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INFLUENCE OF FORMALDEHYDE AND HYDROGEN PEROXIDE AS PRESERVATIVES ON THE PROPERTIES OF BUFFALO'S MILK PROTEINS

BY

Fayed, A.E.*; Metwally, A.I.*; Osman, S.G. **; Hala A.Abd-El Rahman and Meranda A. Tawfek****

* Food Science Department, Faculty of Agriculture. Ain Shams University, Shoubra Kheima, Cairo, Egypt.

** Food Technology Research Institute, Agricultural Research Center, Giza, Egypt.

ABSTRACT

This work was planned to study the effect of formaldehyde (FA) and hydrogen peroxide (H_2O_2) on the coagulation, chemical and biological properties of milk proteins. Thus, raw buffalo's skimmilk samples (100 ml each) were spiked using FA (40%) or H_2O_2 at the level of nil (control), 0.025, 0.050, 0.100, 0.200, 0.300, 0.400 and 0.500%.

The obtained results indicated that the rennetability, curd firmness and curd synersis of milk were significantly weakened by FA more than occurred by H_2O_2. There was a negative relationship between the synersis rate and the preservative level. While, sephadex G100 chromatograms of liquid milk, acid and rennet curds indicated that, opposite to the effect of H_2O_2, the presence of FA led to an increase in the absorbancy measured for the caseins peak positively associated according to its level. That was at the expense of other two peak of whey proteins. However, the milk protein fractionation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the two curds confirmed that, although the level of αs_2- casein (Cn) was not affected, the levels of αs_1-Cn and β-Cn raised in the presence of FA and decreased due to H_2O_2 opposite to that behaved by γ-Cns. The SDS-PAGE electrogram of whey proteins residues remained in the curd exhibited no response whether to the kind or the level of preservative spiked.

Compositionally, the presence of 0.3% preservatives regardless their kinds, decreased significantly the levels of the total amino acids (T.A.A) whether essential or non essential AA.

Nutritionally, this decline in A.A. levels contributed to preservative adding resulted in significant reduction in the calculated percentages of protein efficiency ratio (PER), biological value (B.V) and net protein utilization (NPU). All nutritional aspects were higher in the protein of liquid milk followed by rennet curd, while the acid curd came in last order.

INTRODUCTION

Milk is a highly perishable commodity and difficult to handle, especially in countries with relatively high ambient temperature. Besides, unhygienic conditions under which the animals are milked, small quantity of milk which is delivered by individual producer, long distance between the production and market areas, poor transportation, insufficient or non-availability of milk cooling and chilling system are the main problems of milk collection. These problems lead to a considerable loss in keeping quality and hence reduction in the shelf-life of raw milk so that, it retains its quality for only six hours. Therefore, some milk producers illegally, whether qualitatively or quantitatively add some chemical preservatives to milk to destroy and/or inhibit the microflora, and hence to avoid the probable lacking in its keeping quality. Preservatives most commonly used are formalin (40% formaldehyde) and hydrogen peroxide (35%) for their high efficiency against many microorganisms, cheap prices and easy to obtain. Although they usually used with small quantities, they have bad effects on physical, chemical, technological and nutritional milk properties so hazardous on human health. For those reasons many countries considered using of preservatives illegal (Brunn and Klostermeyer, 1983; Pellegrino and Resmini, 1996 and Mohamed, 2002).

Formaldehyde is a highly reactive chemical which readily combines with deoxyribonucleic acid (DNA), ribonucleic acid (RNA), proteins and amino acids (Siomin *et al.*, 1973; Chaw *et al.*, 1980 and Hemminki 1982). Likewise hydrogen peroxide is considered as a safe preservative for milk and can be legitimately employed at levels between 100-800 ppm (FAO, 1957). But using higher amounts than are considered means being risk effective on health (Kosikowski, 1977 and Jha, 1984), where, some loss in biological value of proteins and availability of sulphur containing amino acids were recorded by Deodhar and Mehta (1980). For that in view, this study was planned to throw some light on some harmful effects on the buffalo's proteins with emphasis on the coagulation, composition and biological properties.

MATERIALS AND METHODS

Materials:

Fresh buffalo's milk was obtained from the herd of the Faculty of Agriculture, Ain Shams University, Maxiren 1800, (100% chymosin purified from *Kluyveromyces maxrianous* biovar. *Lactis*) made by DSM Jist, France, commercial formaldehyde solution (40%) and Hydrogen peroxide solution (35%) from Pfizer Co. Inc. U.S.A. were obtained from the local market at Cairo.

Experimental procedures:

Raw buffalo's skimmilk samples (100 ml each), were spiked using formaldehyde (FA, 40%) or hydrogen peroxide (H_2O_2, 35%) at the level of nil (control), 0.025, 0.050, 0.100, 0.200, 0.300, 0.400 or 0.500% and gently agitated for one min. Samples were left at the frig. temperature (5±2°C) for 1 h., after which they were analysed. Three replicates were carried out for every treatment.

Methods of analysis:

Milk cloting time was determined as described by Fahmi (1952). Curd firmness was measured using penetrometer model SUR, BERLIN, PNR6 as in

Bourne (1982). Syneresis of the resultant curd was measured according to Lawrence (1959). The gel filtration procedure described by Davies (1974) was adopted using sephadex G-100 for protein fractionation. Protein fractionation using sodium dodecyl sulfate (SDS) polyacylmide gel electrophoresis (PAGE) was carried out as described by Weber and Osborn (1969) for protein extraction and by Laemmli (1970) modified by Studier (1973) for SDS-PAGE preparation. Morever, SDS PAGE patterns were identified according to Eigel *et al.*, (1984) and Basch *et al.*, (1985). Amino acids other than tyrosine and tryptophan were determined using High Performance Amino Acid-Analyzer as described by Moore *et al.*, (1958) and Kirsten and Eggum (1966). Biological parameters namely PER (Alsmeyer *et al.*, 1974), BV and NPU (Mitchell and Block, 1946) were calculated. The obtained data were statistically analyzed as in SPSS (1998).

RESULTS AND DISCUSSION

Coagulation properties:

Data presented in Table (1) illustrated that the rennetability of milk was significantly weakened due to adding FA or H_2O_2 ($P< 0.001$) as indicated from the increase in RCT of milk. Similar findings were reported by Metwally (1985); Wahba *et al.*, (1986); El-Abbassy (1987); El-Batawy (1991); Ewais (1995) and Uraz and Yildirim (1995).

Moreover, data given in Tables (1 and 2) appeared also that, as the milk contains either FA or H_2O_2 led to delaying the coagulation and weakening the firmness of resultant curd, also retarding the curd wheying off ($p< 0.001$).

In this respect, the retarding effect of FA at any spiking level was higher than that occurred by H_2O_2 at the same level. Furthermore, the statistical interaction either between the kind and level of preservative or between the preservative type and the experimental period was significant ($P< 0.001$). while, that between the preservative level and experimental period was not significant ($P> 0.05$).

Sephadex G100-chromatogramic properties:

As shown from the elution pattern of milk proteins whether of beginning buffalo's milk or its acid as well as rennet curds as a function of the milk spiking level with FA or H_2O_2 (Figures 1 and 2), three means distinct protein fractions were obtained by gel filtration of Sephadex G100 for the genuine samples (control). The great peak (1) was of caseins while peak (2) was β- lactoglobuline (β-Lg) and peak (3) was α-lactalbumin (α-La). Similar observations were reported by Hill and Hansen (1964); Ganguli (1974); Poznanski *et al.* (1979) and Metwally (1985).

Concerning the corresponding peaks in relation to the kind and level of preservative added, it could be noticed that, there are gradual increases in the absorbancy measured for the mean peak, namely that of caseins, being associated with the proportional increment in the spiking level with FA. That was at the expense of other two peaks as seemed from Figure (1). Aoki and Kako, (1984) fractionated acid casein containing 10 and 20 mM FA through Sephadex G200 and found that, the formation of soluble casein was depressed by the addition of FA.

Table (1): Rennet clotting time (sec.) and penetration value (mm) indicating inversely the curd firmness of buffalo's skimmilk spiked with different levels of formaldehyde (FA) or hydrogen peroxide (H_2O_2) solution.

Property	Kind and level of preservative (%, V/V)														
	Nil		0.025		0.050		0.100		0.200		0.300		0.400		0.500
	Control	FA	H_2O_2	FA	H_2O_2	FA	H_2O_2	FA	H_2O_2	FA	H_2O_2	FA	H_2O_2	FA	H_2O_2
Rennet clotting time (sec.)	18	24	22	30	26	41	30	52	35	61	40	NC	45	NC	52
Penetration value (mm)	235	242	239	247	243	257	247	269	253	278	257	NC	262	NC	269

NC : Milk did not clot through 30 min.

Table (2): Curd synersis percent of buffalo's skimmilk spiked with different levels of formaldehyde (FA) or hydrogen peroxide (H_2O_2) solution.

Experimental time (min.)	Kind and level of preservative (%, V/V)										
	Nil	0.025		0.050		0.100		0.200		0.300	
	Control	FA	H_2O_2	FA	H_2O_2	FA	H_2O_2	FA	H_2O_2	FA	H_2O_2
15	35	31	36	29	33	28	28	21	28	14	25
30	42	38	43	35	41	35	37	28	36	20	33
45	46	41	48	39	47	39	42	32	40	24	38
60	48	44	51	42	51	42	45	36	43	27	41

On the contrary, there are descending relationships between the absorbancy of protein fractions and the level of H_2O_2 added to the milk, whether in the partition chromatograms of whole milk proteins given in Figure (1) or those of its acid-or rennet-curd illustrated in Figure (2). That could be attributed to the oxidizing effect of the H_2O_2 on the protein leading to decompose it forming smaller peptides as could be seemed surrounding the chromatograms of H_2O_2-treated samples (Figures 1 and 2). Where, some small peaks tended to appear near but with following orders to the main one. Uraz and Yildirin (1995) reported that, H_2O_2 treatment of milk led to increase the soluble nitrogen.

SDS-PAGE-electrophoregramic properties:

Data displayed in Figure (3) and Table (3) are the proteins fractions of buffalo's milk curd as affected by the kind and level of preservative added as well as the coagulation method applied whether acidic or enzymatic one.

The results of gel scan of the casein fractions involving α_{s1}-casein (α_{s1}-Cn), α_{s2}-casein (α_{s2}-Cn), β-casein (β-Cn) and its derivatives γ-caseins (γ-Cns) and k-casein (k-Cn) of genuine curds of buffalo's skimmilk are in coincidence with those found in buffalo's milk by Addeo *et al.* (1977); Jenness (1982); Ng-Kwai-Hang and Grosclaude (1992) and Abd El-Salam *et al.* (1992).

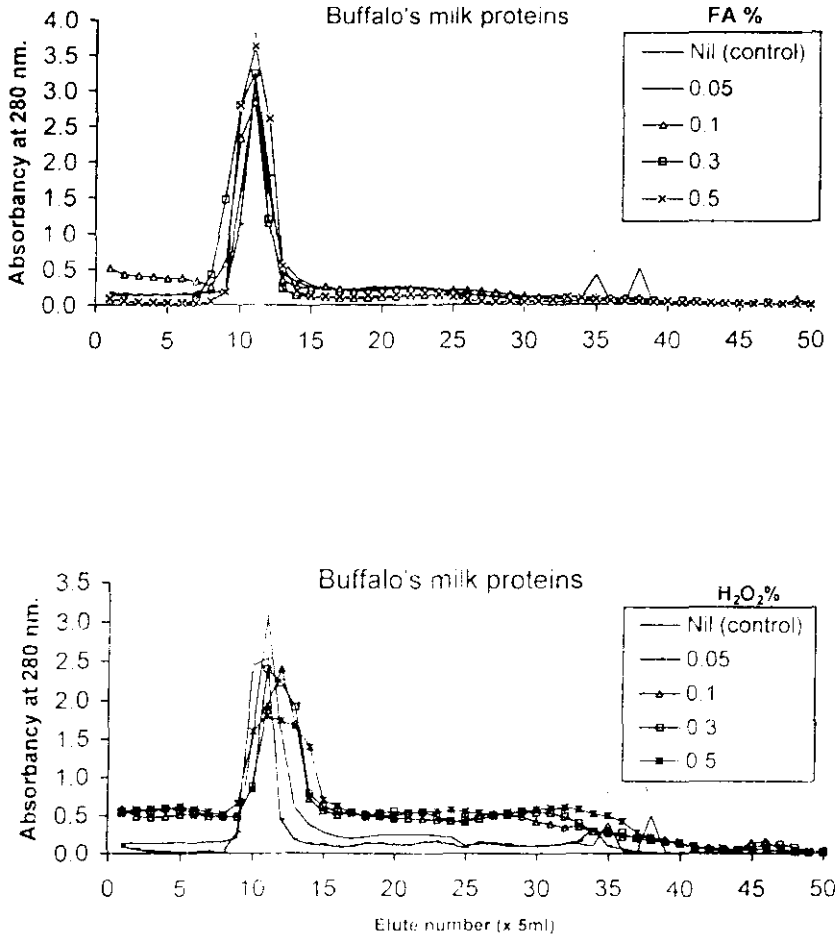

Figure (1) Partitation chromatograms on Sephadex G100 gel filtration of buffalo's milk proteins as a function of the level of formaldehyde (FA) or hydrogen peroxide (H_2O_2) solution added to it

1- Caseins 2- β-Lactoglobulin 3- α-Lactalbumin

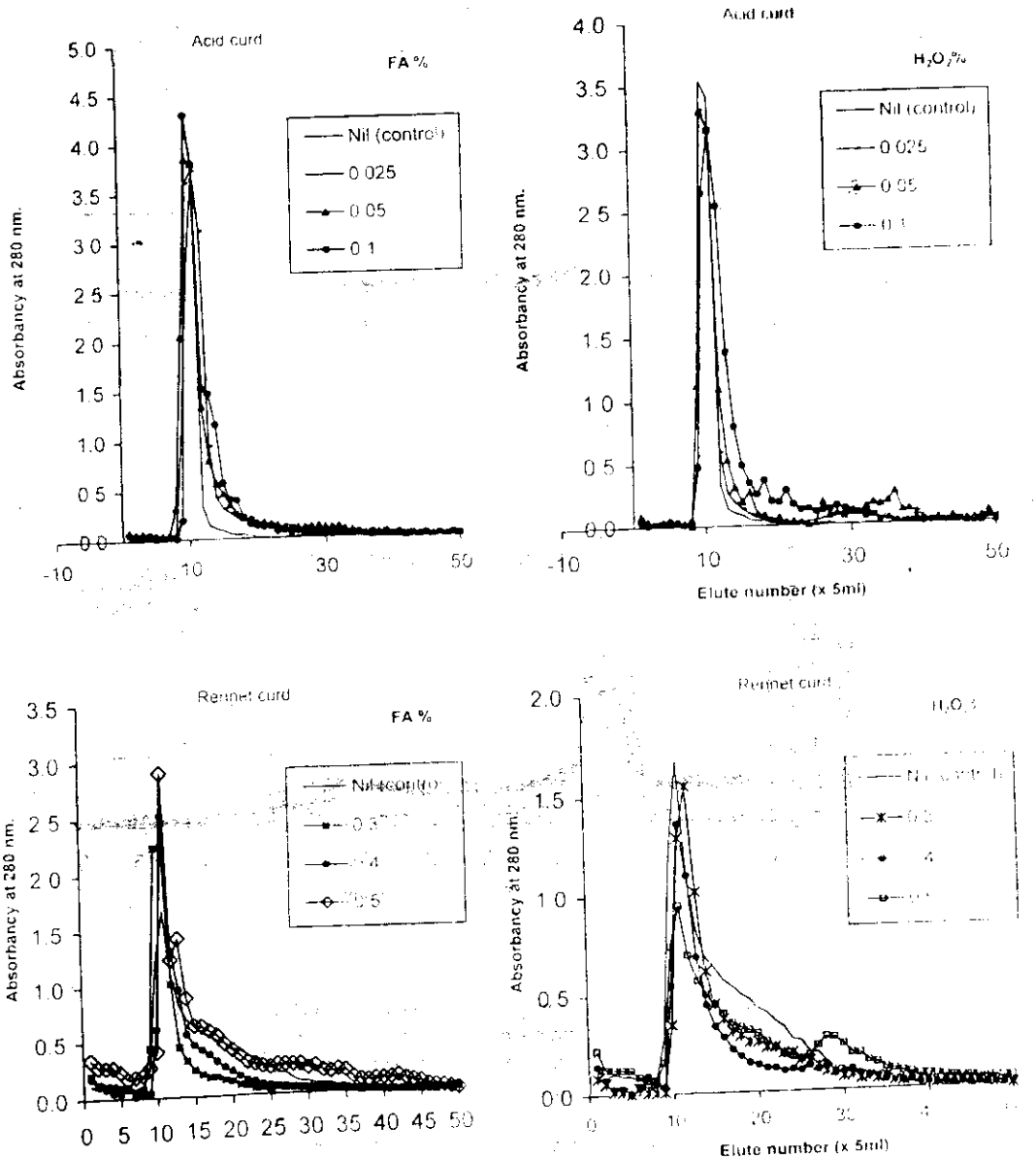

Figure (2) Partitation chromatograms on Sephadex G100 gel filtration of curd proteins of buffalo's milk as a function of the kind of coagulation as well as the level of formaldehyde (FA) or hydrogen peroxide (H_2O_2) solution added to it

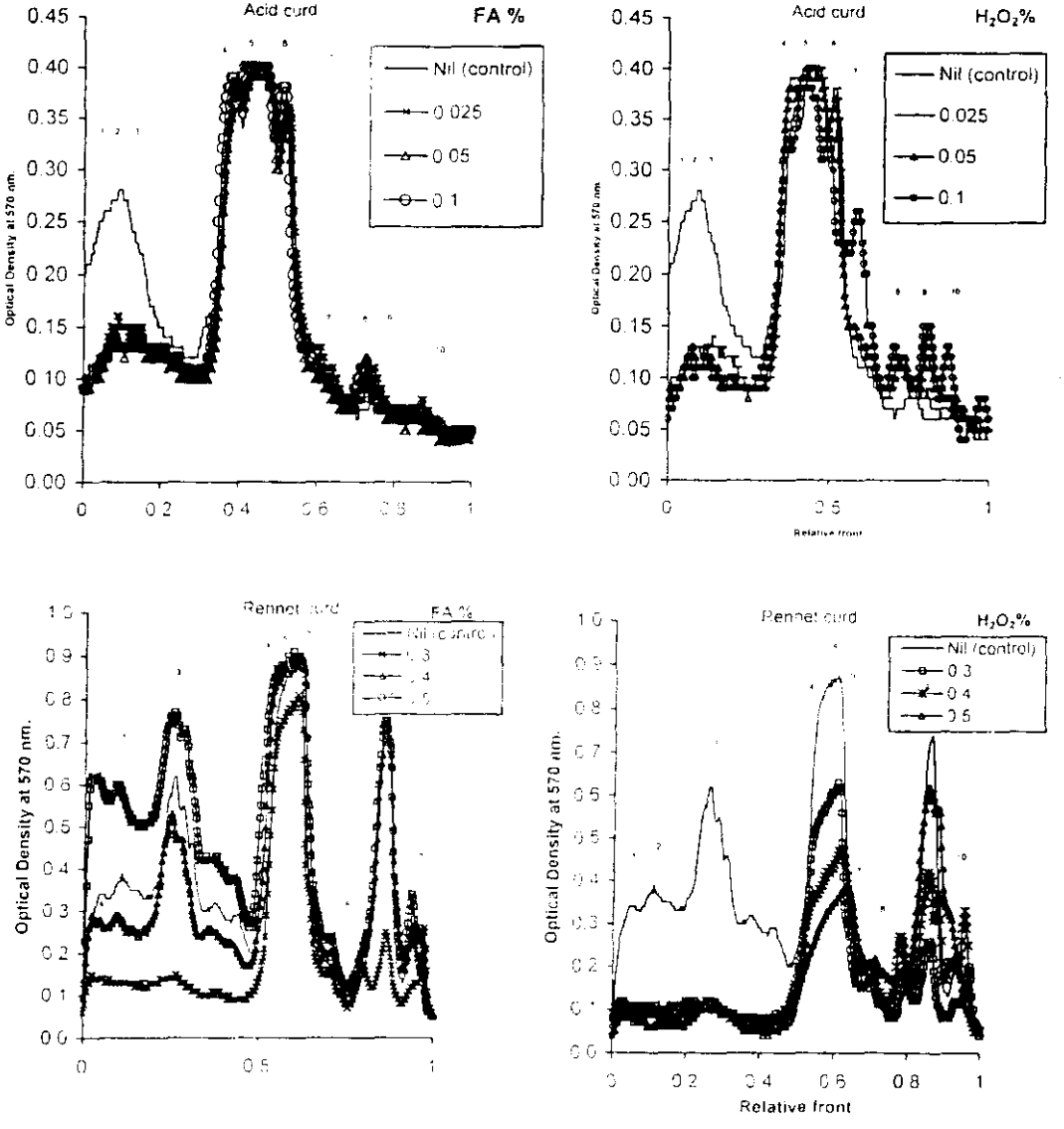

Figure (3) Electrophoretic patterns of SDS-PAGE proteins fractions (mainly caseins) of curd proteins of buffalo's milk as a function of the kind of coagulation as well as the level of formaldehyde (FA) or hydrogen peroxide (H_2O_2) solution added to it

SDS-PAGE Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
1- Lactoferrin 2- Serum-albumin 3- Immunoglobulin 4-αs2-Casein 5-αs1-Casein 6-β-Casein 7-k-Casein 8-γ-Caseins 9- β-Lactoglobulin 10- α-Lactalbumin

Concerning the effect of the kind and level of preservative on the relative patterns levels of caseins fractions, the statistical analysis appeared in details that, opposite to FA, H_2O_2 at any spiking level diminished significantly the level of α_{s1}-Cn as well as β-Cn and increased the level of γ-Cns ($p<0.01$). Korolczuk (1984) and Aoki and Kako (1984) suggested that, FA may have polymerizing effect or promote cross-links among casein component and prevent the formation of soluble casein accompanying the release of k-Cn from micelles, opposite to H_2O_2, which relatively forbids the interaction between β-Lg and k-Cn as reported by Buruiana *et al.* (1983); Marshall (1986); Gaafar (1992) and Özer *et al.* (2003). Moreover, the increased level of γ-Cns associated with H_2O_2 may be occurred at the expense of the reductions in the levels of both α_{s2}-Cn and k-Cn fractions caused by the same reason (H_2O_2).

Table (3): SDS-PAGE proteins fractions of acid and rennet curds of buffalo's skimmilk as affected by milk spiking with different levels of formaldehyde (FA) or hydrogen peroxide (H_2O_2) solution

Fraction %	Kind and level of preservative (%, V/V)							
	Control		FA					
	Acid curd	Rennet curd	Acid curd			Rennet curd		
	Nil	Nil	0.025	0.050	0.100	0.300	0.400	0.500
α_{s2}-casein	17.2	17.5	18.0	18.2	18.4	18.0	18.3	18.2
α_{s1}-casein	27.5	27.3	28.3	28.6	29.5	28.0	28.5	28.8
β-casein	32.8	32.0	33.6	33.5	33.6	33.2	33.4	33.3
k-casein	14.2	11.3	14.6	14.8	15.0	11.9	11.8	11.7
γ-caseins	2.5	2.2	2.8	3.0	3.2	2.0	2.2	2.4
Whey proteins residues	5.8	9.7	2.7	1.9	0.3	6.9	5.8	5.6
	H_2O_2							
α_{s2}-casein	17.2	17.5	18.2	17.9	17.0	17.9	17.8	18.1
α_{s1}-casein	27.5	27.3	25.2	26.0	24.9	27.4	27.0	26.6
β-casein	32.8	32.0	29.6	28.5	28.0	30.5	30.0	29.0
k-casein	14.2	11.3	14.4	14.8	15.0	12.2	12.6	12.8
γ-caseins	2.5	2.2	5.4	4.30	5.8	4.8	7.0	9.7
Whey proteins residues	5.8	9.7	7.2	8.5	9.3	7.2	5.6	3.8

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Regarding the casein fractions in relation to the kind of coagulation, data indicated that, k-Cn was the sole fraction which was influenced by it. Where, some k-Cn was used up due to rennet action ($p<0.001$) in comparison with the acidic coagulation. Such relationship between the rennet attacked casein and the level of resultant PAGE k-Cn fraction was reported by Fayed (1986).

Amino acids composition:

Concerning the essential amino acids (EAA), the results indicated that, significant reductions in the levels of both lysine, methionine, cysteine, leucine and even in the total (T) EAA (except tyrosine and tryptophan) were occurred due

to adding of 0.3% preservatives, regardless their kind (Table, 4). Similar trends were reported by Chaudhry and Qureshi (1979), Brunn and Klostermeyer (1983) and Deodhar *et al.*, (1986).

Table (4): Amino acid composition (g/100g proteins) of each buffalo's skimmilk, its acid and rennet curds as affected by milk spiking with nil and 0.3% formaldehyde (FA) or hydrogen peroxide (H_2O_2) solution.

Amino acids (AA)	Kind and level of preservative (%,V/V)								
	Nil (control)			0.3 % FA			0.3 % H_2O_2		
	Skim milk	Acid curd	Rennet curd	Skim milk	Acid curd	Rennet curd	Skim milk	Acid curd	Rennet curd
Essential AA:									
Lysine	6.76	7.36	7.49	4.83	7.24	6.46	5.94	7.23	6.38
Methionine	3.12	2.94	3.15	2.92	2.98	3.03	2.72	2.71	2.79
Cysteine	0.91	0.53	0.53	0.88	0.37	0.34	0.88	0.50	0.30
Threonine	4.50	4.29	3.42	4.51	4.07	3.57	4.50	4.02	3.39
Isoleucine	5.07	5.07	4.60	5.12	4.95	4.68	5.04	5.02	4.49
Leucine	9.41	8.56	8.80	9.46	8.43	8.61	9.31	8.44	8.67
Phenyl alanine	4.63	4.75	4.60	4.66	4.51	4.68	4.59	4.52	4.49
Valine	6.64	5.51	5.25	6.13	5.54	5.62	5.97	5.52	5.18
Tyrosine	ND	ND	ND	ND	ND	ND	ND	ND	ND
Tryptophan	ND	ND	ND	ND	ND	ND	ND	ND	ND
TEAA	41.04	39.01	37.84	38.51	38.09	36.99	38.95	37.96	35.69
Non-essential AA:									
Histidine	2.52	2.69	2.50	2.38	2.51	2.47	2.43	2.41	2.49
Arginine	2.97	3.01	2.89	2.77	2.74	2.81	2.78	2.91	2.69
Serine	5.66	4.97	4.73	5.25	4.88	4.36	5.28	4.72	4.58
Glutamic acid	21.19	20.47	21.67	21.53	21.07	22.85	21.22	20.79	22.63
Aspartic acid	7.19	8.28	6.91	7.01	5.69	6.13	6.93	5.72	5.58
Glycine	1.76	1.52	1.71	1.96	1.55	1.70	1.79	1.61	1.59
Alanine	3.38	2.81	2.83	3.24	2.59	2.74	3.22	2.71	2.49
Proline	10.17	12.85	13.00	10.25	13.75	13.96	10.12	13.46	13.36
TNEAA	54.83	56.60	56.24	54.39	54.78	57.02	53.77	54.33	55.41
TAA	95.87	95.61	94.08	92.90	92.87	94.01	92.72	92.29	91.10

ND: Not determined

TEAA: Total essential amino acids (summed without tyrosine and tryptophan amino acids)

TNEAA: Total non-essential amino acids

TAA: Total amino acids (summed without tyrosine and tryptophan amino acids).

Moreover, it is worthy to mention that, the conversion of liquid milk protein into acidic or enzymatic coagulated form led to increase only the level of lysine. While, methionine was not thereby affected. Whilst the levels of other

EAA as well as TEAA of protein reduced due to the loss of whey protein *via* the curd synersis. Further, the differences in the levels of lysine, cysteine and valine between acidic and rennet-curds were not significant (P> 0.05). Data also show that, acid-curd contained lower leucine in addition to higher threonine, isoleucine and TEAA levels than those found in rennet curd.

Regarding the non essential (NE) AA; it could be observed that, as previously noticed in the case of EAA, the preservative kind did not play any significant role (p> 0.05) on the levels of any one of NEAA as well as TAA while, the level added (0.3%) of any preservative type caused significant reduction in the levels of histidine, arginine, serine, aspartic acid, alanine and TNEAA as well as TAA and increment in the levels of glycine and proline. Similar findings were reported by Resmini *et al.*, (1988 and 1989); Fox *et al.*, (1993) and Metwalli *et al.*, (1995).

In general, the obtained results of AA composition of genuine buffalo's skimmilk are in agreement with those of whole-protein labneh made from ultrafiltrated buffalo's milk reported by Youssef (2000). Likewise, the trends of AA composition of acid-curd made from preservative-free buffalo's milk are in coincidence with those of conventionally drainaged-whey labneh manufactured from buffalo's milk by El-Samragy *et al.* (1997) and Youssef (2000).

Nutrition aspects:

Data presented in Table (5) indicated that, the addition of 0.3% preservative led to significant reduction in all calculated nutritional parameters (P< 0.05). The harmful effect of FA did not significantly vary from that of H_2O_2 (P>0.05). Similar findings reported by Deodhar *et al.* (1986) and Finlayson and Armstrong (1986).

Moreover, the results also declare that, the technical conversion of milk into curd form caused significant reductions in all expressed nutritive parameters including PER, BV and NPU (P<0.001) especially when the acidic coagulation was applied. The trends of these results are in coincidence with those reported by Youssef (2000).

Table (5): Protein efficiency ratio (PER), biological value (BV) and net protein utilization (NPU) of each buffalo's skimmilk, its acid and rennet curds as affected by milk spiking with nil and 0.3% formaldehyde (FA) or hydrogen peroxide (H_2O_2) solution.

Property %	Kind and level of preservative (%, V/V)								
	Nil (control)			0.3 % FA			0.3 % H_2O_2		
	Skim milk	Acid curd	Rennet curd	Skim milk	Acid curd	Rennet curd	Skim milk	Acid curd	Rennet curd
PER	3.13	2.62	2.72	3.15	2.51	2.59	3.09	2.53	2.64
BV	82.85	77.44	78.52	83.05	76.37	77.13	82.39	76.56	77.72
NPU	78.71	73.57	74.59	78.90	72.55	73.27	78.27	72.73	73.83

Finally, it could be concluded that, there is a lot of considerable harmful changes in the composition, coagulation and nutrition properties of protein due to FA or H_2O_2 makes it possible to design reliable measurement procedures for such preservative detection.

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تأثير الفورمالدهيد وفوق أكسيد الهيدروجين كمواد حافظة على خواص بروتينات اللبن الجاموسى.

عاطف السيد فايد* ، احمد اسماعيل متولى* ، شعراوى جمال عثمان**
هالة عبد المنعم عبد الرحمن ** ، ميرندا عبد المجلى توفيق**
* قسم علوم الأغذية – كلية الزراعة – جامعة عين شمس
** معهد بحوث تكنولوجيا الأغذية– مركز البحوث الزراعية – الجيزة.

الهدف من هذا البحث هودراسة تأثير الفورمالدهيد وفوق أكسيد الهيدروجين على خواص بروتين اللبن ولهذا الغرض تم معاملة عينات من لبن فرز خام جاموسى بحجم ١٠٠مل بالفورمالدهيد (٤٠%) اوفوق أكسيد الهيدروجين (٣٥%) بتركيزات صفر (كونترول) ، ٠,٠٢٥ ، ٠,٠٥٠ ، ٠,١٠٠ ، ٠,٢٠٠ ، ٠,٣٠٠ ، ٠,٤٠٠ ، او ٠,٥٠٠%.

وقد أوضحت النتائج ضعف قابلية اللبن للتجبن بإضافة الفورمالدهيد بصورة معنوية عما حدث عند إضافة فوق أكسيد الهيدروجين وانخفضت صلابة الخثرة ومعدل تصفية الشرش مع الفورمالدهيد بصورة معنوية عما حدث مع فوق أكسيد الهيدروجين. كما وجدت علاقة عكسية بين سرعة معدل طرد الشرش من الخثرة ونسبة إضافة المواد الحافظة وأوضح كروماتوجرام السيفادكس G100 لبروتين اللبن السائل والخثرة الناتجة من التجبن الحامضى والخثرة الناتجة من التجبن الانزيمى انة على عكس تأثير فوق أكسيد الهيدروجين كانت الزيادة فى قراءة الامتصاص المقاس لمنحنى الكازينات مرتبطة بصورة طردية بنسبة الفورمالدهيد المضاف وكان هذا على حساب المنحنين الأخريين لبروتينات الشرش واثبت التفريد الكهربى لفصل بروتين اللبن بواسطة نظام الصوديوم دودوكسيل سلفيت لجل بولى الاكراميد لنوعى خثرة اللبن (الحامض والانزيمى) انه على الرغم من ان نسبة شق الفا اس ٢ كازين لم يتأثر إلا أن نسب الشقوق الفا اس١ كازين وبيتا كازين زادت في وجود الفورمالدهيد وانخفضت في وجود فوق أكسيد الهيدروجين ويظهر عكس ذلك مع جاما كازينات بينما اظهر الالكتروفيروجرام لبروتينات الشرش عدم استجابة سواء لنوع أونسب إضافة المواد الحافظة.

ومن الناحية التركيبية أدى وجود ٠,٣% من اى من المواد الحافظة المستعملة الى التقليل بدرجة معنوية من تركيز الأحماض الامينية الكلية الأساسية والغير أساسية. أما من ناحية القيمة الغذائية فادت هذة التغيرات فى تركيز الأحماض الامينية المصاحبة لاضافة المواد الحافظة الى نقص معنوي فى نسبة كفاءة البروتين ، القيمة الحيوية وصافى المستفاد من البروتين وكانت كل الدلائل الغذائية السابقة أعلى لدى اللبن السائل يلية الخثرة الإنزيمية بينما الخثرة الحامضية جاءت فى المرتبة الاخيرة.