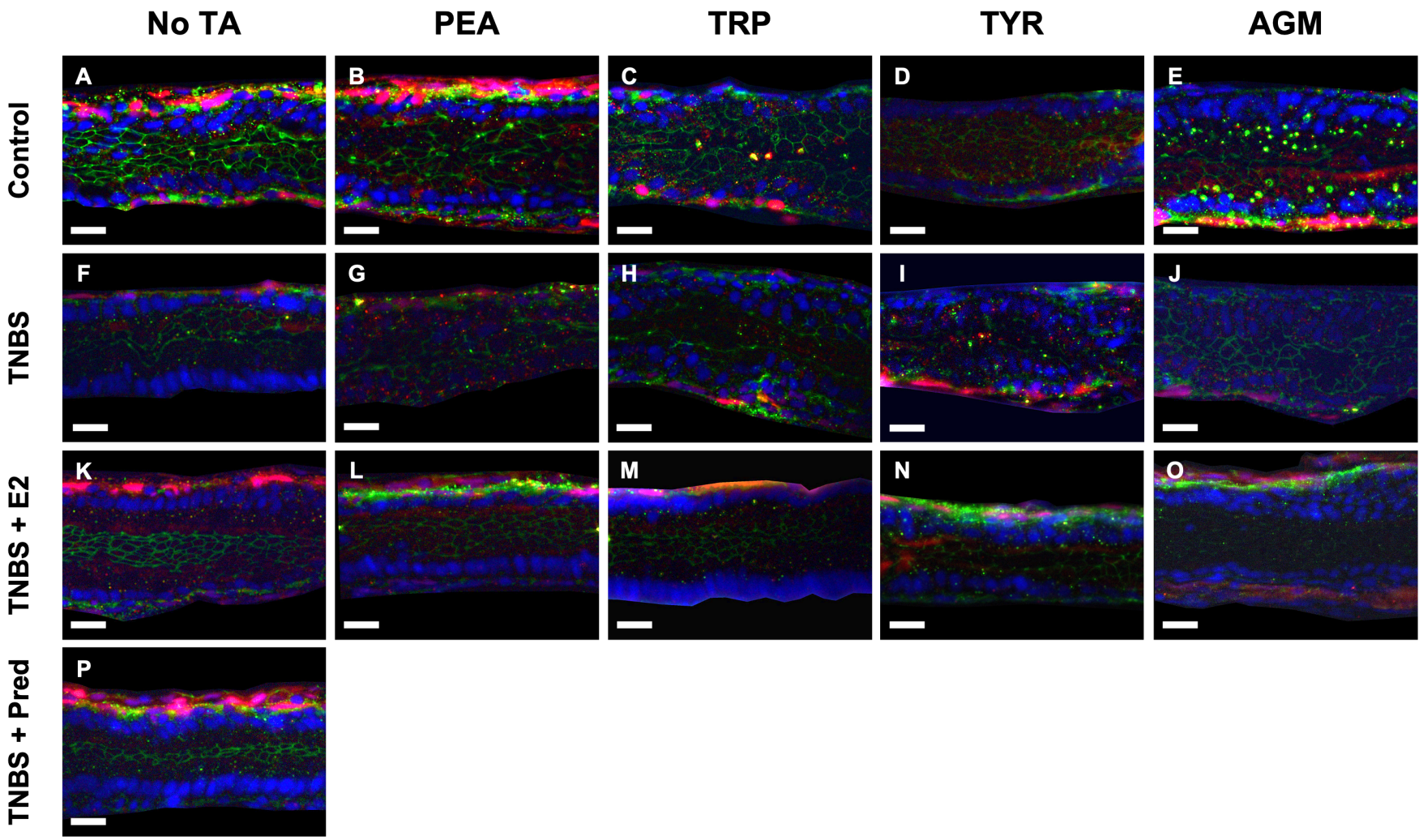


Figure 6.4: Representative fluorescent micrographs showing the effects of selected trace amines (TAs), TNBS, 17β-estradiol (E2), prednisolone, and combined treatments on tight junction proteins in zebrafish larvae (7 dpf) mid-intestines. Green signal = ZO-1, red signal = occludin, blue signal = Hoechst. All micrographs were imaged using a 60x objective; scale bar = 10 μm. Abbreviations: PEA: β-phenethylamine, TRP: tryptamine, TYR: ρ-tyramine, AGM: agmatine.

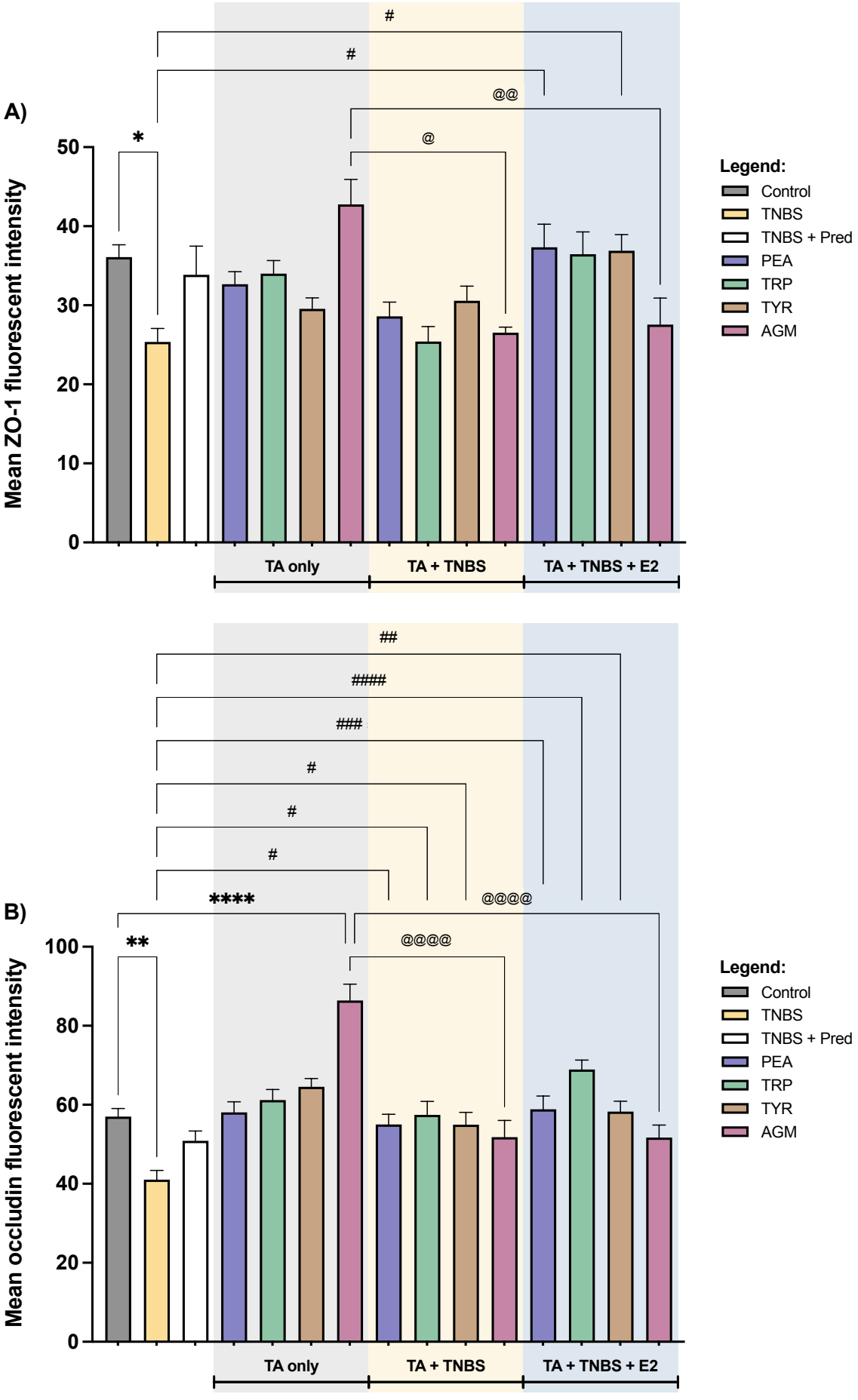


Figure 6.5: Mid-intestinal immunofluorescent tight junction protein signal in stained zebrafish larvae (7 dpf) treated with 25 μM β-phenethylamine (PEA) or tryptamine (TRP) or ρ-tyramine (TYR) or agmatine (AGM), in the absence or presence of TNBS and 17β-estradiol (E2). In addition, the effects of TNBS treatment (yellow bar), and TNBS and prednisolone (Pred) co-administration are represented (white bar). In (A) the effect on ZO-1 intensity is reported while in (B) the effect on occludin intensity is reported. Data are expressed as mean fluorescent intensity ± SEM, n=6. Statistical analyses: One-way ANOVA with Tukey's multiple comparison tests; $^{*/\#/@}$ = p<0.05, $^{**/\#\#/@@}$ = p<0.01, $^{\#\#\#}$ = p<0.001, $^{****/\#\#\#\#\#/@@@@}$ = p<0.0001. Asterisks (*) represent differences compared to the control group, hashtags (#) represent differences compared to the TNBS only group, asperands (@) represent differences compared to the AGM only group.

6.4.4 Differential effect of trace amines to modulate whole-body oxidative profile

In the absence of TA treatment (Fig. 6.6A), TNBS-exposure caused a significant increase in H_2O_2 levels compared to control ($p<0.05$), an effect that was rescued with the co-administration of prednisolone (Fig. 6.6A). Interestingly, this effect was not rescued or worsened with the co-administration of E2, but the addition of TAM resulted in a significant increase in H_2O_2 levels compared to both control ($p<0.0001$) and TNBS ($p<0.05$) groups, potentially suggesting involvement of ER modulation (different to the full agonistic effects of estradiol) in advancement of oxidative stress in the presence of an inflammatory stimulus (TNBS). In response to TA-exposure, dose-related trends to increase in H_2O_2 levels were observed following PEA, TRP and TYR (Fig. 6.6B). In contrast, a U-shaped trend is observed following AGM treatment, with 25 μM of AGM significantly decreasing H_2O_2 levels compared to basal levels ($p<0.01$), while 100 μM and 200 μM had no significant effect (Fig. 6.6B).

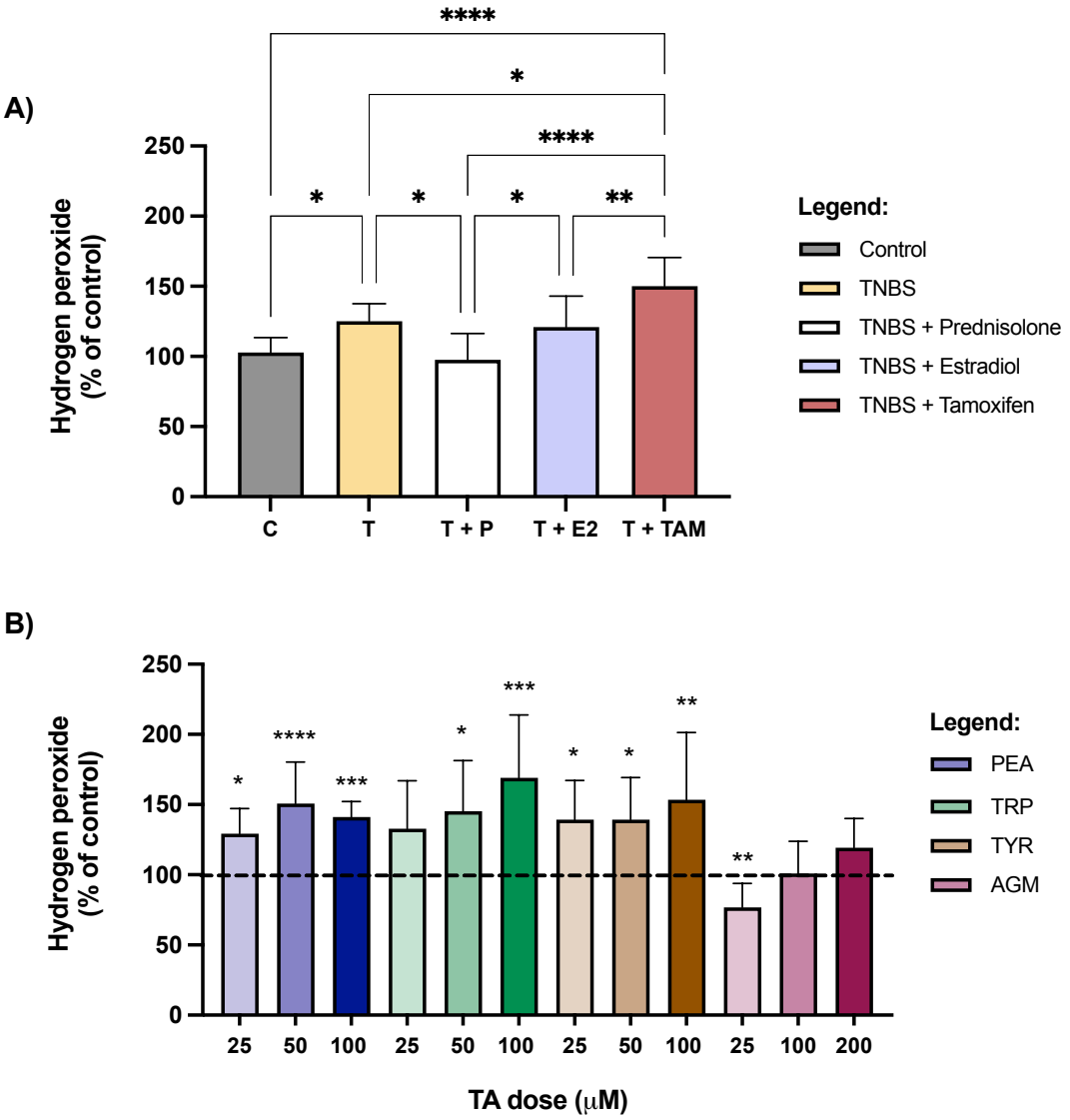


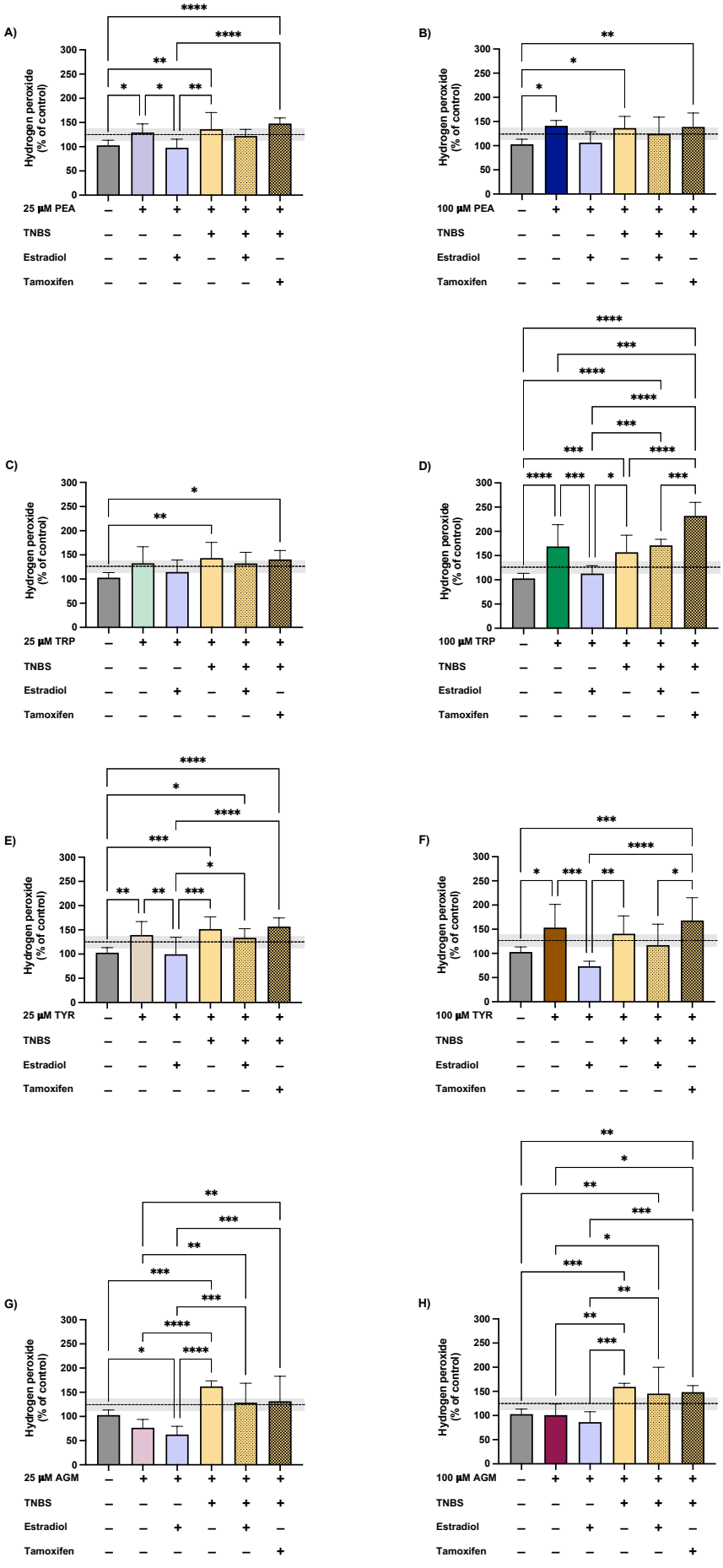
Figure 6.6: Hydrogen peroxide (H_2O_2) levels in pooled zebrafish larvae (7 dpf; n=25 per pooled sample). In (A) the effect of TNBS (25 μg/mL, 2-day exposure) in combination with prednisolone (P: 25 μg/mL, 1-day exposure), 17β-estradiol (E2: 20 nM, 2-day exposure) and tamoxifen (TAM: 2.5 μM, 2-day exposure) are displayed. In (B) H_2O_2 outcome in response to trace amine (TA) dose responses (25 μM, 50 μM, and 100/200 μM) are displayed. Additionally, the basal (control) H_2O_2 level is depicted (black dotted line). Data are expressed as mean concentration per total protein as % of control ± SD, n=6. Statistical analyses: One-way ANOVA with Tukey's multiple comparison tests; * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001. Asterisks (*) represent differences compared to the control group (dotted line). Abbreviations: β-phenethylamine (PEA), (C) tryptamine (TRP), (D) ρ-tyramine (TYR), and (E) agmatine (AGM).

Moreover, H_2O_2 outcome in response to TA exposure combined with TNBS, E2 and TAM are represented in Figure 6.7, with main findings summarised here. PEA, TRP and TYR administration resulted in worse H_2O_2 outcomes, but these effects were generally attenuated in the presence of E2. Moreover, the effect of TA and TNBS exposures did not seem to have a cumulative effect on H_2O_2 production. Nevertheless, in the presence of both TA and TNBS

administration, the presence of E2 exhibited a mild limiting effect on H_2O_2 production, suggesting a direct beneficial effect of E2. Importantly, the addition of TAM (i.e. selective ER modulator) in the absence of E2 seemed to exacerbate the effects of TA/TNBS significantly for following both TRP (Fig. 6.7C, D) and TYR (Fig. 6.7E, F) administrations. In contrast to the other assessed TAs, low-dose AGM treatment seemed to confer protective effects but could not counter TNBS-induced H_2O_2 secretion (Fig. 6.7G, H). In this context, neither E2 nor TAM administration elicited a significant benefit.

*PTO for Figure 6.7

Figure 6.7: Hydrogen peroxide (H_2O_2) levels in pooled zebrafish larvae (7 dpf; n=25 per pooled sample) treated with 25 μM (left column) or 100 μM (right column) of selected trace amines: (A&B) β-phenethylamine (PEA), (C&D) tryptamine (TRP), (E&F) ρ-tyramine (TYR), and (G&H) agmatine (AGM), in the absence (-) or presence (+) of TNBS (25 μg/mL, 2-day exposure), 17β-estradiol (E2: 20 nM, 2-day exposure) and tamoxifen (TAM: 2.5 μM, 2-day exposure). In addition, the mean ± SD of TNBS only condition is represented by the dotted black line and greyed box respectively, located next to the y-axis. Data are expressed as mean concentration per total protein as % of control ± SD, n=6. Statistical analyses: One-way ANOVA with Tukey's multiple comparison tests; * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001.



6.4.5 Differential effect of trace amines to modulate whole-body prostaglandin profile

To assess changes in inflammatory profile in response to the induction of GI inflammation and TA exposure, the levels of prostaglandin E2 (PGE2) are represented in Figure 6.8. In the absence of TA treatment, TNBS treatment significantly increased PGE2 levels compared to basal levels (control) ($p<0.05$). This effect was rescued partially in the presence of prednisolone or TAM, and significantly by E2 ($p<0.05$). In the presence of all TA assessed, PGE2 levels increased when compared to controls, with most significant activation by TYR (Fig. 6.8B).

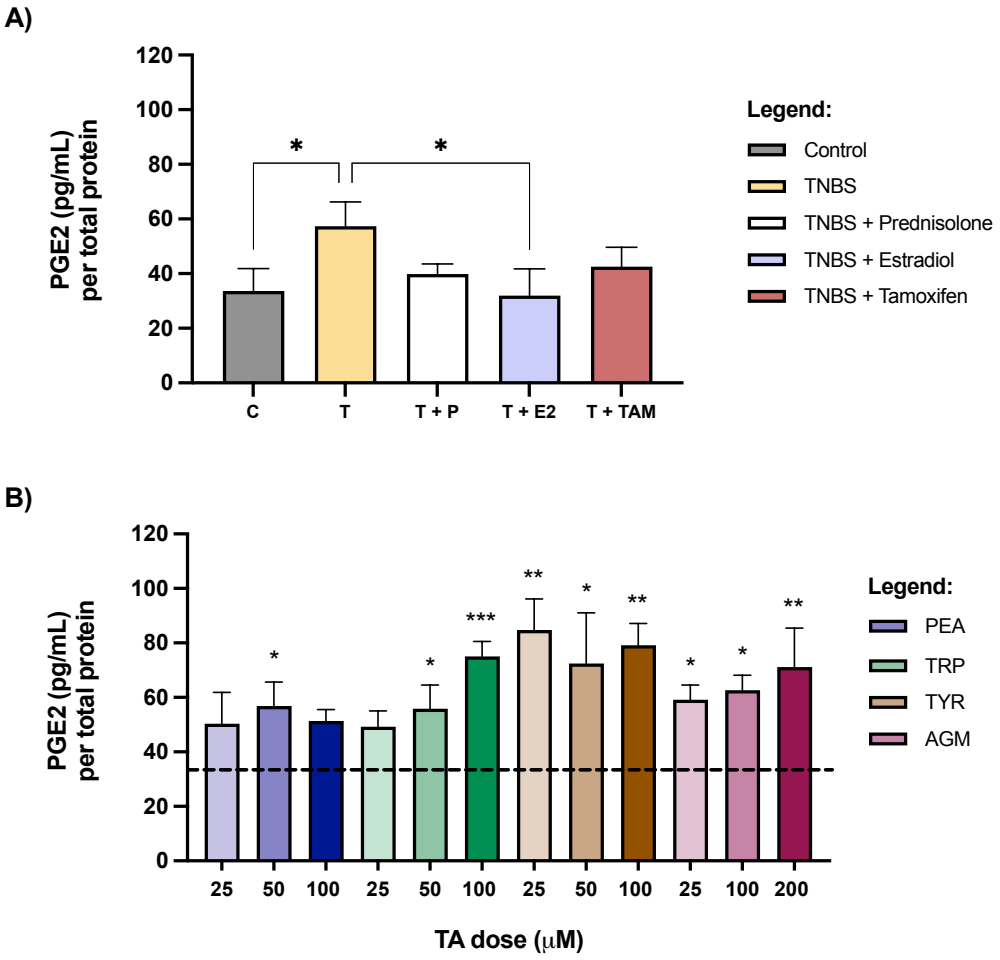
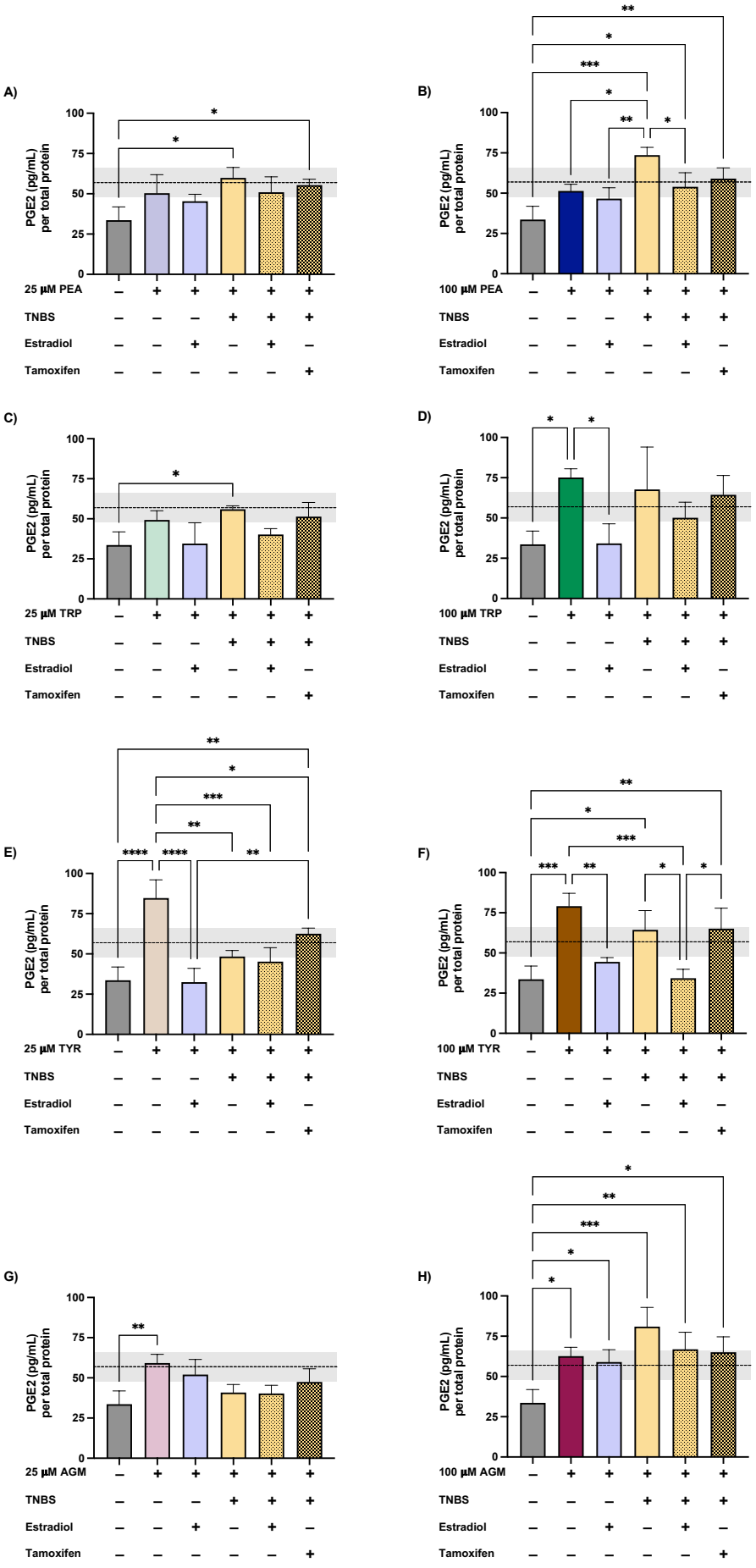


Figure 6.8: Prostaglandin E2 (PGE2) levels in pooled zebrafish larvae (7 dpf; n=25 per pooled sample). In (A) the effect of TNBS (25 μg/mL, 2-day exposure) in combination with prednisolone (P: 25 μg/mL, 1-day exposure), 17β-estradiol (E2: 20 nM, 2-day exposure) and tamoxifen (TAM: 2.5 μM, 2-day exposure) are displayed. In (B) H_2O_2 outcome in response to trace amine (TA) dose responses (25 μM, 50 μM, and 100/200 μM) are displayed. Additionally, the basal (control) PGE2 level is depicted (black dotted line). Data are expressed as mean concentration per total protein as % of control ± SD, n=3. Statistical analyses: One-way ANOVA with Tukey's multiple comparison tests; * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$. Asterisks (*) represent differences compared to the control group (dotted line). Abbreviations: β-phenethylamine (PEA), (C) tryptamine (TRP), (D) ρ-tyramine (TYR), and (E) agmatine (AGM).

When considering PGE2 outcome in response to TA-exposure combined with TNBS, E2 and TAM, profiles were more TA-specific (Fig. 6.9). Firstly, for PEA treatment (Fig. 6.9A & B), at both concentrations a mild protective effect of both E2 and TAM was demonstrated in the context of joint PEA and TNBS-exposure (which seemed to elicit a cumulative PGE2 response). A similar trend was observed following TRP treatment (Fig. 6.9C, D), except that the response to TNBS and TRP did not seem to be cumulative and was not alleviated in the presence of TAM. In contrast, both TYR and AGM resulted in significant PGE2 responses (Fig. 6.9E-H), which was only rescued by E2 in the case of TYR, where E2 (but not TAM) also normalised PGE2 levels in larvae exposed to both TYR and TNBS. Finally, in the presence of low-dose AGM (Fig. 6.9G), the presence of TNBS partially normalised PGE2 levels, with no added benefit of either E2 or TAM. However, at high dose AGM, TNBS seemed to almost elicit a cumulative effect on PGE2 release, again with no evident effect of either E2 or TNBS (Fig. 6.9H).

*PTO for Figure 6.9

Figure 6.9: Prostaglandin E2 (PGE2) levels in pooled zebrafish larvae (7 dpf; n=25 per pooled sample) treated with 25 μM (left column) and 100 μM (right column) of selected trace amines: (A&B) β-phenethylamine (PEA), (C&D) tryptamine (TRP), (E&F) ρ-tyramine (TYR), and (G&H) agmatine (AGM), in the absence (-) or presence (+) of TNBS (25 μg/mL, 2-day exposure), 17β-estradiol (E2: 20 nM, 2-day exposure) and tamoxifen (TAM: 2.5 μM, 2-day exposure). In addition, the mean ± SD of TNBS only condition is represented by the dotted black line and greyed box respectively, located next to the y-axis. Data are expressed as mean concentration per total protein ± SD, n=3. Statistical analyses: One-way ANOVA with Tukey's multiple comparison tests; * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$.



6.5 Discussion

Current data obtained from investigations in a zebrafish larval model of TNBS-induced GI inflammation elucidated a number of key, novel findings. Firstly, excess PEA, TRP and TYR all have deleterious local (gut) and systemic effects to varying degrees, with the actions of TYR being most significant in the context of H_2O_2 and PGE2 responses. Secondly, current data clearly illustrates direct beneficial effects of E2 (or potential ER involvement) in regulating effects of PEA, TRP and TYR (but not AGM) in the context of gut epithelial inflammation, which expands on the literature focused on mechanisms involved in female predominance of GI disorders. Thirdly, in contrast to the other TAs assessed in the current study, AGM displayed potential protective local effects – which we suggest may be importantly related to its subsequent metabolism; however, systemic effects may be less favourable (pro-inflammatory) highlighting the importance of dosage and administration optimisation, as well as comprehensive pharmacokinetics and pharmacodynamics studies to minimise risk of undesired effects, from a therapeutics standpoint.

In line with results from previously mentioned metabolomics studies linking PEA, TRP and TYR to GI disorders, excess PEA, TRP and TYR were also associated with poorer local (gut) and/or systemic effects *in vivo* in the current study. In the context of local (gut) effects, current findings from both the neutral red staining – which is a well-described indicator of intestinal function (Oehlers et al., 2013) – and TJP analyses – which are crucially involved in the dynamic regulation of intestinal barrier integrity and permeability (Odenwald and Turner, 2017) – in addition seem to suggest that of these three TAs, TYR has the potential to mediate the most significant damage. This is in line with our previous results in HT-29 gut epithelial cells in culture, where TYR was also identified as major challenge to TJP integrity and inflammatory outcome (Pretorius and Smith, manuscript under review). Furthermore, PEA treatment alone also reduced functional capacity significantly, which was drastically exaggerated in the presence of TNBS. This effect of PEA and TRP (no neutral red staining in the presence of TNBS) could be explained by the specific actions of TNBS in this context. As described by Oehlers et al (2010), seeing that zebrafish larvae (at the age utilized in the current study) do not yet have an adaptive immune system, TNBS, which normally induces inflammation *via* haptenization, may act by indirectly disrupting mucin production and subsequent mucus-related protection of the intestinal epithelium, resulting in aberrant microbial – or direct TA – contact with epithelial host cells. While in the absence of additional stimuli, the TNBS dose utilised was unlikely to induce apoptosis (Oehlers et al., 2011), we have previously shown prominent cytotoxic actions of direct TA-exposure (TRP>PEA>TYR>AGM) in an *in vitro* colon cell model (Pretorius et al., 2022b), which could explain these findings. Indeed, in our own

experience, in terms of survivability, zebrafish larvae are most sensitive to the effects of TRP>PEA>TYR>AGM. In addition, this reasoning explains the effect of PEA and TRP treatment to increase occludin intensity to a greater extent in the presence of TNBS. As such, a diminished epithelial functional capacity (resulting from the combined treatments) could limit TNBS-induced occludin endocytosis (Li et al., 2008, Nighot and Ma, 2021) and subsequent degradation. Indeed, the same effect was observed for TYR, however, TYR-related loss of functional epithelium – as suggested for PEA and TRP – is not an applicable explanation in this context. Instead, we suggest, a differentiating factor of TYR is potentially related to TYR-mediated increases in occludin internalization, which is an effect we have previously also observed (Pretorius et al., 2022b)(Pretorius and Smith, manuscript under review). As such, it may be feasible that a simultaneous prompt to degrade (TNBS stimuli) and increase (TYR) may have counteracted each other, appearing to 'normalise' occludin levels. In this regard, we have recently suggested a requirement for future studies to elucidate the potential links between TYR and occludin internalisation.

Turning our attention to systemic effects, patients with GI disorders often experience a reduced quality of life and are less productive (Buono et al., 2017, Faresjo et al., 2019). In this regard, behavioural analyses suggested that both TRP and TYR (but not PEA) reduced general locomotion, which could be associated with 'sickness behaviour' in both cases (Kirsten et al., 2018). Notably, TRP treatment also reduced inter-group variation suggesting heightened sensitivity of zebrafish larvae to TRP or its metabolites in this regard. Importantly whole-body analyses revealed that TRP (followed by TYR) administration elicited the most pronounced H_2O_2 effect of all TAs assessed, while TYR (followed by TRP) exposure increased PGE2 levels most prominently. While these findings do not accurately depict the oxidative and inflammatory profiles in the gut, Kristen et al (2018) reported that a peripheral inflammatory event could result in an upregulation of pro-inflammatory cytokine secretion in the brain, which may result in reduced locomotion (assessed in the current study), social interaction and exploration. As such, the effects of both TRP and TYR to increase whole-body H_2O_2 and PGE2 may explain the effect on general activity observed. In this context, the relatively milder (compared to TRP/TYR) effect of PEA on H_2O_2 and PGE2 responses, supports our interpretation that larvae in this group probably had lower levels of discomfort.

Considering both local (gut) and systemic effects observed in the current study, while excess of PEA/TRP/TYR can result in pathological consequences, TYR seems to stand out as potentially the most detrimental in the context of interest and may be the physiologically most relevant. In this regard there are a few factors to consider. Firstly, TYR – in line with our previous work (Pretorius et al., 2022a) – is abundantly produced by lactic acid bacteria *via* L-

tyrosine decarboxylation (Barbieri et al., 2019, Luqman et al., 2018, Pugin et al., 2017). In addition, TYR is also found to be present at high levels in certain foods, while Del Rio et al (2018) reported that TYR, at concentrations readily found in biogenic amine-rich foods, may have genotoxic and oxidative effects *in vitro* (Del Rio et al., 2018). Together, this supports the notion that dietary TYR load could already have potentially negative implications for gut health. Secondly, TYR (besides histamine) has been implicated as the biogenic amine with the highest probability to cause potential health risks (Barbieri et al., 2019, Marcobal et al., 2012). This attribute of TYR is multifaceted. For example, TYR can act indirectly as a sympathomimetic by displacing stored norepinephrine, which can cause adverse systemic effects (migraine, nausea, increased cardiac output etc.). Moreover, most biogenic amine-rich foods would contain more than just TYR, and as such the intestinal capacity for TYR 'detoxification' *via* subsequent monoamine oxidase (MAO) action, could become saturated/reduced in the presence of other TAs. In this case a TA-dense meal could perpetuate the effects of TYR, which links to the high perceived food (biogenic amine-rich) intolerance among patients with IBS (Bohn et al., 2013). Furthermore, patients with altered MAO function or those supplementing with MAO inhibitor (MAOI) drugs, could be at a greater risk in this context (McCabe-Sellers et al., 2006). Indeed, considering the high prevalence of anxiety/depression among patients with GI disorders (Barberio et al., 2021, Zamani et al., 2019) – which are commonly treated with MAOIs – this specific patient population may be most susceptible to the effects of TYR, and as suggested recently (Burns and Kidron, 2022), should be counselled to avoid TYR-containing foods as part of standard care. Also, given that the effects of ingested TYR would be related to the combined food 'matrix', of which the presence of certain substances, such as alcohol, reportedly potentiate the effects of biogenic amines (Linares et al., 2011) a host response to elevated TYR would be largely dependent on the above-mentioned factors, as well as individual host-sensitivity and behaviour. Finally, in terms of inflammation, in line with the current study, TYR reportedly stimulates cytokine secretion from T-lymphocytes and Ig secretion from B-lymphocytes (Babusyte et al., 2013) – while this aspect of TYR cannot be assessed in a larval model, this may indicate that the detrimental effect of TYR in an *in vivo* human scenario may be even worse than illustrated here in zebrafish larvae. In addition, TYR is also chemotactic for a subset of polymorphonuclear leukocytes (Babusyte et al., 2013), which are one of the main sources of enzymes and reactive oxygen species implicated in secondary tissue damage during inflammatory processes (Checa and Aran, 2020, Mittal et al., 2014). Considering that patients with GI disorders have heightened or dysfunctional innate immune responses, an increase in TYR could feasibly promote intestinal immune activation, thereby stimulating or exacerbating intestinal inflammation.

Interestingly, two studies investigating food intolerance in patients with IBS found that female sex predicted a high degree of food-related symptomology/intolerance (Bohn et al., 2013, Simrén et al., 2001). Considering the context of IBS, and specifically the associated female-predominance, it was important to elucidate if E2 – as one of the main female reproductive hormones – could alter the proposed contribution of TA (specifically TYR) to intestinal inflammation. In the current study, across all parameters assessed, the presence of E2 beneficially modulated the undesired effects of PEA, TRP and TYR to somewhat similar extents. Indeed, ZO-1 and occludin expression were normalised in the presence of E2, suggesting a significant effect of E2 to protect against TNBS-induced TJP damage *via* potentially TA-related signalling mechanisms. Importantly, this trend was also apparent in both H_2O_2 and PGE2 analyses as well. Notably, the addition of TAM in this context seemed to suggest that this beneficial effect of E2 may not primarily result from E2 abundance, but rather ER signalling as well, particularly in the context of our H_2O_2 findings. Indeed, involvement of ERs in redox signalling, although highly dependent on receptor isoform ratios and cellular status, is a well-known phenomenon (Kumar et al., 2010a). In this context, it may be possible that TAs (or TA-related downstream signalling) may alter ER-mediated outcomes; however, future studies investigating ER isoform expression, localisation and perhaps homo/heterodimerization in the context of trace aminergic signalling is required to fully elucidated the mechanisms at play. Regardless, the fact that E2 seems to confer protection, is in line with literature reporting GI symptom exaggeration during menses, when E2 levels are at their lowest (Kim and Kim, 2018). Moreover, we suggest that loss of E2-mediated TA modulation, may render specific patient populations (i.e. who have high TA levels or are most sensitive to the effects of TAs) at increased risk for subsequent TA-related GI symptomology. In many chronic inflammatory disorders, as well as in IBS, the female sex has been linked to poorer prognosis related to greater oxidative damage resulting from inflammation (Choghakhori et al., 2017a, Choghakhori et al., 2017b, Flak et al., 2013), although these differences cannot be fully accounted for by hormonal status alone (Casimir et al., 2018). As such, considering the potential implications of TYR exposure, modulation of the trace aminergic system or TA load in the gut, especially during menses, may have important clinical implications regarding symptom management.

Moving on to the effects of AGM, local (gut) effects, such as demonstrated by neutral red and TJP analyses in the current study, suggested that the systemic administration of AGM increased GI epithelial functional capacity, as well as ZO-1 and occludin expression in the mid-intestinal region. From this point of view, AGM supplementation seems beneficial. However, it is important to note, as demonstrated in the activity tracking analyses, AGM treatment was often associated with increased inter-group variation, suggesting that the larvae

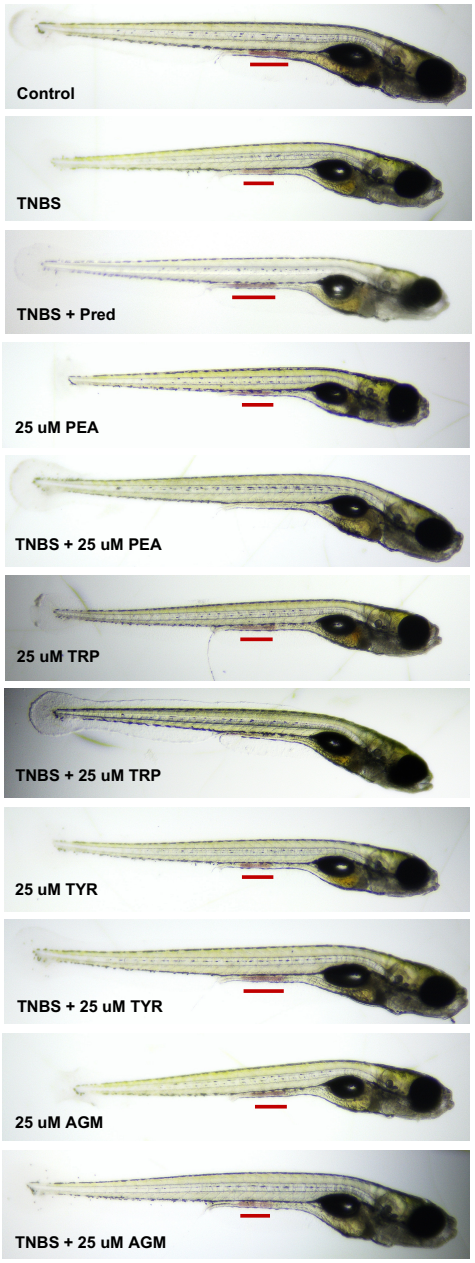
were selectively or differentially sensitive to AGM or its metabolites. Given the fact that redox status is clearly a determining factor in terms of whether a desired or undesired outcome is achieved in the context of intestinal inflammation, this suggests that either individual endogenous antioxidant system capacity showed significant inter-individual variability – similar to that seen in humans, or that different fish had varying sensitivity to AGM and its (antioxidant) metabolites, or both. Indeed, in the context of gut health, Nakamura et al., (2021) recently commented on the importance of 'symbiotic metabolism'. In this regard, microbial derived metabolites (putrescine – downstream metabolite of AGM) are metabolised further (to spermidine and spermine) in colonic epithelium to exert beneficial effects, which assists with maintenance of intestinal homeostasis (Nakamura et al., 2021). Incidentally, excessive AGM intake can disrupt this symbiotic metabolism, and as such excessive supplementation has been cautioned for its potential detrimental effect on polyamine synthesis/action (Oliphant and Allen-Vercoe, 2019).

In terms of behaviour, AGM partially improved general activity levels in the presence of TNBS, suggesting potential for effecting relief from TNBS-induced 'sickness behaviour'. This finding is in line with the reported neuroprotective role of AGM (Ahn et al., 2015). Moreover, AGM seemed to beneficially modulate oxidative status. In this regard, AGMs role in redox balance was dependent on dose, with lower doses having more favourable outcomes and higher doses suggesting that potential excess may lead to pro-oxidative outcomes. Indeed, AGM is identified to have a dual role in apoptosis *via* regulation of mitochondrial permeabilization (Martinis et al., 2020) and as such, emphasises the importance of dose. Indeed, considering that AGM breaks down into antioxidant metabolites (polyamines are classified as non-enzymatic antioxidants (Mironczuk-Chodakowska et al., 2018)), potential pro-oxidant consequences related to excess AGM – or even excess polyamines (Stewart et al., 2018) – reiterates this point. Finally, looking at the effects of AGM on inflammatory status, AGM increased PGE2 levels in a dose-dependent fashion. This discrepancy between the oxidative and inflammatory outcomes could be attributed to the fact that unlike an endogenous antioxidant capacity, there is no endogenous inflammatory 'quenching' mechanism. Taken together, these findings, as well as the interindividual variation of the larvae to AGM, suggests that an individualised approach is required to optimise redox outcome in patients with GI disorder, however, the systemic inflammatory response highlights caution regarding excess or systemic administration of AGM.

6.6 Conclusions

In terms of future perspectives, current data highlight the complexity of trace aminergic signalling, specifically in the context of therapeutic target identification or therapeutics development. AGM, for example, showed promising effects in the gut, but potential AGM-related inflammatory signalling may suggest that specific routes of administration (i.e. slow-release tablet or suppository) or combination treatment with an anti-oxidant agent, should be investigated as therapeutic options in future studies. Also, seeing that current data highlighted the protective actions of E2, and that we have previously demonstrated that E2 indeed alters microbially derived TA levels (Pretorius et al., 2022a, Pretorius et al., 2022b) an effective therapeutic in this context may be required to maintain the intestinal environment (microbially derived TA load) despite cyclic E2 fluctuations (i.e. have a more pronounced effect than E2 on TA status), and thereby may mitigate associated GI symptom exaggeration. Taken together, current findings suggest the importance of dose, however, it remains to be elucidated at what range/threshold do TAs contribute to GI symptomology and how this relates to levels in patients with IBS. As such, data to date warrants an observational study in a human IBS cohort.

6.7 Supplementary material



Supplementary Figure 6.1: Representative micrographs of neutral red stained zebrafish larvae.

Chapter 7

Synthesis

IBS is becoming an increasingly prevalent FGID that negatively impacts quality of life and overall productivity. Moreover, consistently reported female-predominance in this regard (1:2 to 1:5) cannot be solely attributed to differences in reproductive hormone levels. Importantly, the lack of effective therapeutic options is a formidable dilemma in translation of transient symptom alleviation to 'remission'. Of particular significance, the lack of therapies which target inflammation – despite its implications in IBS – is problematic, especially considering literature reporting worse inflammatory and oxidative outcomes in female *vs* males with IBS (Choghakhori et al., 2017a, Choghakhori et al., 2017b). In addition, difficulties in therapeutic advancement are additionally confounded by the individual, yet multifactorial, nature of IBS onset and disease progression. In this regard an extensive review of current literature (Chapter 2) led us to identify the trace aminergic system as a potential key role player, linking sex, diet, and inflammation, which may be exploited as a therapeutic target. In fact, the 'trace' in TA may be a misnomer to a certain extent, considering that relatively high levels (due to direct consumption and/or subsequent microbial metabolism) can be present at the interface of mucosal immunity, the GI epithelium, and the gut microbiota. As such, the loss of TA homeostasis can be associated with altered immune reactivity and GI epithelial modulation towards environmental cues – which is in line with a high perception of food intolerance in this patient population.

However, to the best of our knowledge, the role of an altered/dysregulated trace aminergic system has not been investigated in an IBS context. As previously suggested, this is in part due to the limited knowledge of the peripheral actions of TAs, as well as the lack of a relevant *in vivo* model. Therefore, as stated in the formal hypothesis statement, investigation into the GI modulatory capacity of TAs in the presence of E2 in both *in vitro* and *in vivo* models was warranted.

Considering these unknowns, the novelty of this dissertation is evidenced by the following main findings, which contribute significantly to current literature. I have demonstrated – at least to some degree – that microbial production of TAs is E2-dependent (Chapter 3), yet the nature of this microbial response to E2 is differential (even within probiotic strains). Furthermore, the administration of different E2 concentrations may elicit opposing effects on TA secretion from the same microbe. These results highlighted the dependence of the probiotic secretome profile on host hormonal status, which may provide insight into the lack of probiotic efficacy

for specific patient populations (Lee and Bak, 2011). In addition to these findings, I have also demonstrated the effects of three different rooibos preparations to alter microbial TA secretion capacities to generally decrease TRP and increase PUT output (Chapter 4). While I acknowledge the limitations of microbial monocultures (which cannot accurately simulate the *in vivo* environment), I suggest that these findings may provide potential insight into mechanisms by which rooibos may elicit its anecdotally renowned alleviation of GI discomfort. More importantly, rooibos may be able to negate E2-induced alterations in TA profile, which may have important implications for TA-associated symptom management in female-predominant GI disorders.

To further our understanding of the physiological implications of altered TA homeostasis – specifically an increased TA load – we transitioned into an *in vitro* HT-29 colon cell model. Results from these experiments (Chapter 3 & 5) illustrated that TAs (at levels produced by microbes *in vitro* (Luqman et al., 2018, Pugin et al., 2017)) can act as significant stressors. In this context, the presence of E2 buffered the cytotoxic effects of some, but not all TAs, confirming that fluctuating E2 levels may alter the response of the gut barrier to stressors (Alonso et al., 2008, Alonso et al., 2012). Regarding inflammatory effects in particular, high-dose TYR-exposure (and to a lesser extent AGM as well) stimulated a significant PGE2 response (Chapter 3 & 5), suggestive of a relatively more pro-inflammatory outcome at high TA load. Moreover, TJP analyses demonstrated occludin redistribution in response to TA treatment (Chapter 3). In Chapter 5, the inclusion of pre-treatment conditions (higher [E2] and rooibos), served to illustrate potential differences in mechanisms of actions between TYR and AGM. In this regard, extensive occludin internalisation (i.e. loss of colocalization with ZO-1) was observed following TYR administration, while exposure to AGM promoted the colocalization of ZO-1 and occludin on the cellular periphery in areas of intact monolayer, but with apparent loss of TJP expression in other areas. In the presence of E2, the outcomes of combination of (E2 and TA) treatments were not universal (Chapter 3). In fact, our initial results suggested the different TAs interact differently with E2, and the nature of the interaction not only alters TJP status, but also cell sensitivity/vulnerability to stressors (Chapter 3). Interestingly, at a higher dose of E2 treatment the PGE2 response to TYR only was prevented, normalising TJP levels and distribution (Chapter 5) while rooibos treatment prevented TA-induced PGE2 and IL-8 secretion following both TYR- and AGM-exposure but was only able to normalise TJP levels and distribution profile for AGM (Chapter 5). Taken together, *in vitro* data expanded on the literature implicating TYR as major trigger in GI disorders by suggesting that manipulation of E2 may provide therapeutic effect. Furthermore, my data suggest that combination treatment with AGM and rooibos may have substantial benefit in the context of intestinal inflammation and barrier disruption. As such, the complexity of TA-TJP interplay in

a multi-microbe populated environment was emphasized, necessitating investigation in whole organism models, so that limitations of cell culture models may be overcome.

Moving on to my final study, an established model of GI inflammation in zebra fish larvae was utilised (Chapter 6), which allowed for the comprehensive assessment of the *in vivo* effects of TAs, in the presence of E2 and TAM. The main findings from this study included the deleterious effects of excess PEA, TRP and TYR, which were in line with recent metabolomics studies linking the surplus of these TAs to GI disorders (Jacobs et al., 2016, Ponnusamy et al., 2011, Santoru et al., 2018). Again, TYR was the TA which was highlighted as potentially the most detrimental in this context, specifically in the context of inflammation. As such, I suggest that an increase in TYR may feasibly promote GI immune activation, thereby exacerbating intestinal inflammation. Moreover, in this study, the beneficial effects of E2 to modulate TA became more apparent, with similar protective effects of E2 in the context of TJP status and inflammatory/redox profile extending to PEA, TRP and TYR (but not AGM). Importantly, this expands on literature focused on mechanisms by which female predominance in certain GI disorders may come about. Finally, the transition into an *in vivo* model also shed light on the importance of subsequent AGM metabolism (which was restricted due to a limitation of the *in vitro* HT-29 model utilised). In this regard, a potential protective effect of AGM was observed in both TJP and oxidative aspects. However, the risk of such possible pro-inflammatory systemic effects following AGM treatment, also crucially highlighted the importance of dosage and administration optimisation to minimize adverse side effects from a therapeutics standpoint.

Taken together, these data serves to support my stated hypotheses. Firstly, in line with the first two hypotheses, presented data highlights the importance of estrogen in the IBS context both at the level of TA secretion by gut microbes, as well as in determining effects of TA on host cells. Furthermore, the in vivo data generated in larval zebrafish in particular, demonstrates the importance of investigating dysregulation of regulatory systems – such as the TA system – using in vivo models, as well as supporting my third hypothesis by illustrating the importance of TA metabolism in managing risk profile.

In terms of future perspectives, three main areas of interest have emerged from the current findings. Firstly, the complex interplay of TA and TJP regulation – particularly TA-related occludin redistribution – requires deeper investigations into the molecular mechanisms involved. In this regard, I have recently suggested (Chapters 5 and 6) a requirement for future studies to elucidate the potential links between TYR and occludin internalisation/endocytosis. In Chapters 3 and 5, a potential mechanism of action may have involved the binding of TAs

to the α2-adrenergic receptor (α2-ADR) in HT-29 cells. In a study by Luqman et al (2018), the authors reported that the binding of TAs (PEA, TRP and TYR) to the α2-ADR boosted bacterial adherence and subsequent internalization in HT-29 cells due to downstream actin re-organisation, resulting from downstream α2-ADR signalling (Luqman et al., 2018). Indeed, Shen et al (2005) described that actin depolymerization resulted in decreased transepithelial resistance and concurrent caveolin-1-dependent occludin redistribution and internalisation. Interestingly, in the same study, ZO-1 and claudin redistribution only occurred well after maximum transepithelial resistance was lost (Shen and Turner, 2005), suggesting that an initial effect on occludin-related endocytosis precedes changes in TJP permeability. Considering the feasibility of this hypothesis, HT-29 generally express low levels of caveolin-1 (Felley-Bosco et al., 2000), but these levels can be upregulated in the presence of exogenous stimuli (Díaz-Valdivia et al., 2017). While the role of caveolins in endocytosis is controversial, the morphology of caveolae suggest a role in the concentration and internalisation of substances (de Almeida, 2017), and as such it would be interesting to assess if there are any links to caveolin-1 levels and TA-exposure in future studies.

Secondly, mechanisms involved in TA-E2 interplay remain to be further elucidated. For example, in Chapter 6 I alluded to the fact that it was unknown if the beneficial effects of E2 observed were related to a direct E2 affect or TA-related modulation of downstream E2 signalling (i.e. ER involvement), or both. In this context, future studies investigating ER isoform expression (ratio between ERα and ERβ), localization (membrane, nuclear or mitochondrial) and dimerization status (homo/heterodimerization) can be assessed utilising high-resolution microscopy techniques (i.e. correlative light and electron microscopy). Moreover, correlating these findings to changes in oxidative or inflammatory outcome could inform on potential targets for therapeutic modulation. In addition, seeing that current data highlighted the protective actions of E2, and that we have previously demonstrated that E2 indeed alters microbially derived TA levels (Chapter 3 and 4), an effective therapeutic in this context may be required to maintain the intestinal TA load despite cyclic E2 fluctuations (i.e. have a more pronounced effect than E2 on TA status), in order to effectively mitigate associated GI symptom exaggeration. In this regard, preliminary findings on the use of rooibos (Chapter 4) suggested that the overall effect of E2 on microbial TA production was relatively insignificant in the presence of rooibos supplementation, and as such, there may be scope for further exploration of the potential therapeutic capacity of specific rooibos formulations.

Thirdly, given the known neuroprotective actions of AGM, as well as my *in vivo* findings that show promising effects of AGM in the gut, future studies should elucidate the therapeutic

potential of AGM. These findings, as well as the interindividual variation of the larvae to AGM, suggests that an individualised approach is required to optimise redox outcome in patients with GI disorders. Nevertheless, the systemic inflammatory response I observed in zebrafish cautions against excess or systemic administration of AGM. In this context, specific routes of administration (i.e. slow-release tablet or suppository) or combination treatment with an anti-oxidant agent (such as rooibos), should be investigated.

Finally, even though investigations into TAARs was beyond the scope of this dissertation, future studies targeting TAARs, specifically TAAR-1, may shed light on feasible therapeutic targets, which regulate, at least in part, downstream TA functioning. In this regard, as reviewed in Chapter 2, literature seems to suggest that TAs do not bind exclusively to TAARs, but act as a ligand for several other receptors e.g. α2-ADR. Nevertheless, differential TAAR-1 expression in several leukocyte populations (PMN cells, B- and T-lymphocytes, monocytes, and natural killer cells (Babusyte et al., 2013, Nelson et al., 2007, Sriram et al., 2016, D'Andrea et al., 2003)) suggests that TAAR1 activation may regulate leukocyte differentiation and activation, and thereby regulate inflammatory processes. For example, the activation of TAAR-1 and -2 stimulates both IL-4 and IgE secretion (from T- and B-lymphocytes respectively) (Babusyte et al., 2013), which is of particular relevance to the allergy-like symptomology observed in IBS cohorts (Barbara et al., 2006, Lee and Lee, 2016). Moreover, TAAR1 may have a modulatory/down-regulatory role in trace aminergic signalling (dependent on heterodimerization status). Nevertheless, given the clear role for TAs in IBS aetiology and/or symptomology, and the fact that GPCRs are common targets of pharmacological agents, it is a high priority to elucidate the potential of (at least) TAAR-1 as a therapeutic target.

In conclusion, TAs have a relatively unappreciated role in the regulation of homeostasis in the GI tract. This thesis furthers our understanding of TAs and their physiological role and relevance, as well as highlights previously unknown mechanisms by which E2 may modulate the GI environment, thereby providing insights into the reported female-predominance associated with IBS. While current findings emphasize the importance of dose, it remains to be elucidated at what range/threshold TAs contribute to GI symptomology and how this relates to levels (and symptomology severity) in patients with IBS. As such, data to date warrants an observational study in a human IBS cohort.

Chapter 8

References

ABDUL, N. S. & MARNEWICK, J. L. 2021. Rooibos, a supportive role to play during the COVID-19 pandemic? *J Funct Foods,* 86**,** 104684.

AGUS, A., CLEMENT, K. & SOKOL, H. 2021. Gut microbiota-derived metabolites as central regulators in metabolic disorders. *Gut,* 70**,** 1174-1182.

AHN, S. S., KIM, S. H., LEE, J. E., AHN, K. J., KIM, D. J., CHOI, H. S., KIM, J., SHIN, N. Y. & LEE, S. K. 2015. Effects of agmatine on blood-brain barrier stabilization assessed by permeability MRI in a rat model of transient cerebral ischemia. *AJNR Am J Neuroradiol,* 36**,** 283-8.

AKINRINMADE, O., OMORUYI, S., DIETRICH, D. & EKPO, O. 2017. Long-term consumption of fermented rooibos herbal tea offers neuroprotection against ischemic brain injury in rats. *Acta Neurobiol Exp (Wars),* 77**,** 94-105.

AKIRA, S. & TAKEDA, K. 2004. Toll-like receptor signalling. *Nature Reviews Immunology,* 4**,** 499-511.

ALATAB, S., SEPANLOU, S. G., IKUTA, K., VAHEDI, H., BISIGNANO, C., SAFIRI, S., SADEGHI, A., NIXON, M. R., ABDOLI, A., ABOLHASSANI, H., ALIPOUR, V., ALMADI, M. A. H., ALMASI-HASHIANI, A., ANUSHIRAVANI, A., ARABLOO, J., ATIQUE, S., AWASTHI, A., BADAWI, A., BAIG, A. A. A., BHALA, N., BIJANI, A., BIONDI, A., BORZÌ, A. M., BURKE, K. E., CARVALHO, F., DARYANI, A., DUBEY, M., EFTEKHARI, A., FERNANDES, E., FERNANDES, J. C., FISCHER, F., HAJ-MIRZAIAN, A., HAJ-MIRZAIAN, A., HASANZADEH, A., HASHEMIAN, M., HAY, S. I., HOANG, C. L., HOUSEH, M., ILESANMI, O. S., JAFARI BALALAMI, N., JAMES, S. L., KENGNE, A. P., MALEKZADEH, M. M., MERAT, S., MERETOJA, T. J., MESTROVIC, T., MIRRAKHIMOV, E. M., MIRZAEI, H., MOHAMMAD, K. A., MOKDAD, A. H., MONASTA, L., NEGOI, I., NGUYEN, T. H., NGUYEN, C. T., POURSHAMS, A., POUSTCHI, H., RABIEE, M., RABIEE, N., RAMEZANZADEH, K., RAWAF, D. L., RAWAF, S., REZAEI, N., ROBINSON, S. R., RONFANI, L., SAXENA, S., SEPEHRIMANESH, M., SHAIKH, M. A., SHARAFI, Z., SHARIF, M., SIABANI, S., SIMA, A. R., SINGH, J. A., SOHEILI, A., SOTOUDEHMANESH, R., SULERIA, H. A. R., TESFAY, B. E., TRAN, B., TSOI, D., VACANTE, M., WONDMIENEH, A. B., ZARGHI, A., ZHANG, Z.-J., DIRAC, M., MALEKZADEH, R. & NAGHAVI, M. 2020. The global, regional, and national burden of inflammatory bowel disease in 195 countries and territories, 1990–2017: a systematic analysis for the Global Burden of Disease Study 2017. *The Lancet Gastroenterology & Hepatology,* 5**,** 17-30.

ALBERT, K., PRUESSNER, J. & NEWHOUSE, P. 2015. Estradiol levels modulate brain activity and negative responses to psychosocial stress across the menstrual cycle. *Psychoneuroendocrinology,* 59**,** 14-24.

ALONSO, C., GUILARTE, M., VICARIO, M., RAMOS, L., RAMADAN, Z., ANTOLIN, M., MARTINEZ, C., REZZI, S., SAPERAS, E., KOCHHAR, S., SANTOS, J. & MALAGELADA, J. R. 2008. Maladaptive intestinal epithelial responses to life stress may predispose healthy women to gut mucosal inflammation. *Gastroenterology,* 135**,** 163-172 e1.

ALONSO, C., GUILARTE, M., VICARIO, M., RAMOS, L., REZZI, S., MARTINEZ, C., LOBO, B., MARTIN, F. P., PIGRAU, M., GONZALEZ-CASTRO, A. M., GALLART, M., MALAGELADA, J. R., AZPIROZ, F., KOCHHAR, S. & SANTOS, J. 2012. Acute experimental stress evokes a differential gender-determined increase in human intestinal macromolecular permeability. *Neurogastroenterol Motil,* 24**,** 740-6, e348-9.

ALTEMUS, M. 2006. Sex differences in depression and anxiety disorders: potential biological determinants. *Horm Behav,* 50**,** 534-8.

ALTEMUS, M., SARVAIYA, N. & NEILL EPPERSON, C. 2014. Sex differences in anxiety and depression clinical perspectives. *Front Neuroendocrinol,* 35**,** 320-30.

ALZAMORA, R., O'MAHONY, F. & HARVEY, B. J. 2011. Estrogen inhibits chloride secretion caused by cholera and Escherichia coli enterotoxins in female rat distal colon. *Steroids,* 76**,** 867-76.

AN, F., YANG, G., TIAN, J. & WANG, S. 2012. Antioxidant effects of the orientin and vitexin in Trollius chinensis Bunge in D-galactose-aged mice. *Neural Regen Res,* 7**,** 2565-75.

ANDREWS, C. N., SIDANI, S. & MARSHALL, J. K. 2020. Clinical Management of the Microbiome in Irritable Bowel Syndrome. *Journal of the Canadian Association of Gastroenterology*.

ANHÊ, F. F., ROY, D., PILON, G., DUDONNE, S., MATAMOROS, S., VARIN, T. V., GAROFALO, C., MOINE, Q., DESJARDINS, Y., LEVY, E. & MARETTE, A. 2015. A polyphenol-rich cranberry extract protects from diet-induced obesity, insulin resistance and intestinal inflammation in association with increased Akkermansia spp. population in the gut microbiota of mice. *Gut,* 64**,** 872-83.

ANKOLEKAR, C., JOHNSON, D., PINTO MDA, S., JOHNSON, K., LABBE, R. & SHETTY, K. 2011. Inhibitory potential of tea polyphenolics and influence of extraction time against Helicobacter pylori and lack of inhibition of beneficial lactic acid bacteria. *J Med Food,* 14**,** 1321-9.

ARAI, N., STROM, A., RAFTER, J. J. & GUSTAFSSON, J. A. 2000. Estrogen receptor beta mRNA in colon cancer cells: growth effects of estrogen and genistein. *Biochem Biophys Res Commun,* 270**,** 425-31.

ATKINSON, W., LOCKHART, S., WHORWELL, P. J., KEEVIL, B. & HOUGHTON, L. A. 2006. Altered 5-hydroxytryptamine signaling in patients with constipation- and diarrhea-predominant irritable bowel syndrome. *Gastroenterology,* 130**,** 34-43.

AVRAMIDOU, M., ANGST, F., ANGST, J., AESCHLIMANN, A., ROSSLER, W. & SCHNYDER, U. 2018. Epidemiology of gastrointestinal symptoms in young and middle-aged Swiss adults: prevalences and comorbidities in a longitudinal population cohort over 28 years. *BMC Gastroenterol,* 18**,** 21.

AYMERICH, T., MARTIN, B., GARRIGA, M., VIDAL-CAROU, M. C., BOVER-CID, S. & HUGAS, M. 2006. Safety properties and molecular strain typing of lactic acid bacteria from slightly fermented sausages. *J Appl Microbiol,* 100**,** 40-9.

BABUSYTE, A., KOTTHOFF, M., FIEDLER, J. & KRAUTWURST, D. 2013. Biogenic amines activate blood leukocytes via trace amine-associated receptors TAAR1 and TAAR2. *J Leukoc Biol,* 93**,** 387-394.

BAKER, J. M., AL-NAKKASH, L. & HERBST-KRALOVETZ, M. M. 2017. Estrogen-gut microbiome axis: Physiological and clinical implications. *Maturitas,* 103**,** 45-53.

BALEMANS, D., MONDELAERS, S. U., CIBERT-GOTON, V., STAKENBORG, N., AGUILERA-LIZARRAGA, J., DOOLEY, J., LISTON, A., BULMER, D. C., VANDEN BERGHE, P., BOECKXSTAENS, G. E. & WOUTERS, M. M. 2017. Evidence for long-term sensitization of the bowel in patients with post-infectious-IBS. *Sci Rep,* 7**,** 13606.

BARBARA, G., FEINLE-BISSET, C., GHOSHAL, U. C., QUIGLEY, E. M., SANTOS, J., VANNER, S., VERGNOLLE, N. & ZOETENDAL, E. G. 2016. The Intestinal Microenvironment and Functional Gastrointestinal Disorders. *Gastroenterology*.

BARBARA, G., STANGHELLINI, V., DE GIORGIO, R. & CORINALDESI, R. 2006. Functional gastrointestinal disorders and mast cells: implications for therapy. *Neurogastroenterol Motil,* 18**,** 6-17.

BARBERIO, B., ZAMANI, M., BLACK, C. J., SAVARINO, E. V. & FORD, A. C. 2021. Prevalence of symptoms of anxiety and depression in patients with inflammatory bowel disease: a systematic review and meta-analysis. *Gastroenterology & Hepatology,* 6**,** 359-370.

BARBIERI, F., MONTANARI, C., GARDINI, F. & TABANELLI, G. 2019. Biogenic Amine Production by Lactic Acid Bacteria: A Review. *Foods,* 8.

BEARCROFT, C. P., PERRETT, D. & FARTHING, M. 1998. Postprandial plasma 5-hydroxytryptamine in diarrhoea predominant irritable bowel syndrome: a pilot study. *Gut,* 42**,** 42-46.

BELLONO, N. W., BAYRER, J. R., LEITCH, D. B., CASTRO, J., ZHANG, C., O'DONNELL, T. A., BRIERLEY, S. M., INGRAHAM, H. A. & JULIUS, D. 2017. Enterochromaffin Cells Are Gut Chemosensors that Couple to Sensory Neural Pathways. *Cell,* 170**,** 185-198 e16.

BENNET, S. M., OHMAN, L. & SIMREN, M. 2015. Gut microbiota as potential orchestrators of irritable bowel syndrome. *Gut Liver,* 9**,** 318-31.

BENNETT, A. C., VAN CAMP, A., LOPEZ, V. & SMITH, C. 2018. Sceletium tortuosum may delay chronic disease progression via alkaloid-dependent antioxidant or anti-inflammatory action. *J Physiol Biochem,* 74**,** 539-547.

BERNARDI, S., DEL BO, C., MARINO, M., GARGARI, G., CHERUBINI, A., ANDRES-LACUEVA, C., HIDALGO-LIBERONA, N., PERON, G., GONZALEZ-DOMINGUEZ, R., KROON, P., KIRKUP, B., PORRINI, M., GUGLIELMETTI, S. & RISO, P. 2020. Polyphenols and Intestinal Permeability: Rationale and Future Perspectives. *J Agric Food Chem,* 68**,** 1816-1829.

BERRY, M. D. 2004. Mammalian central nervous system trace amines. Pharmacologic amphetamines, physiologic neuromodulators. *J Neurochem,* 90**,** 257-71.

BERRY, M. D. 2016. Trace Amines and Their Receptors in the Control of Cellular Homeostasis. *In:* FAROOQUI, T. & FAROOQUI, A. (eds.) *Trace Amines and Neurological Disorders: Potential Mechanisms and Risk Factors* Elsevier Inc.

BERRY, M. D., GAINETDINOV, R. R., HOENER, M. C. & SHAHID, M. 2017. Pharmacology of human trace amine-associated receptors: Therapeutic opportunities and challenges. *Pharmacol Ther,* 180**,** 161-180.

BERTIAUX-VANDAELE, N., YOUMBA, S. B., BELMONTE, L., LECLEIRE, S., ANTONIETTI, M., GOURCEROL, G., LEROI, A. M., DECHELOTTE, P., MENARD, J. F., DUCROTTE, P. & COEFFIER, M. 2011. The expression and the cellular distribution of the tight junction proteins are altered in irritable bowel syndrome patients with differences according to the disease subtype. *Am J Gastroenterol,* 106**,** 2165-73.

BETHEA, C. L., GUNDLAH, C. & MIRKES, S. J. 2000a. Ovarian steroid action in the serotonin neural system of macaques. *Novartis Found Symp,* 230**,** 112-33.

BETHEA, C. L., MIRKES, S. J., SHIVELY, C. A. & ADAMS, M. R. 2000b. Steroid Regulation of Tryptophan Hydroxylase Protein in the Dorsal Raphe of Macaques. *Biol Psychiatry,* 47**,** 562-576.

BETTS, J. A., MORADI MARJANEH, M., AL-EJEH, F., LIM, Y. C., SHI, W., SIVAKUMARAN, H., TROPEE, R., PATCH, A. M., CLARK, M. B., BARTONICEK, N., WIEGMANS, A. P., HILLMAN, K. M., KAUFMANN, S., BAIN, A. L., GLOSS, B. S., CRAWFORD, J., KAZAKOFF, S., WANI, S., WEN, S. W., DAY, B., MOLLER, A., CLOONAN, N., PEARSON, J., BROWN, M. A., MERCER, T. R., WADDELL, N., KHANNA, K. K., DRAY, E., DINGER, M. E., EDWARDS, S. L. & FRENCH, J. D. 2017. Long Noncoding RNAs CUPID1 and CUPID2 Mediate Breast Cancer Risk at 11q13 by Modulating the Response to DNA Damage. *Am J Hum Genet,* 101**,** 255-266.

BHARADWAJ, S., BARBER, M. D., GRAFF, L. A. & SHEN, B. 2015a. Symptomatology of irritable bowel syndrome and inflammatory bowel disease during the menstrual cycle. *Gastroenterology Report,* 3**,** 185-193.

BHARADWAJ, S., KULKARNI, G. & SHEN, B. 2015b. Menstrual cycle, sex hormones in female inflammatory bowel disease patients with and without surgery. *J Dig Dis,* 16**,** 245-55.

BHATTARAI, Y., JIE, S., LINDEN, D. R., GHATAK, S., MARS, R. A. T., WILLIAMS, B. B., PU, M., SONNENBURG, J. L., FISCHBACH, M. A., FARRUGIA, G., SHA, L. & KASHYAP, P. C. 2020. Bacterially Derived Tryptamine Increases Mucus Release by Activating a Host Receptor in a Mouse Model of Inflammatory Bowel Disease. *iScience,* 23**,** 101798.

BHATTARAI, Y., WILLIAMS, B. B., BATTAGLIOLI, E. J., WHITAKER, W. R., TILL, L., GROVER, M., LINDEN, D. R., AKIBA, Y., KANDIMALLA, K. K., ZACHOS, N. C., KAUNITZ, J. D., SONNENBURG, J. L., FISCHBACH, M. A., FARRUGIA, G. & KASHYAP, P. C. 2018. Gut Microbiota-Produced Tryptamine Activates an Epithelial G-Protein-Coupled Receptor to Increase Colonic Secretion. *Cell Host Microbe,* 23**,** 775-785 e5.

BLACHIER, F., BEAUMONT, M., ANDRIAMIHAJA, M., DAVILA, A. M., LAN, A., GRAUSO, M., ARMAND, L., BENAMOUZIG, R. & TOME, D. 2017. Changes in the Luminal Environment of the Colonic Epithelial Cells and Physiopathological Consequences. *Am J Pathol,* 187**,** 476-486.

BLACK, C. J. & FORD, A. C. 2020. Global burden of irritable bowel syndrome: trends, predictions and risk factors. *Nat Rev Gastroenterol Hepatol,* 17**,** 473-486.

BLANCHARD, E. B., KEEFER, L., GALOVSKI, T. E., TAYLOR, A. E. & TURNER, S. M. 2001. Gender differences in psychological distress among patients with irritable bowel syndrome. *Journal of Psychosomatic Research,* 50**,** 271-275.

BLUM, I., VERED, Y., LIFSHITZ, A., HAREL, D., BLUM, M., NORDENBERG, Y., HARSAT, A., SULKES, J., GABBAY, U. & GRAFF, E. 1996. The effect of estrogen replacement therapy on plasma serotonin and catecholamines of postmenopausal women. *Isr J Med Sci* 32**,** 1158-1162.

BLY, M. 2005. Examination of the trace amine-associated receptor 2 (TAAR2). *Schizophr Res,* 80**,** 367-8.

BOHN, L., STORSRUD, S., TORNBLOM, H., BENGTSSON, U. & SIMREN, M. 2013. Self-reported food-related gastrointestinal symptoms in IBS are common and associated with more severe symptoms and reduced quality of life. *Am J Gastroenterol,* 108**,** 634-41.

BOND, T. J. & DERBYSHIRE, E. J. 2020. Rooibos Tea and Health: A Systematic Review of the Evidence from the Last Two Decades. *Nutrition and Food Technology: Open Access,* 6.

BOROWSKY, B., ADHAM, N., JONES, K. A., RADDATZ, R., ARTYMYSHYN, R., OGOZALEK, K. L., DURKIN, M. M., LAKHLANI, P. P., BONINI, J. A., PATHIRANA, S., BOYLE, N., PU, X., KOURANOVA, E., LICHTBLAU, H., OCHOA, F. Y., BRANCHEK, T. A. & GERALD, C. 2001. Trace amines: identification of a family of mammalian G protein-coupled receptors. *Proc Natl Acad Sci U S A,* 98**,** 8966-71.

BORTOLATO, M., CHEN, K. & SHIH, J. C. 2008. Monoamine oxidase inactivation: from pathophysiology to therapeutics. *Adv Drug Deliv Rev,* 60**,** 1527-33.

BOWLES, S., JOUBERT, E., DE BEER, D., LOUW, J., BRUNSCHWIG, C., NJOROGE, M., LAWRENCE, N., WIESNER, L., CHIBALE, K. & MULLER, C. 2017. Intestinal Transport Characteristics and Metabolism of C-Glucosyl Dihydrochalcone, Aspalathin. *Molecules,* 22.

BRADAIA, A., TRUBE, G., STALDER, H., NORCROSS, R. D., OZMEN, L., WETTSTEIN, J. G., PINARD, A., BUCHY, D., GASSMANN, M., HOENER, M. C. & BETTLER, B. 2009. The

selective antagonist EPPTB reveals TAAR1-mediated regulatory mechanisms in dopaminergic neurons of the mesolimbic system. *Proc Natl Acad Sci U S A,* 106**,** 20081-6.

BRANISTE, V., LEVEQUE, M., BUISSON-BRENAC, C., BUENO, L., FIORAMONTI, J. & HOUDEAU, E. 2009. Oestradiol decreases colonic permeability through oestrogen receptor beta-mediated up-regulation of occludin and junctional adhesion molecule-A in epithelial cells. *J Physiol,* 587**,** 3317-28.

BREITER, T., LAUE, C., KRESSEL, G., GROLL, S., ENGELHARDT, U. H. & HAHN, A. 2011. Bioavailability and antioxidant potential of rooibos flavonoids in humans following the consumption of different rooibos formulations. *Food Chem,* 128**,** 338-47.

BROADLEY, K. J., AKHTAR ANWAR, M., HERBERT, A. A., FEHLER, M., JONES, E. M., DAVIES, W. E., KIDD, E. J. & FORD, W. R. 2009. Effects of dietary amines on the gut and its vasculature. *Br J Nutr,* 101**,** 1645-52.

BRUGMAN, S. 2016. The zebrafish as a model to study intestinal inflammation. *Dev Comp Immunol,* 64**,** 82-92.

BUHNER, S., LI, Q., VIGNALI, S., BARBARA, G., DE GIORGIO, R., STANGHELLINI, V., CREMON, C., ZELLER, F., LANGER, R., DANIEL, H., MICHEL, K. & SCHEMANN, M. 2009. Activation of Human Enteric Neurons by Supernatants of Colonic Biopsy Specimens From Patients With Irritable Bowel Syndrome. *Gastroenterology,* 137**,** 1425–1434.

BUNZOW, J. R., SONDERS, M. S., ARTTAMANGKUL, S., HARRISON, L. M., ZHANG, G., QUIGLEY, D. I., DARLAND, T., SUCHLAND, K. L., PASUMAMULA, S., KENNEDY, J. L., OLSON, S. B., MAGENIS, E., AMARA, S. G. & GRANDY, D. K. 2001. Amphetamine, 3,4-methylenedioxymethamphetamine, lysergic acid diethylamide, and metabolites of the catecholamine neurotransmitters are agonists of a rat trace amine receptor. *Molecular Pharmacology,* 60**,** 1181-1188.

BUONO, J. L., CARSON, R. T. & FLORES, N. M. 2017. Health-related quality of life, work productivity, and indirect costs among patients with irritable bowel syndrome with diarrhea. *Health Qual Life Outcomes,* 15**,** 35.

BURNS, C. & KIDRON, A. 2022. *Biochemistry, Tyramine,* Treasure Island, (FL), StatPearls Publishing.

BUSTOS, V., NOLAN, A. M., NIJHUIS, A., HARVEY, H., PARKER, A., POULSOM, R., MCBRYAN, J., THOMAS, W., SILVER, A. & HARVEY, B. J. 2017. GPER mediates differential effects of estrogen on colon cancer cell proliferation and migration under normoxic and hypoxic conditions. *Oncotarget,* 8**,** 84258-84275.

CAMILLERI, M. 2009. Serotonin in the Gastrointestinal Tract. *Curr Opin Endocrinol Diabetes Obes.,* 16**,** 53-59.

CAMILLERI, M. 2015. Intestinal secretory mechanisms in irritable bowel syndrome-diarrhea. *Clin Gastroenterol Hepatol,* 13**,** 1051-7; quiz e61-2.

CAMPBELL-THOMPSON, M., LYNCH, J. & BHARDWAJ, B. 2001. Expression of estrogen receptor (ER) subtypes in colon cancer cell lines. *Cancer Research,* 61**,** 632-640.

CANAVAN, C., WEST, J. & CARD, T. 2014a. The epidemiology of irritable bowel syndrome. *Clin Epidemiol,* 6**,** 71-80.

CANAVAN, C., WEST, J. & CARD, T. 2014b. Review article: the economic impact of the irritable bowel syndrome. *Aliment Pharmacol Ther,* 40**,** 1023-34.

CARROLL, I. M., CHANG, Y. H., PARK, J., SARTOR, R. B. & RINGEL, Y. 2010. Luminal and mucosal-associated intestinal microbiota in patients with diarrhea-predominant irritable bowel syndrome. *Gut Pathog,* 2**,** 19.

CASEN, C., VEBO, H. C., SEKELJA, M., HEGGE, F. T., KARLSSON, M. K., CIEMNIEJEWSKA, E., DZANKOVIC, S., FRØYLAND, C., NESTESTOG, R., ENGSTRAND, L., MUNKHOLM, P., NIELSEN, O. H., ROGLER, G., SIMRÉN, M., ÖHMAN, L., VATN, M. H. & RUDI, K. 2015. Deviations in human gut microbiota: a novel diagnostic test for determining dysbiosis in patients with IBS or IBD. *Aliment Pharmacol Ther,* 42**,** 71-83.

CASIMIR, G. J., LEFEVRE, N., CORAZZA, F., DUCHATEAU, J. & CHAMEKH, M. 2018. The Acid-Base Balance and Gender in Inflammation: A Mini-Review. *Front Immunol,* 9**,** 475.

CATALKAYA, G., VENEMA, K., LUCINI, L., ROCCHETTI, G., DELMAS, D., DAGLIA, M., DE FILIPPIS, A., XIAO, H., QUILES, J. L., XIAO, J. & CAPANOGLU, E. 2020. Interaction of dietary polyphenols and gut microbiota: Microbial metabolism of polyphenols, influence on the gut microbiota, and implications on host health. *Food Frontiers,* 1**,** 109-133.

CHADWICK, V. S., CHEN, W., SHU, D., PAULUS, B., BETHWAITE, P., TIE, A. & WILSON, I. 2002. Activation of the Mucosal Immune System in Irritable Bowel Syndrome. *Gastroenterology,* 122**,** 1778–1783.

CHAN, C. W. H., LAW, B. M. H., WAYE, M. M. Y., CHAN, J. Y. W., SO, W. K. W. & CHOW, K. M. 2019. Trimethylamine-N-oxide as One Hypothetical Link for the Relationship between Intestinal Microbiota and Cancer - Where We Are and Where Shall We Go? *J Cancer,* 10**,** 5874-5882.

CHATILA, R., MERHI, M., HARIRI, E., SABBAH, N. & DEEB, M. E. 2017. Irritable bowel syndrome: prevalence, risk factors in an adult Lebanese population. *BMC Gastroenterology,* 17**,** 137-142.

CHATZINIKOLAOU, G., NIKITOVIC, D., STATHOPOULOS, E. N., VELEGRAKIS, G. A., KARAMANOS, N. K. & TZANAKAKIS, G. N. 2007. Protein Tyrosine Kinase and Estrogen Receptor-dependent Pathways Regulate the Synthesis and Distribution of Glycosaminoglycans:Proteoglycans Produced by Two Human Colon Cancer Cell Lines. *Anticancer Research,* 27**,** 4101-4106.

CHECA, J. & ARAN, J. M. 2020. Reactive Oxygen Species: Drivers of Physiological and Pathological Processes. *J Inflamm Res,* 13**,** 1057-1073.

CHEEL, J., THEODULOZ, C., RODRIGUEZ, J. & SCHMEDA-HIRSCHMANN, G. 2005. Free Radical Scavengers and Antioxidants from Lemongrass (Cymbopogon citratus (DC.) Stapf.). *J. Agric. Food Chem.,* 53**,** 2511-2517.

CHEN, Y., ZHOU, J. & WANG, L. 2021. Role and Mechanism of Gut Microbiota in Human Disease. *Front Cell Infect Microbiol,* 11**,** 625913.

CHENG, G., LI, Y., OMOTO, Y., WANG, Y., BERG, T., NORD, M., VIHKO, P., WARNER, M., PIAO, Y. S. & GUSTAFSSON, J. A. 2005. Differential regulation of estrogen receptor (ER)alpha and ERbeta in primate mammary gland. *J Clin Endocrinol Metab,* 90**,** 435-44.

CHENG, Q., YU, X., ZHANG, R. & CHEN, L. 2020. Isoorientin Alleviates Inflammatory Bowel Disease by Inhibiting NLRP3 Inflammasome Activation through Nrf2/NQO1 Pathway. *Current Topics in Nutraceutical Research,* 18**,** 392-397.

CHIELLINI, G., ERBA, P., CARNICELLI, V., MANFREDI, C., FRASCARELLI, S., GHELARDONI, S., MARIANI, G. & ZUCCHI, R. 2012. Distribution of exogenous [125I]-3-iodothyronamine in mouse in vivo: relationship with trace amine-associated receptors. *Journal of Endocrinology,* 213**,** 223-230.

CHOGHAKHORI, R., ABBASNEZHAD, A., AMANI, R. & ALIPOUR, M. 2017a. Sex-Related Differences in Clinical Symptoms, Quality of Life, and Biochemical Factors in Irritable Bowel Syndrome. *Dig Dis Sci,* 62**,** 1550-1560.

CHOGHAKHORI, R., ABBASNEZHAD, A., HASANVAND, A. & AMANI, R. 2017b. Inflammatory cytokines and oxidative stress biomarkers in irritable bowel syndrome: Association with digestive symptoms and quality of life. *Cytokine,* 93**,** 34-43.

CHONG, P. P., CHIN, V. K., LOOI, C. Y., WONG, W. F., MADHAVAN, P. & YONG, V. C. 2019. The Microbiome and Irritable Bowel Syndrome - A Review on the Pathophysiology, Current Research and Future Therapy. *Front Microbiol,* 10**,** 1136.

CHRISTIAN, S. L. & BERRY, M. D. 2018. Trace Amine-Associated Receptors as Novel Therapeutic Targets for Immunomodulatory Disorders. *Front Pharmacol,* 9**,** 680.

CHUANG, L. S., MORRISON, J., HSU, N. Y., LABRIAS, P. R., NAYAR, S., CHEN, E., VILLAVERDE, N., FACEY, J. A., BOSCHETTI, G., GIRI, M., CASTILLO-MARTIN, M., THIN, T. H., SHARMA, Y., CHU, J. & CHO, J. H. 2019. Zebrafish modeling of intestinal injury, bacterial exposures and medications defines epithelial in vivo responses relevant to human inflammatory bowel disease. *Dis Model Mech,* 12.

COEFFIER, M., GLORO, R., BOUKHETTALA, N., AZIZ, M., LECLEIRE, S., VANDAELE, N., ANTONIETTI, M., SAVOYE, G., BOLE-FEYSOT, C., DECHELOTTE, P., REIMUND, J. M. & DUCROTTE, P. 2010. Increased proteasome-mediated degradation of occludin in irritable bowel syndrome. *Am J Gastroenterol,* 105**,** 1181-8.

COLLINS, S. M., DENOU, E., VERDU, E. F. & BERCIK, P. 2009. The putative role of the intestinal microbiota in the irritable bowel syndrome. *Dig Liver Dis,* 41**,** 850-3.

CONDLIFFE, S. B., DOOLAN, C. M. & HARVEY, B. J. 2001. 17b-Oestradiol acutely regulates Cl secretion in rat distal colonic epithelium. *Journal of Physiology* 530**,** 47-54.

CORNEY, R. & STANTON, R. 1990. Physical symptom severity, psychological and social dysfunction in a series of outpatients with irritable bowel syndrome. *J Psychosom Res,* 34**,** 483–91.

COSTELLO, E. K., LAUBER, C. L., HAMADY, M., FIERER, N., GORDON, J. I. & KNIGHT, R. 2009. Bacterial community variation in human body habitats across space and time. . *Science,* 326**,** 1694–1697.

COTON, E. & COTON, M. 2009. Evidence of horizontal transfer as origin of strain to strain variation of the tyramine production trait in Lactobacillus brevis. *Food Microbiol,* 26**,** 52-7.

CRACIUN, S. & BALSKUS, E. P. 2012. Microbial conversion of choline to trimethylamine requires a glycyl radical enzyme. *Proc Natl Acad Sci U S A,* 109**,** 21307-12.

CROUZET, L., GAULTIER, E., DEL'HOMME, C., CARTIER, C., DELMAS, E., DAPOIGNY, M., FIORAMONTI, J. & BERNALIER-DONADILLE, A. 2013. The hypersensitivity to colonic distension of IBS patients can be transferred to rats through their fecal microbiota. *Neurogastroenterol Motil,* 25**,** e272-82.

CROWELL, M. D. 2004. Role of serotonin in the pathophysiology of the irritable bowel syndrome. *Br J Pharmacol,* 141**,** 1285-93.

CUOMO, R., ANDREOZZI, P., ZITO, F. P., PASSANANTI, V., DE CARLO, G. & SARNELLI, G. 2014. Irritable bowel syndrome and food interaction. *World J Gastroenterol,* 20**,** 8837-45.

D'AMATO, M. 2013. Genes and functional GI disorders: from casual to causal relationship. *Neurogastroenterol Motil,* 25**,** 638-49.

D'ANDREA, G., TERRAZZINO, S., FORTIN, D., FARRUGGIO, A., RINALDI, L. & LEON, A. 2003. HPLC electrochemical detection of trace amines in human plasma and platelets and expression of mRNA transcripts of trace amine receptors in circulating leukocytes. *Neuroscience Letters,* 346**,** 89-92.

DAINESE, R., GALLIANI, E., DE LAZZARI, F., DI LEO, V. & NACCARATO, R. 1999. Discrepancies Between Reported Food Intolerance and Sensitization Test Findings in Irritable Bowel Syndrome Patients. *American Journal of Gastroenterology,* 94**,** 1892-1897.

DAMIANI, E., CARLONI, P., ROCCHETTI, G., SENIZZA, B., TIANO, L., JOUBERT, E., DE BEER, D. & LUCINI, L. 2019. Impact of Cold versus Hot Brewing on the Phenolic Profile and Antioxidant Capacity of Rooibos (Aspalathus linearis) Herbal Tea. *Antioxidants (Basel),* 8.

DAVE, M., HIGGINS, P. D., MIDDHA, S. & RIOUX, K. P. 2012. The human gut microbiome: current knowledge, challenges, and future directions. *Transl Res,* 160**,** 246-57.

DE ALMEIDA, C. J. G. 2017. Caveolin-1 and Caveolin-2 Can Be Antagonistic Partners in Inflammation and Beyond. *Front Immunol,* 8**,** 1530.

DE PRETER, V. & VERBEKE, K. 2013. Metabolomics as a diagnostic tool in gastroenterology. *World J Gastrointest Pharmacol Ther,* 4**,** 97-107.

DE SANTIS, S., CAVALCANTI, E., MASTRONARDI, M., JIRILLO, E. & CHIEPPA, M. 2015. Nutritional Keys for Intestinal Barrier Modulation. *Front Immunol,* 6**,** 612.

DEL RIO, B., REDRUELLO, B., LADERO, V., CAL, S., OBAYA, A. J. & ALVAREZ, M. A. 2018. An altered gene expression profile in tyramine-exposed intestinal cell cultures supports the genotoxicity of this biogenic amine at dietary concentrations. *Sci Rep,* 8**,** 17038.

DEL RIO, B., REDRUELLO, B., LINARES, D. M., LADERO, V., FERNANDEZ, M., MARTIN, M. C., RUAS-MADIEDO, P. & ALVAREZ, M. A. 2017. The dietary biogenic amines tyramine and histamine show synergistic toxicity towards intestinal cells in culture. *Food Chem,* 218**,** 249-255.

DEL RIO, B., REDRUELLO, B., LINARES, D. M., LADERO, V., RUAS-MADIEDO, P., FERNANDEZ, M., MARTIN, M. C. & ALVAREZ, M. A. 2019. The biogenic amines putrescine and cadaverine show in vitro cytotoxicity at concentrations that can be found in foods. *Sci Rep,* 9**,** 120.

DEMADY, R. D., JIANMONGKOL, S., VULETICH, J. L., BENDER, A. T. & OSAWA, Y. 2001. Agmatine Enhances the NADPH Oxidase Activity of Neuronal NO Synthase and Leads to Oxidative Inactivation of the Enzyme. *Mol Pharmacol,* 59**,** 24-29.

DIAL, E. J., COOPER, C. & LICHTENBERGER, L. M. 1991. Amino acid- and amine-induced gastrin release from isolated rat endocrine granules. *American Journal of Physiology,* 260**,** G175-G181.

DÍAZ-VALDIVIA, N. I., CALDERÓN, C. C., DÍAZ, J. E., LOBOS-GONZÁLEZ, L., SEPULVEDA, H., ORTÍZ, R. J., MARTINEZ, S., SILVA, V., MALDONADO, H. J., SILVA, P., WEHINGER, S., BURZIO, V. A., TORRES, V. A., MONTECINO, M., LEYTON, L. & QUEST, A. F. G. 2017. Anti-neoplastic drugs increase caveolin-1-dependent migration, invasion and metastasis of cancer cells. *Oncotarget,* 8**,** 111943-111965.

DIDARI, T., MOZAFFARI, S., NIKFAR, S. & ABDOLLAHI, M. 2015. Effectiveness of probiotics in irritable bowel syndrome: Updated systematic review with meta-analysis. *World J Gastroenterol,* 21**,** 3072-84.

DIEBEL, M. E., DIEBEL, L. N., MANKE, C. W. & LIBERATI, D. M. 2015. Estrogen modulates intestinal mucus physiochemical properties and protects against oxidant injury. *J Trauma Acute Care Surg,* 78**,** 94-9.

DINTER, J., MUHLHAUS, J., JACOBI, S. F., WIENCHOL, C. L., COSTER, M., MEISTER, J., HOEFIG, C. S., MULLER, A., KOHRLE, J., GRUTERS, A., KRUDE, H., MITTAG, J., SCHONEBERG, T., KLEINAU, G. & BIEBERMANN, H. 2015. 3-iodothyronamine differentially modulates alpha-2A-adrenergic receptor-mediated signaling. *J Mol Endocrinol,* 54**,** 205-16.

DISTRUTTI, E., MONALDI, L., RICCI, P. & FIORUCCI, S. 2016. Gut microbiota role in irritable bowel syndrome: New therapeutic strategies. *World J Gastroenterol,* 22**,** 2219-41.

DLUDLA, P. V., MULLER, C. J. F., LOUW, J., MAZIBUKO-MBEJE, S. E., TIANO, L., SILVESTRI, S., ORLANDO, P., MARCHEGGIANI, F., CIRILLI, I., CHELLAN, N., GHOOR, S., NKAMBULE, B. B., ESSOP, M. F., HUISAMEN, B. & JOHNSON, R. 2020. The Combination Effect of Aspalathin and Phenylpyruvic Acid-2-O-beta-D-glucoside from Rooibos against Hyperglycemia-Induced Cardiac Damage: An In Vitro Study. *Nutrients,* 12.

DLUGOS, A., CHILDS, E., STUHR, K. L., HILLARD, C. J. & DE WIT, H. 2012. Acute stress increases circulating anandamide and other N-acylethanolamines in healthy humans. *Neuropsychopharmacology,* 37**,** 2416-27.

DLUGOSZ, A., WINCKLER, B., LUNDIN, E., ZAKIKHANY, K., SANDSTROM, G., YE, W., ENGSTRAND, L. & LINDBERG, G. 2015. No difference in small bowel microbiota between patients with irritable bowel syndrome and healthy controls. *Sci Rep,* 5**,** 8508.

DROSSMAN, D. A., TONER, B. B., WHITEHEAD, W. E., DIAMANT, N. E., DALTON, C. B., DUNCAN, S., EMMOTT, S., PROFFITT, V., AKMAN, D., FRUSCIANTE, K., LE, T., MEYER, K., BRADSHAW, B., MIKULA, K., MORRIS, C. B., BLACKMAN, C. J., HU, Y., JIA, H., LI, J. Z., KOCH, G. G. & BANGDIWALA, S. I. 2003. Cognitive-behavioral therapy versus education and desipramine versus placebo for moderate to severe functional bowel disorders 1 1This study was registered with ClinicalTrials.gov (trial registry no. NCT00006157). *Gastroenterology,* 125**,** 19-31.

DUAN, X., WU, T., LIU, T., YANG, H., DING, X., CHEN, Y. & MU, Y. 2019. Vicenin-2 ameliorates oxidative damage and photoaging via modulation of MAPKs and MMPs signaling in UVB radiation exposed human skin cells. *J Photochem Photobiol B,* 190**,** 76-85.

DUBINSKY, M. C., DOTAN, I., RUBIN, D. T., BERNAUER, M., PATEL, D., CHEUNG, R., MODESTO, I., LATYMER, M. & KEEFER, L. 2021. Burden of comorbid anxiety and depression in patients with inflammatory bowel disease: a systematic literature review. *Expert Rev Gastroenterol Hepatol,* 15**,** 985-997.

DUNLOP, S. P., COLEMAN, N. S., BLACKSHAW, E., PERKINS, A. C., SINGH, G., MARSDEN, C. A. & SPILLER, R. C. 2005. Abnormalities of 5-hydroxytryptamine metabolism in irritable bowel syndrome. *Clin Gastroenterol Hepatol* 3**,** 349-357.

EK, W. E., REZNICHENKO, A., RIPKE, S., NIESLER, B., ZUCCHELLI, M., RIVERA, N. V., SCHMIDT, P. T., PEDERSEN, N. L., MAGNUSSON, P., TALLEY, N. J., HOLLIDAY, E. G., HOUGHTON, L., GAZOULI, M., KARAMANOLIS, G., RAPPOLD, G., BURWINKEL, B., SUROWY, H., RAFTER, J., ASSADI, G., LI, L., PAPADAKI, E., GAMBACCINI, D., MARCHI, S., COLUCCI, R., BLANDIZZI, C., BARBARO, R., KARLING, P., WALTER, S., OHLSSON, B., TORNBLOM, H., BRESSO, F., ANDREASSON, A., DLUGOSZ, A., SIMREN, M., AGREUS, L., LINDBERG, G., BOECKXSTAENS, G., BELLINI, M., STANGHELLINI, V., BARBARA, G., DALY, M. J., CAMILLERI, M., WOUTERS, M. M. & D'AMATO, M. 2015. Exploring the genetics of irritable bowel syndrome: a GWA study in the general population and replication in multinational case-control cohorts. *Gut,* 64**,** 1774-82.

EL-SALHY, M., GILJA, O. H., HATLEBAKK, J. G. & HAUSKEN, T. 2014. Stomach antral endocrine cells in patients with irritable bowel syndrome. *Int J Mol Med,* 34**,** 967-74.

EL-SALHY, M., HATLEBAKK, J. G. & HAUSKEN, T. 2020. Possible role of peptide YY (PYY) in the pathophysiology of irritable bowel syndrome (IBS). *Neuropeptides,* 79**,** 101973.

ELMALIKLIS, I. N., LIVERI, A., NTELIS, B., PARASKEVA, K., GOULIS, I. & KOUTELIDAKIS, A. E. 2019. Increased Functional Foods' Consumption and Mediterranean Diet Adherence May Have a Protective Effect in the Appearance of Gastrointestinal Diseases: A Case(-)Control Study. *Medicines (Basel),* 6.

ENCK, P., AZIZ, Q., BARBARA, G., FARMER, A. D., FUKUDO, S., MAYER, E. A., NIESLER, B., QUIGLEY, E. M., RAJILIC-STOJANOVIC, M., SCHEMANN, M., SCHWILLE-KIUNTKE, J., SIMREN, M., ZIPFEL, S. & SPILLER, R. C. 2016. Irritable bowel syndrome. *Nat Rev Dis Primers,* 2**,** 16014.

ENCK, P. & MAZURAK, N. 2018. Dysbiosis in Functional Bowel Disorders. *Ann Nutr Metab,* 72**,** 296-306.

ENDO, Y., SHOJI, T. & FUKUDO, S. 2015. Epidemiology of irritable bowel syndrome. *Annals of Gastroenterology,* 28**,** 158-159.

ERVIN, S. M., LI, H., LIM, L., ROBERTS, L. R., LIANG, X., MANI, S. & REDINBO, M. R. 2019. Gut microbial beta-glucuronidases reactivate estrogens as components of the estrobolome that reactivate estrogens. *J Biol Chem,* 294**,** 18586-18599.

FARESJO, A., WALTER, S., NORLIN, A. K., FARESJO, T. & JONES, M. P. 2019. Gastrointestinal symptoms - an illness burden that affects daily work in patients with IBS. *Health Qual Life Outcomes,* 17**,** 113.

FARRUGGIO, S., RAINA, G., COCOMAZZI, G., LIBRASI, C., MARY, D., GENTILLI, S. & GROSSINI, E. 2019. Genistein improves viability, proliferation and mitochondrial function of cardiomyoblasts cultured in physiologic and peroxidative conditions. *International Journal of Molecular Medicine* 44**,** 2298-2310.

FAYYAZ, M. & LACKNER, J. M. 2008. Serotonin receptor modulators in the treatment of irritable bowel syndrome. *Therapeutics and Clinical Risk Management,* 4**,** 41-48.

FELLEY-BOSCO, E., BENDER, F. C., COURJAULT-GAUTIER, F., BRON, C. & QUEST, A. F. 2000. Caveolin-1 down-regulates inducible nitric oxide synthase via the proteasome pathway in human colon carcinoma cells. *Proc Natl Acad Sci U S A,* 97**,** 14334-9.

FENG, H., GUO, W., HAN, J. & LI, X. A. 2013. Role of caveolin-1 and caveolae signaling in endotoxemia and sepsis. *Life Sci,* 93**,** 1-6.

FENNEMA, D., PHILLIPS, I. R. & SHEPHARD, E. A. 2016. Trimethylamine and Trimethylamine N-Oxide, a Flavin-Containing Monooxygenase 3 (FMO3)-Mediated Host-Microbiome Metabolic Axis Implicated in Health and Disease. *Drug Metab Dispos,* 44**,** 1839-1850.

FERNANDEZ DE PALENCIA, P., FERNANDEZ, M., MOHEDANO, M. L., LADERO, V., QUEVEDO, C., ALVAREZ, M. A. & LOPEZ, P. 2011. Role of tyramine synthesis by food-borne Enterococcus durans in adaptation to the gastrointestinal tract environment. *Appl Environ Microbiol,* 77**,** 699-702.

FERNANDEZ, M., LINARES, D. M., RODRIGUEZ, A. & ALVAREZ, M. A. 2007. Factors affecting tyramine production in Enterococcus durans IPLA 655. *Appl Microbiol Biotechnol,* 73**,** 1400-6.

FISHER, D., THOMAS, K. A. & ABDUL-RASOOL, S. 2020. The Synergistic and Neuroprotective Effects of Alcohol-Antioxidant Treatment on Blood-Brain Barrier Endothelial Cells. *Alcohol Clin Exp Res,* 44**,** 1997-2007.

FLAK, M. B., NEVES, J. F. & BLUMBERG, R. S. 2013. Welcome to the Microgenderome. *Science,* 339**,** 1044-1045.

FLORES, E. M., NGUYEN, A. T., ODEM, M. A., EISENHOFFER, G. T. & KRACHLER, A. M. 2020. The zebrafish as a model for gastrointestinal tract-microbe interactions. *Cell Microbiol,* 22**,** e13152.

FLORICA-HOWELLS, E., HEN, R., GINGRICH, J., LI, Z. & GERSHON, M. D. 2002. 5-HT2A receptors: location and functional analysis in intestines of wild-type and 5-HT2A knockout mice. *Am J Physiol Gastrointest Liver Physiol* 282**,** G877–G893.

FOND, G., LOUNDOU, A., HAMDANI, N., BOUKOUACI, W., DARGEL, A., OLIVEIRA, J., ROGER, M., TAMOUZA, R., LEBOYER, M. & BOYER, L. 2014. Anxiety and depression comorbidities in irritable bowel syndrome (IBS): a systematic review and meta-analysis. *Eur Arch Psychiatry Clin Neurosci,* 264**,** 651-60.

FORD, A. C., LACY, B. E. & TALLEY, N. J. 2017. Irritable Bowel Syndrome. *N Engl J Med,* 376**,** 2566-2578.

FORD, A. C., QUIGLEY, E. M. M., LACY, B. E., LEMBO, A. J., SAITO, Y. A. & SCHILLER, L. R. 2014. Efficacy of prebiotics, probiotics, and synbiotics in irritable bowel syndrome and chronic idiopathic constipation: systematic review and meta-analysis. *Am J Gastroenterol.,* 109**,** 1547-61.

FROHLICH, E. E., FARZI, A., MAYERHOFER, R., REICHMANN, F., JACAN, A., WAGNER, B., ZINSER, E., BORDAG, N., MAGNES, C., FROHLICH, E., KASHOFER, K., GORKIEWICZ, G. & HOLZER, P. 2016. Cognitive impairment by antibiotic-induced gut dysbiosis: Analysis of gut microbiota-brain communication. *Brain Behav Immun,* 56**,** 140-55.

FUJISAWA, S. & KADOMA, Y. 2005. Kinetic Evaluation of Polyamines as Radical Scavengers. *ANTICANCER RESEARCH* 25**,** 965-970.

GADKARI, T. V., CORTES, N., MADRASI, K., TSOUKIAS, N. M. & JOSHI, M. S. 2013. Agmatine induced NO dependent rat mesenteric artery relaxation and its impairment in salt-sensitive hypertension. *Nitric Oxide,* 35**,** 65-71.

GAINETDINOV, R. R., HOENER, M. C. & BERRY, M. D. 2018. Trace Amines and Their Receptors. *Pharmacol Rev,* 70**,** 549-620.

GAO, J., XU, K., LIU, H., LIU, G., BAI, M., PENG, C., LI, T. & YIN, Y. 2018. Impact of the Gut Microbiota on Intestinal Immunity Mediated by Tryptophan Metabolism. *Front Cell Infect Microbiol,* 8**,** 13.

GARDINI, F., MARTUSCELLIB, M., CARUSOB, M. C., GALGANOB, F., CRUDELE, M. A., FAVATI, F., GUERZONI, M. E. & SUZZI, G. 2001. Effects of pH, temperature and NaCl concentration on the growth kinetics, proteolytic activity and biogenic amine production of Enterococcus faecalis. *International Journal of Food Microbiology,* 64**,** 105-117.

GARDINI, F., ROSSI, F., RIZZOTTI, L., TORRIANI, S., GRAZIA, L., CHIAVARI, C., COLORETTI, F. & TABANELLI, G. 2012. Role of Streptococcus thermophilus PRI60 in histamine accumulation in cheese. *International Dairy Journal,* 27**,** 71-76.

GAVAGHAN MCKEE, C. L., WILSON, I. D. & NICHOLSON, J. K. 2006. Metabolic Phenotyping of Nude and Normal (Alpk:ApfCD, C57BL10J) Mice. *J Proteome Res.,* 5**,** 378-384.

GERSEMANN, M., WEHKAMP, J. & STANGE, E. F. 2012. Innate immune dysfunction in inflammatory bowel disease. *J Intern Med,* 271**,** 421-8.

GERSHON, M. D. 2013. 5-Hydroxytryptamine (serotonin) in the gastrointestinal tract. *Curr Opin Endocrinol Diabetes Obes,* 20**,** 14-21.

GERSHON, M. D. & TACK, J. 2007. The serotonin signaling system: from basic understanding to drug development for functional GI disorders. *Gastroenterology,* 132**,** 397-414.

GHARBI, Y., FHOULA , I., RUAS-MADIEDO, P., AFEF, N., BOUDABOUS , A., GUEIMONDE, M. & OUZARI, H.-I. 2019. In-vitro characterization of potentially probiotic Lactobacillus strains isolated from human microbiota interaction with pathogenic bacteria and the enteric cell line HT29. *Annals of Microbiology,* 69**,** 61-72.

GHOSHAL, U. C., PARK, H. & GWEE, K. A. 2010. Bugs and irritable bowel syndrome: The good, the bad and the ugly. *J Gastroenterol Hepatol,* 25**,** 244-51.

GIBSON, P. R., VARNEY, J., MALAKAR, S. & MUIR, J. G. 2015. Food components and irritable bowel syndrome. *Gastroenterology,* 148**,** 1158-74 e4.

GIDDABASAPPA, A., BAULER, M., YEPURU, M., CHAUM, E., DALTON, J. T. & ESWARAKA, J. 2010. 17-beta estradiol protects ARPE-19 cells from oxidative stress through estrogen receptor-beta. *Invest Ophthalmol Vis Sci,* 51**,** 5278-87.

GIL-CARDOSO, K., GINES, I., PINENT, M., ARDEVOL, A., BLAY, M. & TERRA, X. 2016. Effects of flavonoids on intestinal inflammation, barrier integrity and changes in gut microbiota during diet-induced obesity. *Nutr Res Rev,* 29**,** 234-248.

GILAD, L. A., BRESLER, T., GNAINSKY, J., SMIRNOFF, P. & SCHWARTZ, B. 2005. Regulation of vitamin D receptor expression via estrogen-induced activation of the ERK 1/2 signaling pathway in colon and breast cancer cells. *J Endocrinol,* 185**,** 577-92.

GILANI, A. H., KHAN, A., GHAYUR, M. N., ALI, S. F. & HERZIG, J. W. 2006. Antispasmodic Effects of Rooibos Tea (*Aspalathus linearis)* is Mediated Predominantly through K+-Channel Activation. *Basic & Clinical Pharmacology & Toxicology* 99**,** 365–373.

GILLIGAN, L. C., GONDAL, A., TANG, V., HUSSAIN, M. T., ARVANITI, A., HEWITT, A. M. & FOSTER, P. A. 2017. Estrone Sulfate Transport and Steroid Sulfatase Activity in Colorectal Cancer: Implications for Hormone Replacement Therapy. *Front Pharmacol,* 8**,** 103.

GLOSSMANN, H. H. & LUTZ, O. M. D. 2017. Torpor: The Rise and Fall of 3-Monoiodothyronamine from Brain to Gut—From Gut to Brain? *Frontiers in Endocrinology,* 8**,** 118.

GONZALEZ-SARRIAS, A., ROMO-VAQUERO, M., GARCIA-VILLALBA, R., CORTES-MARTIN, A., SELMA, M. V. & ESPIN, J. C. 2018. The Endotoxemia Marker Lipopolysaccharide-Binding Protein is Reduced in Overweight-Obese Subjects Consuming Pomegranate Extract by Modulating the Gut Microbiota: A Randomized Clinical Trial. *Mol Nutr Food Res,* 62**,** e1800160.

GORINSKI, N., BIJATA, M., PRASAD, S., WIRTH, A., ABDEL GALIL, D., ZEUG, A., BAZOVKINA, D., KONDAUROVA, E., KULIKOVA, E., ILCHIBAEVA, T., ZAREBA-KOZIOL, M., PAPALEO, F., SCHEGGIA, D., KOCHLAMAZASHVILI, G., DITYATEV, A., SMYTH, I., KRZYSTYNIAK, A., WLODARCZYK, J., RICHTER, D. W., STREKALOVA, T., SIGRIST, S., BANG, C., HOBUSS, L., FIEDLER, J., THUM, T., NAUMENKO, V. S., PANDEY, G. & PONIMASKIN, E. 2019. Attenuated palmitoylation of serotonin receptor 5-HT1A affects receptor function and contributes to depression-like behaviors. *Nat Commun,* 10**,** 3924.

GRABAUSKAS, G., WU, X., GAO, J., LI, J.-Y., TURGEON, D. K. & OWYANG, C. 2020. Prostaglandin E2, Produced by Mast Cells in Colon Tissues From Patients With Irritable Bowel Syndrome, Contributes to Visceral Hypersensitivity in Mice. *Gastroenterology,* 158**,** 2195–2207.

GREUTER, T., MANSER, C., PITTET, V., VAVRICKA, S. R., BIEDERMANN, L. & ON BEHALF OF SWISS IBDNET, A. O. W. G. O. T. S. S. O. G. 2020. Gender Differences in Inflammatory Bowel Disease. *Digestion,* 101 Suppl 1**,** 98-104.

GRIMSBY, J., TOTH, M., CHEN, K., KUMAZAWA, T., KLAIDMAN, L., ADAMS, J. D., KAROUM, F., GAL, J. & SHIH, J. C. 1997. Increased stress response and β–phenylethylamine in MAOB–deficient mice. *Nature Genetics,* 17**,** 206-210.

GROSHEVA, I., ZHENG, D., LEVY, M., POLANSKY, O., LICHTENSTEIN, A., GOLANI, O., DORI-BACHASH, M., MORESI, C., SHAPIRO, H., DEL MARE-ROUMANI, S., VALDES-MAS, R., HE, Y., KARBI, H., CHEN, M., HARMELIN, A., STRAUSSMAN, R., YISSACHAR, N., ELINAV, E. & GEIGER, B. 2020. High-Throughput Screen Identifies Host and Microbiota Regulators of Intestinal Barrier Function. *Gastroenterology,* 159**,** 1807-1823.

GU, Y., ZHOU, G., QIN, X., HUANG, S., WANG, B. & CAO, H. 2019. The Potential Role of Gut Mycobiome in Irritable Bowel Syndrome. *Front Microbiol,* 10**,** 1894.

GUO, X., RAO, J. N., LIU, L., ZOU, T., KELEDJIAN, K. M., BONEVA, D., MARASA, B. S. & WANG, J. Y. 2005. Polyamines are necessary for synthesis and stability of occludin protein in intestinal epithelial cells. *Am J Physiol Gastrointest Liver Physiol,* 288**,** G1159-69.

GWILT, K. B., GONZALEZ, D. P., OLLIFFE, N., OLLER, H., HOFFING, R., PUZAN, M., EL AIDY, S. & MILLER, G. M. 2020. Actions of Trace Amines in the Brain-Gut-Microbiome Axis via Trace Amine-Associated Receptor-1 (TAAR1). *Cell Mol Neurobiol,* 40**,** 191-201.

HALBREICH, U. & KAHN, L. S. 2001. Role of Estrogen in the Aetiology and Treatment of Mood Disorders. *CNS Drugs,* 15**,** 797-817.

HALPERN, M. 2003. Structure and function of the vomeronasal system: an update. *Progress in Neurobiology,* 70**,** 245-318.

HAN, L., ZHAO, L., ZHOU, Y., YANG, C., XIONG, T., LU, L., DENG, Y., LUO, W., CHEN, Y., QIU, Q., SHANG, X., HUANG, L., MO, Z., HUANG, S., HUANG, S., LIU, Z., YANG, W., ZHAI, L., NING, Z., LIN, C., HUANG, T., CHENG, C., ZHONG, L. L. D., LI, S., BIAN, Z. & FANG, X. 2021. Altered metabolome and microbiome features provide clues in understanding irritable bowel syndrome and depression comorbidity. *ISME J*.

HANNING, N., EDWINSON, A. L., CEULEERS, H., PETERS, S. A., DE MAN, J. G., HASSETT, L. C., DE WINTER, B. Y. & GROVER, M. 2021. Intestinal barrier dysfunction in irritable bowel syndrome: a systematic review. *Therap Adv Gastroenterol,* 14**,** 1756284821993586.

HANYANG, L., XUANZHE, L., XUYANG, C., YUJIA, Q., JIARONG, F., JUN, S. & ZHIHUA, R. 2017. Application of Zebrafish Models in Inflammatory Bowel Disease. *Front Immunol,* 8**,** 501.

HARNISH, D. C., ALBERT, L. M., LEATHURBY, Y., ECKERT, A. M., CIARLETTA, A., KASAIAN, M. & KEITH, J. C. 2004. Beneficial effects of estrogen treatment in the HLA-B27 transgenic rat model of inflammatory bowel disease. *Am J Physiol Gastrointest Liver Physiol* G118–G125.

HARRIS, H. A. 2007. Estrogen receptor-beta: recent lessons from in vivo studies. *Mol Endocrinol,* 21**,** 1-13.

HEINKEN, A., KHAN, M. T., PAGLIA, G., RODIONOV, D. A., HARMSEN, H. J. & THIELE, I. 2014. Functional metabolic map of Faecalibacterium prausnitzii, a beneficial human gut microbe. *J Bacteriol,* 196**,** 3289-302.

HEITKEMPER, M., CAIN, K. C., JARRETT, M. E., BURR, R. L., HERTIG, V. & BOND, E. F. 2003. Symptoms across the menstrual cycle in women with irritable bowel syndrome. *The American Journal of Gastroenterology,* 98**,** 420-430.

HEITKEMPER, M., CARTER, E. & AMEEN, V. 2002. Women with irritable bowel syndrome: differences in patients' and physicians' perceptions. . *Gastroenterol Nurs,* 25**,** 192-200.

HEITKEMPER, M. & JARRETT, M. 2008. Irritable bowel syndrome: does gender matter? *J Psychosom Res,* 64**,** 583-7.

HEITKEMPER, M. M. & JARRETT, M. 1992. Patterns of gastrointestinal and somatic symptoms across the menstrual cycle. *Gastroenterology,* 102**,** 505-513.

HILL, C., GUARNER, F., REID, G., GIBSON, G. R., MERENSTEIN, D. J., POT, B., MORELLI, L., CANANI, R. B., FLINT, H. J., SALMINEN, S., CALDER, P. C. & SANDERS, M. E. 2014. Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat Rev Gastroenterol Hepatol,* 11**,** 506-14.

HIMPE, E., CUNHA, D. A., SONG, I., BUGLIANI, M., MARCHETTI, P., CNOP, M. & BOUWENS, L. 2016. Phenylpropenoic Acid Glucoside from Rooibos Protects Pancreatic Beta Cells against Cell Death Induced by Acute Injury. *PLoS One,* 11**,** e0157604.

HOEFIG, C. S., WUENSCH, T., RIJNTJES, E., LEHMPHUL, I., DANIEL, H., SCHWEIZER, U., MITTAG, J. & KOHRLE, J. 2015. Biosynthesis of 3-Iodothyronamine From T4 in Murine Intestinal Tissue. *Endocrinology,* 156**,** 4356-64.

HOEFIG, C. S., ZUCCHI, R. & KOHRLE, J. 2016. Thyronamines and Derivatives: Physiological Relevance, Pharmacological Actions, and Future Research Directions. *THYROID,* 26**,** 1656-1672.

HONDA, J., IIJIMA, K., ASANUMA, K., ARA, N., SHIROKI, T., KONDO, Y., HATTA, W., UNO, K., ASANO, N., KOIKE, T. & SHIMOSEGAWA, T. 2016. Estrogen Enhances Esophageal Barrier Function by Potentiating Occludin Expression. *Dig Dis Sci,* 61**,** 1028-38.

HOOSEN, M. 2019. The Effects of Aspalathus linearis (Rooibos Tea) on Nitric Oxide (NO) and Cytokine Activity. *International Journal of Human and Health Sciences (IJHHS),* 3.

HORVATH, A., DZIECHCIARZ, P. & SZAJEWSKA, H. 2011. Meta-analysis: Lactobacillus rhamnosus GG for abdominal pain-related functional gastrointestinal disorders in childhood. *Aliment Pharmacol Ther,* 33**,** 1302-10.

HOYLES, L., JIMÉNEZ-PRANTEDA, M. L., CHILLOUX, J., BRIAL, F., MYRIDAKIS, A., ARANIAS, T., MAGNAN, C., GIBSON, G. R., SANDERSON, J. D., NICHOLSON, J. K., GAUGUIER, D., MCCARTNEY, A. L. & DUMAS, M.-E. 2018. Metabolic retroconversion of trimethylamine N-oxide and the gut microbiota. *Microbiome,* 6**,** 73.

HU, Z., LI, M., YAO, L., WANG, Y., WANG, E., YUAN, J., WANG, F., YANG, K., BIAN, Z. & ZHONG, L. L. D. 2021. The level and prevalence of depression and anxiety among patients with different subtypes of irritable bowel syndrome: a network meta-analysis. *BMC Gastroenterol,* 21**,** 23.

ISLAM, J., SATO, S., WATANABE, K., WATANABE, T., ARDIANSYAH, HIRAHARA, K., AOYAMA, Y., TOMITA, S., ASO, H., KOMAI, M. & SHIRAKAWA, H. 2017. Dietary tryptophan alleviates dextran sodium sulfate-induced colitis through aryl hydrocarbon receptor in mice. *J Nutr Biochem,* 42**,** 43-50.

ISWALDI, I., ARRAEZ-ROMAN, D., RODRIGUEZ-MEDINA, I., BELTRAN-DEBON, R., JOVEN, J., SEGURA-CARRETERO, A. & FERNANDEZ-GUTIERREZ, A. 2011. Identification of phenolic compounds in aqueous and ethanolic rooibos extracts (Aspalathus linearis) by HPLC-ESI-MS (TOF/IT). *Anal Bioanal Chem,* 400**,** 3643-54.

JACENIK, D., ZIELINSKA, M., MOKROWIECKA, A., MICHLEWSKA, S., MALECKA-PANAS, E., KORDEK, R., FICHNA, J. & KRAJEWSKA, W. M. 2019. G protein-coupled estrogen receptor mediates anti-inflammatory action in Crohn's disease. *Sci Rep,* 9**,** 6749.

JACOBS, J. P., GOUDARZI, M., SINGH, N., TONG, M., MCHARDY, I. H., RUEGGER, P., ASADOURIAN, M., MOON, B. H., AYSON, A., BORNEMAN, J., MCGOVERN, D. P., FORNACE, A. J., JR., BRAUN, J. & DUBINSKY, M. 2016. A Disease-Associated Microbial and Metabolomics State in Relatives of Pediatric Inflammatory Bowel Disease Patients. *Cell Mol Gastroenterol Hepatol,* 2**,** 750-766.

JANE, Z. Y., CHANG, C. C., LIN, H. K., LIU, Y. C. & CHEN, W. L. 2011. The association between the exacerbation of irritable bowel syndrome and menstrual symptoms in young Taiwanese women. *Gastroenterol Nurs,* 34**,** 277-86.

JEFFERY, I. B., DAS, A., O'HERLIHY, E., COUGHLAN, S., CISEK, K., MOORE, M., BRADLEY, F., CARTY, T., PRADHAN, M., DWIBEDI, C., SHANAHAN, F. & O'TOOLE, P. W. 2020. Differences in Fecal Microbiomes and Metabolomes of People With vs Without Irritable Bowel Syndrome and Bile Acid Malabsorption. *Gastroenterology,* 158**,** 1016-1028 e8.

JEFFERY, I. B., O'TOOLE, P. W., OHMAN, L., CLAESSON, M. J., DEANE, J., QUIGLEY, E. M. & SIMREN, M. 2012. An irritable bowel syndrome subtype defined by species-specific alterations in faecal microbiota. *Gut,* 61**,** 997-1006.

JOOSTEN, H. M. L. J. & NORTHOLT, M. D. 1989. Detection, Growth, and Amine-Producing Capacity of Lactobacilli in Cheese. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY,* 55**,** 2356-2359.

JORANDLI, J. W., THORSVIK, S., SKOVDAHL, H. K., KORNFELD, B., SAETERSTAD, S., GUSTAFSSON, B. I., SANDVIK, A. K. & VAN BEELEN GRANLUND, A. 2020. The serotonin reuptake transporter is reduced in the epithelium of active Crohn's disease and ulcerative colitis. *Am J Physiol Gastrointest Liver Physiol,* 319**,** G761-G768.

JOUBERT, E., BEELDERS, T., DE BEER, D., MALHERBE, C. J., DE VILLIERS, A. J. & SIGGE, G. O. 2012. Variation in phenolic content and antioxidant activity of fermented rooibos herbal tea infusions: role of production season and quality grade. *J Agric Food Chem,* 60**,** 9171-9.

JOUBERT, E. & DE BEER, D. 2011. Rooibos (Aspalathus linearis) beyond the farm gate: From herbal tea to potential phytopharmaceutical. *South African Journal of Botany,* 77**,** 869-886.

JOUBERT, E., WINTERTON, P., BRITZ, T. J. & GELDERBLOM, W. C. A. 2005. Antioxidant and Pro-oxidant Activities of Aqueous Extracts and Crude Polyphenolic Fractions of Rooibos (Aspalathus linearis). *J. Agric. Food Chem. ,* 53**,** 10260−10267.

KALNINS, G., KUKA, J., GRINBERGA, S., MAKRECKA-KUKA, M., LIEPINSH, E., DAMBROVA, M. & TARS, K. 2015. Structure and Function of CutC Choline Lyase from Human Microbiota Bacterium Klebsiella pneumoniae. *J Biol Chem,* 290**,** 21732-40.

KAMADA, N. & ROGLER, G. 2016. The Innate Immune System: A Trigger for Many Chronic Inflammatory Intestinal Diseases. *Inflamm Intest Dis,* 1**,** 70-77.

KANE, S. V., SABLE, K. & HANAUER, S. B. 1998. The menstrual cycle and its effect on inflammatory bowel disease and irritable bowel syndrome: a prevalence study. *Am J Gastroenterol,* 93**,** 1867-1872.

KANO, M., MURATSUBAKI, T., VAN OUDENHOVE, L., MORISHITA, J., YOSHIZAWA, M., KOHNO, K., YAGIHASHI, M., TANAKA, Y., MUGIKURA, S., DUPONT, P., LY, H. G., TAKASE, K., KANAZAWA, M. & FUKUDO, S. 2017. Altered brain and gut responses to corticotropin-releasing hormone (CRH) in patients with irritable bowel syndrome. *Sci Rep,* 7**,** 12425.

KELESIDIS, T. & POTHOULAKIS, C. 2012. Efficacy and safety of the probiotic Saccharomyces boulardii for the prevention and therapy of gastrointestinal disorders. *Therap Adv Gastroenterol,* 5**,** 111-25.

KELLY, J. R., KENNEDY, P. J., CRYAN, J. F., DINAN, T. G., CLARKE, G. & HYLAND, N. P. 2015. Breaking down the barriers: the gut microbiome, intestinal permeability and stress-related psychiatric disorders. *Front Cell Neurosci,* 9**,** 392.

KENNEDY, P. J., CRYAN, J. F., DINAN, T. G. & CLARKE, G. 2014a. Irritable bowel syndrome: a microbiome-gut-brain axis disorder? *World J Gastroenterol,* 20**,** 14105-25.

KENNEDY, P. J., CRYAN, J. F., QUIGLEY, E. M., DINAN, T. G. & CLARKE, G. 2014b. A sustained hypothalamic-pituitary-adrenal axis response to acute psychosocial stress in irritable bowel syndrome. *Psychol Med,* 44**,** 3123-34.

KERLIN, P. & WONG, L. 1988. Breath hydrogen testing in bacterial overgrowth of the small intestine. *Gastroenterology,* 95**,** 982-988.

KHAN, I., SAEED, K., JO, M. G. & KIM, M. O. 2021. 17-beta Estradiol Rescued Immature Rat Brain against Glutamate-Induced Oxidative Stress and Neurodegeneration via Regulating Nrf2/HO-1 and MAP-Kinase Signaling Pathway. *Antioxidants (Basel),* 10.

KIM, D.-Y. & CAMILLERI, M. 2000. Serotonin: A Mediator of The Brain–Gut Connection. *American Journal of Gastroenterology,* 95**,** 2698-2709.

KIM, H. J., VAZQUEZ ROQUE, M. I., CAMILLERI, M., STEPHENS, D., BURTON, D. D., BAXTER, K., THOMFORDE, G. & ZINSMEISTER, A. R. 2005. A randomized controlled trial of a probiotic combination VSL# 3 and placebo in irritable bowel syndrome with bloating. *Neurogastroenterol Motil,* 17**,** 687-96.

KIM, Y. S. & KIM, N. 2018. Sex-Gender Differences in Irritable Bowel Syndrome. *J Neurogastroenterol Motil,* 24**,** 544-558.

KIRSTEN, K., SOARES, S. M., KOAKOSKI, G., CARLOS KREUTZ, L. & BARCELLOS, L. J. G. 2018. Characterization of sickness behavior in zebrafish. *Brain Behav Immun,* 73**,** 596-602.

KLEM, F., WADHWA, A., PROKOP, L. J., SUNDT, W. J., FARRUGIA, G., CAMILLERI, M., SINGH, S. & GROVER, M. 2017. Prevalence, Risk Factors, and Outcomes of Irritable Bowel Syndrome After Infectious Enteritis: A Systematic Review and Meta-analysis. *Gastroenterology,* 152**,** 1042-1054 e1.

KOH, A. & BACKHED, F. 2020. From Association to Causality: the Role of the Gut Microbiota and Its Functional Products on Host Metabolism. *Mol Cell,* 78**,** 584-596.

KOH, A., DE VADDER, F., KOVATCHEVA-DATCHARY, P. & BACKHED, F. 2016. From Dietary Fiber to Host Physiology: Short-Chain Fatty Acids as Key Bacterial Metabolites. *Cell,* 165**,** 1332-1345.

KOPPEL, N., MAINI REKDAL, V. & BALSKUS, E. P. 2017. Chemical transformation of xenobiotics by the human gut microbiota. *Science,* 356.

KRAFCZYK, N. & GLOMB, M. A. 2008. Characterization of Phenolic Compounds in Rooibos Tea. *J. Agric. Food Chem.,* 56**,** 3368–3376.

KREUZ, S., JOUBERT, E., WALDMANN, K. H. & TERNES, W. 2008. Aspalathin, a flavonoid in Aspalathus linearis (rooibos), is absorbed by pig intestine as a C-glycoside. *Nutr Res,* 28**,** 690-701.

KUGAYA, A., EPPERSON, C. N., ZOGHBI, S., VAN DYCK, C. H., HOU, Y., FUJITA, M., STALEY, J. K., GARG, P. K., SEIBYL, J. P. & INNIS, R. B. 2003. Increase in Prefrontal Cortex Serotonin2A Receptors Following Estrogen Treatment in Postmenopausal Women. *Am J Psychiatry,* 160**,** 1522-1524.

KUMAR, S., LATA, K., MUKHOPADHYAY, S. & MUKHERJEE, T. K. 2010a. Role of estrogen receptors in pro-oxidative and anti-oxidative actions of estrogens: a perspective. *Biochim Biophys Acta,* 1800**,** 1127-35.

KUMAR, S., MISRA, A. & GHOSHAL, U. C. 2010b. Patients with irritable bowel syndrome exhale more hydrogen than healthy subjects in fasting state. *J Neurogastroenterol Motil,* 16**,** 299-305.

KUO, W. T., SHEN, L., ZUO, L., SHASHIKANTH, N., ONG, M., WU, L., ZHA, J., EDELBLUM, K. L., WANG, Y., WANG, Y., NILSEN, S. P. & TURNER, J. R. 2019. Inflammation-induced Occludin Downregulation Limits Epithelial Apoptosis by Suppressing Caspase-3 Expression. *Gastroenterology,* 157**,** 1323-1337.

KÜPELI, E., ASLAN, M., GÜRBÜZ, I. & YESILADA, E. 2004. Evaluation of in vivo Biological Activity Profile of Isoorientin. *Z. Naturforsch.,* 59c**,** 787-790.

KWA, M., PLOTTEL, C. S., BLASER, M. J. & ADAMS, S. 2016. The Intestinal Microbiome and Estrogen Receptor-Positive Female Breast Cancer. *J Natl Cancer Inst,* 108.

LA GIOIA, F., RIZZOTTI, L., ROSSI, F., GARDINI, F., TABANELLI, G. & TORRIANI, S. 2011. Identification of a tyrosine decarboxylase gene (tdcA) in Streptococcus thermophilus 1TT45 and analysis of its expression and tyramine production in milk. *Appl Environ Microbiol,* 77**,** 1140-4.

LACY, B. E. & PATEL, N. K. 2017. Rome Criteria and a Diagnostic Approach to Irritable Bowel Syndrome. *J Clin Med,* 6.

LAUWERYNS, J. M. & VAN RANST, L. 1998. Immunocytochemical localization of aromatic L-amino acid decarboxylase in human, rat, and mouse bronchopulmonary and gastrointestinal endocrine cells. *J. Histochem. Cytochem.,* 36**,** 1181-1186.

LAVELLE, A. & SOKOL, H. 2020. Gut microbiota-derived metabolites as key actors in inflammatory bowel disease. *Nat Rev Gastroenterol Hepatol,* 17**,** 223-237.

LAZARIDIS, N. & GERMANIDIS, G. 2018. Current insights into the innate immune system dysfunction in irritable bowel syndrome. *Ann Gastroenterol,* 31**,** 171-187.

LEE, B.-S., YANG, S., LEE, C., KU, S.-K. & BAE, J.-S. 2020a. Renal protective effects of vicenin-2 and scolymoside in a mouse model of sepsis. *Brazilian Journal of Pharmaceutical Sciences,* 56.

LEE, B. J. & BAK, Y. T. 2011. Irritable bowel syndrome, gut microbiota and probiotics. *J Neurogastroenterol Motil,* 17**,** 252-66.

LEE, H., PARK, J. H., PARK, D. I., KIM, H. J., CHO, Y. K., SOHN, C. I., JEON, W. K., KIM, B. I. & CHAE, S. W. 2013. Mucosal mast cell count is associated with intestinal permeability in patients with diarrhea predominant irritable bowel syndrome. *J Neurogastroenterol Motil,* 19**,** 244-50.

LEE, J. S., KIM, S. Y., CHUN, Y. S., CHUN, Y. J., SHIN, S. Y., CHOI, C. H. & CHOI, H. K. 2020b. Characteristics of fecal metabolic profiles in patients with irritable bowel syndrome with predominant diarrhea investigated using (1) H-NMR coupled with multivariate statistical analysis. *Neurogastroenterol Motil,* 32**,** e13830.

LEE, J. Y., KIM, N., PARK, J. H., NAM, R. H., LEE, S. M., SONG, C. H., KIM, G., NA, H. Y., CHOI, Y. J., KIM, J. J. & LEE, D. H. 2020c. Expression of Neurotrophic Factors, Tight Junction Proteins, and Cytokines According to the Irritable Bowel Syndrome Subtype and Sex. *J Neurogastroenterol Motil,* 26**,** 106-116.

LEE, K. N. & LEE, O. Y. 2016. The Role of Mast Cells in Irritable Bowel Syndrome. *Gastroenterol Res Pract,* 2016**,** 2031480.

LEI, M. G., TAN, X., QURESHI, N. & MORRISON, D. C. 2005. Regulation of cellular caveolin-1 protein expression in murine macrophages by microbial products. *Infect Immun,* 73**,** 8136-43.

LI, Q., ZHANG, Q., ZHANG, M., WANG, C., ZHU, Z., LI, N. & LI, J. 2008. Effect of n-3 polyunsaturated fatty acids on membrane microdomain localization of tight junction proteins in experimental colitis. *FEBS J,* 275**,** 411-20.

LI, Y., ZHENG, Y. & WANG, H. 2021a. Anticancer activity of Vicenin-2 against 7,12 dimethylbenz[a]anthracene-induced buccal pouch carcinoma in hamsters. *J Biochem Mol Toxicol,* 35**,** e22673.

LI, Z., ZHU, G., LI, C., LAI, H., LIU, X. & ZHANG, L. 2021b. Which Probiotic Is the Most Effective for Treating Acute Diarrhea in Children? A Bayesian Network Meta-Analysis of Randomized Controlled Trials. *Nutrients,* 13.

LIBERLES, S. D. & BUCK, L. B. 2006. A second class of chemosensory receptors in the olfactory epithelium. *Nature,* 442**,** 645-50.

LIM, J. S., NA, H. S., LEE, H. C., CHOY, H. E., PARK, S. C., HAN, J. M. & CHO, K. A. 2009. Caveolae-mediated entry of Salmonella typhimurium in a human M-cell model. *Biochem Biophys Res Commun,* 390**,** 1322-7.

LIN, R., PIAO, M. & SONG, Y. 2019. Dietary Quercetin Increases Colonic Microbial Diversity and Attenuates Colitis Severity in Citrobacter rodentium-Infected Mice. *Front Microbiol,* 10**,** 1092.

LINARES, D. M., DEL RIO, B., REDRUELLO, B., LADERO, V., MARTIN, M. C., FERNANDEZ, M., RUAS-MADIEDO, P. & ALVAREZ, M. A. 2016. Comparative analysis of the in vitro cytotoxicity of the dietary biogenic amines tyramine and histamine. *Food Chem,* 197**,** 658-63.

LINARES, D. M., FERNANDEZ, M., MARTIN, M. C. & ALVAREZ, M. A. 2009. Tyramine biosynthesis in Enterococcus durans is transcriptionally regulated by the extracellular pH and tyrosine concentration. *Microb Biotechnol,* 2**,** 625-33.

LINARES, D. M., MARTIN, M. C., LADERO, V., ALVAREZ, M. A. & FERNANDEZ, M. 2011. Biogenic amines in dairy products. *Crit Rev Food Sci Nutr,* 51**,** 691-703.

LINDEMANN, L., EBELING, M., KRATOCHWIL, N. A., BUNZOW, J. R., GRANDY, D. K. & HOENER, M. C. 2005. Trace amine-associated receptors form structurally and functionally distinct subfamilies of novel G protein-coupled receptors. *Genomics,* 85**,** 372-85.

LINDEMANN, L. & HOENER, M. C. 2005. A renaissance in trace amines inspired by a novel GPCR family. *Trends Pharmacol Sci,* 26**,** 274-81.

LIU, B., JIANG, X., CAI, L., ZHAO, X., DAI, Z., WU, G. & LI, X. 2019. Putrescine mitigates intestinal atrophy through suppressing inflammatory response in weanling piglets. *J Anim Sci Biotechnol,* 10**,** 69.

LOBIONDA, S., SITTIPO, P., KWON, H. Y. & LEE, Y. K. 2019. The Role of Gut Microbiota in Intestinal Inflammation with Respect to Diet and Extrinsic Stressors. *Microorganisms,* 7.

LOOIJER-VAN LANGEN, M., HOTTE, N., DIELEMAN, L. A., ALBERT, E., MULDER, C. & MADSEN, K. L. 2011. Estrogen receptor-beta signaling modulates epithelial barrier function. *Am J Physiol Gastrointest Liver Physiol,* 300**,** G621-6.

LOPEZ, V., CASEDAS, G., PETERSEN-ROSS, K., POWRIE, Y. & SMITH, C. 2022. Neuroprotective and anxiolytic potential of green rooibos (Aspalathus linearis) polyphenolic extract. *Food Funct,* 13**,** 91-101.

LOPEZ-SILES, M., DUNCAN, S. H., GARCIA-GIL, L. J. & MARTINEZ-MEDINA, M. 2017. Faecalibacterium prausnitzii: from microbiology to diagnostics and prognostics. *ISME J,* 11**,** 841-852.

LORENCOVÁ, E., BUŇKOVÁ, L., MATOULKOVÁ, D., DRÁB, V., PLEVA, P., KUBÁŇ, V. & BUŇKA, F. 2012. Production of biogenic amines by lactic acid bacteria and bifidobacteria isolated from dairy products and beer. *International Journal of Food Science and Technology,* 47**,** 2086-2091.

LOZUPONE, C. A., STOMBAUGH, J., GONZALEZ, A., ACKERMANN, G., WENDEL, D., VAZQUEZ-BAEZA, Y., JANSSON, J. K., GORDON, J. I. & KNIGHT, R. 2013. Meta-analyses of studies of the human microbiota. *Genome Res,* 23**,** 1704-14.

LUCA, S. V., MACOVEI, I., BUJOR, A., MIRON, A., SKALICKA-WOZNIAK, K., APROTOSOAIE, A. C. & TRIFAN, A. 2020. Bioactivity of dietary polyphenols: The role of metabolites. *Crit Rev Food Sci Nutr,* 60**,** 626-659.

LUCAS, P. M., WOLKEN, W. A., CLAISSE, O., LOLKEMA, J. S. & LONVAUD-FUNEL, A. 2005. Histamine-producing pathway encoded on an unstable plasmid in Lactobacillus hilgardii 0006. *Appl Environ Microbiol,* 71**,** 1417-24.

LUNDGREN, O. 1998. 5-Hydroxytryptamine, enterotoxins, and intestinal fluid secretion. . *Gastroenterology,* 115**,** 1009–1012.

LUQMAN, A., NEGA, M., NGUYEN, M. T., EBNER, P. & GOTZ, F. 2018. SadA-Expressing Staphylococci in the Human Gut Show Increased Cell Adherence and Internalization. *Cell Rep,* 22**,** 535-545.

LUVIAN-MORALES, J., VARELA-CASTILLO, F. O., FLORES-CISNEROS, L., CETINA-PEREZ, L. & CASTRO-EGUILUZ, D. 2021. Functional foods modulating inflammation and metabolism in chronic diseases: a systematic review. *Crit Rev Food Sci Nutr*, 1-22.

LYU, M., WANG, Y. F., FAN, G. W., WANG, X. Y., XU, S. Y. & ZHU, Y. 2017. Balancing Herbal Medicine and Functional Food for Prevention and Treatment of Cardiometabolic Diseases through Modulating Gut Microbiota. *Front Microbiol,* 8**,** 2146.

MACHADO, F. S., RODRIGUEZ, N. E., ADESSE, D., GARZONI, L. R., ESPER, L., LISANTI, M. P., BURK, R. D., ALBANESE, C., VAN DOORSLAER, K., WEISS, L. M., NAGAJYOTHI, F., NOSANCHUK, J. D., WILSON, M. E. & TANOWITZ, H. B. 2012. Recent developments in the interactions between caveolin and pathogens. *Adv Exp Med Biol,* 729**,** 65-82.

MAHOMOODALLY, M. F. 2013. Traditional medicines in Africa: an appraisal of ten potent african medicinal plants. *Evid Based Complement Alternat Med,* 2013**,** 617459.

MAHURKAR, S., POLYTARCHOU, C., ILIOPOULOS, D., POTHOULAKIS, C., MAYER, E. A. & CHANG, L. 2016. Genome-wide DNA methylation profiling of peripheral blood mononuclear cells in irritable bowel syndrome. *Neurogastroenterol Motil,* 28**,** 410-22.

MAKKER, J., CHILIMURI, S. & BELLA, J. N. 2015. Genetic epidemiology of irritable bowel syndrome. *World J Gastroenterol,* 21**,** 11353-61.

MALONGANE, F., MCGAW, L. J., NYONI, H. & MUDAU, F. N. 2018. Metabolic profiling of four South African herbal teas using high resolution liquid chromatography-mass spectrometry and nuclear magnetic resonance. *Food Chem,* 257**,** 90-100.

MANGWANA, N. 2020. *The in vitro faecal evaluation of prebiotic effects of rooibos phenolic compounds on the gut microbiota of vervet monkeys (Chlorocebus pygerythrus).* Masters degree, Cape Peninsula University of Technology.

MARCHIANDO, A. M., SHEN, L., GRAHAM, W. V., WEBER, C. R., SCHWARZ, B. T., AUSTIN, J. R., 2ND, RALEIGH, D. R., GUAN, Y., WATSON, A. J., MONTROSE, M. H. & TURNER, J. R. 2010. Caveolin-1-dependent occludin endocytosis is required for TNF-induced tight junction regulation in vivo. *J Cell Biol,* 189**,** 111-26.

MARCINKIEWCZ, C. A., MAZZONE, C. M., D'AGOSTINO, G., HALLADAY, L. R., HARDAWAY, J. A., DIBERTO, J. F., NAVARRO, M., BURNHAM, N., CRISTIANO, C., DORRIER, C. E., TIPTON, G. J., RAMAKRISHNAN, C., KOZICZ, T., DEISSEROTH, K., THIELE, T. E., MCELLIGOTT, Z. A., HOLMES, A., HEISLER, L. K. & KASH, T. L. 2016. Serotonin engages an anxiety and fear-promoting circuit in the extended amygdala. *Nature,* 537**,** 97-101.

MARCOBAL, A., DE LAS RIVAS, B., LANDETE, J. M., TABERA, L. & MUÑOZ, R. 2012. Tyramine and Phenylethylamine Biosynthesis by Food Bacteria. *Critical Reviews in Food Science and Nutrition,* 52**,** 448-467.

MARCOBAL, A., DE LAS RIVAS, B. & MUNOZ, R. 2006a. First genetic characterization of a bacterial beta-phenylethylamine biosynthetic enzyme in Enterococcus faecium RM58. *FEMS Microbiol Lett,* 258**,** 144-9.

MARCOBAL, A., KASHYAP, P. C., NELSON, T. A., ARONOV, P. A., DONIA, M. S., SPORMANN, A., FISCHBACH, M. A. & SONNENBURG, J. L. 2013. A metabolomic view of how the human gut microbiota impacts the host metabolome using humanized and gnotobiotic mice. *ISME J,* 7**,** 1933-43.

MARCOBAL, A., MARTIN-ALVAREZ, P. J., MORENO-ARRIBAS, M. V. & MUNOZ, R. 2006b. A multifactorial design for studying factors influencing growth and tyramine production of the lactic acid bacteria Lactobacillus brevis CECT 4669 and Enterococcus faecium BIFI-58. *Res Microbiol,* 157**,** 417-24.

MARNEWICK, J. L. 2014. Antioxidant Properties of Rooibos (Aspalathus linearis) – In Vitro and in Vivo Evidence. *Systems Biology of Free Radicals and Antioxidants.*

MARNEWICK, J. L., RAUTENBACH, F., VENTER, I., NEETHLING, H., BLACKHURST, D. M., WOLMARANS, P. & MACHARIA, M. 2011. Effects of rooibos (Aspalathus linearis) on oxidative stress and biochemical parameters in adults at risk for cardiovascular disease. *J Ethnopharmacol,* 133**,** 46-52.

MARTINIS, P., GRANCARA, S., KANAMORI, Y., GARCIA-ARGAEZ, A. N., PACELLA, E., DALLA VIA, L., TONINELLO, A. & AGOSTINELLI, E. 2020. Involvement of the biogenic active amine agmatine in mitochondrial membrane permeabilization and release of pro-apoptotic factors. *Amino Acids,* 52**,** 161-169.

MATSUMOTO, M., KIBE, R., OOGA, T., AIBA, Y., SAWAKI, E., KOGA, Y. & BENNO, Y. 2013. Cerebral low¬molecular metabolites influenced by intestinal microbiota: a pilot study. . *Front Syst Neurosci* 7**,** 9.

MAVRANGELOS, C., CAMPANIELLO, M. A., ANDREWS, J. M., BAMPTON, P. A. & HUGHES, P. A. 2017. Longitudinal analysis indicates symptom severity influences immune profile in irritable bowel syndrome. *Gut,* 67**,** 398-9.

MAWE, G. M., COATES, M. D. & MOSES, P. L. 2006. Review article: intestinal serotonin signalling in irritable bowel syndrome. *Aliment Pharmacol Ther,* 23**,** 1067-76.

MAWE, G. M. & HOFFMAN, J. M. 2013. Serotonin signalling in the gut–func- tions, dysfunctions and therapeutic targets. *Nat. Rev. Gastroenterol. Hepatol. ,* 10**,** 473-486.

MAYER, E. A. 2011. Gut feelings: the emerging biology of gut-brain communication. *Nat. Rev. Neurosci.,* 12**,** 453-466.

MAYER, E. A., SAVIDGE, T. & SHULMAN, R. J. 2014. Brain-gut microbiome interactions and functional bowel disorders. *Gastroenterology,* 146**,** 1500-12.

MAYEUR, C., VEUILLET, G., MICHAUD, M., RAUL, F., BLOTTIERE, H. M. & BLACHIER, F. 2005. Effects of agmatine accumulation in human colon carcinoma cells on polyamine metabolism, DNA synthesis and the cell cycle. *Biochim Biophys Acta,* 1745**,** 111-23.

MCCABE-SELLERS, B. J., STAGGS, C. G. & BOGLE, M. L. 2006. Tyramine in foods and monoamine oxidase inhibitor drugs: A crossroad where medicine, nutrition, pharmacy, and food industry converge. *Journal of Food Composition and Analysis,* 19**,** S58-S65.

MCFARLAND, L. V. 2010. Systematic review and meta-analysis of Saccharomyces boulardii in adult patients. *World J Gastroenterol,* 16**,** 2202-22.

MELEINE, M. & MATRICON, J. 2014. Gender-related differences in irritable bowel syndrome: potential mechanisms of sex hormones. *World J Gastroenterol,* 20**,** 6725-43.

METE, R., TULUBAS, F., ORAN, M., YILMAZ, A., AVCI, B. A., YILDIZ, K., TURAN, C. B. & GUREL, A. 2013. The role of oxidants and reactive nitrogen species in irritable bowel syndrome: a potential etiological explanation. *Med Sci Monit,* 19**,** 762-6.

MIJAN, M. A. & LIM, B. O. 2018. Diets, functional foods, and nutraceuticals as alternative therapies for inflammatory bowel disease: Present status and future trends. *World J Gastroenterol,* 24**,** 2673-2685.

MIKOCKA-WALUS, A., KNOWLES, S. R., KEEFER, L. & GRAFF, L. 2016. Controversies Revisited: A Systematic Review of the Comorbidity of Depression and Anxiety with Inflammatory Bowel Diseases. *Inflamm Bowel Dis,* 22**,** 752-62.

MILLER, G. M. 2011. The emerging role of trace amine-associated receptor 1 in the functional regulation of monoamine transporters and dopaminergic activity. *J Neurochem,* 116**,** 164-76.

MILLICHAP, J. G. & YEE, M. M. 2003. The diet factor in pediatric and adolescent migraine. *Pediatric Neurology,* 28**,** 9-15.

MILOSEVIC, K., STEVANOVIC, I., BOZIC, I. D., MILOSEVIC, A., JANJIC, M. M., LAKETA, D., BJELOBABA, I., LAVRNJA, I. & SAVIC, D. 2022. Agmatine Mitigates Inflammation-Related Oxidative Stress in BV-2 Cells by Inducing a Pre-Adaptive Response. *Int J Mol Sci,* 23.

MILUTINOVIC, M., DIMITRIJEVIC-BRANKOVIC, S. & RAJILIC-STOJANOVIC, M. 2021. Plant Extracts Rich in Polyphenols as Potent Modulators in the Growth of Probiotic and Pathogenic Intestinal Microorganisms. *Front Nutr,* 8**,** 688843.

MIQUEL, S., LECLERC, M., MARTIN, R., CHAIN, F., LENOIR, M., RAGUIDEAU, S., HUDAULT, S., BRIDONNEAU, C., NORTHEN, T., BOWEN, B., BERMUDEZ-HUMARAN, L. G., SOKOL, H., THOMAS, M. & LANGELLA, P. 2015. Identification of metabolic signatures linked to anti-inflammatory effects of Faecalibacterium prausnitzii. *mBio,* 6.

MIRONCZUK-CHODAKOWSKA, I., WITKOWSKA, A. M. & ZUJKO, M. E. 2018. Endogenous non-enzymatic antioxidants in the human body. *Adv Med Sci,* 63**,** 68-78.

MITTAL, M., SIDDIQUI, M. R., TRAN, K., REDDY, S. P. & MALIK, A. B. 2014. Reactive oxygen species in inflammation and tissue injury. *Antioxid Redox Signal,* 20**,** 1126-67.

MOAYYEDI, P., YUAN, Y., BAHARITH, H. & FORD, A. C. 2017. Faecal microbiota transplantation for Clostridium difficile-associated diarrhoea: a systematic review of randomised controlled trials. . *Med J Aust,* 207**,** 166-172.

MOBLEY, J. A. & BRUEGGEMEIER, R. W. 2004. Estrogen receptor-mediated regulation of oxidative stress and DNA damage in breast cancer. *Carcinogenesis,* 25**,** 3-9.

MODOUX, M., ROLHION, N., MANI, S. & SOKOL, H. 2021. Tryptophan Metabolism as a Pharmacological Target. *Trends Pharmacol Sci,* 42**,** 60-73.

MOISES, H. W., WALDMEIER, P. & BECKMANN, H. 1985. Phenethylamine and Personality. *In:* BOULTON, A. A., BIECK, P. R., MAITRE, L. & RIEDERER, P. (eds.) *Neuropsychopharmacology of the trace amines.* Clifton, New Jersey: Humana Press.

MONSBAKKEN, K. W., VANDVIK, P. O. & FARUP, P. G. 2006. Perceived food intolerance in subjects with irritable bowel syndrome-- etiology, prevalence and consequences. *Eur J Clin Nutr,* 60**,** 667-72.

MONSEES, T. K. & OPUWARI, C. S. 2017. Effect of rooibos (Aspalathus linearis) on the female rat reproductive tract and liver and kidney functions in vivo. *South African Journal of Botany,* 110**,** 208-215.

MORACANIN, S. V., STEFANOVIC, S., RADICEVIC, T., BOROVIC, B. & DJUKIC, D. 2015. Production of Biogenic Amines by Lactic Acid Bacteria Isolated from Uzicka Sausages. *Procedia Food Science,* 5**,** 308-311.

MORÉ, M. & VANDENPLAS, Y. 2018. Saccharomyces boulardii CNCM I-745 Improves Intestinal Enzyme Function: A Trophic Effects Review. *Clinical Medicine Insights: Gastroenterology,* 11**,** 1-14.

MORENO-ARRIBAS, V. & LONVAUD-FUNEL, A. 1999. Tyrosine decarboxylase activity of Lactobacillus brevis IOEB 9809 isolated from wine and L. brevis ATCC 367. *FEMS Microbiology Letters,* 180**,** 55-60.

MOSES-KOLKO, E. 2003. Widespread increases of cortical serotonin type 2A receptor availability after hormone therapy in euthymic postmenopausal women. *Fertility and Sterility,* 80**,** 554-559.

MOUSSA, Z., JUDEH, Z. M. A. & AHMED, S. A. 2019. Nonenzymatic Exogenous and Endogenous Antioxidants. *In:* DAS, K., DAS, S., BIRADAR, M. S., BOBBARALA, V. & ATA, S. S. (eds.) *Free Radical Medicine and Biology.* London: IntechOpen.

MOZAFFARI, S., ESMAILY, H., RAHIMI, R., BAEERI, M., SANEI, Y., ASADI-SHAHMIRZADI, A., SALEHI-SURMAGHI, M.-H. & ABDOLLAH, M. 2011. Effects of Hypericum perforatum extract on rat irritable bowel syndrome. *Pharmacognosy Magazine,* 7**,** 213-223.

MUJAGIC, Z., LUDIDI, S., KESZTHELYI, D., HESSELINK, M. A., KRUIMEL, J. W., LENAERTS, K., HANSSEN, N. M., CONCHILLO, J. M., JONKERS, D. M. & MASCLEE, A. A. 2014. Small intestinal permeability is increased in diarrhoea predominant IBS, while alterations in gastroduodenal permeability in all IBS subtypes are largely attributable to confounders. *Aliment Pharmacol Ther,* 40**,** 288-97.

MULAK, A., TACHE, Y. & LARAUCHE, M. 2014. Sex hormones in the modulation of irritable bowel syndrome. *World J Gastroenterol,* 20**,** 2433-48.

NAKAMURA, A., KURIHARA, S., TAKAHASHI, D., OHASHI, W., NAKAMURA, Y., KIMURA, S., ONUKI, M., KUME, A., SASAZAWA, Y., FURUSAWA, Y., OBATA, Y., FUKUDA, S., SAIKI, S., MATSUMOTO, M. & HASE, K. 2021. Symbiotic polyamine metabolism regulates epithelial proliferation and macrophage differentiation in the colon. *Nat Commun,* 12**,** 2105.

NAKAMURA, A., OOGA, T. & MATSUMOTO, M. 2019. Intestinal luminal putrescine is produced by collective biosynthetic pathways of the commensal microbiome. *Gut Microbes,* 10**,** 159-171.

NAVARRO, H. A., GILMOUR, B. P. & LEWIN, A. H. 2006. A rapid functional assay for the human trace amine-associated receptor 1 based on the mobilization of internal calcium. *J Biomol Screen,* 11**,** 688-93.

NAYAK, V. & UMA, P. 2006. Antioxidant and Radioprotective Effects of Ocimum Flavonoids Orientin and Vicenin in Escherichia coli. *Defence Science Journal,* 56.

NEIS, E. P., DEJONG, C. H. & RENSEN, S. S. 2015. The role of microbial amino acid metabolism in host metabolism. *Nutrients,* 7**,** 2930-46.

NEISH, A. S. 2009. Microbes in gastrointestinal health and disease. *Gastroenterology,* 136**,** 65-80.

NELSON, D. A., TOLBERT, M. D., SINGH, S. J. & BOST, K. L. 2007. Expression of neuronal trace amine-associated receptor (Taar) mRNAs in leukocytes. . *J. Neuroimmunol. ,* 192**,** 21-30.

NIGHOT, P. & MA, T. 2021. Endocytosis of Intestinal Tight Junction Proteins: In Time and Space. *Inflamm Bowel Dis,* 27**,** 283-290.

NISHIMURA, K., UTSUMI, K., YUHARA, M., FUJITANI, Y. & IRITANI, A. 1989. Identification of puberty-accelerating pheromones in male mouse urine. *J Exp Zool,* 251**,** 300-305.

NOBAEK, S., JOHANSSON, M. L., MOLIN, G., AHRNÉ, S. & JEPPSSON, B. 2000. Alteration of intestinal microflora is associated with reduction in abdominal bloating and pain in patients with irritable bowel syndrome. *Am J Gastroenterol.,* 95**,** 1231-1238.

NOVAES, P., TORRES, P. B., CORNU, T. A., LOPES, J. D. C., FERREIRA, M. J. P. & SANTOS, D. Y. A. C. D. 2019. Comparing antioxidant activities of flavonols from Annona coriacea by four approaches. *South African Journal of Botany,* 123**,** 253-258.

NOVOA, B. & FIGUERAS, A. 2011. Zebrafish: Model for the Study of Inflammation and the Innate Immune Response to Infection Diseases. *Advances in Experimental Medicine and Biology.* AEMB.

NOVOTNY, M. V. 2003. Pheromones, binding proteins and receptor responses in rodents. *Biochem Soc Trans.,* 31**,** 117-122.

NUGENT, S. G., KUMARD, D., RAMPTON, S. & EVANS, D. F. 2001. Intestinal luminal pH in inflammatory bowel disease: possible determinants and implications for therapy with aminosalicylates and other drugs. *Gut,* 48**,** 571-577.

O'MAHONY, F. & HARVEY, B. J. 2008. Sex and estrous cycle-dependent rapid protein kinase signaling actions of estrogen in distal colonic cells. *Steroids,* 73**,** 889-94.

O'MAHONY, L., MCCARTHY, J., KELLY, P., HURLEY, G., LUO, F., CHEN, K., O'SULLIVAN, G. C., KIELY, B., COLLINS, J. K., SHANAHAN, F. & QUIGLEY, E. M. 2005. Lactobacillus and bifidobacterium in irritable bowel syndrome: symptom responses and relationship to cytokine profiles. *Gastroenterology,* 128**,** 541-51.

O'MALLEY, D. 2019. Endocrine regulation of gut function - a role for glucagon-like peptide-1 in the pathophysiology of irritable bowel syndrome. *Exp Physiol,* 104**,** 3-10.

ODENWALD, M. A. & TURNER, J. R. 2017. The intestinal epithelial barrier: a therapeutic target? *Nat Rev Gastroenterol Hepatol,* 14**,** 9-21.

OEHLERS, S. H., FLORES, M. V., HALL, C. J., OKUDA, K. S., SISON, J. O., CROSIER, K. E. & CROSIER, P. S. 2013. Chemically induced intestinal damage models in zebrafish larvae. *Zebrafish,* 10**,** 184-93.

OEHLERS, S. H., FLORES, M. V., OKUDA, K. S., HALL, C. J., CROSIER, K. E. & CROSIER, P. S. 2011. A chemical enterocolitis model in zebrafish larvae that is dependent on microbiota and responsive to pharmacological agents. *Dev Dyn,* 240**,** 288-98.

OFIR, R., TAMIR, S., KHATIB, S. & VAYA, J. 2003. Inhibition of Serotonin Re-Uptake by Licorice Constituents. *Journal of Molecular Neuroscience,* 20**,** 135-140.

OHMAN, L. & SIMREN, M. 2010. Pathogenesis of IBS: role of inflammation, immunity and neuroimmune interactions. *Nat Rev Gastroenterol Hepatol,* 7**,** 163-73.

OLIPHANT, K. & ALLEN-VERCOE, E. 2019. Macronutrient metabolism by the human gut microbiome: major fermentation by-products and their impact on host health. *Microbiome,* 7**,** 91.

ORMSBEE, H. S. & FONDACARO, J. D. 1985. Action of Serotonin on the Gastrointestinal Tract. *Proceedings of the society for experimental biology and medicine,* 178**,** 333-338.

OSTLUND, H., KELLER, E. & HURD, Y. L. 2003. Estrogen Receptor Gene Expression in Relation to Neuropsychiatric Disorders. *Ann. N.Y. Acad. Sci.,* 1007**,** 54-63.

OSWIECIMSKA, J., SZYMLAK, A., ROCZNIAK, W., GIRCZYS-POLEDNIOK, K. & KWIECIEN, J. 2017. New insights into the pathogenesis and treatment of irritable bowel syndrome. *Adv Med Sci,* 62**,** 17-30.

OZDAL, T., SELA, D. A., XIAO, J., BOYACIOGLU, D., CHEN, F. & CAPANOGLU, E. 2016. The Reciprocal Interactions between Polyphenols and Gut Microbiota and Effects on Bioaccessibility. *Nutrients,* 8**,** 78.

PADHY, S. K., SAHOO, S., MAHAJAN, S. & SINHA, S. K. 2015. Irritable bowel syndrome: Is it “irritable brain” or “irritable bowel”? *J Neurosci Rural Pract.,* 6**,** 568-577.

PAIS, P., ALMEIDA, V., YILMAZ, M. & TEIXEIRA, M. C. 2020. Saccharomyces boulardii: What Makes It Tick as Successful Probiotic? *J Fungi (Basel),* 6.

PARK, C. M. & SONG, Y. S. 2019. Luteolin and luteolin-7-O-glucoside protect against acute liver injury through regulation of inflammatory mediators and antioxidative enzymes in GalN/LPS-induced hepatitic ICR mice. *Nutr Res Pract,* 13**,** 473-479.

PARK, J. M., CHOI, M. G., CHO, Y. K., LEE, I. S., KIM, J. I., KIM, S. W. & CHUNG, I. S. 2011. Functional Gastrointestinal Disorders Diagnosed by Rome III Questionnaire in Korea. *J Neurogastroenterol Motil,* 17**,** 279-86.

PATA, C., ERDAL, E., YAZICI, K., CAMDEVIREN, H., OZKAYA, M. & ULU, O. 2004. Association of the -1438 G/A and 102 T/C Polymorphism of the 5-Ht2A Receptor Gene with Irritable Bowel Syndrome 5-Ht2A Gene Polymorphism in Irritable Bowel Syndrome.

PAUL, W. E. & ZHU, J. 2010. How are T(H)2-type immune responses initiated and amplified? *Nat Rev Immunol,* 10**,** 225-35.

PAULOS, M. A. & TESSEL, R. E. 1982. Excretion of beta-phenethylamine is elevated in humans after profound stress. *Science,* 215**,** 1127-1129.

PECINS-THOMPSON, M., BROWN, N. A. & BETHEA, C. L. 1998. Regulation of serotonin re-uptake transporter mRNA expression by ovarian steroids in rhesus macaques. *Molecular Brain Research,* 53**,** 120-129.

PEERAPEN, P. & THONGBOONKERD, V. 2011. Effects of calcium oxalate monohydrate crystals on expression and function of tight junction of renal tubular epithelial cells. *Lab Invest,* 91**,** 97-105.

PEGG, A. E. 2013. Toxicity of Polyamines and Their Metabolic Products. *Chem. Res. Toxicol,* 26**,** 1782-1800.

PEI, Y., ASIF-MALIK, A. & CANALES, J. J. 2016. Trace Amines and the Trace Amine-Associated Receptor 1: Pharmacology, Neurochemistry, and Clinical Implications. *Front Neurosci,* 10**,** 148.

PEREIRA, C. I., MATOS, D., SAN ROMAO, M. V. & CRESPO, M. T. 2009. Dual role for the tyrosine decarboxylation pathway in Enterococcus faecium E17: response to an acid challenge and generation of a proton motive force. *Appl Environ Microbiol,* 75**,** 345-52.

PEREZ, M., CALLES-ENRIQUEZ, M., NES, I., MARTIN, M. C., FERNANDEZ, M., LADERO, V. & ALVAREZ, M. A. 2015. Tyramine biosynthesis is transcriptionally induced at low pH and improves the fitness of Enterococcus faecalis in acidic environments. *Appl Microbiol Biotechnol,* 99**,** 3547-58.

PESSIONE, E. 2012. Lactic acid bacteria contribution to gut microbiota complexity: lights and shadows. *Front Cell Infect Microbiol,* 2**,** 86.

PETECCHIA, L., SABATINI, F., USAI, C., CACI, E., VARESIO, L. & ROSSI, G. A. 2012. Cytokines induce tight junction disassembly in airway cells via an EGFR-dependent MAPK/ERK1/2-pathway. *Lab Invest,* 92**,** 1140-8.

PFANZAGL, B., ZEVALLOS, V. F., SCHUPPAN, D., PFRAGNER, R. & JENSEN-JAROLIM, E. 2019. Histamine causes influx via T-type voltage-gated calcium channels in an enterochromaffin tumor cell line: potential therapeutic target in adverse food reactions. *Am J Physiol Gastrointest Liver Physiol,* 316**,** G291-G303.

PHILIPS, S. R. 1987. B-Phenethylamines: a metabolically and pharmacology active amine. *In:* MOSNAIM, A. D. & WOLF, M. E. (eds.) *Phenylethylamines: Biological Mechanisms and Clinical Aspects.* New York.

PICHE, T. 2014. Tight junctions and IBS--the link between epithelial permeability, low-grade inflammation, and symptom generation? *Neurogastroenterol Motil,* 26**,** 296-302.

PICHE, T., BARBARA, G., AUBERT, P., BRULEY DES VARANNES, S., DAINESE, R., NANO, J. L., CREMON, C., STANGHELLINI, V., DE GIORGIO, R., GALMICHE, J. P. & NEUNLIST, M. 2009. Impaired intestinal barrier integrity in the colon of patients with irritable bowel syndrome: involvement of soluble mediators. *Gut,* 58**,** 196-201.

PLOTTEL, C. S. & BLASER, M. J. 2011. Microbiome and malignancy. *Cell Host Microbe,* 10**,** 324-35.

PONNUSAMY, K., CHOI, J. N., KIM, J., LEE, S. Y. & LEE, C. H. 2011. Microbial community and metabolomic comparison of irritable bowel syndrome faeces. *J Med Microbiol,* 60**,** 817-827.

POZUELO, M., PANDA, S., SANTIAGO, A., MENDEZ, S., ACCARINO, A., SANTOS, J., GUARNER, F., AZPIROZ, F. & MANICHANH, C. 2015. Reduction of butyrate- and methane-producing microorganisms in patients with Irritable Bowel Syndrome. *Sci Rep,* 5**,** 12693.

PRAVEENA, R., SADASIVAM, K., DEEPHA, V. & SIVAKUMAR, R. 2014. Antioxidant potential of orientin: A combined experimental and DFT approach. *Journal of Molecular Structure,* 1061**,** 114-123.

PRETORIUS, L. & SMITH, C. 2020. The trace aminergic system: a gender-sensitive therapeutic target for IBS? *J Biomed Sci,* 27**,** 95.

PRETORIUS, L., VAN STADEN, A. D., KELLERMANN, T. A., HENNING, N. & SMITH, C. 2022a. Rooibos (Aspalathus linearis) alters secretome trace amine profile of probiotic and commensal microbes in vitro. *J Ethnopharmacol,* 297**,** 115548.

PRETORIUS, L., VAN STADEN, A. D. P., VAN DER MERWE, J. J., HENNING, N. & SMITH, C. 2022b. Alterations to microbial secretome by estrogen may contribute to sex bias in irritable bowel syndrome. *Inflammopharmacology,* 30**,** 267-281.

PRICE, D. B., ACKLAND, M. L., BURKS, W., KNIGHT, M. I. & SUPHIOGLU, C. 2014. Peanut allergens alter intestinal barrier permeability and tight junction localisation in Caco-2 cell cultures. *Cell Physiol Biochem,* 33**,** 1758-77.

PUGIN, B., BARCIK, W., WESTERMANN, P., HEIDER, A., WAWRZYNIAK, M., HELLINGS, P., AKDIS, C. A. & O'MAHONY, L. 2017. A wide diversity of bacteria from the human gut produces and degrades biogenic amines. *Microb Ecol Health Dis,* 28**,** 1353881.

QIN, H. Y., ZANG, K. H., ZUO, X., WU, X. A. & BIAN, Z. X. 2019. Quercetin Attenuates Visceral Hypersensitivity and 5-Hydroxytryptamine Availability in Postinflammatory Irritable Bowel Syndrome Rats: Role of Enterochromaffin Cells in the Colon. *J Med Food,* 22**,** 663-671.

QIN, X., LU, Y., PENG, Z., FAN, S. & YAO, Y. 2018. Systematic Chemical Analysis Approach Reveals Superior Antioxidant Capacity via the Synergistic Effect of Flavonoid Compounds in Red Vegetative Tissues. *Front Chem,* 6**,** 9.

QUIGLEY, E. M. 2009. Do Patients with Functional Gastrointestinal Disorders have an Altered Gut Flora? *Therap Adv Gastroenterol,* 2**,** 23-30.

RAAB, S., WANG, H., UHLES, S., COLE, N., ALVAREZ-SANCHEZ, R., KUNNECKE, B., ULLMER, C., MATILE, H., BEDOUCHA, M., NORCROSS, R. D., OTTAWAY-PARKER, N., PEREZ-TILVE, D., CONDE KNAPE, K., TSCHOP, M. H., HOENER, M. C. & SEWING, S. 2016. Incretin-like effects of small molecule trace amine-associated receptor 1 agonists. *Mol Metab,* 5**,** 47-56.

RAO, J. N., RATHOR, N., ZHUANG, R., ZOU, T., LIU, L., XIAO, L., TURNER, D. J. & WANG, J. Y. 2012. Polyamines regulate intestinal epithelial restitution through TRPC1-mediated Ca(2)+ signaling by differentially modulating STIM1 and STIM2. *Am J Physiol Cell Physiol,* 303**,** C308-17.

RAOTE, I., BHATTACHARYA, A. & PANICKER, M. M. 2007. Serotonin 2A (5-HT2A) Receptor Function: Ligand-Dependent Mechanisms and Pathways. *In:* CHATTOPADHYAY, A. (ed.) *Serotonin Receptors in Neurobiology.* Boca Raton, Florida: CRC Press/Taylor & Francis.

RAUSCHER-GABERNIG, E., GROSSGUT, R., BAUER, F. & PAULSEN, P. 2009. Assessment of alimentary histamine exposure of consumers in Austria and development of tolerable levels in typical foods. *Food Control,* 20**,** 423-429.

REDDICK, L. E. & ALTO, N. M. 2014. Bacteria fighting back: how pathogens target and subvert the host innate immune system. *Mol Cell,* 54**,** 321-8.

REGARD, J. B., SATO, I. T. & COUGHLIN, S. R. 2008. Anatomical profiling of G protein-coupled receptor expression. *Cell,* 135**,** 561-71.

REHFELDT, S. C. H., SILVA, J., ALVES, C., PINTEUS, S., PEDROSA, R., LAUFER, S. & GOETTERT, M. I. 2022. Neuroprotective Effect of Luteolin-7-O-Glucoside against 6-OHDA-Induced Damage in Undifferentiated and RA-Differentiated SH-SY5Y Cells. *Int J Mol Sci,* 23.

REVEL, F. G., MOREAU, J. L., GAINETDINOV, R. R., BRADAIA, A., SOTNIKOVA, T. D., MORY, R., DURKIN, S., ZBINDEN, K. G., NORCROSS, R., MEYER, C. A., METZLER, V., CHABOZ, S., OZMEN, L., TRUBE, G., POUZET, B., BETTLER, B., CARON, M. G., WETTSTEIN, J. G. & HOENER, M. C. 2011. TAAR1 activation modulates monoaminergic neurotransmission, preventing hyperdopaminergic and hypoglutamatergic activity. *Proc Natl Acad Sci U S A,* 108**,** 8485-90.

RIDER, J. E., HACKER, A., MACKINTOSH, C. A., PEGG, A. E., WOSTER, P. M. & CASERO, R. A., JR. 2007. Spermine and spermidine mediate protection against oxidative damage caused by hydrogen peroxide. *Amino Acids,* 33**,** 231-40.

RIZZETTO, L., FAVA, F., TUOHY, K. M. & SELMI, C. 2018. Connecting the immune system, systemic chronic inflammation and the gut microbiome: The role of sex. *J Autoimmun,* 92**,** 12-34.

ROAGER, H. M. & LICHT, T. R. 2018. Microbial tryptophan catabolites in health and disease. *Nat Commun,* 9**,** 3294.

ROBLES-MEDRANDA, C., OLEAS, O., VALERO, M., PUGA-TEJADA, M., SORIA-ALCÍVAR, M., OSPINA, J., ALVARADO-ESCOBAR, H., MUÑOZ-JURADO, G., BAQUERIZO-BURGOS, J. & PITANGA-LUKASHOK, H. 2020. Confocal laser endomicroscopy detects colonic inflammation in patients with irritable bowel syndrome: a prospective study. *Endoscopy International Open,* 08**,** E550–E557.

RODGERS, A., MOKOENA, M., DURBACH, I., LAZARUS, J., DE JAGER, S., ACKERMANN, H., BREYTENBACH, I., OKADA, A., USAMI, M., HIROSE, Y., ANDO, R., YASUI, T. & KOHRI, K. 2016. Do teas rich in antioxidants reduce the physicochemical and peroxidative risk factors for calcium oxalate nephrolithiasis in humans? Pilot studies with Rooibos herbal tea and Japanese green tea. *Urolithiasis,* 44**,** 299-310.

RODINO-JANEIRO, B. K., VICARIO, M., ALONSO-COTONER, C., PASCUA-GARCIA, R. & SANTOS, J. 2018. A Review of Microbiota and Irritable Bowel Syndrome: Future in Therapies. *Adv Ther,* 35**,** 289-310.

RODRIGUEZ-DAZA, M. C., DAOUST, L., BOUTKRABT, L., PILON, G., VARIN, T., DUDONNE, S., LEVY, E., MARETTE, A., ROY, D. & DESJARDINS, Y. 2020. Wild blueberry proanthocyanidins shape distinct gut microbiota profile and influence glucose homeostasis and intestinal phenotypes in high-fat high-sucrose fed mice. *Sci Rep,* 10**,** 2217.

RODRIGUEZ-DAZA, M. C., PULIDO-MATEOS, E. C., LUPIEN-MEILLEUR, J., GUYONNET, D., DESJARDINS, Y. & ROY, D. 2021. Polyphenol-Mediated Gut Microbiota Modulation: Toward Prebiotics and Further. *Front Nutr,* 8**,** 689456.

RODRIGUEZ-LAGUNAS, M. J., MARTIN-VENEGAS, R., MORENO, J. J. & FERRER, R. 2010. PGE2 promotes Ca2+-mediated epithelial barrier disruption through EP1 and EP4 receptors in Caco-2 cell monolayers. *Am J Physiol Cell Physiol,* 299**,** C324-34.

ROMANO, A., LADERO, V., ALVAREZ, M. A. & LUCAS, P. M. 2014. Putrescine production via the ornithine decarboxylation pathway improves the acid stress survival of Lactobacillus brevis and is part of a horizontally transferred acid resistance locus. *Int J Food Microbiol,* 175**,** 14-9.

ROOKS, M. G. & GARRETT, W. S. 2016. Gut microbiota, metabolites and host immunity. *Nat Rev Immunol,* 16**,** 341-52.

ROOPCHAND, D. E., CARMODY, R. N., KUHN, P., MOSKAL, K., ROJAS-SILVA, P., TURNBAUGH, P. J. & RASKIN, I. 2015. Dietary Polyphenols Promote Growth of the Gut Bacterium Akkermansia muciniphila and Attenuate High-Fat Diet-Induced Metabolic Syndrome. *Diabetes,* 64**,** 2847-58.

ROSHCHINA, V. V. 2010. Evolutionary Considerations of Neurotransmitters in Microbial, Plant, and Animal Cells. *Microbial Endocrinology.*

ROSSI, F., GARDINI, F., RIZZOTTI, L., LA GIOIA, F., TABANELLI, G. & TORRIANI, S. 2011. Quantitative analysis of histidine decarboxylase gene (hdcA) transcription and histamine production by Streptococcus thermophilus PRI60 under conditions relevant to cheese making. *Appl Environ Microbiol,* 77**,** 2817-22.

ROTHSCHILD, D., WEISSBROD, O., BARKAN, E., KURILSHIKOV, A., KOREM, T., ZEEVI, D., COSTEA, P. I., GODNEVA, A., KALKA, I. N., BAR, N., SHILO, S., LADOR, D., VILA, A. V., ZMORA, N., PEVSNER-FISCHER, M., ISRAELI, D., KOSOWER, N., MALKA, G., WOLF, B. C., AVNIT-SAGI, T., LOTAN-POMPAN, M., WEINBERGER, A., HALPERN, Z., CARMI, S., FU, J., WIJMENGA, C., ZHERNAKOVA, A., ELINAV, E. & SEGAL, E. 2018. Environment dominates over host genetics in shaping human gut microbiota. *Nature,* 555**,** 210-215.

ROWLAND, I., GIBSON, G., HEINKEN, A., SCOTT, K., SWANN, J., THIELE, I. & TUOHY, K. 2018. Gut microbiota functions: metabolism of nutrients and other food components. *Eur J Nutr,* 57**,** 1-24.

ROY-BYRNE, P. P., DAVIDSON, K. W., KESSLER, R. C., ASMUNDSON, G. J., GOODWIN, R. D., KUBZANSKY, L., LYDIARD, R. B., MASSIE, M. J., KATON, W., LADEN, S. K. & STEIN, M. B. 2008. Anxiety disorders and comorbid medical illness. *Gen Hosp Psychiatry,* 30**,** 208-25.

RUAN, Y., WU, S., ZHANG, L., CHEN, G. & LAI, W. 2014. Retarding the senescence of human vascular endothelial cells induced by hydrogen peroxide: effects of 17beta-estradiol (E2) mediated mitochondria protection. *Biogerontology,* 15**,** 367-75.

RUIZ-CAPILLAS, C. 2004. Biogenic amines in meat and meat products. *Critical Reviews in Food Science and Nutrition,* 44**,** 489-499.

RUIZ-CAPILLAS, C. & HERRERO, A. M. 2019. Impact of Biogenic Amines on Food Quality and Safety. *Foods,* 8.

RUTIGLIANO, G., ACCORRONI, A. & ZUCCHI, R. 2017. The Case for TAAR1 as a Modulator of Central Nervous System Function. *Front Pharmacol,* 8**,** 987.

SABA, A., CHIELLINI, G., FRASCARELLI, S., MARCHINI, M., GHELARDONI, S., RAFFAELLI, A., TONACCHERA, M., VITTI, P., SCANLAN, T. S. & ZUCCHI, R. 2010. Tissue Distribution and Cardiac Metabolism of 3-Iodothyronamine. *Endocrinology,* 151**,** 5063–5073.

SALES, A. L., DEPAULA, J., MELLINGER SILVA, C., CRUZ, A., LEMOS MIGUEL, M. A. & FARAH, A. 2020. Effects of regular and decaffeinated roasted coffee (Coffea arabica and Coffea canephora) extracts and bioactive compounds on in vitro probiotic bacterial growth. *Food Funct,* 11**,** 1410-1424.

SALONEN, A., DE VOS, W. M. & PALVA, A. 2010. Gastrointestinal microbiota in irritable bowel syndrome: present state and perspectives. *Microbiology,* 156**,** 3205-15.

SALVEMINI, D., KIM, S. F. & MOLLACE, V. 2013. Reciprocal regulation of the nitric oxide and cyclooxygenase pathway in pathophysiology: relevance and clinical implications. *Am J Physiol Regul Integr Comp Physiol,* 304**,** R473-87.

SAMODIEN, S., KOCK, M., JOUBERT, E., SWANEVELDER, S. & GELDERBLOM, W. C. A. 2021. Differential Cytotoxicity of Rooibos and Green Tea Extracts against Primary Rat Hepatocytes and Human Liver and Colon Cancer Cells - Causal Role of Major Flavonoids. *Nutr Cancer,* 73**,** 2050-2064.

SANTORU, M. L., PIRAS, C., MURGIA, A., PALMAS, V., CAMBONI, T., LIGGI, S., IBBA, I., LAI, M. A., ORRU, S., BLOIS, S., LOIZEDDA, A. L., GRIFFIN, J. L., USAI, P., CABONI, P., ATZORI, L. & MANZIN, A. 2017. Cross sectional evaluation of the gut-microbiome metabolome axis in an Italian cohort of IBD patients. *Sci Rep,* 7**,** 9523.

SANTORU, M. L., PIRAS, C., MURGIA, A., PALMAS, V., CAMBONI, T., LIGGI, S., IBBA, I., LAI, M. A., ORRU, S., BLOIS, S., LOIZEDDA, A. L., GRIFFIN, J. L., USAI, P., CABONI, P., ATZORI, L. & MANZIN, A. 2018. Author Correction: Cross sectional evaluation of the gut-microbiome metabolome axis in an Italian cohort of IBD patients. *Sci Rep,* 8**,** 4993.

SARIKAHYA, N. B., PEKMEZ, M., ARDA, N., KAYCE, P., YAVAŞOĞLU, N. Ü. K. & KIRMIZIGÜL, S. 2011. Isolation and characterization of biologically active glycosides from endemic Cephalaria species in Anatolia. *Phytochemistry Letters,* 4**,** 415-420.

SASAKI, Y., HADA, R., NAKAJIMA, H., FUKUDA, S. & MUNAKATA, A. 1997. Improved Localizing Method of Radiopill in Measurement of Entire Gastrointestinal pH Profiles: Colonic Luminal pH in Normal Subjects and Patients With Crohn's Disease. *Am J Gastroenterol,* 92**,** 114-118.

SATHYANARAYANA RAO, T. S. & YERAGANI, V. K. 2009. Hypertensive crisis and cheese. *Indian J Psychiatry,* 51**,** 65-66.

SCHLOMS, L., SMITH, C., STORBECK, K. H., MARNEWICK, J. L., SWART, P. & SWART, A. C. 2014. Rooibos influences glucocorticoid levels and steroid ratios in vivo and in vitro: a natural approach in the management of stress and metabolic disorders? *Mol Nutr Food Res,* 58**,** 537-49.

SEEMAN, M. V. 1997. Psychopathology in Women and Men: Focus on Female Hormones. *Am J Psychiatry* 154**,** 1641-1647.

SEREGI, A. & HERTTING, G. 1984. Changes in cyclooxygenase activity and prostaglandin profiles during monoamine metabolism in rat bain homogenates. *Prostaglandins Leukotrienes and Medicine,* 14**,** 113-121.

SHEN, L. & TURNER, J. R. 2005. Actin Depolymerization Disrupts Tight Junctions via Caveolae-mediated Endocytosis. *Molecular Biology of the Cell,* 16**,** 3919-3936.

SHI, T., BIAN, X., YAO, Z., WANG, Y., GAO, W. & GUO, C. 2020. Quercetin improves gut dysbiosis in antibiotic-treated mice. *Food Funct,* 11**,** 8003-8013.

SHI, X., WALTER, N. A., HARKNESS, J. H., NEVE, K. A., WILLIAMS, R. W., LU, L., BELKNAP, J. K., ESHLEMAN, A. J., PHILLIPS, T. J. & JANOWSKY, A. 2016. Genetic Polymorphisms Affect Mouse and Human Trace Amine-Associated Receptor 1 Function. *PLoS One,* 11**,** e0152581.

SHIMAMURA, N., MIYASE, T., UMEHARA, K., WARASHINA, T. & FUJII, S. 2006. Phytoestrogens from *Aspalathus linearis*. *Biol. Pharm. Bull. ,* 29**,** 1271-1274.

SHIMIZU, M. 2012. Modulation of Intestinal Functions by Dietary Substances: An Effective Approach to Health Promotion. *Journal of Traditional and Complementary Medicine* 2**,** 81-83.

SIMREN, M., BARBARA, G., FLINT, H. J., SPIEGEL, B. M., SPILLER, R. C., VANNER, S., VERDU, E. F., WHORWELL, P. J., ZOETENDAL, E. G. & ROME FOUNDATION, C. 2013. Intestinal microbiota in functional bowel disorders: a Rome foundation report. *Gut,* 62**,** 159-76.

SIMRÉN, M., MÅNSSON, A., LANGKILDE, A. M., SVEDLUND, J., ABRAHAMSSON, H., BENGTSSON, U. & BJÖRNSSON, E. S. 2001. Food-Related Gastrointestinal Symptoms in the Irritable Bowel Syndrome. *Digestion,* 63**,** 108–115.

SIROTKIN, A. V. 2021. Rooibos (Aspalathus linearis) influence on health and ovarian functions. *J Anim Physiol Anim Nutr (Berl)*.

SMITH, C. & SWART, A. 2018. Aspalathus linearis (Rooibos) - a functional food targeting cardiovascular disease. *Food Funct,* 9**,** 5041-5058.

SMITH, N., J. & MILLIGAN, G. 2010. Allostery at G Protein-Coupled Receptor Homo- and Heteromers: Uncharted Pharmacological Landscapes. *Pharmacol Rev,* 62**,** 701-725.

SNOODY, A. M., HECKATHORN, D. & TESSEL, R. E. 1985. Cold-Restraint Stress and Urinary Endogenous B-Phenylethylamine Excretion in Rats. *Pharmacology Biochemistry & Behavior,* 22**,** 497-500.

SONG, Y. S. & PARK, C. M. 2014. Luteolin and luteolin-7-O-glucoside strengthen antioxidative potential through the modulation of Nrf2/MAPK mediated HO-1 signaling cascade in RAW 264.7 cells. *Food Chem Toxicol,* 65**,** 70-5.

SOUDER, J. P. & GORELICK, D. A. 2017. Quantification of Estradiol Uptake in Zebrafish Embryos and Larvae. *Toxicol Sci,* 158**,** 465-474.

SPERBER, A. D., BANGDIWALA, S. I., DROSSMAN, D. A., GHOSHAL, U. C., SIMREN, M., TACK, J., WHITEHEAD, W. E., DUMITRASCU, D. L., FANG, X., FUKUDO, S., KELLOW, J., OKEKE, E., QUIGLEY, E. M. M., SCHMULSON, M., WHORWELL, P., ARCHAMPONG, T., ADIBI, P., ANDRESEN, V., BENNINGA, M. A., BONAZ, B., BOR, S., FERNANDEZ, L. B., CHOI, S. C., CORAZZIARI, E. S., FRANCISCONI, C., HANI, A., LAZEBNIK, L., LEE, Y. Y., MULAK, A., RAHMAN, M. M., SANTOS, J., SETSHEDI, M., SYAM, A. F., VANNER, S., WONG, R. K., LOPEZ-COLOMBO, A., COSTA, V., DICKMAN, R., KANAZAWA, M., KESHTELI, A. H., KHATUN, R., MALEKI, I., POITRAS, P., PRATAP, N., STEFANYUK, O., THOMSON, S., ZEEVENHOOVEN, J. & PALSSON, O. S. 2021. Worldwide Prevalence and Burden of Functional Gastrointestinal Disorders, Results of Rome Foundation Global Study. *Gastroenterology,* 160**,** 99-114 e3.

SRIRAM, U., CENNA, J. M., HALDAR, B., FERNANDES, N. C., RAZMPOUR, R., FAN, S., RAMIREZ, S. H. & POTULA, R. 2016. Methamphetamine induces trace amine-associated receptor 1 (TAAR1) expression in human T lymphocytes: role in immunomodulation. . *J. Leukoc. Biol. ,* 99**,** 213-223.

SRIVASTAVA, V., DEY, I., LEUNG, P. & CHADEE, K. 2012. Prostaglandin E2 modulates IL-8 expression through formation of a multiprotein enhanceosome in human colonic epithelial cells. *Eur J Immunol,* 42**,** 912-23.

STALMACH, A., MULLEN, W., PECORARI, M., SERAFINI, M. & CROZIER, A. 2009. Bioavailability of C-linked dihydrochalcone and flavanone glucosides in humans following ingestion of unfermented and fermented rooibos teas. *J Agric Food Chem,* 57**,** 7104-11.

STANDER, M. A., VAN WYK, B. E., TAYLOR, M. J. C. & LONG, H. S. 2017. Analysis of Phenolic Compounds in Rooibos Tea (Aspalathus linearis) with a Comparison of Flavonoid-Based Compounds in Natural Populations of Plants from Different Regions. *J Agric Food Chem,* 65**,** 10270-10281.

STEWART, T. M., DUNSTON, T. T., WOSTER, P. M. & CASERO, R. A., JR. 2018. Polyamine catabolism and oxidative damage. *J Biol Chem,* 293**,** 18736-18745.

SUN, L., XU, G., DONG, Y., LI, M., YANG, L. & LU, W. 2020. Quercetin Protects Against Lipopolysaccharide-Induced Intestinal Oxidative Stress in Broiler Chickens through Activation of Nrf2 Pathway. *Molecules,* 25.

SZE, J. Y., VICTOR, M., LOER, C., SHI, Y. & RUVKUN, G. 2000. Food and metabolic signalling defects in a Caenorhabditis elegans serotonin-synthesis mutant. *Nature,* 403**,** 560-564.

TACK, J., CAMILLERI, M., CHANG, L., CHEY, W. D., GALLIGAN, J. J., LACY, B. E., MULLER-LISSNER, S., QUIGLEY, E. M., SCHUURKES, J., DE MAEYER, J. H. & STANGHELLINI, V. 2012. Systematic review: cardiovascular safety profile of 5-HT(4) agonists developed for gastrointestinal disorders. *Aliment Pharmacol Ther,* 35**,** 745-67.

TACK, J., STANGHELLINI, V., MEARIN, F., YIANNAKOU, Y., LAYER, P., COFFIN, B., SIMREN, M., MACKINNON, J., WISEMAN, G., MARCINIAK, A. & GROUP, I.-C. S. 2019. Economic burden of moderate to severe irritable bowel syndrome with constipation in six European countries. *BMC Gastroenterol,* 19**,** 69.

TAKAKI, M., MAWE, G. M., BARASCH, J. M., GERSHON, M. D. & GERSHON, M. D. 1985. PHYSIOLOGICAL RESPONSES OF GUINEA-PIG MYENTERIC NEURONS SECONDARY TO THE RELEASE OF ENDOGENOUS SEROTONIN BY TRYPTAMINE. *Neuroscience,* 16**,** 223-240.

TAN, H. P., LEBECK, L. K. & NEHLSEN-CANNARELLA, S. L. 1992. Regulatory role of cytokines in IgE-mediated allergy. *J Leukocyte Biol,* 52**,** 115-118.

TAP, J., DERRIEN, M., TORNBLOM, H., BRAZEILLES, R., COOLS-PORTIER, S., DORE, J., STORSRUD, S., LE NEVE, B., OHMAN, L. & SIMREN, M. 2017. Identification of an Intestinal Microbiota Signature Associated With Severity of Irritable Bowel Syndrome. *Gastroenterology,* 152**,** 111-123 e8.

THACKRAY, V. G. 2019. Sex, Microbes, and Polycystic Ovary Syndrome. *Trends Endocrinol Metab,* 30**,** 54-65.

THOMAS, M. L., XU, X., NORFLEET, A. M. & WATSON, C. S. 1993. The presence of functional estrogen receptors in intestinal epithelial cells. *Endocrinology,* 132**,** 426-430.

TIPTON, K. F. 2018. 90 years of monoamine oxidase: some progress and some confusion. *J Neural Transm (Vienna),* 125**,** 1519-1551.

TOKUNAGA, A., SAIKA, M. & SENBA, E. 1998. 5-HT2A receptor subtype is involved in the thermal hyperalgesic mechanism of serotonin in the periphery. *Pain,* 76**,** 349-355.

TORRIANI, S., FELIS, G. E. & FRACCHETTI, F. 2010. Selection criteria and tools for malolactic starters development: an update. *Annals of Microbiology,* 61**,** 33-39.

TURVILL, J. L., CONNOR, P. & FARTHING, M. J. 2000. The inhibition of cholera toxin-induced 5-HT release by the 5-HT(3) receptor antagonist, granisetron, in the rat. *Br. J. Pharmacol. ,* 130**,** 1031–1036.

UYANGA, V. A., AMEVOR, F. K., LIU, M., CUI, Z., ZHAO, X. & LIN, H. 2021. Potential Implications of Citrulline and Quercetin on Gut Functioning of Monogastric Animals and Humans: A Comprehensive Review. *Nutrients,* 13.

VALDES, A. M. 2018. Role of the gut microbiota in nutrition and health. *thebmj,* 361.

VANNUCCHI, M. G. & EVANGELISTA, S. 2018. Experimental Models of Irritable Bowel Syndrome and the Role of the Enteric Neurotransmission. *J Clin Med,* 7.

VENDITTI, P., NAPOLITANO, G., DI STEFANO, L., CHIELLINI, G., ZUCCHI, R., SCANLAN, T. S. & DI MEO, S. 2011. Effects of the thyroid hormone derivatives 3-iodothyronamine and thyronamine on rat liver oxidative capacity. *Mol Cell Endocrinol,* 341**,** 55-62.

VERNOCCHI, P., DEL CHIERICO, F. & PUTIGNANI, L. 2016. Gut Microbiota Profiling: Metabolomics Based Approach to Unravel Compounds Affecting Human Health. *Front Microbiol,* 7**,** 1144.

VIDELOCK, E. J., SHIH, W., ADEYEMO, M., MAHURKAR-JOSHI, S., PRESSON, A. P., POLYTARCHOU, C., ALBERTO, M., ILIOPOULOS, D., MAYER, E. A. & CHANG, L. 2016. The effect of sex and irritable bowel syndrome on HPA axis response and peripheral glucocorticoid receptor expression. *Psychoneuroendocrinology,* 69**,** 67-76.

VIEIRA-COELHO, M. A. & SOARES-DA-SILVA, P. 1993. Dopamine formation, from its immediate precursor 3,4-dihydroxyphenylalanine, along the rat digestive tract. *Fundam. Clin. Pharmacol.,* 7**,** 235-243.

VILJOEN, A., CHEN, W., MULAUDZI, N., KAMATOU, G. & SANDASI, M. 2022. *Phytochemical Profiling of Commercially Important South African Plants*, Academic Press.

VILLAÑO, D., PECORARI, M., TESTA, M. F., RAGUZZINI, A., STALMACH, A., CROZIER, A., TUBILI, C. & SERAFINI, M. 2010. Unfermented and fermented rooibos teas (Aspalathus linearis) increase plasma total antioxidant capacity in healthy humans. *Food Chemistry,* 123**,** 679-683.

WADA-HIRAIKE, O., IMAMOV, O., HIRAIKE, H., HULTENBY, K., SCHWEND, T., OMOTO, Y., WARNER, M. & GUSTAFSSON, J. A. 2006. Role of estrogen receptor beta in colonic epithelium. *Proc Natl Acad Sci U S A,* 103**,** 2959-64.

WALLRABENSTEIN, I., KUKLAN, J., WEBER, L., ZBORALA, S., WERNER, M., ALTMULLER, J., BECKER, C., SCHMIDT, A., HATT, H., HUMMEL, T. & GISSELMANN, G. 2013. Human trace amine-associated receptor TAAR5 can be activated by trimethylamine. *PLoS One,* 8**,** e54950.

WALTERS, N. A., DE VILLIERS, A., JOUBERT, E. & DE BEER, D. 2017. Phenolic profiling of rooibos using off-line comprehensive normal phase countercurrent chromatographyxreversed phase liquid chromatography. *J Chromatogr A,* 1490**,** 102-114.

WAN, M. L. Y., LING, K. H., EL-NEZAMI, H. & WANG, M. F. 2019. Influence of functional food components on gut health. *Crit Rev Food Sci Nutr,* 59**,** 1927-1936.

WANG, L., ALAMMAR, N., SINGH, R., NANAVATI, J., SONG, Y., CHAUDHARY, R. & MULLIN, G. E. 2020. Gut Microbial Dysbiosis in the Irritable Bowel Syndrome: A Systematic Review and Meta-Analysis of Case-Control Studies. *J Acad Nutr Diet,* 120**,** 565-586.

WASIK, A. M., MILLAN, M. J., SCANLAN, T., BARNES, N. M. & GORDON, J. 2012. Evidence for functional trace amine associated receptor-1 in normal and malignant B cells. *Leuk Res,* 36**,** 245-9.

WATTS, S. W. 1998. Activation of the mitogen-activated protein kinase pathway via the 5-HT2A receptor. *Ann N Y Acad Sci* 861**,** 162-168.

WHITEHEAD, W. E., CHESKIN, L. J. & HELLER, B. R. 1990. Evidence for exacerbation of irritable bowel syndrome during menses. . *Gastroenterology,* 98**,** 1485-1489.

WIKOFF, W. R., ANFORA, A. T., LIU, J., SCHULTZ, P. G., LESLEY, S. A., PETERS, E. C. & SIUZDAK, G. 2009. Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. *Proc Natl Acad Sci U S A,* 106**,** 3698-703.

WILKINS, T., PEPITONE, C., ALEX, B. & SCHADE, R. R. 2012. Diagnosis and Management of IBS in Adults. *American Family Physician,* 86**,** 419-426.

WILLIAMS, B. B., VAN BENSCHOTEN, A. H., CIMERMANCIC, P., DONIA, M. S., ZIMMERMANN, M., TAKETANI, M., ISHIHARA, A., KASHYAP, P. C., FRASER, J. S. & FISCHBACH, M. A. 2014. Discovery and characterization of gut microbiota decarboxylases that can produce the neurotransmitter tryptamine. *Cell Host Microbe,* 16**,** 495-503.

WILSON, A., TEFT, W. A., MORSE, B. L., CHOI, Y. H., WOOLSEY, S., DEGORTER, M. K., HEGELE, R. A., TIRONA, R. G. & KIM, R. B. 2015. Trimethylamine-N-oxide: A Novel Biomarker for the Identification of Inflammatory Bowel Disease. *Dig Dis Sci,* 60**,** 3620-30.

WISSINK, S., VAN DER BURG, B., KATZENELLENBOGEN, B. S. & VAN DER SAAG, P. T. 2001. Synergistic Activation of the Serotonin-1A Receptor by Nuclear Factor-?B and Estrogen. *Mol. Endo.,* 15**,** 543-552.

WLODARSKA, M., KOSTIC, A. D. & XAVIER, R. J. 2015. An integrative view of microbiome-host interactions in inflammatory bowel diseases. *Cell Host Microbe,* 17**,** 577-91.

WÖHRL, S., HEMMER, W., FOCKE, M., RAPPERSBERGER, K. & JARISCH, R. 2004. Histamine intolerance-like symptoms in healthy volunteers after oral provocation with liquid histamine. *Allergy and Asthma Proc,* 25**,** 305-311.

WOLINSKY, T. D., SWANSON, C. J., SMITH, K. E., ZHONG, H., BOROWSKY, B., SEEMAN, P., BRANCHEK, T. & GERALD, C. P. 2007. The Trace Amine 1 receptor knockout mouse: an animal model with relevance to schizophrenia. *Genes Brain Behav,* 6**,** 628-39.

WOLKEN, W. A., LUCAS, P. M., LONVAUD-FUNEL, A. & LOLKEMA, J. S. 2006. The mechanism of the tyrosine transporter TyrP supports a proton motive tyrosine decarboxylation pathway in Lactobacillus brevis. *J Bacteriol,* 188**,** 2198-206.

WOUTERS, M. M., DAFNE BALEMANS, D., VAN WANROOY, S., JAMES DOOLEY, J., CIBERT-GOTO, V., ALPIZAR, Y. A., VALDEZ-MORALES, E. E., NASSER, Y., VAN VELDHOVEN, P. P., VANBRABANT, W., VAN DER MERWE, S., MOLS, R., GHESQUIÈRE, B., CIRILLO, C., KORTEKAAS, I., CARMELIET, P., PEETERMANS, W. E., VERMEIRE, S., RUTGEERTS, P., AUGUSTIJNS, P., HELLINGS, P. W., BELMANS, A., VANNER, S., BULMER, D. C., TALAVERA, K., VANDEN BERGHE, P., LISTON, A. & BOECKXSTAENS, G. E. 2016. Histamine Receptor H1–Mediated Sensitization of TRPV1 Mediates Visceral Hypersensitivity and Symptoms in Patients With Irritable Bowel Syndrome. *Gastroenterology,* 150**,** 875–887.

WU, J. C. 2012. Psychological Co-morbidity in Functional Gastrointestinal Disorders: Epidemiology, Mechanisms and Management. *J Neurogastroenterol Motil,* 18**,** 13-8.

XU, D., HU, M. J., WANG, Y. Q. & CUI, Y. L. 2019. Antioxidant Activities of Quercetin and Its Complexes for Medicinal Application. *Molecules,* 24.

YANG, X., GUO, Y., HE, J., ZHANG, F., SUN, X., YANG, S. & DONG, H. 2017. Estrogen and estrogen receptors in the modulation of gastrointestinal epithelial secretion. *Oncotarget,* 8**,** 97683-97692.

YANNI, A. & KOURKOUTAS, Y. 2021. Editorial: Functional Foods and Bioactive Compounds for Improving and Maintaining Digestive Health. *Front Nutr,* 8**,** 815370.

YANO, J. M., YU, K., DONALDSON, G. P., SHASTRI, G. G., ANN, P., MA, L., NAGLER, C. R., ISMAGILOV, R. F., MAZMANIAN, S. K. & HSIAO, E. Y. 2015. Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis. *Cell,* 161**,** 264-76.

YAP, I. K. S., LI, J. V., SARIC, J., MARTIN, F.-P., DAVIES, H., WANG, Y., WILSON, I. D., NICHOLSON, J. K., UTZINGER, J., MARCHESI, J. R. & HOLMES, E. 2008. Metabonomic and Microbiological Analysis of the Dynamic Effect of Vancomycin-Induced Gut Microbiota Modification in the Mouse *Journal of Proteome Research,* 7**,** 3718-3728.

YOUNG, E. & KORSZUN, A. 2010. Sex, trauma, stress hormones and depression. *Mol Psychiatry,* 15**,** 23-8.

YU, L. M., ZHAO, K. J., WANG, S. S., WANG, X. & LU, B. 2018. Gas chromatography/mass spectrometry based metabolomic study in a murine model of irritable bowel syndrome. *World J Gastroenterol,* 24**,** 894-904.

YU, M., YANG, S., QIU, Y., CHEN, G., WANG, W., XU, C., CAI, W., SUN, L., XIAO, W. & YANG, H. 2015. Par-3 modulates intestinal epithelial barrier function through regulating intracellular trafficking of occludin and myosin light chain phosphorylation. *J Gastroenterol,* 50**,** 1103-13.

YU, Q., LIU, X., HUANG, H., ZHENG, X., PAN, X., FANG, J., MENG, L., ZHOU, C., ZHANG, X., LI, Z. & ZOU, D. 2019. Mass spectrometry-based metabolomics for irritable bowel syndrome biomarkers. *Therapeutic Advances in Gastroenterology,* 12.

YUAN, L., LI, X., HE, S., GAO, C., WANG, C. & SHAO, Y. 2018. Effects of Natural Flavonoid Isoorientin on Growth Performance and Gut Microbiota of Mice. *J Agric Food Chem,* 66**,** 9777-9784.

YUE, B., YU, Z. L., LV, C., GENG, X. L., WANG, Z. T. & DOU, W. 2020. Regulation of the intestinal microbiota: An emerging therapeutic strategy for inflammatory bowel disease. *World J Gastroenterol,* 26**,** 4378-4393.

ZAMANI, M., ALIZADEH-TABARI, S. & ZAMANI, V. 2019. Systematic review with meta-analysis: the prevalence of anxiety and depression in patients with irritable bowel syndrome. *Aliment Pharmacol Ther,* 50**,** 132-143.

ZAMANI, M. J., SHARIFZADEH, M., REZAIE, A., MASHAYEKHI, F. & ABDOLLAHI, M. 2005. Effects of sildenafil on rat irritable bowel syndrome. *Therapy,* 2**,** 237-242.

ZENG, R., LI, X. & GORODESKI, G. I. 2004. Estrogen abrogates transcervical tight junctional resistance by acceleration of occludin modulation. *J Clin Endocrinol Metab,* 89**,** 5145-55.

ZHANG, J., PACIFICO, R., CAWLEY, D., FEINSTEIN, P. & BOZZA, T. 2013. Ultrasensitive detection of amines by a trace amine-associated receptor. *J. Neurosci.,* 33**,** 3228–3239.

ZHANG, L. S. & DAVIES, S. S. 2016. Microbial metabolism of dietary components to bioactive metabolites: opportunities for new therapeutic interventions. *Genome Med,* 8**,** 46.

ZHENG, D., LIWINSKI, T. & ELINAV, E. 2020. Interaction between microbiota and immunity in health and disease. *Cell Res,* 30**,** 492-506.

ZHENG, Y. Z., DENG, G., LIANG, Q., CHEN, D. F., GUO, R. & LAI, R. C. 2017. Antioxidant Activity of Quercetin and Its Glucosides from Propolis: A Theoretical Study. *Sci Rep,* 7**,** 7543.

ZHOU, Q., ZHANG, B. & VERNE, G. N. 2009. Intestinal membrane permeability and hypersensitivity in the irritable bowel syndrome. *Pain,* 146**,** 41-6.

ZHOU, Z., ZHANG, L., DING, M., LUO, Z., YUAN, S., BANSAL, M. B., GILKESON, G., LANG, R. & JIANG, W. 2017. Estrogen decreases tight junction protein ZO-1 expression in human primary gut tissues. *Clin Immunol,* 183**,** 174-180.

ZHU, S., LIU, S., LI, H., ZHANG, Z., ZHANG, Q., CHEN, L., ZHAO, Y., CHEN, Y., GU, J., MIN, L. & ZHANG, S. 2019. Identification of Gut Microbiota and Metabolites Signature in Patients With Irritable Bowel Syndrome. *Front Cell Infect Microbiol,* 9**,** 346.

ZHU, Y., JAMESON, E., CROSATTI, M., SCHAFER, H., RAJAKUMAR, K., BUGG, T. D. & CHEN, Y. 2014. Carnitine metabolism to trimethylamine by an unusual Rieske-type oxygenase from human microbiota. *Proc Natl Acad Sci U S A,* 111**,** 4268-73.

ZOU, Y., WEI, H. K., XIANG, Q. H., WANG, J., ZHOU, Y. F. & PENG, J. 2016. Protective effect of quercetin on pig intestinal integrity after transport stress is associated with regulation oxidative status and inflammation. *J Vet Med Sci,* 78**,** 1487-1494.

ZUCCHI, R., ACCARINO, A. & CHIELLINI, G. 2014. Update on 3-iodothyronamine and its neurological and
metabolic actions. *Frontiers in Physiology,* 5**,** 1-12.

ZUCCHI, R., CHIELLINI, G., SCANLAN, T. S. & GRANDY, D. K. 2006. Trace amine-associated receptors and their ligands. *British Journal of Pharmacology,* 149**,** 967–978.

Addenda

Addendum A: Ethical approval

UNIVERSITEIT
STELLENBOSCH
UNIVERSITY

Protocol Approval

Date: 04 May 2021

PI Name: Prof C Smith

Protocol #: ACU-2021-21677

Title: Use of zebrafish larval model of IBS for drug screening

Dear C Smith ,

Your response to modifications, was reviewed by the Research Ethics Committee: Animal Care and Use via committee review procedures and was approved. Please note that this clearance is only valid for a period of twelve months. Ethics clearance of protocols spanning more than one year must be renewed annually through submission of a progress report (due 30 days prior to expiry date), up to a maximum of three years.

Approval Date: 04 May 2021 - 03 May 2022

Animal Species: Zebrafish

Animal Numbers: 10080

Applicants are reminded that they are expected to comply with accepted standards for the use of animals in research and teaching as reflected in the South African National Standards 10386: 2008. The SANS 10386: 2008 document is available on the Division for Research Developments website www.sun.ac.za/research.

As provided for in the Veterinary and Para-Veterinary Professions Act, 1982. It is the principal investigator's responsibility to ensure that all study participants are registered with or have been authorised by the South African Veterinary Council (SAVC) to perform the procedures on animals, or will be performing the procedures under the direct and continuous supervision of a SAVC-registered veterinary professional or SAVC-registered para-veterinary professional, who are acting within the scope of practice for their profession.

Please remember to use your protocol number 21677 on any documents or correspondence with the REC: ACU concerning your research protocol.

Please note that the REC: ACU has the prerogative and authority to ask further questions, seek additional information, require further modifications or monitor the conduct of your research.

Any event not consistent with routine expected outcomes that results in any unexpected animal welfare issue (death, disease, or prolonged distress) or human health risks (zoonotic disease or exposure, injuries) must be reported to the committee, by creating an Adverse Event submission within the system.

We wish you the best as you conduct your research.

If you have any questions or need further help, please contact the REC: ACU Secretariat at wabeukes@sun.ac.za or 021 808 9003.

Sincerely,

Winston Beukes

REC: ACU Secretariat

Research Ethics Committee: Animal Care and Use

Addendum B: Mass spectrometry parameters

Trace amine	Standard level	Nominal conc. (ng/mL)	Mean observed conc. (ng/mL)	% CV	% Accuracy	n	R^2	Quadratic weighting factor
PEA	1	10000	10157.38	3.29	101.57	2	0.999	1/c
	2	5000	4906.02	1.26	98.12	2		
	3	2500	2397.14	10.00	95.89	2		
	4	1250	1272.70	11.03	101.82	2		
	5	625	645.96	4.83	103.35	2		
	6	312.5	322.77	8.35	103.28	2		
	7	156.25	157.44	16.26	100.76	2		
	8	78.125	78.04	7.66	99.89	2		
	9	39.06	36.38	6.79	93.13	2		
	10	19.53	19.05	1.82	97.55	2		
	11	9.76	10.40	9.43	106.60	2		
	12	4.88	4.79	12.77	98.07	2		
	13	2.44	2.43	0.00	99.76	1		
	14	1.22	1.24	0.00	101.67	1		
	15	0.61	0.64	0.00	104.83	1		
	16	0.305	0.283	0.00	92.88	1		
	17	0.153	0.15	0.00	95.67	1		
TRP	1	10000	10034.99	1.42	100.35	2	0.999	1/c
	2	5000	4948.71	1.23	98.97	2		
	3	2500	2408.44	5.28	96.34	2		
	4	1250	1294.11	12.64	103.53	2		
	5	625	659.23	4.03	105.48	2		
	6	312.5	351.16	4.45	112.37	2		
	7	156.25	156.65	9.84	100.25	2		
	8	78.125	74.54	0.31	95.41	2		
	9	39.06	32.36	2.35	82.86	2		
	10	19.53	21.11	3.83	108.09	2		
	11	9.76	9.59	17.95	98.24	2		
	12	4.88	4.79	0.00	98.07	1		
	13	2.44	2.39	0.00	98.13	1		
	14	1.22	1.17	0.00	95.89	1		
	15	0.61	0.63	0.00	103.43	1		
	16	0.305	0.29	0.00	94.09	1		
	17	0.153	0.16	0.00	104.31	1		
TYR	1	10000	9936.53	2.52	99.37	2	0.998	$1/c^2$
	2	5000	5212.42	5.03	104.25	2		
	3	2500	2352.24	3.41	94.09	2		
	4	1250	1303.87	5.78	104.31	2		
	5	625	619.72	18.69	99.15	2		
	6	312.5	287.03	8.45	91.85	2		
	7	156.25	127.55	0.00	81.63	1		
	8	78.125	61.56	0.00	78.79	1		
	9	39.06	40.35	3.88	99.26	2		
	10	19.53	17.47	0.00	89.43	1		
	11	9.76	8.35	0.00	85.59	1		
	12	4.88	4.37	0.00	89.47	1		
	13	2.44	2.04	0.00	83.76	1		
AGM	1	10000	9336.62	9.11	93.37	2	0.986	$1/c^2$
	2	5000	5916.94	14.77	118.34	2		
	3	2500	2992.07	6.22	119.68	2		
	4	1250	1459.82	2.34	116.79	2		
	5	625	672.03	13.11	107.52	2		
	6	312.5	310.18	7.15	99.26	2		
	7	156.25	139.73	5.45	89.43	2		
	8	78.125	66.94	6.28	85.69	2		
	9	39.06	36.96	13.37	94.63	2		
	10	19.53	16.17	12.32	82.80	2		

	11	9.76	9.10	5.19	93.26	2		
	12	4.88	5.25	0.00	107.62	1		
	13	2.44	2.1	0.00	98.76	1		
PUT	1	10000	9945.43	1.15	99.45	2	0.999	1/c
	2	5000	5152.00	3.44	103.04	2		
	3	2500	2453.26	2.59	98.13	2		
	4	1250	1198.62	5.38	95.89	2		
	5	625	646.42	5.34	103.43	2		
	6	312.5	305.55	4.58	97.77	2		
	7	156.25	146.23	1.62	93.59	2		
	8	78.125	69.90	6.55	89.47	2		
	9	39.06	39.60	22.38	101.37	2		
	10	19.53	20.68	6.65	105.89	2		