Egypt. J. Chem. Environ. Health, 2 (2):500-515 (2016)
On line ISSN: 2536-9164.

Prevalence of Enterococci and Streptococci in Raw Milk and Some Dairy Products and The Subsequent Alteration on Quality.

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Abstract

The purpose of this research was to study the prevalence of *enterococci* and *streptococci* in milk and some dairy products. Survey included 50 samples of raw milk, 25 kareish cheese and 25 yoghurt samples which were collected from El-Behera governorate markets. The results revealed that the mean of chemical composition was 12.95±0.11, 86.34±0.26, 6.3±0.07 and 0.18±0.002 for total solid, Moisture content, pH values and titratable acidity, respectively. in raw milk samples; 36.8±0.87, 61.76±0.84, 3.87±0.11 and 2.27±0.037 in kareish cheese and 36.8±0.87, 76.48±0.94, 4.33±0.05 and 1.09±0.036 in yoghurt samples, Respectively. incidence of positive samples for aerobic plate count, streptococci and enterococci count were 88 , 26 and 22 % in raw milk samples, respectively . 76 , 36 and 36 % in kareish cheese and 56, 28 and 32 % in yoghurt samples, respectively. with mean values of $1.22x10^5±0.1x10^{5,}$ $4.5x10^3±0.7x10^3$ and $6.3x10^3±1.9x10^3$ cfu/ml in raw milk samples; $8.9x10^4±0.76x10^4$, $4.7x10^3±0.87x10^3$ and $5.7x10^3±1.6x10^3$ cfu/gm in kareish cheese samples and in yoghurt samples were $7.6x10^4±0.59x10^4$, $3.7x10^3±0.6x10^3$ and $5.5x10^3±0.64x10^3$, respectively. All isolated *Enterococcus faecium, Enterococcus faecalis, S. agalactiae* and *S. dysgalatiae* were confirmed at a genus level using specific primer targeting 16s rRNA gene in *S. dysgalatiae and Enter. Faecalis; sod A* gene in *enterococcus faecium* and *cfb* gene in *S. agalactiae*. Desired amplicon for virulence genes were obtained.

Key words: milk, dairy products, *enterococci, streptococci*, PCR, 16s rDNA

Introduction

Enterococci and *streptococci* are environmental organisms commonly found in organic matter, including bedding. *Enterococci* are also commonly found in silage inoculants and are associated with plant matter, such as dairy feed. Because *streptococci* bacteria are common in dairy cattle manure, they are often found in the bedding. Poor udder cleanliness, inadequate stall management, and damaged teat ends also appear to increase the risk of spreading *Enterococcus spp.* and environmental *streptococci* to uninfected cows (**Christina, 2012**).

Enterococci may have a distinctive role as indicators of poor factory sanitation owing to their relatively high resistance to drying, detergents, as well as

freezing temperature, moreover, these organisms are also implicated in food poisoning outbreaks **(Yabaya and Idris 2012).**

The presence of *enterococci* in dairy products has long been considered as an indication of inadequate sanitary conditions during the production and processing of milk **(Giraffa *et al*., 1997).** *Enterococci* organisms have been proposed for hygienic condition inspections in process lines of fermented products **(Vanos, 1991).**

Enterococci have important implication in the dairy industry. They play an important role in the development of sensory characteristics during ripening of many cheeses, probably through proteolysis, lipolysis, and citrate breakdown, hence, contributing to their typical taste and flavor moreover **.** Because of their role in ripening, flavor development, and bacteriocin production in cheese, it has been suggested that *enterococci* with desirable technological and metabolic traits could be included in starter cultures of various cheeses **(Foulquie Moreno *et al.,* 2006).**

The proteolytic and esterolytic activities displayed by some *enterococcal* strains, as well as their ability to metabolize citrate, may contribute to cheese ripening and flavour development. Because of these interesting metabolic properties, *enterococci* have been proposed as part of defined starter culture combinations for different European cheeses, such as Feta, water-buffalo Mozzarella and Cebreiro cheeses **(Morandi *et al.,* 2006).**

Some authors claim that the *enterococci* used as adjunct starters in cheese manufacture contribute to increased breakdown of casein and, thus, to soluble nitrogen production **(Centeno *et al.,* 1999).** However, other studies have shown that proteinase activity in *enterococci* is low, with *E. faecalis* being the most proteolytic species **(Suzzi *et al.,* 2000).**

Streptococcus agalactiae, S. dysgalatiae and S. uberis have been reported as the three most common etiological agents of mastitis **(Khan *et al.,* 2003).** Other *Streptococcal* species such as *S. uberis, S. agalactiae, S. dysgalatiae, S. epidemicus, S. bovis, S. equinus* have been implicated in bovine mastitis, although they are relatively infrequent (**Leigh 1999 and Khan *et al.,* 2003**). *Streptococcus agalactiae* has been widely reported as an important pathogen of both animals and man (**Ko *et al.,* 2001**).

Streptococcus uberis is known worldwide as an environmental pathogen responsible for high proportion of cases of clinical and subclinical mastitis in

lactating cows and is also the predominant organism isolated from mammary gland during the non-lactating period **(Bradley, 2002 and Khan *et al.,* 2003).**

Subclinical mastitis caused by *Streptococcus uberis is an* intramammary infection which was associated with changes of milk compositions including elevated somatic cells and depressed percentages of lactose, fat, total solid and fat protein ratio. *Streptococcus uberis* causing mastitis in certain level may cause economic losses due to not only production loss but also milk quality **(Rerk *et al.,* 2008)**.

Several studies using similar modern taxonomical concepts are needed for classification and species identification in dairy enterococci. These methods based on the analysis of bacterial DNA have been successfully applied **(Delgado and Mayo, 2004).** PCR with species-specific primers is a valuable method, and, this can replace complex molecular clustering techniques and conventional microbiological tests necessary to identify species hard to distinguish by phenotypical approaches **(Jackson *et al.,* 2004).**

There are different strategies to PCR amplification of bacterial DNA in clinical samples. The first based on usage of species-specific primers. This method lacks the ability to determine bacterial infection definitely **(McCabe and McCabe, 1997)**. The second approach involves amplification of sequences found in all bacteria based on universal sequences common in bacteria (**McCabe *et al.* 1995**).

So, the aim of this work was to study the prevalence of *enterococci* and *streptococci* in raw milk and some dairy product as well as application of PCR identification of isolated strains using specific primer for each strain.

Material and Methods

Collection of samples:

One hundred samples of raw milk and some dairy products (50 raw milk, 25 kariesh cheese, and 25 yoghurt samples) were collected randomly from El-Behera Governorate. The collected samples were transported under aseptic conditions in an ice packed container to the laboratory as soon as possible for further evaluation immediately.

Compositional quality of milk, cheese and yoghurt:

- Total solid and moisture of milk samples were analyzed using standard (**Association of Official and Analytical Chemists, 1999**). PH was measured directly with a combination electrode according to **AOAC (2000).**
- Total solids of the yoghurt and cheese samples were measured by forced-draft oven at 105°C until a steady weight was achieved (approximately 24 h) **AOAC (2005).**
- Moisture content and pH of yoghurt and cheese samples were determined according to **AOAC (2000).**
- Titratable acidity of milk, cheese and yoghurt were determined according to **AOAC (2005).**

Bactiological examination:

- **Preparation of samples:** 10 ml of milk and 10 gm yoghurt of each sample were added to 90ml sterile saline while 10 g of each Kareish cheese sample were added to 90 ml sodium citrate 2% to make ten-fold serial dilution.
- **APC:** The aerobic plate count (APC) was carried out as the conventional method, **Food and Drug Administration, (2002)** using plate count agar (Oxoid).
- **Total enterococci count:** *enterococci* in these samples were isolated by standard microbiological methods and selective medium of Kanamycin Esculin Azide Agar (KAA) (**Suzzi *et al.,* 2000**). The isolates were identified biochemically according to(**(Teixeira &Facklam, 2003).**
- **Total *streptococcus* count according to (Amosun *et al.*, 2010):** 1ml from each serial dilution were poured into sterile Brain heart infusion (BHI) Broth and incubated aerobically at 37 °C for 48 hours. Next, the cultures were purified on 7% sheep Blood Agar plates at the same conditions. Suspected colonies were tested by Gram staining method in terms of being *cocci* and gram-positive; in the next phase of identification, the catalase-negative and oxidase negative colonies were isolated and then *Streptococcus agalactiae* strain was differentiated due to Esculin hydrolysis, Carbohydrates utilization containing lactose, maltose, mannitol, raffinose, CAMP test and resistance to bacitracin from two other *streptococci, Streptococcus dysgalactiae and Streptococcus uberis.*

PCR based techniques according to (Goldenberger, *et al.*, 1995):

- **DNA extraction.** DNA extraction from purified cultural suspension of tested isolates was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 20 µl of proteinase K and 200 µl of lysis buffer at 56°C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged

following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

- **Oligonucleotide Primer.** Primers used were supplied from **Metabion (Germany)** are listed in table (1):
- **PCR amplification.** Primers were utilized in a 25- µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentrations, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an applied biosystem 2720 thermal cycler.
- **Analysis of the PCR Products.**
- The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the products was loaded in each gel slot. A Gelpilot 100 bp Ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

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Table (1): Primers sequences, target genes, amplicon sizes and cycling conditions.

Target Agent	Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
					Secondary denaturation	Annealing	Extension		
E. fecalis	***16S rRNA***	GTT TAT GCC GCA TGG CAT AAG AG	310	94˚C 5 min.	94˚C 30 sec.	50˚C 45 sec	72˚C 45 sec	72˚C 10 min.	**Zoletti *et al*., 2006**
		CCG TCA GGG GAC GTT CAG							
E. faecium	***sodA***	GAAAAAACAATA GAAGAATTAT	215	94˚C 5 min.	94˚C 30 sec.	50˚C 30 sec	72˚C 30 sec	72˚C 7 min.	**Jackson *et al*., 2004**
		TGCTTTTTTGAAT TCTTCTTTA							
S. agalactiae	***cfb***	TTTCACCAGCTGT ATTAGAAGTA	153	94˚C 5 min.	94˚C 30 sec.	55˚C 30 sec	72˚C 30 sec	72˚C 7 min.	**Ke *et al*., 2000**
		GTTCCCTGAACAT TATCTTTGAT							
S. dysgalactiae	***16S rRNA***	GGAGTGGAAAAT CCACCAT	549	94˚C 5 min.	94˚C 30 sec.	49.5˚C 45 sec	72˚C 45 sec	72˚C 10 min.	**Prabhu *et al*., 2013**
		CGGTCAGGAGGA TGTCAAGAC							

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Results and Discussion

Enterococci are natural inhabitants of the intestine of warm-blooded animals **(Devriese *et al.*, 1992**) and are widely distributed in various plant surfaces (**Cai *et al.*, 1998**). Moreover, *Enterococcus* species generally appear and participate in food fermentations (**Franz *et al.*, 1999**). Regarding dairy products, the occurrence of *enterococci* has been reported in milk as well as in cheeses; *Enterococcus faecalis* and *Enterococcus faecium* are the most frequent species detected in raw milk, pasteurized milk and cheese **(Citak *et al.*, 2004)**. *Enterococci* have also been isolated from natural milk starters (**Centeno, *et al.*, 1999).**

Result illusterated in table (2) revealed that the mean of chemical composition of examined milk samples was 12.95±0.11, 86.34±0.26, 6.3±0.07 and 0.18±0.002 for total solid , Moisture content, pH values and titratable acidity, respectively.

Fresh milk has a titratable acidity of 0.14 to 0.16% expressed as lactic acid and loses its keeping quality when a critical acidity of 0.200 ± 0.01% is reached **(Al-Zenki, *et al.,* 2007)**. Our results agree with **(Popescu and Angel, 2009)**, While higher results obtained by **Marimuthu, *et al.*, (2013) and Meshref and Meshref (2013)**.

Results in table (3) illustrated that the mean of chemical composition of examined kareish cheese samples was 36.8±0.87, 61.76±0.84, 3.87±0.11 and 2.27±0.037 for total solid , Moisture content, pH values and titratable acidity, respectively. While in table (4) showed that the mean of chemical composition of examined yoghurt samples was 36.8±0.87, 76.48±0.94, 4.33±0.05 and 1.09±0.036 for total solid,. Moisture content, pH values and titratable acidity, respectively.

The average titratable acidity of kareish cheese samples obtained in this study was 2.27±0.037 and ranged between 1.35 and 3.60 which were higher than that reported by **(Mohammed, *et al.*, 2009).**The relatively high acidity percent may be attributed to the method of Kareish cheese production in which milk is kept for about 36 hours at room temperature giving the chance for lactic acids bacteria to grow and produce acids, while Average acidity percent of yoghurt samples was 1.09±0.036, which nearly agree with **(Abo El-Makarem, 2013)** who reported that average titratable acidity of examined yoghurt samples at small scales was 1.12 ± 0.023.

Titratable acidity is one of the most important parameters with respect to the shelf-life of fermented milk products and also a reasonable indication of the performance of the starter culture **(Tamime and Robinson, 2001).**

The obtained results in table (5) showed that the incidence of aerobic bacterial count,(TBC) *streptococci* and *enterococci* count in examined milk samples were 88,

26 and 22 %, respectively with mean values of $1.22x10^5±0.1x10^{5,}$ $4.5x10^3±0.7x10^3$ and $6.3x10^3±1.9x10^3$cfu/ml.

Total aerobic counts are a good indicator of general hygiene, permitting the appreciation of microbial pollution and the general quality of the milk and milk products (**Aggad *et al.,* 2010**). The number of bacteria in aseptically drawn milk was low, but infection occurred subsequently from the skin of animals, milker's hands, with cow shed and milking utensils (**Khan *et al.,* 2008**).

Data in table (6) reveals the incidence of APC, *streptococci* and *enterococci* count in examined kareish cheese samples was 76, 36 and 36 %, respectively. The aerobic bacterial count ranged between $2.5x10^4$ and $1.65x10^5$ with mean value $8.9x10^4±0.76x10^4$ cfu/gm, also the number of *streptococci* ranged between $2x10^3$ and $9x10^3$ with mean value $4.7x10^3±0.87x10^3$ cfu/gm, finally the *enterococci* count ranged from $2x10^3$ to $1.8x10^4$ with mean values of $5.7x10^3±1.6x10^3$, respectively. Higher results of total bacterial count for Kareish cheese samples were reported by **Kaldes (1997) and Amin, *et al.* (2001)** as they reported $2.6x10^8$ and $2.9x10^9$ cfu/g, respectively. While, **Omar (2006)** recorded that, the mean of total bacterial count is $1x10^9$ for opened Kareish cheese samples and $2.40x10^8$ for packed Kareish cheese samples.

Higher incidence of *enterococci* (86.6%) in kareish cheese samples reported by (**Ahlam, *et al.* (2015)**, while lower incidence were reported by **Halawa and Moawad (1999).**

Results presented in Table (7) revealed that total bacterial streptococci and *enterococci* count were present in 14(56%), 7 (28%) and 8 (32%) of examined yoghurt samples with mean values $7.6x10^4±0.59x10^4$, $3.7x10^3±0.6x10^3$ and $5.5x10^3±0.64x10^3$, respectively. Our results of *enterococci* count agree with **(El-Ansary, 2014)** who reported that the incidence of *enterococci* in examined yoghurt sample was 28 %, while higher results were obtained by **El-Malt, *et al.* (2013)** who reported that incidence of *enterococci* in small scale yoghurt samples was 58 % with an average count $1.72\ x10^4$. Higher incidence of *enterococci* (60%) in examined yoghurt samples reported by (**Ahlam, *et al.* 2015**).

The existence of *Enterococci* in yoghurt is considered an indication of neglected sanitary control measures during production. Moreover, *Enterococci* count is considered an index of sanitary quality of yoghurt as they are able to survive the unfavorable microenvironment as the low pH value of yoghurt.

Fig (1) showed that PCR amplification of 549 and 310 bp *16S rRNA* of *S. dysgalatiae* and *Enter. Faecalis,* also show the PCR amplification of 215 bp *sod A*

gene of *enterococcus faecium* and 153 bp *cfb* gene of *S. agalactiae* using specific primer for detection.

In order to overcome problems associated with biochemical testing, molecular methods for identification have been developed. Genus-specific PCR primers to 16S rRNA have already been designed and found useful for distinguishing strains of *Enterococcus* (**Deasy *et al.*, 2000).**

We focused on 16S rRNA gene-based assays as these genes are part of multiple operons and therefore the detection limits (i.e., per genome copy) of these assays are expected to be higher than single copy genes.

A previous report identified the manganese-dependent superoxide dismutase gene *sodA* as an ideal gene for species identification of *enterococci* **(Poyart *et al.*, 2000).** The superoxide dismutase gene has been used to distinguish genera and species of *streptococci*, and *enterococci* **(Poyart *et al.*, 2001).**

Cell surface protein like pore forming protein encoded by CAMP factor/*cfb* gene, was found to produce a classical CAMP phenomenon with typical half-moon forming haemolytic zones on cattle or sheep blood agar plates by the influence of β-lysin of exosusbtances of non-hemolytic *streptococci***.** CAMP factor genes are described to be fairly widespread among *streptococci* at least serogroups A, B, C, G, M, P, R, and U **(Gase *et al.* 1999).**

Public health significance of *streptococci* and *enterococci* :

In humans, *S. agalactiae* has been described as one of the most common factors of invasive infections in neonates, but it also causes invasive and non-invasive infections in adults **(Schuchat 2001).** *S. agalactiae* also causes significant morbidity and mortality in humans, both infants and adults, all over the world **(Blumberg et al. 1992).** In neonates*, S. agalactiae* is mostly acquired from the mother's vagina in early-onset disease, although community and breast milk transmissions have been reported **(Bingen et al. 1992).** In adults, *S. agalactiae* occurs preferentially in certain individuals, such as diabetics, pregnant and post-partum women, and immunocompromised patients, emphasizing the opportunistic nature of the infection **(Lerner et al. 1977).** One of the reasons for the rise of nosocomial infections related to *enterococci* might be their ability to develop resistance against a wide variety of antibiotics. *Enterococci* have also been recognized as nosocomial pathogens causing infections such asnbacteremia and endocarditis. **(Semedo, et al. 2003).**

Results are illustrated in tables (2-7) and Fig 1

Table (2): Statistical analytical results of compositional quality of examined raw milk samples (n=50)

Parameter	Minimum	Maximum	Mean ±SEM
Total solid percent	12.20	14.50	12.95±0.11
Moisture percent	81	89	86.34±0.26
pH value	5.20	7.20	6.3±0.07
Titratable acidity %	0.14	0.21	0.18±0.002

Table (3): Statistical analytical results of compositional quality of examined Kareish cheese samples (n=25).

Parameter	Minimum	Maximum	Mean ±SEM
Total solid percent	31	45	36.8±0.87
Moisture percent	51	70	61.76±0.84
pH value	2.90	5.20	3.87±0.11
Titratable acidity %	1.35	3.60	2.27±0.037

Table (4): Statistical analytical results of compositional quality of examined yoghurt samples (n=25).

Parameter	Minimum	Maximum	Mean ±SEM
Total solid percent	31	45	36.8±0.87
Moisture percent	65	82	76.48±0.94
pH value	3.38	5.20	4.33±0.05
Titratable acidity %	0.58	1.63	1.09±0.036

Table (5): Statistical analytical results of APC, streptococci and enterococci count , of examined raw milk samples (n=50).

Counts (CFU/ml)	Positive samples				
	No	%	Minimum	Maximum	Mean± SEM
Total bacterial count	44	88	$4.3x10^4$	$2.5x10^5$	$1.22x10^5±0.1x10^5$
Streptococci count	13	26	$1x10^3$	$9x10^3$	$4.5x10^3±0.7x10^3$
Enterococci count	11	22	$1x10^3$	$2.4x10^4$	$6.3x10^3±1.9x10^3$

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On line ISSN: 2536-9164.

Table (6): Statistical analytical results of total bacterial count, *streptococci* and *enterococci* count of examined Kareish cheese samples (n=25).

Counts (CFU/ml)	Positive samples				
	No	**%**	**Minimum**	**Maximum**	**Mean± SEM**
Total bacterial count	19	76	$2.5x10^4$	$1.65x10^5$	$8.9x10^4±0.76x10^4$
***Streptococci* count**	9	36	$2x10^3$	$9x10^3$	$4.7x10^3±0.87x10^3$
***Enterococci* count**	9	36	$2x10^3$	$1.8x10^4$	$5.7x10^3±1.6x10^3$

Table (7): Statistical analytical results of total bacterial count, *streptococci* and *enterococci* count of examined yoghurt samples (n=25).

Counts (CFU/ml)	Positive samples				
	No	**%**	**Minimum**	**Maximum**	**Mean± SEM**
Total bacterial count	14	56	$4.2x10^4$	$1.11x10^5$	$7.6x10^4±0.59x10^4$
***Streptococci* count**	7	28	$1x10^3$	$7x10^3$	$3.7x10^3±0.6x10^3$
***Enterococci* count**	8	32	$2x10^3$	$9x10^3$	$5.5x10^3±0.64x10^3$

S. dysgalactiae			*S. agalactiae*			L	*E. faecium*			*E. fecalis*		
Pos	S	Neg	Pos	S	Neg		Pos	S	Neg	Pos	S	Neg

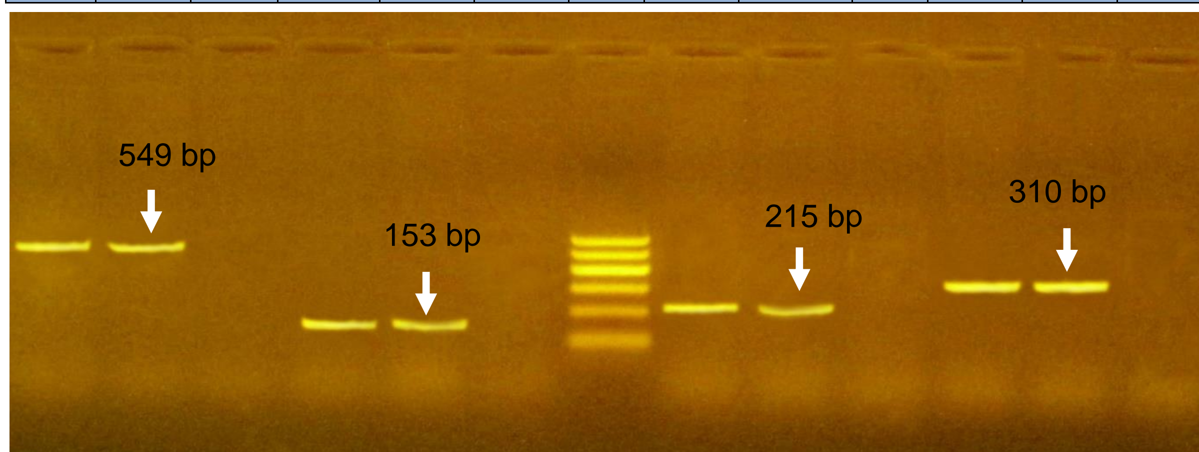

Fig (1): Amplicon of the 16S rRNA gene of *S. dysgalatiae and Enter.Faecalis* with a size of 549 and 310 bp using the specific primers, also, show amplicon of sod A and cfb gene of *enterococcus faecium* and *S. agalactiae* with a size 215 and 153 bp using specific primer sequences. L = a 100 bp ladder served as size marker.

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