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A research on existence and special activities of *Acinetobacter* in different cheese

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ABSTRACT

The objective of this study was to identify *Acinetobacter* in a traditional cheese from west Azarbaijan, Iran, and to study the lipolytic and proteolytic activities of these bacteria in ripening of white brine cheese. *Acinetobacter* is a gram negative bacteria which can be found in different sources as dairy products. Lypolysis and proteolysis characteristic of this bacteria and thus its role in creating special odor and taste in traditional cheeses was proved in previous researches. A different province in Azarbaijan, Iran has its own traditional cheese, namely poosti, lighvan and koozeh cheese. Koozeh cheese is a traditional cheese of west Azarbaijan which spends about 3-6 months underground before consuming. In this research the bacteria was extracted and identified from Koozeh cheese from west Azarbaijan and was applied in specified amount and in three different formula as starter bacteria in manufacturing white brine cheese. Biochemical and Microbiologic methods (such as catalase, oxidase, KOH test, Protease and Lipase test, measuring TN, NPN and SN, Lypolysis) were applied. Based on significance of the results obtained from the research, it was revealed that this bacteria has suitable lipolytic and proteolytic activities and it can be used with other bacteria in cheese production in industry in order to achieve fermented cheese with suitable odor, texture and taste like the traditional products.

Key words: *Acinetobacter*, Koozeh cheese, Lipolytic, Proteolytic

INTRODUCTION

Cheese has been largely used as one of the most important sources of calcium in people's diet. Among varieties of traditional cheeses in Iran, Koozeh cheese is considered important in Iranian dishes because of acceptance of its special odor and taste among local people. Koozeh cheese is kept in plastic or pottery container for a period of 3-6 months under ground and in an unaerobic condition for ripening (HessamiNejad and Khosroshahi, 2006). Salt is used as a proteolytic agent, and it is also employed to keep the food healthy due to its anti-bacterial activity. In addition, it provides taste and odor and helps some enzymes to destitute through cheese texture (Geurts, Walstra, and Mulder, 1980). According to the surveys conducted recently, there are proteolytic and lipolytic factors that play important role in creating odor and taste in traditional cheese (Vicente,

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Ibanez, Barcina and Barron, 2001). In separating different traditional cheese flora, special species of different flora were obtained as well as *Acinetobacter* in addition to Lactic acid bacteria. It is identified that *Acinetobacter* has a role in creating special odor in such cheese types and also to improve proteolytic and lipolytic activities (Roseline , Regina, Ayodeji, 2006). Sable, Portrait, Gautier, Letellier, and Cottenceau (1997) reported that among extracted species from French cheese prepared of goat raw milk, the *Acinetobacter* percentage in cheese compared with other species was accounted as 3.2%. Larpin, Bonaïti, Bora, Gelsomino, Goerges, and Desmasures(2004) studied Smear Livariot cheese and identified 450 species of bacteria which among *Acinetobacter* was accounted as cheese's flora profile. Some species of *Acinetobacter* were found from two traditional dairy products of Nano and Wara (Roselin et al, 2006), which are known to cause ropiness of milk and secretion of extracellular enzymes both at psychrophilic and mesophilic temperatures. In a research by Fuka, Engel, Skelin, Redžepović, and Schloter (2010) some species of *Acinetobacter* were characterized which had some secondary activities in the process of Isterian cheese and it was revealed that they interfered in the taste of such cheese as a result of Citrate catabolism and their lipolytic and proteolytic activities. *Acinetobacter* is a gram negative, non motile, Oxidase negative, Nitrate negative bactria which belongs to Gammaproteobacteria and occur in pairs under magnification. They are important soil organism where contribute to mineralization and create aromatic compounds. Species of the genus *Acinetobacter* are strictly aerobic nonfermentative gram-negative bacilli. They show preponderantly a coccobacillary morphology on nonselective agar (Dijkshoorn, L.2008).

MATERIAL AND MTHODS

15 samples of Koozeh cheeses (150-250 gr each) were collected from 3 different varieties (namely Mahabad, Bookan, and Salmas) from local markets in Urmia City during winter and fall sessions. Koozeh cheese samples were collected using sampling sterile plastic and were kept in 4^0C refrigerator till the time of experiments. Nutrient agar culture media (Micromedia, JÓZSEF KÖZ 4. HUNGARY, MM0073) was used to identify and separate *Acinetobacter* species. Chemicals such as Hydrogen peroxide, N, N, N´, N´ Tetramethyl-P-Phenylen di-amin, Granul Hydroxide Potassium, Rodamin B., NaoH, Chloridric Acid, Trichloro Acetic Acid, Hydrated Calcium Chloride,Tween 80, Olive oil, Tributyrin, Skimmed milk, Sodium Citrate, Kjeldal catalyzer tablet and Sulfuric Acid were used.

Microbial and Biochemical testing Methods

Using physiological serum, different dilutions of cheese samples were prepared. For the first dilution 10g of cheese and 90 cc of physiological serum were used but for the rest of dilutions only 1g of cheese and 9cc of serum is needed, and the procedure were repeated to reach a final 10^{-6} dilution. Then the tubes were properly shaken to homogenize the dilutions. Finally, 1 cc from each container was transferred into petridishes 2/3 full with nutrient agar. Nutrient agar solution was prepared following the instruction recommended by the manufacturer, sterilized in an autoclave at 121^0C and 1.5 bars, and finally kept at 48^0C water bath until it is transferred to petridishes (Iran standard, No 356). The nutrient agar solution was transferred, before setting, into petridishes and let them set there. Different dilutions of cheese samples were transferred into petridishes. Next, they were put into the incubators for 48 h at 30^0C. In order to identify and separate *Acinetobacter* species, some biochemical tests, namely, oxidase, catalase, KOH, protease and lipase tests were conducted. Following morphological (eggshape appearance under microscope) and biochemical (the result of catalase, oxidase, proteolysis and lipolysis activities) confirmation and studying the

lipolytic and proteolytic activity of this bacteria, *Acinetobacter* bacteria were chosen and kept in slant Nutrient Agar at 4^0 C and species No. 9, with the highest amount of proteolytic activity, was employed in manufacturing of some types of Iranian white brine cheese as below. Three different types of white brine cheese were prepared namely 1) cheese made of 0.04 gr/lit starter mesophilic bacteria (Choozit feta B, danlac,Canada) 2) cheese made of 10 CFU/gr *Acinetobacter* species (Species No. 9- Using Mcfarland method), and 3) cheese made of both the starter and *Acinetobacter* species as the amount stated above for both (Species No. 9) Considering that based on the information of the manufacturer for starter bacteria used, it was required to add them in gram as 0.04 gr/lit to reach the specified mentioned CFU since it was in powder form. After drainage, the cheese samples were kept for storing and ripening at 4^o C temperature of refrigerator, in sterilized and air-tighted containers and then lipolytic and proteolytic procedure in these cheese types were studied during a peiod of 60 days. Nitrogen and free fatty acids were measured every 15 days. The kjeldal procedure was applied to measure nitrogen in the samples, and using different formula would reach us to total nitrogen, non protein nitrogen and soluble nitrogen.To measure free fatty acid, anhydride sodium sulphate and ethanolic KOH were applied.

Statistical Analysis

One way ANOVA test at significant level of 0.05 was used to study the significance of the factor effects using SPSS software.

RESULTS AND DISCUSSION

Following confirmation of biochemical tests, it was revealed that 86.5% of cheeses from different regions in autumn and winter contained *Acinetobacter*. Collins, McSweeneyb, and Wilkinson (2013) identified *Acinetobacter* in another traditional cheese which was influential in creating special odor, taste and texture in cheese. Roselin et al. (2006) worked on two local dairy products such as Nono and Wara and identified *Acinetobacter* that caused secretion of some extracellular enzymes. Larpin et al. (2004) identified 450 bacteria on Smear Livariot cheese in France and announced *Acinetobacter* as one of these separated species. Fuka et al. (2010) separated some species of *Acinetobacter* which had some secondary activities in Isterian cheese ripening interfering in Citrate catabolism and lipolytic and proteolytic activities. Sable et al. (1997) studied on French ripened cheese from goat cheese and concluded that *Acinetobacter* species were accounted as 3.2% comparing to other separated species. Marrakchi, Tantaoui-Elaraki, Hamama, and Grini(1988) in a study considering specific flora involved in the lipolysis of smen, a kind of traditional Morrocan cheese, during ripening reported that *Acinetobacte*r, Pseudomonas and Flavobacterium spp. predominated the micro-flora of smen during the early phases of ripening, and then they are rapidly outnumbered by other bacteria species. Separating *Acinetobacter* from traditional cheese of west Azarbaijan has not been yet reported and neither proteolysis nor lipolysis process of such bacteria from mentioned cheeses. Based on the results obtained all separated species were catalase positive, oxidase negative and didn't have any motility under microscope and had Coccobacillus (egg shape) morphological figure under the microscope(Table 1). In KOH test, all the tested species had adhesive property.

Table1: Experiments done on different species of separated *Acinetobacte*r

Shape	TestProtease	Oxidase	Gram test	Motility	Catalase	Species
Coccobacillus	*	Negative	red	mobile	Positive	*Acinetobacter B1*
Coccobacillus	*	Negative	red	immobile	Positive	*Acinetobacter B2*
Coccobacillus	**	Negative	red	immobile	Positive	*Acinetobacter B3*
Coccobacillus	**	Negative	red	immobile	Positive	*Acinetobacter B4*
Coccobacillus	**	Negative	red	immobile	Positive	*Acinetobacter B5*
Coccobacillus	**	Negative	red	immobile	Positive	*Acinetobacter B6*
Coccobacillus	***	Negative	red	immobile	Positive	*Acinetobacter B7*
Coccobacillus	**	Negative	red	immobile	Positive	*Acinetobacter B8*
Coccobacillus	***	Negative	red	immobile	Positive	*Acinetobacter B9*
Coccobacillus	*	Negative	red	immobile	Positive	*Acinetobacter B10*
Coccobacillus	NO	Negative	red	immobile	Positive	*Acinetobacter B11*
Coccobacillus	**	Negative	red	immobile	Positive	*Acinetobacter B12*
Coccobacillus	**	Negative	red	immobile	Positive	*Acinetobacter B13*

No. of * shows the intensity of Proteolysis activity.

In this research, all separated species appeared red in gram staining which showed their gram negative characteristic and this place these bacteria in Moraxellaceae family. But in Bergey's systematic bacteriology, this bacteria belongs to Neisseriaceae with species as *Acinetobacter* Calcoaceticus which divides to sub-species as anitratus (former name Herellea vaginicola) and lwoffi (former name Mima Polymorpha). Vicente, Ibanez, Barcina, and Barron(2001) and Madrau, Mangia, Murgia, Sanna, Garau, Leccis, Caredda, and Deiana(2006) studied proteolytic and lipolytic activities of *Acinetobacter*and also conducted a research on creating special odor and taste as a result of proteolytic and lipolytic activities. In this regard, in studying Lipolytic activity of *Acinetobacter*, little lipolytic activity was observed when applying 0.01% hydrated calcium chloride and 1% Tributyrin, as only B7 and B8 species showed very little activity(small halo around colonies) (Suzuki, T., Nakayama, T., Kurihara, T., Nishino, T., and Esaki, N. 2001). However, proteolytic activity of these bacteria appeared to be rather appropriate (Table1) as the highest activity belonged to B9, which was used as a selective species to be used as starter for cheese making. After biochemical and morphological confirmation and studying the lipolytic and proteolytic activity of this bacteria, B9 species among other *Acinetobacter* colonies was chosen because of its higher proteolytic activities and kept at 4^0 C in refrigerator and was used later in making Iranian white brine cheese. Lipolysis and fatty acids oxidation regarded as one of the most

important reaction in hard cheeses. Meanwhile, proteolysis has remarkable role in creating odor and taste in cheese which is the result of making free peptides and amino acids that are aromatic material such as amines, acids, tiols, tioesters. First, stored caseins in the curd would be changed to large and medium size peptides during cheese making and ripening as a result of rennet and natural plasmin in milk. More proteolysis would lead to small peptides and free amino acids due to peptidases of starter lactic acid bacteria (Khosroshahi et al., 2006). There are proteolytic factors in cheese namely: Coagulant (such as chymosine and pepsin), internal proteinases of milk like plasmin, enzymes from starter and non-starter and secondary cultures and also exogenous proteinases. These factors create proteolysis activity in cheese(Beresford et al., 2001) and thus develop favorable odor and taste in cheese which would be destroyed under pasteurization heat, however they remain safe in traditional cheeses(Buffa et al., 2001). In addition as mentioned above, some microorganism show lipolytic activities which can either exist in milk or would be included as secondary microflora of cheese making milk which are responsible for triglycerides hydrolysis to di- and mono- glycirides, fatty acids and glycerols(Atasoy et al., 2008). Before this research, a test conducted by Hesamie, Nejad, and Khosroshahi(2006) worked on *Ecoli.* in traditional cheese of west Azarbaijan and confirmed its existence in such cheese. As was described, three different brine cheeses were produced using the extracted *Acinetobacter.*The results showed that in primary proteolysis of cheese displayed by SN/TN%, *Acinetobacter* did not show an active role. The $p<0.05$ showed the significant relations between cheeses with different starters. Meanwhile, starter bacteria along with rennet and the enzyme of the milk itself had a higher proteolytic activity compared to that of *Acinetobacter* itself. In cheese with a mixed starter bacteria a lower level of proteolysis was seen compared to the starter bacteria alone which may showed an antagonism effect of *Acinetobacter* in the first step of proteolysis.

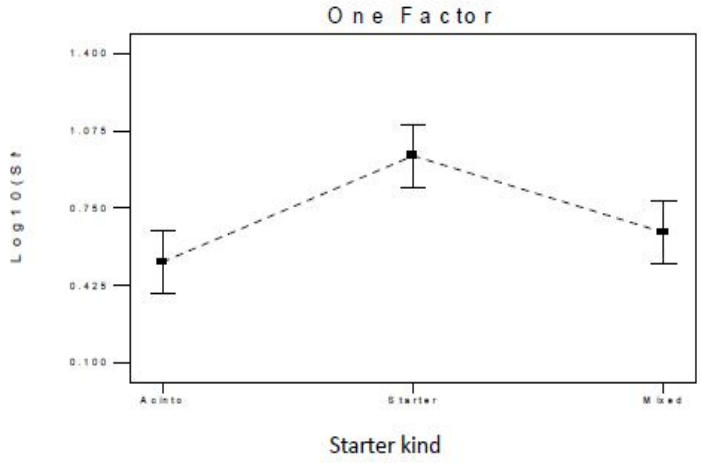

Figure1-Comparison for SN/TN ratio in cheese resulted from *Acinetobacter,* Starter and mixed

However, the results obtained from secondary proteolysis in cheese shown by NPN/TN%, revealed that *Acinetobacter* can be employed along with starter bacteria to create proteolytic effects in secondary part of cheese ripening. The $p<0.05$ showed the significant relations between cheeses with a variety of starters in the second step of cheese ripening. Cheeses with mixture starter showed proteolytic activities the same level as cheeses with only starter bacteria. It can be

concluded that *Acinetobacter* can have similar effects as starter bacteria in creating proteolytic impact during cheese ripening (Figure2).

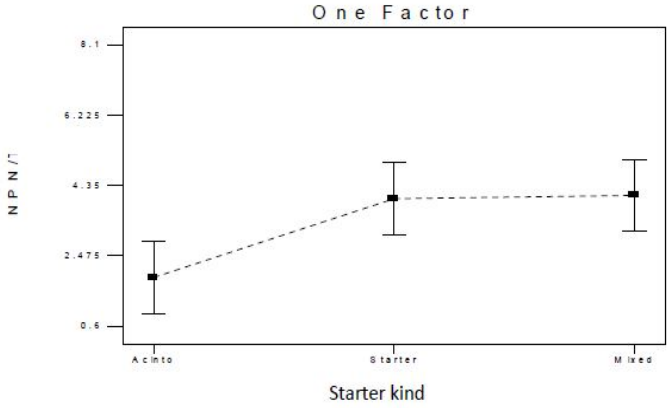

Figure2-Comparison for NPN/TN ratio in cheese resulted from *Acinetobacter*, Starter and mixed

In studying FFA which shows free fatty acid in miliequavalantgram unit every 100 gram of fat. The P<0.05 shows significant relations between all three types of cheeses with the mentioned starters. Although there were some delicate differences at the beginning of the ripening period but by reaching the end of this period no differences could be observed. Which can be inferred that lipolytic activity of *Acinetobacter* may increase by reaching the end of ripening period (Figure2).Thus, *Acinetobacter* may be applied along with other starter bacteria in cheese making.

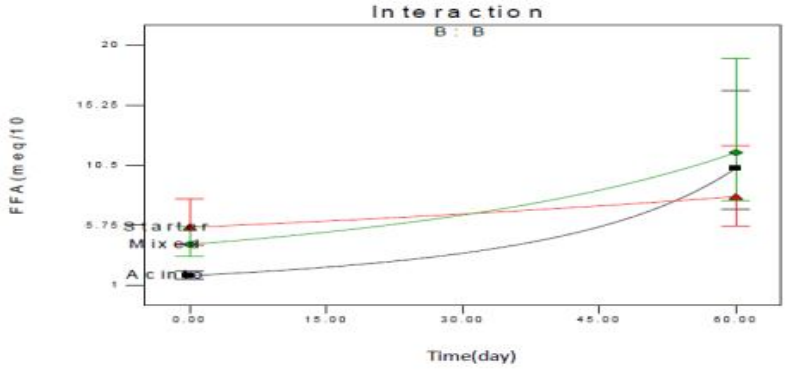

Figure3-Comparison for FFF (milliequivallant in 100 gr) in cheese resulted from *Acinetobacter*, Starter and mixed

Based on previous results of other researchers and according to the results obtained from the recent research, it can be concluded that *Acinetobacter* is among bacteria participating in creating special odor and taste in Koozeh cheese and it can probably be employed along with starter bacteria or as secondary microbial flora in ripened cheese with strictly secondary proteolysis. Fresh cheese has a mild acid taste and flavor which shows taste and flavor of ripened cheese during ripening. Taste and flavor of ripened cheese us a mixture of different flavors such as diacetyle in mild cheese,

butyric acid and caproic acid smelling, alcoholic ester, propionic acid and acetic acid salts in rather ripened cheeses and sharp ammonium flavor and in some cases hydrogensulfide in very old cheeses (Fox, P.F, Lucey, J.A, and Logan, T. M.1990). According to the results obtained, it can be inferred that *Acinetobacter* may be applied along with starter bacteria in cheese making. However, it should be mentioned that *Acinetobacter* in tradiotional Koozeh cheese which is prepared by non-hygienic method, can be entered cheeses from different ways as infected containers, infected air or hands, infected mamma and some other similar ways. Since the milk used in cheese making of this kind of cheese is only pasteurized so lightly, the bacteria would remain there in cheese and if they are not outnumbered by other bacteria, they can have an influential role in creating odor and flavor which can be implied by the experiments' results above. Anyway, based on the results of this research and mentioned conclusion of other researchers, *Acinetobacter* can be used in industry in producing industrial cheese while keeping the odor and taste similar to that of local cheese.

We hope that in future other features of this bacteria such as adhesive property and producing pigments in some of species of this bacteria would be studied in creating similar products according to customer‘s requirements.

CONCLUSION

Regarding proteolytic and lipolytic activities of proteolysis and lipolysis enzymes in creating taste and odor and also in order to make appropriate texture in cheese, and in addition to proving such activities of *Acinetobacter* which are resulted from different researches, and by obtaining the results of the present research, that shows the synergistic characteristic of *Acinetobacter* on lipolytic and proteolytic activities of other proteolysis and lipolysis enzymes, it can be stated that *Acinetobacter* is among the bacteria which are participated in creating granule texture and special odor and taste of traditional cheese of west Azarbaijan and it may probably be applied along with microbial starter bacteria or as secondary flora in ripened cheese with strictly secondary proteolysis activity.

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