Microbial community of natural starter of fermented dairy product

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Abstract. The study of natural starter cultures in different geographical areas showed a high biochemical activity of lactic acid bacteria. They have spontaneous variability being in a state of stress that determines their morphological, cultural physiological and biochemical properties. Adaptive evolution of lactic acid bacteria under the influence of adverse natural factors in its growth process affects their ability to metabolize important functional compounds. It leads to the activation of adaptive genes that provide the cell development and stability. They act as chemical agents with defined functions in the nature. Their functions affect the natural history of the organism in biochemical status. It has been established that the directed synthesis of the products depends on the producer strain, the activity of corresponding enzymes and the physic-chemical state of the nutrient medium. It has been known that the function of microorganisms is more specialized, more clearly delineated in nature than in the laboratory environments. The surface layer pigmentation of fermentable dairy products increases in density due to the transition of lactic acid bacteria cells in hypometabolism conditions. These conditions are defined by functional specialization in bacteria and the redox conditions.

1. Introduction
Proper selection of microbiological cultures for fermentation, their safety, and resistance to bacteriophage will greatly improve the quality of starter (leaven). Furthermore, it will also extend the range of fermented dairy products for different layers of the population and their health status. Regarding to the last suggestions local strains of lactic acid bacteria, yeast and its mutants widespread in the nature might be used. The mutants are insensitive to seasonal changes in climate and other geophysical stresses.

The microbiological culture properties influence the palatability of a product and duration of storage and most importantly the healing properties of a product. The isolation and examination of microorganisms obtained from the homemade products from the different geographic areas have always been the main methods of obtainment of bacterial cultures with valuable biochemical properties.

Research of leavens, their properties, peculiarities of interaction in microbial associations and the production is one of the urgent problems of technical microbiology.

The effect of climatic conditions on the formation of microorganism geographical races has been established in the last century. Explored microorganisms possessed a sustainable hereditary characteristic. Some of the numerous selected lactic bacteria strains possess many valuable
technological characteristics [1,2].

Strains must have high biochemical activity such as acid, agrometeorologia, proteolysis, resistance to drugs, frostiest, antagonistic action against pathogens and extraneous microflora (hard - and non-culturable forms), etc., to have wide practical application. It is preferable to have lactic bacteria strains produced bacteriocinin and capable of quickly lactose using as well as inhibiting the harmful microorganisms [3].

Microorganism strains selection for leaven must guarantee their activity, purity, product consistency, pleasant aroma and other characteristics which determine the value of the fermented milk product.

2. The purpose of the research
The objective was exploring the microbial community of the naturally fermented milk product, its structure, successional changes and trophic rearrangements of lactic acid bacteria during its store for 12 months. The research tasks involved monitoring the status of a product bunch, growth dynamics of lactic acid bacteria and yeast, microorganisms’ distribution in the profile (0-12 cm) and in time, the direction of the microorganisms’ growth and the functioning of individual structures of natural microbial milk communities.

3. The object of the research
The object of the study was a natural leaven from Tambov region and isolated strains of lactic acid bacteria and yeast.

4. Material and methods
We used the methods modified by us: application, direct light microscopy, staining to define the lactobacillus metabolism products. The resistance of lactic acid bacteria was determined by the disco diffusion method, etc. All the research work was based on the methods accepted in microbiology [4-6].

5. Discussion of the results
The results of the experiment have been partially published [1,2,6,7]. A brief summary of the results is that after 24 hours the pigment with weakly cream-color has been found on the surface of the product which has been fermented by natural microbiological culture. Furthermore, the pigment consolidation and formation of a film has been established after 72 hours. The microscopic examination of the film revealed the presence of yeast and lactic acid bacteria (Figure 3).

Figure 1. Cream pigment on the surface of the fermented milk product

Figure 2. Original sample of lactic acid product
Moreover, the *Lactococcus* is attracted by the yeast cells, thus there have been some changes of their form. The cytokinesis of *Lactococcus* is due to the genotype but the microbe shape is the phenotypic trait in the specific conditions of its habitat in the current situation. The processes in the experiment are due to biochemical conditions linked to the synthesis of cell walls. The phenotype of lactic acid bacteria has been varying later (by 12 months of the product storage).

![Cells of Lactococcus and yeast](image)

**Figure 3.** Cells of Lactococcus and yeast

Since the environmental conditions are relevant to the growth and development of microorganisms, the species always exhibit their inherent characteristic form and function. Unstable transient changes affect the organism adaptation to the changing environmental conditions.

Fermented milk has formed a “film” after 12 months storing under laboratory conditions at an ambient temperature around +15-20°C. We found no microorganisms on the film surface. It also had a high density, pure brilliance (not soluble) and a brown color known as melanin.

Apparently, this pigment film keeps fermented dairy product for 12 months due to its anti-bacterial activity. A microbial population of lactic acid bacteria in the form of "autoplasty" has emerged on the surface of the lactic acid product. Autoplasy is genetically determined cells of the same species forming a complex self-regulating and self-developing system. This microbiological population localized on the surface actively grows undergoing developmental cycle and metabolic type, determined by a range of catalytic activities.

Lactic acid bacteria activate mechanisms of anti-stress protection in the response of changes in temperature (from 4 to 20 °C) and storage duration (about 12 months). Anti-stress protection is a set of biochemical and physicochemical reactions that form the physiological cell response. The latter can have either cell death (autolysis) or inclusion of the genetic program that encourages creating reproductive resting forms, which have a unique type of metabolism - hibernation. These processes in combine can occur simultaneously as well as can affect the morphological features of the cells (cytodifferentiation) and their biochemical state called the bactericidal action.

At the same time, the bacteria on the film surface undergoes a rather complicated cycle of development, retains their properties within the development cycle and transmits them to its offspring. Colonies on solid medium have an "S-shape" - a smooth surface with smooth edges and a relatively fragile structure. Any change in the external environment breaks a complex system of many opposing processes in the cell and the latter is committed to establish its equilibrium with the environment in a new, altered form.

The underlying layers of the clot were unlike the surface as they had white color, smooth density on the substrate. Collaborative development of lactobacilli and yeasts has been found at the bottom of the bunch after the 12-month storage (at a depth of 12 cm) (Figure 4).
Therefore, the synthesis process of the pigment may serve as a marker to determine the beginning of the active lactic acid bacteria metabolism and their redistribution along the profile of the bunch. In its turn the redistribution depends on the degree of aerobic respiration, type of metabolism and other biochemical features. This phenomenon can be considered as the formation and the separation of lactic acid bacteria producing bacteriocin, quickly using lactose and inhibiting the vital functions of foreign microflora.

The symbiosis of lactic acid bacteria and yeast is quite strong and, apparently, is beneficial for both. The phenomenon of symbiotic relationship between lactic acid bacteria (cocci and rods) and yeast is due to the assimilation of certain compounds (growth substances) which are necessary for those bacteria races. Perhaps, these races of lactic acid bacteria have a lack of nitrogen compounds in the form of amino acids, which they are not able to obtain from the casein fission. The appearance of the yeast (for instance, Saccharomyces cerevisiae) in fermented milk is a rare phenomenon because they are usually not able to ferment lactose. However, lactic acid causes the hydrolysis of milk sugar and thus yeast can actively grow. At the same time, small amount of the gas (carbon dioxide) and small amount of the alcohol have been found at the bottom of the vessel. An optimal environment for the yeast and high acidity for the acidophilic bacteria (acid tolerant group) have been formed at the bottom of the clot. Typical microorganisms cannot grow under these conditions. The clot has not almost changed its dense structure. Gaps and syneresis have not been observed.

Intensive development of the yeast may lead to the reduction of the partial pressure of oxygen in the medium or perhaps, lactic acid bacteria dispose of extracellular capsular polysaccharides of yeast and its metabolites.

Chemical transformations in the cultural medium have been changing during the development of lactic acid bacteria and the redistribution of redox reactions in space as well as the formation of certain micro-watersheds on the clotting profile have been observed. The figure shows that the large yeast cells act as formation centers for the bacterial and yeast consortium.

Besides the bactericidal activity, one of the important characteristics of lactic acid bacteria from a natural leaven is its proteolytic activity. The enzymes which have capacity to hydrolyze proteins are often entered in the raw material processing. They provide normal conditions for yeast activity which improves the entire technological process. Strains with proteolysis are the most valuable as they can break down the milk proteins to amino acids and evolve in the milk more than others. The starter with active proteolysis is characterized by large number of microorganisms than a starter with weak proteolytic agents. Apparently, it can affect the quality of cheese. Therefore, this fact has to be taken in account in the selection of starter cultures for cheese production.

The development of dairy products with a reduced content of allergenic milk proteins is an important direction in the dairy production. Such products have lack of the high molecular weight...
proteins due to the partial proteolysis of milk. The products obtained by such strains of *Lactococcus* will be available for preventive nutrition.

Obtaining functionally active proteins and peptides is possible by using limited proteolysis. In those conditions, one or several peptide bonds in the protein molecule is selectively hydrolyzed and the reaction is catalyzed by specific bonds [8].

Apparently, the reason of different proteolytic activity can be associated mainly with biochemical activity of lactic acid bacteria isolated from milk or leaven from different geographical areas. I. V. Tsaregradskaya, A. K. Maksimov and others (1968) approved that the decrease in the ferment activity is associated with a season of the year and a geography zone of raw materials. The total number of lactic acid bacteria with non-starter origin in raw milk in the south and in the middle zones of Russia is higher from 100 to 1000 times than in the northern areas [9].

Nowadays the possibility of obtaining lactic acid bacteria with enhanced biochemical activity by mutagens exposure (of physical nature) and by adaption. The latter is the type of variability, but changes passed on from generation to generation are already mutations. Under γ-ray radiation on lactic acid bacteria, the mutants have been obtained. They were significantly different from the original parental strains by their morphological and physiological properties [6].

There are some principles for the Lactobacillus culture selection of poly-strains starters which are the energy of acid formation, phage resistance, aroma formation, the lack of antagonism between cultures, inhibiting the growth of extraneous lactic acid non-starter microflora. Apparently, these signs of lactic acid bacteria are naturally incorporated in local starter cultures. Natural resistance of lactic acid bacteria is due to the bacteria species that can have multiple resistance genes encoding resistance to multiple drugs of the same class (cross-resistance) or groups of genes determining resistance to several classes of antimicrobial drugs (associated resistance). Such strains have a great importance in biotechnology to produce new functional dairy food products.

Selected strains of *Lactobacillales* do not reduce biochemical activity within 12 months of storage at a temperature of 18 - 20°C. This is due to the adaptive properties of the cultures, the stability, and reversibility of the enzymatic mechanism. Therefore, the most valuable strains of lactic acid bacteria are isolated from natural sources of different geographical zones. North and Central zone are favorable for the selection of aroma-forming *Lactobacillales*, South zone contributes to the development of drug-resistant streptococci and yeast.

In our experiment symbiotic trophic linkages between Lactococcus and yeasts have been constantly observed in milk fermentation with natural leaven.

The formation of some by-products (acetic, succinic, formic acid, alcohol, etc.) in a small amount (rarely exceeding 2-3%) has been observed in the long course of lactic acid fermentation. In many cases these by-products have a secondary origin, i.e. they have been formed not from milk sugar, but from lactic acid obtained in the first period of fermentation. Thus, the more acid was accumulated the more concentration of H-ions increased, therefore the development of bacteria stopped. This could explain the gradual weakening, and then complete termination of the activity of the forming acid microorganisms.

*Lactococcus* proteinases use peptides and milk proteins (i.e. casein) as substrates. As it has been known casein is a heterogeneous compound of phosphoproteins consisting of approximately 30 fractions. The individual molecules of casein form micelles whose average size is about 90 nm.

Casein coagulation process is carried out in several stages: hydrolytic cleavage, then the formation of curd in the presence of calcium ions and separation of whey. In the beginning, a change in viscosity (it falls) and the aggregation of particles occur simultaneously. Then, coagulation, compression of the sediment (compaction) and the release of whey take place. The aggregation of casein micelles, which have a negative charge is partly due to lactic acid bacteria which destabilizes the micelle or reduce intercellular repulsive forces. Calcium ions also reduce the negative charge of the micelles, so their aggregation is enhanced.
The storage temperature of the fermented product was +20°C that was significantly important. In this mode, clot densification and clearly formation or separation of microorganisms into layers have been established, it has been visibly observed at a temperature of 10-15°C.

By 12 months of storage of the product, the softening, dissolution and formation of whey but not the separation have been noticed at the bottom of the curd (height 0-12 cm). At the same time, there was active development of *Lactobacillus acidophilus* and yeast and seal pigment (thicker cream) on the surface. The active life of the pure culture of *Lactococcus* (Figure 5) has been established in the form of a thick "layer" having a *bactericidal action*. It can be observed by microscopy of the surface "layer": complete absence of growth of extraneous gram-positive, gram-negative bacteria and yeast.

**Figure 5.** Lack of growth of extraneous microorganisms on the *Lactococcus* film.

Bacteriocinogeny is an important property of most bacteria, which is determined by the antagonistic orientation of their metabolites which are bacteriocins, representing a heterogeneous antibacterial complex. This complex might be different depending on activity, action mechanisms, molecular weight, biochemical properties, genetic origin [10,11]. Bacteriocin protein can be associated with oligosaccharide of the cell membrane, but the protein itself of this complex has antibacterial activity [12]. The search for natural strains which possesses high biochemical activity, resistance to extreme pH values, temperature, photodynamic changes, etc. opens great opportunities in the development of functional products for the treatment of certain people groups [12].

We should also highlight another feature of the pigment bactericidal activity, which is the lethality of microbes’ producers. Perhaps, bactericidal activity is the adaptation of individual cells that die and form a dense bactericidal film. At the same time, the density of pigmentation can be due to a temporary transition cell in hypometabolism condition that causes the limited growth of the culture. It is known that Lactococci is pleomorphic and can exist not only in the form of normal cells usually observed in each research. They pass a special stage, in which a change in shape and size become visible. After a certain period and under certain conditions, they pass in the usual form (Fig. 6).

Morphogenesis, as it is known, is associated with controlling of the changes in chemical composition and cell functions as well as the changes in enzymes that are under direct genetic control.

Morphogenesis and differentiation of vegetative yeast forms can be expressed in diverse forms which are largely dependent on environmental conditions (nutrient medium, aeration). The biochemical nature of the morphogenesis is not completely researched, but there is certainly influence of appropriate conditions on the activation of differential expression of genes. Bacteria that produces
bacteriocin in fermented foods can be found in natural microflora of feed used by animals for centuries. The synthesis of bacteriocins is an inherited feature of organisms. Bacteriocins are accumulated inside the cells which are located on the surface. They were not found below the nanometric surface layer of the curd; the underlying layers are white. Besides, the antibiotic function of melanin is not no longer in dispute by researchers nowadays. Brown pigments of many bacteria, actinomycetes, mycobacteria, etc. are proved to be antimicrobial agents.

![Figure 6. The modified form of streptococci](image)

The resistance of lactic acid bacteria and milk yeast to antibiotics is a good indicator of protective and corrective actions on the gastrointestinal tract. It is significantly important that Lactobacillales were resistant to the antibiotics and retain all the basic physiological properties at the same time. International health organizations recommend the wise use of antimicrobial agents for food safety i.e. minimize drug toxicity and sustainable formation of a product. For this purpose, it is necessary to improve the composition of probiotics, prebiotics or to use dairy products that heal intestinal flora and limit the colonization of intestinal pathogenic microorganisms.

Such natural lactic acid bacteria from a natural starter have been distinguished [2]. They have differed by various sensitivity to different classes of antibiotics. Researched natural leaven and fermented dairy products have contained resistant microbiological culture of varying sensitivity (Figure 7).

![Figure 7. Resistance of selected cultures of lactic acid bacteria](image)
Thus, the research of complex natural starters has both scientific and great practical interest as the symbiotic community of prokaryotes called eukaryotes.

6. Conclusions
The strains of lactic acid bacteria of the natural products keep their activity and vitality for a long time. They have increased proteolytic activity as well.

Redox conditions determine:
- the formation and localization of lactic acid bacteria and yeast through the micro areas;
- the biosynthesis of the pigment;
- morphological and physiological - biochemical properties.

During the selection of valuable productional cultures of lactic acid bacteria with high biochemical activity strains should be isolated from natural starter cultures from different geographical areas in Russia.

The composition of the new starter cultures will have to consist mainly of the strains with high proteolytic activity, increased resistance to antibiotics, the ability to synthesize bacteriocins and stable biochemical activity.

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