

Determination of antioxidant potential of human milk peptides and amino acids and effects of tryptophan on bacterial growth in formula

By Andrea Romanowski

A thesis submitted to the Faculty of Graduate and Postdoctoral Affairs in partial fulfillment of the requirements for the degree of Master of Science

Carleton University

Ottawa, Ontario

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Your file *Votre référence*
ISBN: 978-0-494-83153-3
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List of abbreviations

5-HTP	serotonin
AA	amino acids
BBB	blood-brain barrier
BPD	bronchopulmonary dysplasia
EEAs	essential AAs
GPx	glutathione peroxidase
GSH	glutathione
H ₂ O ₂	hydrogen peroxide
NEC	necrotizing enterocolitis
O ₂ ^{•-}	superoxide anion
OH•	hydroxyl radical
OONO-	peroxynitrite
PCAP	continuous positive airway pressure
PI	premature infants
ROS	reactive oxygen species
SOD	superoxide dismutase
TRP	tryptophan

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Acknowledgements

The completion of this thesis would not have been successful without support and guidance of several individuals. I would like to thank the members of my thesis committee, Dr. Apollinaire Tsopmo, Dr. Tyler Avis, and Dr. Alfonso Abizaid. A special thanks to my supervisor, Dr. Apollinaire Tsopmo, whose constant encouragement and guidance from the initial to final research period enabled me to complete my project. Dr. Tsopmo's support and trust has made me strive for excellence and has made my project enjoyable. I would like to show my gratitude to Dr. Tyler Avis for his advice and help during the conduction of my study. I would like to thank Shari Levac for her assistance.

I offer my regards and blessings to all of those who supported me in any respect during the completion of the project. In particular, this list includes my family and my mother, Walentina Piotrowska, whose confidence in me and positivity throughout my education has allowed me to persevere. I would like to thank my friends and colleagues Sara Jodayree, Tamara Desroches, Mark Sumarah, and the Miller Lab for their faith in me and keeping me company and uplifted during long hours.

Lastly, it is with deep gratitude that I thank the Canadian Institutes of Health Research and Carleton University for providing me with the funds to continue and complete my studies.

Abstract

Oxidative stress is associated with many infant diseases. Antioxidants have the ability to scavenge free radicals and reduce oxidative stress. Novel breast milk peptides demonstrated the ability to inhibit the formation of lipid peroxides using linoleic acid emulsion system and showed potential to scavenge free radicals using oxygen radical absorbance capacity assays. All peptides, except for 23-8-8, significantly reduced the generation of lipid hydroperoxides compared to control. Peptide 23-8-11 showed greatest antioxidant activity compared to other human milk peptides and was able to decrease hydroperoxides produced from autoxidation of linoleic acid. ORAC assay suggest that the most active peptides were 23-8-11(ISELGW [-NH₂]) and 23-8-10 ([ACE]-ISELGW), followed by 23-8-5 (WISELG), 23-8-4 (ISELGW) AND 23-8-7 (ISELWG). The effects of two of peptides 23-7 and 23-8 on quality mother's milk and infant's milk formulas were evaluated using the electronic nose. There is no significant difference in quality between milk samples with and without peptides as graphs depicted a high amount of overlap of principle component analysis data points between all samples. Addition of peptides ISELGW and YGYTGA to human milk and Infant formula milk had no significant difference in odour profile. These results demonstrate potential in the involvement of human milk peptides and tryptophan as a means of reducing diseases related to oxidative stress, and may be included into infant formulas following further analysis. Resazurin tests do not suggest antimicrobial activity of peptides nor tryptophan, although further evaluation is necessary. One-way ANOVA on peptides analyzing Total Plate Count suggests 23-6, 23-7, and 23-8 show a

significant difference. LSD test shows that peptides 23-6, 23-7, and 23-8 have antimicrobial properties of the same potency. Antioxidant properties of amino acids were evaluated using ORAC, where the amino acids showing highest antioxidant activity are cysteine (589 TE/mM \pm 22.1 TE/mM), tryptophan (193 TE/mM \pm 1.94 TE/mM), tyrosine (171 TE/mM \pm 2.11 TE/mM), methionine (111 TE/mM \pm 4.25 TE/mM), histidine (60.0 TE/mM \pm 1.80 TE/mM), and phenylalanine (8.72 TE/mM \pm 6.0 TE/mM); the remaining amino acids showed values close to zero. The stable free radical diphenylpicrylhydrazyl (DPPH) was used to evaluate scavenging of free radicals. Controls were Vitamin C and glutathione at 97 \pm 0.06% at 2 mg/ml and a 95 \pm at 6 mg/ml respectively. Tryptophan had 90 \pm 11.1% at 6 mg/ml demonstrating that it is an effective at scavenging free radicals.

1. Background

1.1. Nutrient and antioxidant properties of formula and breast milk

Breast milk provides the best source of nutrients for a growing infant. Human milk is distinct from other mammalian milks due to its unique complex system host defence factors that aids in infant protection (Tsopmo *et al.*, 2009). Part of the defence system in breast milk includes antioxidants; however, the complete list of active antioxidants in human breast milk is unknown (Friel *et al.*, 2002). Two main groups of antioxidants exist in milk protein, which are those from casein and those from whey. Caseins are the principal class of protein in milk from most species, except in human milk. Caseins in human milk comprise 10 - 50% of total protein, and whey proteins make up the remainder (Armaforte *et al.*, 2010). Human milk does not contain b-lactoglobulin. Infant formula milk contains approximately 11% protein, with the caseins and whey proteins at a ratio of 40:60 respectively (Armaforte *et al.*, 2010). Sometimes human breast milk is not available from the infant's mother, and another source must be used. Banked milk from donor mothers can be used as an alternative, but donor human breast milk has been found to contain a lower content of protein and host defences than that from the infant's own mother (Korchazhkina *et al.*, 2006). Feeding with unfortified donor human breast milk, especially when compared with nutrient supplemented formula milk is linked to lower rates of growth in preterm infants (PI) in the short term (Fanaro *et al.*, 2010).

1.1.2. Antioxidants in breast milk and formula milk

It has been reported that antioxidants in breast milk help eliminate reactive oxygen species in infants. Formula milk has less antioxidants present than human breast milk (Friel *et al.*, 2002). The lower amount of antioxidants in formula milk has been linked to an increase in infant diseases and even death (Tsopmo *et al.*, 2011). Formula-fed infants have a higher incidence of infant illnesses such as necrotizing enterocolitis (NEC), sepsis, and respiratory illness. Although NEC, for example, can be due to causes such as abnormal bacterial colonisation, immature intestinal circulatory regulation, and immature intestinal barrier function, it has also been linked to feeding with formula milk (Lin and Stoll, 2006). Diseases such as NEC can be attributed to oxidative stress when the formation of reactive oxygen species (ROS) is not counteracted by an antioxidant defence system to neutralize the damaging effects of oxidants (Korchazhkina *et al.*, 2006).

Preliminary data on both tryptophan and novel peptides isolated from human milk has demonstrated antioxidant potential. Supplementation of tryptophan and novel peptides into formula milk can potentially strengthen the antioxidant defence system in premature infants; an ability to quench free radicals is associated to a decrease in diseases and will allow infants to develop and grow properly and healthy. In addition, a decrease in infant hospitalization can save billions in the annual cost savings for the national health care system (Russell *et al.*, 2007).

1.2. Sources of free radicals in premature infant

1.2.1. Oxidative stress associated with supplemental oxygen

Premature babies or infants born with heart or lung problems require supplemental oxygen (Weinberger *et al*, 2002) such a continuous positive airway pressure (CPAP) machine, a breathing machine, or ventilator (Neil, 2005). A high concentration of supplemental oxygen causes lung, eye, and brain injury (McCord, 1985; Saugstad and Aasen, 1980) if the infant has an unbalanced antioxidant-defence system (Figure 1). Any oxygen delivery system that delivers oxygen to an infant at an elevated pressure may cause oxygen toxicity. Prolonged exposure to higher oxygen concentrations causes oxidative damage to the cell membrane, retinal detachment, and collapse of alveoli in the lungs. Due to the imbalance of free radical species and antioxidants, damage from using oxygen supplementation can be long-term and lead to health complications which is partially attributed to an underdeveloped immune system (Hummler and Schulze, 2009). Oxidative stress caused by supplemental oxygen is directly linked to infant diseases.

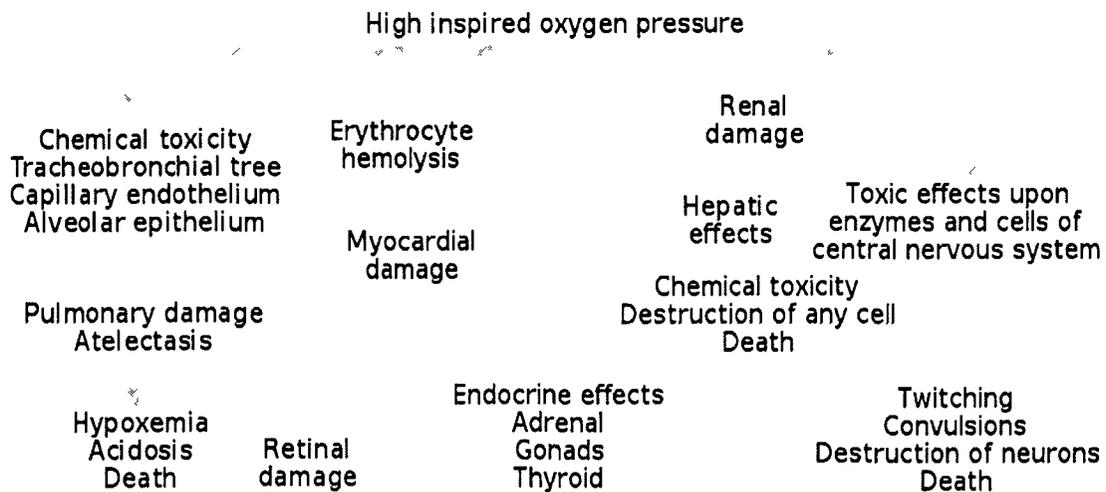


Figure 1. Flow chart demonstrating the toxicity of oxygen (Clark, 1974)

When not enough oxygen is delivered, the infant is under hypoxic conditions; excess oxygen will put the infant under hyperoxic conditions. It has been demonstrated that both a hypoxic and a hyperoxic environment create oxidative stress conditions that can harm the infant (Baker, 2004; Saugstad, 2003). Although it is possible to monitor the delivery system and decrease the settings on the ventilator when possible to reduce the severity of oxygen toxicity, there is no known method for eliminating oxidative stress damage from this form of oxygen toxicity (Richmond and Goldsmith, 2008). In order to overcome or improve the problems associated with oxidant exposure, it would be beneficial to boost the antioxidant defence system to protect the vulnerable premature infant population.

1.2.2. Radical formation

A free radical is any species that is able to exist on its own which contains one or more unpaired electrons, which usually increases the chemical reactivity of the molecule (Karlsson, 1997). This is a general and broad definition and it encompasses a wide range of species, where some examples are summarized in the table below (Table 1). Free radicals are highly reactive and both environmental radiation and physiological processes in the body can cause free radicals to form (Halliwell, 1992).

Table 1. Free radical types and examples (Halliwell, 1992)

Type of radical	Example
Hydrogen centred	Hydrogen atom, H [•]
Carbon centred	Trichloromethyl, CCl ₃ [•]
Sulphur centred	Glutathione thiyl, GS [•]
Oxygen centred	Superoxide, O ₂ ^{•-} Hydroxyl, OH [•] (often written as [•] OH) Lipid peroxy, lipid -O ₂ [•]
Electron delocalized	Phenoxy, C ₆ H ₅ O [•] (electron delocalized into benzene ring) Nitric oxide, NO [•] (often written as NO)

The superscript dot (•) denotes the presence of one or more unpaired electrons. Carbon-centred and sulphur-centered radicals usually react rapidly with oxygen.

Although there are numerous types of free radicals that can be formed within the body, the focus is on oxygen-centred free radicals (Table 2) which can act as a pro-oxidant to damage biological targets such as DNA, lipids, and proteins (Kohen and Nyska, 2002).

Table 2. Radical and nonradical oxygen metabolites (Kohen and Nyska, 2002)

Name	Symbol
Oxygen radicals	
Oxygen (bi-radical)	O ₂
Superoxide ion	O ₂ ⁻
Hydroxyl	OH
Peroxy	ROO
Alkoxy	RO
Nitric oxide	NO
Nonradical oxygen derivatives	
Hydrogen peroxide	H ₂ O ₂
(Organic peroxide)	ROOH
Hypochlorous acid	HOCL
Ozone	O ₃
Aldehydes	HCOR
Singlet oxygen	¹ O ₂
Peroxynitrite	ONOOH

In vivo, the production of free radicals is closely linked with the participation of redox-active metals such as chromium, copper, and iron (Rahmen, 2007). Very reactive hydroxyl radicals are generated during the Fenton reaction (Prousek, 2007) as seen in Figure 2.

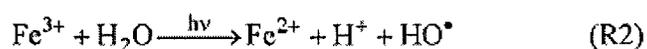
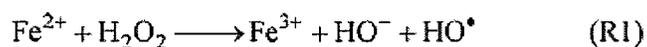
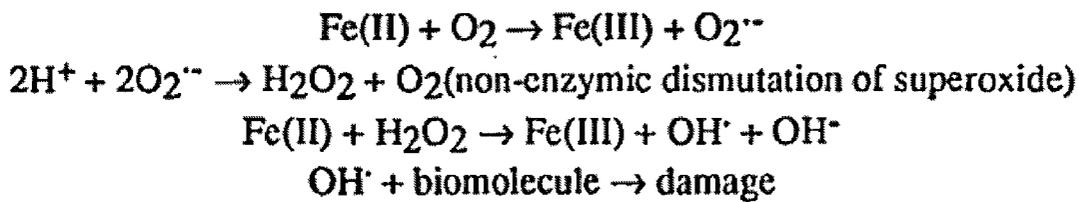


Figure 2. Fenton reaction (Kohen and Nyska, 2002)

Reactions R1 and R2 generate the hydroxyl radical that is able to oxidize organic compounds, according to R3. The Fenton reaction is efficient due to the reactivity of HO[•], which can react with multiple species when in the liquid phase (Jomovaa and Valko, 2011). Although Fenton chemistry is also present *in vitro*, its significance under physiological conditions is not fully

understood (Kell, 2009). Hydroxyl radical is produced in living organisms by two mechanisms: reaction of transition metal ions with H_2O_2 and homolytic fission of water due to background exposure to ionizing radiation (Halliwell and Glutteridge, 1999). Hydroxyl radical is a very reactive species and attacks all biological molecules, which initiates the free radical chain-reactions (Halliwell and Glutteridge, 1999) as seen in the reaction below.



The ferrous ions are soluble in biological fluids and produce, in the presence of oxygen, damaging hydroxyl radicals. The ferrous ions are unstable in aqueous media and tend to react with molecular oxygen. However, metals do play important roles in a wide variety of biological processes of living systems and is critical for life. Iron is crucial for cell growth, oxygen utilization, and immune responses. Homeostasis of metal ions must be maintained through tightly regulated mechanisms of uptake, storage and secretion (Rahman, 2007).

1.2.3. Reactive oxygen species

Excess production of ROS can damage protein, lipids and DNA, which could lead health problems. ROS can be generated by oxidases when they produce incompletely reduced oxygen species-superoxides (when O accepts a single electron) and hydroxyl radicals (Apel and Heribert, 2004). Hydroxyl radicals are able to cause injury to the cell membrane by initiating the oxidation of fatty acid in the cell membrane lipids, which is a process termed lipid peroxidation.

Damage also occurs to the nucleic acids by disrupting the strand and by changing the structure of DNA bases. The superoxide, although less toxic can also cause lipid peroxidation due to its unpaired electron. It can also bind to another free radical, such as nitric oxide. Nitric oxide is a free radical that is released by several cell types, such as phagocytes, and the binding of nitric oxide and the superoxide results in peroxynitrite (OONO-) that damages proteins and also results in lipid peroxidation. It may also be directly cytotoxic by oxidizing thiol groups and may decompose to become hydroxide (Halliwell, 1992).

1.2.4. Reactive oxygen species in infants

Oxygen is essential to human life, yet it can cause tissue injury when administered in relative excess to infants with compromised antioxidant defenses (Weinberger *et al*, 2002). Studies have shown that premature neonatal animals are highly susceptible to oxidative stress partially due to the inability to sufficiently upregulate antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase (Frank and Sosenko, 1991). In addition, deficiencies in selenium, taurine, and glutathionine decrease the free radical trapping capacity in plasma of premature infants. Conditions following premature birth are hyperoxic relative to *in utero* where fetal haemoglobin is saturated with 90% oxygen where arterial oxygen tensions (PaO₂) are approximately 40 Tor; however, oxygen supplementation or mechanical ventilation generally maintain a PaO₂ of over 50 Tor in order to supply sufficient pulmonary vasodilation and tissue oxygenation. Preterm infants mechanically ventilated can have 50-100 Tor (Emond *et*

al., 1993). Exposure to these levels of oxygen in premature babies can be physiologically challenging.

The detrimental effects of direct oxygen toxicity due to highly reactive free radicals derived from oxygen (Weinberger *et al.*, 2002) damages tissues from the formation of reactive oxygen intermediates and the peroxidation of membrane lipids. The toxicity from supplemental oxygen indicates damage to the airway, alveolar epithelium and capillary endothelium which leads to the infiltration of neutrophils to the tissue (Halliwell and Gutteridge, 1999). Normally, oxygen is reduced in the mitochondria to two water molecules carried out by reactions in electron-transport, which are catalyzed by the cytochrome oxidase enzyme complex. Reactive oxygen intermediates are produced as by-products of these reactions, and can lead to damage in excess amounts. One of the intermediates generated by the transfer of an electron to molecular oxygen is superoxide anion ($O_2^{\bullet-}$), which can act as both an oxidant and a reductant (Jenkinson, 1993). An extremely reactive oxygen intermediate, H_2O_2 , can be produced by the addition of an electron to the superoxide anion; this can also occur when two $O_2^{\bullet-}$ are reacted together. When H_2O_2 , and $O_2^{\bullet-}$ react together, they generate an extremely reactive oxygen intermediate known as the hydroxyl radical ($OH\bullet$). These aforementioned free radical species initiate damage to cells, and promote lipid peroxidation by reacting with polyunsaturated fatty acid side chains of membrane lipids (Weinberger *et al.*, 2002). The lipid hydroperoxide products produced can directly harm membranes and proteins, as well as being a strong inhibitor of cellular enzymes. Oxygen free radicals damage DNA directly and cause protein sulfhydryl oxidation.

Due to the extremely reduced antioxidant defence system, premature infants are sensitive to excess oxygen, which can lead to toxicity. Enhancing the antioxidant defences in a premature infant would decrease risks such as chronic lung disease, as well as maintain homeostasis of protease expression and growth factors.

1.2.5. Antioxidant protection

Antioxidants can offer defence against free radicals by donating electrons to their unpaired electrons, and can delay or inhibit oxidation (Halliwell, 1990). An example is Vitamin E, which is a lipid-soluble antioxidant found in cell membranes. Vitamin E is able to protect against lipid peroxidation as it acts directly with a variety of oxygen radicals, including singlet oxygen, lipid peroxide products, and the superoxide radical, to produce a fairly harmless tocopherol radical (Bisby *et al*, 2008). Similarly, vitamin C, a water soluble vitamin, has the ability to act on the tocopherol radical to regenerate reduced tocopherol. Vitamin C can also directly react with superoxide, hydroxyl radicals, and singlet oxygen (Valko *et al.*, 2007). Antioxidant defence systems can be enzymatic or non-enzymatic. Humans have a variety of general antioxidant defences, including enzymatic antioxidant defences including proteins such as catalase, glutathione peroxidase (GPx), superoxide dismutase (SOD), and metal-binding proteins (Christen *et al.*, 1990). Ascorbic acid (vitamin c), α -tocopherol (vitamin E), glutathione (GSH), flavonoids and many other antioxidants are small molecules or non-enzymatic (Valko *et al.*, 2007). For example, GSH has several protective roles against oxidative stress, but it can act as an antioxidant to scavenge hydroxyl radicals and singlet oxygen directly. Similarly, GSH has the ability to regenerate some antioxidants back to their active forms. Previous studies have

demonstrated that low-molecular weight antioxidants are involved directly in the conversion of ROS to less reactive species (Valko *et al.*, 2007).

PIs are at a greater risk for oxidative stress and require greater antioxidant protection in order to maintain homeostasis within the body. Since human milk contains bioactive components and several protective qualities in relation to the infant's immune system and growth, it has been demonstrated that human milk has greater protective mechanisms than infant formula milk (Tsopmo *et al.*, 2011). Human milk can be protective with a three- to ten-fold risk reduction in infants fed human milk compared with those fed formula milk (Lin and Stoll, 2006). Addition of antioxidant properties to formula milk can potentially strengthen the immune defence system and protect the infant from diseases associated with oxidative stress.

1.2.6. Tryptophan

Tryptophan (Trp) is a limiting amino acid in infant nutrition. Human milk contains a relatively higher content of tryptophan than do both soy- and milk-based infant formulas. To increase its concentration in the infant diet, α -lactalbumin enriched formulae may be given to term infants (Sandstrom *et al.*, 2008). Melatonin, an endogenously produced Trp metabolite was found to be a scavenger of a number oxygen reactive species (hydroxyl radical, singlet oxygen, and hydrogen peroxide) and nitrogen reactive species (nitric oxide and peroxy nitrite anion), when analyzed *in vitro* and *in vivo* (Reiter *et al.*, 2003). Katayama and Mine reported that Glutathione S-transferase, a key antioxidant enzyme was induced by cells pre-treated with tryptophan (Katayama and Mine, 2007).

Studies have demonstrated that, *in vitro*, tryptophan released from digested human milk mimicking PI gastrointestinal tract has free radical-scavenging ability (Tsopmo *et al.*, 2009). It was assumed that delocalization of electron of tryptophan aromatic played a role in its scavenging activity. Other studies report that tryptophan can help control oxidant effects when working together with vitamins C and E (Feksa *et al.*, 2006). However, tryptophan's interaction with other molecules must be further evaluated to support its potential antioxidant role in PI diet.

Because tryptophan is not available in sufficient amounts in infant formula or other alternative milks, it may be necessary to supplement formula with tryptophan to a level similar to what is present in human milk (Allegri *et al.*, 1993) which can be considered safe. High doses of tryptophan in adults 100mg/kg/day cause gastric irritation, vomiting, and head twitching. Levels of 200mg/kg/day tested in rats showed that they lost 25% of their body weight and had reduced liver weights of 35-40% with distinct liver atrophy, which was diagnosed as toxic hepatitis (Benevenga and Steele, 1984). Toxic levels of tryptophan have not yet been established for infants.

1.2.6.1. Tryptophan in breast milk and formula

Tryptophan is found in all mammalian milk products. Human breast milk contains approximately 33 mg of tryptophan per kilo calorie as shown in Table 3 (Koletzko *et al.*, 2005), which is higher than that found in infant formula milk. This is due to the higher concentration of α -lactalbumin present in human milk compared to formula. The α -lactalbumin is a dominant

protein found in human milk and is rich in tryptophan (Food and Agricultural Organization of UN, 2005). Human milk has a concentration of approximately 2.2- 2.4 % tryptophan, whereas infant formula milk supplemented with protein has about 1.7% (Tsopmo *et al.*, 2009). Literature suggests that tryptophan containing proteins are found in greater concentrations in soybean infant formula milk than those from bovine milk, yet its total tryptophan content is still significantly lower than that found in human milk (Food and Agricultural Organization of UN, 2005). Addition of tryptophan to infant formula milk has been documented, and infant formula milk supplemented with 10mg/dL demonstrated similar tryptophan concentrations of an infant fed mother's milk (Tsopmo *et al.*, 2009). It is also interesting to note that the non-protein tryptophan content of colostrum is greater than that of both bovine milk and mature breast milk, but does decrease in the first week of lactation (Allegrì *et al.*, 1993). However, this higher level of free tryptophan in colostrum in the initial week post-birth could be attributed to supplying the central nervous system and to allow for homeostasis mother-fetus adjustment after removal from the placenta (Allegrì *et al.*, 1993). Soy-based formulas actually have a higher content of non-protein tryptophan than that in bovine milk placenta (Allegrì *et al.*, 1993). In addition, casein hydrolysates are occasionally used as a source of nutrition in infants with gastrointestinal disturbances. The free tryptophan levels in casein hydrolysates are high and may result in vitamin B6-deficiency (Allegrì *et al.*, 1993).

Table 3. Proposed values for amino acid content in the human milk reference protein expressed as g/100g protein and as mg/100 kcal (Koletzko *et al.*, 2005)

Amino Acid	g/100g protein	mg/100kcal
Cysteine	2.1	38
Histidine	2.3	41
Isoleucine	5.1	92
Leucine	9.4	169
Lysine	6.3	114
Methionine	1.4	24
Phenylalanine	4.5	81
Threonine	4.3	77
Tryptophan	1.8	33
Tyrosine	4.2	75
Valine	5.0	90

1.2.7. Bacteria in milk and infant formula

Milk is an excellent growth medium for microorganisms found in the food environment and in favorable conditions they can multiply quickly and dramatically (Rasolofo *et al.*, 2010). Bacteria are metabolically active, and some are known to cause disease while others are used in the medical field (Angert, 2005). The microbial cell has a variety of genotypic and phenotypic changes that can occur to maintain its growth and survival, and thus can endure nutrient/biochemical and environmental stresses and exist under a large range of physical conditions (Roszakt and Colwell, 1987). The microbiological quality of milk is controlled using treatments such as the addition of CO₂, microfiltration, pasteurization, and thermization to eliminate or inhibit organism growth (Champagne *et al.*, 1994; Rasolofo *et al.*, 2010). Despite

treatments, harmful bacteria can still be present in pasteurized milk and cause outbreaks, though rare, from pathogenic organisms. For example, *Staphylococcus aureus* is commonly found in milk (Peles *et al.*, 2007) and produces enterotoxins in contaminated milk leading to food poisoning (Asao *et al.*, 2003). Vulnerable groups such as preterm infants cannot afford to be infected with bacteria with their pre-existing health complications. Infant formula milk has had bacterial outbreaks as well, and the newborn infant has a sterile gastrointestinal tract that can be quickly colonized through oral ingestion (Mackie *et al.*, 1999). Specific microbes commonly tested for in IF are *Staphylococcus aureus*, *Bacillus cereus*, *Clostridium perfringens*, Enterobacteriaceae (or coliforms) and *Salmonella* and their presence can have serious health complications for an infant (Forsythe, 2005). Enterobacter sakazakii infections in neonates cause bacteraemia, NEC and infant meningitis and has had serious outbreaks in recent years (Iversen and Forsythe, 2003). A direct count for Enterobacteriaceae is used to measure the quantity of enteric-like organisms within infant formula samples. Enterobacteriaceae are common in the large intestines of animals and are not necessarily harmful. Therefore, their presence is used only as an indication of the presence of serious intestinal pathogens (Forsythe, 2005).

E. coli is an enteric bacterium and is part of the Enterobacteriaceae bacterial family and is anaerobic, gram-negative rod, versatile to its environment and has no growth factor requirements. *E. coli* outbreaks, such as with strain O157:H7, have occurred from consumption of both improperly pasteurized and unpasteurized milk which causes hemorrhagic colitis and other health complications (Guodong *et al.*, 1997; Clough *et al.*, 2009). In addition, it has been found in cross-contamination with HM and IFM (Benno *et al.*, 1984; Penders *et al.*, 2004).

Studies have shown that compounds in human milk have been able to decrease *E.coli* growth (Newburg, 1997), and that infants fed with human breast milk had lower *E.coli* numbers in fecal matter compared to infants fed infant formula milk (Benno *et al.*, 1984; Penders *et al.*, 2004). *Escherichia coli* is used in these experiments since it is known to be a “model organism” due to its ability to reproduce quickly, its basic nutritional requirements, its ease of maintenance, and its documented genomic sequence (Blattner *et al.*, 1997).

1.3. Antioxidant potency of amino acids

1.3.1. Immune and antioxidant properties of amino acids

Amino acids (AAs) are organic substances containing both an amine group and a carboxylic acid group (Wu, 2009). There are over 300 amino acids found in nature and they are comprised of carbon, hydrogen, oxygen and nitrogen; they are characterized by their side-chains. These side-chain variations account for the AAs having a variety of functions and biochemical properties. Only 20 of the amino acids are essential, and serve as building blocks of protein (Curis *et al.*, 2007).

It has been established that a lack of protein weakens the functioning of the immune system, which makes an animal more prone to disease (Li *et al.*, 2007). Proteins are a potential source of antioxidant capacity (Wayner *et al.*, 1987). The availability of amino acids found in plasma is decreased, when a deficiency in dietary protein is present. The most affected amino acids in relation to the decrease are: cysteine, glutamine, methionine, and arginine; it has been

determined that these four amino acids strengthen the immune function (Li *et al*, 2007; Tan *et al*, 2008a; Van Brummelen and du Toit, 2007). Although not established, tryptophan and proline are potentially involved in immune functions. Tryptophan has been shown to play a role involving lymphocytes and macrophages when catabolized (Melchior *et al*, 2003). Similarly, literature has stated that a lack of proline catabolism leads to a weakened gut immunity *in vivo* (Sun *et al*, 2002). Antioxidants play a role in the immune system by reducing oxidative stress. Methionine and cysteine have demonstrated effective antioxidant effects in some oils such as from rice or corn (Ahmad *et al.*, 1983). Little research is present, yet the studies on amino acid antioxidant activity have shown some contradiction with the different orders of antioxidant effectiveness (Ahmed *et al*, 1983; Meucci and Mele, 1997).

1.4. Rationale

Premature infants are new-borns that have been delivered before the completed gestation period of 37 weeks (Russell *et al*, 2007). Premature birth is associated with chronic conditions, neurodevelopmental disorders and morbidity (Hack *et al.*, 2002; Gray *et al.*, 2004; McCormick and Richardson., 2002). Rates of preterm birth continue to rise and are 12.3% of 4 million annual births in the United States (Russell *et al*, 2007). However, the survival of PT infants is increasing as well despite the 15- to 75-fold infant mortality rate in preterm and very preterm babies (Mathews *et al*, 2004). Despite improvements in feeding and oxygen supplementation, preterm infants are still susceptible to oxidative stress (Halliwell and Gutteridge, 1999).

Optimal nutritional support is extremely important for newborns for short-term and long-term health and for their survival. Nutrient supplementation or fortification of infant formula is required to meet an infant's nutritional requirements (Fanaro *et al.*, 2010). However, bioactive compounds must also be considered as a potential additive to formulas, since nutrients alone cannot imitate the composition of human breast milk nor its beneficial effects.

Preterm infants have nutritional supplementation as well as oxygen supplementation. However, preterm babies are vulnerable to oxidative stress due to their immature immune systems (Saugstadt, 1990). In order to reduce the damaging effects from diseases such as NEC, the compromised immune system of the preterm infant must be strengthened. However, IF must be made as close as possible to human milk. Thus, additives of bioactive components from human milk may reduce damage resulting from oxidative stress and decrease the incidence of disease if found to have antioxidant qualities. Additives to IF that have the ability to quench free radicals would be beneficial.

Studies on human breast milk peptides are scarce, and as such, known bioactive peptide sequences from human breast milk are minimal. Breast milk peptide sequences are comprised of amino acids, but neither essential nor all amino acids' antioxidant capacity is listed comparing each other. Human breast milk peptide sequences with amino acids that have a higher antioxidant potential will have a greater ability to quench free radicals.

Public data on additives to IF and its relationship to bacterial growth is lacking. Studies have shown that some antioxidants also have antimicrobial properties. Bioactive compounds that are both antioxidant and antimicrobial will allow for infant protection against the many health challenges they face.

1.5. Hypothesis

1. Human milk peptides can effectively prevent oxidative deterioration of milks
2. Quality of milks supplemented with peptides and tryptophan is similar to un-supplemented milks.
3. Some essential amino acids have peroxy radical scavenging activities
4. Tryptophan and human milk peptides will alter bacterial growth

1.6. Objectives

1. Determine antioxidant capacity of human peptides and whether it has antimicrobial potential.
2. Determine sensory quality of milks (human milk, Formulas) supplemented with human milk peptides and tryptophan.
3. Determine the effects of added tryptophan on bacteria growth in formulas.
4. Determine peroxy radical scavenging activity of essential amino acids to begin to form a database.

2. Materials and methods

2.1. Chemicals and reagents for antioxidant assay

AAPH (2,2-Azobis), Amonium thiocyanate, Glycine, Iron (II) chloride, L- Glutathionine reduced, L-Alanine, L-Asparagine, L-Cysteine, L-Glutamic acid, L-Glutamine , L-Histidine , Linoleic acid, L-Isoleucine, L-Serine, L-Threonine, L-Tryptophan , L-Tyrosine, Potassium phosphate monobasic, Rutin trihydrate, α -Tocopherol, were purchased from Sigma Aldrich (Oakville, ON). Acetone, ethanol, fluorescein, L-Ascorbic acid, sodium hydroxide, Trolox, disposable 1.5 ml semi-micro UV-cuvettes were purchased from Fisher Scientific (Nepean, ON). L-Arginine, L-Aspartic Acid, L-Leucine, L-Methionine, L-Phenylalanine, L-Proline, L-Valine, Potassium phosphate dibasic were purchased at Bioshop (Burlington, Ontario). Ethyl alcohol was purchased from Commercial Alcohols (Boucherville, QC). Hydrochloric acid was purchased at Anachemia (Kirkland Lake, ON). Methanol was purchased at Caldeon (Georgetown, ON).

Human milk samples were previously obtained from three volunteer breastfeeding mothers (Tsopmo, *et al.*, 2011), and collected using a mechanical breast pump (Ameda-Egnel Lincolnshire, Illinois) over the initial four months of lactation. The human milk samples were stored at 20°C and were transferred in dry ice to the laboratory and kept at -80 °C. The protocol for peptides was previously identified and small molecular weight peptides from 4 to 6 amino acids Table 4) were selected for synthesis by ProlImmune Inc. (Bradenton, FL, USA) (Tsopmo *et al.*, 2011).

2.2. Chemicals and reagents for antimicrobial assays

Agar, D-glucose, Tryptone, Yeast extract were purchased from Bioshop. L-Tryptophan was purchased from Sigma Aldrich. Tryptic soy broth and tryptic soy agar was purchased from Becton Dickinson (Sparks, MD, USA). Resazurin was purchased from Sigma Aldrich. *Escherichia coli* strain DH5 α was purchased from Invitrogen (city, province). Similac Regular powdered infant formula, Similac Advance liquid formula, Isomil Advance soy infant powder, and liquid Enfamil A IF were purchased from a local retailer.

Small molecular weight peptides from 4 to 6 amino acids were previously synthesized, where peptide 23-8 (ISELGW) had demonstrated free radical scavenging activity. Since tryptophan was contained within the sequence, tryptophan's importance in the peptide 23-8's activity can be assessed as well as whether its position within the sequence alters activity; these derivatives are described in Table 4.

Table 4. Human breast milk peptide sequences and ID

ID	Sequence	MW
23-1	HNPI	479
23-2	VPVQA	512
23-3	PLAQPA	595
23-4	VPYPQ	602
23-5	VPNSYP	675

23-6	NPYVPR	744
23-7	YGYTGA	630
23-8	ISELGW	703
23-8-4	ISELGW	703
23-8-5	WISELG	703
23-8-6	WISELGW	890
23-8-7	ISELWG	703
23-8-8	ISELGF	665
23-8-9	ISELGY	681
23-8-10	[ACE]-ISELGW	746
23-8-11	ISELGW-[NH ₂]	703
23-8-12	IS*ELGW	882

*Phosphorus group on serine (S)

2.3. Lipid peroxidation assay

The ability of the novel human breast milk peptides to inhibit the formation of lipid peroxides (LP) using linoleic acid emulsion system are evaluated as well as its potential to scavenge free radicals using oxygen radical absorbance capacity assays according to literature (Osawa and Namiki, 1985) with several modifications, and was compared to alpha-tocopherol. Linoleic acid solution was prepared from 30µL linoleic acid into 2.5 mL 99.5% ethanol. Human milk peptide samples (table 4) were prepared at 250 µM in 50mM phosphate buffer (pH 7.0) and were incubated in the dark at 50°C in screw cap vials for 4 days in the Precision 280series

waterbath, analyzed for 4-7 days with Cary 50- Bio UV-Vis spectrophotometer Varian (Mississauga, ON). Degree of oxidation measured using ferric thiocyanate values (Sakanaka *et al.*, 2004). Reaction solution (50µL of sample) was mixed with 2.35mL 75% ethanol and 50µL 30% ammonium thiocyanate added to 0.02 M ferrous chloride solution in 3.5% HCL. Thiocyanate value was measured after 10 min at absorbance of 500 nm on Cary 50- Bio UV-Vis spectrophotometer Varian during 4 days of incubation.

The percentage inhibition for lipid peroxidation was calculated using the following equation:

$$\% \text{ Inhibition} = ((A_0 - A_c) / A_0) * 100$$

A₀ = initial absorbance (control)

A_c = Sample Absorbance

2.4. Oxygen radical absorbance assay

The oxygen radical absorbance capacity (ORAC) assay measures the free radical scavenging activity against the peroxy radical, which is the most common reactive oxygen species (ROS) found in the body. Potassium phosphate buffer (75 mM, pH 7.4) was used to prepare all reagents, standards, amino acid and peptide samples, and the control. All solutions were vortexed until dissolved or were sonicated for ten minutes. Rutin trihydrate at 10 µM was used as the antioxidant control. Trolox concentrations used were 6.25, 12.5, 25, 50, and 100 µM were used to establish the calibration curve. Twenty essential amino acid samples (0.5-20mg/ml) and the 17 human milk peptides (7.5-15 µM) were individually analyzed at four

replicates each with repeat experiments. Fluorescein at 0.082 μM and AAPH at 0.15 M was prepared immediately before use. Gen5 Software was used on the fluorescence reader model FLx800 purchased from Bio-Tek Instruments, Winooski, VT.

2.5. Antimicrobial assay

2.5.1. Resazurin test

When preparing the bacterial suspension to inoculate the infant formula milk an isolated 24 hour culture of *E. coli* on TSA was transferred to 25 mL TSB broth with an inoculation loop and grown at 37°C for 48 hours at 150 rpm (incubator shaker MaxQ4450 Thermo Scientific). Varying concentrations (10^5 to 10^{11} cells/ml) and volumes (range of volumes) of *E. coli* were inoculated into 10 mL infant formula milk samples. *E. coli* is inoculated into varying samples at varying concentrations and is diluted up to $10^6\times$ with inoculations of 100 μl – 4000 μl to determine at which concentration and volume *E. coli* can be detected by the resazurin test. At the determined detection level (concentration and volume), the effect of tryptophan on bacterial growth or inhibition can be evaluated. Tryptophan (0.1 mg/ml) solution is sonicated (sonicator VWR) and filtered using a low protein binding syringe filter (0.2 μm) and added to infant formula milk samples from 250 μl - 2500 μl . Formula is prepared by adding 1.6 g infant formula powder to 10 ml autoclaved tap water nanopure water. Resazurin solution was prepared from 5.5 mg (Sigma Aldrich certified by Biological Stain Commission) and adding 100 ml of boiling nanopure water. Resazurin is added to tubes (ratio of 1:10) before samples. Infant formula milk samples with and without inoculation and additives are added to resazurin solution. Infant formula milk with tryptophan and *E. coli* addition are incubated for 24

hours. Four successive hourly intervals are analyzed, followed by a 24 h qualitative reading by observing colour change. *E.coli* is inoculated into samples at varying concentrations and is diluted up to 10^6 x with inoculations of 100 μ l – 4000 μ l.

Table 5. Resazurin test: colour as an indication of bacterial levels

Colour of solution	Quality of milk	Bacteria Presence	Score
Blue (no colour change)	Excellent	Extremely low numbers	0
Light blue	Very good	Very low numbers	1
Purple	Good	Low numbers	2
Purple-pink	Fair	Medium numbers	3
Light pink	Poor	High numbers	4
Pink	Bad	Very high numbers	5
White	Very bad	Extremely high numbers	6

2.5.2. Total plate count

To determine effect of peptides on growth of *E.coli*, peptides were plated with bacteria and compared to positive and negative controls using ANOVA one-way on determined CFU/ml counts. *E.coli* suspensions were prepared as described previously (Section 2.5.1.). Peptides 23-6, 23-7, 23-8 were measured out at 7.3 mg, 6.2 mg, and 6.9 mg respectively and added to 0.5 ml nanopure water before filtering. Peptides are plated onto TSA plates where 150µl of each peptides was plated with *E.coli* (5 µl at a concentration of 10^5 cells/ml). *E.coli* and water acted as the positive control, and the negative control was water only. Each peptide sample and control plate had three replicates and a duplicate with three replicates. Plates were incubated at 37°C for 22 hours at 150 rpm. Plate count was performed and expressed as colony forming unit (CFU)/ml.

2.6. Sensory analysis

1ml of powdered infant formula (27 mg/ml), industry prepared liquid formula and control were injected into vials with up to 11 replicates. Peptides (12.4mg/ml) are added to infant formula. Each vial was heated to 80°C and then agitated at 500rpm for 480 s immediately prior to injection. A sample headspace volume of 1 mL was drawn from the vial at 0.5 mL/s using a syringe maintained at 90°C to avoid condensation. Using this syringe, the sample headspace was injected into the e-nose at a speed of 1mL/s, and delivered to the sensors with a pure air carrier gas ($O_2 + N_2 > 99.95\%$, $H_2O < 5\text{ppm}$, $C_nH_m < 5\text{ ppm}$, $CO_2 < 5\text{ ppm}$). After 24 hours, PCA and DFA graphs are generated.

3. Results

3.1. Lipid peroxidation assay: antioxidant potency of human breast milk peptides

Prevention of lipid peroxidation, a major contributor to oxidative stress, is analyzed by using the linoleic acid emulsion system and compared to alpha-tocopherol. Human milk peptide 23-8 (ISELGW) and its derivatives 23-8-8 (ISELGF), 23-8-9 (ISELGY), 23-8-10 ([Ace]-ISELGW), 23-8-11 (ISELGW-[NH₂]) and 23-8-12 (IS*ELGW) were measured for their antioxidant activity at 250 µl against one another. Peptide 23-8-11 has the greatest free radical scavenging potential compared to the other peptides in the experiment, but did not exceed the scavenging potential of the standard, Vitamin E; most peptides displayed a potential for inhibiting formation of lipid hydroperoxides that results from autoxidation of linoleic acid over a four day period and were able to maintain a higher antioxidant activity over 4 days compared to the control. Peptide 23-8-8 demonstrated a very weak ability to inhibit lipid peroxidation, and had no difference

between the control on day 1.

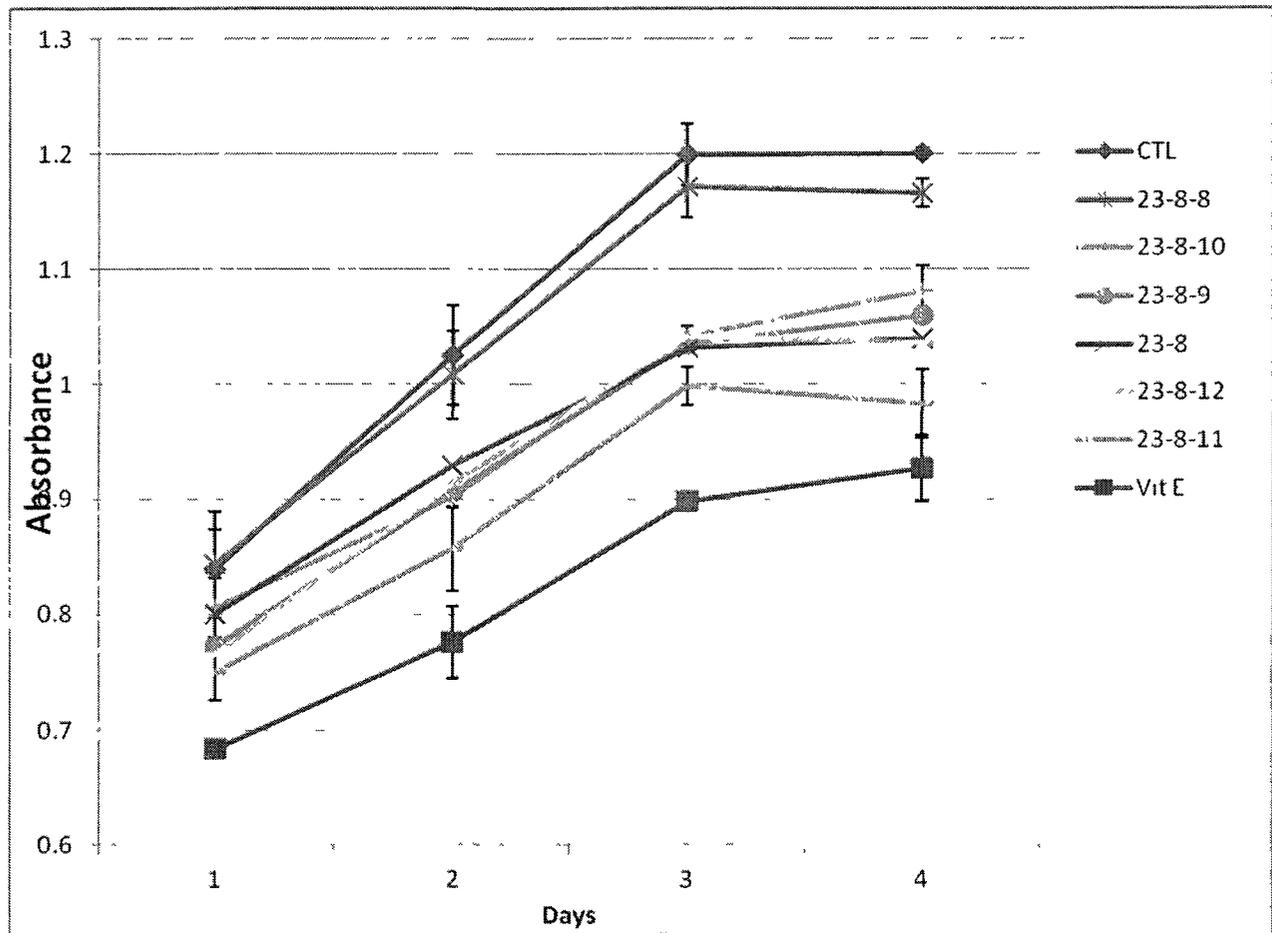


Figure 3. Bioactivity of breast milk peptides over 4 day period at 250 μM

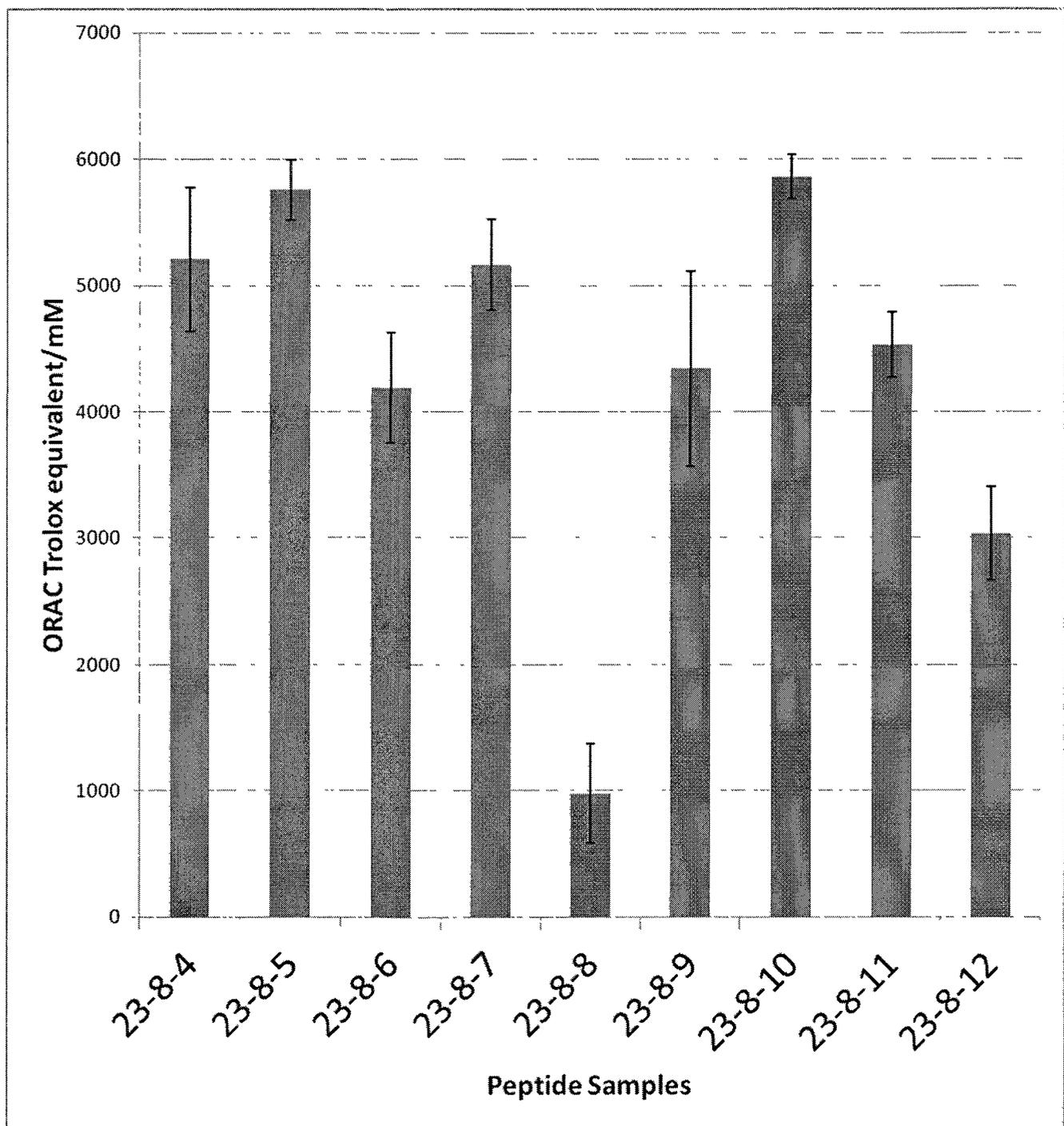


Figure 4. Human milk peptides demonstrating antioxidant potential

The ability of human breast milk peptides to scavenge peroxy radical was measured in the ORAC assay (Figure 4), where 23-8-10 ({ACE]-ISELGW) and 23-8-5 (WISELG) showed the highest

activity followed by 23-8-4 (ISELGW) and 23-8-7(ISELWG). Results suggest that 23-8-8 has lowest antioxidant potential.

3.2. ORAC assay demonstrating the antioxidant potential of 20 essential amino acids

The ability of twenty amino acids to scavenge peroxy radical was measured in the ORAC assay. The amino acids that exhibited the highest antioxidant activity from highest to lowest were cysteine, tryptophan, tyrosine, methionine, histidine, leucine, phenylalanine and are shown in bold. The remaining amino acids (alanine, arginine, asparagine, aspartic acid, glutamic acid, glutamine, glycine, isoleucine, lysine, proline, serine, valine, and threonine) displayed negligible amount of antioxidant activity. Cysteine demonstrated 3 times higher antioxidant potential than tryptophan and tyrosine, 5 times higher than methionine and 10x higher than histidine. Although leucine and phenylalanine had some antioxidant activity, it was 63-75 times less than cysteine. Tryptophan and tyrosine have similar levels of antioxidant activity. Tryptophan is 1.5 times the antioxidant potential of methionine, 3 times histidine, and 20-22 times higher than leucine and phenylalanine.

Table 6. Oxygen radical absorbance capacity of 20 essential amino acids

Sample	ORAC (TE/mM)	Stdev
1: alanine	0.18	0.09
2: arginine	0.75	0.04
3: asparagine	0.57	0.08
4: aspartic acid	0.75	0.41
5: cysteine	589.00	22.10
6: glutamic acid	0.35	0.03
7: glutamine	0.73	0.17

8: glycine	0.05	0.09
9: histidine	59.8	1.80
10: isoleucine	0.78	0.29
11: leucine	9.23	6.59
12: Lysine	0.88	0.03
13: Methionine	111.32	4.25
14: Phenylalanine	8.72	6.00
15: Proline	0.37	0.02
16: Serine	0	0.00
17: Tryptophan	192.91	1.94
18: Tyrosine	171.42	2.11
19: Valine	0	0.00
20: Threonine	0.30	0.03

3.3. Effects of tryptophan on bacterial growth in formula milk

3.3.1. Resazurin test: *E. coli* addition to formula

Resazurin method tested the quality of infant formula milk samples in relation to bacterial population. All results demonstrated a colour change over time, including controls. Colours vary from blues, lavender, purple, pinks, and white which indicates amount of bacteria present (Table 5). In order to be able to detect whether tryptophan has an effect on bacterial growth it is necessary to determine at which concentration and cell number *E.coli* can be detected using the resazurin test.

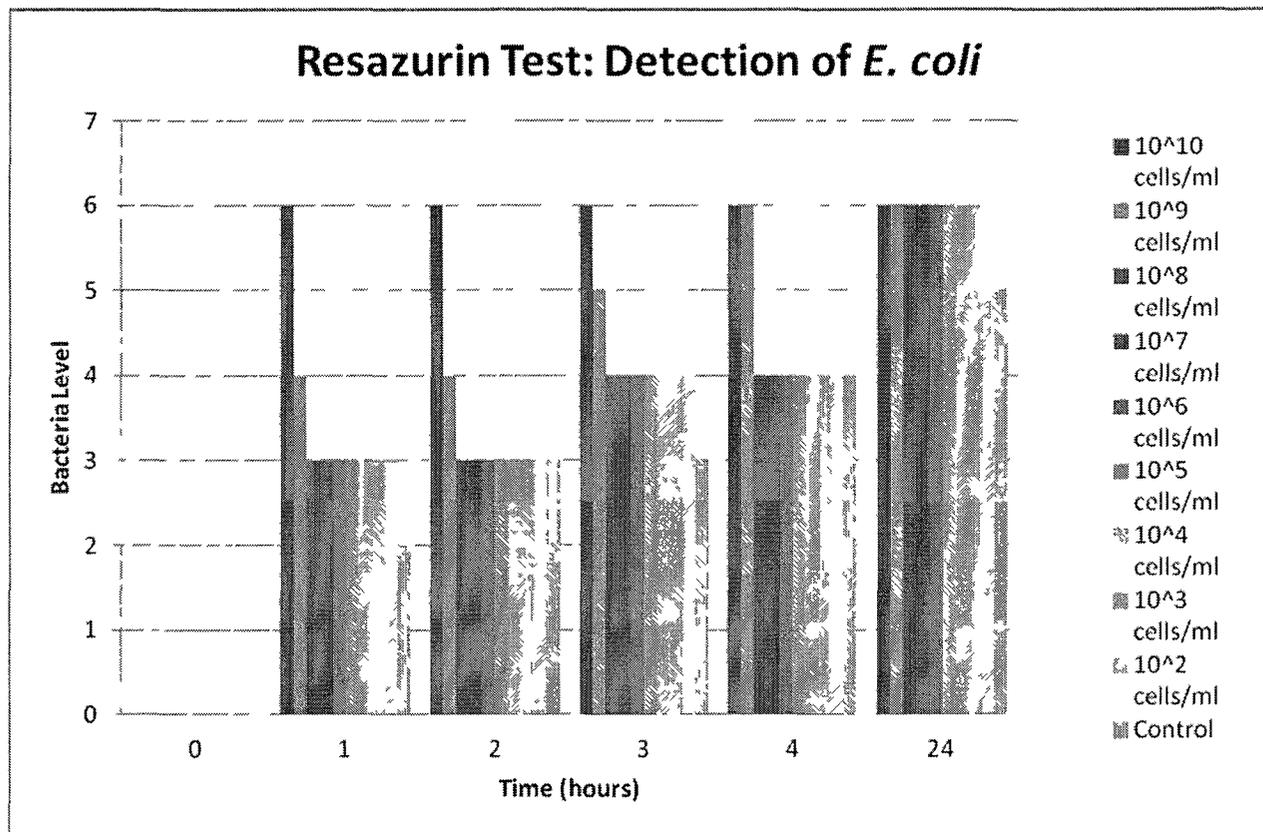


Figure 5. Resazurin test for high levels of *Escherichia coli* cells inoculated at greater volume for Group C

Group C: tubes 1-9 are all inoculated with 1000 μ l *E. coli*. Stock concentration (10^{10} cells/ml) is diluted to 10^{-2} cells/ml times. At 10^{10} cells/ml at 1 hour, the solution had turned white which indicated extremely high bacterial numbers and a “very bad” milk quality, which is true as it has the highest concentration of *E. coli*. 10^9 cells/ml was pink after an hour, resulting in high bacterial numbers. 10^8 cells/ml were purple-pink indicating medium bacterial counts and a “fair” milk quality; control was borderline purple-pink to purple and indicated “low levels” of bacteria. After 2 hours, the initial 10^9 cells/ml increased visibly in bacterial numbers since it changed to a pink colour; control demonstrated some bacterial growth as well. After 3 and 4 hours the same trend followed for all tubes, except the initial 10^9 cells/ml which increased again

in bacterial numbers, where the tubes initially at 10^{3-8} cells/ml changed from medium bacteria numbers to high bacteria numbers. The control followed the same trend, but was slower to increase in bacteria numbers maintaining mostly medium bacterial numbers until the 4th hour where bacteria increased to high levels. After 24 hours, the control did increase to very high bacteria numbers. *E.coli* can be detected quickly at very high cell counts of 10^{10} cells/ml.

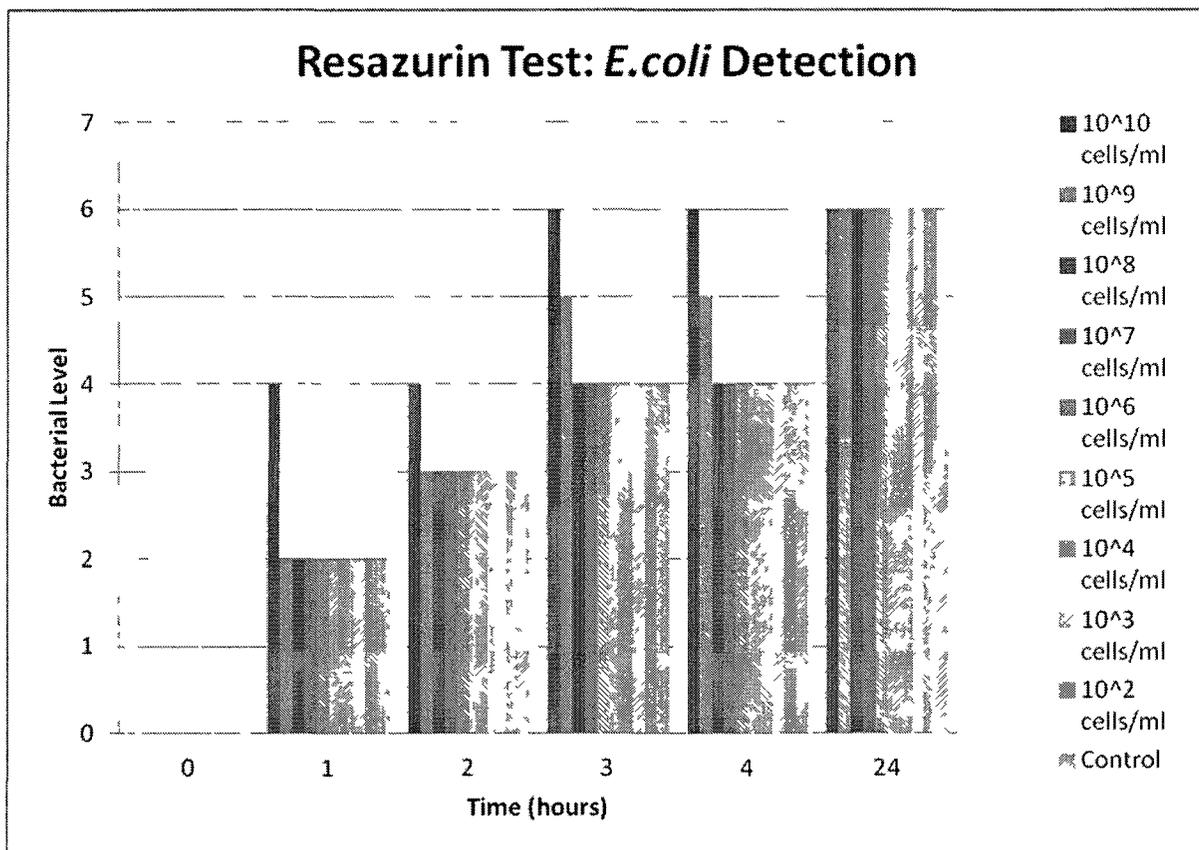


Figure 6. Resazurin test for high levels of *Escherichia coli* cells inoculated at lower volume for Group A

Group A had 100 μ l *E. coli* inoculated into formula from a 10^{10} cells/ml stock solution into 9 tubes ranging from 10^0 to 10^{-9} dilution series. After 1 hour only, it can be seen that 10^{10} cells/ml in the first tube has the highest bacterial count as it was pink, showing "high numbers" of

bacterial cells present. All other tubes showed “low numbers” of bacteria. After 2 hours, all other tubes that initially were $10^{2.9}$ cells/ml and control all increased in bacteria to “medium” number. All tubes increased in bacteria after 3 hours; the initial 10^{10} cells/ml turned white indicating “extremely high” levels of bacteria; tube 2 had “very high” levels. At 4 hours no change was visible. At 24 hours all tubes, except for control, had “extremely high level” of bacteria present and had turned white; control has “high level” of bacteria and remained pink. *E. coli* can be detected at high bacterial numbers of 10^{10} cells/ml.

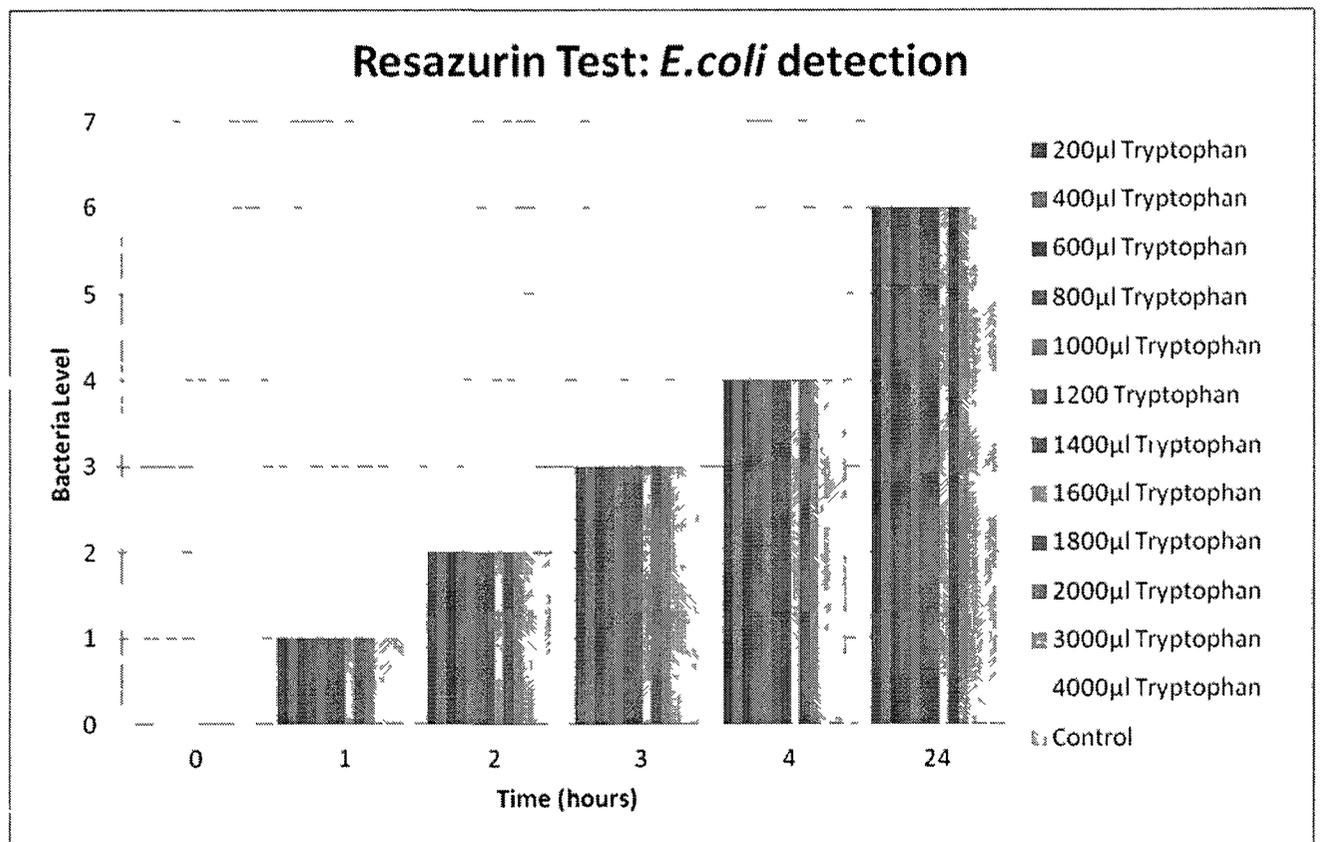


Figure 7. Resazurin test for detection of low levels of *Escherichia coli* at a range of dilutions for Group B

Group B, $100 \mu\text{l } 10^5$ cells/ml of *E. coli* were diluted up to 10^{13} . After one hour, it appears that all tubes have “very low” bacteria levels. At two hours all the tubes visibly had more bacteria and indicated “low numbers” for bacteria level. At 3 hours, “medium numbers” of bacteria was

visible according to colouration. High numbers of bacteria was apparent after four hours in all tubes. After 24 hours, the bacteria level evolved from “high numbers” to “extremely high” numbers in all tubes except control; although control also had bacterial growth, its level was deemed at “very high numbers”. *E. coli* inoculated into IFM at 10^5 cells/ml at any dilution is not able to be detected using resazurin test.

Groups A, B, and C are comparable. Group A and C only differ by the volume of *E. coli* inoculated into IFM. Group A and C displayed very similar results, except that group C indicated bacteria contamination at a faster rate than group A. Group B used 10^5 cells/ml at volumes ranging from 0-4000 μ l of tryptophan and although demonstrated some indication of the presence of bacteria overtime, there was no change in bacterial numbers or score in tryptophan treatments on infant formula milk inoculated with *E. coli*.

3.3.2. Resazurin test: *E. coli* and tryptophan addition to formula

This test was the first resazurin test which was determining whether tryptophan is able to increase or reduce bacterial growth. Tryptophan at 4mg/ml was added to formula at either 250 μ l or 2500 μ l. Group X did not have *E. coli* inoculation and group Y had 10^5 cells/ml *E. coli* inoculated at 100 μ l. Since all repetitions had the same colour and thus same bacteria level, each group of repetitions will be represented and discussed as a whole and thus as “one” tube

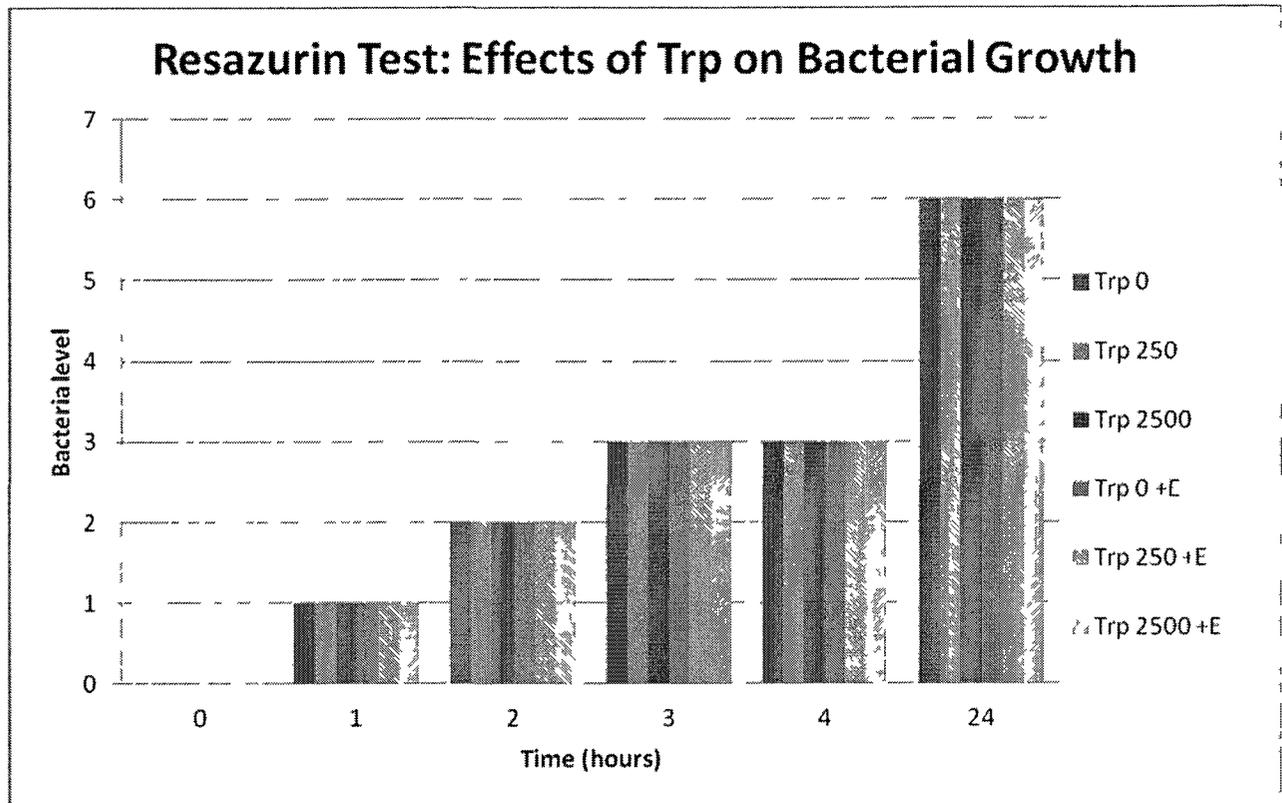


Figure 8. Resazurin test with lower *Escherichia coli* cell number and varying tryptophan volume

Tryptophan's effect on bacterial growth was tested at 4mg/ml at either 500 µl or 1000 µl. Stock *E.coli* was inoculated at either 500 µl or 1000 µl into infant formula milk. Duplicates matched the colour and thus the bacterial numbers present, and so each treatment will be discussed as a single entity. This result lead to the *E.coli* detection resazurin test ; in order to accurately screen for growth or reduction of *E.coli* cells it is imperative to determine whether they can be first detected.

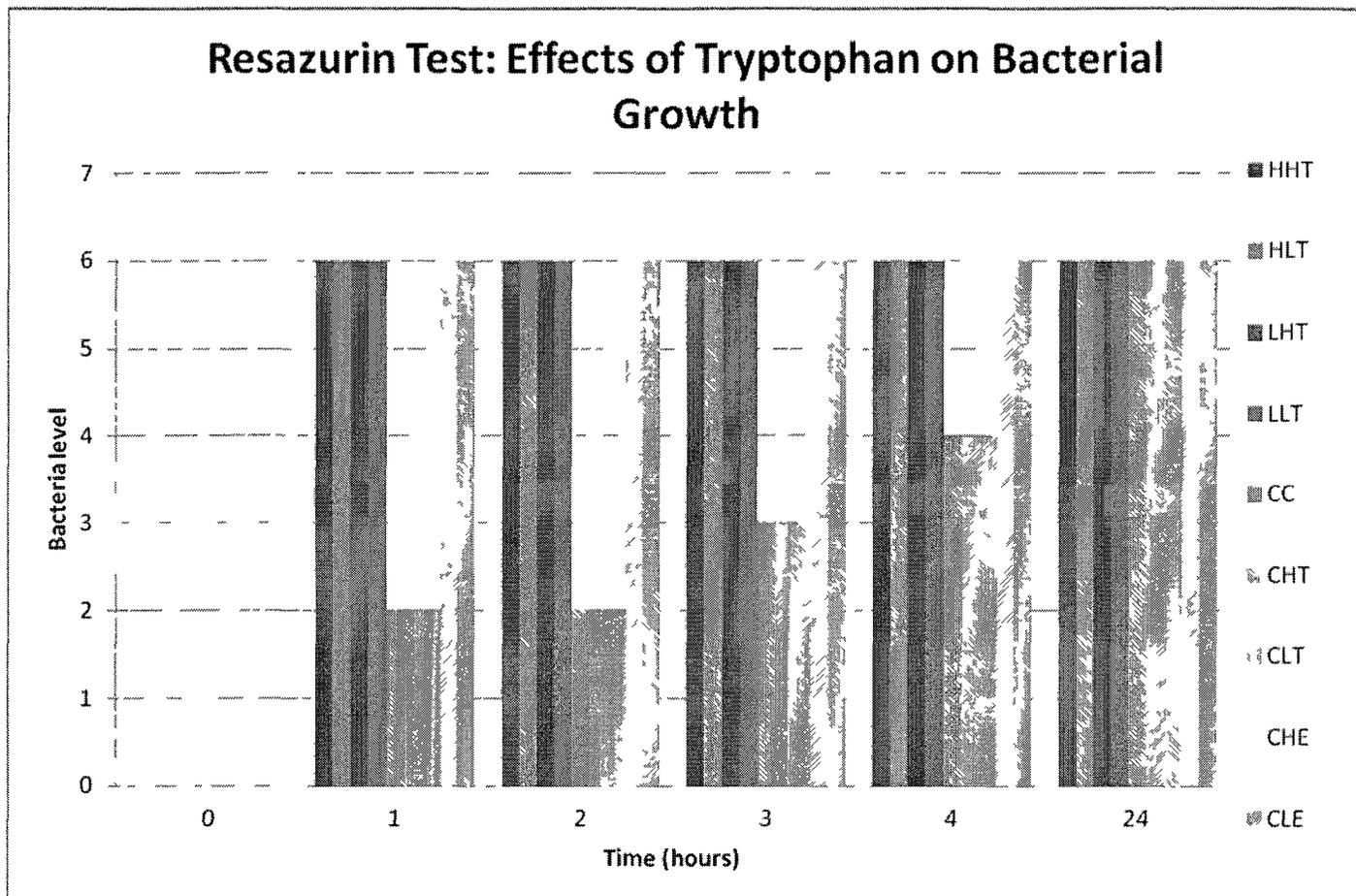


Figure 9. Resazurin test with high level of *Escherichia coli* numbers at varying volumes and tryptophan's effects on bacterial growth.

Treated groups contained both *E. coli* (at 10^{10} cells/ml) and tryptophan (4mg/ml) at varying volumes of either 500 μ l or 1000 μ l. High bacteria and high tryptophan volume (HHT) and high bacteria and lower tryptophan volume (HLT) have the greatest amount of bacteria present and are detected within an hour at a score of 6 indicating high bacterial numbers. Tryptophan did not inhibit bacterial growth. Lower bacteria and high tryptophan volume (LHT) as well as lower bacteria and lower tryptophan volume (LLT) demonstrated no difference in comparison to lower bacteria volume at 10^{10} cells/ml by showing the same score of 6. Control (CC) showed minimal bacterial growth at score 2. Controls with only tryptophan present (CHT and CLT) did not show an increase in growth for bacteria present and also showed a score of 2. Controls with

only *E.coli* (CHE and CLE) demonstrate that tryptophan had no effect on inhibition of bacteria cell numbers when comparing to HLT and LHT and showed a score of 6 indicating very high levels of bacteria.

3.3.3. Total plate count: tryptophan addition to formula

One-way ANOVA SPSS test on *E.coli* control and peptides (23-6, 23-7, 23-8) treated with *E.coli* shows a difference.

Table 7. Oneway ANOVA analysis on human milk peptides 23-6, 23-7, 23-8 treated with *Escherichia coli* and control

Fit Y by X Group, Oneway analysis of CFU/mL by treatment

Oneway Anova Summary of Fit

Rsquare	0.595881
Adj Rsquare	0.535263
Root Mean Square Error	4433.029
Mean of Response	4069.458
Observations (or Sum Wgts)	24

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	3	579536524	193178841	9.8301	0.0003
Error	20	393034846	19651742		
C. Total	23	972571370			

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
23-6	6	1277.8	380.50	155.3	878.5	1677
23-7	6	1388.8	642.79	262.4	714.3	2063
23-8	6	1033.3	333.97	136.3	682.9	1384
Control	6	12577.8	8828.22	3604.1	3313.2	21842

The ANOVA shows an F value of 9.83 and a p value of less than 0.0003 showing a difference exists. The Least Significant Difference test (LSD) determines differences between groups.

Table 8. Least significant difference test on peptides 23-6, 23-7, 23-8 and control

**Means Comparisons
Comparisons for each pair using LSD**

Level		Mean
Control	A	12577.833
23-7	B	1388.833
23-6	B	1277.833
23-8	B	1033.333

Levels not connected by same letter are significantly different

From the LSD table it can be seen that peptides 23-6, 23-7, and 23-8 are all different from the control but not from one another. These peptides are antimicrobial as there is a difference between peptides and the control, but the peptides are the same antimicrobial strength.

Oneway Analysis of CFU/ml By Treatment

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Treatment	2	1189333333	594666667	1.4960	0.2556
Error	15	5962666667	397511111		
C. Total	17	7152000000			

Means and Std Deviations

Level	Number	Mean	Std Dev	Std Err Mean	Lower 95%	Upper 95%
Control	6	52333.3	21546.8	8796.5	29721	74945
Trp 25 ul	6	71000.0	14099.6	5756.2	56203	85797
Trp 250 ul	6	55666.7	23010.1	9393.9	31519	79814

Means Comparisons

Level		Mean
Trp 25 ul	A	71000.000

Level	Mean
Trp 250 ul A	55666.667
Control A	52333.333

Levels not connected by same letter are significantly different

Table 9. Oneway ANOVA on treatments of tryptophan with *Escherichia coli*

For tryptophan, no significant difference was found as the F value is 1.496 but the p value is 0.256. The p value is greater than 0.05 and groups can be considered to be the same.

Equation 1. Colony Forming Units

$$\text{CFU/ml (CFU/g)} = \frac{\text{average number of colonies from replicates}}{(\text{dilution factor}) \times (\text{volume plated})}$$

Table 10. Colony forming units per ml on peptides 23-6, 23-7 and 23-8 treated with *Escherichia coli* and control

Treatment	CFU/ml	Stdev
Peptide 23-6	1200 ^b	380.4
Peptide 23-7	1389 ^b	642.7
Peptide23-8	1033 ^b	333.9
<i>E.coli</i> control	12578 ^a	8828

Peptides 23-6, 23-7, and 23-8 suggests having antimicrobial potential with the same level of antimicrobial strength denoted by *b* between peptides. Colony forming units are lower than that of the control. ANOVA on treatments suggested a significant difference in CFU/ml, whereas the LSD test further explained that the difference occurs only between control and peptides, but not within peptides.

3.4 Electronic nose

Electronic Noses (eNoses) is a device used to detect and recognize odours. It is a machine olfaction device with an array of chemical sensors that simultaneously respond to the volatile chemicals present in a gas sample (Amalia *et al.*,2009).

Effects on sensory properties when novel human milk peptides and tryptophan are added to either human breast milk or powdered and liquid infant formulas are evaluated using electronic nose and are portrayed using principle component analysis (PCA) graphs or discriminant function analysis (DFA) graphs. Peptide ID can be referred to from table 4.

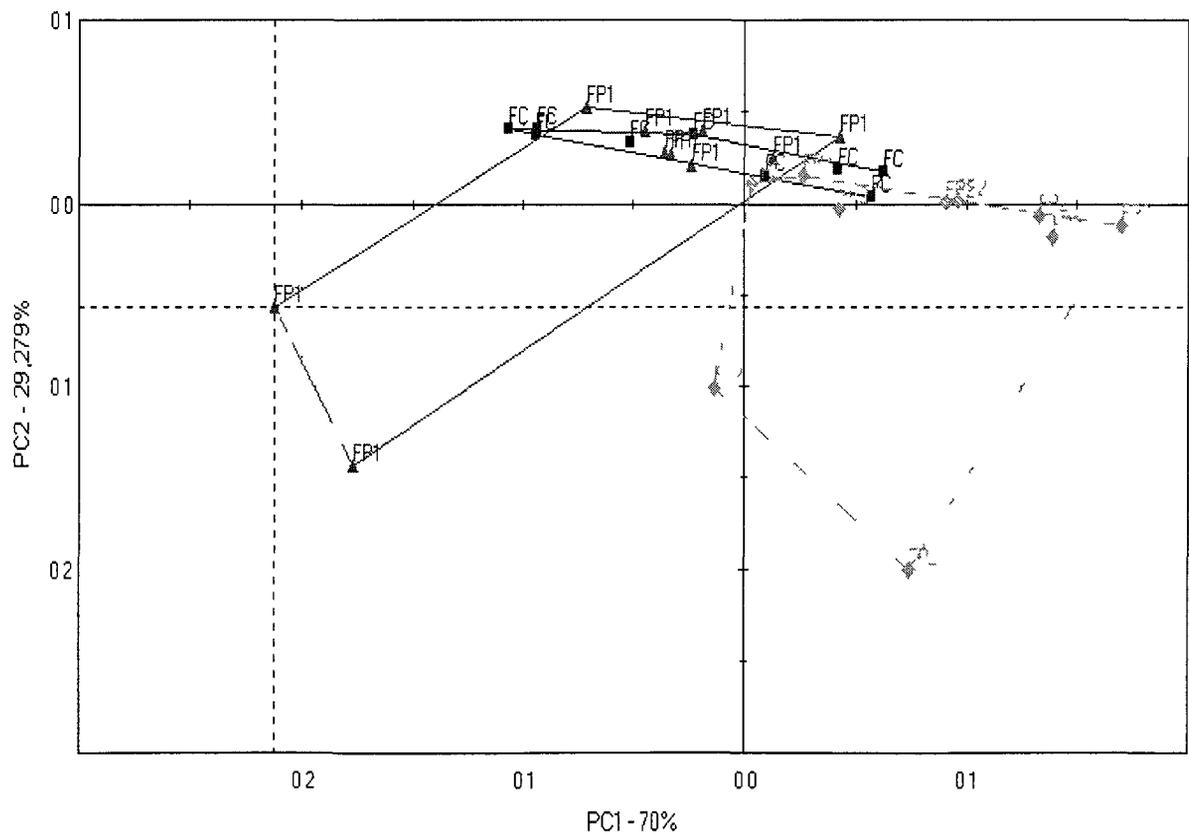


Figure 10. Principle component analysis for formula milk with and without breast milk peptide additives

PCA (principle component analysis) for FC: formula milk control in blue; FP1: formula milk with peptide 23-8-8 in red; FP2: formula milk with peptide 23-8-8' in green.

When comparing sensory characteristics of infant formula milk with infant formula milk with peptide additives, there was enough of an overlap to demonstrate that sensory characteristics are similar in terms of aroma. They have no significant difference in terms of aroma, and can be stated to have the same "smell" or sensory composition.

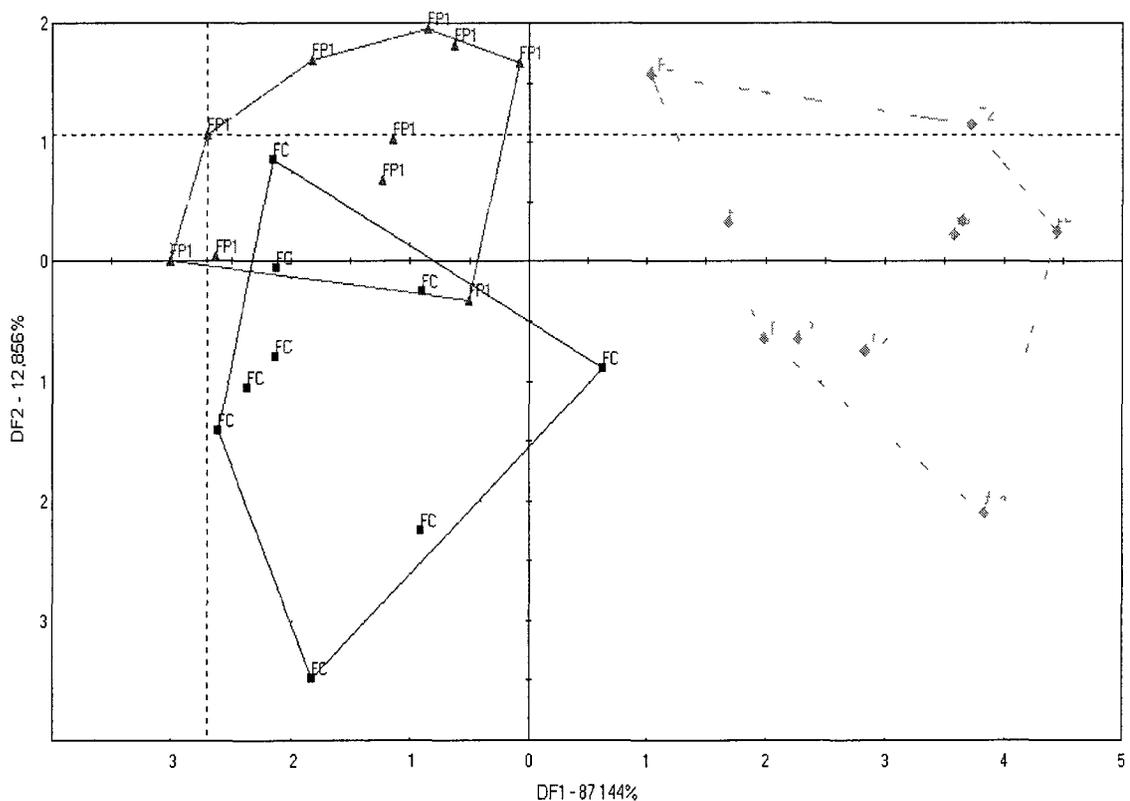


Figure 11. Discriminant analysis for formula with and without peptide addition

DFA for FC: formula milk control in blue; FP1: formula milk with peptide 23-8-8 in red; FP2: formula milk with peptide 23-8-8' in green.

DFA graphs group samples by differences in sensory characteristics by looking to differentiate between samples. Infant formula milk control and infant formula milk with peptide 23-8-8 show more sensory similarity than infant formula milk with peptide 23-8-8'. Although this graph generates the differences between samples, all samples had no significant difference in aroma. Since the peptide 23-8-8' is further removed from the other groups of data it is slightly more different in sensory composition than the other two groups, however, no significance between sensory composition exists.

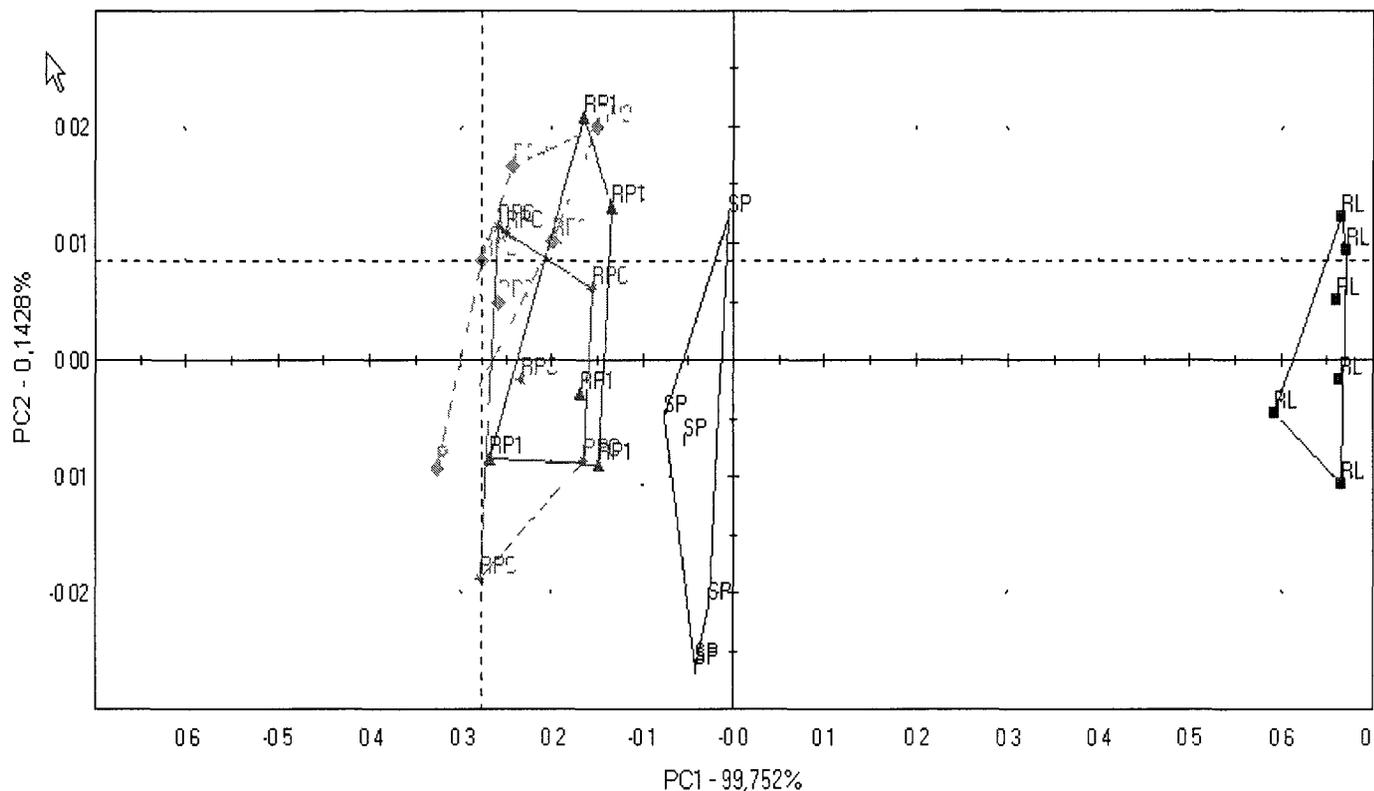


Figure 12. Principle component analysis graph for comparison between powdered regular, powdered soy, and liquid infant formula with peptide additives

PCA (principle component analysis) for RPC: regular powder (control); RP1: regular powder with peptide 23-8-4; RP2: regular powder with peptide 23-8'-6; SP: soy powder, RL: regular liquid

PCA graph of liquid infant formula milk and powdered soy and bovine milk formulas are compared to one another for similarities and shows that the liquid formula is far removed from powdered formulas. The liquid formula has the greatest difference as it is seen spatially separated on the graph so it has the most “different” aroma or sensory composition. Soy infant formula milk is more similar in terms of smell and sensory

composition to bovine-milk powdered infant formula milk but can also be seen to be spatially different and sensory characteristics do not overlap with bovine-milk powdered infant formula milk with and without peptide addition. Overlap exists between infant formula milk with peptide 23-8-4 and peptide 23-8'-6 addition. These two peptides have very similar sensory properties and their addition does not greatly vary the sensory characteristics and composition of infant formula milk when added, meaning that they have the same aroma.

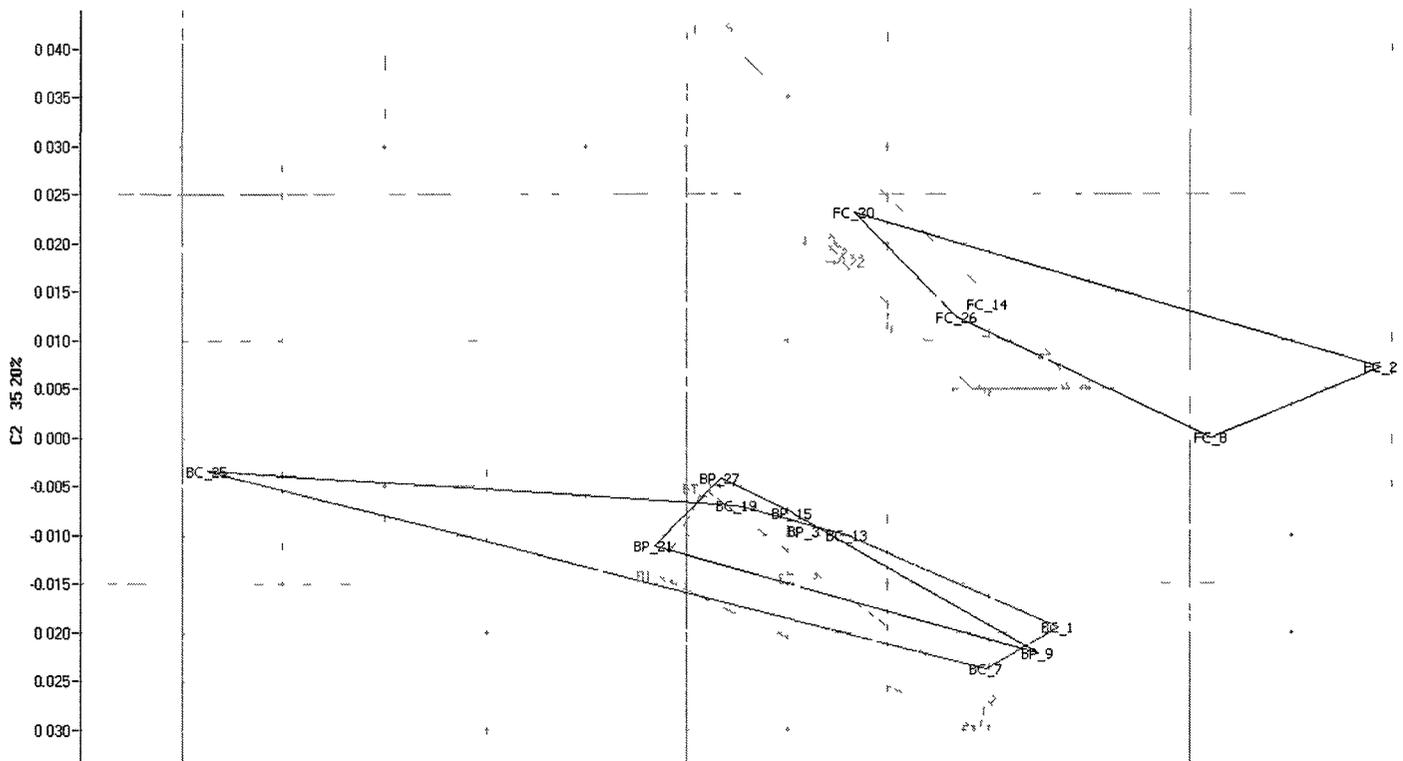


Figure 13. Principle component analysis of breast milk and infant formula milk with and without tryptophan or breast milk peptide addition

PCA showing BC: breast milk control (red), FC: infant formula control (black), BP: breast milk with peptide 23-7 (dark blue), FP: formula milk with peptide 23-7 (pink), BT: breast milk with tryptophan (green), FT: formula milk with tryptophan (light blue).

Infant formula milk with and without peptide and tryptophan addition is spatially separated from human breast milk with and without peptide and tryptophan addition. All additives showed overlap with control and demonstrate close sensory composition and same aroma.

All figures include milk-based infant formula milk powder samples with peptide additives. In all these PCA graphs, there is overlap between data for the addition of human milk peptides to infant formula milk milk-based powder with the controls. Figure 10 had 2 outliers in the peptide additive samples as well as a human breast milk sample outlier in Figure 13, which can be ignored as they are due to error. Overlap in the PCA demonstrates that human milk peptide additives do not alter the sensory composition of infant formula milk powder samples. Similarly, addition of peptides also maintains a similar sensory composition of infant formula milk with additive. The discrimination in DFA graph shows that using peptide 23-8-8 or its derivative will have no great effect on sensory composition and supports the PCA graph findings. In Figure 12 PCA graph, there was also infant formula milk soy-based and infant formula milk liquid. Both of these did not overlap with the infant formula milk powder, and were spatially separate from the infant formula milk with and without peptides. As PCA searches for similarities between samples and data points from soy and liquid infant formula milk are separate they are distinctly different in smell and composition compared to the infant formula milk milk-based powder. Breast milk and formula milk were compared (Figure 13) with

the addition of tryptophan or human milk peptides 23-8-4 and 23-8'-6 to them. Although the peptides did not change the composition or smell of the samples compared to the control, the human breast milk and formula milk were spatially separate and did not overlap. All results supported one another in determining that addition of peptides or tryptophan to infant formula milk or human breast milk does not make a significant difference in terms of aroma and can be stated to all have the same smell and sensory composition.

4. Discussion

4.1. Antioxidant potential of breast milk peptides and tryptophan

Human milk has many health benefits to an infant and has protective properties that can stimulate the immune system and play a role in endogenous defence mechanisms. Human colostrum has demonstrated antioxidant properties, but its antioxidant components are not all defined (Friel *et al.*, 2002).

The antioxidant activities of peptides measured in linoleic acid emulsion system with α -tocopherol as reference (Fig 8), peptide 23-8-11 has the greatest free radical scavenging potential; all peptides display potential for inhibiting formation of lipid hydroperoxides that results from autoxidation of linoleic acid. Linoleic acid is used since the fatty acid radical is not a stable molecule and can react readily with molecular oxygen, thus creating a peroxy-fatty acid radical commencing the chain reaction for radicals. The antioxidant potential of human milk peptides in Figure 3 suggests that peptide 23-8-11 has the radical quenching activity; all human breast milk peptides evaluated exceeded α -tocopherol antioxidant activity. However, a

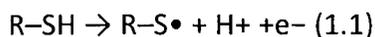
positive control must be added in order to determine the human milk peptides' effectiveness in free radical scavenging in comparison with a known potent antioxidant. Peptide 11 (23-8-11) has sequence Ile-Ser-Glu-Leu-Gly-Trp-amine; the higher antioxidant potential could be attributed to containing tryptophan within its sequence. Tryptophan has been discussed in literature as having antioxidant effects (Jiang *et al.*, 2010; Meucci and Mele, 1997). Peptide 11 (23-8-11) is unique from the other human milk peptides in that it has an amine group attached; amines have displayed antioxidant properties (Vukovic *et al.*, 2010). Peptide 23-8-10 exhibits the ability to inhibit lipid peroxidation with its sequence being [ACE]-ISELGW; it is acetylated. Peptides 23-8-4 (ISELGW) and 23-8-7 (ISELWG) showed an ability to inhibit lipid peroxidation. It is also possible that the arrangement or order of the amino acids in sequence could play a role in determining the antioxidant potential of these human milk peptides; however, 23-8-7 has the tryptophan shifted inward and still displayed similar antioxidant activity as those peptides with tryptophan attached last. The shift of tryptophan in this peptide did not appear to make a large difference. In Figure 4, peptide 23-8-5 (WISELG) demonstrated a higher antioxidant activity and has its tryptophan shifted, and it is comparable to 23-8—10 which is acetylated. Removing tryptophan completely does make a large difference as seen in Figure 3 for peptide 23-8-8 (ISELGF) which had a very low antioxidant capacity and no tryptophan present. Human milk peptides that have a tryptophan within its sequence are effective in preventing lipid peroxidation. Still, tryptophan supplementation in milks should be monitored since tryptophan can compete with other amino acids for crossing the blood brain barrier.

4.2. Antioxidant potential of 20 essential amino acids

According to Meucci and Mele (1997), no antioxidant activity was demonstrated with solutions of arginine, aspartic acid, histidine, isoleucine, glutamic acid, leucine, lysine, methionine, phenylalanine, proline, and serine. Cysteine, tryptophan, and tyrosine showed some antioxidant activity in the Meucci and Mele (1997) study where all amino acids were analyzed using the 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) Antioxidant Assay. However, in this study, the amino acids that displayed the greatest antioxidant potential in order of greatest free radical scavenging activity are: cysteine, tryptophan, tyrosine, methionine, histidine, leucine, phenylalanine. The aforementioned AAs, with the exception of leucine, either contain sulfur or are aromatic.

Cysteine and methionine are sulphur-containing compounds; sulphur-containing compounds are termed "thiols" (Gungor *et al.*, 2011). Thiol-type antioxidants contain sulfhydryl (-SH) functional groups that have a critical role in protecting cells from oxidative damage and are a play a major role in the antioxidant defence system. A greater number of -SH groups leads to greater antioxidant potential. The -SH groups are able to interact with electrophilic groups from the ROS during oxidative stress, which can reduce damage (Gungor *et al.*, 2011). Thiol compounds are essential to an organism and a reduced level of it can have serious health implications such as neurological disorders, liver failure, and stroke (Yardim-Akaydin *et al.*, 1990). Thiols have negative standard reduction potentials (-0.34V), making them reducing agents. When thiols and ROS interact with one another, the thiol is able to convert the ROS into a less toxic byproduct while the thiol itself oxidizes to a disulphide (Wardman and Sonntag, 1995). The formation of thiyl radicals (R-S•) occurs when the thiol undergoes electron

oxidation and loses the hydrogen atom from the sulfhydryl group or from the sulphur, as well as proton loss or two electron oxidation with the sulphenic acid (R-SOH).



Thiyl radicals could recombine to form disulfides under certain pH, since they are unstable (Wardman and Sonntag, 1995).

Cysteine, being a sulfur containing-compound, had the greatest antioxidant activity with a value of 589 TE/mM. The sulfur atoms in cysteine form disulfide bridges between two cysteine molecules but also play a role in determining the antimicrobial potential of some sulfides (Atmaca, 2004). Glutathione, has been shown to prevent oxidative stress, and is a tripeptide and it is synthesized from cysteine, glutamate, and glycine (Braake *et al.*, 2009). An oxidatively stressed cell can result when there is a decrease in the antioxidant capacity, which generally occurs when there is a reduction in the GSH (glutathione) (Atkuri *et al.*, 2007). Oxidative stress *in vivo* can be correlated to a deficiency in GSH since it is the most dominant intracellular free thiol, or a deficiency in its precursor cysteine (Atkuri *et al.*, 2007). There are numerous studies that support cysteine as having antioxidant properties. It has even been found that cysteine has a role in controlling antioxidant defences in response to electrophilic lipid oxidation products where a cysteine-rich protein is a sensor of oxidative stress (Levonen *et al.*, 2004). Cysteine can be produced from methionine via the transsulfuration pathway. It is of interest to note that the human fetal liver does not appear to have activity in the

transsulfuration pathway, which translates to a lack of cysteine being produced (Kalhan and Bier, 2008). This finding suggests that cysteine is only a conditionally essential amino acid for the fetus and neonate (Kalhan and Bier, 2008).

Methionine is an efficient scavenger of almost all oxidizing molecules under physiological conditions, such as, H₂O₂, hydroxyl radicals, peroxynitrite, chloramines and hypochlorous acid (Berlett and Stadtman, 1997). Methionine residues may act as endogenous antioxidants. Methionine residues play a role in the antioxidant defence system (Levine *et. al*, 1999), which was apparent from Figure 9 where it demonstrated a high value of 111 TE/mM from the ORAC assay. Oxidants react with methionine to form methionine sulfoxide. Methionine residues within a protein exhibit variability in their susceptibility to oxidation, which can correlate with the surface exposure of the residue. Surface-exposed methionine residues effectively scavenge oxidizing agents while generally preserving the biological function of the molecule (Levine *et. al*, 1999).

Both studies support one another, except that the present study shows that methionine does exhibit antioxidant potential which was not displayed in the study done by Meucci and Mele (1997). Both ABTS and ORAC assays show high correlation between each other. However, the ORAC assay has shown more significance since it utilizes a biologically relevant radical source (Thaiponga *et al*, 2006).

Tryptophan, tyrosine, histidine, leucine, and phenylalanine are all phenols. Tryptophan had the highest antioxidant activity with an ORAC value of 193 TE/mM; tyrosine had a value of 171 TE/mM; phenylalanine had a low value of 8.72 TE/mM but it was still not zero. One class of

antioxidants is the group of phenolic antioxidants composed of at least one aromatic ring and one or more hydroxyl groups. Although known for their importance in flavour, they are also now known for their ability to quench free radicals (Escarpa and Gonzales, 2001). Phenols are more acidic than alcohols and can donate protons, which is one of the reasons they are able to scavenge free radicals. They have delocalized π -electrons which allow them to transfer electrons while being stable (Edreva, 2008). A free radical removes hydrogen from the phenol's hydroxyl group and creates a radical (Arora *et al.*, 1998; Sakihama *et al.*, 2000). The reaction of the radical continues as a chain reaction until it is stabilized as it is energetically unflavoured. Phenolic compounds have antioxidant action, in part, because of their ability to chelate metals especially iron and copper due to the nucleophilic character of the benzene ring (Michalak, 2006). Literature shows that tryptophan and tyrosine have been observed to have antioxidant effects (Jiang *et al.*, 2010; Galisteo and Herraiz, 2004; Meucci and Mele, 1997; Nimalaratne *et al.*, 2011).

Tryptophan's antioxidant activity is likely due to its indole group (Galisteo and Herraiz, 2004), which is able to stabilize radicals through delocalization or through resonance (Figure 14) and stopping radical chain reactions (Tsoptom *et al.*, 2009). Tyrosine has also been determined to act as a peroxidation protector and demonstrate hydrogen peroxide scavenging activity (Glucin, 2007). Tyrosine was able to inhibit superoxide radical generation, where superoxide anions plays a major role in oxygen toxicity (Glucin, 2007). Literature reports that phenylalanine does not appear to exhibit antioxidant activity (Meucci and Mele, 1997) which contradicts with the findings from the ORAC assay. Literature is scarce for values of antioxidant capacity of amino acids. Tyrosine and phenylalanine both have one benzene ring, whereas tryptophan has two;

these structural differences are related to antioxidant strength which is supported by the ORAC values found.

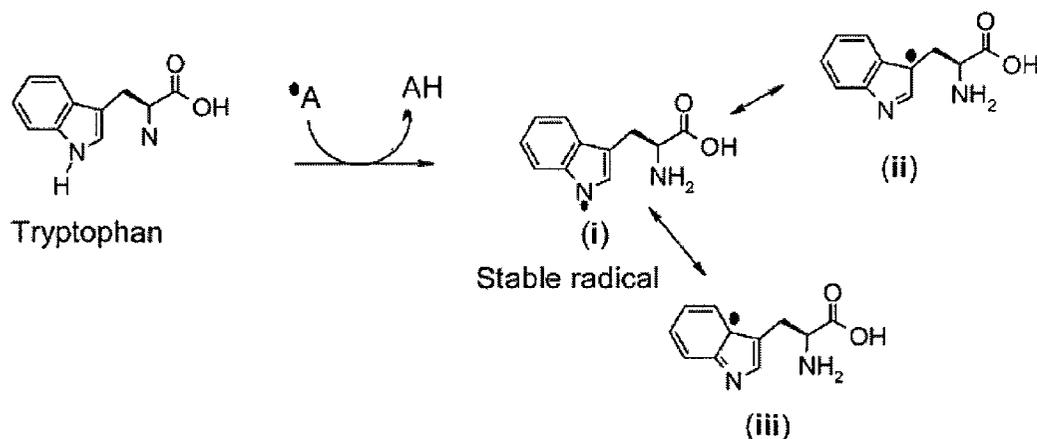


Figure 14. Resonance forms of the tryptophan radical. (i) tryptophan is a stable radical due to either unpaired, delocalized electron or due to its resonance forms (i-iii) (Tsopmo et al., 2009)

Histidine showed an ORAC value of 59.8 TE/mM and leucine had an antioxidant value of 9.23 TE/mM. Literature values for antioxidant potential are limited for histidine and leucine. However, antioxidative dipeptides have been found with histidine (Wu, 2009). Histidine has a pentene ring which can still participate in donating protons. Both leucine and histidine require further research to discover any potential antioxidant properties.

The remaining amino acids are alanine, arginine, asparagine, aspartic acid, glutamic acid, glutamine, glycine, isoleucine, lysine, proline, serine, valine, and threonine; these AAs display either no antioxidant activity or a negligible amount.

4.3. Effects of tryptophan on bacterial growth in formula milk

Milk contains many nutrients that allow for a good medium for the growth of microorganisms, which partly explains the relatively short shelf life for dairy products. Moulds, yeasts, and a broad spectrum of bacteria can grow in milk; microorganisms grow especially at temperatures above 16°C, and once they enter the milk, the microbes increase in number rapidly if not kept at 4°C. In order to grow bacteria in the laboratory (or in nature), it must have water, an energy source and a tolerant range of physical conditions such as oxygen concentration, temperature, and pH. It is extremely important to evaluate the microorganisms in products directed at premature infants, as these infants have an immature defence system which puts them at high risk to health complications.

The microbiological quality of dry-milk mixes and milk substitute infant formulas in Canada and the United States were compared in literature, and showed that the total aerobic microflora was actually lower in US-made dried infant formula products. However, both countries met the microbiological criterion that is recommended by the International Commission for Microbiological Specification (Nazarowec-White and Farberb, 1997). In a study with over 141 powdered samples from 35 countries, the Enterobacteriaceae family was found in just over 52% of the samples. The most common bacteria isolated from the samples are *E. agglomerans*, *E. cloacae*, *E. sakazakii*, and *Klebsiella pneumoniae*.

It is of interest to work with a non-harmful *E. coli* strain DH5- α to see the effects of tryptophan and human milk peptides on the growth of bacteria using the model organism *E. coli*.

4.3.1. Resazurin test: *Escherichia coli* and tryptophan addition to infant formula milk; tryptophan's effect on bacterial growth

Resazurin, an electron acceptor dye, has a blue compound (7-hydroxy-3H-phenoxazin-3-one 10-oxide) which acts as a redox indicator. As microbial activity uses up available oxygen, the dye becomes reduced. The rate of colour change, the colour itself, or the extent of change in a given time, is a semi-quantitative measure of the microbial activity (Table 4). Similarly, the colour itself can qualitatively indicate the amount of bacteria found in the formula. The experiment uses *Similac Regular Formula*, a dry powder, which has non-fat milk as its base, since the color standards are made to compare with skim milk-resazurin mixtures rather than with whole milk-resazurin mixtures; experiments using whole milk will have a different colouration which can alter the shades of resazurin, making the qualitative evaluation (score) of the milk less effective.

The experiment focuses on the comparison between the general number of bacteria in infant formula milk versus the number of bacteria in infant formula milk with tryptophan addition. Most bacteria can produce its own amino acids; however, it would be of interest to determine whether addition of the amino acid tryptophan would contribute to the growth of microorganisms. Tryptophan may act as another source of nutrient for the bacteria, as its molecular formula contains both carbon and nitrogen. The experiment focuses on evaluating

whether having an additional readily available amino acid in the growth media will increase the speed of bacterial reproduction. *E. coli*, was inoculated into the infant formula milk to first determine at what concentration and volume the organism would be detected by the resazurin test. In order to determine if tryptophan has an effect on bacterial growth, the concentration and volume of *E.coli* that exhibits a score of level 6 high bacterial numbers must be determined first. Once *E.coli* can be detected at a known cell number and value, it can be evaluated during tryptophan treatment.

The test can detect high populations of bacteria at 10^{10} cells/ml at any volume. This test is not sensitive enough for real life application as such a high population of bacterial organisms would cause serious medical health implications to a premature infant with low immune defences, since a much lower population of organisms would already be able to initiate health problems as mentioned previously from Nazarowec-White and Farberb (1997). The 10^{10} cells/ml of *E.coli* was used for most of the Resazurin Tests to see if there were any changes in bacterial growth with tryptophan addition. However, although 10^{10} cells/ml *E.coli* will always be able to be detected, it may have been more beneficial to use 10^9 cells/ ml *E.coli* to better evaluate if tryptophan has an effect on bacterial growth. Figure 5 shows that 10^{10} cells/ml *E.coli* has a score of 6 meaning that it is already at the highest bacteria number and score, and if tryptophan does increase growth of *E.coli* , then this score will not reflect that growth as it is already at its maximum score. Using a lower bacterial count of 10^9 cells/ml *E.coli* may not show

detection as rapidly or as accurately as 10^{10} cells/ml *E.coli*, but as it is at a score of 4 (Figure 5) which is lower than a score of 6, there is room for bacterial growth and thus a higher score if tryptophan were to increase its growth. On the other hand, Figure 9 does show that when only tryptophan is added to infant formula milk and is not inoculated with *E.coli* it does appear to have some low counts of bacterial growth at a score level of 2. This bacteria may be due to bacteria that is already present in the container and may also have resulted in faster reduction due to light in the room, as it is light sensitive. Since there does appear to be some level of bacteria in these tubes with solely infant formula and tryptophan, it does not appear that tryptophan increased the existing bacterial numbers present since it had the same score, and thus bacterial numbers, as the control without tryptophan (Figure 9). Figure 9 also demonstrates that tryptophan did not appear to inhibit growth of bacteria when viewing the controls with both high and low volumes of 10^{10} cells/ml *E.coli* and comparing it to those score of *E.coli* with tryptophan addition.

Literature states that phenolic compounds, such as tryptophan, may possess a variety of functions such as antibacterial, antiviral, anti-inflammatory, and antioxidant properties (Cai et al., 2006). The concentration of tryptophan (4mg/ml) was chosen to be higher than the approximate amount found in breast milk to test for bacterial effects and the possible amount that may be added to infant formula milk. Addition of varying volumes of tryptophan did not demonstrate any additional bacterial growth as all tubes, including control after the 4 hour incubation period. Addition of tryptophan to infant formula milk not inoculated with bacteria showed no effect on growth of microorganisms. It is possible that an even greater concentration of tryptophan could have antimicrobial effects, however the solution is saturated

at 4 mg/ml and a higher concentration would require other methods for effectively dissolving the tryptophan into solution. Thus, it would be more beneficial to test these organisms with a more sensitive test or additional observation by microscopic count after the test has finished. The total plate count (TPC) is a reliable and accurate method that can be used to further analyze potential antibacterial properties.

4.3.2. Total plate count method: addition of human milk peptides and *Escherichia coli*

Peptides from human milk that were plated in the experiment ranged from 6-8 amino acids. Plates were counted at a dilution of 10^{-2} to count within the 30-300 range. Addition of 200 μ l of each *E.coli* solution and tryptophan did appear to inhibit bacterial growth. The TPC method was also used to observe effects of some human milk peptides on bacterial growth. Peptides 23-6 (WISEELGW), 23-7 (ISELWG), and 23-8 (ISELWG) did imply to have an inhibitory effect on the growth of *E.coli* compared to the control from LSD test. There was no difference in antimicrobial strength between peptides. However, based on F and p values for tryptophan, there was no significance found meaning that tryptophan did not have an inhibitory effect on bacterial growth. Whether the human milk peptides is an effective antimicrobial is yet to be further analyzed. Most active molecules synthesized are composed of hydrophilic, hydrophobic and cationic amino acids. These human milk peptides are composed of tryptophan (W), isoleucine (I), serine (S), glutamic acid (E), leucine (L), and glycine (G). Tryptophan, isoleucine, and leucine are all hydrophobic amino acids. Glycine and serine are both hydrophilic. However, glutamic acid is not cationic (having a positive charge) and is acidic rather than basic. Another

interesting note is that natural peptides that are composed of D-amino acids instead of L-amino acids preserve complete antibiotic potency. When a peptide is synthesized in the lab, the mixture of D- and L- amino acids can generate a range of antimicrobial potency. In addition, *E.coli* is a gram negative bacteria; its outer membrane consists of lipopolysaccharide that is maintained by calcium and magnesium ions which bridge negatively charged phosphosugars. When a peptide is cationic, it is able to displace the metal bridge which disrupts the outer membrane of bacteria. This allows for the inflow of molecules from the exterior such as the peptide, and in this way, the peptide may be able to integrate into the cytoplasmic membrane. Since antimicrobial peptides target the bacterial membrane, most microbial species forgo changing its composition or structure of membrane as it is a costly solution for most species. Recalling that peptide 23-8-11 had an amine within its sequence, it is of interest to further test this peptide since amines exhibit some antimicrobial properties (Vukovic *et al.*, 2010). Although peptides 23-6, 23-7, and 23-8 did display some antimicrobial potential, addition of an amino acid sequence with cationic properties such as found in histidine, lysine, and arginine could display greater potency.

4.4. Electronic nose: sensory properties of milks supplemented with active peptides

The e-nose technology uses comparative analysis of volatile compounds (Xu, 2006) and is valuable for quality assurance and control (Li *et al*, 2009). It is a machine olfaction device with an array of chemical sensors that simultaneously respond to the volatile chemicals present in a gas sample (Amalia *et al.*, 2009). The two main components of an electronic nose are automated pattern recognition system and the sensing system. The sensing system can have an

assortment of gas sensors, or it could be a single device. The gas sensors respond quickly, in less than ten seconds, and are extremely sensitive to organic vapours. The gas sensors are based on chemical sensitivity of semiconducting metal oxides. Usually a heating element is also present within the machine. Oxygen from the air dissolves in the semiconductors' lattice, which sets an electrical resistance to a background level. Molecules from any element are adsorbed on the surface of the semiconductor, and the electrical conductivity changes due to the volatiles reacting with the dissolved oxygen species (Amalia *et al.*, 2009). The use of the electronic nose is important as a quality control tool to determine whether there are sensory differences in aroma and sensory composition between groups. When analyzing odour quality, the infant formula milks without additives and those with additives should have the same or very similar sensory composition. Additives that alter the aroma is not sought after since additives should solely improve infant milk quality without changing aroma. Not only are infants sensitive to aroma but mothers can be resistant to altered infant milk smells; additives, in this case, should not reformulate the aroma but are desired to maintain the infant formula as close as possible to its current aroma composition or else to match it to human breast milk.

Using PCA, as a non-supervised technique, was employed to look at general relationships between formula samples while retaining most of the variance within data. DFA was employed on the raw data values to study the differences and to discriminating between infant formula milk and human milk treatments. Firstly, it is of interest to identify whether the aroma of human breast milk and infant formula milk overlap to determine initial sensory characteristics. In addition, it has been documented that human newborns are able to recognize their mother's human breast milk (Russell, 1976; Varendi *et al.*, 1996) and prefer it over infant formula milk

(Nishiitani, 2009). Results supported that human breast milk and infant formula milk are distinct from one another. If human milk peptides were added to infant formula milk and were able to more closely mimic the aroma of human milk, then the infant formula milk with additives is preferred. It is also important to determine whether addition of human milk peptides or tryptophan to infant formula milk would greatly alter the composition and sensory properties of infant formula milk distinct from both infant formula milk and human breast milk control groups, which is not a desired outcome. PCA graphs showed overlap for data clusters when human milk peptides were added to either human breast milk or infant formula milk milk-based powder. This signifies that all the human milk peptide additives tested do not alter the aroma composition of the infant formula milk powder samples and demonstrates that there is no true sensory difference. These findings show that there is potential for these human breast milk peptides to be added to infant formula milk and that regardless of addition to either soy- or bovine-based infant formula milk powders, these additives will continue to maintain the overall sensory composition of the infant formula milk. Similar findings for tryptophan addition to human breast milk and powdered infant formula milk were observed from the PCA graph. Tryptophan addition does not show a significant change in sensory composition when compared to controls, and thus does not alter the aroma composition of powdered infant formula milk. In addition to PCA, a DFA graph was also used to support the PCA graph and to map out differences between sample sensory smells. In accordance with the PCA result trends, the DFA showed no significance between peptide 23-8-8 and its derivative, once again establishing that there is no difference in aroma quality when added to powdered infant formula milk. Soy-based infant formula milk and bovine-based infant formula milk powders

were distinct from one another and from liquid infant formula milk and have a greater difference in aroma due to processing differences. It is of interest to note whether they were similar aroma or distinct. Differences between smells of powder and liquid infant formula may be attributed to several factors, one of them being the types of processing the infant formulas undertake. Processing of infant formulas can induce lactose isomerization or the Maillard reaction; the protein alterations and protein denaturing, and the Maillard-reaction products can play a role in aroma differences (Sarría *et al.*, 2001). Further analysis would include whether peptide additives to liquid formula will have any difference on sensory aroma. Human peptides and tryptophan were only added to bovine-based infant formula milk to more closely relate to human milk; whether a sensory difference exists with the addition of HM-derived peptides and tryptophan to soy-based infant formula milk remains unknown.

It can be suggested that human breast milk peptides 23-8-8, 23-8-8', 23-8-4, 23-8'-6 and tryptophan can be added to formula without a significant sensory change.

5. Summary

The antioxidant human breast milk peptides and tryptophan may be added to infant formula to reduce oxidative stress in infants and improve their health and development. The most active peptides were 23-8-11(ISELGW [-NH₂]) and 23-8-10 ([ACE]-ISELGW), followed by 23-8-5 (WISELG), 23-8-4 (ISELGW) AND 23-8-7 (ISELWG). Peptides 23-6, 23-7, and 23-8 displayed antimicrobial potential for decreasing the presence of microbes in formula. Further analysis of the peptides and *in vivo* studies are required to allow for the addition of human milk peptides and tryptophan into infant formula milk in order to reduce oxidative stress in premature infants.

All phenolic and sulphur-containing amino acids demonstrated antioxidant properties using ORAC assay. The total antioxidant activities of chemical compounds cannot be evaluated by any single method because the antioxidant capacity of a compound occurs through different mechanisms, such as, activated oxygen species scavenging and metal chelation. Further comparative analysis using other antioxidant assays are required to verify results since literature data is scarce.

The resazurin is not sensitive enough for industry application, but still allowed for qualitative analysis when large bacterial populations were present. Tryptophan did not appear to affect bacterial growth, and neither decreased nor increased growth of *E.coli*. Human milk peptides 23-6, 23-7 and 23-8 appeared to have inhibited some bacterial growth when

inoculated into contaminated *E.coli* infant formula milk samples. Other quantitative tests should be used to further evaluate whether tryptophan has effective antimicrobial properties at higher concentrations. Total plate count test appeared to suggest that the peptides tested have antimicrobial potential. It is also of interest to test antioxidant human milk peptides ISELGW-[NH₂] and ISELGW (with phosphorous group on serine) for its antimicrobial properties since literature suggests that amines and phosphorous may have antimicrobial properties; it would be beneficial to use these two peptides in infant formula milk if they have antimicrobial properties since they both demonstrate a high level of antioxidant activity in comparison to the other human milk peptides.

Addition of human milk peptides and L-tryptophan to human breast milk, infant formula milk based powders, infant formula milk liquids, and infant formula soy-based powders did not exhibit a significant sensory change of samples. These additives will not alter infant formula milk composition and may be added to infant formula milk. It would also be beneficial to test whether these peptides will increase the shelf-life of infant formula milk, since antioxidants are associated with prolonging shelf-life of lipid-containing foods and the human milk peptides have demonstrated antioxidant activity.

The discrimination in DFA graph is not statistically significant, which shows that using peptide 23-8-8 or its derivative will make no true difference to formula "smell".

6. References

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