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Towards the identification of metabolite markers of nipple pain and inflammation in human milk

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

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‘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.

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

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

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, 29th August 2015.

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List of Units

Abbreviation	Meaning
L	Litre
ml	Millilitre
µl	Microliter
g	Gram
mg	Milligram
µg	Microgram
ng	Nanograms
°C	Degree/s celsius
rpm	Revolutions per minute
mM	Millimolar
mmol	Millimole
N	Normal (molarity)
eV	Electron volt
m	Metres
mm	Millimetres
µm	Micrometres
min	Minutes
hr	Hour
amu	Atomic mass units
g	Gravity (centrifuge)

Abbreviations

Abbreviation	Meaning
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	Decoulourizer 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

HHLRG	Hartmann Human Lactation Research Group
HMO	Human milk oligosaccharide
K^+	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^+	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

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

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

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^+ concentration of the human milk samples (8.04 ± 2.40 mM), significantly highly than the control group (4.32 ± 1.18 mM; $p<0.001$). There was no significant difference in Na^+ concentration between the TG and PG subgroups. The Na^+/K^+ 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

difference in ratio between the PG and TG subgroups (p=0.10). No sample recorded a Na^+/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.