

**A traditional Sudanese fermented camel's
milk product, *Gariss*, as a habitat of
Streptococcus infantarius subsp. *infantarius***

By

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Introduction:

- ▶ *Streptococcus bovis* is a lactic acid bacterium inhabits the rumen of ruminants.
- ▶ It is a human pathogen associated with endocarditis, gastroenteritis, meningitis, endophthalmitis, sepsis and gastrointestinal tract cancer.
- ▶ It carries the Lancefield group D antigen shared by members of enterococci.

- ▶ In terms of physiological characteristics, it more closely resembles the viridans streptococci. •
- ▶ *S. bovis* strains of human origin are said to be biotype I if they ferment manitol and produce glucan and biotype II if they cannot ferment mannitol or produce glucan. •
- ▶ *S. bovis* biotype II strains are further divided into type II/1 and type II/2 by the ability of the latter to ferment trehalose but not glycogen. •

▶ Based on 16S rRNA gene sequencing, ribotyping and whole-cell protein electrophoresis pattern, it was suggested that *S. infantarius* forms a separate species closely related to *S. bovis* with 1.8% 16S rRNA gene sequence difference to *S. bovis* type strain which is phenotypically like *S. bovis* biotype III/1.

▶ In a later study ribotyping patterns were used to differentiate closely related species from *S. infantarius* and distinguished two subspecies namely *S. infantarius* subsp. *infantarius* and *S. infantarius* subsp. *coli*.

- ▶ *S. infantarius subsp. infantarius* has been isolated from faeces of infants, clinical specimens including cases of endocarditis and from dairy products and frozen peas. •
- ▶ Camel's milk is produced in certain areas of Sudan under nomadic conditions (33,000 tons/year), and is not available to urban or village residents. •
- ▶ The camel herders have to prepare Gariss (a fermented product) on which they sustain living for several months as the sole source of nutrients. •

- ▶ **Semi-continuous fermentation is carried out in two leather bags of tanned goat skin embedded in green wet grass carried on the back of the camel (continuous shaking).**
- ▶ **Gariss made from camel's milk has only been sparsely microbiologically and biochemically characterized.**

Aim of the study:

- ▶ To enumerate and identify the microbial population present in Gariss produced from camel's milk at household level under nomadic condition using phenotypic and molecular biology-based methods with special attention to LAB and yeast**

Materials and methods:

- ▶ Samples were collected from traditional (Si'in) tanned goat skin container from 9 production sites in different geographical areas of Sudan.**
- ▶ They were aseptically transferred to 250ml screw-capped bottles.**

- ▶ From remote areas, collected over a period of 2-3 days, kept at 4°C until being brought to Khartoum by plane on ice over a period of 4-6 hours.
- ▶ Upon receipt pH and microbiological examination were performed.
- ▶ All samples were collected at the end of fermentation and correspond to the starter culture used for subsequent fermentation.

Media

- ▶ **Plate Count Agar, incubated at 37 °C for 48 h for enumeration of aerobic mesophilic bacteria**
- ▶ **MRS and M17 incubated anaerobically for 48 h at 37 °C for enumeration of lactobacilli and lactococci respectively (anaerobic jars and Gas Pak anaerobic system).**
- ▶ **Potato Dextrose Agar incubated for 72 h at 25 °C for the enumeration of yeast.**

▶ After incubation, all colonies from a segment of the highest dilution (15-20 colonies) were further purified by successive streaking on the corresponding agar.

▶ Stock cultures were stored at -80 °C (20% (v/v) glycerol, 80% (v/v) MRS, M17 and YPG broth).

Characterization of LAB and Yeast

- ▶ All isolates were macro- and microbiologically characterized.
- ▶ For Gram +ve catalase –ve rods and cocci, growth at 10 °C and 45 °C and production of gas from glucose in MRS& M17 broth were determined.
- ▶ A total of 180 LAB and 100 yeast isolates were obtained.
- ▶ Presumptive LAB and yeast were screened into groups by rep-PCR (GTG₅).

▶ Representative isolates of LAB and yeasts were selected for the 16S rRNA gene (bacteria) and D1/D2 region of the 26S rRNA gene (yeast)

▶ Determination of the carbohydrates fermentation profile using API 50CHL and API ID 32C kit .

▶ *S. bovis* suspects were identified by PCR amplification and sequencing of the 4 housekeeping genes.

- The gene encoding D-alanine:D alanine ligase (*ddl*)
- Manganese-dependant superoxide dismutase (*sodA*)
- Glutamate dehydrogenase (*gdh*)
- The β subunit of RNA polymerase (*rpoB*)
- Streptococcal glucosyltransferase (*gtf*)
 - ▶ Production of ammonia from arginine, bile tolerance and acid resistance were also tested.
 - ▶ The sequences in the present study have been assigned nucleotide accession no. EU420145-EU420-179.

Table 1: Production (sampling) sites, pH of the samples and log(CFU/g) of lactic acid bacteria (LAB, enumerated on MRS and M17, respectively) and yeast

Production site	Kab.H.	Kab.N.	Sha. H.	Khw.D	Kass.	Gad.	But.	Don.	Sal.
pH	3.90	3.93	3.85	3.79	4.32	3.99	4.43	3.90	4.28
Log (CFU/ _{LAB, MRS} /g) % of LAB _{MRS} population	8.31	8.08	8.66	8.13	8.27	8.49	7.76	8.53	8.00
<i>S. infantarius subsp, infantarius</i>	36	18	54	31	11	38	14	29	27
<i>Lb. fermentum</i>	57	46	23	54	89	54	86	71	64
<i>Lb. helveticus</i>	N.D. ^a	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	9
<i>E.faecium</i>	7	36	23	15	N.D.	8	N.D.	N.D.	N.D.
Log(CFU _{LAB, M17} /g) % of LAB _{M17} population	8.23	8.13	8.32	7.92	7.39	8.18	7.81	8.37	7.34
<i>S. infantarius subsp, infantarius</i>	100	100	100	100	100	100	100	100	100
Log (CFU/ _{yeast} /g), MRS/g) % of yeast population	7.06	7.43	6.93	6.62	7.79	6.14	6.32	6.10	6.05
<i>K. marxianus</i>	86	61	62	46	88	75	33	67	63
<i>I. orientalis</i>	14	39	38	54	12	25	67	33	37

Abbreviations: S: Streptococcus, Lb: Lactobacillus, E: Enterococcus, K: Kluyveromyces.

I: Issatchenkia, * N.D Not detected

Kab.H: kababish hamdab, Kab.N: Kababish norab, Sha. H: Shanabla hawal, Khw.D: Al Khway donki,

Kass: Kassla, Gad: El Gadarif, But: Butana area, Don: Dongola, Sal: El Salhea

Results:

- ▶ Similar levels of microbial counts were observed on MRS {log (cfu/g) = 7.76-8.66} compared to M17 {log (cfu/g) = 7.34-8.37}.
- ▶ Aerobic mesophilic count were in the same range {log (cfu/g) = 7.11-8.36}.
- ▶ yeasts were detected in high numbers in all Gariss samples {log (cfu/g) = 6.05 – 7.79}.
- ▶ The pH of the analyzed samples ranged from 3.79-4.43 with 6 out of 9 samples having a pH less than 4.

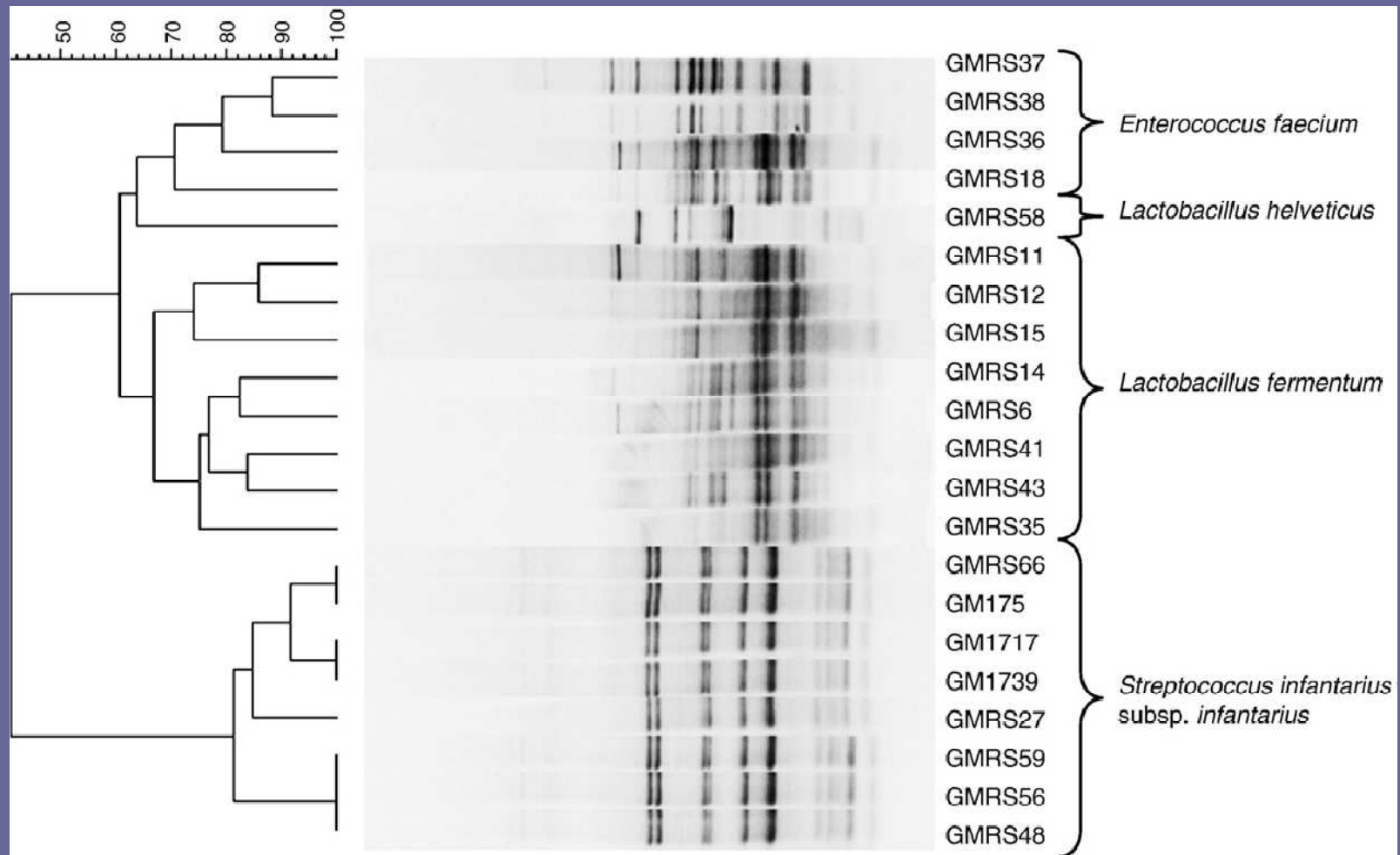


Fig. 1: Dendrogram obtained by cluster analysis of rep-PCR (GTG₅) fingerprints of LAB isolates originating from Gariss. The Dendrogram is based on Dice's coefficient of similarity with the unweighted pair group method with arithmetic average clustering algorithm (UPGMA).

Identification of LAB:

- ▶ Genotypic grouping by rep-PCR and subsequent cluster analysis showed a clear separation of 180 isolates of LAB into 4 clusters.
- ▶ 123 isolates (the majority of the homofermentative cocci) were grouped in one cluster with high similar profiles.
- ▶ They grew at 45°C but not at 15°C.
- ▶ Representatives of this group were identified by 16S rRNA gene sequencing as belonging to *S. bovis*.

- ▶ For unambiguous identification, strains were identified by PCR mediated detection and sequencing of *ddl*, *gdh*, *rpoB*, *sodA*, and *gtf* genes.
- ▶ The primers specific for the *ddl* and *gdh* failed to produce an amplicon in all tested strains of streptococci.
- ▶ The *rpoB*, *sodA* specific primers yielded amplicons from all the tested strains.

▶ PCR analysis with primer specific for *gtf* resulted in an amplicon for 10 out of 13 tested strains (77%), although the amplicon of 7 strains were weak.

▶ By comparing the sequences of the PCR products from *rpoB*, *sodA* and *gtf* (3 strong bands) with sequences deposited in the Genbank database, it was found that all strains were closely related to *S. infantarius subsp, infantarius* (99.1-100%) sequence similarity to *rpoB* from *S. infantarius subsp, infantarius*, (98.0-99.4) similarity to *sodA* from *S. infantarius subsp, infantarius* and (99.1-99.6) similarity to *gtf* from *S. infantarius subsp, infantarius*

Table 2: Identification of representative isolate identified as belonging to the streptococcus bovis group by detection (PCR) sequencing of the genes *ddl*, *rpoB*, *soda* similarity to streptococcus infantarius subsp. Infantarius (closest relative in the Genbank database) stated

Isolate	Target gene (accession no.)							Bile	pH=2.77
	<i>ddl</i>	<i>gdh</i>	<i>rpoB</i>		<i>sodA</i>		<i>gtf</i>		
GM1712	- ^a	-	99.7	EU420158	98.0	EU420145	-	*****	*****
GM1734	-	-	99.3	EU420159	99.1	EU420146	-	*****	**
GM1737	-	-	99.1	EU420160	99.1	EU420147	+ ^b	*****	*
GM1750	-	-	99.8	EU420161	98.8	EU420148	+	*****	*
GM1761	-	-	100	EU420162	99.1	EU420149	+	*****	*
GM1769	-	-	99.2	EU420163	99.1	EU420150	+	*****	*****

GM1774	-	-	100	EU420164	99.2	EU420151	99.6	EU420171	****	*
GM1780	-	-	99.4	EU420165	99.4	EU420152	99.6	EU420172	****	*
GMRS2	-	-	99.4	EU420166	99.2	EU420153	-		****	****
GMRS47	-	-	99.5	EU420167	99.1	EU420154	+		****	****
GMRS55	-	-	99.9	EU420168	99.2	EU420155	+		****	**
GMRS59	-	-	99.9	EU420169	99.1	EU420156	99.1	EU420173	****	**
GMRS89	-	-	99.9	EU420170	99.1	EU420157	+		****	****

- All isolate were furthermore tested for survival in M17 containing oxgall (0.3% w/v) with pH adjusted to 2.7 for 1 h (*), 3h (**), and 4 h (****), respectively.
- ^a- Not detected
- ^b+ Detected, But weak amplicon.

Carbohydrates fermentation profiles:

- ▶ All (13) isolates produced acid from esculin and lactose.**
- ▶ Six out of 13 isolates (46%) produce acid from tagatose and 4 (31%) produced acid from melibiose and only one isolate (8%) produce acid from raffinose, starch, glycogen and D-arbitol.**
- ▶ None of the isolates produce acid from manitol, trehalose, inulin, melezitose and sorbitol.**

- ▶ All the 13 isolates were found to be arginine negative.
- ▶ According to Schlegel *et al* (2000) this pattern correspond well with *S. infantarius*.
- ▶ On exposure to bile and low pH of 2.7, all tested isolates were tolerant to 0.3% oxgall and survive even after 4 h of exposure, whereas the isolates showed variable susceptibility to survival at pH 2.7.

▶ A minor group of homofermentative cocci growing at 15 °C and 45 °C showed high 16S rRNA gene similarity (100%) to *E. faecium*.

▶ All isolates of this group were found to produce acid from L-arabinose, ribose, maltose, mannitol and melibiose, but not melezitose, xylose and sorbitol.

▶ The heterofermentative rod-shaped isolates grouped in one rep-PCR cluster, grew at both 15°C and 45 °C, identified as *Lb. fermentum* by the 16S rRNA gene sequencing (100% similarity) corroborated by API 50CHL carbohydrate assimilation profiling

▶ A single homofermentative rod growing at 45 °C but not at 15 °C was identified as *Lb. helveticus* by both 16S rRNA gene sequencing (99.8%) and carbohydrate assimilation profiling.

▶ Yeast isolates were grouped in two clusters based on rep-PCR, identified as *K. marxianus* and *I. orientalis* by sequencing of 26S rRNA gene confirmed by the carbohydrate assimilation profiling.

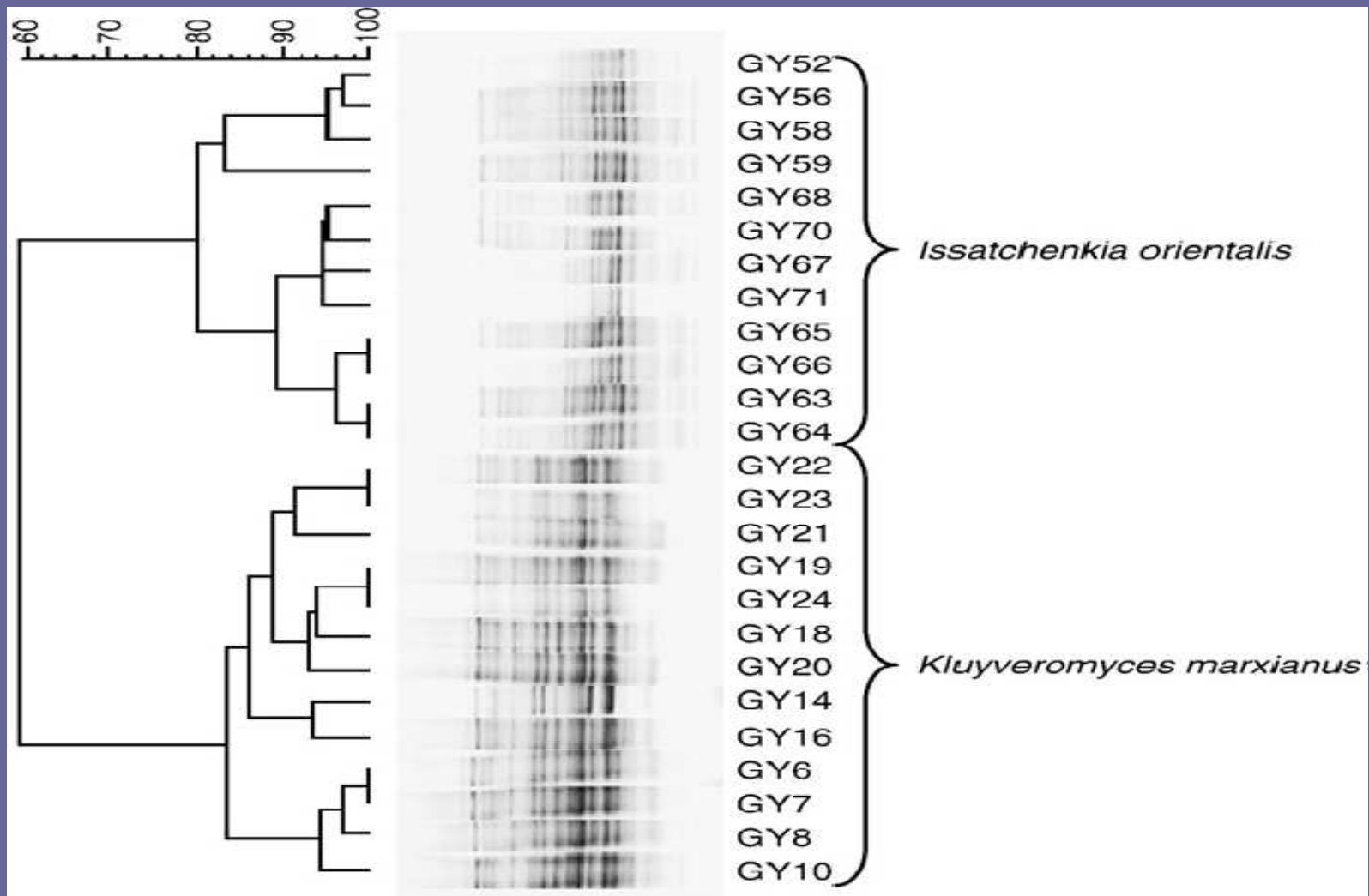


Fig. 1: Dendrogram obtained by cluster analysis of rep-PCR (GTG₅) fingerprints of yeast isolates originating from Gariss. The Dendrogram is based on Dice's coefficient of similarity with the unweighted pair group method with arithmetic average clustering algorithm (UPGMA).

▶ *S. infantarius subsp, infantarius* and *Lb. fermentum* were the dominant LAB in the 9 investigated samples of Gariss, *E. faecium* constituted between 7 and 36% of the LAB isolated from MRS from 5 out of 9 samples (not detected in 4 samples), *Lb. helveticus* was detected in only one sample.

▶ *K. marxianus* was the dominant yeast in 7 out of 9 samples.

Discussion:

- ▶ LAB and yeast cell count found were comparable to those reported in previous studies on fermented milk products in Sudan and neighboring countries.
- ▶ *S. infantarius subsp. Infantarius* and *Lb. fermentum* were the dominant LAB in Gariss.
- ▶ *Lb. fermentum* is frequently isolated from African fermented milk products, but this study is the first report on the occurrence of high counts of *S. infantarius subsp. Infantarius* in fermented milk products in Sudan and elsewhere.

- ▶ The similarity of the rep-PCR profiles of the *S. infantarius* subsp. *infantarius* indicates that the isolates of the nine samples belong to the same subspecies
- ▶ The potential human pathogenicity of *S. infantarius* subsp. *infantarius* is indicated by the previous isolation of the bacterium from blood, endocarditis and the detection of the *gtf* gene.

- ▶ The glucosyltransferase enzyme encoding gene *gtf* detected in 77% of the tested strains has been suggested as a virulence factor in systemic infection, being responsible for biosynthesis of the capsule-like extracellular polysaccharide.
- ▶ It has also been reported in adhesion, invasion and killing of cultured human umbilical endothelial cells by other streptococci than *S. infantarius* subsp. *Infantarius*.

▶ The tolerance of all tested strains to 0.3% bile indicates their ability to survive and establish within the human and animal gastrointestinal tract.

▶ Despite the high counts of *S. infantarius* subsp. *Infantarius* in Gariss, no incidence of transmitted disease was reported among the consumers.

▶ On the contrary, fermented camel's milk is used to cure leishmaniases or kala-azar and other kinds of infections in Sudan.

▶ The presence of high yeast counts, indicate that not only LAB but also yeast contribute to the fermentation of camel's milk to produce Gariss.

▶ Both yeast species have previously been reported isolated from indigenous fermented cow and camel's milk products in Africa and elsewhere.

▶ Many strains of *K. marxianus* are capable of metabolizing lactose whereas *I. orientalis* is lactose negative. Both species are able to metabolize lactate.

▶ Yeast growth in Gariss is thus probably positively influenced by the metabolic activities of the LAB present.

Conclusion

- ▶ The dominant microflora were *S. infantarius* subsp. *Infantarius*, *Lb. fermentum*, *K. marxianus* and *I. orientalis*.
- ▶ *S. infantarius* subsp. *Infantarius* is a potential pathogen and has been isolated from septicemic patients and some food items as contaminants.
- ▶ Some strains in this study were found to contain a gene encoding the virulence determinant *gtf* and pose a potential safety risk and hence the practice of using Gariss as inoculum for subsequent fermentation (back-slopping) is questionable.



