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PROBIOTIC ACTIVITY OF MIXED CULTURES OF KEFIR'S LACTOBACILLI AND NON-LACTOSE FERMENTING YEASTS

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Abstract: Kefir is a fermented milk drink produced by lactic acid bacteria and lactose or non-lactose fermenting yeast, which are growing in a strong relationship, and is reported to have a unique taste and properties. During fermentation, peptides and exo-polysaccharides with bioactive properties are formed. Moreover, by *in vitro* tests and animal trials, kefir and its constituents have shown to have anticarcinogenic, antimutagenic, antiviral and antifungal properties.

INTRODUCTION

Kefir is refreshing, self-carbonated fermented milk with a slightly acidic taste, yeasty flavour and creamy consistency and when agitated, the beverage foams and fizzes. This led to kefir being named "the champagne of cultured dairy products". The history of Kefir is shrouded by legend, with the drink dating back many centuries. The word "kefir is derived from the Turkish word "keif" which means "good-feeling"(Kaufmann,1997) and the drink originated in the Caucasian mountains of Russia, between the Black Sea and the Caspian Sea. Today, kefir is still manufactured in Russia and Europe under variety of names, such as Kephir, Kiaphur, Kefer, Kepi and Kippi (Kwack et al.,1996). It is also popular in Eastern European countries and is produced in small quantities in Czekia, Poland, Hungary, Romania, Finland, Sweden and Germany. It is also available in the United States and is growing in popularity in Japan.The beverage is manufactured by fermenting milk with kefir grains, comprised microorganisms, polysaccharides and milk proteins. The microbial population of kefir grains primaly includes lactic acid bacteria (LAB), namely lactococci and lactobacilli, yeasts, acetic bacteria and filamentous fungi.

Kefir exhibits antimicrobial activity *in vitro* against some fungi and Gram-positive and Gram-negative bacteria. The exact cause of this inhibition is not known but now, is well known the ability of LAB to inhibit the growth of closely related bacteria. This inhibitions of pathogenic and spoilage microbes may be due to the production of organic acids, hydrogen peroxide, acetaldehyde, diacetyl, carbon dioxide or bacteriocins. The microbiological and chemical composition of kefir indicates that it is a much more complex probiotic, as the large number of different bacteria and yeast found in it distinguishes it from other probiotic products. Since the yeasts and bacteria present in kefir grains have undergone a long association, the resultant microbial population exhibits many similar characteristics, making isolation and identification of individual species difficult. Many of these microorganisms are

only now being identified by using advanced molecular, biological techniques. The study of kefir is made more difficult,
because it appears that many different sources of kefir grains that are being used to produce kefir. The production of kefir depends on the synergistic interaction of the microflora in kefir grains. During the fermentation process, the yeasts and bacteria in kefir grains produce a variety of ingredients that give kefir its unique taste and texture. After fermentation, the finished kefir product contains many ingredients which proved to be bioactive. At least one exopolysaccharide-kefiran- has been identified in kefir, although others may be present. Many bacteria found in kefir have been shown to have proteinase activity, and a large number of bioactive peptides have been found in kefir (Farnworth, 2005).

MICROBIOLOGY OF KEFIR GRAINS

Kefir is a product manufactured by starter culture prepared from kefir grains. The grains were kept in leather bags or horseback, only added milk. In the warm climate fermentation began. Kefir grains are very complex, and as they vary from region to region, from dairy to dairy, no thorough and unambiguous identification of its components are possible. However studies show that all kefir grains consist of lactic acid bacteria and lactose fermenting and non-lactose fermenting yeasts, growing in a strong relationship (Farnworth, 2005).

1. Bacteria found in kefir grains and kefir :

Lactobacilli
Lactobacillus kefir [a,c,j,n,o,p,r] *Lactobacillus delbrueckii* [a,h,p]
Lactobacillus kefiranofaciens l,[n,p]
Lactobacillus rhamnosus [a,r]
Lactobacillus kefirgranum [n] *Lactobacillus casei* [h]
Lactobacillus parakefir [n,o] *Lactobacilli paracasei* [p]
Lactobacillus brevis [g,h,p,r] *Lactobacillus fructivorans* [k]
Lactobacillus plantarum [o,p] *Lactobacillus hilgardii* [k]
Lactobacillus helveticus [a,b,h] *Lactobacillus fermentum* [r]
Lactobacillus acidophilus [g,p,r]
Lactobacillus viridescens [r]
Lactococci
Lactococcus lactis subsp. lactis [a,c,e,f,g,h,k,o,r]
Lactococcus lactis subsp. cremoris [a,e,f]
Streptococci
Streptococcus thermophilus [e,h]
Enterococci
Enterococcus durans [d*,e*]
Leuconostocs
Leuconostoc sp. [r]
Leuconostoc mesenteroides [a,b,g*,o]
(reported as Leuconostoc kefir in ref. g)
Acetic acid bacteria
Acetobacter sp. [o]
Acetobacter pasteurianus [g*]
(reported as Acetobacter rancens in ref. g)
Acetobacter aceti [a,d]
Other bacteria
Bacillus sp. r Micrococcus sp. r
Bacillus subtilis g Escherichia coli r

([a]Koreleva 1991; [b]Lin et al. 1999; [c]Pintado et al. 1996; [d]Rosi 1978; ref.,[e]Yu¨ksekdag et al. 2004; [f]Dousset and Caillet 1993; [g]Ottogalli et al. 1973;[h]Simova et al. 2002; [j]Kandler and Kunath 1983; [k]Yoshida and Toyoshima 1994; [l]Fujisawa et al. 1988; [n]Takizawa et al. 1994; [o]Garrote et al. 2001)

2. Yeasts found in kefir grains and kefir:

Kluyveromyces marxianus [a,b,f*,g,h,i,j,k,m*,n] *Candida friedrichii* [n]*(reported as Saccharomyces lactis in ref. f; reported as Kluyveromyces lactis in ref. m)*

Saccharomyces sp. [k] *,Candida pseudotropicalis* [f]*,Saccharomyces cerevisiae* [a,d,e,f*,g,j,m,n] *Candida tenuis* [f] *(reported as Saccharomyces carlbergensis in ref. f),Saccharomyces unisporus* [c,h,j,m]*, Candida inconspicua* [g]*, Saccharomyces exiguus* [l*]
Candida maris [g] *(reported as Torolopsis holmii in ref. l)*
Saccharomyces turicensis [h]*, Candida lambica* [j]*,Saccharomyces delbrueckii* [d]*,Candida tannotelerans* [e]*,Saccharomyces dairensis* [n]*, Candida valida* [e]*,Torulaspora delbrueckii* [a,h,m] *Candida kefyr* [a,j,n]*,Brettanomyces anomalus* [h] *Candida holmii* [j,m]*,Issatchenkia occidentalis* [j] *Pichia fermentans* [b,m,n] ([a]Koreleva 1991; [b]Lin et al. 1999; [c]Pintado et al. 1996; [d]Rosi 1978; [e]Dousset and Caillet 1993; ref. [f]Ottogalli et al. 1973; [g]Simova et al. 2002; [h]Wyder and Puhan 1997, 1999; [i]Yoshida and Toyoshima 1994; [j]Engel et al. 1986; [k]Garrote et al. 2001; ref. [l]Iwasawa et al. 1982; [m]Angulo et al. 1993; [n]Rohm).

The kefir grains are insoluble in water and common solvents, gelatinous and irregular in size, varying from 0.3-3.5 cm in diameter. Kefir grains resemble small cauliflower florets: they measure1–3 cm in length, are lobed, irregularly shaped, white to yellow-white in color, and have a slimy but firm texture (Kosikowski and Mistry 1997).Grains are kept viable by transferring them daily into fresh milk and allowing them to grow for approximately 20 hours; during this time, the grains will have increased their mass by 25% (Halle´ et al. 1994). Grains must be replicated in this way to retain their viability, since old and dried kefir grains have little or no ability to replicate.

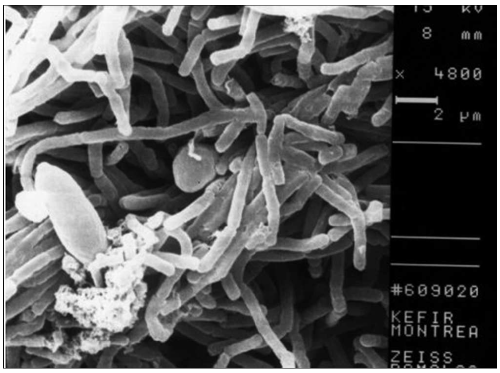

Fig. 1. Electron micrograph of a kefir grain.

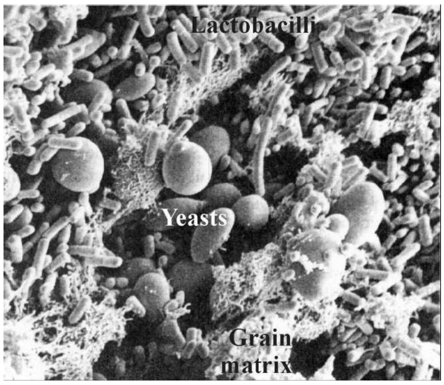

Fig. 2 Scanning electron micrograph of a kefir grain

Today's modern technologies have made it possible to produce DVS (Direct Vat Set) cultures consisting of the same strain families as the ones found in some kefir grains. Based on many years of experience, scientists have developed a range of thermophilic and mesophilic lactic acid bacteria cultures which can be combined with various yeasts strains and used for kefir products. It has been recognized that yeasts play an important role in the preparation of fermented dairy products, where they can provide essential growth nutrients such as amino acids and vitamins, alter the pH, secrete ethanol and produce CO_2 (Viljoen 2001). The yeasts in kefir have been less well studied than kefir bacteria, although it is obvious that the yeasts in kefir grains provide an environment for the growth of kefir bacteria, producing metabolites that contribute to the flavor and mouthfeel of kefir.To prevent excessive CO_2 production (particularly after fermentation), Kwak et al. (1996) suggested a two stage fermentation process starting with a non-lactose fermenting yeast such as *Saccharomyces cerevisiae*. The role of yeasts is not only limited to their contribution to kefir flavor. Yeasts also promote symbiosis among microorganisms by providing LAB with growth stimulants. On the other hand, LAB produces β-galactosidase which splits lactose into glucose and galactose. Nearly all the yeasts are able to utilize either glucose or galactose or both.

During the alcoholic fermentation by the yeast, CO_2 and ethanol are formed. Both compounds are among others responsible for the characteristic flavor of kefir products. Also, the metabolism in the bacteria culture results in flavor formation. The acidity from lactic acid and the flavor characteristics from diacetyl, acetaldehyde and acetate come from the metabolism. The bacteria culture converts lactose into lactic acid and into flavor compounds. A thermophilic culture will beside lactic acid produce acetaldehyde and lactate from lactose, whereas diacetyl will be formed from the metabolism of some mesophilic cultures. Furthermore, these mesophilic cultures are able to metabolize citrate. The yeast cultures are responsible for the alcoholic fermentation where ethanol and CO_2 are formed. The lactose fermenting yeasts degrades lactose directly, whereas the non-lactose fermenting yeasts are dependent on lactic acid bacteria to hydrolyze lactose into galactose and/or glucose which is subsequently metabolized by the yeast into ethanol.

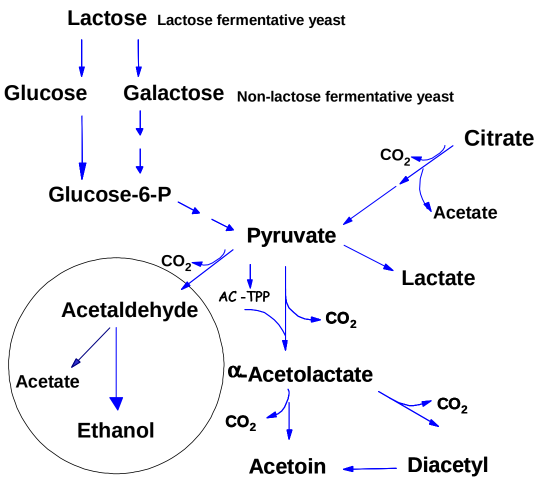

Fig.3 Simplified model of lactose metabolism (and citrate co-metabolism in mesophilic cultures) in bacteria cultures and yeast cultures

Ethanol-The largest amount is produced during the alcoholic fermentation. Ethanol gives the characteristic alcoholic and sharp flavor of a kefir product.

Acetic acid-Acetic acid contributes to both the taste and flavor. It is produced during degradation of citrate and also in lactose catabolism. However, excess of acetate is not desirable as it could cause a sharp taste.

Diacetyl-Diacetyl is recognized as a compound contributing to the specific “buttery” flavor of buttermilk, sour cream and cream cheeses. In fresh products fermented by a mixed culture, diacetyl is present in low concentration, not more than 1-3 mg/l. Generally, this concentration gives a good flavor in fermented dairy products. Diacetyl is an unstable compound; it is subjected to degradation to compounds with no aromatic properties.

Acetaldehyde-Acetaldehyde is a flavor component desirable in yoghurt types, and gives "green-apple" or "fruity" flavor. In order to gets a balanced flavor in mesophilic products where this flavor is normally not wanted, strains belonging to *Leuconostoc* species are commonly used as a component of the mixed mesophilic cultures. Their role is to reduce the level of acetaldehyde. However, in kefir products, acetaldehyde has been reported to be an important volatile compound contributing to flavor.

Carbon dioxide-Carbon dioxide is formed mainly during milk alcoholic fermentation. By itself, CO_2 is tasteless but it has an influence on taste and contributes to perceived freshness. Furthermore, it causes a sparkling/tingling effect, which is characteristic for the kefir products. The formation of CO_2 is desirable to a certain extent. Lack of CO_2 causes a so-called flat flavor, but a surplus of CO_2 or post-fermentative development of CO_2 is undesirable, as it can cause packing deformation.

PROBIOTIC ACTIVITY OF MIXED CULTURES

Kefir possesses antimicrobial activity *in vitro* against a wide variety of Gram-positive and Gram-negative bacteria, as well as some fungi (Garote et al., 2000). Some coliforms are actively inhibited by kefir microorganisms, and pathogenic bacteria such as *Shigella and Salmonella* do not grow when they are introduced to kefir. Van Wyk (2001) showed that kefir possesses an inhibitory activity against *Staphylococcus aureus, Bacillus cereus, Escherichia coli, Clostridium tyobutyrivum and Listeria monocytogenes.* The exact cause of inhibition is not known, but may be due to the antagonist action of various species of LAB, which are also capable to prevent the adherence, establishment, replication, and/or pathogenic action of certain enteropathogenes. The precise mechanism is not clear, but may include the activity of lactic acid or volatile acids, hydrogen peroxide,carbon dioxide, acetaldehyde and diacetyl or bacteriocin and bacteriocin-like products(Powell,2006).

Lactic acid and volatile acids- the un-dissociated forms of lactic and acetic acid penetrates the microbial cell membrane; this results in acidification of the cytoplasm and the formation of inhibitions, especially against enzymes, by salt excesses. At a higher intracellular pH these acids dissociate to produce hydrogen ions, which interfere with important metabolic functions such as oxidative phosphorylation. This acids are known to inhibit *E. coli and B. cereus.*

Hydrogen peroxide- in the presence of oxygen, H_2O_2, is produced by LAB throught electron transport via flavin enzymes. In the presence of H_2O_2, superoxide anions form destructive hydroxyl radicals ($^{\cdot}OH$), leading to increased membrane permeability and to the peroxidation of membrane lipids. Bactericidal oxygen metabolites cause the destruction of nucleic acids and cell proteins, and have strong oxidizing effects on the bacterial cells. Hydrogen peroxide inhibits *Pseudominass spp and S. aureus*.

Carbon dioxide- is produced in substantial volumes by heterofermentative LAB and yeasts as an end-product of hexose fermentation or by metabolising arginine (deaminase pathway) or by decarboxylation of the histidine and tyrosine. CO_2 contributes to the antimicrobial activity by replacing existing molecular oxygen, creating an anaerobic environment.

Acetaldehyde and diacetyl- they are responsible for the typical aroma. They elicits antimicrobial activity against *S.aureus,E.coli,S.typhimurium,*yeasts,moulds, but they required high concentrations in medium.

Bacteriocin and bacteriocin-like products. Bacteriocins are bacterial proteins or peptides with bactericidal or bacteriostatic activity against genetically closely species (Schillinger,1996) They can be produced spontaneously or induced and the genetic determinants of most bacteriocins are located on plasmid. The majority of bacteriocins produced by LAB have been characterized according to their activity as a proteinaceous inhibitor, on the estimation molecular mass, and on the determination of their spectrum of inhibition. They are divided into distinct classes.

Class I-Lantibiotics- are small, membrane-active peptides of less than 5KDa, contain 19 to 50 amino acids. They are ribosomally synthesized as pre-peptides containing an unusual and structurally homologous leader sequence that may be involved in post-translational modification. Examples: nisin A and Z, subtilin, sublancin, cytolisin and staphylococcin.
Class II-are small, membrane-active,heat-stable,hydrophobic peptides that are less than 10Kda.They form amphiphilic helices with varying p-sheet structures. Example include leucocin A, mesentericin Y105, sakacin A nad P, enterocin A and P, pediocin AcH.
Class III- are large, heat-stable, hydrophilic peptides less than 30Kda. Example includes acidophilin A, lacticin A and B, helveticin J and V.
Class IV-is a complex composed of a protein and one or more chemical moieties such a carbohydrate or lipid. Examples include pediocin SJ-1, leucocin S, plantaricin S and lactocin.

The mechanism of LAB bacteriocins suggested that the peptide accumulates at the surface of membrane, through ionic interactions with the phospholipids groups. The presence of these peptides results in significant membrane thinning in these regions, caused by the localized displacement phospholipids. Other bacteriocins form an ion-permeable channel in the cytoplasmic membrane of target cells(Garneau,2002). This results in an increase in membrane permeability, resulting in a disturbance of the membrane potential and causing an efflux of ATP, essential ions and amino acids. Biosynthesis of macromolecules and energy production is thus inhibited, ultimately resulting in cell death.

CONCLUSION

Since kefir has a pH of 4.2-4.6 after fermentation and maturation, one can suppose that the inhibitory activity of this beverage and grains is due to the production of acids by LAB. However, a number of investigators have reported that acid is not the only contributor to the antimicrobial activity. Kefir is a complex microbial system that has been found to not only be nutritionally beneficial, but has proven also to inhibit a number of food-borne pathogens and spoilage microorganisms.

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