Antimicrobial Activity of Sheep Yoghurt Prepared by Different Commercial Starter Strains

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ABSTRACT

The antimicrobial activities of sheep milk yoghurt prepared by different starter strains were evaluated against different pathogenic microbial strains using different techniques. Sheep yoghurt prepared with LAB starters YC-183 and probiotic starter ABT-3, manifested anti-*Staphylococcus aureus* activities. Most antimicrobial activities were associated with yoghurt soluble proteins fraction and originating from high molecular weight peptide fragments (more than 3000 Dalton) if the milk yoghurt was pretreated at 85°C / 10 min, but from low molecular weight peptides (lower than 3000 Dalton) if the milk used was pretreated at 96°C/ 5 min. Yoghurt prepared by all starter cultures exhibited antimicrobial activity against *E.coli* HB101. This activity was more pronounced in the casein fraction of the yoghurt prepared after heating at 96°C/5 min especially those prepared with ABT-3 culture. Comparable or slightly lower activities were associated with soluble protein fractions separated from different starter-yoghurt prepared from sheep milk preheated at 85°C/10 min which loosed their activities after ultrafiltration through 3000 Da membrane. This inhibitory action was particularly evident against *Staphylococcus aureus* and *E. coli* HB101. An antibacterial peptide against *E. coli* which generated from β-casein by the proteolytic system of starter culture of yoghurt was isolated and this peptide has a molecular weight 4.6KDa.

Key words: sheep milk; yoghurt proteins; bioactive activity; antibacterial peptides; pathogenic bacteria.

INTRODUCTION

Antimicrobial activity of milk is mainly associated with minor whey proteins, namely lactoferrin. This protein has bacteriostatic and bactericidial properties attributed to its ability to chelate iron or to bind to bacterial surfaces. Tomita *et al.* (1995) found that pepsin digestion of bovine lactoferrin produces potent bactericidial peptide, and that the antimicrobial potency of hydrolysate was higher than that of undigested lactoferrin. Dionysius and Milne (1997) have identified two peptides from the N-terminal of lactoferrin which displayed antimicrobial activity toward a number of pathogenic and food spoilage microorganisms. These results indicated that the bactericidial mechanism is independent of iron because the identified peptides are distinct from the iron-binding site of the molecule. It is possible that the active peptides have an affinity for the bactericidal cell surface and act by disrupting the essential membrane functions. No effect has been detected against *Bifidobacterium*, therefore lactoferrin derived peptides may positively affect the intestinal flora. δ_{s1}-casein f1-23 obtained from chymosin hydrolysis has been shown to have antibacterial activity against *Satphylococcus aureus* and *Candida albicans* (Lahov and Regelson, 1996).

The yoghurt and different fermented acidophilus milks have been considered as healthy probiotic diets due to viable lactobacilli, which could inhibit food borne enteric pathogenic microorganisms by producing lactic acid or antimicrobial substances. Moreover, milk proteins are considered as a well balanced dietary protein source for infants, athletes and clinical formulas. Bioactive peptides are encrypted in milk proteins and are only released by enzymatic hydrolysis *in vivo* during gastrointestinal digestion, food processing or by microbial enzymes in fermented products. A variety of naturally formed bioactive peptides have been found in fermented dairy products, such as yoghurt, sour milk and cheese. In particular, antihypertensive peptides have been identified in fermented milks, whey and ripened cheese. Some of these peptides have been commercialized in the form of fermented milks. Bioactive peptides have the potential to be used in the formulation of health-enhancing nutraceuticals, and as potent drugs with well defined pharmacological effects (Haque & Chand, 2006). Bioactive peptides have been defined as specific protein fragments that have a positive impact on body functions or conditions and may ultimately influence health. Upon oral administration, bioactive peptides, may affect the major body systems—namely, the cardiovascular, digestive, immune and nervous systems. The beneficial health effects may be classified as antimicrobial, antioxidative, antithrombotic, antihypertensive, antimicrobial or immunomodulatory (Korhonen & Pihlanto, 2003, and Matar *et al.*, 2003). The activity of these biofunctional peptides is based on their inherent amino acid composition and sequence. The size of active sequences may vary from two to twenty amino acid residues, and many peptides are known to have multifunctional properties (Meisel & FitzGerald, 2003). Peptides from

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the sequence 60-70 of β-casein show immunostimulatory, opioid and angiotensin-I-converting enzyme-inhibitory activities. Hydrolysis of ovine and caprine lactoferrin by pepsin resulted in antibacterial hydrolysates (Qian *et al.* 1995, Recio *et al.* 2000). However, no much research was conducted on the bactericidal activity of different sheep protein components or their enzymatic hydrolysates.

The objective of the present work was to study the effects of heate treatment of yoghurt milk and the starter cultures on the growth inhibition and behaviour of some pathogenic bacteria. Also, isolation amd characterization an antibacterial peptide which is generated from β-casein by the proteolytic system of starter culture of yoghurt.

MATERIALS AND METHODS

Starter cultures

Two starter cultures obtained from (Christian Hansen, Denmark) were used for the preparation of yoghurt samples. The LAB starter was Yo-flex culture (YC-183) which containing *Streptococcus thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus*. The probiotic culture ABT-3 was consisted of mixed strains of *Streptococcus thermophilus* TH4, *Lactobacillus acidophilus* LA5 and *Bifidobacterium bifidum* Bb12.

Pathogenic bacteria strains

The antibacterial activity of yoghurt was evaluated against seven strains of pathogenic bacteria: *Escherichia coli* HB101 was obtained from the Institute of Microbiology of the Academy of Sciences of Bulgaria; *Escherichia coli* 812 *Streptococcus mutans* 103220T, *Staphylococcus aureus* 9973 and *Salmonella enterica* 5858 were obtained from the collection of Institute Pasteur (Paris, France), *Bacillus subtilis* 6633 was from the American type culture collection and *Listeria innocua* R1007 was obtained from ENITIAA, Nantes, France.

Manufacture of yoghurt

Raw sheep milk was obtained from healthy animals at the dairy farm of Faculty of Veterinale, Zagazig University, and defatted by centrifuging at 3000xg /30 min at 30°C. Then skimmed sheep milk was heated at 85°C/ 10 min or 96°C/ 5 min. Yoghurt was prepared according to the methods of Anifantakis (1990).

Antibacterial activity of yoghurt fractions

Preparation of yoghurt fraction

One mL of yoghurt was mixed with 4 mL sterile distilled water and the pH was readjusted to 4.6 with 1N HCl, then non soluble yoghurt fraction was removed by centrifuging at 6000xg at 4°C / 30 min. Separated casein was rediscovered in 5 mL distilled water and the pH was adjusted to 4.5, 5.0, 5.5, 6.0 and 6.5 with NaOH before drawing with 80 µL to evaluate the antimicrobial activity using the surface diffusion technique.

Determination of antibacterial activity

The antibacterial activity was estimated by following the bacterial growth by means of optical turbidity in the liquid media and by observing the inhibition zone in the solid media around the applied substance according to Chevalier *et al.*, (2001).

Liquid media technique

A pre-culture of 24 hrs of each studied pathogenic bacteria was prepared using Brain Heart Infusion media BHI (Oxoid, Hampshire, England). An aliquot of 50 µL of this pre-culture was mixed with 50 mL of sterilized BHI media in a 250 mL Erlenmeyer flask and the mixture was kept at the optimal temperature of each studied bacterial strain under continuous agitation until the optical absorption at *600 nm* of the media reached 0.5 using a spectrophotometer 6405 UV/Vis (Jenway LTD, Felsted, Dunmow, Essex, UK). Then 4 mL of the obtained suspension was added to each of 5 test-tubes containing 80µL of the tested substance (5mg mL^{-1}), so that final substance concentration was 100µg mL^{-1}. The five tubes were maintained at the optimal temperature for every studied strain for 0, 1, 2, 3 and 4 hours, respectively. The optical absorption at *600 nm* was measured and taken as an index of the bacterial growth where its reduction was considered as bacterial inhibition.

Solid media technique

Mass diffusion

An aliquot of 10 µL of a 24 hrs- BHI pre-culture of each pathogenic strains were mixed with 10 mL of sterile distilled water. The obtained suspension was mixed with 90mL of plate count agar (Oxoid, Hampshire, England) maintained at 55°C. The bacterium-containing medium was poured in Petri dishes and left to solidify at 45°C during 30 min. Wells of 5 mm diameter were induced in the solidified media using sterilized suitable tool. The amount of the tested substances (80 µL adjusted at different pH) were sterilized by passing through a 0.22 µm micro-membrane and applied in each well (in duplicate), then the Petri dishes were placed at 4°C during two hours for diffusion, followed by incubation at 37°C / 24 hrs.

Purification procedure

The studied yoghurt was centrifuged at 3000 xg/15min at 37°C, in order to be defatted. After that the caseins were removed by centrifugation at 10000 rpm / 1 h at 4°C. The received fraction was used in the purification of the peptide. Ammonium sulphate was added to a final concentration of 80 % w/v and stirred

/24 hrs. The precipitate was collected by centrifugation at 10000g for 60 min and redisolved in 2mL of 3M urea. After that, the pellet was applied on equilibrated with 5 volumes of 0,1% trifluoroacetic acid (TFA) in 10 % acetonitrile in water Hypersep C_{18} cartridge and eluted with different % of acetonitrile (10, 20, 60%). Every fractions were tested for antibacterial activity after convenient pH adjustment (see Assay for antibacterial activity).The active fraction (60%) was analysed by HPLC (Waters) using an analytical column Nucleosil C_{18}, (250 x 4,5 mm) and eluted with the following mobile phases: A (0.1% TFA in water) and B (0.09% TFA, in 80% acetonitrile, 19.91% H_2O) at a flow rate 0.6ml/min. Peptides were monitored spectrophotometrically at 220 nm. The fractions with highest anti *E .coli* and *Staphylococcus aureus* activity were mixed and evaporated on a Speed-vac concentrator. The rechromatography of the active fraction was done under the same conditions.

N-Terminal Sequencing Analysis

The N-terminal amino acid sequence was determined using an Applied Biosystem model 477A sequencer with on-line identification of the phenyl thiohydantoin derivatives. Reagents used for sequencing were purchased from Perkin - Elmer.

RESULTS AND DISCUSSION

Turbidity-based bacterial growth

Figs. (1-3) represent the relative growth of different pathogenic bacterial strains based on turbidity measurement (A_{600}) as subjected to 100 μg mL^{-1} of whole yoghurt, casein fraction and soluble protein fraction. Generally it can be noticed that the presence of the substance tested in the bacterial media did not inhibit the growth of *E. coli* 812, *Bacillus subtilis* 5265, *Salmonella centerica* 5858, *Listeria innocna* R1007, and *Salmonella mutants* 103220T during 4 hours of incubation at optimum temperature of each strain. On the contrary, they rather promoted the microbial growth of *E. coli* HB101 *and Staphyloccocus aureus* 9973 strains. This phenomenon can be explained by the fact that the milk, whole yoghurt, soluble protein fraction and casein of yoghurt added a high quality protein to the nutrients of the media. The whole yoghurt and the soluble protein fraction prepared with both starters (YC-183 and ABT-3), with relatively low or higher heat pretreatment of milk (85°C/10 min or 96°C/ 5min) showed inhibitory effect on the microbial growth of *Staphylococcus aureus* 9973 and *E. coli* HB101, but not on *E. coli* 812, *Bacillus subtilis* 5265 *Salmonella centerica* 5858, *Listeria innocna* R1007 or *Streptococcus mutants* 103220T. This inhibitory action appeared after 1 hour of incubation and lasted until the end of incubation after 4 hours. No distinct difference can be observed between the inhibitory potency of the two substances. Casein fraction of yoghurt prepared by different starters (YC-183 and ABT-3) exhibited antimicrobial activity against *Staphylococcus aureus* and *E. coli* HB101. This activity was more pronounced in the casein fraction after heat pretreatment of milk at 96°C/5 min with *E. coli*, contrarily after heated at 85°C/10 min with the strain *Staphylococcus aureus* (Fig. 2). The observed inhibitory action of the yoghurt, casein fraction and soluble protein fraction contrasted to inactivity of the sheep milk proteins may be due to the fact that the small peptide fragments can have more feasible access into the microbial membranes than the bigger-sized intact protein, and is in agreement with the conclusion of Schanbacher *et al.*,(1998). Alternatively, some released peptides may have particular peptide primary structure more suited to the antimicrobial action; it may have higher content of positively charged amino acid residues, hydrophobic amino acid residues or some amino acid residues of particular importance for the antimicrobial action (Pellegrini *et al.*, 1999, 2003).

Anti-Staphyloccocus aureus activity of sheep yoghurt

Fig.(4) represents the antimicrobial activities of sheep yoghurt against *Staphyloccocus aureus*. Whole YC-183 yoghurt showed slight antimicrobial activity (samples 4&5) while casein fraction extracted from this yoghurt manifested more pronounced activity (sample 6) where the heat pretreatment was 85°C/10 min. More pronounced antimicrobial activities can be observed with YC-183 yoghurt soluble protein fraction (samples 8&9). Sample 8 prepared after relatively preheat at 85°C/10 min seems more powerful than sample 9 prepared after preheated at 96°C/5 min indicating a negative effect of the higher heat treatment on the antimicrobial activity. Ultrafilteration of sample 8 led to the disappearance of the antimicrobial activities (sample 10), indicating that high molecular mass peptides more than 3000 Da, were responsible for this activity. On the contrary, ultrafilteration of samples 9 gave rise to sample 11, with retained antimicrobial activity suggesting that low molecular peptides fragments were responsible for the anti-microbial activities in this fraction (Vorland *et al.*, 1999). Similar results were obtained with ABT-3 yoghurt (Fig. 4) confirming that most antimicrobial activity is derived from whey proteins. This antimicrobial activity is originating from high molecular weight peptide fragments (more than 3000 Da) if the milk used for fabricating yoghurt was preheated at and 85°C/10 min as can be deduced from samples 8&10. Relatively pretreatment of milk at and 96°C/5 min concentrated the antimicrobial activities in

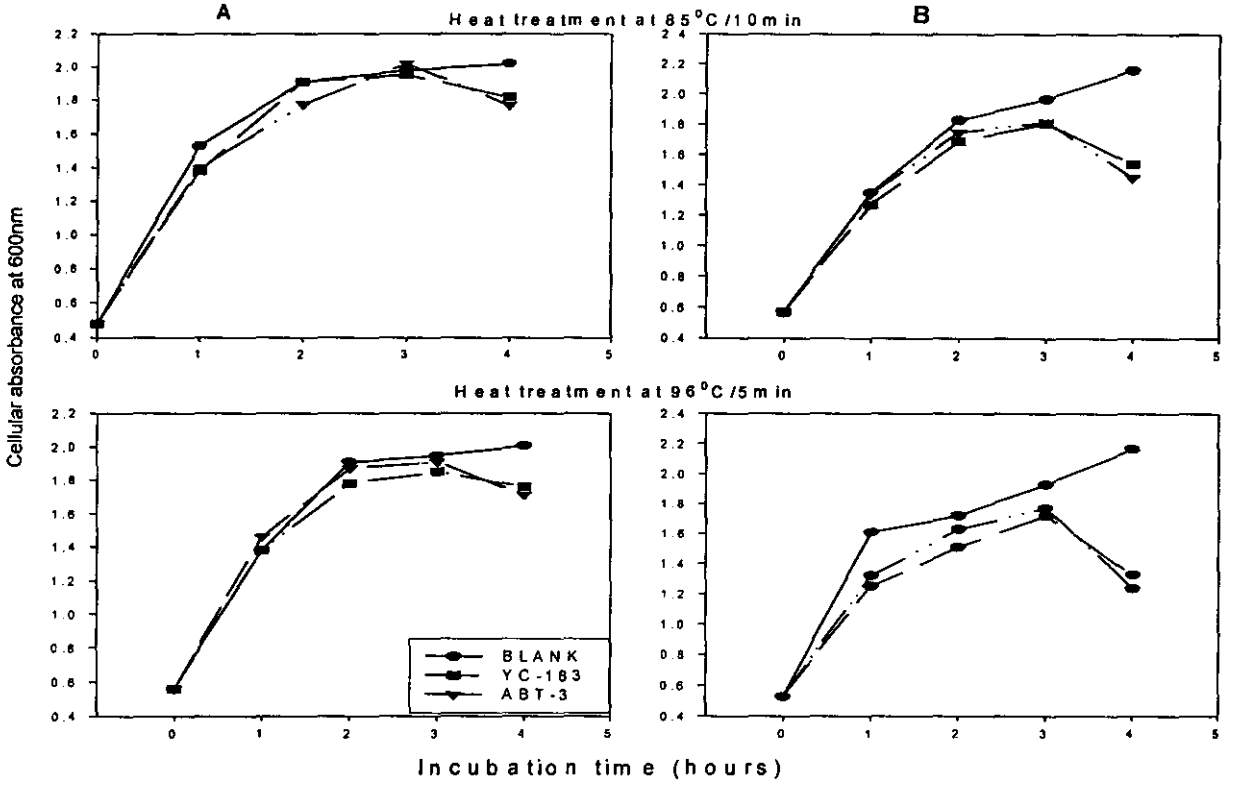

Fig 1. Antibacterial activity of whole ewe's yoghurt prepared by different starter culture against *staphylococcus aureus* 9973 (A) and *E. coli* HB 101 (B)

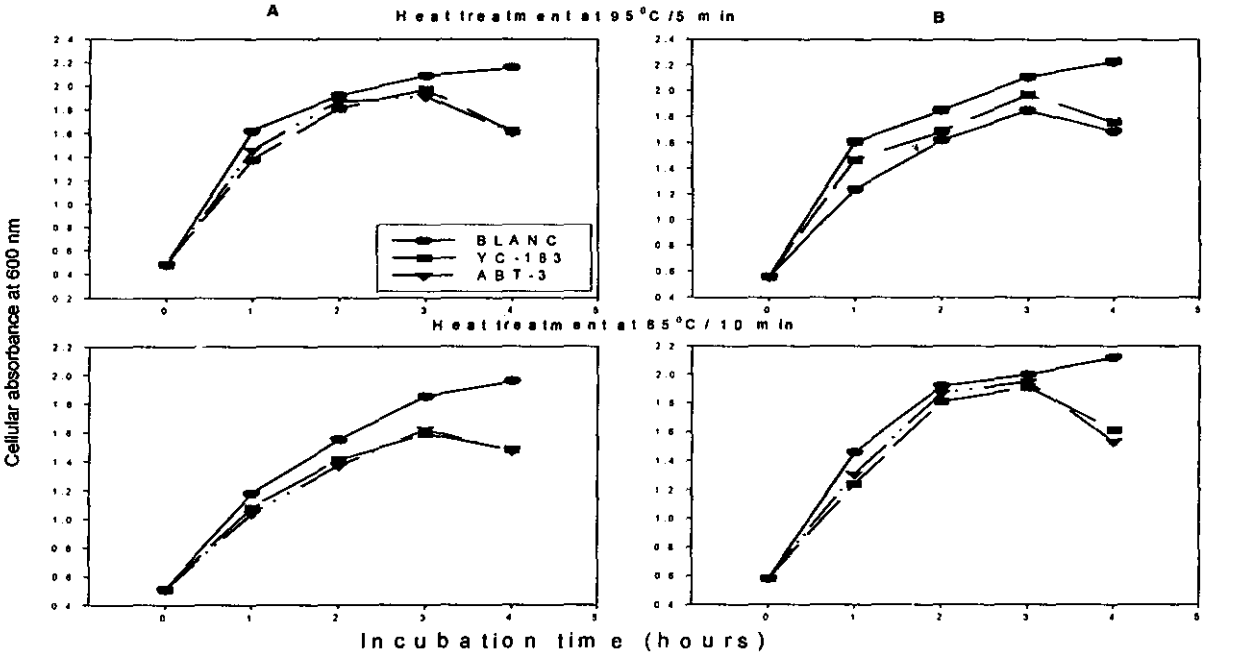

Fig 2. Antibacterial activity of non-soluble protein of yoghurt prepared by different starter cultures against *staphylococcus aureus* (A) and *E. coli* (B)

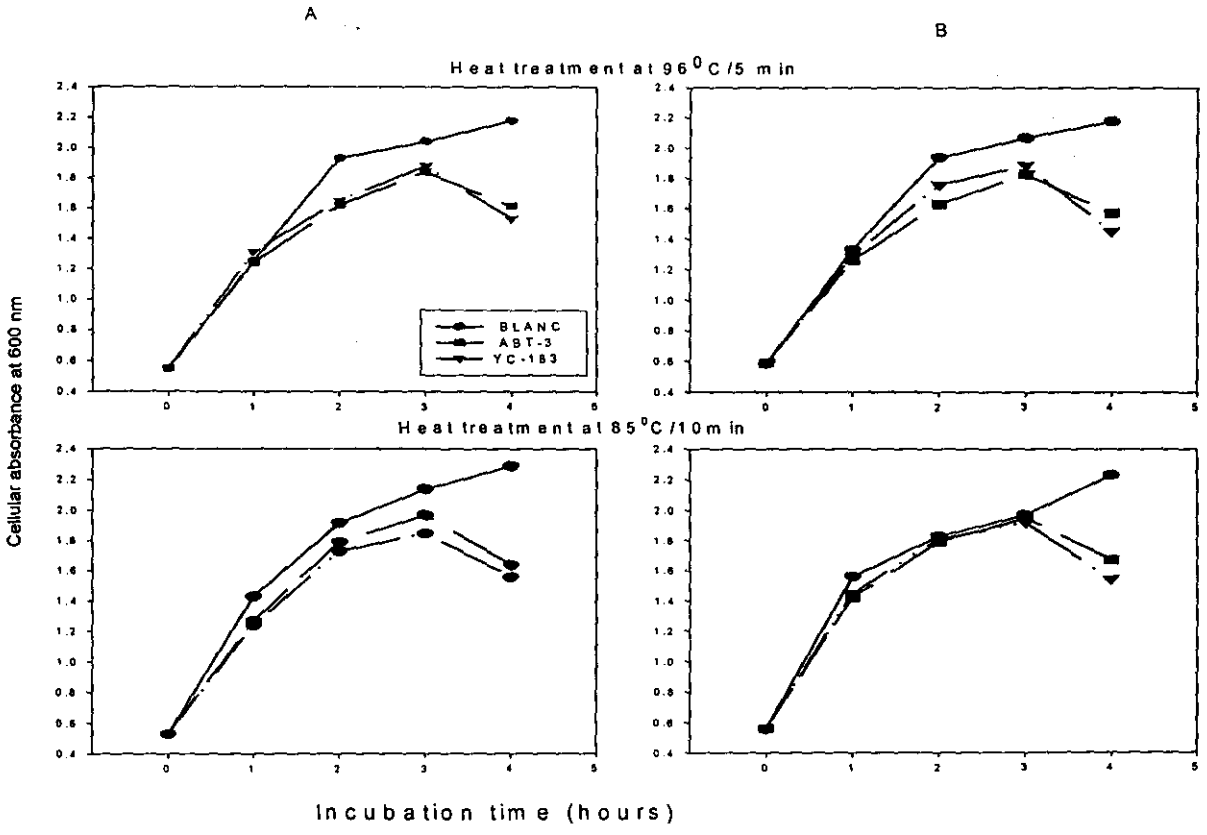

Fig. 3. Antibacterial activity of soluble protein fraction of yoghurt prepared by different starter culture againt *Staphylococcus aureus* (A) and *E. coli* (B)

ABT-3 Yoghurt YC-183 Yoghurt

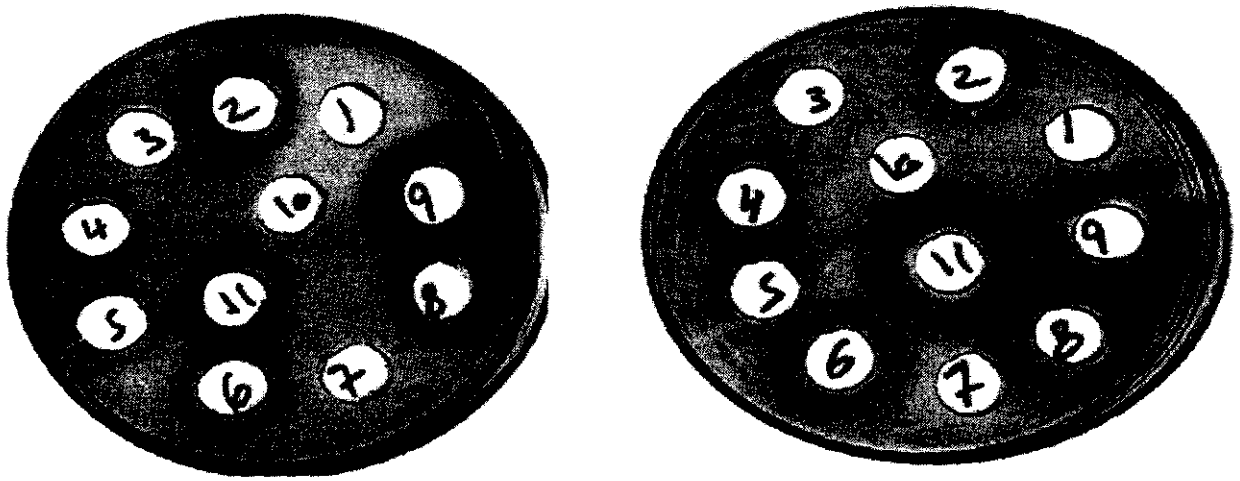

Fig. 4. Antimicrobial activity of ewe 's yoghurt against Staphylococcus aureus
Samples obtained from milk preheated at 85^{0}C/10 min (even numbers)
Samples obtained from milk preheated at 96^{0}C/5 min (odd numbers)
(1)Buffer pH 5.5, (2,3) milk; (4,5) whde yoghurt; (6,7) non soluble fraction; (8,9) soluble protein fraction with out filtration, (10,11) soluble protein fraction filtrated on menbrane 30000Da

the low molecular peptides (lower than 3000 Da) as the ultrafilterated samples (sample11) retained the antimicrobial activity of the non-ultra filtrated samples (sample 9).

Anti-E. coli activity of sheep yoghurt

The antimicrobial activity of sheep yoghurt prepared by different starters (YC-183 and ABT-3) was tested against *E.coli* HB101 and the results are shown in Fig. (5). It can be observed that whole yoghurt prepared by different starters exhibited antimicrobial activity against *E.coli* HB101. The antimicrobial activity of yoghurt was more pronounced in the casein fraction of the yoghurt prepared after milk heated at 96°C/5 min as manifested in samples 7 with ABT-3 yoghurt more tan with YC-183. Antimicrobial activities associated with casein fraction may reveal that casein fraction have some amino acid sequences with potential antimicrobial activities against *E.coli* HB101, which are triggered by the enzymatic action during yoghurt development. Specific enzymatic action exerted in case of ABT-3 may account for the more evident antimicrobial action against *E.coli* HB101 associated with these two yoghurt starters. Comparable or slightly lower activities are associated with soluble protein fractions separated from different starter-yoghurt preheated at 85°C/10 min as seen in samples 8, indicating that the higher preheated had negative effect on the antimicrobial activity of soluble fraction against *E. coli* HB101 as manifested in samples 9. This soluble protein fraction activity resides probably in high molecular weight peptides fragments since the ultrafilteration of this separated soluble protein fraction resulted in abolished antimicrobial activities (samples 10&11). This agrees with the fact that degradation of milk proteins during yoghurt processing occurs at a very limited degree of hydrolysis. This limited degradation may impart the released peptides with the hydrophobic nature required for the antimicrobial activity and may also dispose some cationic fragments that promote this activity to meet the requirements of antimicrobial activity (Wilde *et al.* 1989, Shafer *et al.* 1991, and Pellegrini *et al.* 1997, 2.03).

Antibacterial peptides isolated

Preliminary study of casein and whey fractions of all yoghurt samples for inhibitory activity of *E. coli* and *Staphylococcus aureus* showed that the whey fraction expressed more activity. Therefore, our attention to further purification of antibacterial activity was given to whey fraction. After the ammonium sulfate precipitation of whey fraction, the recovery of the antibacterial active substance was approximately 10%. Further, the precipitate was resolved in 2 M urea and was subjected to Hypersep C_{18} cartridge. The elution was carried out with step by step gradient at 10, 20 and 60 % acetonitrile; the active fraction was eluted at 60% acetonitrile. The active fractions were separated by HPLC on C_{18} reversed phase column. The eluted peaks were collected and checked for activity against *E. coli* and *Staphy. aureus*. For the anti *E. coli,* the result indicated that the activity corresponded to the peak eluted at 33.7 min, which consist from one major peptide and one or to minor peptides (Fig. 6a,b). The N terminal of major peptide was present below (Fig 7) and has a molecular weight of 4.6kDa according to Tricine SDS-PAGE and N-terminal sequencing. The research of identity of obtained amino acids sequences in the ExPasy Swiss-Prot data base shows that this peptide correspond to 57 to 99 residues of β-casein with theoretical pI 8.2. It is interesting to note that the found sequence of the peptide from 57 to 65 residues enter in the sequence of several casomormorphins and immunostimulating peptides (Meisel 1998). The residues 60 to 70 of bovine β-casein play an important biological role and could be considered as a strategic zone of β-caseins (Meisel and Schlimme, 1996). The peptides derived from β-casein have been shown to exhibit opioid activity (Clare and Swaisgood, 2000), antihypertensive (Yamamoto, 1997), immunostimulant (Laffineur *et al.*, 1996) but the antibacterial activity has not been previously described.

For the anti *Staphylococcus aureus* the result indicated that the activity corresponded to the same peak eluted at 33.7 min, which consists from one major peptide and tow minor peptides and the peak eluted at 25min (Fig. 6a,b). Often, we loose the antibacterial activity during the chromatoghraphical stage of the purification.

In conclusion, the observed inhibitory action of sheep yoghurt samples, casein fraction and soluble protein fraction of yoghurt contrasted to inactivity of the sheep milk proteins against *E.coli* HB101 and *Staphyloccocus aureus* may be due to the fact that the small peptide fragments can have more feasible access into the microbial membranes than the bigger-sized intact protein. Released peptides may have higher content of positively charged amino acid residues, hydrophobic amino acid residues or some amino acid residues of particular importance for the antimicrobial action. As a result of our study, dairy products can be considered as therapeutic food and raw material for preparation of biopeptides.

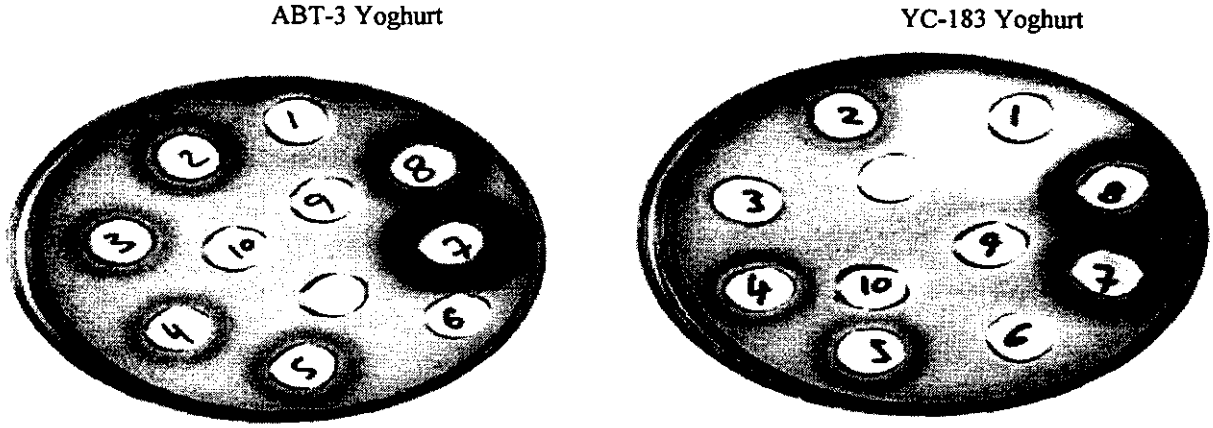

Fig. 5. Antimicrobial activity of ewe 's yoghurt against *E. Coli HB 101.*
Samples obtained from milk preheated at 85^0C/10 min (even numbers)
Samples obtained from milk preheated at 96^0C/5 min (odd numbers)
(1)Buffer pH 5.5; (2,3) milk; (4,5)whole yoghurt; (6,7) non soluble fraction; (8,9) soluble protein fraction with out filtration, (10,11)soluble protein fraction filtrated on membrane 3000Da.

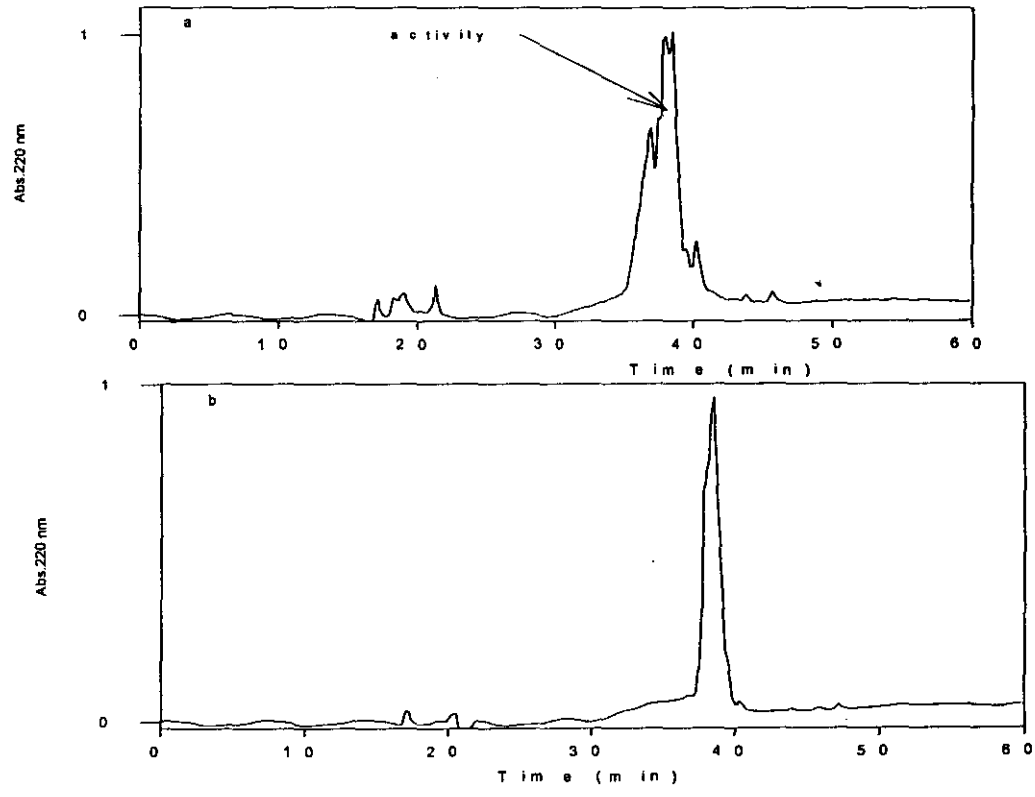

Fig.6a. RP-HPLC separation of 60% fraction active against *E. coli*
b. Rechromatography of the active peak

1 10 20 30 40 50
MKVLILACLV-ALALA REQEE - LNVVGETVES - LSSSE ESITH - INKKIEKFQS-
60 70 80 90 100
EEQQQTEDEL – QDKIHPFAQA – Q SLVYPFTGP-IPNSLPQ NIL – PLTQTPVVVP –
110 120 130 140 150
PFLQPEIMGV- PKVKETMVPK – HKEMPFPKYP- VEPFTESQSL - TLTDVEKLHL –
160 170 180 190 200
PLPLVQSWMH – QPPQPLPPTV- MFPPQSVLSL - SQPKVLPVPQ – KAVPQRDMPI -
210 220
QAFLLYQE PV - LGPVRGPF PI - LV

Fig. 7. -Sheep β -casein primary structure

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الملخص العربي

النشاط التثبيطى لليوجورت المصنع من لبن الأغنام المصنع باستخدام سلالات ميكروبية مختلفة

خالد مغاورى الزهار

تم تقييم التأثير المثبط لألبان الأغنام واليوحورت المصنع منها بأستخدام سلالات ميكروبية مختلفة لنموبعض الميكروبات المرضية المعروفة فى الأغذية والألبان, تم تقييمه من خلال استخدام طريقتين مختلفتين للتقييم. لوحظ التأثير المثبط لنمو الميكروبات المرضية لعينات اليوجورت ضد البكتريا العنقودية *Staphylococcus aureus* على وجه الخصوص مع شقوق البروتين الذائبة (الشرش) خصوصا الببتيدات ذات الوزن الجزئى الاعلى من ٣٠٠٠ دالتون الناتجة من اليوجورت المصنع من لبن معامل حراريا على درجات حرارة ٨٥ °م/ ١٠ق وكذلك الببتيدات ذات الوزن الجزئى الاقل من ٣٠٠٠ دالتون فى عينات اليوجوت المصنع من لبن معامل حراريا على ٩٦ °م/٥ق. عينات اليوجهورت المصنع من بادئات مختلفة (YC-183 , ABT3) كانت تتمتع بقدرة تثبيطية عاليه لميكروبات القولون المرضية*E. coli* HB10. وهذه القدرة التثبيطية كانت واضحة مع شقوق الكازين من عينات اليوجورت المصنع من لبن معامل حراريا على ٩٦ °م/٥ق خاصة العينات المصنعة باستخدام بادىء ABT-3 .وعند مقارنة النشاط الثبيطى لشقوق الكازين المفصولة من عينات اليوجورت المصنعة باستخدام البادئات الاربعة المختلفة من لبن الأغنام المعامل حراريا على ٩٦ °م/٥ق نجد انها تفقد قدرتها التثبيطية عند امرارها خلال أغشية الترشيح الفوقى(٣٠٠٠ دالتون). النشاط التثبيطى لعينات اليوحورت كان واضحاً بصفة خاصة ضد بكتريا القولون *E. coli* HB10 والبكتريا العنقودية *Staphylococcus aureus* وقليلا ما لوحظ هذا النشاط مع بقية الميكروبات المرضية محل الدراسة. وتعتمد القدرة التثبيطية للببتيدات على الجرعة المستعملة منها ضد كل انواع البكتيريا المرضية المدروسة. تم فصل احد الببتيدات من شق البيتا كازين لديه القدرة على تثبيط بكتيريا القولون ووزنه الجزيئ ٤,٦ كيلو دالتون، كما وجد ان الببتيدات المثبطة للبكتيريا العنقودية تفقد قدرتها التثبيطية عند محاولة فصلها بالطريقة الكروماتوجرافية.