Advances on equine milk and derivatives for human consumption

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New findings regarding mare's milk

Around 30 million people consume mare milk regularly throughout the world. It is an important foodstuff for the people of Central Asia steppes: Turks, Bashkirs, Kazakhs, Kyrygyz, Mongols, Yakuts and Uzbeks *(Zhou et al., 2009)*.

It is consumed also in Eastern Europe, in particular in Hungary.

In the last years of the 20th century, in Europe the studies about equine milk regarded especially **protein compounds: identification and characterization of caseins and whey proteins with some interest for a possible use as a substitute of cow milk** for children with intolerance or allergy.

While most studies in the world regard horse milk, **in Italy there is a particular interest regarding donkey's milk,** traditionally used in the past for orphan children, when formulas were not yet available.

In the first years of 2000, many articles by Chinese researchers, mainly from Inner Mongolia, start to appear in scientific journals presenting studies regarding koumiss, sometimes with an emphasis on probiotic aspects.

Recently, some studies regarding **clinical use of mare's milk or colostrum are appearing**

Recent studies about equine milk: main issues

- **Human nutrition and health:**
  - Identification of lactic acid bacteria and yeasts strains in **fermented mare's milk**
  - Studies about potential **probiotic properties** of koumiss for several health problems
  - Identification and characterization of **caseins, whey proteins and bioactive factors**
  - Clinical use of mare's milk
  - New production biotechnologies

- **Non-nutritional uses for human health and welfare:**
  - Cosmetology
  - Dermatology

- **Biomolecular archaeology**

- **Foal nutrition**:
  quality of colostrum (IgG – amyloid A3)

Biomolecular archaeology: Isotopic signature of mare's milk on 5500-year-old pottery fragments from Kazakhstan

- The oldest accepted evidence for horse domestication dated to 2000 B.C.E.
- Animal fat survives in archaeological pottery and its sources can be classified on the basis of the $\delta^{13}C$ values of the major fatty acids
- Testing ancient potsherds by carbon-isotope analysis, horse-milk deuterium signature was found
- Identification of **mare's milk in pottery vessels is clear evidence of horse domestication**
- This may extend Northern Kazakhstan's equine roots another 1500 years

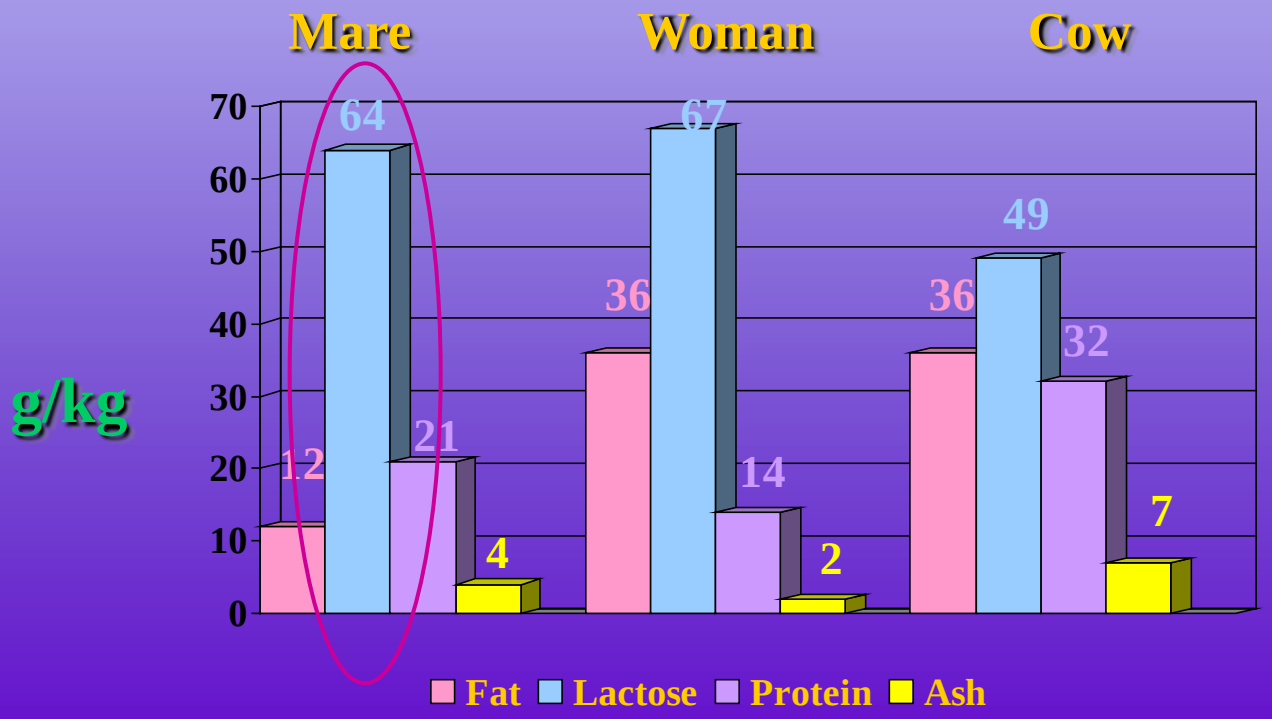
(Outram et al., 2009)

why fermented milk?

Acidifying fermentation is the most ancient method of milk conservation.

Mare milk is rich in **lactose** content, and since most populations in Asia have lactose malabsorption mare milk is consumed mainly as fermented milk, named **koumiss** (East Europe, former Soviet Union Republics), **airag** (Mongolia) or **chigee** (Inner Mongolia, China), alcoholic dairy products obtained by means of a mixed culture of yeasts and bacteria.

During fermentation, the lactose is converted into **lactic acid, etanol** and **carbon dioxide**, and the milk becomes an accessible source of nutrition for people who are lactose intolerant.



Martuzzi *et al.*, 2001

Koumiss, a fermented milk product

- Milk contains up to 10^9 microbes/L. The most abundant organisms cultured in mammalian milk include staphylococci, streptococci, corynebacteria, lactobacilli, propionibacteria, and bifidobacteria.
- These commensal organisms originate from the nipple and surrounding skin as well as the milk duct within the mammary gland
- Koumiss is a lactic acid-alcoholic beverage produced traditionally by fermentation of mare's milk by its indigenous organisms or by a starter culture and is the product first of **lactic acid and then alcoholic fermentation of the residual sugar content by yeasts.**
- In recent years, many studies have been carried out regarding identification of microorganisms in koumiss
- **Koumiss generally contains about 2% alcohol, 0.5–1.5% lactic acid, 2–4% milk sugar and 2% fat.**

A mare being milked in Suusamyr valley, Kirghizistan. Source: Wikipedia photo taken in June 2008

Koumiss: differences

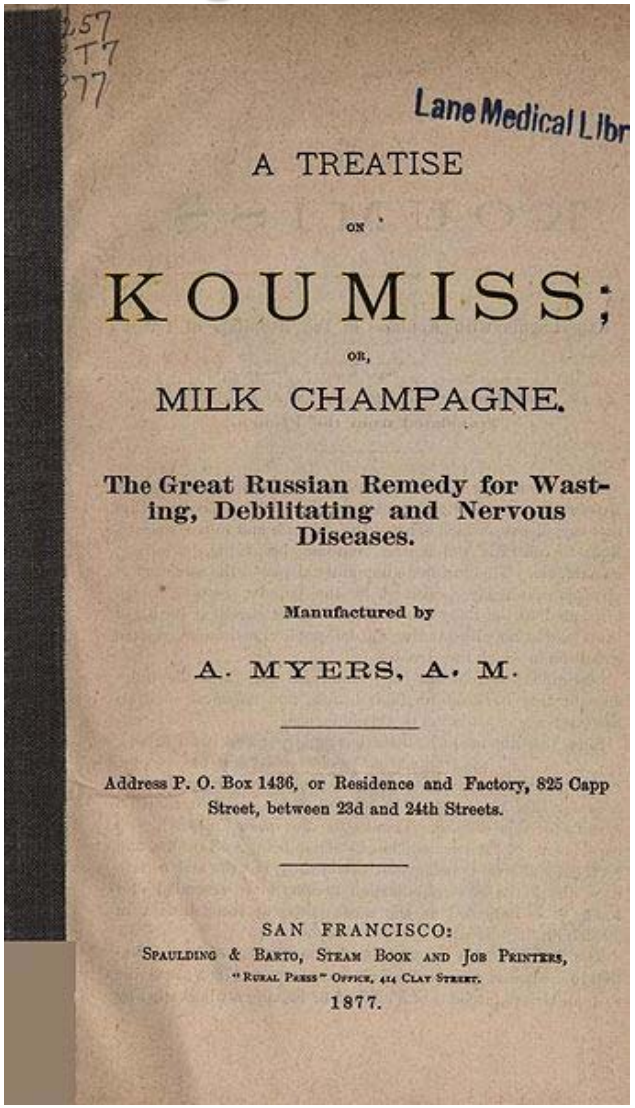
- Traditionally, cultures were maintained by **inoculating fresh milk with a small volume of already fermented milk.**
- From a microbiological point of view, the studies have detected the presence of a number of mesophilic **lactic acid bacteria** and **yeasts**.
- A wide variability in species and strains was observed by many Authors, and results show the tendency that the dairy **products from each family had their original microbiota**
- According to Danova et al., **three types of koumiss exist: so-called 'strong', 'moderate' and 'light' koumiss depending on the lactic acid content: from pH 3.6–3.3 to 4.5-5.0**
- "Moderate" koumiss seems to have the best fragrance and taste

(Danova et al.,2005; Uchida et al., 2007)

Koumiss: a sparkling beverage!!

- The alcohol content is known to reach levels of 3.5%.
- In Russia it is differentiated between weak koumiss with 0.7-1% ethanol, normal koumiss with 1-1.75% and strong koumiss with 1.75-2.5%
- Similar to kefir (fermented cow milk), one can also have **blowing problems with koumiss**: when opening the bottle, up to one third of the content can foam over!!!

(Seiler, 2003).



Yeasts in koumiss

- The yeasts in koumiss are lactose-fermenting species and strains belonging to the genus ***Kluyveromices*** and *Candida kefir* and also non-lactose fermenting yeasts of the genus ***Saccharomyces.***
- In the manufacture of koumiss, the yeasts produce a considerable amount of free amino acids, especially essential amino acids *(Seiler, 2003).*
- While the genus *Kluyveromices* is often the most prevalent yeast (*Kluyveromices marxianus sp)*,
- in a study carried out in Kazakhstan, it was observed a difference in the prevalence of the genus *Saccharomyces versus Kluyveromices* in **relation to the altitude, which selected lactic acid bacteria enriching the medium of galactose**

(Montanari et al., 1997)

Photo taken by Montanari *et al.*, 1997

Lactobacillus

- The genus *Lactobacillus* has a long history of use in the dairy industry.
- It plays a major fermentation role affecting the **aroma, texture and acidity** of the product.
- In recent research, *Lactobacillus* are considered to be probiotic bacteria because of their multiple benefits to human beings
- As the advantages exhibited by probiotics are known by more and more people, more attention has been focused on the usage and safety of these beneficial strains.
- Therefore, the **first point is identifying and characterizing a potential probiotic accurately**.
- Various techniques have been used to characterize lactic acid bacteria and these techniques include whole-cell protein analysis, cell wall composition analysis, and morphological, physiological, and biochemical analyses. *(Wang et al., 2008)*
- The expert consultation jointly set up by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) has **strongly advised the use of molecular biology techniques for the identification of probiotic bacteria**

(Del Piano et al., 2006).

Lactic acid bacteria (LAB) in koumiss: a living, silent competition…

- Recently, many studies are appearing in scientific journals regarding isolation and characterization of LAB in fermented mare milk
- Mostly by Japanese and Chinese research groups. Very active:
- **The Center of Dairy Research of Inner Mongolia College of Food Science and Engineering, Inner Mongolia Agricultural University Huhhof, China**.
- **The Yakult Central Institute for Microbiological Research, Tokio, Japan**
- Since 2004, the Chinese group isolated 240 *Lb* strains from koumiss *(Wu et al., 2009)*
- In 2005, a study was published in "**China Dairy Industry**" regarding **a new *Lactobacillus* strain found by the Chinese group in koumiss** from Inner Mongolia.
- Combined with analysis of phylogenetic dendrogram and partial sequences of 16S rDNA, it was classified as ***Lb casei* subsp.*casei* and called Zhang** *(Wu et al., 2005)*
- Since then, in the Chinese journals many articles were published about studies regarding this strain, with a particular emphasis on its **probiotic properties**.
- I could found news about *Lb* Zhang in European scientific journals for the first time in year 2008 *(Eur Food Res Technol; BMC Immunology)*
- …and the Japanese group found two new strains representing a single, novel *Bifidobacterium* species, for which the name ***Bifidobacterium mongoliense*** was proposed….*(Watanabe et al, 2009)*

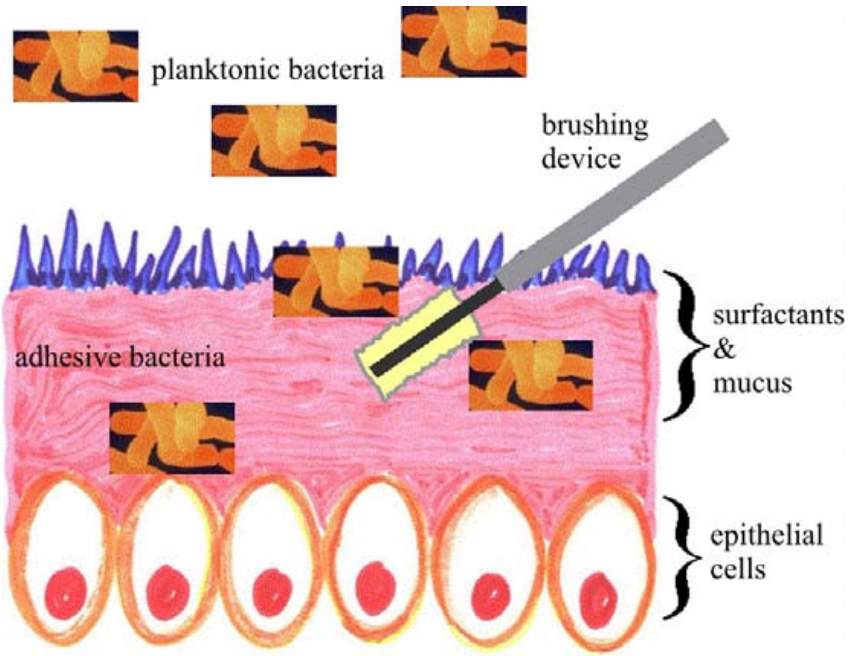
What is a probiotic?

- Probiotics are defined as "living microorganisms, which upon ingestion in certain numbers, exert health effects beyond inherent basic nutrition" *(Guarner and Schaafsma, 1998).*
- it is believed that probiotics **compete with and suppress the growth of undesirable microorganisms** in the colon and small intestine
- probiotics must be able to **survive passage through the stomach and upper intestine** and be present in sufficient amount to impact the colon microenvironment.
- they must **tolerate the acidic and protease-rich conditions of the stomach and survive and grow in the presence of bile acids**. *(Del Piano et al., 2006)*
- The microorganisms need to be **viable, active and abundant**, with a concentration of at least 10^6 cfu/g in the product, throughout the specified shelf life

(Guo et al., 2009).

The long way to become a "probiotic": sequential steps

1. Sample withdrawal by brushing
2. Strain isolation
3. Strain identification
4. Typing
5. Biotype selection
6. Growth capacity
7. Resistance to gastric juice
8. Resistance to bile
9. Resistance to pancreatic secretion
10. Safety evaluation
11. Resistance to freeze-drying
12. Stability of freeze-dried strain
13. Microencapsulation
14. In vitro studies
15. Animal investigation
16. Human clinical investigation



from: Del Piano et al., 2006

Some experiments *in vitro* and *in vivo* to test probiotic properties of Lb from koumiss

- ...so, many recent studies regard koumiss as a source of microorganisms with potential probiotic properties, and experiments are performed to test these properties...
- For example, in a study published in 2009 by the Chinese group in the J of Basic Microbiology:
- ...among the *Lb* collected in koumiss, *Lb casei* Zhang, *Lb helveticus* ZL 12-1 and *Lb plantarum* BX6-6 were selected as potential probiotic strains according to the **resistance to acid and bile** *in vitro*, the properties of **antimicrobial activities** and the **viability in cold storage** *(Wu et al, 2009)*
- Also animal investigation:
- A study was performed in four separate experiments via oral administration of live and heat-killed Lc Zhang to BALB/c mice for several consecutive days. They investigated the **immunomodulating capacity** of Lc Zhang *in vivo* by analyzing the profile of **cytokines, T cell subpopulations,** and **immunoglobulin** concentrations induced in blood serum and intestinal fluid in mice. Only live bacteria elicited a **wide range of immune responses**:
- increased production of interferon-γ (IFN-γ), and depression of tumor necrosis factor-α (TNF-α) levels.
- They also observed that Lc Zhang was capable of inducing gut mucosal responses by enhancing the production of secretory Immunoglobulin A (sIgA) as well influencing the systemic immunity via the cytokines released to the circulating blood *(Ya et al., 2008).*

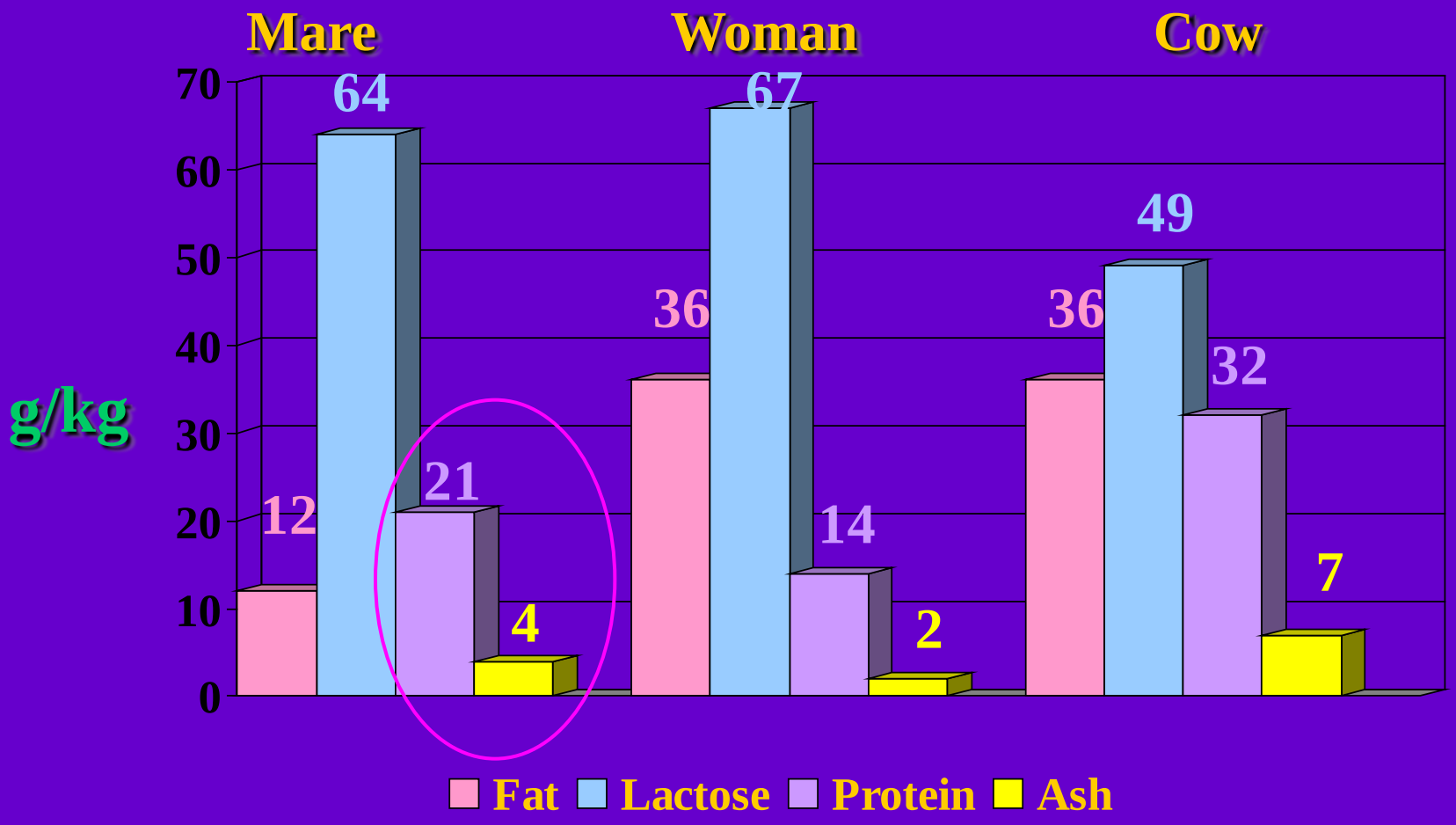
Lb from koumiss for human health: a study for application in the management of hypertension

- Recently there is a great interest in the **peptides that can lower the blood pressure of hypertensive patients**. Angiotensin I-converting enzyme (ACE; dipeptidyl carboxypeptidase) plays an important role in the regulation of blood pressure
- ACE inhibitors may exert an antihypertensive effect. Various **LAB produce specific peptides that possess an inhibitory activity on the ACE** *in vitro* and demonstrate a hypotensive effect in the hypertensive rat model.
- **γ-aminobutyric acid (GABA)** has been proved to be **effective for lowering the blood pressure** of mammals.
- Milk products fermented by LAB producing GABA decreased the blood pressure in spontaneously hypertensive rats and in mildly and moderately hypertensive patients. Therefore, the effects of GABA on human health are also of current interest in food production.
- Eighty-one strains of *Lactobacillus* were isolated from koumiss collected in Xinjiang, China. Screening results revealed that ACE inhibitory activity of 16 strains was higher than 50% and two strains produced GABA. **The *L. helveticus* ND01 strain produces both the high ACE inhibitory activity and GABA and was resistant to acidic condition.**
- The results suggest that ***L. helveticus* ND01 showed good potential for application in the management of hypertension** *(Sun et al., 2009).*

Bioactive factors

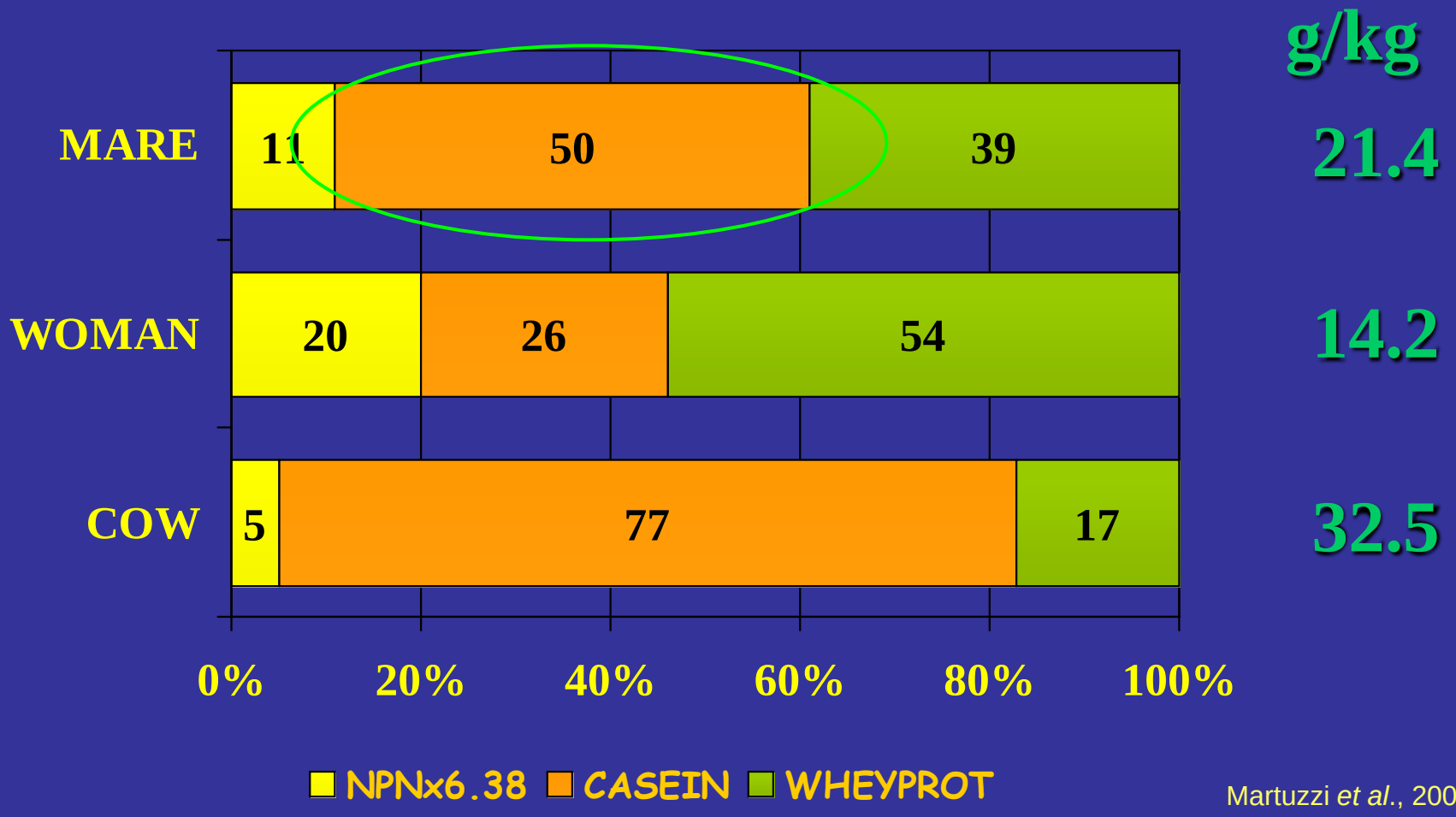
- Mare's milk and colostrum contain several bioactive factors: trophic hormones, growth factors, enzymes...
- Cortisol, insulin, thyroid hormones, insulin-like growth factors (IGF), epidermal growth factor (EGF), lysozyme and lactoferrin are important in maturation of gut enterocytes and provide protective mechanisms for infection, disease, and gastric ulceration *(Lawrence and Lawrence, 2008)*
- **Recently several studies have been carried out to assess structure and properties of some of these factors:**
- The **high content of lysozyme and lactoferrin** compared to cow milk could be involved in the beneficial aspects observed in mare milk consumption. As in human milk, antimicrobial defence in mare milk is mainly due to lysozyme, and its activity is highly thermo-stable *(Di Cagno et al., 2004)*.
- In human milk, the most abundant growth factor is lactoferrin (1-7 g/L), a glycoprotein, which is involved in iron absorption, infection control and growth stimulation of both epithelial cells and fibroblast.
- **In mare's colostrum and milk the concentrations of EGF are lower than in the human counterparts (7-13 µg/L) but higher than in cow's colostrum and milk** *(Murray et al., 1992)*.

Gross composition



Martuzzi *et al.*, 2001

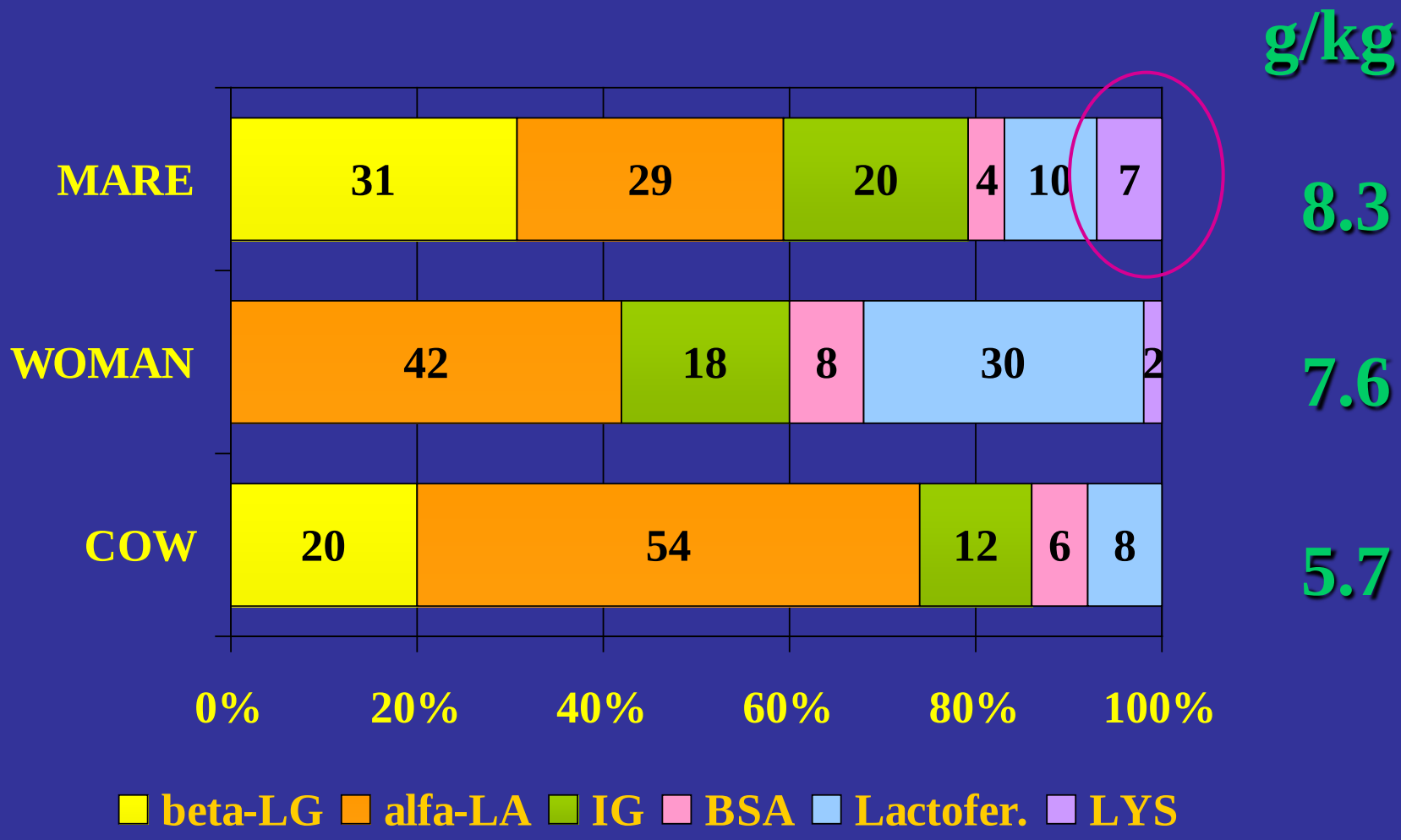
Main nitrogen fractions



Martuzzi *et al.*, 2001

In the past, the proportion of casein in mare milk may have been underestimated: most determinations made by precipitation at **pH 4.6** as in cow's milk, whereas for mare's milk the **maximum of casein precipitation is at pH 4.2** (Egito *et al.*, 2002).

Wheyproteins distribution



Martuzzi *et al.*, 2001

lysozyme

- In mare's milk lysozyme reaches hundreds of mg/L
- Horse lysozyme possesses **bacteriolytic activity** of all lysozymes, but it contains a calcium-binding site similar to the a-lactalbumin site.
- For this reason, horse lysozyme was suggested to be an evolutionary bridge between these two families
- horse lysozyme has an **amyloid structure with filaments and closed rings**
- The study of cytotoxic properties of ring amyloid structure is of particular importance, because the amyloid rings of several peptides inducing neuronal death have been recently proposed to play a key role in the pathogenesis of several diseases

Scheme for the horse lysozyme molecule produced using RasMol software. *(Malisauskas et al., 2006)*

Clinical trials:
wound repair function of mare's milk and colostrum

- Mare's milk as the active principle in a palm oil emulsion was tested for its effects on tissue repair
- Ten persons (38-80 years old) suffering from **diabetic ulcers** received treatment with mare's colostrum formulation twice a day for 20 weeks until complete wound recovery
- After experiments *in vitro* and *in vivo*, mare's colostrum proved to be an interesting tissue repai agent with higher activity than mare's milk: **7 of the 10 patients achieved complete healing of the diabetic ulcer within 2-10 weeks** of treatment, 2 showed a reduction, 1 obtained no benefit.
- The **biological action is located mainly in the fat globule fraction;** the bioactive molecules may be lipids or proteins, of which **lactadherin** and **adipophylin** appear to be the most likely candidates for **fibroblast-activating** action.
- **Lactadherin contains EGF-like domains**

(Topic use)

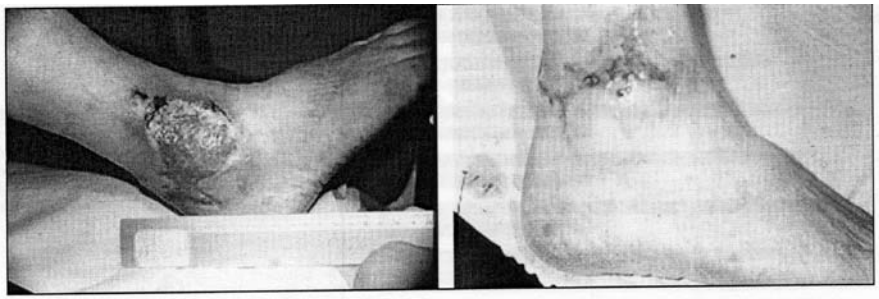


FIG. 1. (**Left**) In an 83-year-old man a chronic ulcer of vascular origin had been present for 10 years in the external perimalleolar area. He was treated with colostrum emulsion (see Table 1) once a day. (**Right**) A 60% wound reduction was achieved after 6 weeks of treatment.

(*Zava et al., 2009*)

Clinical trials: use of mare's milk

Activity of the Jena University group (Germany)

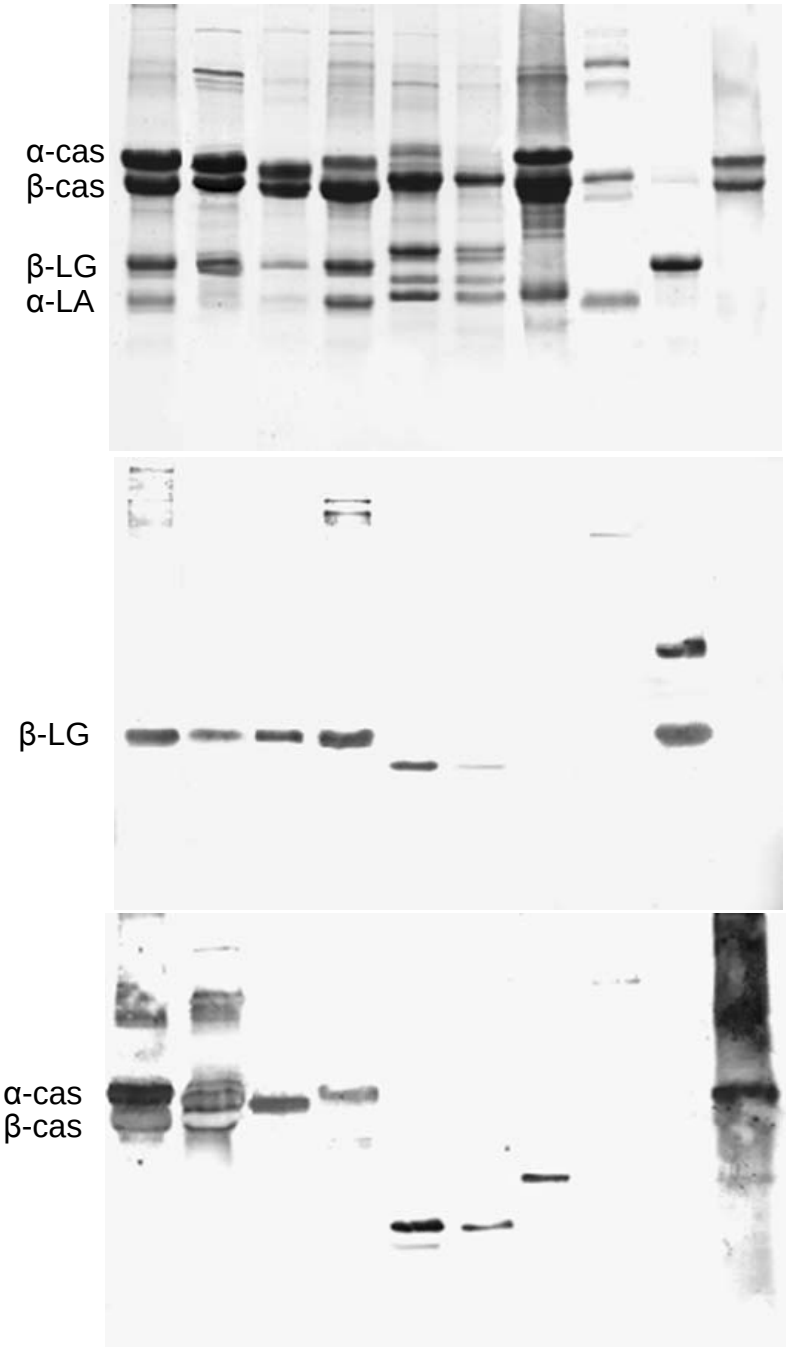
- Since the year 2000, the group of Prof. G. Jahreis, of the Dept. of Nutritional Physiology, performed several studies to investigate the effects of **oral intervention** with mare's milk. Two of these trials are reported:

❖ In a double-blind, placebo-controlled cross-over trial, 23 patients consumed 250 mL mare's milk or placebo for 16 weeks. The aim was to examine:

- **the effects of mare's milk on the characteristics of atopic dermatitis** (AD) **on faecal microbiota and on clinical and immunological parameters**.
- Results: during the mare's milk period, **the mean Severity Scoring of Atopic Dermatitis (SCORAD) value of patients decreased** from 30.1 to 25.3 after 12 weeks ($P<0.05$) and to 26.7 after 16 weeks ($P<0.1$). In a subgroup (n=7) the SCORAD index and especially **the pruritus decreased by 30%** ($P<0.01$). In this subgroup, the **faecal bifidobacteria increased** during the mare's milk period from 4.6% to 11.9% of eubacteria ($P<0.05$). The immunological parameters, except C-reactive protein, were unchanged. *(Foekel et al., 2009)*

❖ In a randomized, placebo-controlled, double-blind, cross-over intervention study, 8 **Crohn's desease** patients and 9 **ulcerative colitis** patients received daily 250 mL mare's milk or placebo for 8 weeks. The aim was to investigate:

- the **effects of oral intake of mare's milk in adolescent patients with cronic bowel inflammatory diseases**
- Results: Consumption of mare's milk caused a **lower abdominal and extraintestinal pain, reduced the occurrence of visible blood in the faeces and required lower medication to reduce the symptoms of bowel diseases** compared to the placebo drink. The parameters of blood, urine and faeces as well as the Crohn's disease activity index and ulcerative colitis activity index were not influenced. *(from: http://clinicaltrials.gov)*



SDS-PAGE of milk from different mammalian species (*A*), and corresponding **immunoblottings obtained by incubation with an anti-β-lactoglobulin monoclonal antibodies (B) and anti-total casein polyclonal antibodies (*C*).**

Both antibodies strongly react with proteins from cow, buffalo, goat and sheep milks, while **very mild immunoreactivity is observed with camel, horse and donkey milks.**

α-cas: α-casein;

β-cas: β-casein;

α-LA: α-lactalbumin;

β-LG: β-lactoglobulin

β-lactoglobulin is absent in human milk

(Restani et al., 2009)

Pub. No.:	WO/2005/074950		International Application No.:	PCT/AT2005/000038	
Publication Date:	18.08.2005		International Filing Date:	04.02.2005	

IPC: [illegible] (A 194/2004; 10.02.2004; AT)

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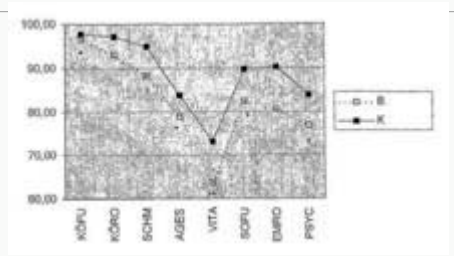
Priority Data:

Title:

(EN) UTILIZATION OF MARE'S MILK FOR THE PRODUCTION OF A PREPARATION TO IMPROVE SPERM QUALITY
(DE) VERWENDUNG VON STUTENMILCH ZUR HERSTELLUNG EINES PRÄPARATS ZUR VERBESSERUNG DER SPERMIENQUALITÄT

Abstract:



(EN) The invention relates to the utilization of mare's milk for the production of a preparation to treat infertility.
(DE) Es wird eine Verwendung von Stutenmilch zur Herstellung eines Präparats zur Behandlung von Unfruchtbarkeit zur Verfügung ge-stellt.

Designated States:

AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.
African Regional Intellectual Property Org. (ARIPO) (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW)
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African Intellectual Property Organization (OAPI) (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Biotechnology techniques: new findings and applications for mare's milk

Despite the great interest in mares' milk for human nutrition and apart from the traditional manufacture of koumiss, very **few studies have been carried out on the technological characteristics** of mares' milk.

The production of fermented milks based on a highly standardized protocol of manufacture is also of considerable interest for increasing the market and consumption of mares' milk products in countries where this milk is not consumed traditionally

Several recent research were carried out to study different production protocols, storage temperature effects, rheological and sensorial properties and so on.

(Kücükcetin et al., 2003; Di Cagno et al, 2004; Batdorj et al., 2006; Pelizzola et al., 2006;

Marchand et al., 2009)

Sakha villagers share fermented mare's milk during an *yhsakh* ceremony in 1902. Eastern Siberia

Biotechnology techniques: new findings and applications for mare's milk

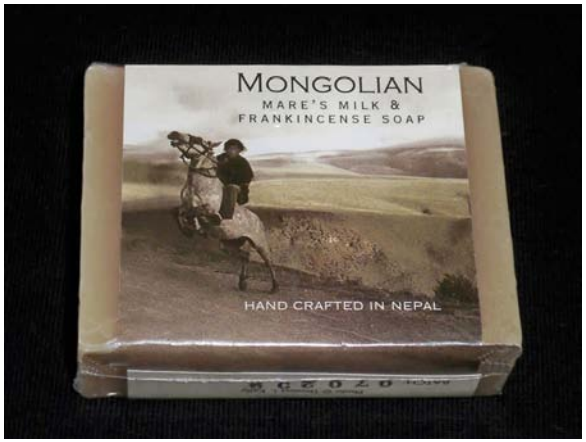
Mare's milk in cosmetology

Mare's milk oil is considered as an ingredient in Mongolian cosmetics because of its high content of polyunsaturated fatty acids which make it readily absorbed by human skin.

Mare's milk oil was prepared from natural milk by a centrifuging technique. However, this oil has an **undesirable yellow colour** unsuitable in its raw state for cosmetic applications. The yellow colour is caused by the presence of the β-carotene which has to be eliminated.

In two studies, efforts are paid to elucidate the effects of porous structures on the decolourization of the mare's milk oil.

(Temuujin et al., 2006a and b)



Biotechnology techniques: new findings and applications for mare's milk

Bacteriocins from LAB isolated from airag

- Bacteriocins are ribosomally synthesized substances of proteinaceous nature produced by bacteria that **kill or inhibit the growth of other bacteria.**
- Many LAB found in numerous fermented foods produce a high diversity of different bacteriocins.
- Class II bacteriocins of LAB have emerged in recent years as the most promising bacteriocin candidates for food preservation
- the spectrum of inhibitory activity of these bacteria suggests a potentially useful means for **controlling the growth of spoilage bacteria**, without suppressing the growth of yeasts
- bacteriocins produced by LAB used in the fabrication of fermented mare's milk were isolated and characterized.
- *Enterococcus durans* A5-11 strain showed the highest antimicrobial activity
- It was assumed that **bacteriocins A5-11A and B could be used in the food preservation** as they were obtained from 'GRAS' LAB strain

(Batdorj et al., 2006).

Conclusions

Research about mare's milk and koumiss for human consumption is more than ever...in ferment, especially in Asian Countries

- Identification and characterization of LAB and yeasts was the aim of many studies
- Some probiotic properties of LAB in fermented milk have been demonstrated
- Structure and properties of some bioactive factors in equine milk and colostrum have been in part unveiled
- New biotechnology techniques for mare's milk use have been set up
- Clinical studies are confirming some of the healing properties which were until now only legend

Perspectives for future research

Even if fermented mare's milk is a very ancient product, a lot of research is still needed:

While the yeasts flora recovers no more secrets, a wide range of scientific activity remains for the clarification of the interdependency and cooperation of the various **microbial components,** because in koumiss really complex population structures of yeasts, white moulds, lactic acid bacteria and acid acetic bacteria are present.

For particular LAB strains in koumiss, all the **steps to be considered a probiotic** have to be performed

Problems for the **industrial production** of koumiss need to be solved

As regard milk, **bioactive factors** can present further areas of investigation, in particular regarding the role of the growth factors

Woman pouring koumiss during the spring festival. Yakuts.

Photoarchive of the Russian Museum of Ethnography


Thank you for your attention