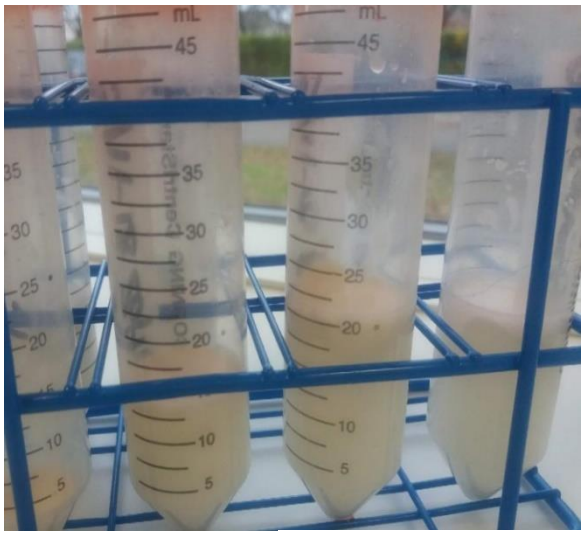
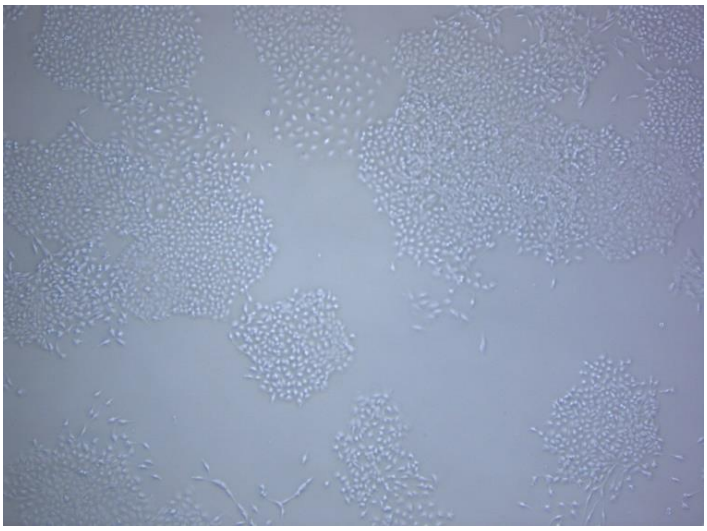
AALBORG UNIVERSITY
DENMARK

Mare milk

bioactive whey fraction

and the effect on the intestinal epithelial cells





Anca Diana Cerescu – June 2019

Master Thesis Biomedicine - Medicine with Industrial Specialisation, Aalborg University Denmark

External supervisor Senior researcher Stig Purup, Aarhus University Denmark

Internal supervisor Meg Duroux, assistant professor Aalborg University Denmark

Preface

Taking the decision to have my thesis to another University wasn't an easy decision but for sure now at the end of this time spent at Aarhus University Foulum I can say that it was worthy.

The team here was a balanced mix of professionalism and friendly-communicative people from whom I had a lot to learn.

I was lucky I stepped upon this chance by browsing the internet before choosing a subject for my thesis and found Prof. Stig Purup Senior researcher Department of Animal Science Aarhus University and his team.

First of all, I would like to thank both Janne B. Funch Adamsen and Kasper V. Poulsen for the technical and practical skills into laboratory. Many thanks for the mare's milk and mare's colostrum samples provided by Eva Søndergård, fundraiser at Foulum Research Centre Aarhus University Denmark.

For all the time used at Aarhus University Foulum Denmark in the laboratory or in the office, I want to thank to my external supervisor Stig Purup for being ready to answer my questions and guide me through the process of writing and planning the thesis. His previous experience with cell-culturing and bovine milk bioactive components gave me a good structure to keep during laboratory experiments.

Thanks to my internal supervisor Meg Duroux Associate Professor, Department of Medicine and Health Technology, Faculty of Health Science Aalborg University for her input and encouragement in choosing this project and organising the information gained during the process of learning by doing.

I need to thank also to post-doctoral researcher Yuan Yue for the statistical analysis skills which have helped me a lot for this thesis.

The subject of mare milk is not only about a food niche product but also a relatively new and with not so much research touched upon it but for sure it is interesting and it can bring many useful scientifically information in the future.

Abstract

Mare milk composition is very well documented in the research literature and it mimics in general breast milk content. Moreover, mare milk is well tolerated in human diet and it is an increased interest for health quality nutrition in the world by using other milk-borne peptides than bovine ones. Mare milk and its fermentation beverages are interesting products which might bring diversity and develop new industries from dairy mares' farms in the proximal future.

Mare milk containing peptides and growth factors in the whey fraction after the enzymatic activation during digestion prove its complex role as both nutrient with a trophic effect for the intestinal epithelium and nutraceutical with multifactorial bioactive effects for the human health. As nutrients are critical for neonatal nutrition mare milk may be important as substitute to human or cow milk for the premature or allergic to cow's milk neonates. As functional food, mare milk could be important particularly for the intestinal cellular turnover and not the least in the case of treatment and prevention of diseases which impair the intestinal mucosal continuity and permeability or as adjuvant for wellbeing support. Supplementary could be important in the case of immaturity of the intestinal barrier for enteral nutrition of premature neonates.

In the present thesis studies whey of mare milk was shown to have mitogenic effect *in vitro* in intestinal epithelial cell lines of rodent and human origin. Furthermore, the whey was shown to have effect on migration of intestinal epithelial cells in a wound healing assay. Concentration of the growth factors insulin growth factor I (IGF-I), epidermal growth factor (EGF) and transforming growth factors β1 and β2 (TGF- β1 and TGF-β2) analysed in this study was not very high and seemed not to be correlated with the horses age, lactation time or lactation number. The highest concentration of growth factors was IGF-I. All of these components from the whey fraction have specific pathways of action and are coupled with cellular receptors *in vivo*. They can have mitogenic effect and migration effect on the small intestine level.

Mare milk is an interesting complex biological fluid with potential health beneficial effects in the intestine.

Abbreviations

ALA – α linolenic acid

ARA – arachidonic acid

ACE – angiotensin converting enzyme inhibitor

Acetyl CoA – acetyl coenzyme A

BSA – bovine serum albumin

CMA – cow milk allergy

CD – Crohn's disease

CXCR4 – Fuxin, CD 184, chemokine receptor type 4,

CD 184 – cluster of differentiation 184

CXCL12 – gene encoding stromal cell-derived factor 1 precursor protein (chemoattractant active on T lymphocytes and monocytes)

DHA – docosahexaenoic acid

DPP-IV – dipeptidyl peptidase IV

DBPCFC – double blind placebo-controlled food challenge

ECM – extra cellular matrix

EPA – eicosapentaenoic acid

EFA – essential fatty acids

EGF – epidermal growth factor

ERK – extracellular-signal-regulated kinase

FGF – fibroblast growth factor

GLP – glucagon-like peptide

G-I – gastro-intestinal

HMO – human milk oligosaccharides

IBD – inflammatory bowel disease

IEC – intestinal epithelial cells

IFN - interferon

IGF – insulin-like growth factor

KGF – keratinocyte growth factor

LA – linoleic acid

LCFA – low chain fatty acids

Lf – lactoferrin

LY – lucifer yellow dye

LAB – lactic acid bacteria

LPS – lipopolysaccharide (endotoxins from Gram – bacteria membrane)

LNFP – lacto-N-fucopentaose

MUFA – monounsaturated fatty acids

MFGM – milk fat globule molecules

MAP-kinase – mitogen activated protein kinase

MDP – (bacterial)muramyl dipeptide

mRNA – RNA messenger

NANA – N-acetyl neuraminic acid

NEEA – non-essential amino-acids

NfKB – nuclear factor kappa B

OAA - oxaloacetate

PMA – phorbol 12 myristate 3 acetate

PUFA – polyunsaturated fatty acids

PDGF – platelet derived growth factor

PG – prostaglandin

P-gP – P glycoprotein

RER – rough endoplasmic reticulum

SCFA – short chain fatty acids

SFA – saturated fatty acids

SER – smooth endoplasmic reticulum

SAMP1/YitFc mouse strain – a model of Crohn's disease

Tx – thromboxane

TEER – transepithelial electrical resistance

TLR – toll-like receptors

TGF – transforming growth factor

TFF – trefoil factors

THP – human leukaemia monocytic cell line (used for study of macrophage or monocyte functions and nutrient or drug transport)

TCA cycle – tricarboxylic acid cycle (Krebs cycle)

UVB – ultra violet B rays

UC – ulcerative colitis

VEGF – vascular endothelial growth factor

βCN – beta casein

Table of Content

1. Introduction

1.1 Mare milk and colostrum

Appreciated and well known in ethnomedicine, mare milk and fermented mare milk kumis, trace back to the pastoral nomad tradition in the central Asian steppe (Carrick, 1881).

The countries with the greater dairy mares are Mongolia, Kazakhstan, Kyrgyzstan, Tajikistan, Uzbekistan, Tibet and Chinese region Xinjiang. Smaller dairy farms have Europe, Belarus and Ukraine and local dairy breeds are representative in Eurasia, France and Belgium (Pieszka et al., 2016) .

However, during the early and late nineties (1912, respective 1959) in Russia respective Germany more kumis and mare milk therapy centres for human tuberculosis were opened. In France heavy breed horses were preserved and nutritionally designed for milk production. The interest in mare milk production for human nutrition increased in Western Europe. Italy introduced the concept of using mare milk as cow milk substitute for allergic children nutrition (Park et al., 2017).

Human interest in mare milk consumption is very old. Professor of Archaeological Science from Exeter University UK found evidence of horse domestication earlier than Bronze Age about 5000 years ago in the shape of bones, teeth's and pottery with mare milk traces (Outram et al., 2009).

Taking in consideration all of these historical information about mare milk and bringing out the importance of milk in general in human nutrition, because it provides 8-9 % of the dietary energy supply in Europe and Oceania; 19% of the dietary protein supply in Europe and 11-14% of the dietary fat supply in Europe, Oceania and Americas one can easily recognise the usage of mare milk in human nutrition and health and the necessity of its study (Burgess, 2014a, Pieszka et al., 2016).

It is expected to have an increased demand of milk of 25 percent by 2025 in developing countries (Center, 2016).

Mare milk is characterised by a whitish-translucent colour, more fresh and sweeter than cow's milk with a coconut flavour. It has a lower viscosity than cow's milk because of a lower casein content. Compared to cow's milk, it has a lower cholesterol and fat content (mostly MUFA and PUFA) and because of that, a 30%

lower calorie level (45 kcal/ 100 g) than cow's milk. As for the Ca: P ratio of 1.7, this recommend it in human consumption. The safe Ca to P ratio for an infant development is 1-2:1 and the average of Ca : P for breast milk has the same value as for mare's milk (Loughrill et al., 2017, Malacarne et al., 2002a).

Colostrum in horses is the source of passive immunity from mares to foals and besides IgG contains factors that improve the microbiota of the digestive tract of foals and prevents septic shock. 24 hours after foaling the permeability of the intestinal epithelial cells to macromolecules and IgG ceases and therefore colostrum administration needs to be done in 12 hours postpartum for the best absorption of the IgG. The factors from the colostrum can delay the intestinal "closure" but no one can prolong the time when colostrum can be supplied to foals, it has to be done within 12 h postpartum for a complete absorption of the IgG (Raidal et al., 2000).

Immunoglobulins levels in colostrum and passive foal immunization can be controlled by pre-foaling vaccination (homologous transfer of passive immunity) (Burns, 2007, Hurley and Theil, 2011) along with mares hyperimmunization in treatment of human diseases or vaccines producing (Zheng et al., 2016, Santos et al., 2013). IgG, IgM and IgA concentration in mares' mammary secretions increased before foaling with IgG the main immunoglobulin present in colostrum before and after foaling, dropping sharply one day after foaling (Koke et al., 2017).

Mares placenta is epitheliochorial with no antibodies transfer to the developing foal so the passive transfer of or activation of immunity is done by colostrum in the first days postpartum and it can be completed by amniotic fluid and finally in time by the foal's body itself. The physiology and development of the neonate is influenced by colostrum factors which can be hormones, cytokines, growth factors, enzymes (Mariella et al., 2017).

Colostrum contains a cocktail of pro-inflammatory cytokines namely TNFα, IFNγ, IL-6 and IL-8 which can determine an immune response from the foal. T cells and specifically $CD8^+$ and CD^{4+} lymphocytes from the colostrum are produced after *in vitro* stimulation with PMA/ionomycin IFNγ and colostrum T cells produced IFNγ and IL-17 pro-inflammatory cytokines (Perkins et al., 2014).

Total dry matter (proteins) in the colostrum was superior in the first day compared to the second day postpartum (96.7 ± 19.5 g/L, 63.5 ± 23.6 g/L) with the same tendency for the somatic cell count (SCC) 89 x

10^3 in the first day and 72 x 10^3 in the second day. There was no correlation between these values and the one in the blood serum of the foals concluding that the time of getting the first suckling of colostrum and the quantity of it is very important. The milk and serum proteins content are becoming stable after 5 days postpartum with a minimum for survival of 8 g/L Ig in the foals' blood serum. Normally this process of Ig transfer (pinocytosis) lowers after 20 hours at 1% because the neonate enterocytes of the intestinal barrier are getting matured quickly being ready for the life outside the womb (Giguere and Polkes, 2005) , (Bobos et al., 2013).

1.2 Milk proteins

Caseins and whey proteins are components of milk protein fraction. The high supply of essential amino acids from the whey protein fraction 40% in mare's milk (circa 50% in human milk and less than 20% in cow's milk) and low content of caseins (40 to 45% of the total protein content) makes it similar to human milk (albumineaux type) and indicates it in human nutrition. Components of the whey protein fraction like serum albumin, α lactalbumin, β lactoglobulin, lactoferrin, lysozyme, lactoperoxidase and G immunoglobulins are the bioactive components of mare's milk and confer a high biological value to this type of milk in particular (Pieszka et al., 2016) (Malacarne et al., 2002a, Lönnerdal, 2003) (Potočnik et al., 2011), (Kusumaningtyas et al., 2018), (Detha et al., 2019b).

Kyrgyz native breed mares have an amino-acid composition of milk with a high leucine, glutamic acid and lysine, with almost the same percentage of amino-acids from May to August (46 – 51%) with a peak in July (51%). Glutamic acid is 16.8 -21.2%, leucine 8.2 – 10.1%, lysine 8.9 – 10.1% and only 0.1 – 0.7% ornithine. The quality profile of this amino-acids depends on lactation and grazing on meadows (Mazhitova and Kulmyrzaev, 2016, Mazhitova et al., 2015).

Amino-acids profile of kumis (fermented mare milk, called airag in Mongolia) from herders of Mongolian steppe it is different compared to the one of raw mare milk with less abundant amino-acids for the last. The highest concentration is coming from one of the province (Burgan) with 21 mg/100ml aspartic acid, 33.4 mg/100ml threonine, 55.1 mg/100ml serine, 13.2 mg/100ml glutamic acid,, 15 mg/100ml glycine, 72.6

mg/100ml alanine, 3.1 mg/100ml cysteine, 45.6 mg/100ml valine, 25.4 mg/100ml methionine, 96.7 mg/100ml leucine, 7.8 mg/100ml tyrosine, 43.3 mg/100ml phenylalanine, 65.5 mg/100ml % lysine, 7.2 mg/100ml tryptophan, 45.3 mg/100ml % proline, 50.4 mg/100ml isoleucine. Leucine was 24.5 mg/100ml and proline 29.22 mg/100ml in other two Mongolian provinces area. 3 mg/100ml acid aspartic, 13 mg/100ml glutamine, 2 mg/100ml valine, 2 mg/100ml leucine, 2 mg/100ml lysine for six days of lactation (day 6 to day 14) (Ishii et al., 2014).

Branched chain amino-acids (leucine, isoleucine, valine) are essential amino-acids degraded in the skeletal muscles not in the liver which stimulates protein metabolism and muscle protein synthesis (transamination). Bioactivity of BCAA comprise fat mobilisation and degradation for muscle energy, stimulation of insulin secretion and modulation of insulin circulation, regulation of the protein metabolism with decreasing catabolism in the case of trauma and increased catabolism in obesity. BCAA balances leucine kinetics, leucine being an amino-acid not produced by the body which is brought by food important for protein synthesis, in wound healing, muscle and bone tissue repair and haemoglobin formation (Database) , (Park, 2009) .

Equine milk protein digestion

In the stomach (pH 2.5) equine caseins are degraded with only 30% of them left intact (for cow casein 69% and for human casein 39% is left intact). As for the pancreatic enzymatic digestion in human duodenum (pH 8), bovine and equine casein are almost integral digested with a better digestibility for equine milk compared to cow milk. Lysozymes after gastric and duodenal digestion is more than a half percentage undigested in equine milk. Bovine β lactalbumin is very resistant to gastrointestinal enzymes opposite to the equine one. This explains the bioavailability of mare milk proteins and the similarity to the human milk protein digestion (Inglingstad et al., 2010).

Casein structure with a bigger size of casein micelles can form a softer precipitate in the human stomach than cow's milk coagulum and it can explain the better digestibility of equine's milk compared to cow's milk (Malacarne et al., 2002a) (Uniacke-Lowe, 2011), (Uniacke-Lowe et al., 2010, Potočnik et al., 2011).

Oligosaccharides like NANA (N-acetylneuraminic acid), galactose and N-acetyl galactosamine are glycosylated with k caseins making it resistant to chymosin digestion and this together with a high proline

content can confer antibacterial effect and additional prebiotic because mars milk oligosaccharides are digested only by bifidogenic or lactobacilli. This explains the bioactive role of most of the protein components from mares' milk. (Zivkovic and Barile, 2011),(Uniacke-Lowe, 2011).

1.3 Milk fat and oligosaccharides

Phospholipids, triglycerides and cholesterol droplets are coated with a 3-layer molecular structure named milk fat globule membrane MFGM which contains an internal protein layer, a phospholipid membrane and an external layer of glycoproteins. On the surface of MFGM is a branched oligosaccharides layer to help binding lipases and milk fat digestion. MFGM is derived from the mammary secretory cells. There is a similar globular structure in human and equine fat milk globules. PUFA, MUFA, SFA and EFA (ALA and LA) content is similar in human and mares' milk but PUFA are in greater amount in mare's milk compared to cow's milk because of the FA enzymatically saturation in the rumen in the case of cows (different type of digestion for ruminants and monogastric) (Park et al., 2017), (Pieszka et al., 2016).

Mare's milk is rich in essential fatty acids linoleic acid and linolenic acid (α linoleic acid, an omega 3 fatty acid and γ linolenic, an omega 6 fatty acid) which cannot be produced by humans so they are required in the diet. Linoleic acid (LA) is a precursor of eicosanoids (prostaglandins, thromboxane, leukotrienes, prostacyclin), important in the inflammatory responses, pain, fever, blood clothing, blood pressure. (Calder, 2013), (Di Cagno et al., 2004).

Mare's milk is richer in phospholipids compared with both human and cow's milk. For example, phosphatidylserine (mare milk 16%, human milk 8%, a double content for mare's milk), phosphatidylethanolamine (31% vs 20%) but less in phosphatidylcholine (19% vs 28%) and phosphatidylinositol (trace vs 5%) and with sphingomyelin proportion, the same for mares and human milk. Sterols - cholesterol proportion is almost the same for cow and mare milk and for mare milk higher than that for humans (Malacarne et al., 2002b).

In conclusion, the fat profile of mare milk it is quite similar to human milk (like the protein content) with richer number of phospholipids and the exclusive presence of omega fatty acids group which, of course adds more nutritional value to horse milk.

Oligosaccharides, not a major nutritional carbohydrate from mares' milk, have a slightly different role. Two of the oligosaccharides found in mares' and human milk HM-3b and HM-5b inhibits bacterial growth and are Bifidobacterium prebiotics. 16 oligosaccharides from mares' colostrum of different breeds have been screened and they have 63% structural homology with human milk bioactive oligosaccharides (Difilippo et al., 2015).

As for HMOs (human milk oligosaccharides, low lacto-N- fucopentaose (LNFP) III was associated with cow milk allergy (CMA) at 18 months age infants 6 weeks and 12 weeks of age gastroenteritis and respiratory tract inflammation was reduced in the case of higher LNFP II concentration). Less frequent infectious diseases at 2 years old was in the case of fucosyloligosaccharides. HMOs are only 1% intestinal absorbed so they are found intact in the colon. Besides HMOs' prebiotic role (competition for nutrient supply), they mimic mucosal intestinal receptors with antimicrobial, antiviral and antiprotozoal effect (Entamoeba histolytica – amoebic dysentery and amoebic liver abscess) (Doherty et al., 2018).

Eighty percent of breast-fed infants from HIV positive mothers are still HIV negative. Not only the intestinal mucosa but also upper respiratory mucosa is protected at breast-fed child's against otitis (Pseudomonas aeruginosa, Hemophilus influentzae, Streptococcus pneumoniae) or RSV (respiratory synovial viruses) due to sialylated HMOs (Sia), glycoproteins and glycolipids contents in the breast milk. There is a high demand of Sia in pre and postnatal brain development for Sia gangliosides and poly Sia glycoproteins (Bode, 2012).

1.4 Milk lactose and kumis

In 1253 William Rubruquis, a missionary monk wrote about trying for the first-time fermented mare milk during his travelling to Tartary region in Russia. He found it very savoury, with the taste of the almond milk and besides that induces relaxation and it is a good diuretic. In 1859 Dr. N. V. Postnikof started using kumis in a special institution for the treatment of dyspepsia, phthisis, chronic bronchitis, laryngitis, haemopthisis, and anaemia, tuberculosis and chronical pulmonary diseases, in a special sanatorium near Samara Russia. His success spread the rumours to entire Russia and surroundings. The increase in appetite with weight gaining and a stimulatory effect for the entire metabolism explain the fact that, the nutritive value of this product it is high enough to assure the replacement of the digestive waste eliminated (less faeces) along with neutralising the urine and a better digestion and absorption of food taken with kumis or after it. This explains physiological effect of kumis. Hyperaemia by vasodilation and increased blood supply of the mucous layer of the respiratory and digestive track comes with a better sleep consecutive with the relief of the symptoms for all of these above mentioned respiratory chronical diseases. The clinical outcome with the improvement of the symptoms of the chronical diseases is explained by lactic acid, alcohol and carbonic dioxide combined action. In this way kumis has a beneficial effect in the therapy of pulmonary diseases (Carrick, 1881).

Table 1 - Lactic acid bacteria (LAB) content in kumis, after (Park, 2009)

Type of kumis	LAB (starter) predominant content	% of lactose to LAB conversion	Milk acidity (pH)
"strong"	Lactobacillus bulgaricus Lactobacillus rhamnosus	80-90%	3.3 - 3.6
"moderate"	L.acidophillus L.plantarum L.casei L.fermentum	50%	3.9 - 4.5
"light"	Streptococcus thermophilus Streptococcus cremoris	< 50%	4.5 - 5

The milk carbohydrate lactose, together with milk proteins, is secreted by reverse pinocytosis in Golgi apparatus of the cell of mammary gland and is higher in mare milk than in human and cow´s milk (mare milk is sweet) and it helps in colonisation of neonate intestines with microflora, which can digest it (Davies Morel, 2003), (Park, 2009).

In the studies by (Doreau et al., 2009) it was showed that that feeding mares with mostly concentrate diet increased the lactose content in milk compared with milk from horses fed a forage diet. This explains the different chemistry of the horse milk based on its diet.

Besides the prophylactic purpose for malnutrition, stress or anaemia, after surgery to help recovery, kumis is indicated as treatment in some diseases of the pulmonary tract, inflammatory bowel diseases and allergy. Indication is one glass of milk per day increasing it gradually, until ½ litres daily. Local communities (Kazakhs, Mongolians) during summer drink up to 10-15 litres daily. (Hui, 2006).

High nutritional value of mares' milk based on essential amino-acids' content for milk proteins (glutamic acid, glutamine, arginine, cysteine, valine, proline, threonine, glycine, alanine, lysine) confer fermented milk more amino-acids (2-10 times more) during kumis maturation.

Lactobacilli and yeasts from fermented mares' milk use milk lactose as energy source with lactic acid and alcohol as fermentation by-products (Carrick, 1881), (Nurtazin et al., 2016).

Traditionally, fresh raw mare milk is mixed with pre-existent old kumis by fermentation carried by LAB-lactic acid bacteria (Lactobacillus casei, Lactobacillus lactis subsp. lactis, Lactobacillus delbrueckii subsp. bulgaricus) and yeasts (Saccharomices unisporus, Kluyveromyces fragilis) for about 8 h, to produce fermented mare milk, kumis. Pure cultures of LAB and yeasts is used for the industrial kumis production (Batt and Tortorello, 2014). The process of fermentation brings flavour, taste and improves the digestibility of the product besides the fact that without the pathogenic bacteria which are inhibited for growing in the process, the shelf life is assured. At the industrial scale, production of kumis is controlled using starter culture of lactic acid bacteria (LAB) and yeasts. Lactose content being higher than cows' milk (63,7g/kg), its fermentation gives rise to increased lactic acid (0, 7-1, 8 %) and alcohol (0, 6-2, 5 %) (Behera et al., 2017).

Nomadic milking makes kumis a source of income for many traditional minority groups for example Xinjiang Kazakhstan (960.000 horses in 2005) with the best in quality and taste kumis during autumn when there is a perfect condition of temperature (20-25°C) for the fermentation process (Boca Raton, 2011).

Kumis is already a functional food in China. Bacteriocins (organic acids, H_2O_2, cholic and deoxycholic acids, alcohol) resulted from LAB e.g. Lactobacillus fermentum SM-7 and yeasts (Saccharomyces cerevisiae, Candida sp.) fermentation processes showed to have antibiotic effect against Gram negative (E. coli) and positive (Staphylococcus aureus) bacteria and decreased cholesterol activity at a low ph. Cholesterol co-precipitate and assimilate at a low pH cholic acid, L. fermentum SM-7 showing in this way to reduce cholesterol by 66.8%. Moreover, in hyper-lipidemic diet induced mice, total serum cholesterol, LDL cholesterol and atherogenic index decreased, serum HDL cholesterol did not increase and it was a good liver to body weight ratio compared to high fat induced diet mice (Dong Pan et al., 2011).

Lactobacillus plantarum J 23 from Chinese traditional yoghurt, was found out to be a bacteriocin strain with sensitivity to proteinases K and E and trypsin. Cu^{2+}, Ca^{2+}, K^{+}, Zn^{2+}, HCl inhibited its activity and Fe^{2+}, alcohol and Mn^{2+}enhanced its activity. The most important point, this strain has anti – Listeria monocytogenes (foodborne pathogen) activity (Zhang et al., 2018).

Lactobacillus acidophilus -La-5, Bifidobacterium longum and strains of Lactobacillus plantarum from a traditional Egyptian yoghurt, showed anti-Staphylococcus aureus, anti- E. coli O 157-H7 and anti-Listeria monocytogenes activity, decreasing by four times these foodborne pathogens inoculated in yoghurt. The storage of yoghurt for 15 days at 4°C lowered only by 8% Bifidobacterium longum strains colonies and only by 6.5% colonies of Lactobacillus acidophilus (El-Kholy et al., 2014).

All of this shows the importance of LAB studies in kumis and other fermented milk products for human health.

Kumis, being a probiotic product because of the LAB and yeasts components improve the intestinal microflora, has an antibiotic effect, can produce free fatty acids important in human metabolism and CLA (conjugated linoleic acid) from linoleic acid with a role in reducing carcinogenesis, obesity and diabetes and stimulates vitamin group B synthesis important for the energy balance , nervous system, skin disorders kidneys and liver function (Park, 2009). Its bioactivity is beneficial for human health in general and the role of mare milk and kumis as functional food is treated in another chapter of the actual project.

To sum up, mare's milk chemistry, it is similar to human milk and quite different from cow's milk. This can be a clue for future investigation with focus on mare milk and human health and the next point "Growth factors and bioactive components in mares' milk" can further point out the importance of mare milk not only in human nutrition but in the prevention and treatment of diseases.

Table 2 - Milk composition for 100 g of milk with the inspiration from (Burgess, 2014b), can clearly show the similarity of mare milk composition compared to human milk composition

	Cow milk	**Human milk**	**Mare milk**
Total fat(g)	3.3	4.4	1.6
Total protein(g)	3.3	1	2
Lactose(g)	4.7	6.9	6.6
Calcium(mg)	112	32	95
Iron(mg)	0.1	traces	0.1
Magnesium(mg)	11	3	7
Potasium(mg)	145	51	51
Sodium(mg)	42	17	16
Cooper(mg)	traces	0.1	0.1
Niacin(mg)	0.13	0.18	0.07
Riboflavin(B_2) (mg)	0.2	0.04	0.02
Thiamin(mg)	0.04	0.01	0.03
Vitamin C(mg)	1	5	4.3

1.5 Growth factors and bioactive components in mares' milk

Growth factors are signalling molecules between cells that can also be found circulating the intestinal tract when ingested in food and secreted by different cells like endothelial cells, fibroblasts, mesenchymal cells, smooth muscle cells, mast cells or blood cells like macrophages, neutrophils or platelets. They can

exist in food such as bioactive peptides in milk like EGF, TGFβ, IGF I and are involved in intestinal wound healing by stimulating different intestinal cells (chemotaxis and attract cytokines). The importance of growth factors includes intestinal growth (hence the name) and may influence IBD and gastro-intestinal anastomotic healing. Their receptors are located in the mucosal cells on the basolateral membrane and when the intestinal permeability is increased (pathological state or physiologically after passing the colostrum) the receptors can shift to the luminal (apical) membrane of the mucosal intestinal cells (Rijcken et al., 2014), (Macdonald et al., 2000).

Under the pathological conditions, growth factors are used in applications for patients with inflammatory bowel diseases (IBD), after intestinal surgery or helping wound healing for those with unsuccessful surgery because of the immunosuppressive, inflammatory disorders or prolonged steroid therapy (anastomotic leakage) (Rijcken et al., 2014).

Physiological anastomotic healing is a three phases process modulated by cytokines and growth factors where breaking down and building up the tissue in the wound site needs to be in balance. Exudative, proliferative and reparative phases have as purpose mucosal and submucosal wound healing where an elastic mechanical resistant mesh layer rebuild the intestinal barrier. The first phase (1-4 days) is dominated by platelets, macrophages and the cocktail of inflammatory cytokines and growth factors (IL-1,IFNγ, TNFα,PDGF,TGFβ) , the second stage (up to 2 weeks) it is still orchestrated by growth factors (VEGF,IGF I, bFGF, TGFβ) and fibroblasts with angiogenesis plus granulation tissue formation and the last much slower phase (14-180 days but it can last for years) is conducted by growth factors like PDGF and FGF with remodelling of the collagen mesh, cross-linking of the fibres and their maturation (Rijcken et al., 2014).

In the case of the physiological conditions, mucosal layer integrity has to be a balance between production, migration and elimination (apoptosis) of intestinal mucosal cells (mucosal turnover) and milk growth factors has been proved to be ligands for the receptors on the mucosal intestinal cells with signalling apoptosis of the cells or crypts renewal. Milk and colostrum have both growth factors and bioactive peptides, which can enhance cell proliferation, mucosal mass function and maturation (Macdonald et al., 2000).

One can’t separate systemic growth factors from the mammary growth factors or those who are found in human milk and mammals’ milk for example horse, but for sure it can be clear that different stages in

development of the body needs them and that's why milk is useful in foal nutrition and human nutrition likewise in all the stages of development starting with neonates and ending with elderly people.
The influence of both systemic growth factors and mammary growth factors (EGF, FGF, TGFβ) and its mitogenic bioactivity on the mammary gland and on the cell level have been tested *in vitro*, confirming their modulatory role for the mammary growth in the pubertal period bringing to reality the fact that growth factors act together with hormones like oestrogens and growth hormone (GH) for the growth and development of mammary gland. A 3D collagen gel *in vitro* model of mammary epithelial cells from prepubertal heifers (a young cow that never have had a calf before) was used to demonstrate the role of growth factors in mammary growth and researchers concluded that FGF2 and TGF-β1 have a stimulating effect at low concentration and inhibitory effect at higher concentration while FGF1, EGF and IGF growth factors families have a stimulatory effect on these cells (Purup et al., 2002).
A general description of the growth factors present in mare milk and their activity in human health is presented below in Table 3. Review of (Rijcken et al., 2014) was used for inspiration together with the book chapter 7 (Park, 2009) and (Murray et al., 1992),(Marjanovic and Hopt, 2011) and (Said, 2018).

Table 3- Growth factors in mare milk and their activity in humans

Growth factor	**Origin**	**Effect**
EGF epidermal growth factor	Macrophages, platelets, fibroblasts, epithelial cells, neutrophils, salivary glands, Brunner's glands of duodenum, Paneth cells in the crypts, in mare milk colostrum and first week milk, in human breast milk	Via EFG(ErB-1) receptor dimerization and tyrosine kinase activity on the cell plasma membrane; Stimulatory mammary cell effect; Angiogenesis, cell migration and proliferation, ECM synthesis, intestinal wound healing

IGF insulin-like growth factor	Hepatocytes, epithelial cells, neutrophils, fibroblasts, mesenchymal cells	Angiogenesis, migration and proliferation, ECM synthesis, central nervous system protection (CNS), growth promotion and cell development
TGF-β transforming growth factor beta	White blood cells	Inhibition of ECM degradation, ECM synthesis, migration, proliferation, intestinal mucosal restitution
FGF fibroblast growth factor	Endothelial cells, smooth muscle cells, mast cells, keratinocytes, fibroblasts	ECM synthesis, migration, proliferation, angiogenesis, remodelling
PDGF platelet derived growth factor	Platelets, endothelial cells, fibroblasts, macrophages	ECM synthesis, migration, proliferation, angiogenesis

Bioactive compounds of milk contain a variety of substances like vitamins (A, D_3, E, K_2 ,C, B_1, B_2, B_3, B_6, B_{12}, nicotinic, pantothenic, folic acids and vitamin C) lipids, lactose and oligosaccharides, growth factors and cytokines, minerals, enzymes and milk proteins – casein and whey proteins (α_{s-1} , α_{s-2} , β and k caseins and whey proteins -α lactoglobulin, lactoferrin and β lactoglobulin) which are called bioactive peptides , BPs. (Pieszka et al., 2016) (Lucarini, 2017)). BPs are inactive and encrypted in protein sequences and by enzymatic proteolysis (gastrointestinal digestion, in vitro hydrolysis or fermentation using starter cultures or enzymes from plants or microorganisms), BPs are released and activated and they have modulatory effects and act as regulatory compounds of some physiological processes like opioid, mineral – binding, immunomodulation, anti-hypertensive effects (Uniacke-Lowe et al., 2010).

In Table 4 an overview about the bioactivity of milk is giving with focus on those components presents in mares' milk.

Table 4 - Bioactivity of mare milk - inspired (Schanbacher et al., 1998)

1. Gastro-intestinal development and function	Casomorphins (β casein)
• Lower gastric and intestinal motility with slower emptying rate;	Casomorphins (β casein)
• Greater absorption of amino-acids, minerals and electrolytes from the milk;	Casokinins (ACE I) (casein)
• Hyperaemia and increased blood flow to the intestinal level (antihypertensive effect);	Lactoferrin
• Intestinal cell proliferation (intestinal wall turnover)	GF (IGF I, TGFα, EGF)
• Growth of intestinal track	Lactoferrin
2. Antibacterial-antiviral activity	
• Bacteriostatic- iron binding competition with bacteria	
• Viral adherence	Lactoferrin
• Bactericidal against Enterobacteriaceae – gut protection	
3. Probiotic activity	Lactoferrin, K casein
• LAB and Bifidobacterial intestinal growth stimulation	IgA, IgG, IL-1, IL-2, IL6, IFNγ, TGFα, TGFβ
4. Immunomodulatory	
• Passive immunity and immunity development	Lactoferrin
• CD4+ (T helper cell) development	Growth factors: IGF I, TGFα, TGFβ, EGF
• NK cells activation	
• Humoral immune response activation, phagocytosis	

• Inflammatory response activation to bacterial endotoxins) 5. Infant development • Organs (e.g. liver) development	

Table 5 give a better understanding about the whey proteins fraction in the composition of milk from mares, humans and cows. For inspiration it was used a comprehensive review by (Potočnik et al., 2011) who cited reports from literature between 90s' and 2000.

Table 5 - Whey fraction composition of mare milk

Whey proteins	**Mare**	**Human**	**Cow**
α lactoglobulin % mean	9.89	30.26	8.38
B lactoglobulin % mean	30.75	20.10	-
Ig % mean	19.77	18.15	11.73
Lysozyme % mean	6.59	-	1.66
Serum albumin % mean	4.45	7.56	6.20
Lactoferrin % mean	9.89	30.26	8.38

To conclude, growth factors are a part of bioactive peptides from the mare milk, particularly present in the whey fraction and their physiological effect for human or horses' body is evident after the enzymatic acid action and with receptor activation pathways which can be generally pinpointed in vitro using cell culturing models for the intestinal epithelium.

1.6 Mare milk as a functional food

International Food Informational Council defines functional food as "Foods or dietary components that may provide a health benefit beyond basic nutrition and may play a role in reducing or minimizing the risk of certain diseases and other health conditions." Functional food or nutraceuticals have besides the nutritional effect, a bioactive function being a growing industry of prevention and slowing the progress of diseases, improving the quality of life. It comprises probiotics and prebiotics which help in food intolerance or sensitivity prevention, lowering the food allergies. Their effects can be gathered together as protection of the bones, low cholesterol (anti-obesity or obesity prevention) content, immunomodulation, antioxidative, antimicrobial and antihypertensive (Boye, 2015).

The physiological effects of probiotics have direct influence of the lactacid bacteria and yeasts on the organism named probiotic effect and an indirect effect via their metabolites named biogenic effect. Sixty five percent of the world functional food market is represented by probiotics.

Food labels are important for the consumers to make good healthy choices and for manufacturers to communicate their nutritional and health benefits. Either way they do it, nutrition content claims has to be made together with structure-function claims and health claims if the case. Health claims has to inform about the physiological role of the nutrient and its composition relevant to its role acceptable by the competent authorities of that country when and where the product is sold. It was shown that split claims with a health message on the front of the package and a more detailed one on the back for example, it has better an outcome. Regulatory agencies have to check and allow nutrient profiles scoring systems in order to choose nutrition and health claims with both negative and positive nutrients named on the pack and also nutrient density. Food legislation is variable in different countries but all have to align to the same standard rules to be followed by functional food/food to a common international code, namely Codex Alimentarius (Latin "food law") for consumer protection regulated by Food and Agriculture Organisation - FAO of the United Nation and the World Health Organisation -WHO (Park, 2009).

In USA only more than 60 therapeutic peptides have been approved by FDA, 140 in clinical trials and 500 in pre-clinical animal testing or at in vitro studies level. Multifunctional peptides with a plus of functionality like wound healing for example, e.g. GLP-1 agonists group, have a great commercial impact and focus for the pharma industry (Fosgerau and Hoffmann, 2015).

Functional food research connects nutrition and health as not only a consistent part of public health but also one important part of the pharmaceutical industry and mare's milk has a large variety of components with bioactive function in a convenient proportion for the human health for preventing diseases (Mills et al., 2011).

Next there is an enumeration of each type of bioactive component groups which can be found in mare's milk and their effect for human health.

In a study by (Chen et al., 2010) it was found that in kumis one bioactive peptide, P1 (βCN(f217-241)) with ACE inhibitory (antihypertensive) activity comprising 27 amino-acids but in general in milk bioactive peptides can have an antibacterial-antiviral effect or cyto-modulatory, opioid or mineral binding, antioxidant or dipeptidyl peptidase 4 inhibitors (anti-diabetes type 2) (El-Salam and El-Shibiny, 2013, Sharifi-Rad et al., 2015).

Phospholipids (MFGM components in mare milk) regulates the inflammatory reaction in the human body. Sphingomyelin and sphingolipids are the components of the myelin sheath with the role in the myelination process and with importance for the neurobehavior for the premature babies. Sphingolipids metabolites ceramides and sphingosines (e.g. sphingosine 1 phosphate) are messengers for cell growth, proliferation, differentiation and apoptosis as well as lymphocyte circulation and angiogenesis as part of immunomodulation.

Essential fatty acids (EFA) (linoleic and α linolenic acid) transform in the liver in LCPUFA (EPA, DHA, ARA) which are cell membrane components with an inhibiting role of pro-inflammatory cytokines production. LCFA by cyclooxygenase and lipoxygenase pathways become eicosanoids (PG), thromboxane (Tx), leukotrienes and lipoxins. Eicosanoids are mediators for diseases like thrombosis, cancer, atherosclerosis, asthma or rhinitis. ALA's final metabolite DHA docosahexaenoic acid, is abundant in central nervous system in the composition of phosphoglycerates together with arachidonic acid (ARA). DHA has its peak in the last

trimester of foetal development and in the first half year after birth when the brain develops. EFA inhibits tuberculosis bacteria growth which is not the case for the cow milk fat where they can grow. Administration of ALA (α linolenic acid) in human diet improved skin roughness in atopic eczema when the conversion of LA to ALA is poor during atopic dermatitis. This illustrates once more the importance of fat components in mare milk for human health.

β-lactoglobulin (β-Lg) (absent in humans) can bind hydrophobic molecules like retinol, steroids and long chain fatty acids; it can also bind cell surface receptors and it has enzymatic regulation role and also a role in the neonatal development of passive immunity. It can be carrier for resveratrol and folic acid.

α-La, equine α-lactalbumin is a part of the enzymatic complex which regulates lactose synthesis in the breast gland and lactose absorption in the intestine. It has anti-inflammatory and antinociceptive functions and its proteolytic digest release 3 peptides with bactericidal properties against G + bacteria.

Lysozyme hydrolytic enzyme breaks the bacterial wall by cleavage of the glycoside bond and it has an antiviral activity by inactivating the viral toxins.

Lactoferrin is a glycoprotein that binds iron with bacteriostatic (binding iron and delaying the growth of bacteria), bactericidal (binding lipopolysaccharides A from the porins of the cell wall of Gram – bacteria) and viricidal effect. Anti-inflammatory, anti-tumoral and immunomodulatory effects by inhibiting pro-inflammatory cytokines like TNF α, IL1β, IL6. Lf is upregulated in diseases like inflammatory bowel disease, neurodegenerative diseases, arthritis.
The most important peptide from the digestion of Lf is lactoferricin with bactericidal, viricidal, anti-fungal and anti-protozoa activity and more specific anti-tumoral by apoptosis in THP1 human monocyte leukemic cells.
Immunoglobulins the natural defence against infection, IgG in humans is transferred to foetus in utero through placenta and IgA is present in colostrum, as for mammals, they need colostrum to assure the natural defence system – IgA is present in equine milk and IgG in colostrum. IgA is a minor presence in colostrum.

Free oligosaccharides (glycans found in mare milk bound to k casein) have a role in gut microbiome's development in early life of mammals and infants ((Karav et al., 2015).

(Contarini and Povolo, 2013); (Haeggström et al., 2010); (Colombo et al., 2016); (Orlandi et al., 2003),(Orlandi et al., 2002, Olivry et al., 2001) (Vincenzetti et al., 2007),(Uniacke-Lowe et al., 2010); (Karav et al., 2018).

To sum up all of this components and subcomponents of mare's milk can regulate physiological processes in human body or can stop the pathological processes from spreading from it.

However, for scientifically proving the use of mare's milk as a functional food many more studies need to be performed both in vitro and in vivo.

Mare milk EFA diet was administrated for 6-8 weeks in rats and researchers (Fotschki et al., 2016) concluded that mare milk increases non-specific resistance to antigens like sheep 5% erythrocytes after 8 weeks of administration. Antimicrobial studies continued using raw milk and heated mare milk to check for the effect on Salmonella thyphimurium gene expression with positive antibacterial effect for the raw milk against Salmonella thyphimurium together with an anti-proliferative effect of the same raw milk against adenocarcinoma cancer colon cells, Caco2 cells (Corredig et al., 2015).

This time Salmonella enteritidis (Detha et al., 2019a) was used to study the antibacterial effect of mare milk (specifically whey) explaining the importance of the whey fraction bioactive peptides (lactoferrin and lysozyme) for mare's milk bactericidal effect against G negative bacteria.

Oral administration of mare milk gave negative reaction to the allergy skin prick test in a double-blind placebo controlled orally challenge (DBPCFC) using Ig-E mediated milk cow allergy (CMA) subjects- 25 children around 36 months old. 96% of the children had no allergic reaction to orally administrated mare milk, only one child having a positive reaction. In this way, mare milk can be used safely in infants and children's nutrition, especially for those with CMA explaining mare milk immunomodulatory effect in general (C Curadi et al., 2001).

Mare milk as a functional food is an interesting research subject of a great importance in human health.

1.7 Mammogenesis and lactogenesis

To understand the content of bioactive components in mare milk and its production it is important to understand the background of development of mare's mammary gland, how it starts producing milk and how this process results into lactation and further on how lactation can be regulated.
Mares have 2 pair of glands one larger and one smaller, each pair being on both sides of the medial line that ends in a single teat, two quarters exit via one teat. The parenchyma is made of lactiferous ducts and alveolar tissue (one layer of lactating epithelial cells) with role in milk secretion which groups into lobules and lobes and two gland cisterns. The teat opening has a sphincter (rosette of Furstenberg) to prevent milk leakage between suckling.

Myoepithelial cells (milk ejection, "myoepithelial basket") and nerve supply surround the alveolar tissue together with a capillary and lymphoid system which supply with milk precursors and give the hormonal control. The blood supply of the mare udder (two external pudendal arteries) contain precursors of the milk components which diffuse through the basal membrane into the lactating epithelial cells from the alveoli. Chemical reactions generate fat, protein and lactose components of the milk and the liquid is secreted from the alveoli to the lumen and further away to be delivered. Fat, lactose and non-essential amino acids of the milk are synthesized inside the epithelial cells of the mammary gland (alveolar tissue).

Nitrogen, non-essential amino acids and essential amino acids are incorporated into proteins. The non-essential amino acids are synthesized within the cells and build up into proteins within the ribosomes of RER (rough endoplasmic reticulum) during mRNA translation. The essential amino acids pass unchanged the basal membrane of the lactating cells together with the non-essential ones and are all incorporated into proteins.

Glucose and galactose are enzymatically (α-La and galactosyltransferase) converted into lactose in the secretory vesicles of Golgi apparatus. Lactose cannot diffuse from Golgi apparatus and creates osmotic pressure disbalance which becomes stable by water coming into the cells and in this was milk as a liquid is formed during the osmotic pressure regulation (Morel, 2015); (Wu, 2018).

Amino acid transport system helps in the uptake of amino acids and covalent chemical reactions help for bounding them into proteins in the RER. The proteins (milk proteins, membrane bound proteins and enzymes) are moved to Golgi apparatus where post-translational processing take place. Lactose and milk proteins in the secretory vesicles (pinocytosis vesicles) are transported in the alveolar apical lumen by fusion of the vesicles with the apical membrane and an opening is made for the discharge of them in the alveolar lumen. The process is called reverse pinocytosis. Casein is secreted as micelles inside Golgi apparatus and it associates with Calcium and Phosphorus.

Milk triglycerides are synthesized in SER (smooth endoplasmic reticulum) as droplets that will fuse into bigger lipid globule toward the apical membrane in the alveolar lumen. Milk fat globules are made by glycerol and short chain fatty acids derived from free fatty acids and glucose. Glucose C absorbed in the lactating cells is chemical converted into Acetyl Coenzyme A (CoA) and Malonyl CoA in the cytosol of the cells. Malonyl CoA and several enzymes form free fatty acids and short chain fatty acids are transported in the alveolar lumen by pinocytosis.

Immunoglobulins (Ig= bodies of Donne) are not synthesized inside the lactating cells but they pass the epithelial cell barrier from the blood by receptor binding to the basolateral membrane and endocytic transport vesicles which fuses with the apical membrane of the cells and are internalized in the alveolar lumen.

Iron bound to Lactoferrin is secreted with proteins by reverse pinocytosis.

Free ions (Ca, Mg, P, Na, K) and lactose increase the osmotic pressure and water from the cells flows into the milk and brings all of the components together fat, proteins and carbohydrates as raw milk (Hurley, 2009), (Morel, 2015).

From the birth to puberty mammary gland growth (fat and connective tissue) is isometric with the body growth and during puberty is allometric with the body growth and after puberty is cyclic depending upon dioestrus phase with the lobular-alveolar development which is short and high progesterone levels which will suppress lactogenesis. Puberty for horses is at one, one and a half years old in order for them to develop their entire genetic potential. Progesterone secreted by corpus luteum on the ovary together with prolactin,

oestrogen and somatotropin stimulates mammary ductal growth and differentiation into lobular-alveolar system.

During lactation, epithelial cell division on the parenchyma increases until the maxim productivity is reached with the size of the udder is returning to the non-lactating state which was post-weaning (Husvéth, 2011).

1.8 Lactation and galactopoiesis (lactation regulation)

1.8.1 Lactation

Lactation is the process of milk secretion, passive cisterns withdrawal and active ejection from the alveolar tissue (Husvéth, 2011).

The mare udder has a low capacity of less than 2 litres of milk and for this reason the foal needs to be fed with milk several times a day and the milking process is also repetitive, 5-7 times a day with 2-3 hours break (Salimei and Fantuz, 2012).

It lasts in general one year with an early, mid and late lactation time. 2-3 months post-partum the mare milk yield increases (10-18 l /day for Thoroughbred; 14-17 l/ day for draft mares and 8-10 l/day for ponies). After three months the foal will not depend only on milk for its nourishment, grass and hay will continue its diet. Consecutive, yield drops off will be seen and of course the weaning process (normally humans are weaning the foals at six months of age) before the due of delivery of the next foal if the mare is pregnant again.

All the nutrients from the mare milk decline during lactation encouraging the foal to get the nourishment from elsewhere (Husvéth, 2011), (Hurley, 2009) (Morel, 2015).

1.8.2 Galactopiesis (lactation regulation)

The maintenance of the lactation requires functional alveolar cells and an efficient milk ejection reflex.

The pituitary gland hormones STH, ACTH, TSH, insulin, parathyroid hormone serve in the process of lactation regulation (Husvéth, 2011).

An important role in the maintenance of lactation is played by the constant milk removal either by foal suckling or by mechanical milking removal knowing the fact that if the milk will stay longer inside the mammary gland it will have a negative feedback for lactation and galactopoiesis will fail to work properly. For the production animals galactopiesis is understood more like a milk yield management of course with human intervention with hormonal products which can help keeping the milk flow even without the need of the foal (Morel, 2015).

2. Cell culture and milk bioactive components

2.1 Cell culture using intestinal cells and milk

The cellular level mechanism for bioactive components in (mare) milk interaction with epithelial intestinal cells is the base for linking nutrition, molecular biology, diseases mechanism and public health.

Cultured cell lines can generate their own extra cellular matrix (ECM) important in cell line sub-culturing and adherence of cells to the plastic bottle substrate, for 2D cell monolayers. Cell differentiation in cell cultured consists of cell proliferation and is induced by an increased density of cells, a high cell to cell and cell to matrix interactions (integrins α, β linked with matrix molecule receptors for the transmembrane proteins - fibronectin, laminin and collagen by RGD -arginine- glycine- aspartic acid motifs). Integrins and cadherins (cell adhesion molecules) interact with vinculin, another transmembrane protein, generating a signalling

pathway to the nucleus. On the other hand, a high density of cells and cell to cell contact inhibits cell proliferation, producing less spreading and morphological changes of cells. However, a low density of cells plus growth factors (EGF, PDGF, FGF) supplements can induce cell proliferation and controlling this critical point helps having reliable and strong scientifically results from using cell culturing both for food safety and public health (Freshney, 2005).

Cell models in general can be used in the cosmetic and pharmaceutical industry and their culturing being improved by modern technique, cell models have been standardised and validated (cell lines) so they can be used in large settings, being cost efficient and avoiding animal testing ethical concerns. Being mini-biological models, cell lines are useful for research in general. Food safety and microbiology, metabolism and nutrition and toxicology can beneficiate from using intestinal cell models (Langerholc et al., 2011).

A similar in vivo environment can be mimicked by using in vitro cell culture inserts (trans wells) in cell culture monolayer to check for the transcellular transport of drugs. This can be useful in studying the time transfer of therapeutic drugs from the plasma blood to milk and to created cellular models for therapeutic drugs metabolism (Athavale et al., 2013).

Caco-2 monolayer monoculture model of human intestinal barrier, do not produce mucin layer and therefore can't be a good model for mucin producing Goblet cells. Furthermore, a low paracellular permeability and a low cellular permeability in general is based on an overexpression of P-glycoprotein, an efflux membrane transporter (Amin, 2013). In order to improve that, it was necessary to add HT29-MTX cells in a trans well co-culture. Rhodamine 123 was used to check for the cellular efflux proving the existence of tight junctions between the cells and verapamil HCl to block P-gp and lucifer yellow (LY) as a marker for the paracellular transport. A transport buffer (TB) was used to show the cells permeability and TEER parameter being determined afterwards. After 7 days of co-culture transepithelial resistance (TEER) increased at its peak with the two cell lines monolayers seeded one after the other- HT29-MTX 2 days after Caco-2 cells (Béduneau et al., 2014).

Caco-2 cell were co-cultured with THP-1 cells differentiated with PMA (phorbol 12 myristate 13 acetate) - to mimic the intestinal barrier for both physiological state or inflamed condition (IFNγ, LPS). PMA differentiated THP-1 cells have been used as a macrophages model and together with Caco-2 cells, gave the possibility to study different state of the intestinal integrity (Kämpfer et al., 2017).

These cell models can probably be used to study the whey bioactive peptides from mare milk and their effect on intestinal barrier.

Bioactive substances in milk like growth factors, whey proteins, caseins and its metabolites, minerals or vitamins, and their effect for animal or human gastro-intestinal tract cells (different cell lines) can be tested in order to improve human health and diagnostic of human diseases. Different assays using cell functions can be of use and have been tested for cow milk. Mammary gland cell lines can be used to test bioactive components from milk or fermented milk linking different diseases of mammary gland with the nutritional implication for diagnostic or treatment (Purup and Nielsen, 2011).

Milk bioactive compounds can be useful in future for treatment, prevention or just for improving nutrition and wellbeing. The focus on cow milk can or was shifted to other products like camel milk proteins or food matrix dairy proteins and its proteolysis metabolites in vitro so the researchers could prove once again the importance of milk bioactive components for human health by showing anti-inflammatory and antioxidant effect of these bioactive peptides for human health. They used mostly in vitro proteolytic digestion by different enzymatic equipment combined with RP-HPLC for fractioning the resulted peptides to scientifically conduct their studies (Rafiee Tari et al., 2019), (Ibrahim et al., 2018).

A two-compartment cell model mimicking the human intestine using Caco2 cell line (Caco2 cell surface peptidase for peptide hydrolysis) together with RP-HPLC and MS/MS peptide sequencing were used to test some peptides resulted from in vitro digestion found in human k casein and bovine β and k casein and prove their antioxidant effect. Caco2 cells were treated with H_2O_2 to create the oxidative stress and after the peptide's pre-exposure, the antioxidant effect was measured using glutathione and thiol groups (redox signalling role) enzymatic reduction as parameters for the antioxidant effect. The tested peptides showed protection against oxidative stress for Caco2 cells and a role in signalling pathways of the cellular oxidative stress (Tonolo et al., 2018).

The two-compartment system have a microporous membrane where the epithelial intestinal cells prior polarised (IEC-6, IEC-18, FHs 74 Int) or transformed cell lines of cancer origin like Caco2, T84, HT-29 (which can differentiate into small intestinal cells and propagate) can grow, above which is the apical -lumen side and below which is the basolateral – intestinal content side. This system has been used in different studies

to show the human and bovine-milk derived bioactive substances (Cencic and Chingwaru, 2010), (Takeda et al., 2004), (Purup et al., 2006).

The gastro-intestinal tract is a target organ for milk and milk products because it is the point where pre-digested peptides and components from whey/raw mare milk/fermented mare's milk enters the intestinal lumen having their positive action for the human body by absorption of its active amino-acids and micro components into the blood. Furthermore, therapeutically drugs transport and their residual excretion into the mammary gland and milk, together with the metabolism of new food matrices or products, can be tested using epithelial intestinal cell-based models (Purup and Nielsen, 2011).

2.2 Advantages and disadvantages of using cell culture

One of the advantages for using cell-based models for searching of novel bioactive substances in milk and not only, can be that several concentrations of treatments or bioactive compounds can be tested at the same time on different and specific cells. Moreover, different cell functions can be checked to see the effect of this substances for the human physiology. Examples for this cell functions that can be tested are, morphology, metabolism, signalling pathways, gene expression, apoptosis, cell viability and migration.

Another reason can be the costs which are less than for using animal models and avoiding their sufferance and respecting the 3Rs principle (replacement, reduction, refinement) for animal welfare used in experiments (Purup and Nielsen, 2011) (Langerholc et al., 2011).

For the fact that milk components and metabolites are reaching the intestinal barrier, generic epithelial intestinal cell lines are proper for studies with milk as the main focus. Their main quality is that these cells are simple, robust, repeatable interlaboratory data can be obtained from using them in experiments and not the least, they have a high-scale testing feature (Langerholc et al., 2011).

Cultured cells change the microenvironment from *in vivo* to *in vitro* and this is one of the major disadvantages of using them for testing in vivo processes. The hormonal and growing stimuli are lower *in*

vitro than *in vivo*, no cell-matrix interactions are seen, low heterogeneity and 3D spatial distribution of cells *in vitro* is missing, being replaced with a 2D plastic cell culturing bottles monolayer which can migrate and proliferate as **undifferentiated cells**. On the other side the culturing conditions (incubation temperature, gas phase, media culture, added supplements for cell growth, cell density and adhesion plus heterotypic cell interactions on the culture bottles) can be fair enough controlled based on the desire and usage of the experiments. More than that, 3D cell culture can be created from 2D monolayer on collagen gel, gelatine sponge or other matrices with filter well inserts (Freshney, 2005).

Nevertheless, *in vitro* systems cannot copy in vivo conditions which are more complex or which require homeostasis regulation and a dynamic metabolism, signalling and interaction between organs and the human body as a whole. Furthermore, beforehand researchers need to plan the experiments in a manner that they will get a high throughput testing and of course data, good predictive and results interpretation, to choose the right target organ (cell line) with the established cell line giving reproducible results conform with the human physiology. Taking into account the possible cell dedifferentiation process which can occur during sub culturing the cell line it is necessary to have the right cell growth medium under the right incubation condition during the right amount of time. As for the milk samples to be analysed, they need to reflect the end-points to be measured and avoid cytotoxicity and lack of selectivity of the bioactive components in the fraction that will be the active treatment for the cell-based chosen (Purup and Nielsen, 2011).

In order to develop good cell-based models for diseases one might be opened to try alternative cell-based models coupled with translational medicine reports where clinical practice has the main role. 3D co-culture, organ on-a-chip systems, usage of induced pluripotent stem cells prove to be a multilevel plan with a combination of scientifically data from genetics, imaging, engineering, molecular biology, immunology, computer science and medical marketing domains. Principles of physiological relevance and disease-relevant assays in preclinical models are proposed to work cost efficiently if financial investment in drug research and personalised medicine is added (Horvath et al., 2016). On the other hand, cell-culturing it is a repetitive set of procedures with steps which cannot work on an ideal model or where the conditions can’t change every assay performed in different laboratories settings. This is an important factor to be taken into account when using in vitro cell culturing models testing.

3. Wound healing and the role of milk

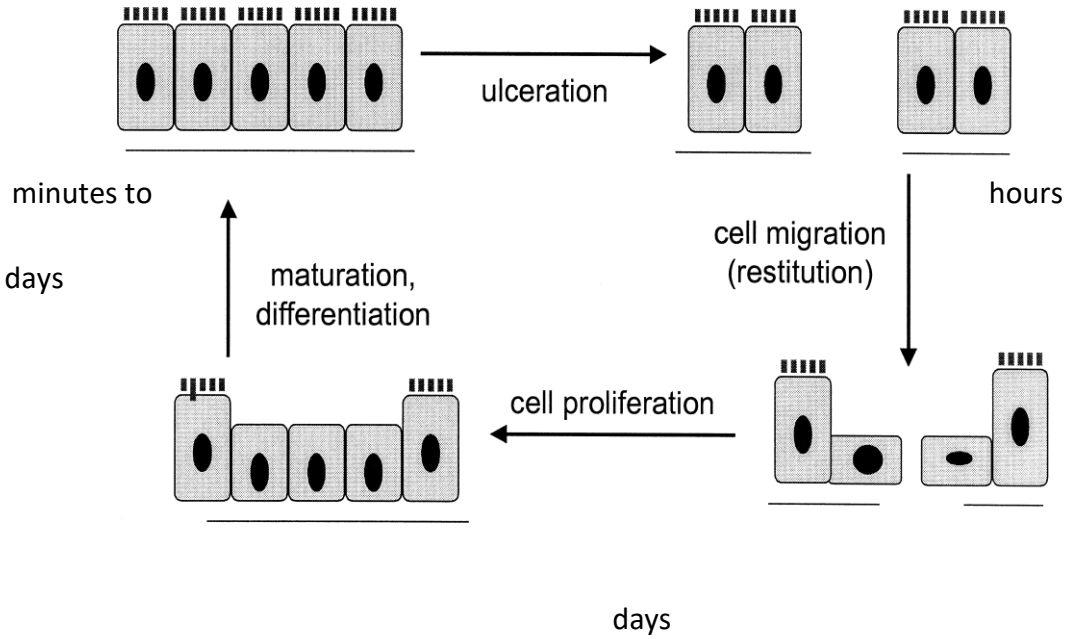
3.1 Wound healing

Mucosal intestinal healing (proliferation and differentiation) and epithelial restitution (migration) is required to the wound site to stop the chaotic inflammatory process. Epithelial barrier function is affected and commensal bacteria are transferred in the intestinal wall with hyperimmune response from T cell and subsequent chronic inflammation of the intestinal wall. Intestinal bowel diseases (IBD) have this general pathogenesis scenario. Cellular microscopical level of epithelial intestinal barrier restitution in the gap produced by the wound requires a balance between proliferation, migration and differentiation of intestinal epithelial cells (IECs) (Neurath and Travis, 2012).

Regulatory growth factors and peptides (chemokines like CXCR4, CXCL12, defensins, cytokines IL-1β, IL-6, IL-22, IL-2, trefoil peptides, TGFα, TGFβ, EGF, FGF, KGF, IGF-I and IGF-II, HGF), soluble peptides and cytokines and TLR (toll-like receptor) signalling pathways are working together in the process of intestinal wound healing.

From the human intestinal lining point of view, this signalling process in wound healing can be explained by TFF peptides' (trefoil factor family) action from the apical membrane of the epithelial intestinal cells together with mucins glycoproteins in an independent TGF-β signalling. Trefoil 3 factor regulation prevents apoptosis of the intestinal epithelial cells. In a TGF-β depending way IECs migration is activated by FGF, EGF, VEGF, GLP-2, HGF growth factors and IL-1, IL-2, IFN-γ with TGF-β activation and synthesis. Epithelial cells restitution is depending on several regulatory peptides which activity can be enhanced, glycoproteins like mucins which form a protective gel for the intestinal barrier together with phospholipids (phosphatidylcholine) and short chain fatty acids (SCFA) in a form of hydrophobic pre-epithelial layer. The epithelial lining is a simple unstratified epithelium brought together by tight junction with a turnover of 24 to 96h. IECs proliferate and differentiate as a functional villus layer in the crypts. TLR signalling enhance the innate immunity and regulates IECs homeostasis. TGF-β and EGF stimulate IECs proliferation 5 to 10 times higher than IL-2, HGF and FGF in in vitro studies (Neurath and Travis, 2012) (Sturm and Dignass, 2008).

TLR9 recognises CpG motifs on bacterial DNA and stimulates innate immunity in IL-12 and IFN production with wound healing. On the surface of myeloid cells (macrophages, immature dendritic cells and osteoclasts) is receptor Trem2 and this signalling pathway activates IL-4 and IL-13 production and mucosal healing. Proliferation of IECs is enhanced by NfkB pathway activation. NfKB gut immune responses is activated by bacterial muramyl dipeptide (MDP) on NOD2 intracellular receptor encoded by CARD15/NOD2- IBD1 gene. This gene is associated with CD in humans. Wound healing signalling at the epithelial level is not the least regulated by TGFα signalling with ERK1 and ERK2 kinases and mitogen activated protein kinase activation (MAP-kinase). Mucosal intestinal healing is a part of the three types of necessary healing of the intestinal wall important in IBD (Inflammatory bowel disease like ulcerative colitis -UC and Crohn's disease- CD) namely ultrastructural, functional and histological healing, an essential key in the treatment of CD and UC. Despite the fact that intestinal mucosa represents less than 15% from the intestinal wall, mucosal healing is associated with a good prognostic for IBDs which last a life time. Reducing hospitalisation, surgery risk and preventing relapse, mucosal healing is a therapeutic endpoint in clinical trials. Newly approaches to understand IBD pathogenesis using mouse models like Tnf $^{\Delta ARE/+}$, SAMP1/YitFc of CD ileitis is important for studying the mucosal healing and advanced targeted drug technologies (biologics or monoclonal antibodies, computational biology) are of a great importance for this all-life time diseases. (Neurath and Travis, 2012, Sturm and Dignass, 2008),(Cominelli et al., 2017).



Simplified wound healing picture of the stages of superficial epithelial layer repair in the case of small injuries. After (Dignass and Sturm, 2001)

3.2 The role of milk in wound healing

The cows' whey milk showed growth stimulation on human intestinal cells based on lactation phase and in a range of concentration up to 10%. The growth stimulation of the intestinal cells decreased during the first week postpartum, being high in colostrum and increased in mature milk (higher than in colostrum).However no correlation between IGF-I, TGF-β1, TGF-β2 and IGFBP concentration variation and growth promoting factor of the whey on human small intestine epithelial cells couldn't be found (Purup et al., 2006).

Epithelial cell migration and proliferation was stimulated in an *in-vitro* study using low concentration casein (casein glycomacropeptide and casein phosphopeptide) and whey protein (with α Lactalbumin) preparation products based on cow milk (Li et al., 2018).

Genomic prediction model of IBD (Peters et al., 2017) and a System Pharmacology network computational model for IBD can be of great help for new biomarkers as therapeutic targets ,a mark of personalised medicine using quantitative models (Balbas-Martinez et al., 2018) that could use mare milk and personalised medicine in intestinal wound healing.

A randomised placebo-controlled, double-blind, cross-over intervention study on adolescents with IBD was done using orally mare milk of 250 mL daily. Abdominal and extraintestinal pain was released, no dramatic symptomatology with less medication to reduce it was observed and less blood in the faeces was observed after the mare milk intake. Blood, urine and faeces parameters were unchanged along with CD and UC's activity index. They concluded that consumption of a glass of mare milk daily improved the clinical state of patients with CD and UC (Schubert et al., 2009).

4. Mare milk in human health

4.1 Previous studies of beneficial effect

Mare milk variate composition with a big nutritive value and an important bioactivity for human health can be considered a functional food and both in vitro testing and in vivo testing using human subjects have started to be cited in the scientific literature in order to demonstrate it.
Milk functional peptides are released by its digestion and can have an angiotensin-converting enzyme inhibition (ACE) role (antihypertensive) or dipeptidyl peptidase IV (DPP-IV) inhibition role or maybe an opioid activity, antioxidative, antimicrobial, antithrombotic, immunomodulating or mineral binding role in the human body (Nielsen et al., 2017a). DPP IV have been tested in clinical trials for type 2 diabetes keeping the activity of GLP-1 and GIP after the food intake and blood glucose control through insulin secretion stimulation (Flatt et al., 2008).

Gene expression (hilA, ssrB2) of Salmonella Typhimurium was tested using raw mare's milk and moreover its effect on Caco2 cells proliferation. All mare milk supernatants had a decrease effect on S. Typhimurium gene expression and a decrease in Caco2 cell proliferation (Corredig et al., 2015).
A Milk Bioactive Peptide Database – MBPDB for human milk and animals' milk (http://mbpdb.nws.oregonstate.edu/) was created based on the literature review with 944 entries, 76% cow, 11% human, 7% goat, 5% sheep, 1% buffalo, camel, rabbit, donkey, yak and pig. Other databases based on bioactive peptides exists as well like MilkAMP (antimicrobial peptides), AHTPDB (milk peptides with ACE-inhibitory activity) and BIOPEP (sensory peptides and amino-acids). ACE and antimicrobial peptides (327) are the most abundant ones in the literature, 64 have DPP-IV-inhibitory activity, 25 opioid, 41 antioxidant, 10 anti-inflammatory, 70 miscellaneous, 14 anti-cancer and 9 immunomodulatory (Nielsen et al., 2017a).

In Germany at Institute of Animal Breeding and Genetics of Justus Liebig University Giessen (Sames et al., 2018) was performed an in silico analysis using BIOPEP of mare milk digested with trypsin, chymotrypsin and pepsin and from whey proteins β-lactoglobulin 1 and 2 ,α-lactalbumin, αS1-casein, αS2-casein, β-casein and κ-casein of mare´s milk they discovered 17 bioactive peptides with antihypertensive effect, among them IVR (αS2 casein), HHL (αS2 casein), EY (αS1 casein) and VY (βlactoglobulin 1 and 2) being most abundant and with the greatest effect as antihypertensive substance in very low concentration. It was observed also that the genetical variance of whey proteins do not affect the ACE-inhibitory activity of mares' milk.
At the Department of Animal Diseases and Veterinary Public Health, Faculty of Veterinary Medicine in Kupang Indonesia, A. Detha and her team (Detha et al., 2019b) showed the antimicrobial activity of mares' milk against Salmonella enteritidis. Lactoferrin and lysozyme from Sumba mare's whey milk inhibits S. Enteritidis with an inhibition zone similar (around 19 mm) to penicillin. Equine milk contains double lysozyme (3% of the total whey proteins) compared to human milk and much greater than cows' milk. This gives it a high resistance to bacterial growth and protection against enterically inflammation (Fox and Kelly, 2006).

Mare milk in cosmetics – in Slovenia 2012 was tested a tonic and a cream based on mare milk for three different ages ladies with dry skin for two months along with placebo treatment. Multiskin Test Centre MC 750and Visioface Quick tested different skin properties like elasticity, pH, pores, moisture, dark spots of the face skin. Wrinkle and dark spots reduction, a good moisture and face skin elasticity was observed

using mare milk products during two months. There is no information about the products, only the fact that they are based on mare milk and they are a tonic and a cream and this study was presented to a Dermatology Congress in 2014. It was stressed out the need for clinical studies in dermatology and certified products based on mare milk which can economically improve dairy horse breeding in Slovenia (Gavric, 2014) .

Furthermore, from fermented milk had positive scavenging tests ($ABST^{+}$, DPPH) and had inhibited melanin production in B16F10 melanoma cells. Topical treatment with NS8-FS in hairless mice SKH-1, reduced UVB skin photodamage, epidermal dehydration and activated BFE2L2 pathway and antioxidant enzymes in oxidative stress (Rong et al., 2017).

Allergy to mare's milk it happens not so often (Fanta and Ebner, 1998), (Gall et al., 1996),(Doyen et al., 2013, Verhulst et al., 2016) and mostly CMA (cow milk allergy) is implied among infants and children (Businco et al., 2000, C Curadi et al., 2001, Heine et al., 2017, RANGEL et al., 2016), (Host, 2002)

In Kazakhstan, in 2017 another study using mare milk and yogurt made of mare milk was tested and accepted to meet the nutritional needs in the diet of 12-23 months old children (milk) and for 2-11 years old children for the care of yoghurt made of mare milk. Omega 6, omega 3, vitamin C content from both raw milk (15-34%, 15-23%, 39,6-57,3%) and fermented mare milk (4,3-14,5%, 6,4-12%, 23,8-31,7%) was bringing a good percentage of the daily recommended necessary dose (V. Yakunin et al., 2017).

Mare's milk chemical composition it is supposed to influence human epigenetics with a focus on aging, the review is in Russian (Grishina, 2018).

Because of the lipid profile of mare milk, it can be used mixed with mineral water and herbal tea for balancing the fat metabolism in the human body being proposed to help as therapy in atherosclerosis- application patent number RU20120115603 20120418, applicant GIL MUTDINOVA LIRA TALGATOVNA [RU].

Low molecular-weight peptides from mare's milk together with a low % of protein and a high quality of free amino-acids made researchers from Kazakhstan to propose the use of mare's milk for sports nutrition production (protein hydrolysates with the addition of antioxidants and vitamins A, C, E). The product (rich in active peptides and hypo-allergic fermentation protein hydrolysates) was tested on mice subjected to daily swimming physical effort for one month. The results showed a decrease in bloods'

lactate and urine an antioxidant enzymes activation and a reduction in the concentration of lipid peroxidation residues(Yakunin et al., 2016).

Due to its balanced composition and nutritive value, mare's milk can be used in different percentage with cow's milk to make ice-cream observing that the combination of these two types of milk added amino-acids (proline, valine, histidine, methionine, proline etc.) and minerals improving ice-cream nutritive and biological value (Akanova et al., 2017)

Casein and whey proteins from milk of different animals (cow, sheep, mare, donkey, camel and goat) were tested with another cell line, namely MCF7 (breast cancer cell line) combined with *in silico* bioinformatic tools to show the anticancer effect of milk. The anticancer activity was explained by high positive and negative charges and a high α helix structure and it was evident for the mare milk together with donkey and camel. Sheep and goat milk did not show antiproliferative cellular activity. Caseins from mare milk showed the highest cytotoxic activity explained by the high percentage of α helix casein followed by camel whey proteins. The highest anticancer activity of mares 'milk casein was observed followed by camel, donkey and cow caseins with no effect for sheep and goat milk. Casein structure was primarily implied for the anticancer activity of the bioactive peptides from mare milk (Shariatikia et al., 2017). Human leukaemia cells lines Raji and CEM-SS viability tests were performed to prove the cytotoxic effect of milk from various species inclusive human and mares milk and 10 % mare milk showed statistically significant cytotoxic effect against human leukaemia cell lines among cow, human and goat milk (Rahmat, 2006).

4.2 Mares' milk in clinical studies

Table 6 Mare milk studies in clinical trials after(GCP)

Clinical trial no.	**Type of product**	**Disease**	**Parameters**	**End of completion**
NCT03669835	Sublimated mare milk supplement (dried powder of mare milk) along with the standard treatment (Kazakhstan)	Hepatitis C for 18-65 years old patients	- liver function, - the degree of liver fibrosis, -change in the gut microbiota, - changes in the immune status, -phospholipids of the lymphocyte membranes (every two weeks, up to week 8)	01.11.2020
NCT03657836		Acute bronchitis for 4-5 years old patients	- changes in intestinal microbiota, - changes in the immune status biomarkers, - changes of stool quality and frequency (up to 60 days)	01.12.2020
NCT03664596		NASH (non/alcoholic steatohepatitis	- changes in the liver indicators,	01.06.2019

		16-60 years old patients	- degree of steatosis and fibrosis by fibroelastometry and ultrasound, - changes in blood biochemistry results (cholesterol, glucose) and weight, - general symptoms of NASH (for two months every two weeks)	
NCT03665519		Biliary cholangitis (primary biliary cirrhosis) 18-75 years old patients	- changes in liver function, - asthenia intensity, - liver histology, - hepatic encephalopathy changes, - inflammatory biomarkers changes (erythrocytes sedimentation rate), - presence of cholestasis and stage, - changes in prothrombin time, fibrinogen and albumin,	01.07.2020

			- changes in LDL and total protein, - changes in platelet count, - anaemia, - hepatic hypertension (for 4 months)	
NCT03594877		Psoriasis (gut microbiome role) 30-45 years old patients	- changes in stool microorganism composition, - changes for intestinal inflammation biomarkers, - vitamin D level changes, - changes in PASI psoriasis indicator	30.01.2020
NCT00753805 Double blind placebo-controlled crossover trial	Mare's milk 250 ml daily for 16 weeks, 4 weeks washout period and placebo drink (Germany)	Atopic dermatitis (AD) 23 patients 18-54 years old	- SCORAD test after 4,8,12,16 weeks and stool samples, - immunological parameters – Ecp, SE selectin, MDC, IL-16, CRP at 16 weeks, faecal microbiota test.	Completed
NCT00940576	Mare's milk 250 ml daily for 8 weeks	Chronic IBD (Crohn's disease and	- Score of Crohn's disease and Ulcerative colitis,	completed

	and placebo drink (Germany)	Ulcerative colitis) 10-54 years old but mostly adolescents	- extraintestinal pain control for 8 weeks	

5. Aim

The aim of this project was to study the whey of mare and its growth factors on the viability of two epithelial cell lines and furthermore to check the wound healing's effect of the mare's milk's whey using one of the cell lines. Six mare milk sample were collected at about four to six months of lactation (late lactation time) and used for the assays. Female horses were warmblood type used for riding, jumping and dressage. Moreover, a quantification of some of the growth factors from the whey of colostrum and the whey of mare milk was done trying to explain the trophic effect upon cellular epithelial intestinal lines both in the case of the mitogenic effect and wound healing.

6. Hypothesis

We hypothesised that peptides and growth factors from the whey of mare's milk will help wound healing in an IEC-6 – based model for wound healing and moreover, different concentrations of mare milk can enhance proliferation of these cells together with human small intestinal cells FHs 74 Int showing the beneficial role of mare milk in human metabolism and health. In this way, a proof of bioactivity of mare's milk or mare's milk as a functional food can be implied from the point of human health with the focus on the gastro-intestinal tract.

7. Materials and methods

Mare milk was obtained from six heathy mares, warmblood type, riding horses bred for dressage and jumping show with none used for competition. Milk sampling was done six times but it was analysed for two of the milking time, one during month 4-5 and one during months 6-7 of lactation (milking samples one and four). One of the mares was at its first lactation (4 years old) and the others were older than 10 years and at six, five and third lactation time.

For the colostrum study, samples were collected from five mares (4 Thoroughbred and 1 warmblood) mostly used for breeding foals.

The milk whey was prepared and analysed as neutralized acidic whey.

Cooled milk, after centrifugation and fat layer removal was acidified to pH 4.2 by titrating acetic acid 5M, in this way, casein was precipitated from the whey fraction. After another round of centrifugation and removal of the casein layer, the acidic whey was neutralized by adding sodium hydroxide 3M to reach 7.4 acidity. A final centrifugation was done and the supernatant was sterile filtrated. The vials were kept at -80˚C before using.

Mare colostrum was analysed as neutralised acidic whey of colostrum using the same method as for the mare milk samples and the vials were kept at -80˚C before usage.

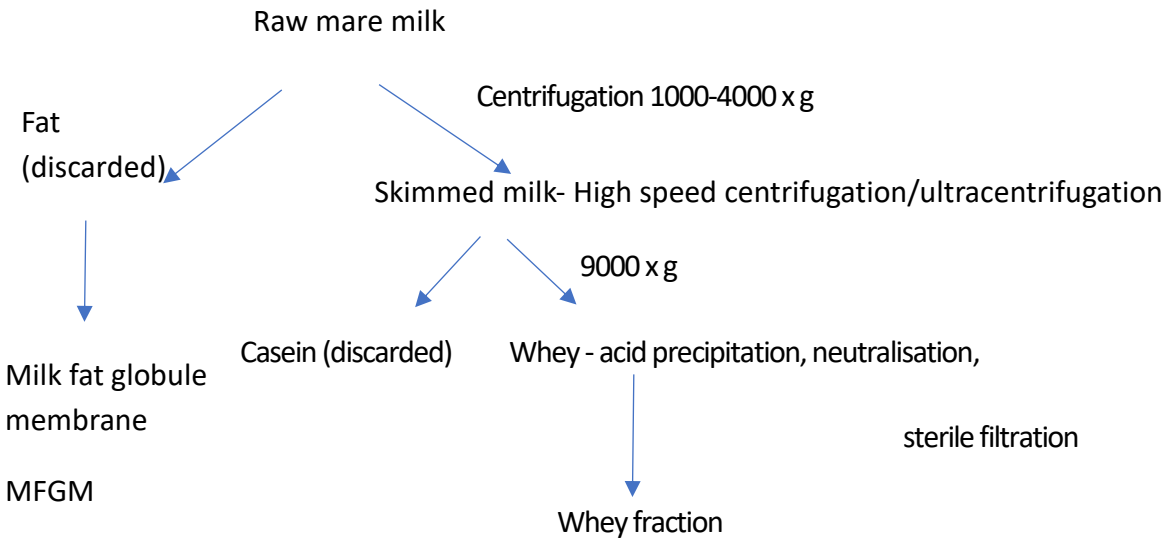
No scratch wound assay was performed using colostrum. Colostrum as neutralised acidic colostrum was analysed for cell viability using IEC-6 cells.

The cell-based model used was first IEC6 cells (DSMZ, ACC 111, Braunschweig, Germany) rat small intestine epithelial cells and second, FHs 74 Int cells (ATCC® CCL-241™), human small intestine epithelial cells.

As endpoint measurements in general was used the cell viability assay, AlamarBlue® BioSource, AH diagnostics, Aarhus, Denmark for two cell lines, both small intestinal epithelial cell lines from rats and humans and the cell migration scratch wound assay only for the rats' intestinal epithelial cell line. The effect was calculated after one hour of exposure to the florescent dye.

Cell viability AlamarBlue® assay was further performed for 5 colostrum samples from different mares (H1-H4 four mares Thoroughbred breed and H5 one Warmblood mare from the stud department of Aarhus University – Foulum (used for breeding foals) to check the viability effect of colostrum on intestinal epithelial cells.

In addition, ELISA and TR-FIA assays were performed by the laboratory assistants from Animal Science Department at Aarhus University Foulum to measure the concentration of growth factors for the first 6 mare milk samples and for the next 5 colostrum samples (different animals than the first 6).



Mare milk fractions, in the lab experiment for the actual project it was used neutralised acidic whey (without fat and casein fraction)

7.1 Cell viability using IEC-6 cells

Monolayers of IEC-6 cells (rat small intestine cells) from P7 to P11 (first time milking samples) and from passage 13 to 20 (fourth time milking samples) were cultivated and treated with different concentration

of whey of mare milk (0.05, 0.1, 0.25, 0.5, 1, 2.5, 5 and 10%) from 6 mares with different age and lactation number at 2 different sampling time points (see *Table8* from the Appendix section). To check if the assays were correct, controls were used every time a negative and a positive control.
Cell viability assay, namely AlamarBlue® using IEC-6 cells was done three times for all six samples of whey of mare's milk for both milk sampling time and relative values of the means were calculated.
Culture media as growth media for IEC6 cells was used Dulbecco's Modified Eagle Medium (DMEM 11960-044; Gibco, Life Technologies, Nærum, Denmark) supplemented with 10% foetal calf serum (FCS, 10%; Cambrex Bio Science, Copenhagen, Denmark) plus sodium pyruvate (11360/039 100mM, Gibco Life Technologies), Glutamax (Gibco Life Technologies), and penicillin and streptomycin (P0781 100mM, Sigma-Aldrich A/S, Brøndby, Denmark) on 37°C and 5% CO_2 atmosphere incubator. For cell culturing it was used 25 cm^2 and 75 cm^2 cell culture plastic bottles depending on the number of cells needed for an assay and how many assays on the same time are needed to be done.

Culture media as dilution media for the whey samples in the concentration range, was almost the same as the growth medium but without foetal calf serum.

Foetal calf serum or foetal bovine serum is normally obtained from the calves' blood after coagulation and centrifugation with the red blood cells, fibrin and clothing factors discarded. It contains bovine serum albumin (BSA), hormones, growth factors, minerals, amino acids, fatty acids, glucose etc. It is useful for the cell's growth and it has less antibodies and more growth factors than non-foetal serum.

IEC6 cells from DSMZ (Braunschweig Germany AC 111) (passage 9 to 22) were plated into 96 wells plates 5000 cells per well per 100 µL in culture media for 24 hours (50000 cells per ml).

In order to get the right number of cells per well, there is need of sub culturing the cells and counting. Sub culturing has been done when the cells in monolayer were 80-90 % confluent. After thawing them out, they require at least 2 passages before usage.

A cell line is grown (culturing the cells for maintenance) in several passages under a pattern until it stopes. At the beginning a lag phase is seen after seeding and a log phase comes right after the lag phase when cells suffer an exponential growth. When the cell density (cell/cm^2 substrate) or cell concentration (cell/ml of specific growth medium) exceeds the capacity of the medium or of the plastic cell-culture bottle surface, the process of growth decreases or it stopes and the cells can be divided or growth medium can be changed if

they are still having space to grow. Everything is done before the confluence is obtained and with a medium change every two days (Wang, 2006).

In this case, for sub culturing or passage the cells, the small plastic cell culturing bottles of 25 cm^2 were used to grow the cells together with the culture medium (5ml) incubated at 37°C with 5% CO_2 for up to 96 (48-96 hours) with the growth medium changed after 24 hours. The growth media was discarded and the cells were washed twice with PBS (Dulbecco Phosphate-Buffered Saline DPBS 14200-067 Gibco Life Technologies) without $CaCl_2$ and $MgCl_2$ to remove any FCS left in the medium before trypsinisation. For loosening the cells from the bottom of the plastic bottle, 2 ml of 0,05% Trypsin-EDTA (25300-062 Gibco) was added to the cells for 2 minutes. Trypsin is a serine protease which cleaves proteins from ECM and detached the cells from the bottom of the plastic cell culture bottles. Trypsin cleaves arginine and lysine amino-acids on the C- termini and is inhibited by the FCS proteases. 10 % FCS from 5ml of growth medium stopes the reaction of cells with trypsin and EDTA so the cells don't risk to be over-digested by trypsin, keeping them morphologically intact and good for usage further in the viability cell proliferation experiment. EDTA binds Ca^{2+} and Mg^{2+} ions and helps detaching the cells from the plastic substrate After centrifugation for 5 minutes at 0.5 rpm, the supernatant is discarded and the cells are re-suspended in 5 ml growth medium ready for counting. As for counting, 10 µl of re-suspended cells is being mixed with trepan blue dye, non-toxic, and pipetted in 50 cell counting chamber slides (Countess Invitrogen) and the counting (per ml of cells in culture medium) was done using an automated counting system, by Countess life II FL. Knowing the cells number per ml, it was determined the ml of cells plus growth medium needed for each 96 well plate.

After plating, the second day (after 24 hours) when the cells attach to the plastic bottom of the wells and they spread evenly on the entire surface , they are washed with phosphate-buffered saline (Gibco by life technologies; 14080-048) supplemented with $CaCl_2$ and $MgCl_2$ and Sodium hydroxide 0,3 M and incubated for 72 hours (days necessary for the cell proliferation on the wound site as in the model designed by Dignass et al.2001) at 37°C, 5% CO_2 with whey samples diluted in culture medium without FCS (200 µl per well) in concentration of 10%, 5%, 2.5%, 1%, 0.5%, 0.25%, 0.1% and 0.05%. Culture medium with FCS was used as negative control and without FCS (dilution medium) as positive control. Water 200 µl per well was added to the edges of the plates to avoid static electricity.

To quantify cells proliferation was used AlamarBlue® fluorometry assay on Envision Multilabel Reader 2103 (Perkin Elmer). Resazurin, a non-fluorescent (λabs=604 nm) compound from AlamarBlue in contact with the

cells in the mitochondria, if cells are viable, is being metabolized to resorufin which is fluorescent (λabs/λem=571/585 nm) and can be quantified.

Alamarblue were diluted 1 to 10 with PBS plus $CaCl_2$ and $MgCl_2$ is added as 110 µl per well after the culture medium removal.

Reading the plates using Envision Multilabel Reader 2103 was done after incubation of 1 hour, 2 hours and 3 hours with AlamarBlue but the data used for the statistical analyses was the measurement of the fluorescence after one hour because the effect was more consistent. The data was processed using Microsoft Excel and SAS version 9.4 mixed model for statistical analyses.

7.1.1 Reagents

For the growth medium it was used DMEM 11960-044 (Dulbecco's Modified Eagle Medium, with high glucose and no glutamine); 10% FCS (foetal calf serum), 0.44% Na-pyruvate; 0.88% penicillin and streptomycin and 1% Glutamax. Sodium pyruvate is a source of energy (carbon source) being an intermediate in glycolysis process. Penstrep contains penicillin and streptomycin antibiotics essential to keep the cell culture media free of bacterial contamination.

Glutamax by Gibco is a dipeptide formed by L-alanine and L-glutamine, another energy source for the cell growth. It has the advantage of being more stable in solution than L glutamine and it is good for cells which are growing fast. Hydrolysis of this dipeptide release the two amino-acids for the cell usage in TCA cycle or protein synthesis (Thermofisher).

Dilution medium had almost the same composition without FCS and it was used for the dilutions (concentration range of the whey of mare milk used as treatment for the IEC-6 cell line). AlamarBlue® (Invitrogen by Thermo Fisher Scientific) the dye used for the cell viability test (5000 cells, a non-toxic variant of MTT 3-(4,5- Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a colorimetric assay for cell viability.

Resazurin is the dye from AlamarBlue and inside the mitochondria, it is reduced to resorufin during REDOX processes which is pink and fluorescent and the number of the viable cells is direct proportional with the colour intensity of it measured by Envision software (Envision Multilabel Reader 2103- Perkin Elmer).

Trepan blue dye (Invitrogen by Thermo Fisher Scientific) was used for the automated cell counting using Countess FL II.

FCS (foetal calf serum) South America Bio Whittaker used as supplement for improved cell growth.

Trypsin plus EDTA (25300-062 by Gybco) is important for the cell detachment essential for cells sub culturing.

PBS without $CaCl_2$and $MgCl_2$ (PBS-/-) Dulbecco Phosphate-Buffered Saline DPBS 14200-067 Gibco Life Technologies for sub culturing the cells.

PBS with $CaCl_2$and $MgCl_2$ (PBS +/+) Dulbecco Phosphate-Buffered Saline DPBS 14080-048 Gibco Life Technologies for washing the cells from the FCS residues.

7.1.2 Plating and dilution schema

Table 7 Whey dilutions samples used as treatment for the IEC-6 cells the dilutions were plated in 4 replicates for each of them and for calculation an average was considered. At the beginning a plate using FCS in the same range of concentrations was used for calibration

Concentration	Amount	Dilution media amount
10%	250µl FCS/mare whey sample	2250µl
5%	1000µl 10%	1000µl
2,5%	1000µl 5%	1000µl
1%	250µl 10%	2250µl
0,5%	1000µl 1%	1000µl
0,25%	1000µl 0,5%	1000µl
0,1%	250µl 1%	2250µl
0,05%	1000µl 0,1%	1000µl
0% = negative control		1000µl

Positive control = growth medium, Negative control = dilution medium

Plate 1

An example of a plate – using one from the total 6 whey milk samples

	1	2	3	4	5	6	7	8	9	10	11	12
A				H_2O			H_2O					
B		NC	0,05%	0,1%	0,25%	0,5%	1%	2,5%	5%	10%	PC	
C		NC	0,05%	0,1%	0,25%	0,5%	1%	2,5%	5%	10%	PC	H_2O
D	H_2O	NC	0,05%	0,1%	0,25%	0,5%	1%	2,5%	5%	10%	PC	
E		NC	0,05%	0,1%	0,25%	0,5%	1%	2,5%	5%	10%	PC	
F				H_2O				H_2O				
G												
H												

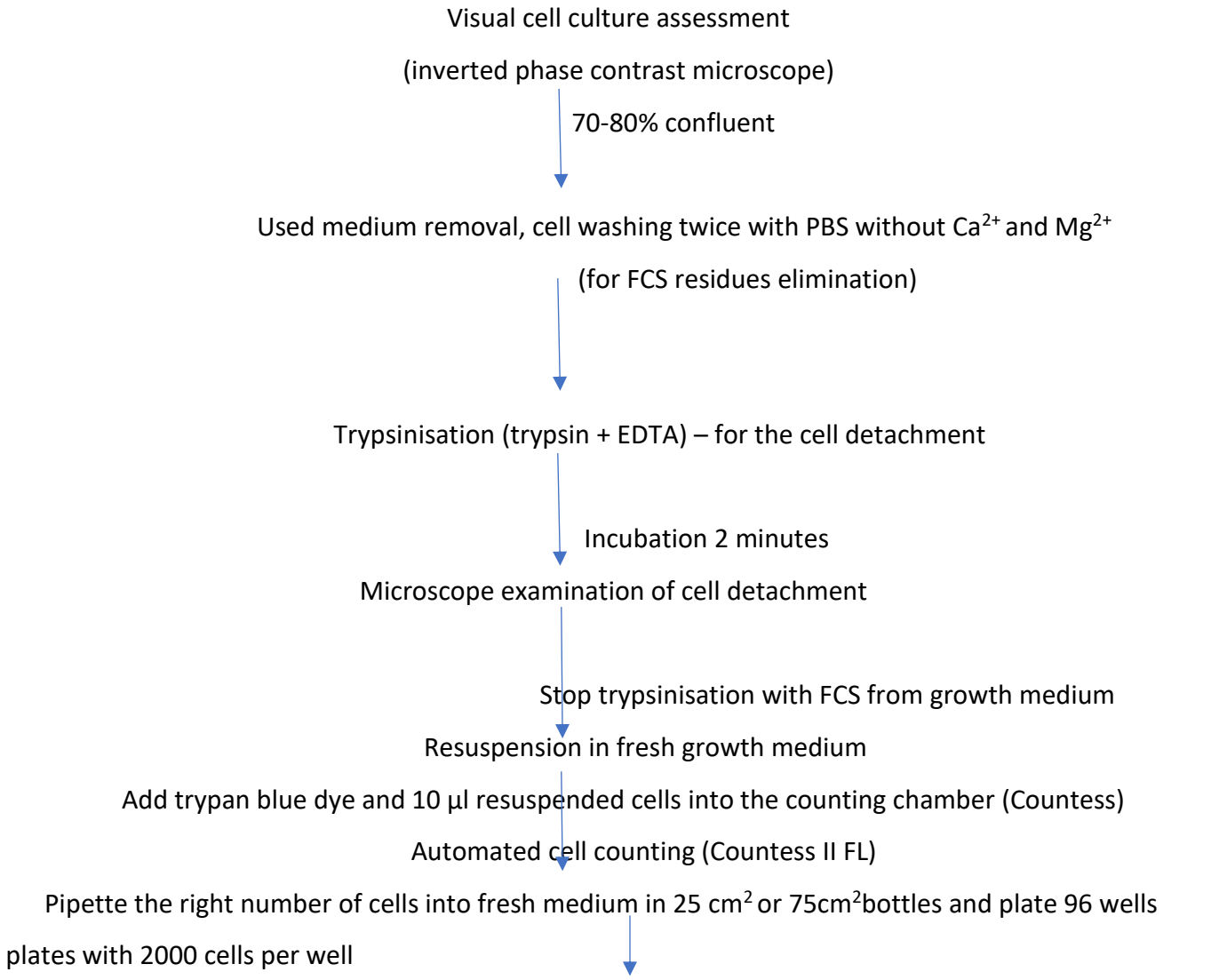
Whey samples dilutions plated in 96 wells 5000 cells/100ml/well, 2 controls positive and negative control, negative control was used to check the results

7.2 Cell viability using FHs 74 Int cells

FHs cells monolayers were cultured and treated with the whey of mare milk from passage 7 to passage 11. Two pre-passages were used before the assays.

For culturing FHs cells (ATCC CCL-241, FHs74 lot 1914408), a growth medium special for them (DMEM 42430 -025) was used with bovine Insulin I 5500 (10 mg/ml) and human EGF (50 ng/µl) supplementation as well as penicillin and streptomycin antibiotic mix, FCS -South America DE 14-801F**,** Glutamax 100x 35050 200 mM, NCTC 135 41350-26 medium with salts which influences amino-acids solubility, amino-

acids and vitamins to support the epithelial cell growth, Non-essential amino-acids (NEAA) 11140-035 important as well for the cell cultures, Na-pyruvate 100mM 11360-039, oxaloacetate (OAA).
To be cell-available, components from the cellular culture needs to be in solution together with the cell being cultured.
When the cell culture developed the right confluency (70% - 80 %) the usage of an inverted phase contrast microscope assessed that, the sub culturing (passaging) the cells in fresh medium to create a new fresh pool of cells and to plate the 96 wells plates to assess for cell viability AlamarBlue® (2000 cells/100µl/well with four replicates, three controls and eight dilution of the whey of mare milk, like in the schema). AlamarBlue dye was diluted 1+10 with PBS plus Ca^{2+} and Mg^{2+} and incubated with the cells after treatment adding for one hour.
After thawing the cells, two passages were used before beginning to assess for cell viability and proliferation. Cell viability was assessed three times for all six horses on both lactation time (at month 4-5 and 6-7 respectively).
Dilution medium was different from the growth medium based on the insulin and EGF content much lower than for the growth medium (100 times lower).
The same dilutions and plates were used for the FHs cells assay with an extra positive control for a self-control of using the right medium for culturing and dilution of the samples as well as for assuring correct results at the end.
Envision Multilabel Reader 2103 by Perkin Elmer was used to quantify cell viability like for IEC-6 cells (with AlamarBlue dye 1/10 with PBS with Ca^{2+} and Mg^{2+} salts) one-hour incubation at 37° C and 5% CO_2), excel and SAS 9.4 for the data interpretation.



A schematic protocol for sub culturing the cells and cell plating for AlamarBlue® viability assay

7.2.1 Reagents

Dulbecco Modified Eagle Medium 42430-025 with high glucose and L glutamine, with red phenol as a cellular metabolism indicator dye was used as a growth medium for the human epithelial intestinal cells CCL-241(FHs 74 Int cells) supplemented with bovine Insulin I5500 Sigma/Aldrich A/S Vallenbæk Strand, Denmark, human recombinant EGF Austral Biologicals San Ramon CA, USA, FCS 10% -South America DE 14-801F**,** NCTC-135 41350-26 with L-glutamine, Invitrogen A/S, non-essential amino-acids 100x 11140-035 Invitrogen A/S and oxaloacetate (O-7753 Sigma Aldrich A/S) together with the same supplements as for IEC-6 cells but in different quantity (Glutamax 100x 35050 200mM, Na-pyruvate and PenStrep P-0781-antibiotic solution).

PBS with and without calcium and magnesium and trypsin with EDTA were the same as for the other cell line used for the study.

The dilution-treatment medium contained lower insulin (Insulin I 5500 0.I mg/ml) and EGF human (5 ng/µl) and no FCS compared with growth-pre-treatment medium (Insulin I 5500 1 mg/ml, EGF human 50 ng/µl).

The laminar flow bench was used for the experiments, alcohol 70% to spray on the surfaces, 75 $cm^2$and 25cm^2plastic cell culture bottles and vacuum aspirator for the cell culture and 37°C and 5% CO_2 incubator. Disinfection of the flow bench and incubator was performed one a month for safety consideration considering working with cells and to avoid a possible contamination.

AlamarBlue dye 1/10 diluted using PBS with Ca^{2+} and Mg^{2+} salts were used for cell viability assay, reading the plates on Envision Multilabel Reader Perkin Elmer after one hour. Excel and SAS 94 were used for the data interpretation using PROC MIX model.

7.2.2 Plating and dilution schema

Table 8 Whey dilutions samples used as treatment for the FHs 74 Int cells, the dilutions were plated in 4 replicates for each of them and for calculation an average was considered

NC =DM,PC1 = GM,PC2 = DM + FCS 10%

concentration	amount	Dilution media amount
10%	250µl FCS/mare whey sample	2250µl
5%	1000µl 10%	1000µl
2,5%	1000µl 5%	1000µl
1%	250µl 10%	2250µl
0,5%	1000µl 1%	1000µl
0,25%	1000µl 0,5%	1000µl
0,1%	250µl 1%	2250µl
0,05%	1000µl 0,1%	1000µl
0% = negative control		1000µl

Plate 1

An example of a plate – using one from the total 6 whey milk samples

	1	2	3	4	5	6	7	8	9	10	11	12
A				H_2O			H_2O					
B		NC	0,05%	0,1%	0,25%	0,5%	1%	2,5%	5%	10%	PC1	
C		NC	0,05%	0,1%	0,25%	0,5%	1%	2,5%	5%	10%	PC1	H_2O
D	H_2O	NC	0,05%	0,1%	0,25%	0,5%	1%	2,5%	5%	10%	PC1	
E		NC	0,05%	0,1%	0,25%	0,5%	1%	2,5%	5%	10%	PC1	
F		PC2	PC2	PC2	PC2			H_2O				
G												
H												

Whey samples dilutions plated in 96 wells 2000 cells/100ml/well, 2 positive controls and 1 negative control, negative control was used to check the results

7.3 Cell migration – scratch wound assay

Wound healing model using 2D monolayer of rat epithelial intestinal cells (IEC-6 cell line P13 to P20) was done 2 times using this cell line using the two times of milk sampling, the same as for the cell viability assay. The migration distance was measured after performing the scratch of the cell culture monolayer as time point 0 and adding the whey dilutions (0,5%, 1%, 5% and 10%) and after 6 hours of incubation with

the dilutions (37°C, 5%CO_2). This wound healing model is a simple reproducible and non-expensive test for intestinal cell migration model.

Having the cells sub cultured and counted like described earlier (IEC-6 cells), 27 x 10^4 cells (1ml of cells, dilution medium and BSA 10%) in each well of 12 wells plates (Nunclon Delta surface 150628 Thermo Scientific) were used for this assay. Concentration of 10%, 5%, 1% and 0.5% were done by diluting the whey samples with culture medium without FCS and 10% bovine serum albumin (BSA) and incubated with the cells for 24 hours in duplicate. The wells were marked with 4 lines in two different colours to simplify the counting. Eight measurements correspond for one well and one picture. The positive control was growth medium with 10% FCS and the negative control was growth medium without FCS (dilution medium).

The scratch was done in each well using a 10 µl pipette tip and pictures (photomicrographs) were taking using a camera Leica digital camera (DFC 4500-00550003316) connected with an inverted microscope (Leica DMIL) immediately after the surface scratch and 6 hours after adding the whey treatment in order to calculate the migration length after the whey was added knowing that for wound healing is necessary some hours for the epithelial cell proliferation to the wound site to clear the superficial interrupted epithelial intestinal cell barrier.

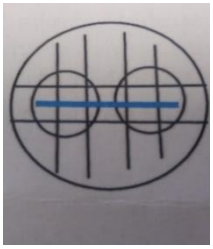


Figure 1 The representation of one well with two consecutive measurement area with 4 measurements for each area. The blue line represents the scratch line right in the middle of the well corresponding to a further cellular migration.

0 h 6 h

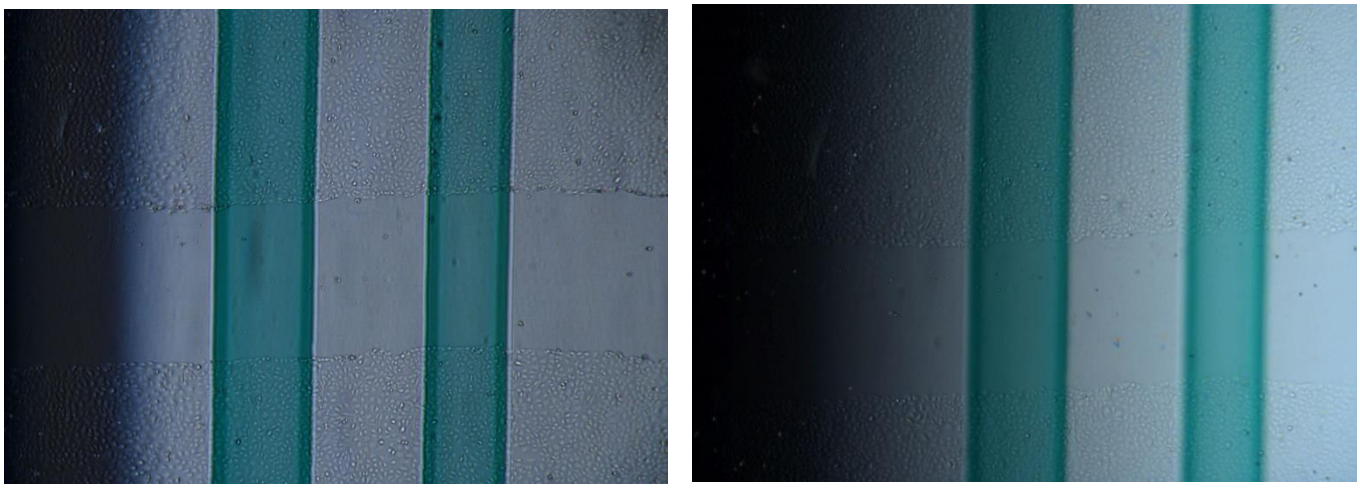


Figure 2 Example of one of the wells at 0 and 6 hours after adding the diluted whey treatment and the scratch, each well had 8 measurements of the length line of cells migration

Using LAS V4.9 by Leica Microsystem software, the measurements for every each well of the 12 wells plates was assessed with 8 measurements of the line length in µm per well for both time frame 0h after the treatment ad and the scratch is performed and after 6h of incubation at 37°C and 5% CO_2.

Microsoft excel was used to get the data namely the measurements of the difference of lengths (migration length) and plot the graphs and SAS version 9.4 for statistical analyses of the data in a proc mixed model.

7.3.1 Reagents

The 12 wells plates were used for the scratch wound healing assay pre-seeded with 270.000 cells in each well.

Growth medium used as a pre-treatment for scratch wound assay on IEC-6 cells was the same as for the IEC-6 cell proliferation assay – DMEM 11960-044, 10% FCS – South America DE 14-801F, 0.44% Sodium

pyruvate 11360-+39 100 mM, 0.88% Pen Strep P3539, 1% Glutamax 100X 35050. The dilution medium is having been used together with the treatment to check the IEC-6 cells migration effect of the whey of mare milk supplemented with 10% bovine serum albumin (BSA A1662). The concentration range of the treatment medium and whey of mare milk was 0.5%, 1%, 5% and 10% in duplicate. The positive and negative control was added for having the quality control of every assay supplemented with 10% bovine serum albumin (BSA).

7.3.2 Plating and dilution schema

Table 9 Whey dilutions samples used as treatment for the IEC-6 cells, the dilutions were plated in 2 replicates for each of them and for calculation an average was considered

Conc. %	Amount whey	Amount dilution media
10	500µl mare whey	4500µl
5	1500µl mare whey 10%	1500µl
1	300µl mare whey 10%	2700µl
0,5	300µl mare whey 5%	2700µl

The dilutions are ready for plating after they get mixed with BSA 10%, namely 2450 µl of both dilutions and controls and 50 µl BSA 10%. Furthermore, they get warmed in the heating cabinet at 37° C before plating them on the cells.

Plate 1

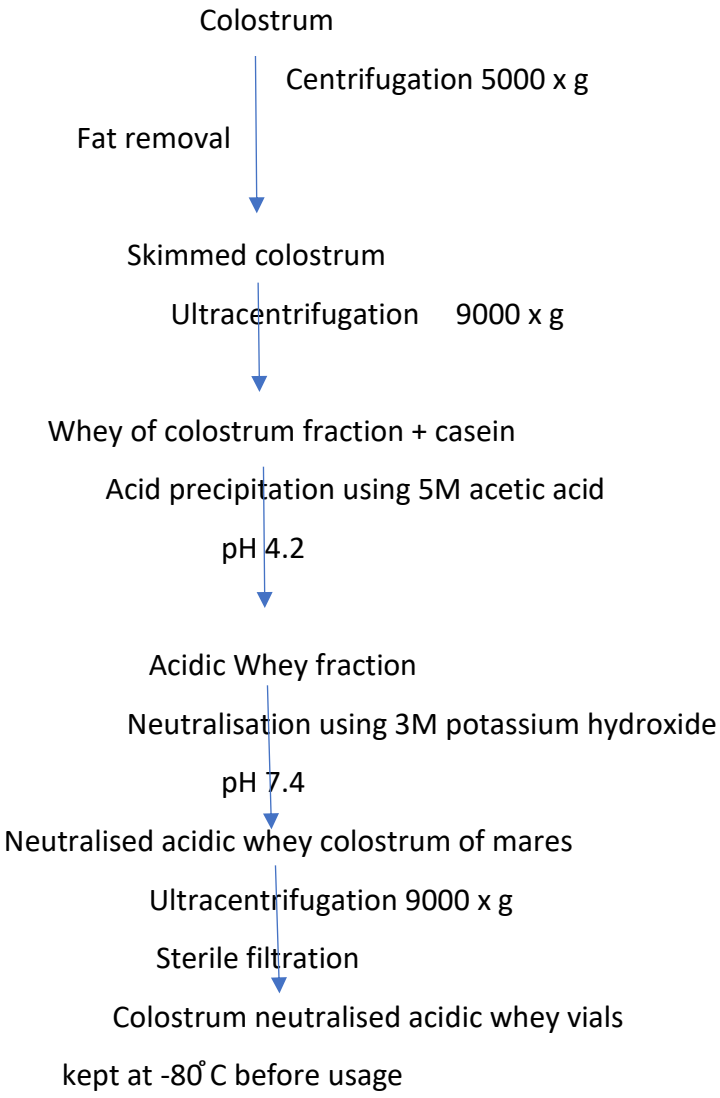
An example of a plate – using one from the total 6 whey milk samples

NC	PC	0,5%	0,5%
1%	1%	5%	5%
10%	10%	NC	PC

Whey samples dilutions plated in 12 wells 270.000 cells/100ml/well, 1 positive control and 1 negative control also in duplicates, negative control was used to check the results

7.4 Cell viability using IEC-6 cells and whey of colostrum

The whey of colostrum was prepared using exactly the same method as for the milk and for cell culturing, dilutions, plating and reading the plates it was indicated the same protocol as for the milk and IEC-6 cells. Fat was removed after the sample centrifugation. Casein was precipitated and discarded. Samples were analysed as neutralised acidic whey of colostrum as sterile filtrated vials kept at -80°C before usage.



IEC-6 cells monolayers were incubated with whey of colostrum from 5 mares (see Table 9 from the Appendix) in the same concentration range (dilutions 0.05%,0.1%,0.25%,0.5%,1%,2.5%,5% and 10%) for the same amount of time namely 3 days (which in vitro mimics the in vivo step of cell proliferation for the intestinal wound healing process (Sturm and Dignass, 2008)) as for the whey of mare milk samples and the results were rad on Envision Multilabel Reader 2103 Perkin Elmer to test the cell viability. Relative values of the means were calculated, controls were used in order to have correct assays all the time.

7.4.1 Reagents

IEC-6 cells 5000 per well (96 wells plates) from DSMZ ACC 111, from passage 10 and 12 used after two consecutive pre-passages.

The growth medium used was specific to IEC-6 cells and the same as the one used to check their viability using the whey of mare milk samples. DMEM 11960-044 supplemented with 10% FCS – South America DE 14-801F, sodium pyruvate 11360-039 100 mM, PenStrep P0781 and Glutamax 100X 35050. Growth medium was changed every two days and it was used as positive control. Dilution medium is similar to growth medium but without FCS supplement. PBS with and without Ca^{2+} and Mg^{2+} salts for AlamarBlue dye dilution 1/10 and cells washing before adding the whey treatment and without salts for cells washing when passaging. Trypsin and ETDA it's useful for the cells detachment from the plastic bottles when sub-culturing (passaging). Trypsinization (2ml for a small 25 cm^2 bottle and 4 ml for a big 75 cm^2 bottle) for 2 minutes at 37° C. Trepan blue dye was used to count the viable cells using Countess II. AlamarBlue dye 1/10 diluted with PBS with Ca^{2+} and Mg^{2+} salts were used for the cell viability assay – reading the plates. For the dilutions of the whey of colostrum was used growth medium without FCS. Dilution medium was used as well as negative control for every assay.

7.4.2 Plating and dilution schema

Table 10 Whey colostrum dilutions samples used as treatment for the IEC-6 cells the dilutions were plated in 4 replicates for each of them and for calculation an average was considered.

Concentration	Amount	Dilution media amount
10%	250µl FCS/mare whey sample	2250µl
5%	1000µl 10%	1000µl
2,5%	1000µl 5%	1000µl
1%	250µl 10%	2250µl

0,5%	1000µl 1%	1000µl
0,25%	1000µl 0,5%	1000µl
0,1%	250µl 1%	2250µl
0,05%	1000µl 0,1%	1000µl
0% = negative control		1000µl

Positive control = growth medium, Negative control = dilution medium

Plate 1

An example of a plate – using one from the total 5 whey colostrum samples

	1	2	3	4	5	6	7	8	9	10	11	12
A				H_2O			H_2O					
B		NC	0,05%	0,1%	0,25%	0,5%	1%	2,5%	5%	10%	PC	
C		NC	0,05%	0,1%	0,25%	0,5%	1%	2,5%	5%	10%	PC	H_2O
D	H_2O	NC	0,05%	0,1%	0,25%	0,5%	1%	2,5%	5%	10%	PC	
E		NC	0,05%	0,1%	0,25%	0,5%	1%	2,5%	5%	10%	PC	
F				H_2O				H_2O				
G												
H												

Whey colostrum samples dilutions plated in 96 wells, 5000 IEC-6 cells/100ml/well, 2 controls positive and negative control, negative control was used to check the results

7.5 Growth factors in mare milk and colostrum

Given the fact that is an in vitro assessment of the whey of mare milk and colostrum, the composition of these two can be influential upon the cell viability / proliferation and migration of the used cell lines for the experiments. Growth factors play a fundamental role in both human and animal physiology and development. IGF-I, TGFβ1, TGFβ2 and EGF were the growth factors measured both in the late lactation mare milk and colostrum of mare milk.

The laboratory assays used for determining the growth factors concentration in whey of mare milk and colostrum were TR-FIA (for IGF-I) and ELISA (for TGF-β1, TGF-β2 and EGF) and the plates reader was Envision Multilabel Reader 2103 by Perkin Elmer. Measurements were done in duplicate.

TGF-β1 and TGF-β2 were quantify by Quantikine ELISA DB100 and DB250 R&D Systems, Minneapolis MN, USA using TGF-β1R II and a polyclonal specific antibody TGF-β1 and TGF-β2 antibody pre-coated microplates and TGF-β2 antibodies conjugated to horse-radish peroxidase.

8. Results

Effect of whey of mare milk on IEC-6 cells viability

All of the figures are correspondent with the relative values of the results of cell viability from all of 6 samples for both time milking. All of the whey samples showed an increase in cell viability of IEC-6 cells compared with the negative control which is considered to have no effect. This is a positive effect of the whey of mare milk on IEC-6 cell line viability in vitro. No negative effect (inhibition of IEC-6 cells viability) was observed.

Ten percent concentration ($P< 0.0001$) of the whey of mare milk for the first time milking increased IEC-6 cell viability with up to 110% (sample no. 2 and 3) to 120% (sample no. 1). Ten percent of whey of mare milk from sample no. 4 increased cells viability with 100% during the first-time sampling. Samples no. 7 and 5 had an increase of cells viability with 40% and 60% respectively. Sample no. 5 and 7 gave cellular viability stimulation effect using a different range of whey concentration during the first-time milking.

Ten percent concentration ($P< 0.0001$) of the whey of mare milk for the fourth time milking samples increased IEC-6 cell viability with 30% (sample no.5) to 100% (sample no. 1) (see table 11 from the Appendix).

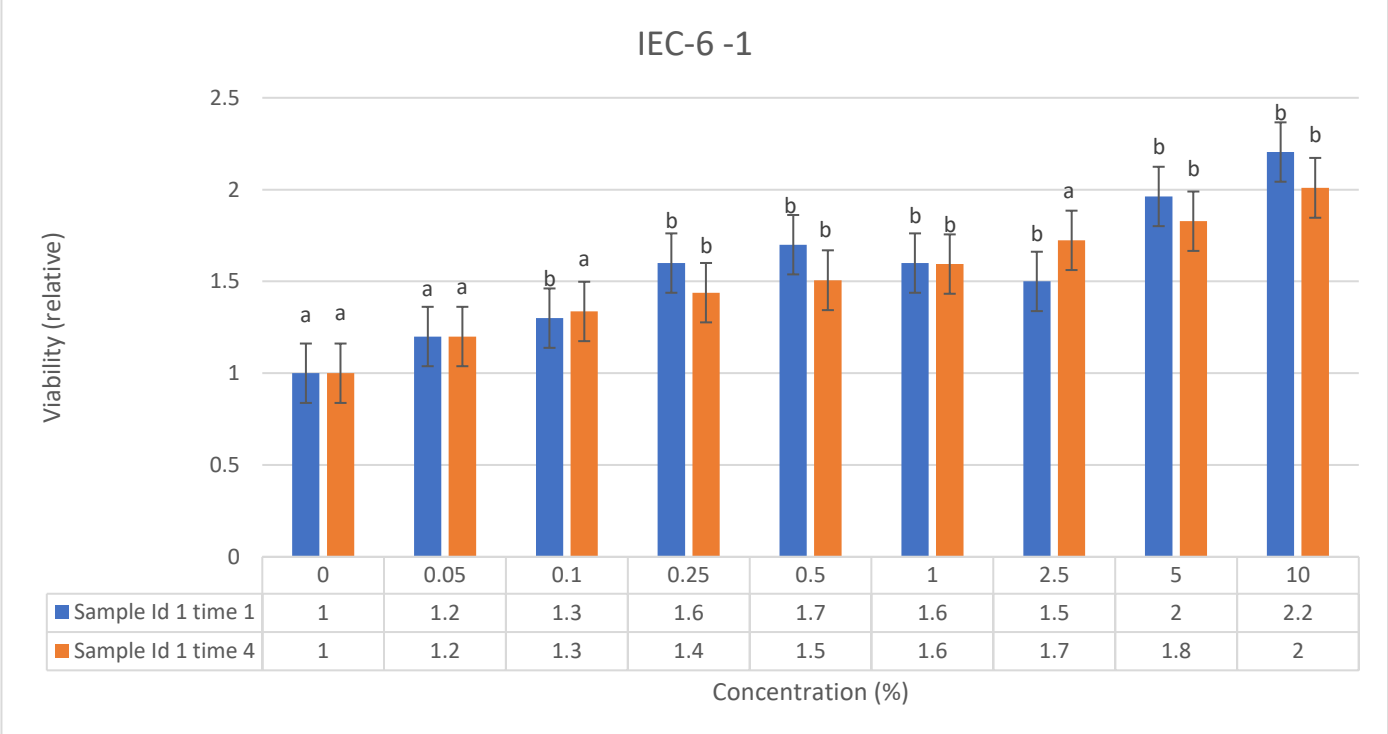
Sample no. 1 gave the maximum increasing viability effect on IEC-6 cells using 10% concentrated whey fraction for both time milking (100-120%) compared with the negative control ($P< 0.0001$). Sample no. 2 (110% - 90%), 4 (100% - 80%) and 3 (110% - 60%) followed closely sample no. 1 based on cell viability enhancement compared with the negative control. Sample no. 7 had the minimum increasing viability effect on IEC-6 cells using 10% concentrated whey of mare milk of 40% first time milking and 70% for the fourth time milking ($P< 0.05$) compared with the negative control. Sample no. 5 had a 60% increasing viability effect on the same cell line using 10% whey ($P< 0.0001$) and down to a half, namely 30% viability increasing on the IEC-6 cell line the fourth time milking compared with the negative control. Sample no. 7 had a 30% increasing of the cell viability the fourth time milking compared with the first time compared

with the negative control. Samples no. 1, 2 and 4 for the fourth time milking increased the cellular viability of IEC-6 cells line 20% less than the first time and sample no. 3 with 50% respectively compared with the negative control. The only sample which gave an increase of cell viability greater for the fourth time milking than the first time was sample no. 7 compared with the negative control (see table 11 from the Appendix).

Almost all the whey samples (except sample 5 and 7) have doubled IEC-6 cell viability in concentration of 10% ($P<0.0001$) compared with the negative control (see table 11 from the Appendix).

If a mean of both milk samples is considered, then, sample no. 5 and 7 showed an increase of cell viability for around 50% and sample no. 1, an 110% increase compared with the negative control.

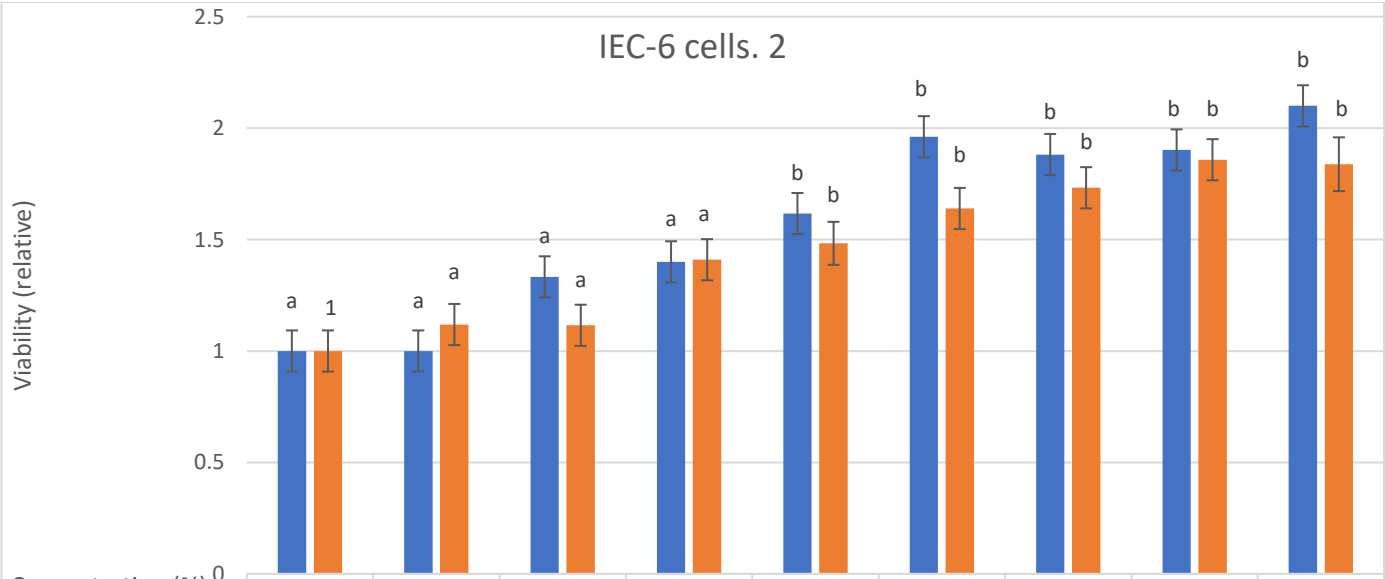
Statistical analyses have been done using SAS 9.4, a PROC MIXED DATA model for cell viability using both cell lines, IEC-6 and FHs 74 Int with CLASS assay time sampleid conc. replicate; MODEL relviab= time conc conc.*time/PDIFF=all adjust= tukey; Where sampleid=1,2,3,4,5,7.



	0	0.05	0.1	0.25	0.5	1	2.5	5	10
Sample Id 1 time 1	1	1.2	1.3	1.6	1.7	1.6	1.5	2	2.2
Sample Id 1 time 4	1	1.2	1.3	1.4	1.5	1.6	1.7	1.8	2

Figure 3 Effect of 6 whey of mare milk samples on cell viability of IEC-6 cells. Results are from 3 experiments with 4 replicates showed as mean values ± SEM relative to the negative control. Different lowercase letters show significant differences from the negative control at $P < 0.05$

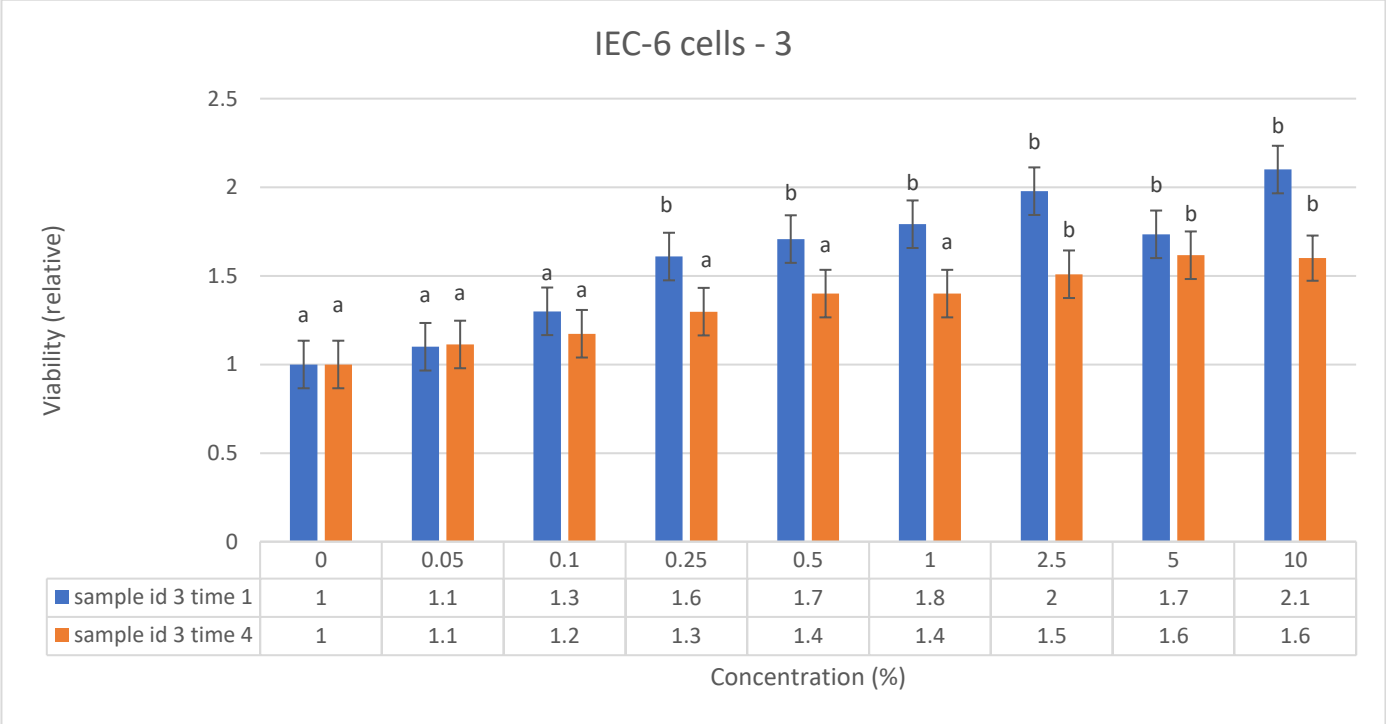
For sample no. 1 during the first-time milking, the cell viability stimulation had an ascending growth compared with the negative control with a relative value from 1 (NC) to 2 (10% whey) so a growth of cell viability of 100%. Whey of mare milk from the first sample on both time milking, have doubled the cells viability of rat small intestinal cell line. During the fourth time milking, the ascending growth of cell viability of IEC-6 cells was more regular and linear compared with the first-time milking sample using the whey concentration range from 0.05% to 10%. Both time frames stimulated the cell viability for up to a mean of 110% when using a concentration of 10% whey.



Concentration (%)	0	0.05	0.1	0.25	0.5	1	2.5	5	10
sample id 2 time 1	1	1	1.3	1.4	1.6	2	1.9	1.9	2.1
sample id 2 time 4	1	1.1	1.1	1.4	1.5	1.6	1.7	1.9	1.8

Figure 4 Effect of 6 whey of mare milk samples on cell viability of IEC-6 cells. Results are from 3 experiments with 4 replicates showed as mean values ± SEM relative to the negative control. Different lowercase letters show significant differences from the negative control at $P < 0.05$

Whey of mare milk (from 0.05% - to 10%) increased rat epithelial cell viability compared with the negative control by using the second sample. A concentration of 1% whey gave also a doubled cell viability by using sample no.2. Concentrations of 2.5% and 5% were having closer to 100% (doubled) cell viability enhancement effect. A concentration greater than 0.5% whey of mare milk increased the cellular viability with almost 100% the first-time milking. The fourth time sampling milk had an upward growth effect of cell viability with a plateau of 80% growth compared with the negative control at 2.5% to 10% whey.

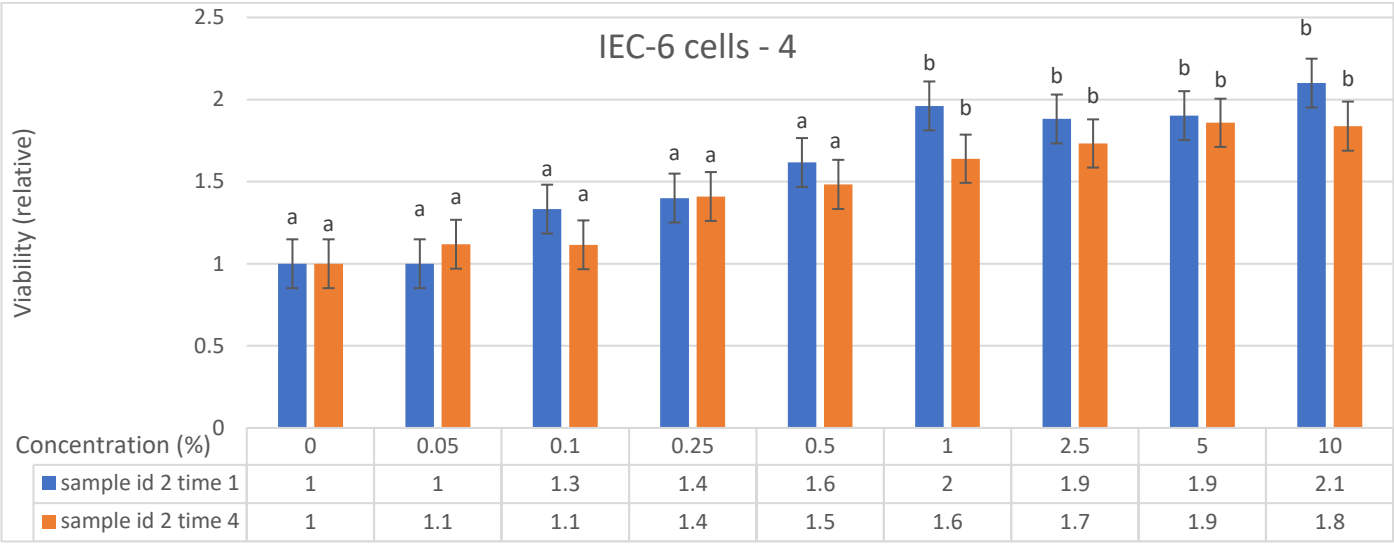


	0	0.05	0.1	0.25	0.5	1	2.5	5	10
sample id 3 time 1	1	1.1	1.3	1.6	1.7	1.8	2	1.7	2.1
sample id 3 time 4	1	1.1	1.2	1.3	1.4	1.4	1.5	1.6	1.6

Figure 5 Effect of 6 whey of mare milk samples on cell viability of IEC-6 cells. Results are from 3 experiments with 4 replicates showed as mean values ± SEM relative to the negative control. Different lowercase letters show significant differences from the negative control at $P < 0.05$

A concentration greater than 0.5% whey of mare milk from sample no. 3 had a cell viability growth of 90% for the first-time milking. Fourth time milking using sample no. 3 had a plateau of 60% growth of cell viability with 5 to 10% whey fraction.

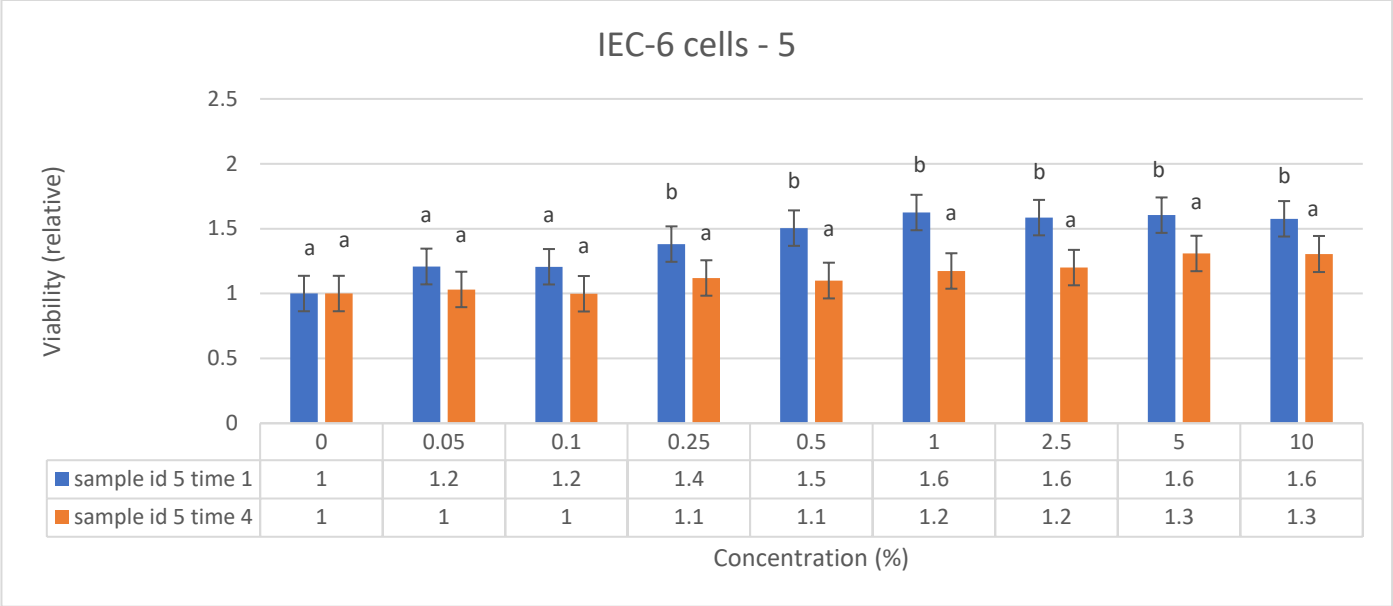
Approaching the doubling of the cell viability growth with increasing the concentration of the whey of mare milk was seen for almost all of the 6 samples (except sample no. 5 and 7).



Concentration (%)	0	0.05	0.1	0.25	0.5	1	2.5	5	10
sample id 2 time 1	1	1	1.3	1.4	1.6	2	1.9	1.9	2.1
sample id 2 time 4	1	1.1	1.1	1.4	1.5	1.6	1.7	1.9	1.8

Figure 6 Effect of 6 whey of mare milk samples on cell viability of IEC-6 cells. Results are from 3 experiments with 4 replicates showed as mean values ± SEM relative to the negative control. Different lowercase letters show significant differences from the negative control at $P < 0.05$

Whey of mare milk greater than 0.5% approached doubled cell viability growth on IEC-6 cell line using sample no. 4. First time milking whey showed an irregular upward growth of cellular viability compared with the fourth time milking which had a steadier growth with increasing the whey concentration on the cells.

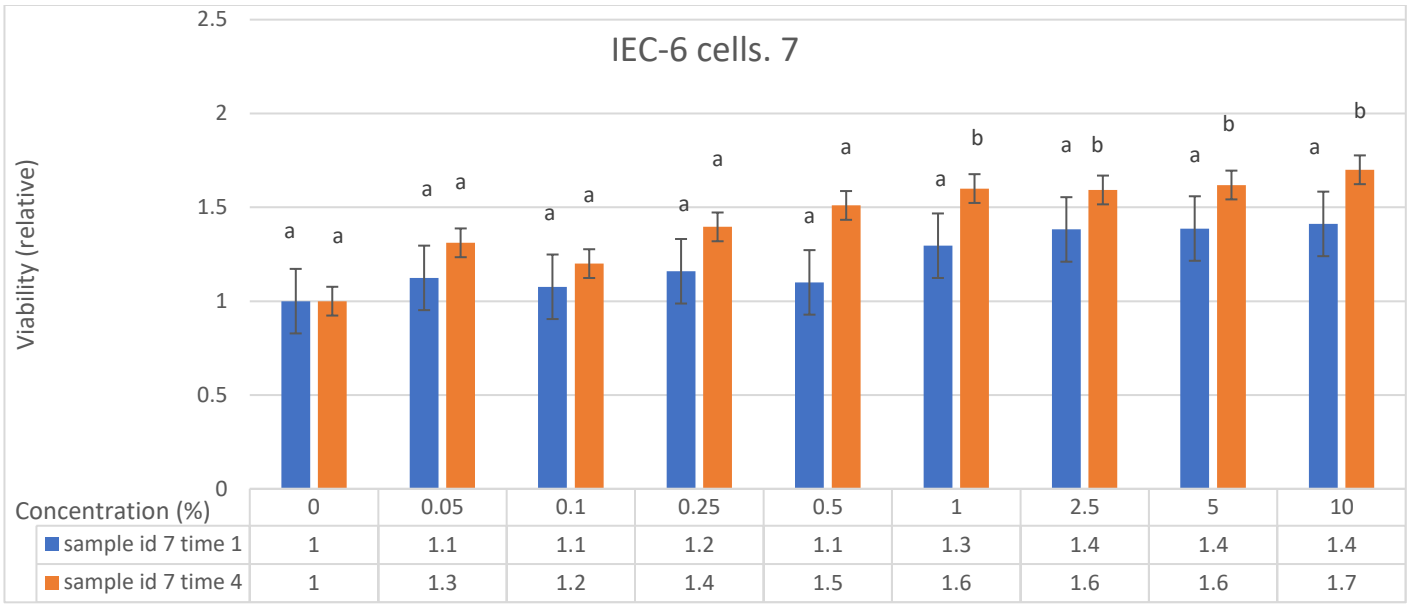


	0	0.05	0.1	0.25	0.5	1	2.5	5	10
sample id 5 time 1	1	1.2	1.2	1.4	1.5	1.6	1.6	1.6	1.6
sample id 5 time 4	1	1	1	1.1	1.1	1.2	1.2	1.3	1.3

Figure 7 Effect of 6 whey of mare milk samples on cell viability of IEC-6 cells. Results are from 3 experiments with 4 replicates showed as mean values ± SEM relative to the negative control. Different lowercase letters show significant differences from the negative control at $P < 0.0001$

First time milking whey samples (1% to 10% whey) had a lower and steadier growth effect on the rat intestinal epithelial cells then the rest of the samples reaching a plateau of 60 % growth. Concentration of 0.05% and 0,1% whey of mare milk sample 5 had no effect on the cells.

Concentration of 0.25% and 0.5% whey had only 10% increase of the cell's viability. A value of a half of this growth was showed with the whey of milk sample from the fourth time milking.



Concentration (%)	0	0.05	0.1	0.25	0.5	1	2.5	5	10
sample id 7 time 1	1	1.1	1.1	1.2	1.1	1.3	1.4	1.4	1.4
sample id 7 time 4	1	1.3	1.2	1.4	1.5	1.6	1.6	1.6	1.7

Figure 8 Effect of 6 whey of mare milk samples on cell viability of IEC-6 cells. Results are from 3 experiments with 4 replicates showed as mean values ± SEM relative to the negative control. Different lowercase letters show significant differences from the negative control at $P < 0.001$

A plateau of 40% of increased cell viability was reached by the whey of sample no. 7 on the first-time milking sampling and a 60% increase of the cell viability was showed during the second assays using the fourth time milking sample.

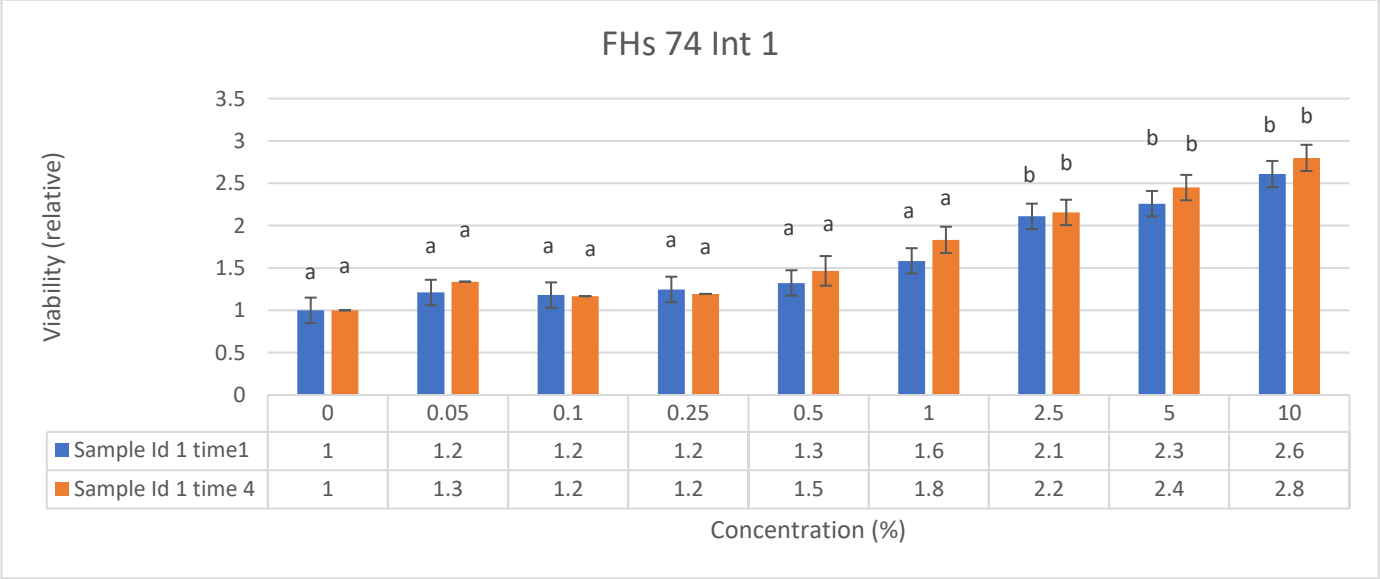
First time milk had a slower and lower increase of the cell viability compared with the fourth time and with the rest of the samples except sample no 5 which had a slower and lower growth the first time.

Can be considered that samples no. 5 and 7 mirror each other with the increase cell viability of IEC-6 cell line but with a reversed time frame for first- and fourth-time milk sampling.

Effect of whey of mare milk on FHs 74 Int cells viability

As for the cell viability assay using human epithelial intestinal cells (FHs 74 Int cell line), the results showed a close resemblance to that using IEC-6 cell line but way much greater cell viability increase from 20% for 0.05% whey for one of the samples to 210% for 10% whey and 250% 5% whey for another one of the samples. In the case of human intestinal epithelial cell line, the viability increased with up to 250% so 3 ½ time compared to negative control ($P<0.0001$).

This is an almost triple effect on cell viability of the intestinal cell line after 3 days of cellular incubation of the 10% whey fraction samples (mean of 172.45 %).



	0	0.05	0.1	0.25	0.5	1	2.5	5	10
Sample Id 1 time1	1	1.2	1.2	1.2	1.3	1.6	2.1	2.3	2.6
Sample Id 1 time 4	1	1.3	1.2	1.2	1.5	1.8	2.2	2.4	2.8

Figure 9 Effect of 6 whey of mare milk samples on cell viability of FHs 74 Int cells. Results are from 3 experiments with 4 replicates showed as mean values ± SEM relative to the negative control. Different lowercase letters show significant differences from the negative control at $P < 0.0001$

Sample no. 1 had a greater increase cell viability for the fourth time sampling compared to the first time. An increase of up to 180% was showed for 10% whey for the fourth time sampling and 160% for 10% whey for the first-time sampling.

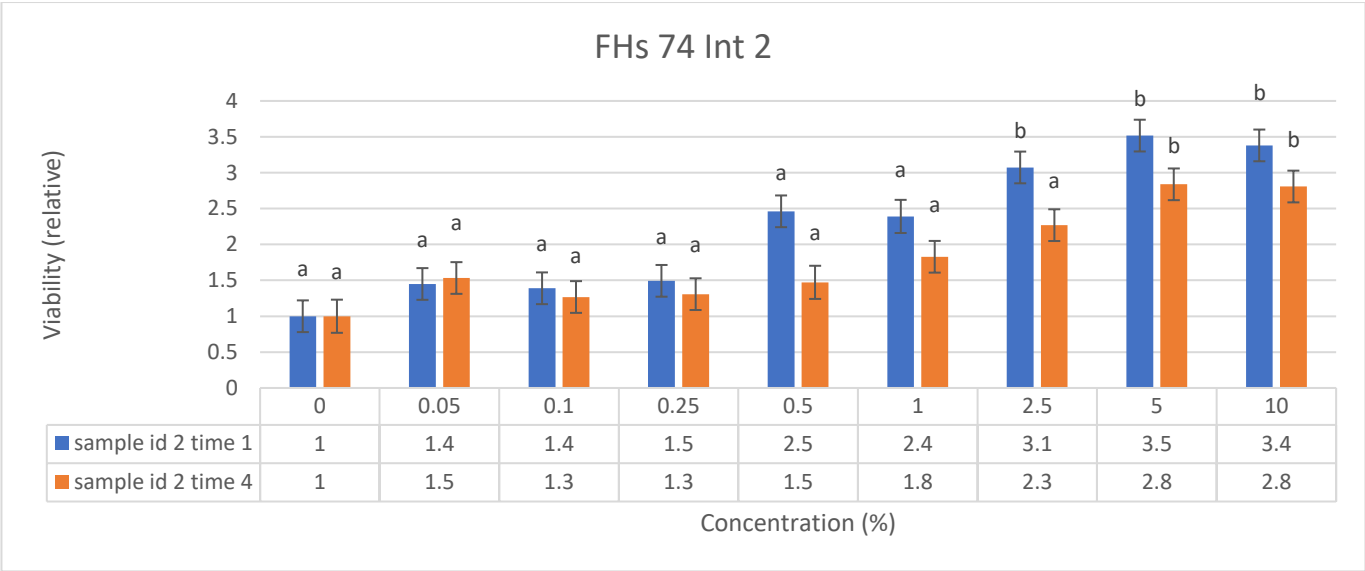


Figure 10 Effect of 6 whey of mare milk samples on cell viability of FHs 74 Int cells. Results are from 3 experiments with 4 replicates showed as mean values ± SEM relative to the negative control. Different lowercase letters show significant differences from the negative control at $P < 0.0001$

Sample no. 2 started to show a great increase of the cell viability using 0.5% - 10% whey fraction on the both time sampling but more for the first-time sampling. An increase of 250% cell viability was seen by 5% whey fraction, 240% by 10% whey on the first sampling and 180% for 5% and 10% whey fraction for the fourth time sampling.

The fourth time sampling had a much lower increase effect on the human intestinal epithelial cell line compared with the first-time sampling.

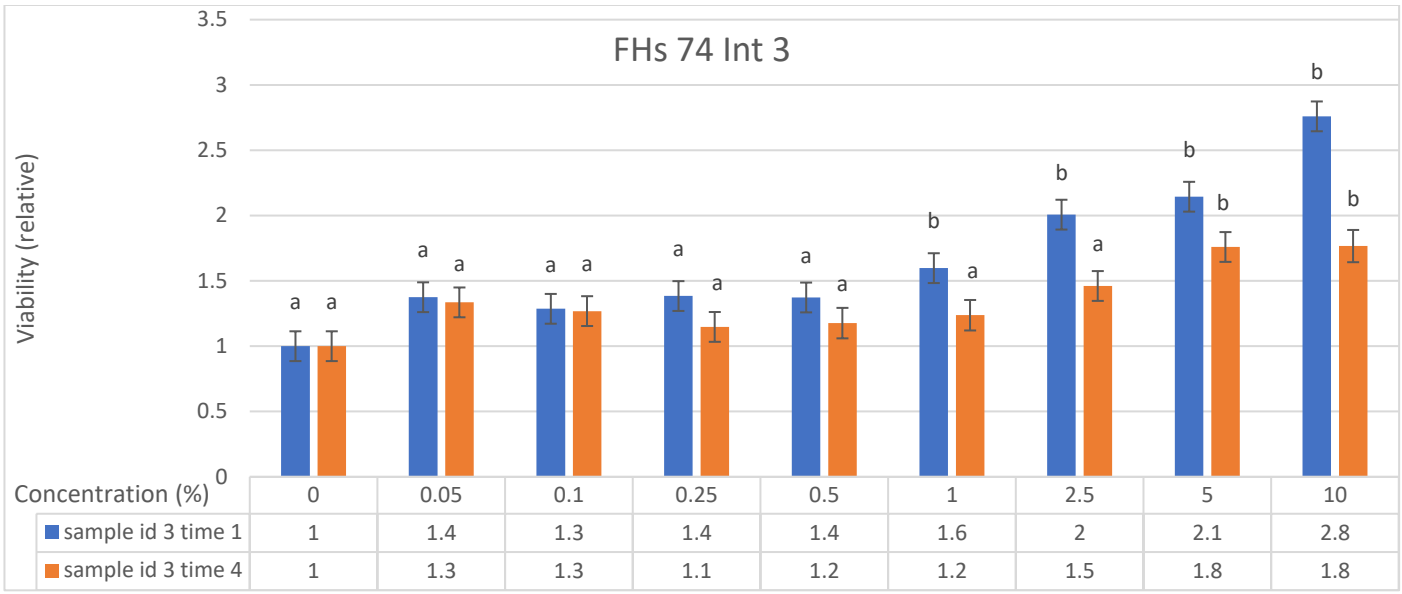


Figure 11 Effect of 6 whey of mare milk samples on cell viability of FHs 74 Int cells. Results are from 3 experiments with 4 replicates showed as mean values ± SEM relative to the negative control. Different lowercase letters show significant differences from the negative control at $P < 0.0001$

Sample no. 3 showed a cell viability increased with 40% to up to 180% first time sampling and 30% to up to 80% for the fourth time sampling.

The fourth time sampling had a much lower increase effect on the cell viability using the whey of mare milk fraction.

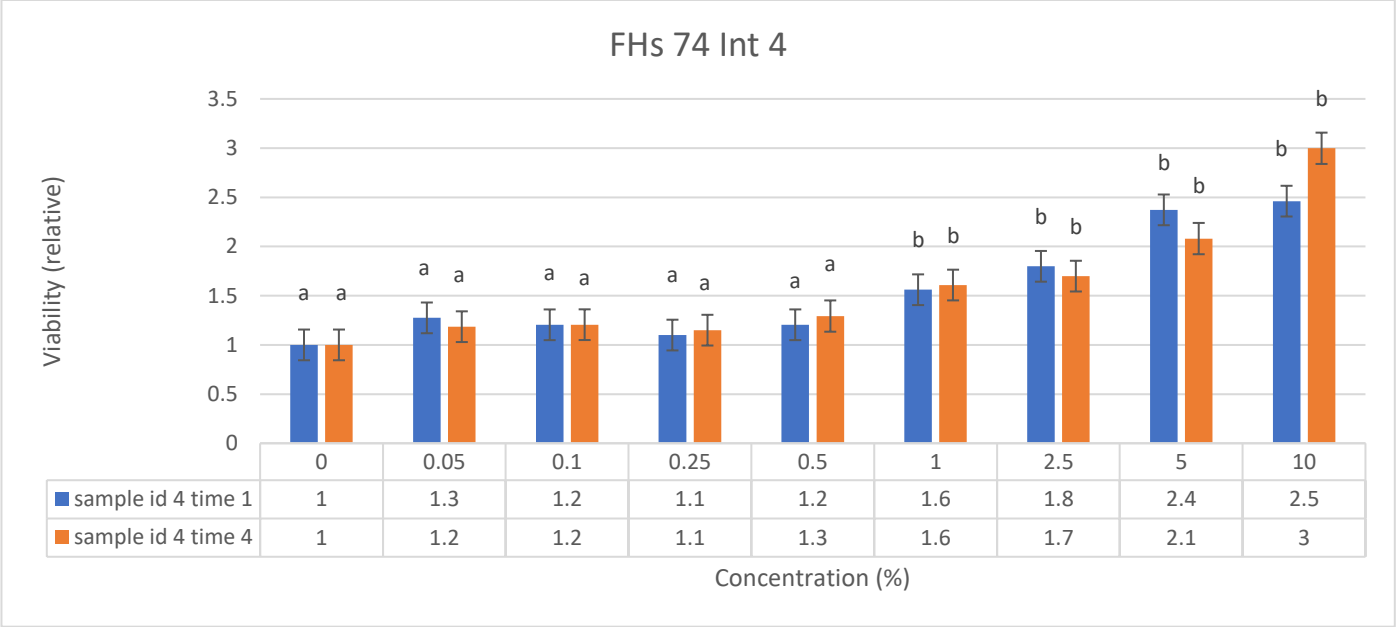


Figure 12 Effect of 6 whey of mare milk samples on cell viability of FHs 74 Int cells. Results are from 3 experiments with 4 replicates showed as mean values ± SEM relative to the negative control. Different lowercase letters show significant differences from the negative control at $P < 0.05$

Sample no. 4 showed a more linear increase of the cell viability compared with the first three samples on both time sampling. A peak of 200% (triple effect) was seen on the fourth time sampling for 10% whey fraction. The fourth time sampling had a sharper increase of the cell viability of the 10% whey then the first-time sampling yet in general the cell viability increased lower and slower than the first-time sampling. Both time sampling had a plateau of low and steady increase for the less concentrated whey dilutions (0.05% - 0.5%). This plateau was seen as well for the other first 3 samples.

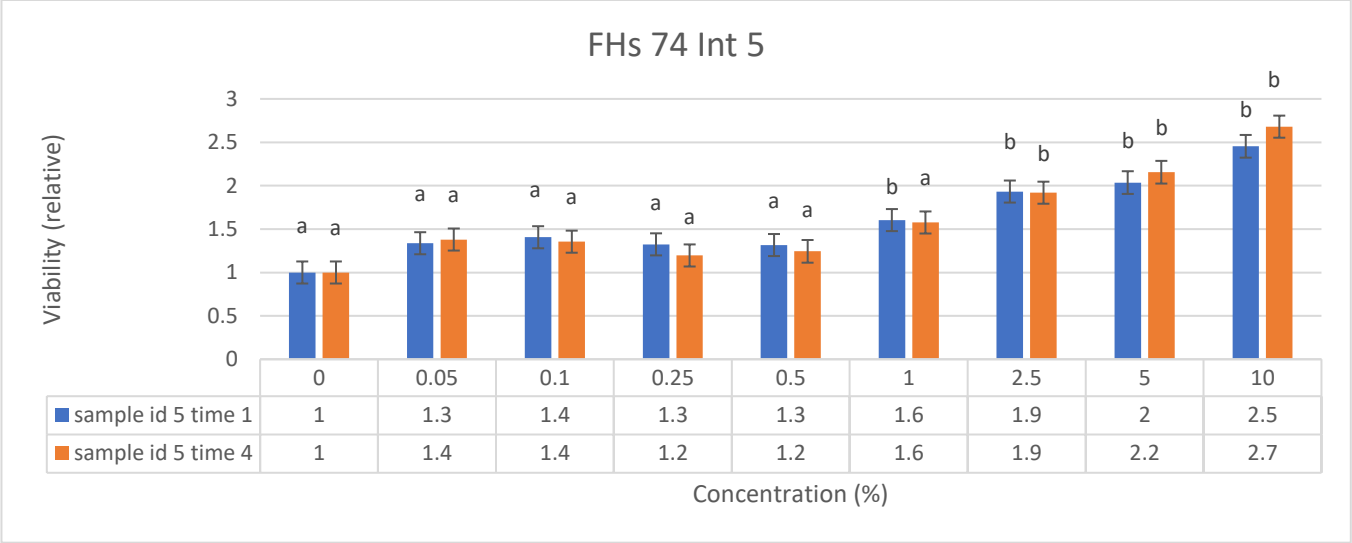


Figure 13 Effect of 6 whey of mare milk samples on cell viability of FHs 74 Int cells. Results are from 3 experiments with 4 replicates showed as mean values ± SEM relative to the negative control. Different lowercase letters show significant differences from the negative control at $P < 0.05$

The pattern of a plateau of low and steady increase of cell viability for less concentrated whey was kept for sample no. 5 (0.05% - 0.5% whey with 20% - 40% increase) for both time sampling. From 1% to 10% whey the cell viability increased from 60% to up to 170% (first time sampling) 150% (fourth time sampling).

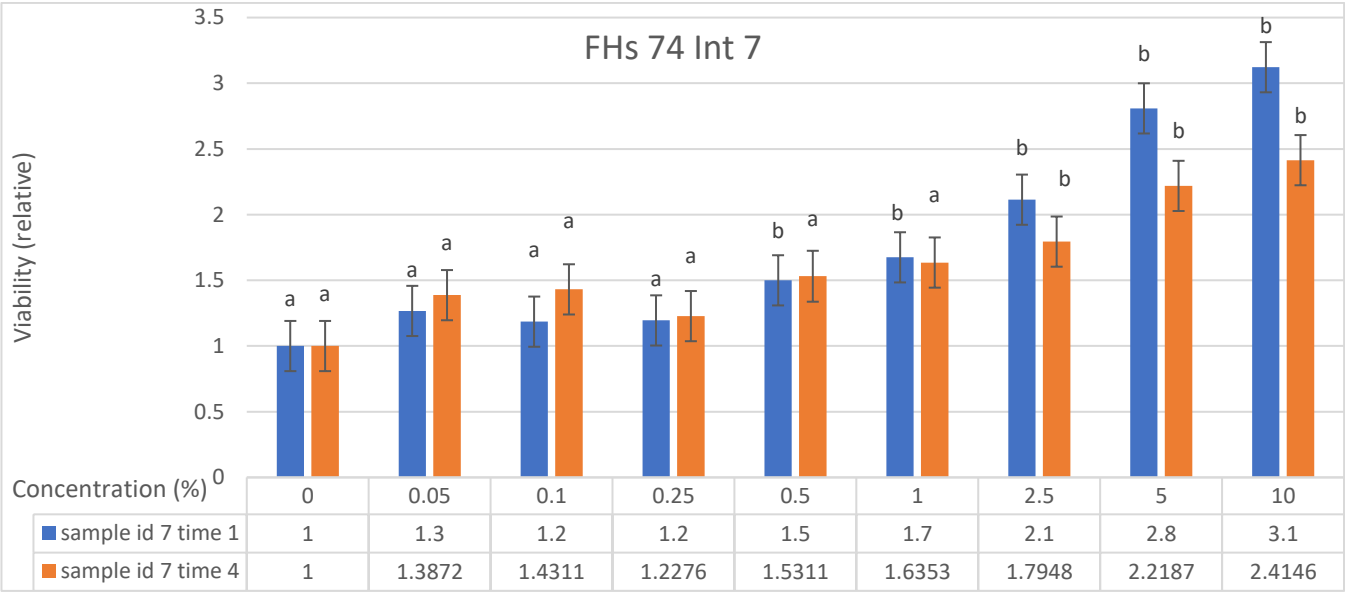


Figure 14 Effect of 6 whey of mare milk samples on cell viability of FHs 74 Int cells. Results are from 3 experiments with 4 replicates showed as mean values ± SEM relative to the negative control. Different lowercase letters show significant differences from the negative control at $P < 0.05$

Concentrations of 0.05% to 0.5% whey from sample no.7 showed a steady low increase of cell viability of 20% to 50% for both time sampling. First time sampling had peaks of 210% increase of 10% whey and the fourth time sampling 140% increase of 10% whey fraction. The triple effect of 10% whey is showed on the first-time sampling. A mean of 175% increase of cellular viability can be observed for 10% whey on the both time sampling.

Concentrations of 2.5%, 5% and 10% whey gave a greater cell viability (more than double) first time sampling than the fourth time.

Effect of whey of mare milk on IEC-6 cells migration

The figures are correspondent with the average values of the results of cell migration from all of the 6 samples for both time sampling. Both time sampling was analysed twice with 2 replicates and average values of the migration length ± SEM was used to evaluate the migration of the IEC-6 after 6 hours of incubation with the whey of mare milk in concentration of 0.5%,1%,5% and 10%.

Sample no.3 represents a mare of 15 years old, at its 6th lactation time, days 117 and 179 respectively after foaling. As for sample no.4, the oldest mare from the group, 17 years old and 3rd lactation time, migration of the intestinal cells was increased by 20% using a dilution of 5% whey of mare milk (not significant increase). Sample no.7 represented by the whey of the youngest mare from the group (4 years old, first lactation) showed an increased migration of the rat enterocytes cultured in monolayers of 70% at 122 days postpartum and 40% at 178 days of lactation (P<0.05).

Statistical analyses have been done using SAS 9.4 PROC MIXED DATA model with CLASS assay time sampleid conc replicate; MODEL relmigration= time conc time*conc./SOLUTION influence OUTPM=stat; RANDOM=assay; LSMEANS time conc conc*time/PDIFF=all adjust=tukey; Where sampleid=1,2,3,4,5,7.

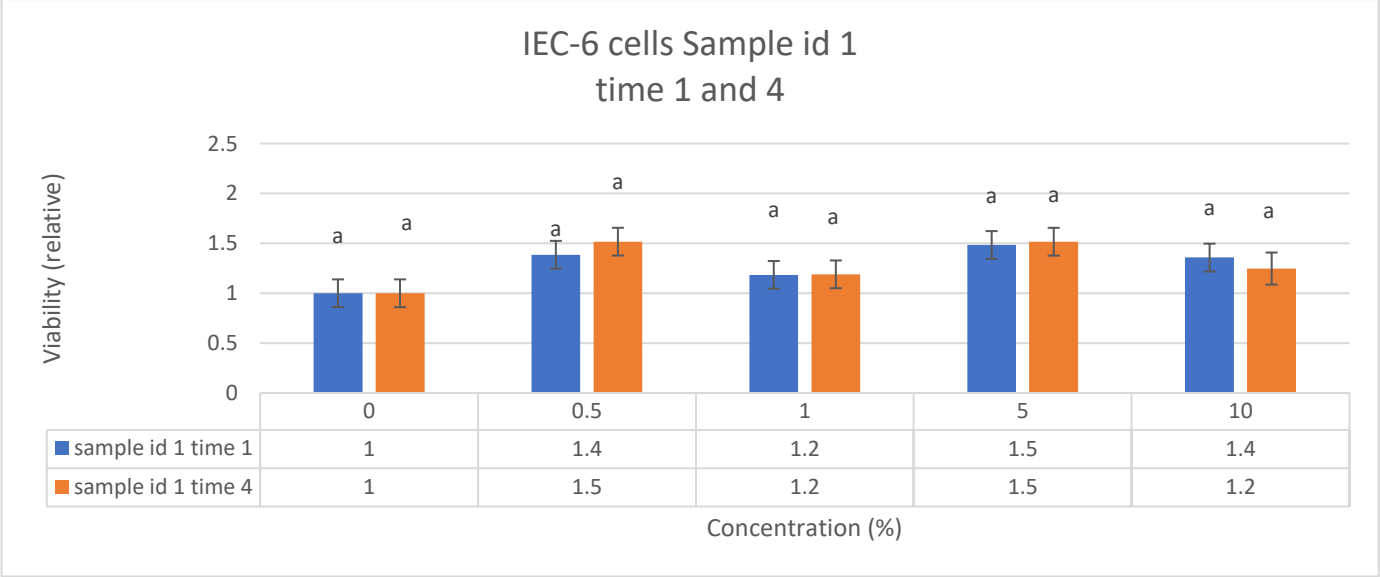


Figure 15 Effect of 6 whey of mare milk on migration of IEC-6 cells. Results are from 2 experiments with 2 replicates showed as average values ± SEM relative to the negative control. Different lowercase letters show significant differences from the negative control at $P < 0.05$

The whey fraction of the first sample in concentration of 5% increased the migration of the cells with 50% on both time sampled milk. A concentration of 0.5% had almost a similar trend with the 5% whey fraction. One percent whey gave a cell response migration in the wound after 6 hours of 20% increase during the both time sampled milk. The maximum concentration of 10% whey in sample no 1 increased cells' migration after 6 hours with 40% the first-time sampled milk and a half less, 20% after 2 months on the fourth time milk sampled of the horse milk during late lactation.

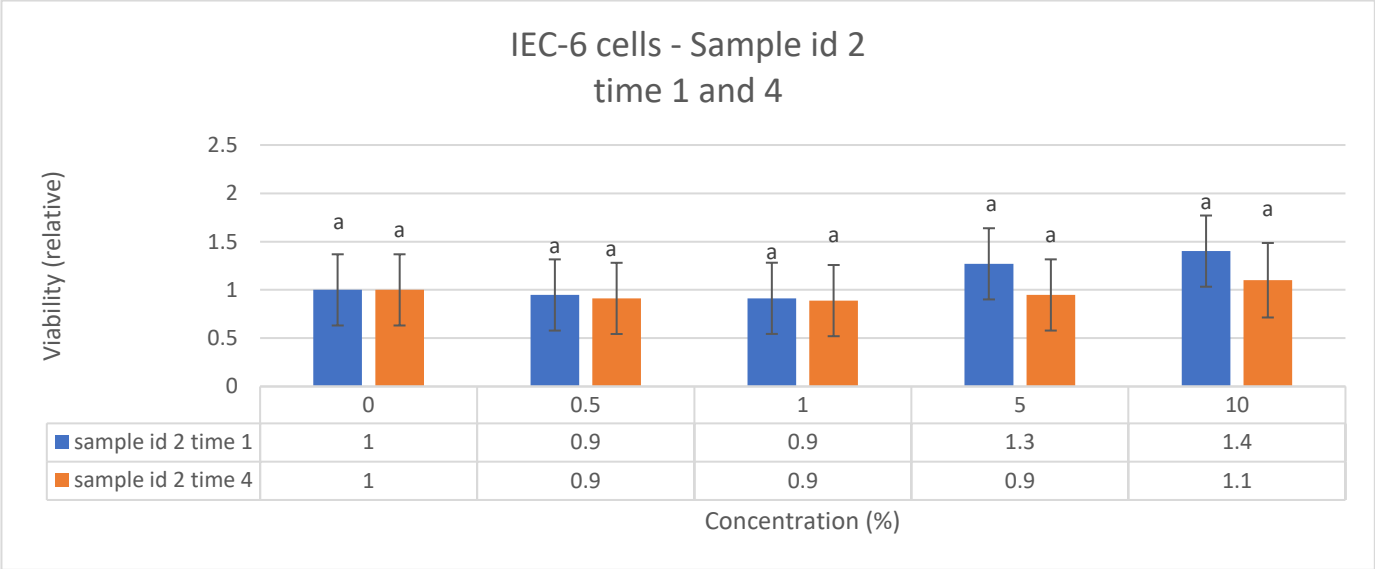


Figure 16 Effect of 6 whey of mare milk on migration of IEC-6 cells. Results are from 2 experiments with 4 replicates showed as mean values ± SEM relative to the negative control. Different lowercase letters show significant differences from the negative control at $P < 0.05$

Both time milk sampling had 0.5% and 1% whey inhibitory effect on IEC-6 cells in intestinal wound healing *in vitro* cell model. The whey fraction in concentration 5% and 10% increased the cell migration with 30%

and 40% for the first-time milk sampled but at the second time cellular migration was inhibited with 10% for 0.5%, 1% and 5% whey.

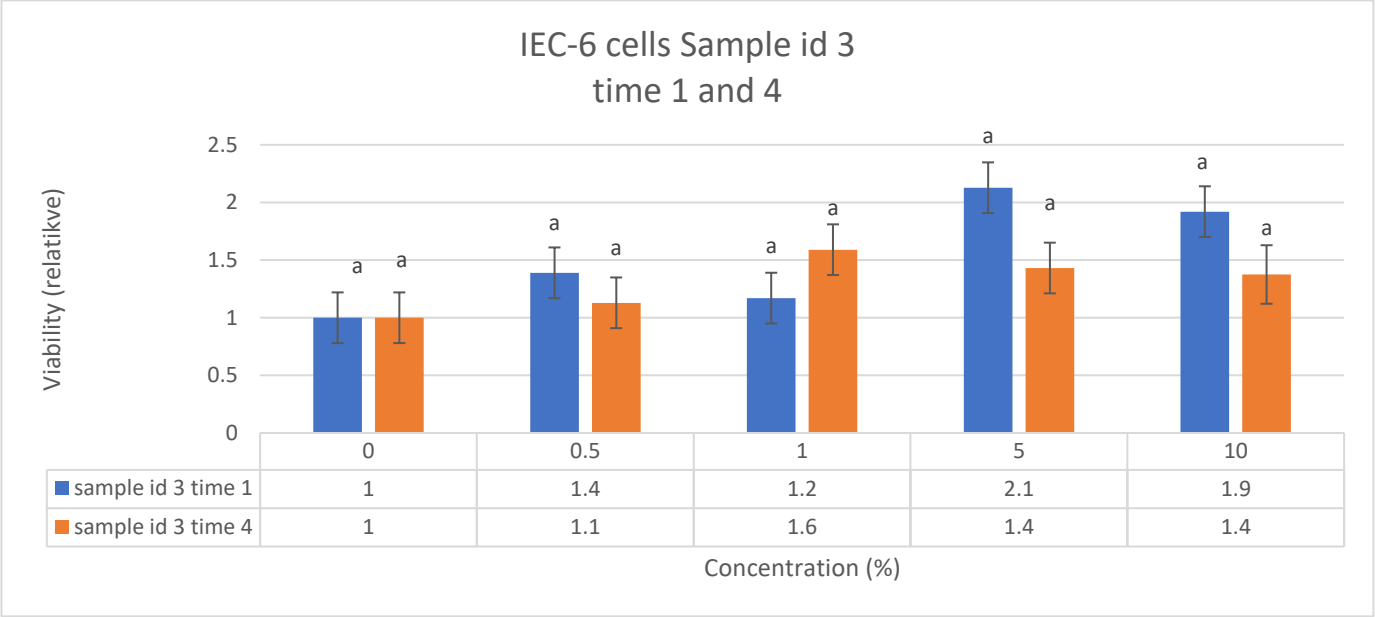


Figure 17 Effect of 6 whey of mare milk on migration of IEC-6 cells. Results are from 2 experiments with 4 replicates showed as mean values ± SEM relative to the negative control. Different lowercase letters show significant differences from the negative control at $P < 0.05$

The whey of the third mare milk sample in concentration of 5% increased the cell migration with 110 % during the first-time milk sampling (more than double positive effect). First time as well, 10% of the whey of sample 3 stimulated the cell migration with 90%. The fourth time milk sample increased the cell migration with minimum 10% (0.5% whey) and maximum 60% (1% whey) and 40% for both 5% and 10% whey fraction. One percent whey of sample 3 increased cell migration of IEC-6 cells with 20% on the first-time sampling and 60% on the fourth time sampling. Whey 5% had the maximum effect on cell migration after six hours, in average 75%, so more than a half, compared with the negative control. The wound between the intestinal epithelial layer became smaller with a half of the length of the wound in six hours after the whey treatment on the cells.

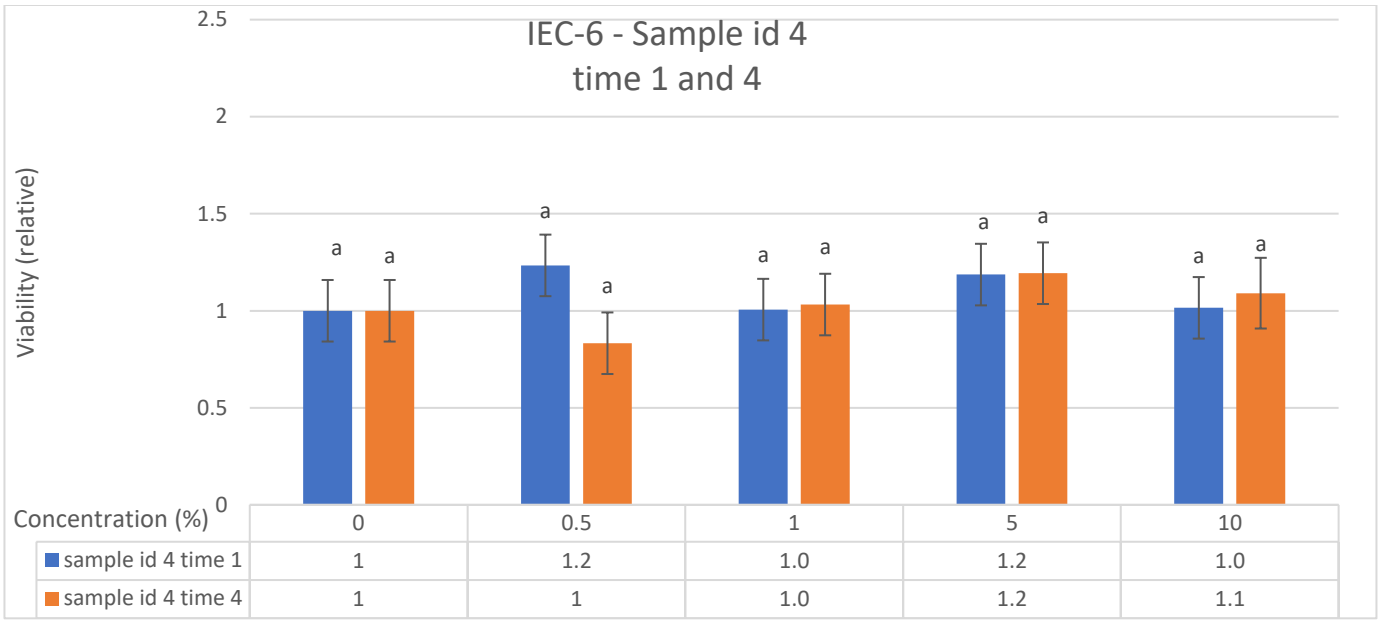
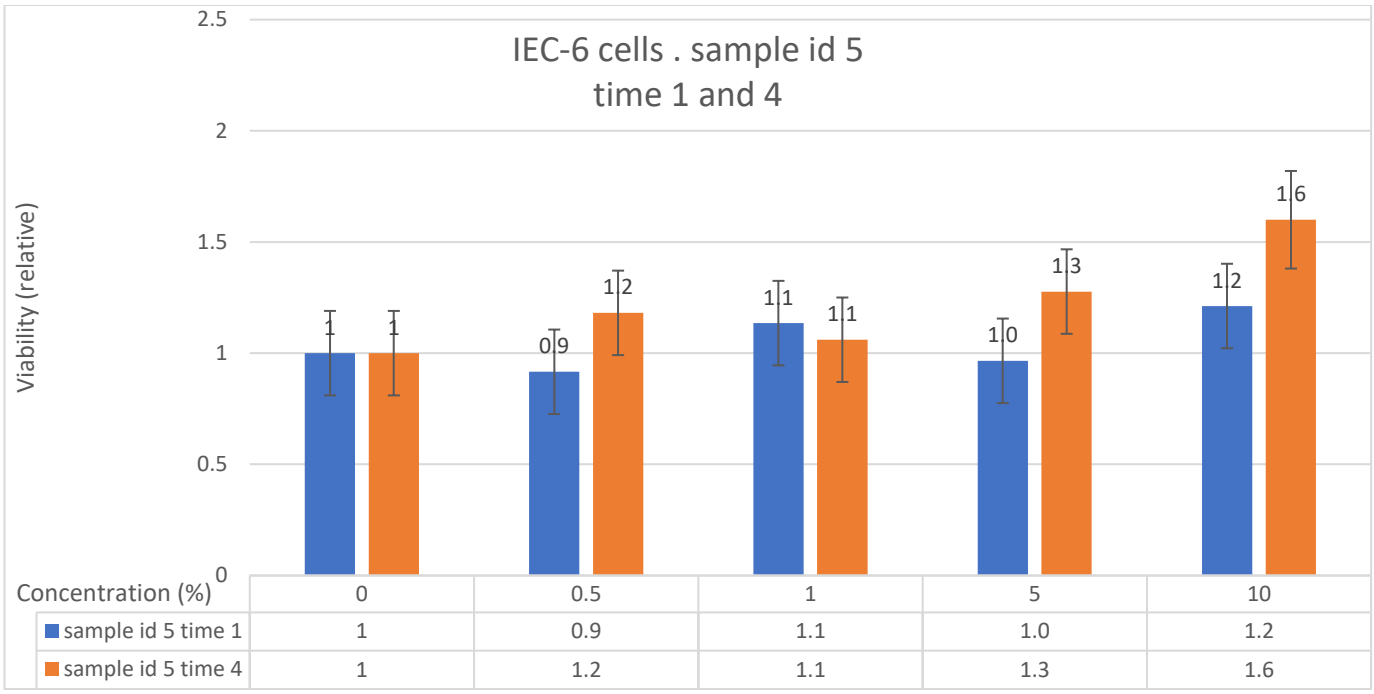


Figure 18 Effect of 6 whey of mare milk on migration of IEC-6 cells. Results are from 2 experiments with 4 replicates showed as mean values ± SEM relative to the negative control. Different lowercase letters show significant differences from the negative control at $P < 0.05$

First time milk sampled increased the cell migration process with up to 20% due to a whey concentration of 0.5% and 5%. Fourth time milk sampled increased the cell migration with 20% only due to a whey concentration of 5% and no effect was seen by using 0.5% and 1% whey. First time milk sampled of 1% and 10% whey fraction had no effect on the IEC-6 cell migration after 6 hours. Five percent of whey of the sample 4 had the constant increase of cell migration of 20% for the time frame of sampling as for 1% whey had no effect on cell migration for the time frame of sampling.



Concentration (%)	0	0.5	1	5	10
sample id 5 time 1	1	0.9	1.1	1.0	1.2
sample id 5 time 4	1	1.2	1.1	1.3	1.6

Figure 19 Effect of 6 whey of mare milk on migration of IEC-6 cells. Results are from 2 experiments with 4 replicates showed as mean values ± SEM relative to the negative control. Different lowercase letters show significant differences from the negative control at $P < 0.05$

As for sample 5, first time sampling of 10% whey increased cell migration with 20% and 1% with 10% and 5% was neutral, with no effect. Concentration of 0.5% whey inhibited the cell migration with 10%. Fourth time milk sampled of mare milk number 5 had 10% whey the most active for the migration of rat intestinal cell line, increasing the cell migration after 6 hours with 60%. All 4 concentration of the whey of mare milk 5 had an increase of cell migration during the fourth time milk sampled. The fourth time milk sampled had a better effect on intestinal cell migration compared with the first time with 2 months before.

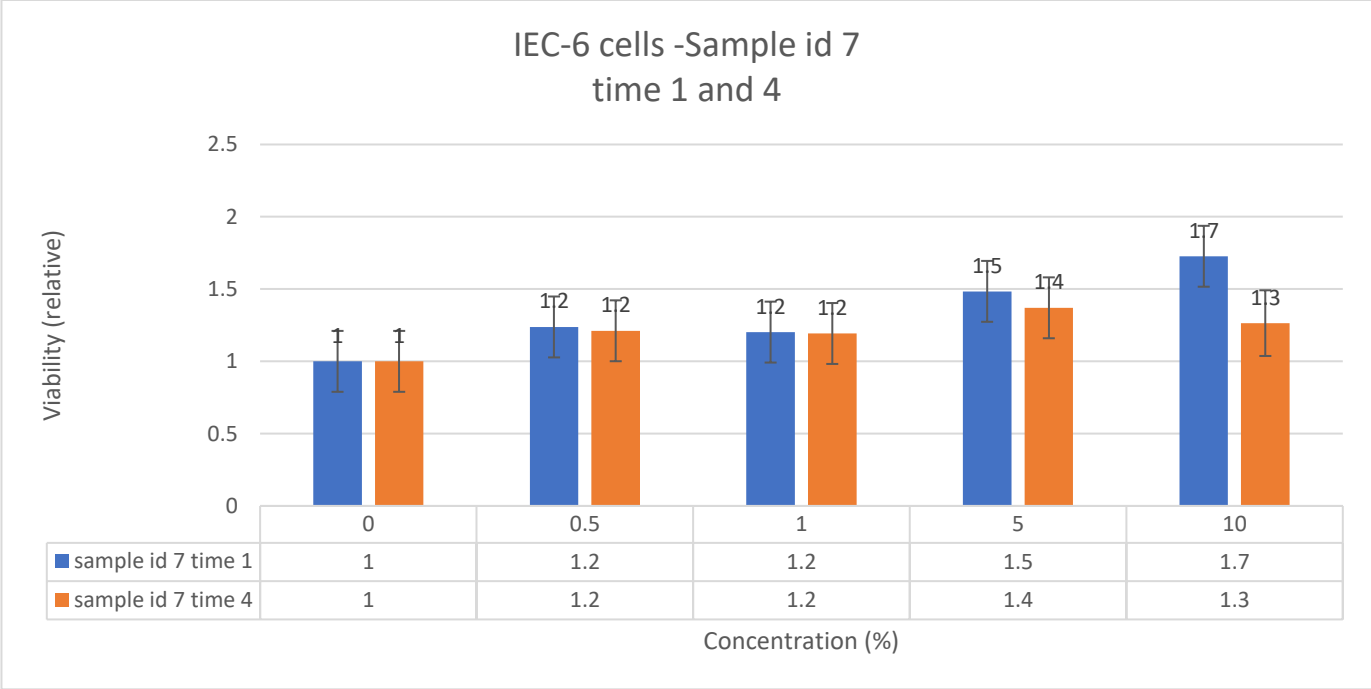


Figure 20 Effect of 6 whey of mare milk on migration of IEC-6 cells. Results are from 2 experiments with 4 replicates showed as mean values ± SEM relative to the negative control. Different lowercase letters show significant differences from the negative control at $P < 0.05$

Sample 7 had the whey second most effective (after sample 3) for helping intestinal cellular migration in the cell model of scratch wound healing model. Ten percent of the way had increased the wound healing with 70% after 6 hours during the first-time milk sampled (the only significant increase) and 30% on the fourth time and 0.5% and 1% on both time frame sampling close the wound by increasing the cell migration with 20%.

Effect of colostrum of mare milk on IEC-6 cells viability

Samples 2 and 3 had a steady upward increase effect on IEC-6 cells viability *in vitro* for maximum 260% - 280% (5%, 10% whey) from 100%, 120% (0.05% whey). For sample 1, 0.25% to 5% whey increased rat enterocyte (IEC-6) viability with up to 310% - 300% (fourth time effect). Five percent doubled the cell viability and 10% whey inhibited cell viability with 40%. Sample 1 was the only one from all 5 colostrum samples which inhibited IEC-6 cell viability *in vitro.* Sample 4 had a constant increase of cell viability for up to 320% (fourth time effect) and sample 5 had the greatest cell viability stimulation for up to 630% (7 times more viable cells compared with the negative control).

Statistical analyses has been done using SAS 9.4 , a PROC MIXED DATA with CLASS sampleid assay conc replicate; MODEL reviab=conc/SOLUTION influence OUTPM=stat; RANDOM=assay; LSMEANS conc/PDIFF=all adjust=tukey; Where sampleid=1,2,3,4,5.

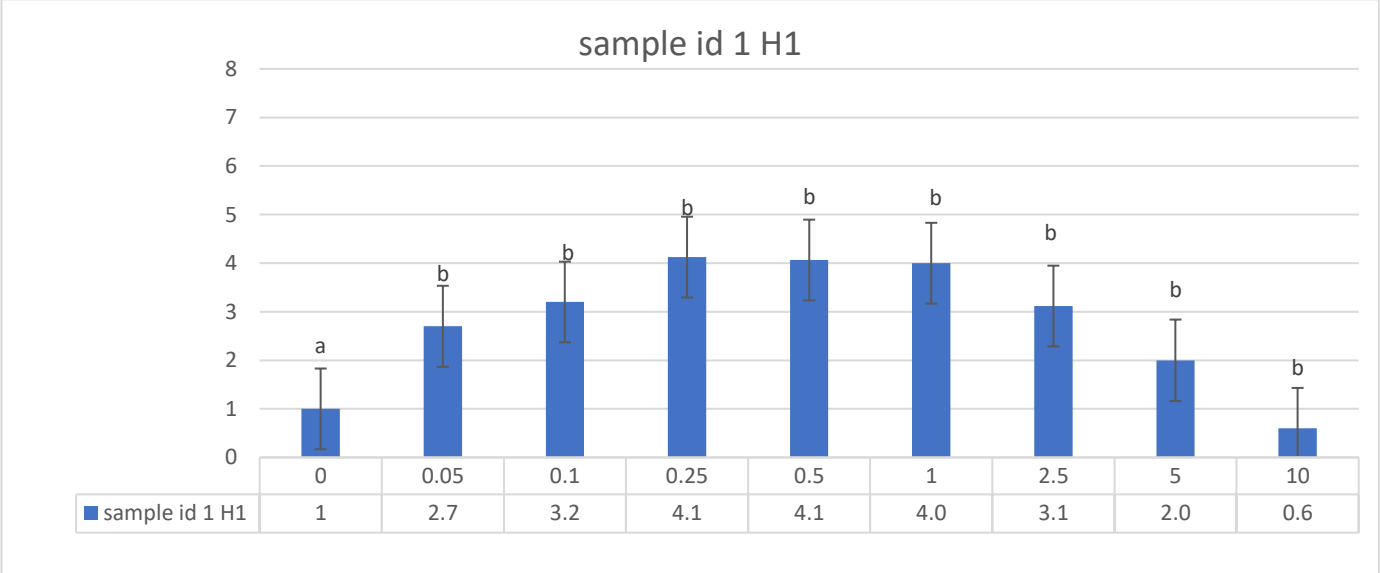


Figure 21 Effect of whey of 5 mare colostrum samples on cell viability of IEC-6 cells. Results are from 2 experiments with 4 replicates showed as mean values ± SEM relative to the negative control. Different lowercase letters show significant differences from the negative control at $P < 0.0001$

The minimum effect on cell viability had 5% whey from colostrum sample 1, double increased the number of viable cells (100%). Concentration of 0.25% and 0.5% whey of colostrum had a maximum cell viability

increase by 310% (fourth time effect). Ten percent whey of colostrum inhibited cell viability by 40%. If increased from 1% to 5% concentration, the whey of mare colostrum started to have a lower cell viability compared with the lower concentrations of it (0.05% - 0.5%).

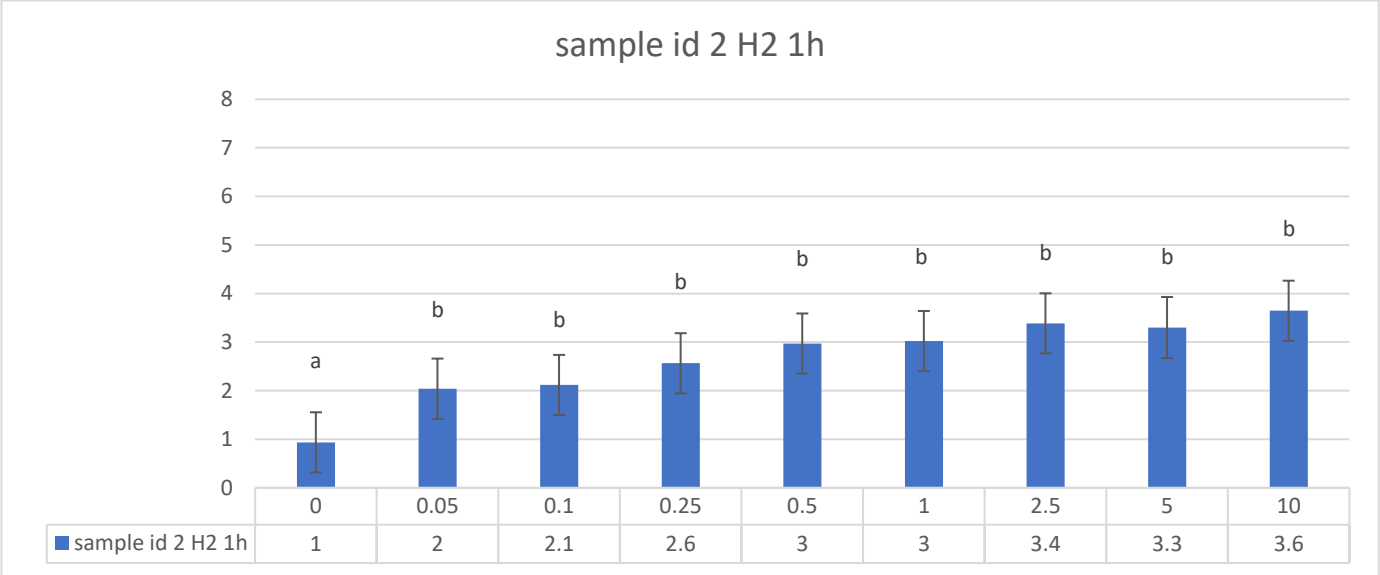


Figure 22 Effect of whey of 5 mare colostrum samples on cell viability of IEC-6 cells. Results are from 2 experiments with 4 replicates showed as mean values ± SEM relative to the negative control. Different lowercase letters show significant differences from the negative control at $P < 0.0001$

Whey 0.05% concentrated from sample 2 increased the cell viability with 100% (double effect) which was minimum and 10% concentrated. No inhibition of viable cells was seen for this sample. Whey with a concentration of 0.5% and 1% had a 200% increase of cell viability (triple effect).

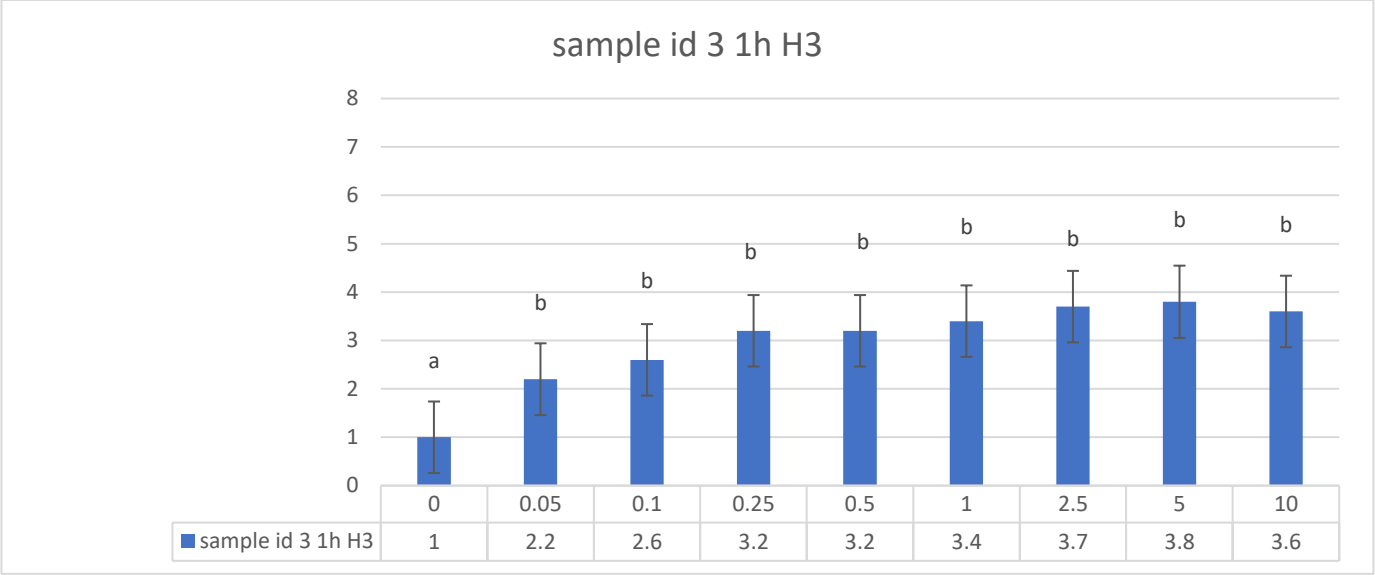


Figure 23 Effect of whey of 5 mare colostrum samples on cell viability of IEC-6 cells. Results are from 2 experiments with 4 replicates showed as mean values ± SEM relative to the negative control. Different lowercase letters show significant differences from the negative control at $P < 0.0001$

Ten percent of the whey of colostrum 3 had the same increase of cell viability with the second sample (260% increase) (triple plus effect). Cell viability increased when the whey concentration increased from 0.05% (110% increase) to 10% whey (260% increase).

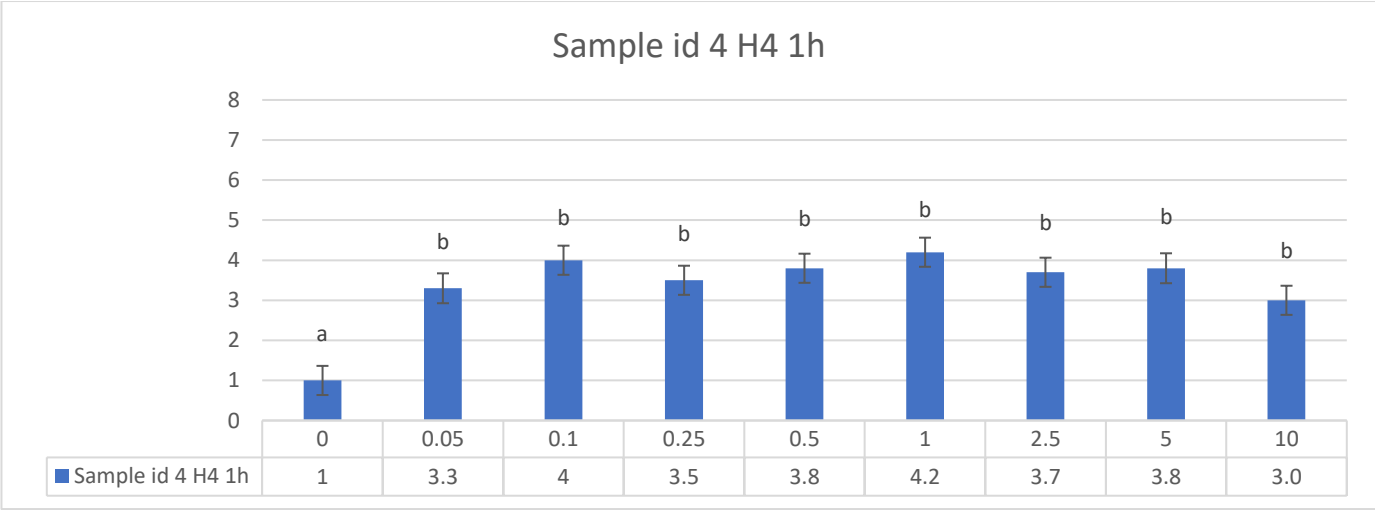


Figure 24 Effect of whey of 5 mare colostrum samples on cell viability of IEC-6 cells. Results are from 2

experiments with 4 replicates showed as mean values ± SEM relative to the negative control. Different lowercase letters show significant differences from the negative control at P < 0.0001

One percent whey of colostrum sample 4 increased the cell viability with 320% (fourth plus effect) and 0.1% concentration of whey with 300%. Less concentrated whey of colostrum gave a greater effect. Ten percent whey had 200% increase of the viability of the cell line. No inhibitory effect was seen in the case of this sample.

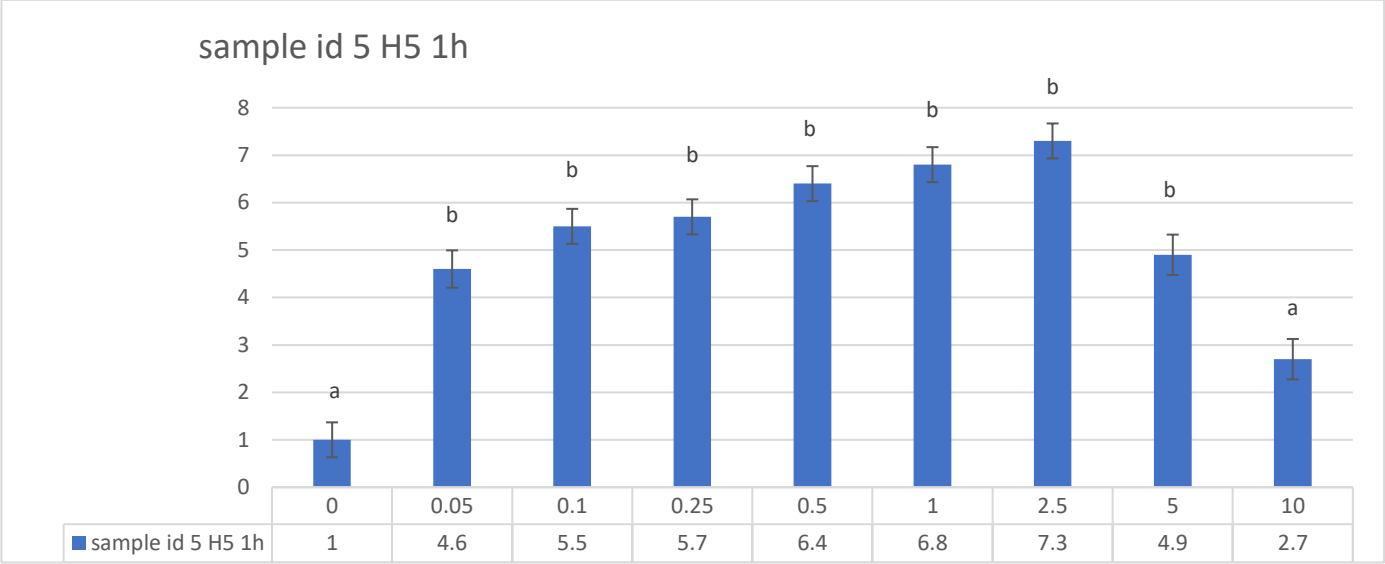


Figure 25 Effect of whey of 5 mare colostrum samples on cell viability of IEC-6 cells. Results are from 3 experiments with 2 replicates showed as mean values ± SEM relative to the negative control. Different lowercase letters show significant differences from the negative control at P < 0.0001

The same pattern with lower concentrated whey of colostrum which greater increased the cellular viability was the case for sample 5. Cell viability increased from 0.05% concentrated whey 360% increase, to 2,5% whey of 630% increase with reducing the cell viability increase for 5% whey and 10% concentrated whey to 390% and 170% respectively.

Growth factors' measurement and their possible effect on cell migration and proliferation

IGF -I ranged from 4.8 ng/ml to 9.4 ng/ml first time and from 7.7 ng/ml to 10.5 ng/ml the second time sampling. Greater values for IGF-I the second time later in lactation have been seen. The lower values were for samples 2 and 4 and the highest for 5 and 7 in both cases (milk sampling 1 and 4). The growth factors presence is independent of age and lactation time and number.
TGFβ1 and TGFβ2 was less than 30 pg/ml for all the 6 mares for both milk sampling and EGF < 25 pg/ml for the whey of mare milk at both time points of lactation (see Table 28 and 29 from the Appendix).
However, colostrum had the highest amount of growth factors. IGF-I in colostrum ranged from 11.4 ng/ml (H2) up to 106 ng/ml (H3). TGFβ1 had a lower range than IGF-I in colostrum, from 2.9 ng/ml (H1) to 6.2 ng/ml (H3). TGFβ2 was again highest 6.2 ng/ml for H3 and the lowest 1 ng/ml for H5. In the case of EGF, which showed very low values, the highest one was for H4 543 pg/ml and for the rest less than 63 pg/ml (for the whole overview of these growth factors in colostrum see Table 30 in the Appendix).

9. Discussion and conclusion

The choice of using IEC-6 cell culture monolayer for this study came because of the need to have a robust and replicable model to test for specific cellular endpoints like intestinal cell viability and proliferation along with integrity of the 2D monolayer in the case of wound healing mimicked scratch model. The choice proved to be scientifically reliable to stress out the importance of bioactive peptides from the whey fraction of mare's milk for the intestinal barrier of epithelial cell lining. The need of using FHs 74 Int human epithelial intestinal cells came because it can create a view about the importance of mare milk for human health opening a path of future scientifically studies and research in this particular field using and marketing mare milk and its products as a food niche product.

Some characteristics of IEC-6 cell line is worth of mention considering the fundamental of laboratory tests used for the current study. They represent a mix of proliferating (crypt region) cells and differentiated non-proliferating cells (villus region). Crypt intestinal cells when matured, develop a brush border at the luminal side and a columnar shape. These cells are growing in monolayers showing their epithelioid trait, have a polygonal shape with microvilli, Golgi apparatus and tight junction as ultrastructure. With a diploid karyotype, they develop growth inhibition when seeded too dense hence they have a good adherence on the plastic wall of the bottle and a good covering when plated dense enough. They are cultured by incubation at 37^0 C with low CO_2 5% (Quaroni et al., 1979).

FHs 74 Int cells are human small intestine cells (ATCC® CCL241™) from normal tissue, non tumorgenic, with expressed EGF receptor, negative for keratin by immunoperoxidase staining. Similar to IEC-6 cells but a longer shape and with special culturing conditions regarding the growth medium composition (insulin and EGF addition) (see the cells in the Appendix Figure 46 and Figure 47).

Both epithelial cell lines were having a positive effect in contact with the whey fraction of mare milk.

The positive effect on intestinal cell viability of the whey bioactive fraction of mare milk can of course be further studied using more complicated cell models and algorithms but the main idea is that the bioactive peptides enclosed in the whey fraction in vitro give a proliferative effect of the epithelial intestinal layer necessary for the gut physiology and metabolism. In the case of diseases and intestinal dismicrobism which affects the human intestinal barrier cell viability enhancement it is of course very important. Growth factors alone couldn't explain this effect and of course they are not present only those growth factors analysed in this study present in the mare milk or colostrum composition.

A database for bovine milk bioactive peptides already exists and its biological functions are diverse and treated individually (Nielsen et al., 2017b).

Rat small intestine epithelial cell line (IEC-6) were used in a cell-based model for proliferation and wound healing together with different bovine whey protein powder made as casein enzymatic hydrolysates (Purup et al., 2018) with stimulation of migration and cell proliferation in low concentration as effect for the casein hydrolysates showing the importance of casein digestion for releasing the functional bioactive substances from the milk.

For the first time (in this study) the whey fraction of mare milk and colostrum was tested in vitro to check the viability of intestinal epithelial cells in culture monolayers and it opened the perspective of investigation of mare milk properties.

For all 6 horses the tendency is to enhance the cell viability (from 30% to up to 120-100%) for both sets of samples (time 1 and 4) after 3 days of incubation with the whey of mare milk. This is showing the fact that rat epithelial cells viability is enhanced by the components of the whey of mare's milk from the mid to late lactation. However, milk was sampled not so early during lactation but IEC-6 cells showed an increased viability in contact with different concentrations of the whey solution.

The importance of using human intestinal cells for in vitro studies using active whey of mare milk draw the line of the positive role of mare milk both in human intestinal health and for neonatal to infancy development of the human intestinal barrier.

Cell viability was stimulated more than 3 times after 3 days of incubation of human intestinal epithelial cell line with one of the whey of mare milk. As in general, a mean for all 6 whey of mare milk samples gave for the first time sampling a peak of 186.6 % increase of cell viability and for the fourth time sampling a peak of 158.3 % increase of the cell viability (see Table 25 in the Appendix).

In general, all six-sample increased cellular migration compared to the negative control. For the first time milking, sample 1 and 2 gave 40% increased migration at 10% dilution of the whey samples (not significant increase). The peak was 110% for the first-time sampling and 60% for the fourth time sampling. Concentrations of 5% and 10% whey gave the maximum increase of the cell migration in general.

Sample no. 2 concentration 0.5% and 1% (first time sampled milk) and 0.5%, 1% and 5% (fourth time sampled milk) had 10% inhibitory cell migration effect. First time sampled milk no. 5 concentration 0.5% had 10% inhibitory effect on cellular migration of the rat small intestinal epithelial cells.

Sample no.4 - 1% for both time sampled milk had no effect on the IEC-6 cell migration in the *in vitro* cell model of scratch wound healing. Sample 5 5% had no effect on migration of IEC-6 cells in the cell model of cellular migration after 6 hours of cellular contact with the whey of mare milk. Almost none of the results was significant compared with the negative control except for the 10% first time sampled milked no.7 which increased cells' migration with 70% ($P<0.05$).

Mares being used in this present study were not of a significant representative or great number neither represent a group of age or dairy specialised horses, but their late lactation milk being analysed as

neutralised acidic whey showed wound healing properties and enhanced proliferation of the intestinal epithelial cells important in mucosal wound healing and in the studies of human intestinal inflammation. Other cell culture scratch models had been used in the literature, important for the study of pathogenesis of inflammatory bowel diseases for drug research and development, for example in Turkey 2017 (Erol et al., 2018).

Additional in vitro inflammatory conditions were created using Cytomix (TNFα, IFNγ, IL-1β) and LPS and cell migration was photomicrograph from 2 to 2 hours up to one day. Nitric oxide synthase 2 (iNOS) and cyclooxygenase-2 (COX-2) protein expression confirmed the inflammation.12 h serum starvation was used to stop the cell proliferation. After scratching 75 cm^2 bottles were incubated with LPS and Cytomix for another 12 hours, supernatants cell lysates after cold and detergent treatment were measured at spectrophotometer (Erol et al., 2018).

The present study didn't have the purpose of showing a cell model for wound healing for the perspective of pharmaceutical industry but testing an intestinal wound healing model using whey of mare milk to show the complexity of mare milk as a functional food and the consecutive positive impact of consuming it for human health.

The cell culturing didn't require a cell proliferation inhibition step because the cells were prior twice washed with PBS with Ca_2 and Mg_2 to extract the traces of FCS from the cell supernatant. However, IEC-6 cells were cultured and incubated for 24 hours before adding the treatment and performing the scratch until they got just enough confluent and covered evenly the bottom of the plastic cell culture bottle and avoided the contact inhibition when plated too dense.

Migration of the cells is time restricted to hours (this study considered 6 hours) because the intestinal epithelial layer in case of small injuries needs minutes to hours for the phase of cell migration before cell proliferation and maturation which takes days and weeks (Sturm and Dignass, 2008).

Human intestinal cell viability is regulated by Wnt - β catenin signalling pathways and p38 is a necessary ligand for the EGF stimulation of cell migration and proliferation (intestinal restitution) within a MAPK signalling pathway. P38 activation in conjunction with EGF switch the intestinal restitution from proliferation of the cells to migration. The intestinal balance is kept through these pathways of cellular signalling and different ligands modulation or antagonists (DKK-1 for Wnt β catenin pathway) can influence the wound healing process and intestinal epithelial cell viability (Koch et al., 2009, Frey et al.,

2004). Considering the fact that 10% whey of mare milk stimulated cell viability on both epithelial intestinal cells line and IEC-6 migration in general, in the case of the whey of colostrum it was unusual to observe a 10% whey of colostrum inhibition of the cell viability. Probably, some components from the colostrum in concentration of 10% could modulate the cells proliferation switching cell viability to inhibition which can be corelated also with apoptosis activation.

A fewer constant cells migration was an effect of IEC-6 cells in wound healing model. Some of the whey samples concentration gave a less substantial cell migration, had no effect, or even inhibited cell migration (see the results section) even though the same cell line had an increased viability using the whey treatment proving the mechanism of cell migration is depending on the bioactivity of the whey (composition), time of contact (hours for migration and days for cell viability). More concentrated bioactive components in the colostrum can bring the cells in contact with some milk-borne ligands, receptors antagonists for cell proliferation which can modulate apoptosis and proliferation with negative effect on the cell viability switched from the greater cell viability general trend. However, some components of the whey fraction can have a direct effect on cell viability or migration *in vitro* and some of them can have a more complicated modulation effect which requires further studies.

This study showed the mare's milk mid to late lactation whey fraction increased cell viability on 2 epithelial cell line (IEC-6 and FHs 74 Int) and migration on one cell line (IEC-6) *in vitro*. Moreover, whey of colostrum in general, increased cell viability on IEC-6 cell line *in vitro.*

Colostrum had a greater stimulatory effect on cell viability compared to the whey samples knowing that the colostrum is essential for mammals' neonates being rich in nutritional and bioactive factors.

General trend for IEC-6 cell proliferation measured as cell viability in vitro was stimulation, an increased cell number could be detected visual (check the photomicrographs in the Appendix) and be measured using fluorometry.

In the case of sample no. 1 which in concentration of 10% whey of colostrum inhibited cell viability with 40%, 0.05% whey increased IEC-6 cells viability with 170% and 5% with 100% but the photomicrographs can show the cell morphology quite different, 5% whey of colostrum treatment made the cells looking more like 10% whey of colostrum treatment on the cells, with cellular debrides and less viable cells with unchanged morphology (see Appendix).

In the case of sample no. 3 and 4, cell viability increased direct proportional with the concentration range of the whey of colostrum and the cells can be seen in the Appendix section as well for some of the concentration treatment.

Some cell debrides and less cells can be seen in the Figure 41 10% sample no.4 (200% increase cell viability) compared with Figure 39 2.5% sample no. 4 (270% increase od cell viability) and Figure 40 5% sample no. 4 (280% increase of cell viability).

More concentrated whey of colostrum, 2.5%, 5% and respectively 10% whey of colostrum for samples no. 4 and 5 had a tendency to a less increased effect on the IEC-6 cell viability compared with the other concentration (less concentrated whey of colostrum). This tendency can be observed on the photomicrographs which appears to have less cells and some cellular debrides for the last 3 concentration range of the treatment added to the cells *in vitro* (see the Appendix).

For example, Figure 43 correspondent to sample 5 2.5% concentration which gave the highest cell viability increase of 630% looks different than Figure 44 correspondent to sample 5 5% concentration of whey of colostrum which showed less cells and some cellular debrides and increased IEC-6 viability with 390%. Figure 45 with 10% whey of colostrum, sample no.5 showed 220% less viable cells compared with Figure 44 5% concentrated whey of colostrum. The more concentrated the colostrum is the less viable cells and more debrides are showed in the case of sample no. 1, 4 and 5. This direct cellular effect can be seen mostly for the colostrum whey fraction which composition is different than the mature milk in general (Csapó-Kiss et al., 1995).

Previous studies have been more frequently done to assess the bioactivity of bovine whey of milk in different cells lines namely, bovine mammary cells (Sejrsen et al., 2001) and human epithelial cell line (FHs) (Purup et al., 2006). Both studies showed the correlation of IGF-I, TGFβ1 and TGFβ2 and IGF binding proteins (IGFBP) content from bovine whey milk with the increased cell viability. Moreover, a correlation of this effect of the treatment with the stage of lactation (greater concentration of growth factors later during lactation) was made. A decreased cell viability due to the existence of IGFBP which can bind the IGF receptors until saturation can be considered as well from these previous studies. Because of the presence of TGFβ and the post-translational modification like phosphorylation of glycosylation of these peptides (IGF-I, TGFβ1 and TGFβ2 and IGF binding proteins IGFBP) cell viability can be inhibited. But none of this can explain the uncorrelated effect of growth factors and increased cell viability effect of the whey

of bovine milk. IGFBP can have an IGF independent manner of action modulating cell survival and mitosis, migration, apoptosis and angiogenesis. It can be considered also the free hormone hypothesis which states that the unbound form of a hormone give its biological activity rather than its total concentration or protein bound concentration in the plasma (Mendel, 1992) (Bach, 2017).

IGF -I ranged from 4.8 ng/ml to 9.4 ng/ml first time and from 7.7 ng/ml to 10.5 ng/ml the second time milking after 2 months (4 month of lactation to 7 month of lactation period, namely mid to late lactation). This shows greater values for IGF-I the second time later in lactation demonstration for time of lactation not being a determinant factor for growth factors composition in mare milk. The lower values were for samples 2 and 4 and the highest for 5 and 7 in both cases (milk sampling 1 and 4) (see the Appendix). The growth factors presence is independent of age and lactation time and number.

IGF-I concentration in mare's milk around week 12 of lactation had a value of 4.8 ng/ml increasing to up to 10.5 ng/ml around 28 weeks compared to cow's milk which was 2.3 ng/ml at 12 weeks lactation and went up to 2.7 ng/ml at 24 weeks of lactation. Cow's and mare's lactation are for sure different and the milk yield is lower in the case of mares but one can definitely see the greater value of IGF-I growth factor in mare milk. As for colostrum the range of IGF-I colostrum was from 11.4 ng/ml to 106 ng/ml and in the case of cow's colostrum IGF-I ranged from 312 ng/ml at the first day to 5.9 ng/ml after one week postpartum. TGFβ1 for cow colostrum ranged from 6.3 to 0.55 ng/ml and for mature milk was 1 ng/ml. TGFβ2 for cow colostrum ranged from 143 to 12 ng/ml and in colostrum 12 ng/ml. Mare milk had very low values for TGFβ1 and TGFβ2 (<30 pg/ml) and colostrum ranged from 2.9 ng/ml to 6.2 ng/ml for TGFβ1 and from 1 ng/ml to 6.2 ng/ml for TGFβ2 (Purup et al., 2006).

EGF and TGFα stimulate primary intestinal epithelial culture in 2.5% FCS with insulin supplement but without EGF with a concentration of 5-10 ng/ml EGF and TGFα 5-20 ng/ml as for primary intestinal epithelial culture in 0.5% FCS was necessary only 1-5 ng/ml of both growth factors to enhance the cell proliferation (Booth et al., 1995). As for cellular migration, 50 ng/ml EGF and TGFα (lower effect than EGF) stimulates cell migration. Insulin 0.25 UI and IGF-I 20 ng/ml stimulates cell proliferation but inhibits intestinal cellular migration. This was a collagen cell model with small intestinal epithelium and the mesenchymal additional layer which was recommended as a superior in vitro cell model to study mesenchymal factors necessary for epithelial cells to grow and proliferate. PDGF was proved to be the

mesenchymal cell stimulatory growth factor and subsequent epithelial cell proliferation stimulator by upregulating IGF-I receptor. TGFβ1 inhibited intestinal epithelial cell proliferation (Booth et al., 1995).
The presence of other growth factors in mare milk can be implied for a complete overview about the bioactivity of mare milk in general in order to correlate the cell viability and proliferation along with IECs migration in the case of wounding which increases after pre-digested mare milk comes in contact with the small intestine enterocytes. Moreover, the whey bioactive fraction of mare milk contains cytokines, lysozymes, lactoferrin and other bioactive peptides which can stimulate cellular turnover in the intestine. Definitely, fermented mare milk can be an important part of the bigger picture of mare milk bioactive components and their usage in human health. Further studies are necessary for the whey of mare milk properties in vitro and in vivo.
The choice of cell lines for in vitro studies might be less accurate but is less expensive and les work-loaded and more reproductible than primary cell culture.
IGF-I in human colostrum was measured since 1984 by Baxter and his colleagues in their experiments showing a value of 28 ng/ml with mitogenic effect and IGF-I unbound to proteins at pH 7 (Baxter et al., 1984).

Some **conclusions** can be briefly driven from this study as like the whey of mare milk has a mitogenic effect on intestinal epithelial cell lines of rats and humans in vitro , the peptides from the whey of mare milk can help in the process of intestinal restauration after an injury, the growth factors concentration analysed in this study was not very high or correlated with the horses age, lactation time and number and the highest content in growth factors had IGF-I. All of these components have specific pathways of action and are coupled with cellular receptors in vivo which can stimulate the cellular proliferation and migration on the small intestine level.

10. Future perspectives

Generally, from monolayer cell culture of animal and human cell lines to organoids and human clinical trials, there are some steps to be done to prove mares' milk bioactivity and its health promoting role.

Animal models' studies are further required in order to expose mare milk bioactive substances to animal gut microflora and see its effects.

The next necessary level to be reached, is clinical research using mare milk for enhancing mucosal intestinal wound healing in the case of intestinal bowel diseases and intestinal ulcers patients.

Computational biology using ligand-based methods and structure-based models namely QSAR (quantitative structure activity relationship) and docking based scoring for the prediction of the bioactive compounds from mare milk can be a future approach for finding these substances but also for predicting their action and creating computer models to validate bioactive peptides from mare's milk into drug discovery and pharmacology. In this way the animal testing stage can be avoided and more reliable data can be obtained (Vilar and Costanzi, 2012), (Nongonierma and FitzGerald, 2018).

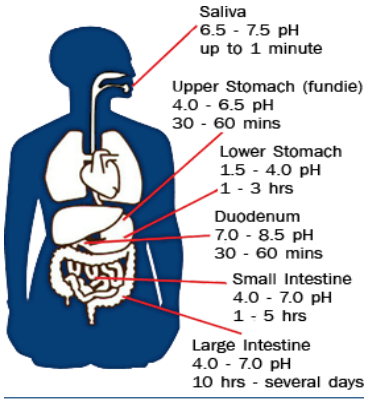
In particular, using fermented mare milk together with cell-culturing can bring another vision about how these released bioactive peptides can simulate the viability of intestinal epithelial cells important in mucosal intestinal healing and in human health of digestive tract.

The digestibility of a fermented product it's greater than of a raw one, because the acidity creates a larger enzymatic area during digestion and less lactose from the fermented milk needs less enzymes to digest it. More than that, LAB using their beta-galactosidase enzyme, improve lactose digestion. LAB can also synthesize vitamins based on their profile. The general amino-acid composition it is the same for fermented milk but a larger quantity of free amino-acids, essential amino-acids and peptides make the difference in the case of fermented milks. The bioavailability of vitamins and minerals in the G-I tract needs to be studied better, being known that in developing countries fermented milk can be a cheap source of peptides and amino-acids (McKevith and Shortt, 2003).

These future studies are relevant because LAB fermentation decrease the immunoreactivity of mare milk peptides, moreover the digestive profile of mare milk proteins makes it a good substitute of cow's milk in

human nutrition (Fotschki et al., 2014), (Inglingstad et al., 2010) and of course to recognise the therapeutic properties of fermented mare's milk, kumis in human health. Kumis is absorbed 2.8 times faster than other fermented milks in one hour (Dhewa et al., 2015).

Figure 26 The pH of the human intestinal tract and the time spent by food in it from (Specialist, 2019)



Further studies are required to test human mammary cells in contact to see if the bioactive peptides from the mare milk whey fraction or from the casein fraction as well can influence in a way these cells and maybe parturient mares can be immunised for milk fraction suitable for human nutrition. FHs 74 cells are human intestinal cells original from 3 to 4 months foetus after therapeutic abortion (Smith et al., 1979) and a milk formula containing mare milk fraction can be further studied on these cells. The neonatology collaboration with the food safety and food technology requires future projects and bench to bedside studies.

11. References

ThermoFisher - Mammalian cell culture media supplements [Online]. Available: https://www.thermofisher.com/dk/en/home.html [Accessed 27.05 2019].

AKANOVA, A., KIKEBAYEV, N., SHAIKENOVA, K., SEIITKAZHY, Z. & OKUSKHANOVA, E. 2017. *Nutritive and Biological Value of Mare*[?]*s Milk Ice Cream*.

AMIN, M. L. 2013. P-glycoprotein Inhibition for Optimal Drug Delivery. *Drug target insights,* 7**,** 27-34.

ATHAVALE, M. A., MAITRA, A., PATEL, S., BHATE, V. R. & TODDYWALLA, V. S. 2013. Development of an in vitro cell culture model to study milk to plasma ratios of therapeutic drugs. *Indian journal of pharmacology,* 45**,** 325-329.

BACH, L. A. 2017. What Happened to the IGF Binding Proteins? *Endocrinology,* 159**,** 570-578.

BALBAS-MARTINEZ, V., RUIZ-CERDÁ, L., IRURZUN-ARANA, I., GONZÁLEZ-GARCÍA, I., VERMEULEN, A., GÓMEZ-MANTILLA, J. D. & TROCÓNIZ, I. F. 2018. A systems pharmacology model for inflammatory bowel disease. *PloS one,* 13**,** e0192949-e0192949.

BATT, C. A. & TORTORELLO, M. L. 2014. Encyclopedia of food microbiology.

BAXTER, R. C., ZALTSMAN, Z. & TURTLE, J. R. 1984. Immunoreactive somatomedin-C/insulin-like growth factor I and its binding protein in human milk. *J Clin Endocrinol Metab,* 58**,** 955-9.

BÉDUNEAU, A., TEMPESTA, C., FIMBEL, S., PELLEQUER, Y., JANNIN, V., DEMARNE, F. & LAMPRECHT, A. 2014. A tunable Caco-2/HT29-MTX co-culture model mimicking variable permeabilities of the human intestine obtained by an original seeding procedure. *European Journal of Pharmaceutics and Biopharmaceutics,* 87**,** 290-298.

BEHERA, D. S., PANDA, S., KAYITESI, E. & MULABA-BAFUBIANDI, A. 2017. Kefir and Koumiss Origin, Health Benefits and Current Status of Knowledge.

BOBOS, S., JOVANOVIC-PANIC, L., TRAILOVIC, D., PAJIC, M., RASIC, Z., RADINOVIC, M., GALFI, A. & SIMIN, S. 2013. *Concentration of total immunoglobulins in blood and milk of periparturient mares and their transfer to foals via colostrum*.

BOCA RATON, T. F. 2011. Functional foods of the East.

BODE, L. 2012. Human milk oligosaccharides: every baby needs a sugar mama. *Glycobiology,* 22**,** 1147-1162.

BOOTH, C., EVANS, G. S. & POTTEN, C. S. 1995. Growth factor regulation of proliferation in primary cultures of small intestinal epithelium. *In Vitro Cellular & Developmental Biology - Animal,* 31**,** 234-243.

BOYE, J. I. 2015. Nutraceutical and Functional Food Processing Technology.

BURGESS, K. 2014a. Milk and Dairy Products in Human Nutrition (2013), by E. Muehlhoff, A. Bennett and D. McMahon, Food and Agriculture Organisation of the United Nations (FAO), Rome. E-ISBN: 978-92-5-107864-8 (PDF). Available on web-site (publications-sales@fao.org). *International Journal of Dairy Technology,* 67**,** 303-304.

BURGESS, K. 2014b. *Milk and Dairy Products in Human Nutrition (2013), by E. Muehlhoff, A. Bennett and D. McMahon, Food and Agriculture Organisation of the United Nations (FAO), Rome. E-ISBN: 978-92-5-107864-8 (PDF). Available on web-site (publications-sales@fao.org)*.

BURNS, T. 2007. CHAPTER 63 - Colostrum. *In:* SAMPER, J. C., PYCOCK, J. F. & MCKINNON, A. O. (eds.) *Current Therapy in Equine Reproduction.* Saint Louis: W.B. Saunders.

BUSINCO, L., GIAMPIETRO, P. G., LUCENTI, P., LUCARONI, F., PINI, C., DI FELICE, G., IACOVACCI, P., CURADI, C. & ORLANDI, M. 2000. Allergenicity of mare's milk in children with cow's milk allergy. *J Allergy Clin Immunol,* 105**,** 1031-4.

C CURADI, M., G GIAMPIETRO, P., LUCENTI, P. & ORLANDI, M. 2001. *Use of mare milk in pediatric allergology*.

CALDER, P. C. 2013. Chapter 4 - Omega-6 and Omega-3 Polyunsaturated Fatty Acids and Inflammatory Bowel Diseases. *In:* WATSON, R. R. & PREEDY, V. R. (eds.) *Bioactive Food as Dietary Interventions for Liver and Gastrointestinal Disease.* San Diego: Academic Press.

CARRICK, G. L. 1881. *Koumiss, or fermented mare's milk, and its uses in the treatment and cure of pulmonary consumption and other wasting diseases. With an appendix on the best methods of fermenting cow's milk,* Edinburgh, Blackwood.

CENCIC, A. & CHINGWARU, W. 2010. The Role of Functional Foods, Nutraceuticals, and Food Supplements in Intestinal Health. *Nutrients,* 2**,** 611-625.

CENTER, D. R. 2016. *The dairy growth is expcted to continue until 2025* [Online]. Available: https://ifcndairy.org/ifcn-forecasts-the-dairy-growth-is-expected-to-continue-until-2025/ [Accessed 26.05. 2019].

CHEN, Y., WANG, Z., CHEN, X., LIU, Y., ZHANG, H. & SUN, T. 2010. Identification of angiotensin I-converting enzyme inhibitory peptides from koumiss, a traditional fermented mare's milk. *Journal of Dairy Science,* 93**,** 884-892.

COLOMBO, J., GUSTAFSON, K. M., GAJEWSKI, B. J., SHADDY, D. J., KERLING, E. H., THODOSOFF, J. M., DOTY, T., BREZ, C. C. & CARLSON, S. E. 2016. Prenatal DHA supplementation and infant attention. *Pediatric research,* 80**,** 656-662.

COMINELLI, F., ARSENEAU, K. O., RODRIGUEZ-PALACIOS, A. & PIZARRO, T. T. 2017. Uncovering Pathogenic Mechanisms of Inflammatory Bowel Disease Using Mouse Models of Crohn's Disease-Like Ileitis: What is the Right Model? *Cellular and molecular gastroenterology and hepatology,* 4**,** 19-32.

CONTARINI, G. & POVOLO, M. 2013. Phospholipids in milk fat: composition, biological and technological significance, and analytical strategies. *International journal of molecular sciences,* 14**,** 2808-2831.

CORREDIG, M., GURI, A., GRIFFITHS, M. W., PIEDBOEUF, B., DAUBE, G., CLAES, J., PALIGOT, M., CRÈVECOEUR, S. & DELCENSERIE, V. 2015. In vitro screening of mare's milk antimicrobial effect and antiproliverative activity. *FEMS Microbiology Letters,* 363.

CSAPÓ-KISS, Z., STEFLER, J., MARTIN, T. G., MAKRAY, S. & CSAPÓ, J. 1995. Composition of mares' colostrum and milk. Protein content, amino acid composition and contents of macro and micro-elements. *International Dairy Journal,* 5**,** 403-415.

DATABASE, N. C. F. B. I. P. *Leucine, CID=6106* [Online]. Available: https://pubchem.ncbi.nlm.nih.gov/compound/6106 [Accessed 15.05 2019].

DAVIES MOREL, M. C. G. 2003. *Equine reproductive physiology, breeding and stud management,* Wallingford, CABI.

DETHA, A., SAPUTRA, A. & OLA, A. 2019a. *Antimicrobial activity of whey mare's milk against Salmonella enteritidis*.

DETHA, A., SAPUTRA, A. & OLA, A. 2019b. Antimicrobial activity of whey mare's milk against Salmonella enteritidis. *Journal of Physics: Conference Series,* 1146**,** 012027.

DHEWA, T., KUMAR, N., MISHRA, V. & SANGU, K. 2015. Koumiss: The Nutritional and Therapeutic Values.

DI CAGNO, R., TAMBORRINO, A., GALLO, G., LEONE, C., DE ANGELIS, M., FACCIA, M., AMIRANTE, P. & GOBBETTI, M. 2004. *Uses of mares' milk in manufacture of fermented milk*.

DIFILIPPO, E., WILLEMS, H. A., VENDRIG, J. C., FINK-GREMMELS, J., GRUPPEN, H. & SCHOLS, H. A. 2015. Comparison of milk oligosaccharides pattern in colostrum of different horse breeds. *J Agric Food Chem,* 63**,** 4805-14.

DIGNASS, A. U. & STURM, A. 2001. Peptide growth factors in the intestine. *European Journal of Gastroenterology & Hepatology,* 13**,** 763-770.

DOHERTY, A. M., LODGE, C. J., DHARMAGE, S. C., DAI, X., BODE, L. & LOWE, A. J. 2018. Human Milk Oligosaccharides and Associations With Immune-Mediated Disease and Infection in Childhood: A Systematic Review. *Frontiers in pediatrics,* 6**,** 91-91.

DONG PAN, D., ZENG, X. & TING YAN, Y. 2011. *Characterisation of Lactobacillus fermentum SM-7 isolated from koumiss, a potential probiotic bacterium with cholesterol-lowering effects*.

DOREAU, M., BOULOT, S. & CHILLIARD, Y. 2009. Yield and composition of milk from lactating mares: effect of body condition at foaling. *Journal of Dairy Research,* 60**,** 457-466.

DOYEN, V., LEDUC, V., CORAZZA, F., MAIRESSE, M., LEDENT, C. & MICHEL, O. 2013. *Protein contact dermatitis and food allergy to mare milk*.

EL-KHOLY, A. M., EL-SHINAWY, S. H., MESHREF, A. M. S. & KORNY, A. M. 2014. Screening of Antagonistic Activity of Probiotic Bacteria against Some Food-Borne Pathogens. *Journal of Applied & Environmental Microbiology,* 2**,** 53-60.

EL-SALAM, M. H. A. & EL-SHIBINY, S. 2013. Bioactive Peptides of Buffalo, Camel, Goat, Sheep, Mare, and Yak Milks and Milk Products. *Food Reviews International,* 29**,** 1-23.

EROL, A., TIRYAKI, S. & ERGUN, O. 2018. A simplified cell culture model for research on intestinal inflammation. *Turk J Med Sci,* 48**,** 435-440.

FANTA, C. & EBNER, C. 1998. Allergy to mare's milk. *Allergy,* 53**,** 539-540.

FLATT, P., BAILEY, C. & GREEN, B. 2008. *Dipeptidyl peptidase IV (DPP IV) and related molecules in type 2 diabetes*.

FOSGERAU, K. & HOFFMANN, T. 2015. Peptide therapeutics: current status and future directions. *Drug Discovery Today,* 20**,** 122-128.

FOTSCHKI, J., SZYC, A. & WRÓBLEWSKA, B. 2014. *Immunoreactivity of lactic acid-treated mare's milk after simulated digestion*.

FOTSCHKI, J., SZYC, A. M., LAPARRA, J. M., MARKIEWICZ, L. H. & WRÓBLEWSKA, B. 2016. Immune-modulating properties of horse milk administered to mice sensitized to cow milk. *Journal of Dairy Science,* 99**,** 9395-9404.

FOX, P. F. & KELLY, A. 2006. *Indigenous Enzymes in Milk: Overview and Historical Aspects - Part 2*.

FRESHNEY, R. I. 2005. Culture of animal cells : [a manual of basic technique].

FREY, M. R., GOLOVIN, A. & POLK, D. B. 2004. Epidermal growth factor-stimulated intestinal epithelial cell migration requires Src family kinase-dependent p38 MAPK signaling. *J Biol Chem,* 279**,** 44513-21.

GALL, H., KALVERAM, C. M., SICK, H. & STERRY, W. 1996. Allergy to the heat-labile proteins alpha-lactalbumin and beta-lactoglobulin in mare's milk. *J Allergy Clin Immunol,* 97**,** 1304-7.

GAVRIC, D. 2014. *Mare milk in cosmetics preliminary tests* [Online]. Available: http://old.eaap.org/Previous_Annual_Meetings/2014Copenhagen/Papers/Published/S32_08.pdf [Accessed 15.05 2019].

GCP, I. *Clinical trials using sublimated mare milk* [Online]. Available: https://ichgcp.net/clinical-trials-registry/research/list?term=sublimated+mare%27s+milk [Accessed 17.05. 2019].

GIGUERE, S. & POLKES, A. C. 2005. Immunologic disorders in neonatal foals. *Vet Clin North Am Equine Pract,* 21**,** 241-72, v.

GRISHINA, N. I. 2018. *Some aspects of a prospective use of mare's milk for prevention of age-associated pathology*.

HAEGGSTRÖM, J. Z., RINALDO-MATTHIS, A., WHEELOCK, C. E. & WETTERHOLM, A. 2010. Advances in eicosanoid research, novel therapeutic implications. *Biochemical and Biophysical Research Communications,* 396**,** 135-139.

HEINE, R. G., ALREFAEE, F., BACHINA, P., DE LEON, J. C., GENG, L., GONG, S., MADRAZO, J. A., NGAMPHAIBOON, J., ONG, C. & ROGACION, J. M. 2017. Lactose intolerance and gastrointestinal cow's milk allergy in infants and children - common misconceptions revisited. *The World Allergy Organization journal,* 10**,** 41-41.

HORVATH, P., AULNER, N., BICKLE, M., DAVIES, A. M., NERY, E. D., EBNER, D., MONTOYA, M. C., ÖSTLING, P., PIETIÄINEN, V., PRICE, L. S., SHORTE, S. L., TURCATTI, G., VON SCHANTZ, C. & CARRAGHER, N. O. 2016. Screening out irrelevant cell-based models of disease. *Nature Reviews Drug Discovery,* 15**,** 751.

HOST, A. 2002. Frequency of cow's milk allergy in childhood. *Ann Allergy Asthma Immunol,* 89**,** 33-7.

HUI, Y. H. 2006. Handbook of food science, technology, and engineering.

HURLEY, W. L. 2009. *Milk Composition and Synthesis*

Resource Library [Online]. Available: http://ansci.illinois.edu/static/ansc438/Milkcompsynth/milkcompsynthresources.html [Accessed 15.05 2019].

HURLEY, W. L. & THEIL, P. K. 2011. Perspectives on immunoglobulins in colostrum and milk. *Nutrients,* 3**,** 442-474.

HUSVÉTH, F. 2011. PHYSIOLOGICAL and REPRODUCTIONAL ASPECTS OF ANIMAL PRODUCTION.

IBRAHIM, H. R., ISONO, H. & MIYATA, T. 2018. Potential antioxidant bioactive peptides from camel milk proteins. *Animal nutrition (Zhongguo xu mu shou yi xue hui),* 4**,** 273-280.

INGLINGSTAD, R. A., DEVOLD, T. G., ERIKSEN, E. K., HOLM, H., JACOBSEN, M., LILAND, K. H., RUKKE, E. O. & VEGARUD, G. E. 2010. Comparison of the digestion of caseins and whey proteins in equine, bovine, caprine and human milks by human gastrointestinal enzymes. *Dairy Science & Technology,* 90**,** 549-563.

ISHII, S., HOSHINO, B., KOMIYAMA, H., UEHARA, A. & NURTAZIN, S. 2014. *Study on Production and Properties of Kumiss of Herders in Mongolian Dry Steppe*.

KÄMPFER, A. A. M., URBÁN, P., GIORIA, S., KANASE, N., STONE, V. & KINSNER-OVASKAINEN, A. 2017. Development of an in vitro co-culture model to mimic the human intestine in healthy and diseased state. *Toxicology in vitro : an international journal published in association with BIBRA,* 45**,** 31-43.

KARAV, S., LEITE NOBREGA DE MOURA BELL, J., LE PARC, A., LIU, Y., MILLS, D., BLOCK, D. & BARILE, D. 2015. *Characterizing the release of bioactive N- glycans from dairy products by a novel endo- β - N-acetylglucosaminidase*.

KARAV, S., SALCEDO, J., FRESE, S. A. & BARILE, D. 2018. Thoroughbred mare's milk exhibits a unique and diverse free oligosaccharide profile. *FEBS open bio,* 8**,** 1219-1229.

KOCH, S., CAPALDO, C. T., SAMARIN, S., NAVA, P., NEUMAIER, I., SKERRA, A., SACKS, D. B., PARKOS, C. A. & NUSRAT, A. 2009. Dkk-1 Inhibits Intestinal Epithelial Cell Migration by Attenuating Directional Polarization of Leading Edge Cells. *Molecular Biology of the Cell,* 20**,** 4816-4825.

KOKE, K., MCGRATH, S. & COLE, K. 2017. Immunoglobulin concentrations in pre- and post-foaling mammary secretions. *Journal of Equine Veterinary Science,* 52**,** 59-60.

KUSUMANINGTYAS, E., WIDIASTUTI, R., KUSUMANINGRUM, H. D. & SUHARTONO, M. T. 2018. Bioactivities and analysis of peptides from Sumbawa horse milk generated by Bacillus thuringiensis protease. *Jurnal Ilmu Ternak dan Veteriner; Vol 21, No 4 (2016): DECEMBER 2016*.

LANGERHOLC, T., MARAGKOUDAKIS, P. A., WOLLGAST, J., GRADISNIK, L. & CENCIC, A. 2011. Novel and established intestinal cell line models – An indispensable tool in food science and nutrition. *Trends in Food Science & Technology,* 22**,** S11-S20.

LI, Y., NGUYEN, D. N., OBELITZ-RYOM, K., ANDERSEN, A. D., THYMANN, T., CHATTERTON, D. E. W., PURUP, S., HECKMANN, A. B., BERING, S. B. & SANGILD, P. T. 2018. Bioactive Whey Protein Concentrate and Lactose Stimulate Gut Function in Formula-fed Preterm Pigs. *Journal of Pediatric Gastroenterology and Nutrition,* 66**,** 128-134.

LÖNNERDAL, B. 2003. Nutritional and physiologic significance of human milk proteins. *The American Journal of Clinical Nutrition,* 77**,** 1537S-1543S.

LOUGHRILL, E., WRAY, D., CHRISTIDES, T. & ZAND, N. 2017. Calcium to phosphorus ratio, essential elements and vitamin D content of infant foods in the UK: Possible implications for bone health. *Maternal & Child Nutrition,* 13**,** e12368.

LUCARINI, M. 2017. Bioactive Peptides in Milk: From Encrypted Sequences to Nutraceutical Aspects. *Beverages,* 3**,** 41.

MACDONALD, C. E., JOHNSON, W. S. & PLAYFORD, R. J. 2000. Colostrum and milk-derived peptide growth factors for the treatment of gastrointestinal disorders. *The American Journal of Clinical Nutrition,* 72**,** 5-14.

MALACARNE, M., MARTUZZI, F., SUMMER, A. & MARIANI, P. 2002a. Protein and fat composition of mare's milk: some nutritional remarks with reference to human and cow's milk. *International Dairy Journal,* 12**,** 869-877.

MALACARNE, M., MARTUZZI, F., SUMMER, A. & MARIANI, P. 2002b. *Protein and fat composition of mare's milk: Some nutritional remarks with reference to human and cow's milk*.

MARIELLA, J., CASTAGNETTI, C., PROSPERI, A., SCAGLIARINI, A. & PELI, A. 2017. Cytokine levels in colostrum and in foals' serum pre- and post-suckling. *Veterinary Immunology and Immunopathology,* 185**,** 34-37.

MARJANOVIC, G. & HOPT, U. T. 2011. [Physiology of anastomotic healing]. *Chirurg,* 82**,** 41-7.

MAZHITOVA, A. T. & KULMYRZAEV, A. A. 2016. Determination of amino acid profile of mare milk produced in the highlands of the Kyrgyz Republic during the milking season. *Journal of Dairy Science,* 99**,** 2480-2487.

MAZHITOVA, A. T., KULMYRZAEV, A. A., OZBEKOVA, Z. E. & BODOSHEV, A. 2015. Amino Acid and Fatty Acid Profile of the Mare's Milk Produced on Suusamyr Pastures of the Kyrgyz Republic During Lactation Period. *Procedia - Social and Behavioral Sciences,* 195**,** 2683-2688.

MCKEVITH, B. & SHORTT, C. 2003. FERMENTED MILKS | Other Relevant Products. *In:* CABALLERO, B. (ed.) *Encyclopedia of Food Sciences and Nutrition (Second Edition).* Oxford: Academic Press.

MENDEL, C. M. 1992. The free hormone hypothesis. Distinction from the free hormone transport hypothesis. *J Androl,* 13**,** 107-16.

MILLS, S., STANTON, C., HILL, C. & ROSS, R. P. 2011. New Developments and Applications of Bacteriocins and Peptides in Foods. *Annual Review of Food Science and Technology,* 2**,** 299-329.

MOREL, M. C. G. D. 2015. *Equine Reproductive Physiology, Breeding and Stud Management, 4th Edition*, CABI.

MURRAY, M. J., SCHAUDIES, R. P. & CAVEY, D. M. 1992. Epidermal growth factor-like activity in mares' milk. *American journal of veterinary research,* 53**,** 1729-31.

NEURATH, M. F. & TRAVIS, S. P. L. 2012. Mucosal healing in inflammatory bowel diseases: a systematic review. *Gut,* 61**,** 1619-1635.

NIELSEN, S., BEVERLY, R., QU, Y. & DALLAS, D. 2017a. *Milk bioactive peptide database: A comprehensive database of milk protein-derived bioactive peptides and novel visualization*.

NIELSEN, S. D., BEVERLY, R. L., QU, Y. & DALLAS, D. C. 2017b. Milk bioactive peptide database: A comprehensive database of milk protein-derived bioactive peptides and novel visualization. *Food Chem,* 232**,** 673-682.

NONGONIERMA, A. B. & FITZGERALD, R. J. 2018. Enhancing bioactive peptide release and identification using targeted enzymatic hydrolysis of milk proteins. *Anal Bioanal Chem,* 410**,** 3407-3423.

NURTAZIN, S., ISHII, S. & HOSHINO, B. 2016. *Mare's milk and kumys*.

OLIVRY, T., MARSELLA, R. & HILLIER, A. 2001. The ACVD task force on canine atopic dermatitis (XXIII): are essential fatty acids effective? *Veterinary Immunology and Immunopathology,* 81**,** 347-362.

ORLANDI, M., GORACCI, J. & CLAUDIA CURADI, M. 2002. *Essential fatty acids (EFA) in Haflinger and Thoroughbred mare's milk*.

ORLANDI, M., GORACCI, J. & CLAUDIA CURADI, M. 2003. *Fat composition of mare's milk with reference to human nutrition*.

OUTRAM, A. K., STEAR, N. A., BENDREY, R., OLSEN, S., KASPAROV, A., ZAIBERT, V., THORPE, N. & EVERSHED, R. P. 2009. The Earliest Horse Harnessing and Milking. *Science,* 323**,** 1332-1335.

PARK, Y. 2009. *Bioactive Components in Milk and Dairy Products*.

PARK, Y. W., HAENLEIN, G. F. W. & WENDORFF, W. L. 2017. Handbook of milk of non-bovine mammals.

PERKINS, G. A., GOODMAN, L. B., WIMER, C., FREER, H., BABASYAN, S. & WAGNER, B. 2014. Maternal T-lymphocytes in equine colostrum express a primarily inflammatory phenotype. *Vet Immunol Immunopathol,* 161**,** 141-50.

PETERS, L. A., PERRIGOUE, J., MORTHA, A., IUGA, A., SONG, W.-M., NEIMAN, E. M., LLEWELLYN, S. R., DI NARZO, A., KIDD, B. A., TELESCO, S. E., ZHAO, Y., STOJMIROVIC, A., SENDECKI, J., SHAMEER, K., MIOTTO, R., LOSIC, B., SHAH, H., LEE, E., WANG, M., FAITH, J. J., KASARSKIS, A., BRODMERKEL, C., CURRAN, M., DAS, A., FRIEDMAN, J. R., FUKUI, Y., HUMPHREY, M. B., IRITANI, B. M., SIBINGA, N., TARRANT, T. K., ARGMANN, C., HAO, K., ROUSSOS, P., ZHU, J., ZHANG, B., DOBRIN, R., MAYER, L. F. & SCHADT, E. E. 2017. A functional genomics predictive network model identifies regulators of inflammatory bowel disease. *Nature genetics,* 49**,** 1437-1449.

PIESZKA, M., ŁUSZCZYŃSKI, J., ZAMACHOWSKA, M., AUGUSTYN, R., DŁUGOSZ, B. & HĘDRZAK, M. 2016. Is mare milk an appropriate food for people? – a review. 16**,** 33.

POTOČNIK, K., GANTNER, V., KUTEROVAC, K. & ANGELA, C. 2011. *Mare's milk: composition and protein fraction in comparison with different milk species*.

PURUP, S., NIELSEN, S., LE, T., BERTELSEN, H., SØRENSEN, J. & LARSEN, L. 2018. *Wound Healing Properties of Commercial Milk Hydrolysates in Intestinal Cells*.

PURUP, S. & NIELSEN, T. S. 2011. Cell-based models to test the effects of milk-derived bioactives. *animal,* 6**,** 423-432.

PURUP, S., VESTERGAARD, M., O PEDERSEN, L. & SEJRSEN, K. 2006. Biological activity of bovine milk on proliferation of human intestinal cells. *Journal of Dairy Research,* 74**,** 58-65.

PURUP, S., VESTERGAARD, M. & SEJRSEN, K. 2002. Involvement of Growth Factors in the Regulation of Pubertal Mammary Growth in Cattle. *In:* MOL, J. A. & CLEGG, R. A. (eds.) *Biology of the Mammary Gland.* Boston, MA: Springer US.

QUARONI, A., WANDS, J., TRELSTAD, R. L. & ISSELBACHER, K. J. 1979. Epithelioid cell cultures from rat small intestine. Characterization by morphologic and immunologic criteria. *The Journal of cell biology,* 80**,** 248-265.

RAFIEE TARI, N., ARRANZ, E. & CORREDIG, M. 2019. Effect of protein composition of a model dairy matrix containing various levels of beta-casein on the structure and anti-inflammatory activity of in vitro digestates. *Food & Function*.

RAHMAT, A. 2006. *Comparative evaluation of cytotoxic effects of milk from various species on leukemia cell lines*, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia.

RAIDAL, S., RURAL INDUSTRIES, R. & DEVELOPMENT, C. 2000. *Immunity in foals : a report for the Rural Industries Research and Development Corporation,* Barton, A.C.T., Rural Industries Research & Development Corp.

RANGEL, A. H. D. N., SALES, D. C., URBANO, S. A., GALVÃO JÚNIOR, J. G. B., ANDRADE NETO, J. C. D. & MACÊDO, C. D. S. 2016. Lactose intolerance and cow's milk protein allergy. *Food Science and Technology,* 36**,** 179-187.

RIJCKEN, E., SACHS, L., FUCHS, T., SPIEGEL, H.-U. & NEUMANN, P.-A. 2014. Growth factors and gastrointestinal anastomotic healing. *Journal of Surgical Research,* 187**,** 202-210.

RONG, J., SHAN, C., LIU, S., ZHENG, H., LIU, C., LIU, M., JIN, F. & WANG, L. 2017. Skin resistance to UVB-induced oxidative stress and hyperpigmentation by the topical use of Lactobacillus helveticus NS8-fermented milk supernatant. *Journal of Applied Microbiology,* 123**,** 511-523.

SAID, H. M. 2018. Growth factors in the intestinal tract chapter 3. *In:* SAID, H. M. (ed.) *Physiology of the Gastrointestinal Tract (Sixth Edition).* the 6th ed.: Academic Press.

SALIMEI, E. & FANTUZ, F. 2012. *Equid Milk for human consumption*.

SAMES, L., WEIMANN, C. & ERHARDT, G. 2018. *Mare's milk as a source of ACE-inhibitory peptides for human nutrition*.

SANTOS, K. S., STEPHANO, M. A., MARCELINO, J. R., FERREIRA, V. M. R., ROCHA, T., CARICATI, C., HIGASHI, H. G., MORO, A. M., KALIL, J. E., MALASPINA, O., CASTRO, F. F. M. & PALMA, M. S. 2013. Production of the First Effective Hyperimmune Equine Serum Antivenom against Africanized Bees. *PLOS ONE,* 8**,** e79971.

SCHANBACHER, F. L., TALHOUK, R. S., MURRAY, F. A., GHERMAN, L. I. & WILLETT, L. B. 1998. Milk-Borne Bioactive Peptides. *International Dairy Journal,* 8**,** 393-403.

SCHUBERT, R., KAHLE, C., KAUF, E., HOFMANN, J., HOBERT, I. & GRUHN, B. 2009. *Dietetic efficacy of mare's milk for patients with chronic inflammatory bowel diseases - clinical study*.

SEJRSEN, K., PEDERSEN, L. O., VESTERGAARD, M. & PURUP, S. 2001. Biological activity of bovine milk: Contribution of IGF-I and IGF binding proteins. *Livestock Production Science,* 70**,** 79-85.

SHARIATIKIA, M., BEHBAHANI, M. & MOHABATKAR, H. 2017. *Anticancer activity of cow, sheep, goat, mare, donkey and camel milks and their caseins and whey proteins and in silico comparison of the caseins*.

SHARIFI-RAD, J., HOSEINI-ALFATEMI, S., SHARIFI-RAD, M., SHARIFI-RAD, M., IRITI, M., SHARIFI-RAD, M., SHARIFI-RAD, R. & RAEISI, S. 2015. Phytochemical Compositions and Biological Activities of Essential Oil from Xanthium strumarium L. *Molecules Molecules,* 20**,** 7034-7047.

SMITH, H. S., SPRINGER, E. L. & HACKETT, A. J. 1979. Nuclear ultrastructure of epithelial cell lines derived from human carcinomas and nonmalignant tissues. *Cancer Res,* 39**,** 332-44.

SPECIALIST, A. N. T. E. 2019. *The Human Digestive Tract pH Range Diagram* [Online]. Available: https://www.alleganynutrition.com/supporting-pages/the-human-digestive-tract-ph-range-diagram/ [Accessed].

STURM, A. & DIGNASS, A. U. 2008. Epithelial restitution and wound healing in inflammatory bowel disease. *World journal of gastroenterology,* 14**,** 348-353.

TAKEDA, T., SAKATA, M., MINEKAWA, R., YAMAMOTO, T., HAYASHI, M., TASAKA, K. & MURATA, Y. 2004. *Human milk induces fetal small intestinal cell proliferation – involvement of a different tyrosine kinase signaling pathway from epidermal growth factor receptor*.

THERMOFISHER. Available: https://www.thermofisher.com/dk/en/home.html [Accessed 27.05 2019].

TONOLO, F., SANDRE, M., FERRO, S., FOLDA, A., SCALCON, V., SCUTARI, G., FELLER, E., MARIN, O., BINDOLI, A. & RIGOBELLO, M. P. 2018. Milk-derived bioactive peptides protect against oxidative stress in a Caco-2 cell model. *Food & Function,* 9**,** 1245-1253.

UNIACKE-LOWE, T. 2011. *Studies on Equine Milk and Comparative Studies on Equine and Bovine Milk Systems.*

UNIACKE-LOWE, T., HUPPERTZ, T. & F. FOX, P. 2010. *Equine milk proteins: Chemistry, structure and nutritional significance*.

V. YAKUNIN, A., A. SINYAVSKIY, Y. & S. IBRAIMOV, Y. 2017. *ASSESSMENT OF THE NUTRITIONAL VALUE OF MARE'S MILK AND FERMENTED MARE'S MILK PRODUCTS AND THE POSSIBILITY OF THEIR USE IN BABY FOOD*.

VERHULST, L., KERRE, S. & GOOSSENS, A. 2016. The unsuspected power of mare's milk. *Contact Dermatitis,* 74**,** 376-377.

VILAR, S. & COSTANZI, S. 2012. Predicting the biological activities through QSAR analysis and docking-based scoring. *Methods in molecular biology (Clifton, N.J.),* 914**,** 271-284.

VINCENZETTI, S., POLIDORI, P. & VITA, A. 2007. NUTRITIONAL CHARACTERISTICS OF DONKEY'S MILK PROTEIN FRACTION.

WANG, F. 2006. Culture of animal cells: A manual of basic technique, fifth edition. *In Vitro Cellular & Developmental Biology - Animal,* 42**,** 169-169.

WU, G. 2018. *Principles of animal nutrition*.

YAKUNIN, A. V., SINYAVSKIY, Y. A., TORGAUTOV, A. S., VYSKUBOVA, V. G. & IBRAIMOV, Y. S. 2016. Assessment of oxidation factors for dry mare milk during storage. *Food Processing: Techniques and Technology,* 43**,** 96-101.

ZHANG, J., YANG, Y., YANG, H., BU, Y., YI, H., ZHANG, L., HAN, X. & AI, L. 2018. Purification and Partial Characterization of Bacteriocin Lac-B23, a Novel Bacteriocin Production by Lactobacillus plantarum J23, Isolated From Chinese Traditional Fermented Milk. *Frontiers in microbiology,* 9**,** 2165-2165.

ZHENG, X., WONG, G., ZHAO, Y., WANG, H., HE, S., BI, Y., CHEN, W., JIN, H., GAI, W., CHU, D., CAO, Z., WANG, C., FAN, Q., CHI, H., GAO, Y., WANG, T., FENG, N., YAN, F., HUANG, G., ZHENG, Y., LI, N., LI, Y., QIAN, J., ZOU, Y., KOBINGER, G., GAO, G. F., QIU, X., YANG, S. & XIA, X. 2016. Treatment with hyperimmune equine immunoglobulin or immunoglobulin fragments completely protects rodents from Ebola virus infection. *Scientific reports,* 6**,** 24179-24179.

ZIVKOVIC, A. M. & BARILE, D. 2011. Bovine milk as a source of functional oligosaccharides for improving human health. *Advances in nutrition (Bethesda, Md.),* 2**,** 284-289.

12. Appendix

Micrographs:

Figure 27

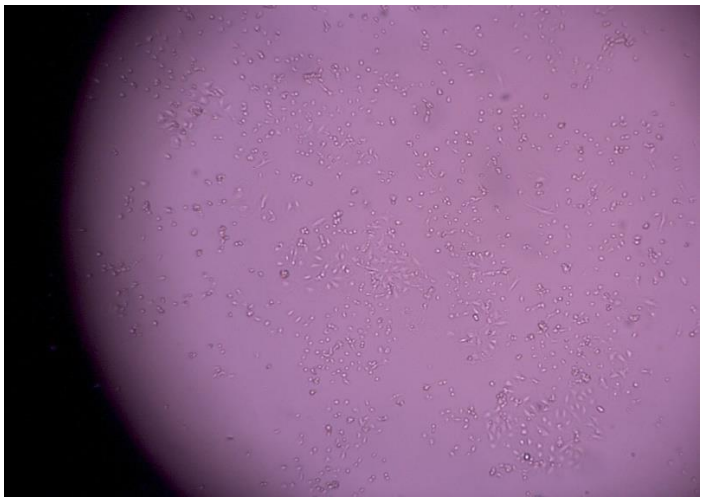


Figure 27- 1 IEC-6 cells culture monolayer treated for 72 hours with 0.05% whey of colostrum from sample 1 H1 - 0.05%, magnification 40x

Figure 28

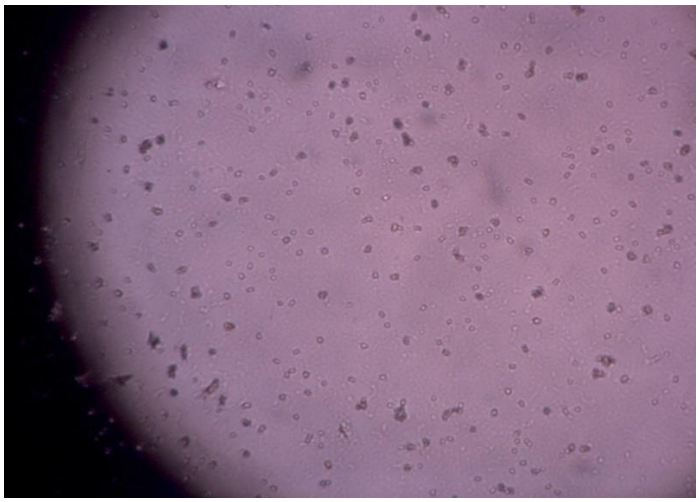


Figure 28 IEC-6 cells culture monolayer treated for 72 hours with 10% whey of colostrum from sample 1 H1 - 10%, magnification 40x

Figure 29

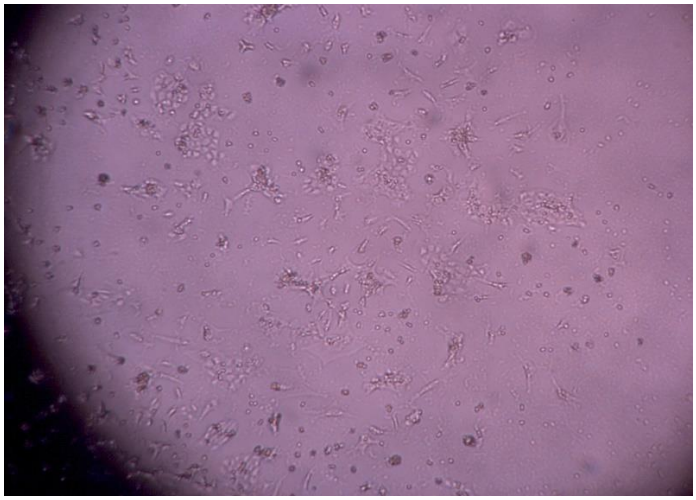


Figure 30

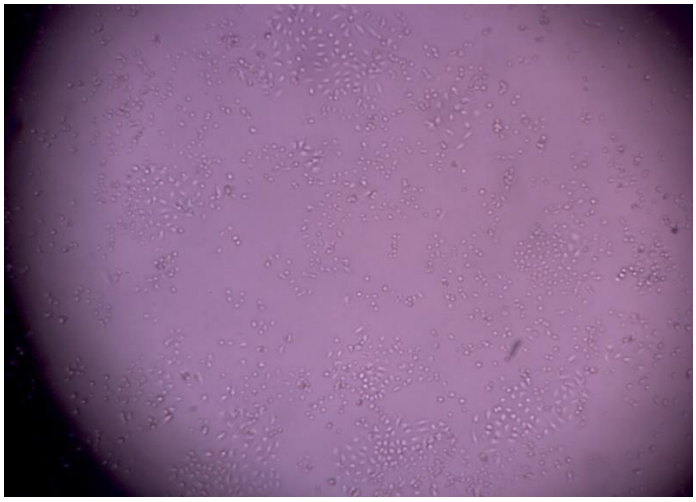


Figure 29 IEC-6 cells culture monolayer treated for 72 hours with 5% whey of colostrum from sample 1 H1 - 5%, magnification 40x

Figure 30 IEC-6 cells culture monolayer treated for 72 hours with 0.05% whey of colostrum from sample 2 H2 - 0.05%, magnification 40x

Figure 31

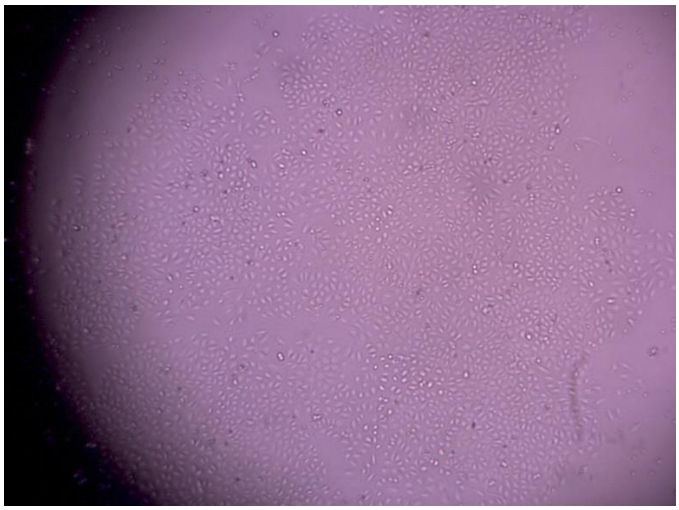


Figure 32

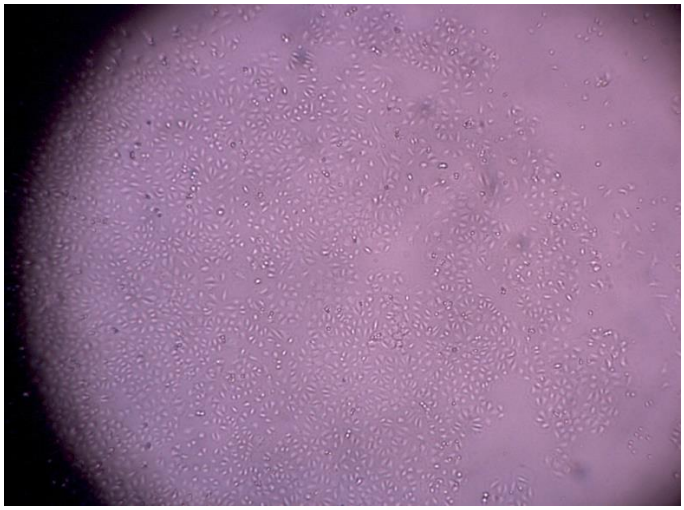
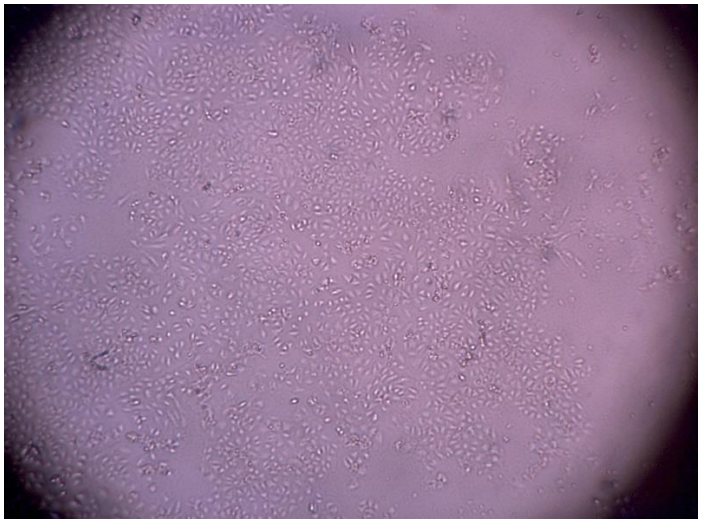


Figure 31 IEC-6 cells culture monolayer treated for 72 hours with 2.5% whey of colostrum from sample 2 H2 – 2.5%, magnification 40x

Figure 32 IEC-6 cells culture monolayer treated for 72 hours with 5% whey of colostrum from sample 2 H2 - 5%, magnification 40x

Figure 33 *Figure 34*



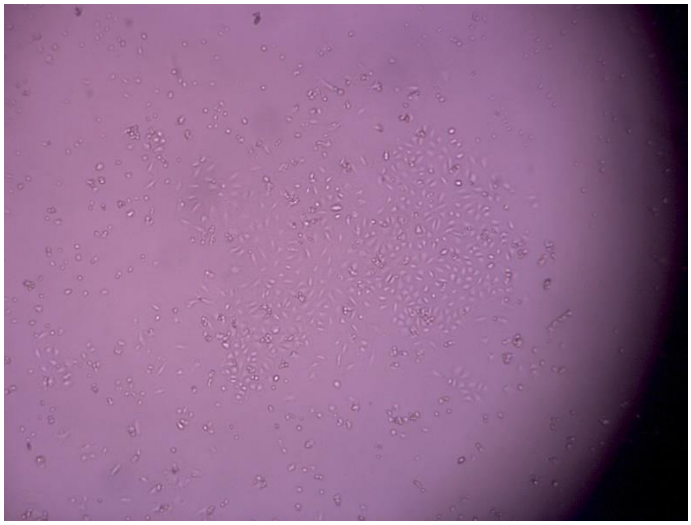


Figure 33 IEC-6 cells culture monolayer treated for 72 hours with 10% whey of colostrum from sample 2 H2 - 10%, magnification 40x

Figure 34 IEC-6 cells culture monolayer treated for 72 hours with 0.05% whey of colostrum from sample 3 H3 – 0.05%, magnification 40x

Figure 35

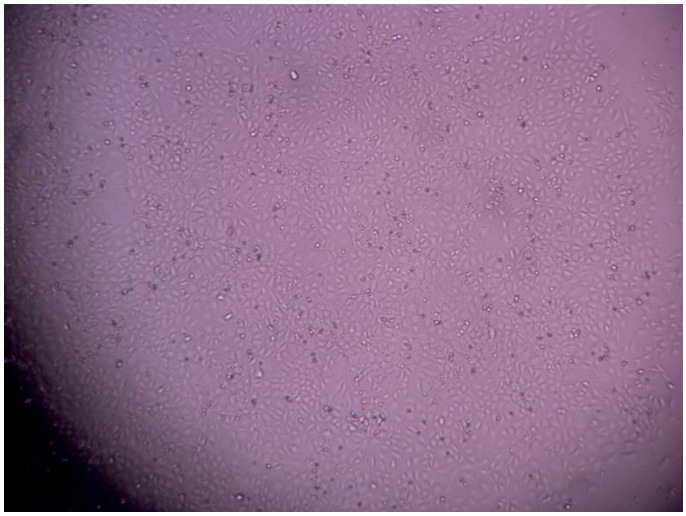


Figure 35 IEC-6 cells culture monolayer treated for 72 hours with 2.5% whey of colostrum from sample 3 H3 – 2.5%, magnification 40x

Figure 36

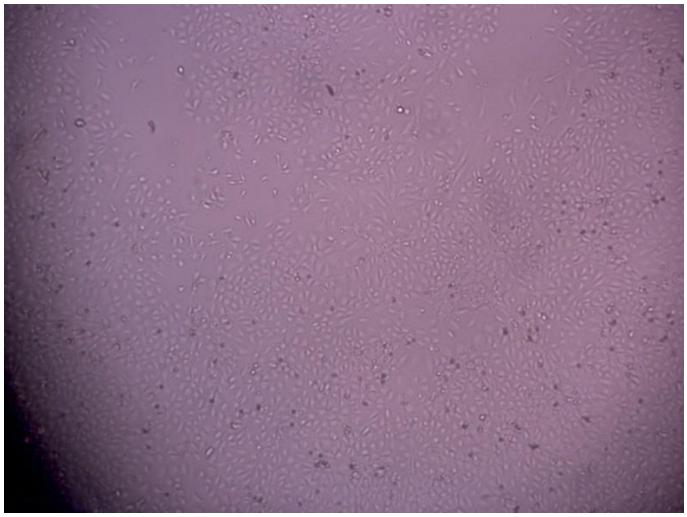


Figure 36 IEC-6 cells culture monolayer treated for 72 hours with 5% whey of colostrum from sample 3 H3 – 5%, magnification 40x

Figure 37

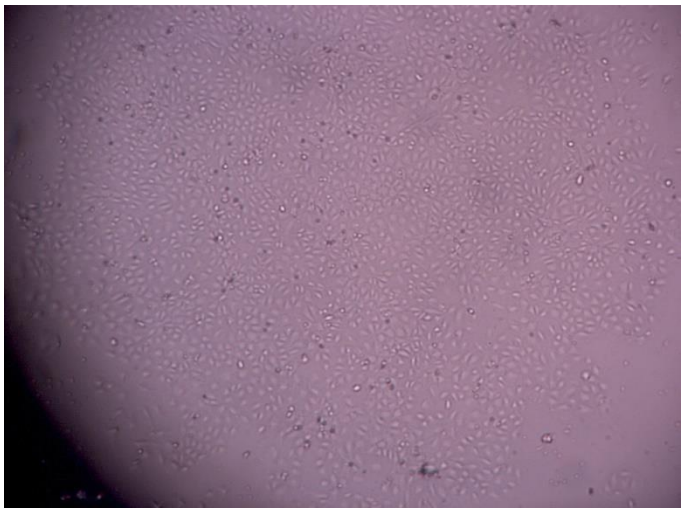


Figure 38

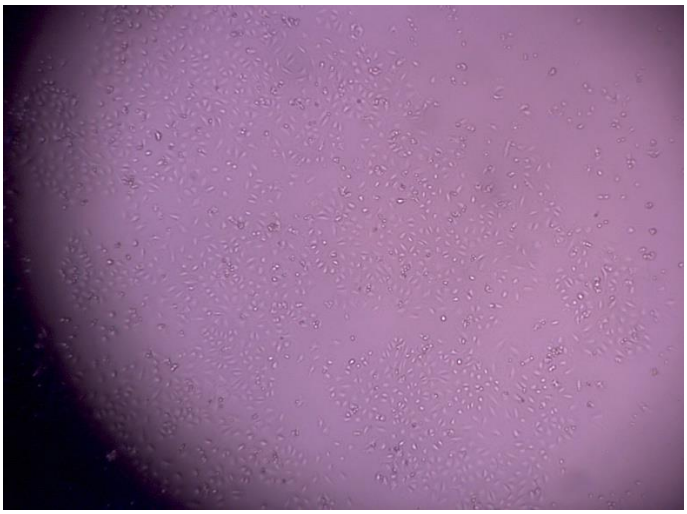


Figure 37 IEC-6 cells culture monolayer treated for 72 hours with 10% whey of colostrum from sample 3 H3 – 10%, magnification 40x

Figure 38 IEC-6 cells culture monolayer treated for 72 hours with 0.05% whey of colostrum from sample 4 H4 – 0.05%, magnification 40x

Figure 39

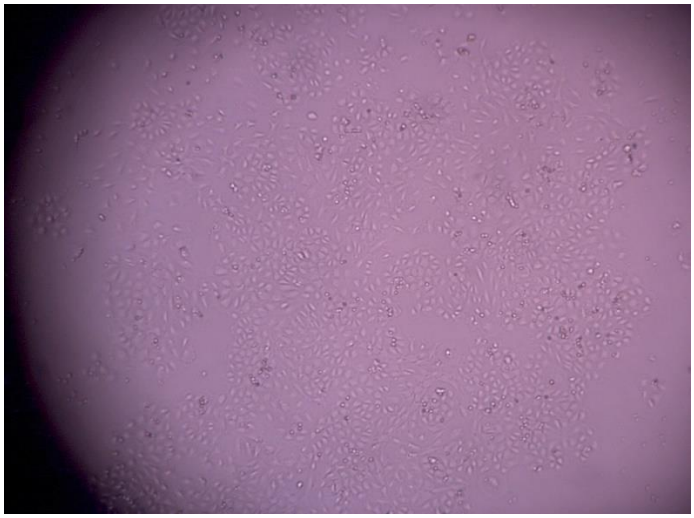


Figure 40

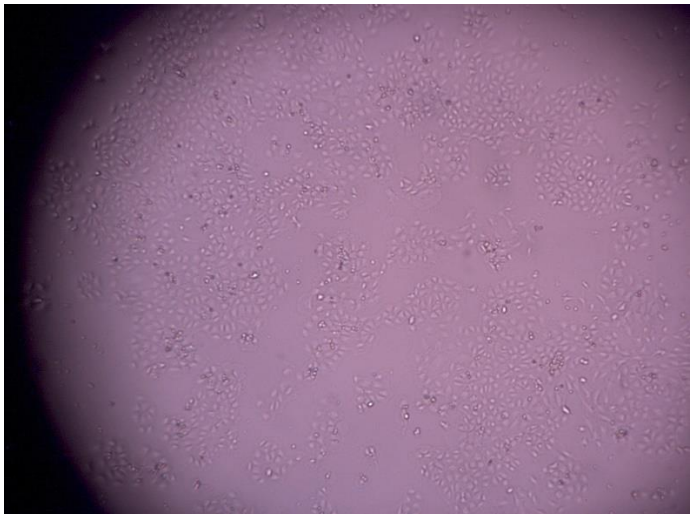


Figure 39 IEC-6 cells culture monolayer treated for 72 hours with 2.5% whey of colostrum from sample 4 H4 – 2.5%, magnification 40x

Figure 40 IEC-6 cells culture monolayer treated for 72 hours with 5% whey of colostrum from sample 4 H4 – 5%, magnification 40x

Figure 41

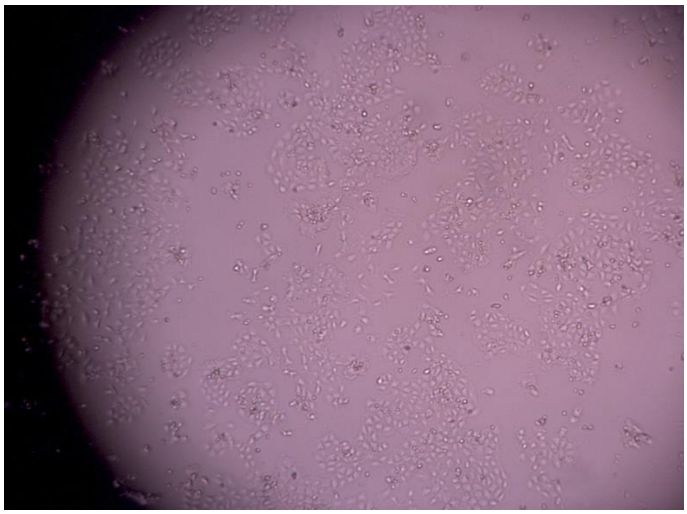


Figure 41 IEC-6 cells culture monolayer treated for 72 hours with 10% whey of colostrum from sample 4 H4 – 10%, magnification 40x

Figure 42

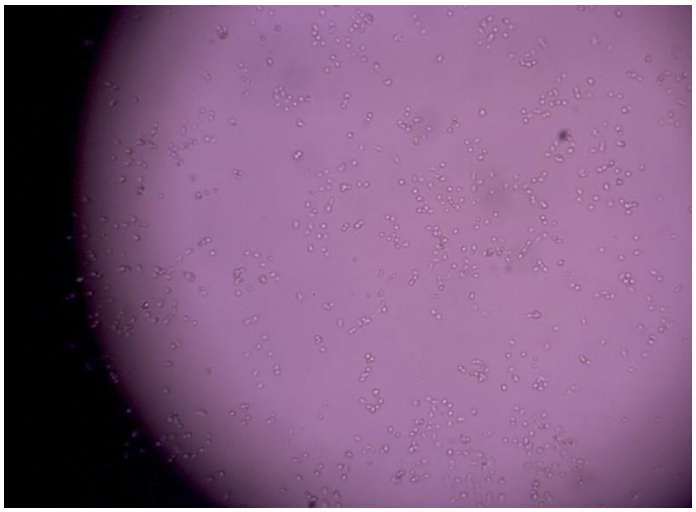


Figure 42 IEC-6 cells culture monolayer treated for 72 hours with 0.05% whey of colostrum from sample 5 H5 – 0.05%, magnification 40x

Figure 43

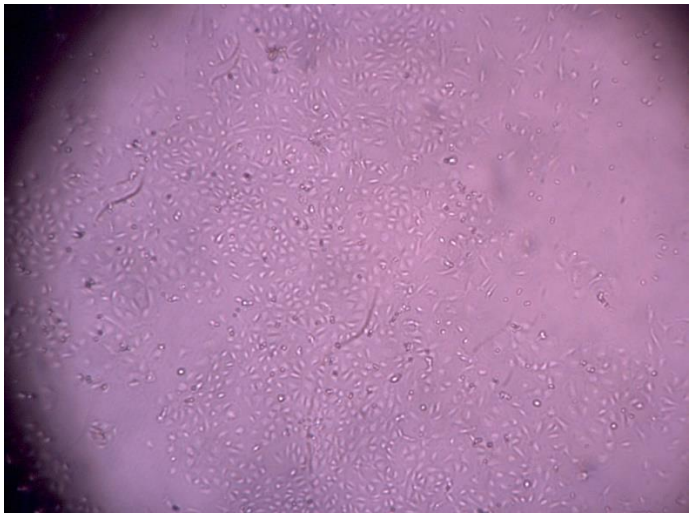


Figure 44

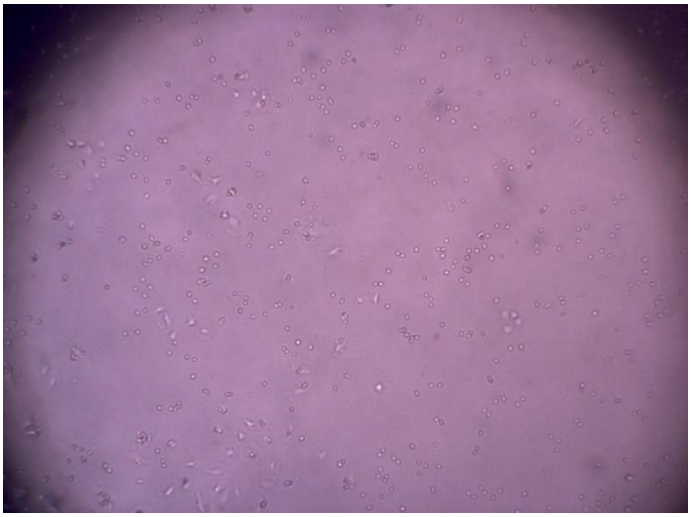


Figure 43 IEC-6 cells culture monolayer treated for 72 hours with 2.5% whey of colostrum from sample 5 H5 – 2.5%, magnification 40x

Figure 44 IEC-6 cells culture monolayer treated for 72 hours with 5% whey of colostrum from sample 5 H5 – 5%, magnification 40x

Figure 45

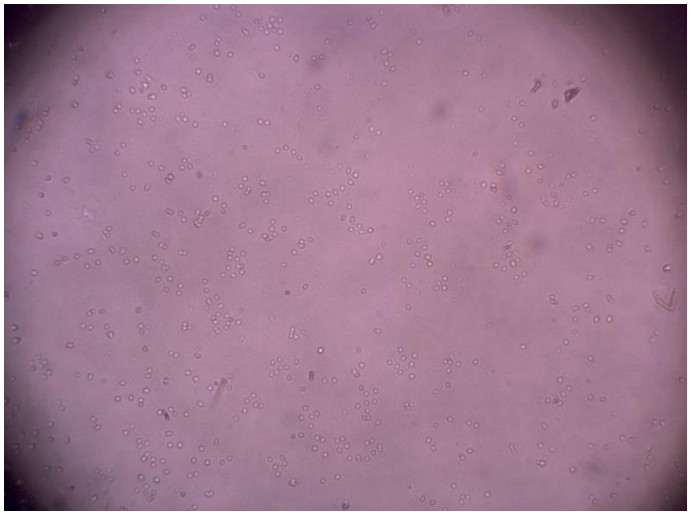


Figure 45 IEC-6 cells culture monolayer treated for 72 hours with 10% whey of colostrum from sample 5 H5 – 10%, magnification 40x

Figure 46 *Figure 47*

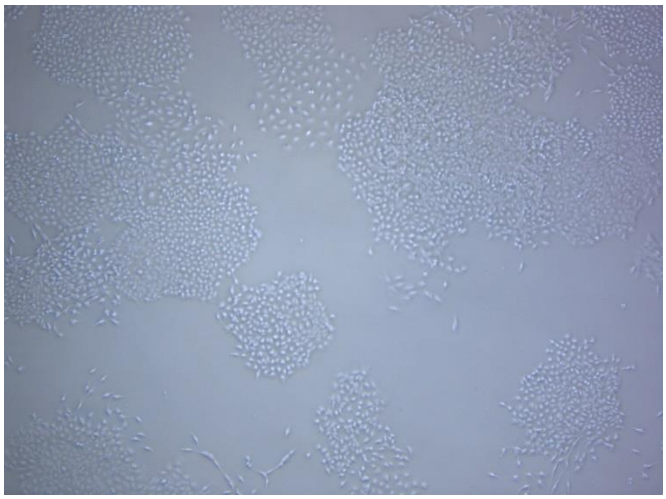


Figure 46 FHs 74Int cells (human small intestinal cells) culture monolayer 60% confluency magnification 40x

Figure 47 IEC-6 cells (rat small intestinal cells) culture monolayer 60% confluency magnification 40x

Sample id/ Horse no.	Age (yrs.)	Milking time	Lactation day – 1st sampling	Lactation day - 4th sampling	Lactation no.	Maximal activity (relative values)	Conc.with maximal activity (%)	P value		
								Time	Conc.	Time* Conc.
2	11	1	117		3	2.1	10	0.03	0.0001	0.3309
		4		179		1.9	5			
3	15	1	117		6	2.1	10	0.0001	0.0001	0.0613
		4		179		1.6	5, 10			
4	17	1	121		3	2	10	0.6251	0.0001	0.0607
		4		177		1.8				
7	4	1	122		1	1.4	2.5, 5, 10	0.0001	0.0001	0.5204
		4		178		1.7	10			
5	10	1	131		5	1.6	1, 2.5, 5, 10	0.8221	0.0001	0.0082
		4		190		1.3	10			
1	15	1	152		6	2.2	10	0.0374	0.0001	0.0216
		4		209		2				

Table 24 Relative values of IEC-6 cells viability under the treatment (72 hours) with different concentration of whey of mare milk for 2 times milk sampled points (1 and 4)

Sample id/ Horse no.	Age (yrs.)	Milking time	Lactation day – 1st sampling	Lactation day - 4th sampling	Lactation no.	Maximal activity (relative values)	Conc.with maximal activity (%)	P value		
								Time	Conc.	Time* Conc.
2	11	1	117		3	3.5	5	0.03	0.0001	0.1849
		4		179		2.8	10			
3	15	1	117		6	2.8	10	0.0001	0.0001	0.0001
		4		179		1.8	5, 10			
4	17	1	121		3	2.5	10	0.5434	0.0001	0.0199
		4		177		3				
7	4	1	122		1	3.1	10	0.0001	0.0001	0.5204
		4		178		2.4				
5	10	1	131		5	2.5	10	0.8489	0.0001	0.8083
		4		190		2.7				
1	15	1	152		6	2.6	10	0.1477	0.0001	0.9618
		4		209		2.7				

Table 25 Relative values of FHs 74 Int cells viability under the treatment (72 hours) with different concentration of whey of mare milk for 2 times milk sampled points (1 and 4)

Sample id/ Horse no.	Age (yrs.)	Milking time	Lactation day – 1st sampling	Lactation day - 4th sampling	Lactation no.	Maximal activity (relative values)	Conc.with maximal activity (%)	P value		
								Time	Conc.	Time* Conc.
2	11	1 4	117	179	3	1.4 1.1	10	0.2711	0.4596	0.8445
3	15	1 4	117	179	6	2.1 1.6	5 1	0.2033	0.0070	0.155
4	17	1 4	121	177	3	1.2 1.2	5	0.5977	0.7485	0.5272
7	4	1 4	122	178	1	1.7 1.4	10 5	0.2084	0.0174	0.5599
5	10	1 4	131	190	5	1.2 1.6	10	0.1743	0.3662	0.7333
1	15	1 4	152	209	6	1.4 1.5	10 5	0.8726	0.0072	0.9484

Table 26 Relative values of IEC-6 cells migration under the treatment (6 hours) with different concentration of whey of mare milk for 2 times milk sampled points (1 and 4)

Sample id/Horse no.	Age (yrs.)	Lactation no.	Maximal activity (relative values)	Conc. with maximal activity (%)	P value- - concentration
1	13	7	4.1	O.25; 0.5	0.0001
2	9	4	3.6	10	0.0001
3	9	2	3.6	10	0.0001
4	15	6	4.2	1	0.0001
5	11	6	7.3	2.5	0.0001

Table 27 Relative values of IEC-6 cell viability under the treatment (72 hours) with different concentration of 5 whey of mare colostrum

Sampleid	IGF- I ng/ml (mean)	TGFβ1 pg/ml	TGFβ2 pg/ml	EGF pg/ml
1-1	6.2	*< 30*	*< 30*	*< 25*
2-1	4.8			
3-1	7.6			
4-1	6.8			
5-1	9.7			
7-1	9.4			

Table 28 Growth factors in 6 whey of mare milk samples- first time milking

Sampleid	*IGF-I ng/ml (mean)*	*TGF β1 pg/ml*	*TGF β2 pg/ml*	*EGF pg/ml*
1-4	*10*	*<30*	*<30*	*< 25*
2-4	*7.7*			
3-4	*8*			
4-4	*7.8*			
5-4	*10.5*			
7-4	*9.6*			

Table 29 Growth factors in 6 whey of mare milk samples- fourth time milking

Sampleid	*IGF-I ng/ml (mean)*	*TGF β1 pg/ml (ng/ml)*	*TGF β2 pg/ml*	*EGF pg/ml*
H1	*60*	*2868.9 (2.8689)*	*6224 (6.224)*	*< 63*
H2	*11.4*	-	-	
H3	*105.9*	*6151 (6.151)*	*4672 (4.672)*	
H4	*66.9*	*3132.9 (3.1329)*	*2404 (2.404)*	*543*
H5	*88.2*	*2879.9 (2.8799)*	*1034 (1.034)*	*< 63*

Table 30 Growth factors in 5 whey of colostrum of mare

Mare	Foaling date	Age	Lactation no	10.09	12.09	17.09	5.11	6.11	7.11
Nutte 1.	10/04/2018	15	6	: 1-1	:1-2	:1-3	:1-4	:1-5	:1-6
Georcia 5.	03/05/2018	10	5	:1-5	:2-5	:3-5	:4-5	:5-5	:6-5
Betty 3.	18/05/2018	15	6	:3-1	:3-2	:3-3	:3-4	:3-5	:3-6
Baronessa 4.	12/05/2018	17	3	:4-1	:4-2	:4-3	:4-4	:4-5	:4-6
Fortaleza 7.	11/05/2018	4	1	:7-1	:7-2	:7-3	:7-4	:7-5	:7-6
Zoftware 2.	18/05/2018	11	3	:2-1	:2-2	:2-3	:2-4	:2-5	:2-6

1-1 -age 15, foal 10.04,6th lactation, milking 10.09	all 6 horses first milking
2-1- age 11, foal 18.05, lactation 2, milking 10.09	
3-1- age 15, foal 03.05, lactation 6, milking 10.09	
4-1- age 17, foal 12.05, lactation 3, milking 10.09	
5-1- age 10, foal 03.05, lactation 5, milking 10.09	
7-1 age 4, foal 11.05, lactation 1, milking 10.09	
1-4 age 15, foal 10.04,6th lactation, milking 5.11	all 6 horses 4th milking
2-4 age 11, foal 18.05, lactation 2, milking	
3-4 age 15, foal 03.05, lactation 6, milking 05.11	
4-4 age 17, foal 12.05, lactation 3, milking 05.11	
5-4 age 10, foal 03.05, lactation 5, milking 05.11	
7-4 age 4, foal 11.05, lactation 1, milking 05.11	

Table 31 Data about the mares – 6 mare's milk was analysed at two time point of sampling 1 and 4

Mare	Foaling date	Age	Foals no.
H1 Pietra Santa	12.04.19	13	7
H2 Kokoosint	29.03.19	9	4
H3 Whistler	03.04.19	9	2
H4 Bea apache	14.04.19	15	6
H5 Liva	09.04.19	11	6

Table 32 Data about the mares – colostrum was milked in the first 24 hours pospartum