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THE UNIVERSITY OF  
**NOTRE DAME**  
A U S T R A L I A

**Towards the Identification of Metabolite Markers  
of Nipple Pain and Inflammation in Human Milk**

Erin Fee

Masters of Philosophy (Health Science)

Primary supervisor: Professor Naomi Trengove

Co-supervisors: Associate Professor Robert Trengove, Dr Joel Gummer,  
Winthrop Professor Peter Hartmann, Research Associate Professor Donna Geddes  
and Dr Ching Tat Lai

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This thesis is presented as a requirement for Masters Of Philosophy in Health Science  
research at the University of Notre Dame, Fremantle Western Australia

Submitted February 2016.

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## Acknowledgements

Thank you to Professor Naomi Trengove for her kind nature, honesty and encouragement. Thank you for persisting with me and providing the stern yet caring words that kept me going. Your determination and will power inspire me.

To the University of Notre Dame thank you for this wonderful opportunity and for the ongoing support and encouragement that a close-knit university provides. Thank you to the research office and all the health science staff members, with special mention to Professor Gerard Hoyne for his continuous interest and support of my project.

Thank you to Associate Professor Robert Trengove for your advice and guidance, your incredible knowledge of the metabolomics field is unmatched. Thank you for allowing me to be part of a world-class research group that is Metabolomics Australia. Thank you also to the other members of the group, specifically to Catherine Rawlinson for your constant help and assistance, you're a valued member of Metabolomics Australia.

To the Hartmann Human Lactation Research Group thank you for the opportunity and for planting the original seed which started my love of research and my endeavour to apply to the postgraduate program. To Winthrop Professor Peter Hartmann you're a wealth of knowledge, yet so humble and grounded, your advice and insight has been invaluable. Thank you to Research Associate Professor Donna Geddes and Dr Ching Tat Lai for your help getting my project off the ground, for your help with recruitment and with technical assistance. Thank you to Anna Hepworth for your statistical assistance and the time you spent teaching and advising me, you were forever patient.

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I would like to acknowledge the generous financial support of Medela AG, without which my project would not have been possible.

Thank you to Senior Medical Scientist Cristina Farrar from Princess Margaret Hospital for your microbiology expertise and technical assistance for the bacteriology portion of my research. You were beyond helpful and I would have been lost without your guidance, thank you for taking me under your wing.

Thank you to all the donating mothers, many of which were in considerable amounts of pain yet still found the time and will to donate in the hope of helping other mothers in the future. Your individual stories and struggles touched me and gave me a new admiration for all mothers, your selflessness and generosity is greatly appreciated.

Thank you to my friends for their constant understanding and tenancy to keep in touch, it has been a tough year on friendship but I'm looking forward to spending time with you all now that I've finished.

To my partner Paul, thank you for your endless support and encouragement. Thank you for providing comfort when time spent with my family was scarce and trips home were few and far between. You have taught me that home isn't a place, but more so it's the people around you and I feel at home with you.

I'm forever grateful to my family, despite the large geographical distance between us I feel we are closer than ever. To my parents my voices of sanity and reassurance, the

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reason I aspire to improve and better myself comes from you both. To my grandparents, your unconditional love and support has meant the world.

Finally to Dr Joel Paul Aloysius Gummer, you have played an irreplaceable role in my postgraduate experience. Thank you for your optimism, good humour and patience. Thank you for lifting my spirits in times of stress and for your constant reassurance when difficulties arose. I thank my lucky stars every day that I was lucky enough to be your first student, any future post-graduate students will be very fortunate to have you.

‘Up and at them’

---

## **Preface**

The work in this thesis was supervised by Professor Naomi Trengove, University of Notre Dame Fremantle, Associate Professor Robert Trengove and Dr Joel Gummer, Metabolomics Australia at Murdoch University, and Winthrop Professor Peter Hartmann, Research Associate Professor Donna Geddes and Dr Ching Tat Lai, Hartmann Human Lactation Research Group at the University of Western Australia. My candidature was financially supported by Medela, AG, Baar, Switzerland.

### **Approvals**

Human and Animal ethics was obtained through the University of Notre Dame and University of Western Australia.

Initial human ethics approval (ref number- 013014F) was received on the 21<sup>st</sup> of February 2013. Due to significant changes an ethics amendment was submitted and approval received on the 3<sup>rd</sup> of August 2013 (ref number 013014F)

Notification of use of animal tissue/cadaver approved 14<sup>th</sup> of November 2013, file reference number RA/3/500/ at the University of Western Australia listing Donna Geddes as chief investigator.

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## **Facilities**

The Hartmann Human Lactation Research Group provided the majority of equipment and materials required for bacterial and human milk analysis. Additional equipment for sample analysis was provided by Metabolomics Australia, Murdoch University.

## **Presentations:**

Part of this work has been presented at a scientific conference in poster format;

Fee, E.L., Gummer, J.P.A, Trengove, R.D., Hartmann, P.E., Geddes, D.T., Lai, C.T. and Trengove, N.J. 2014. Sodium and potassium concentration in human milk samples of mothers experiencing nipple pain. Combined Biological Science Meeting, 29<sup>th</sup> August 2015.

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## List of Units

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<b>Abbreviation</b>	<b>Meaning</b>
L	Litre
ml	Millilitre
$\mu$ l	Microliter
g	Gram
mg	Milligram
$\mu$ g	Microgram
ng	Nanograms
$^{\circ}$ C	Degree/s celsius
rpm	Revolutions per minute
mM	Millimolar
mmol	Millimole
N	Normal (molarity)
eV	Electron volt
m	Metres
mm	Millimetres
$\mu$ m	Micrometres
min	Minutes
hr	Hour
amu	Atomic mass units
g	Gravity (centrifuge)

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## Abbreviations

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<b>Abbreviation</b>	<b>Meaning</b>
ABA	Australian Breastfeeding Association
ACN	Acetonitrile
B01-04	Bovine 01-04
BC	Bovine control group
BM	Bovine mastitis group
BV	Bovine vat sample group
C01-22	Human control samples 01-22
CG	Control group (human)
CI	Confidence interval
CLED	Cysteine Lactose Deficient agar
CFU	Colony forming unit
CNS	Coagulase negative staphylococci
CV	Crystal violet
DDI	Distilled deionised water
DECL	Decolourizer solution
DNase	Deoxyribonuclease
DRBC	Dichloran Rose Bengal Chlortetracycline
EI	Electron ionisation
FDA	Food and Drugs Administration
GC	Gas chromatography
GC-MS	Gas chromatography mass spectrometry
GI	Gram's iodide

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HHLRG	Hartmann Human Lactation Research Group
HMO	Human milk oligosaccharide
K <sup>+</sup>	Potassium ion
KCl	Potassiumchloride
LC-MS	Liquid chromatography mass spectrometry
MS	Mass spectrometry
MSA	Mannitol Salt agar
MSTFA	N-Methyl-N-(trimethylsilyl)trifluoroacetamide
m/z	Mass to charge ratio
Na <sup>+</sup>	Sodium ion
NaCl	Sodium chloride
NHMRC	National Health and Medical Research Council
NIST	National Institute of Standards and Technology
NMR	Nuclear magnetic resonance spectroscopy
NP	Nipple pain
NP01-11	Human nipple pain samples 01-11
OR	Odds ratio
PCA	Principal component analysis
PCR	Polymerase chain reaction
PG	Pain group (without evidence of trauma) (human)
QC	Quality control
RSD	Relative standard deviation
SF	Safranin aqueous stain
SIDS	Sudden infant death syndrome
SIM	Selected Ion monitoring

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SPE	Solid phase extraction
TG	Trauma group (human)
TM	Thermomix
TMS	trimethylsyl
V01-02	Bovine vat (pooled) samples 01-02
VAS	Visual analog scale
YGC	Yeast Extract Glucose Chloramphenicol

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## **Abstract**

### **Background**

Human milk is considered the best source of nutrition for all newborns as it contains important growth, developmental and immunological factors. The WHO (2003) recommends exclusive breastfeeding for the first six months of age, with complementary breastfeeding up to two years and beyond. However, some women experience complications of the breast that lead to early cessation of breastfeeding, which can adversely affect the well being of the developing infant and her own health.

Nipple pain is the most commonly cited reason for weaning in the first week post partum. Nipple pain is also linked to mastitis from milk stasis and possible bacterial infection, although the influence of bacteria is still largely unknown. However, it is known that the presence of bacteria and fungi along with their metabolites contribute to the composition of the milk as the baby receives it.

Metabolomics is increasingly being utilised in the dairy industry to determine spoilage as a result of teat trauma and mastitis. Given the current diagnostic application of metabolomics in clinical medicine uses blood and urine samples, it has been proposed as a potential tool for detecting biomarkers and determining compositional changes in human milk. Measuring the composition of milk from human mothers experiencing persistent nipple pain, with or without evidence of trauma, and identifying the influence of this condition on endogenous and exogenous metabolites may determine the relationship between milk composition and nipple pain.

### **Aims**

The aims of this study were to source the appropriate human and bovine milk samples; to identify and quantify bacterial and fungal species using traditional culture and microscopy techniques; to measure the effect of nipple pain on the paracellular pathway of the breast by measuring the sodium and potassium concentration and ratio in the milk; to optimise GC-MS methodology for the measurement of milk metabolites; and to use untargeted metabolomics to identify compositional differences

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in the metabolite profile in human milk from mothers presenting with nipple pain compared to healthy control mothers.

## **Results**

Two groups were recruited; a control group of mothers not experiencing nipple pain (n=22 samples) and a group of mothers experiencing persistent nipple pain during breastfeeding (n=11 samples); mothers with unilateral nipple pain supplied a milk sample from their affected and non-affected breast (n=4). The nipple pain group (n=11) was divided into two subgroups; persistent nipple pain without evidence of trauma (PG) (n=6) and persistent nipple pain with evidence of trauma (TG) (n=5). Additionally 9 bovine samples were collected, 3 from healthy cows (control), 4 from cows presenting with mastitis and 2 from a single storage vat, to be used as positive controls throughout the study.

All 42 samples were tested for the presence of microbial and fungal species, sodium and potassium concentrations and ratio were determined and untargeted metabolomics analysis of the milk metabolome was performed.

Overall there was no significant difference in microbe content between the human control and nipple pain group (1, 623 CFU/ml vs. 1, 503 CFU/ml); the TG subgroup had the highest colony count of 2, 778 CFU/ml. The bovine mastitis group had a higher colony count than the bovine control group, 2, 173 CFU/ml vs. 473 CFU/ml. Coagulase negative staphylococcus ssp. were the most frequently isolated microorganisms and was found in 91% of human milk samples and 100% of bovine milk samples. *Staphylococcus aureus* were identified in one human milk sample from a mother in the PG subgroup and in one bovine sample from a cow suffering from untreated mastitis as well as both pooled bovine vat samples. *Streptococcus* ssp. and yeast were only found in bovine samples.

The TG subgroup had the highest Na<sup>+</sup> concentration of the human milk samples (8.04 ± 2.40 mM), significantly higher than the control group (4.32 ± 1.18 mM; p<0.001). There was no significant difference in Na<sup>+</sup> concentration between the TG and PG subgroups. The Na<sup>+</sup>/K<sup>+</sup> ratio was significantly higher in the TG subgroup (0.55 ± 0.14) compared to the control group (0.34 ± 0.09) (p<0.001); there was no significant

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difference in ratio between the PG and TG subgroups ( $p=0.10$ ). No sample recorded a  $\text{Na}^+/\text{K}^+$  ratio above 1, consistent with the physiological observations and indicative of no mother presenting with mastitis.

Untargeted metabolomic analysis found compositional differences between the human control and nipple pain groups, in particular samples from the TG subgroup. Compositional variations between milk from the control and nipple pain subgroups was identified using principal component analysis and PC4 best represented the differences in metabolite composition between the groups. This result is consistent with the subtlety of the nipple pain condition. A list of the most influential metabolites based on their correlation loadings (explained within 50-100% of the model) was determined. The most influential metabolites with respect to the TG milk samples were included isoleucine, proline, galactose and some as yet unidentified metabolites.

### **Conclusion**

As nipple pain is often a precursor to mastitis the results from this study will form a basis for further development using metabolomics as a tool for more efficient detection and treatment of breast infection and inflammation within the nipple and breast.