Raw goat's milk fermented Anbaris from Lebanon: insights into the microbial dynamics and chemical changes occurring during artisanal production, with a focus on yeasts

Marie-José Ayoub, Pamela Bechara, Mariana Habchi, Rachelle Hosry, Mohamad Akl, Sandy Haj Hassan and Pierre Abi Nakhoul

SUPPLEMENTARY FILE

Graphical Abstract

Materials and Methods

Microbial counts

20 grams of Anbaris or 20 ml of milk were diluted and homogenized in 180 ml of sterile buffered peptone water (Scharlau, Spain). Serial duplicated 10-fold dilutions were made in sterile buffered peptone water and plated on general or specific media incubated according to the conditions specified hereafter for each microbial group. Enumeration results were expressed in $\log_{10}$ cfu/g of product or ml of milk. Total aerobic mesophilic microorganisms were grown on Plate Count Agar (PCA, Merck, Germany) and enumerated after 48/72 h of incubation at 30°C. Mesophilic Presumptive Lactococci and Streptococci were grown on M17 agar (Fluka, Switzerland) and enumerated after 48 h of incubation at 30°C. Mesophilic Presumptive Lactobacilli were grown on de Man, Rogosa and Sharpe or MRS agar (Fluka, Switzerland) supplemented with cycloheximide and enumerated after 72h of incubation under anaerobic conditions at 30°C. Enterobacteriaceae were grown on Violet Red Bile Glucose agar (VRBG, Sharlau, Spain) and Coliforms on Violet Red Bile Lactose agar (VRBL, Sharlau, Spain) using the pour-plate and overlay technique for both media. Bacteria were enumerated after 24h of incubation at 37°C. Yeasts were grown on Yeast-Extract Glucose Chloramphenicol agar (YGC, Bio-Rad, France) and enumerated after 3–5 days of incubation at 28°C.

Yeast species molecular identification

PCR amplifications of the ITS1-5.8S-ITS2 rDNA region were performed with the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') according to the following program: an initial denaturation step at 95°C for 5 min followed by 35 cycles of 95°C for 1 min, 55.5°C for 2 min, and 72°C for 2 min, and a final extension step of 10 min at 72°C. The success of the amplifications was monitored on 1.4% agarose gels in 1xTBE buffer. Restriction fragments were separated on 2%

agarose gels in 1xTBE buffer. All gels were stained using Ethidium bromide, visualized under UV light and their photos captured and integrated using the Digidoc-It Imaging System (UVP/Analytik Jena, Germany*).* Bands sizes were estimated in comparison to the 100 bp DNA ladder (Solis BioDyne, Estonia) using the VisionWorks-V8 software (UVP/Analytik Jena, Germany). For sequencing, amplicons were purified (PerfectPrep Gel Cleanup, Eppendorf, Germany) and then sequenced using the BigDye Terminator (v1.1) Cycle Sequencing kit (Applied Biosystems, USA) on an ABI 3130 Genetic Analyser (Applied Biosystems, Thermo Fisher Scientific, USA).

Kluyveromyces marxianus Typing

Inter-LTR PCR was carried out for 60 isolates of the *K. marxianus* species with the primers KM1 (5'-GTTGGTATAATATCTGG-3') and KM2 (5'-TTCTAAGGTCCCTACTAC-3') and using the following program : an initial denaturation step at 94°C for 4 min followed by a first series of 4 cycles at 94°C for 30 sec, 38°C for 30 sec and 72°C for 2 min then a second series of 30 cycles at 94°C for 30 sec, 41°C for 30 sec, and 72°C for 2 min, and a final extension step of 5 min at 72°C. PCR amplification products were separated on 1.7% (w/v) agarose gels in 1xTBE buffer and the gels stained, visualized and analysed as mentioned previously.

Proteolysis and Lipolysis indices determination

To determine the water-soluble nitrogen (WSN), 10 g of product were homogenized with 50 mL of deionized water. The homogenate was incubated at 40^{o}C for 1 h, centrifuged at 4^{o}C (3000g, 3min) and filtered through Whatman N^{o}40 filter paper. The content of water-soluble nitrogen (WSN) was determined by the Kjeldahl method and expressed as percentage of total nitrogen (TN). For the 12% (w/v) Trichloroacetic acid-soluble nitrogen (TCA-SN), 10 ml of 24% (w/v) TCA (trichloracetic acid) solution were mixed with 10 ml of the WSN fraction. The mixture was allowed to stand for 2 h at room temperature before it was filtered through Whatman N^{o}.

40 filter paper to eliminate the precipitate. Nitrogen content of the filtrate was also determined by Kjeldahl method and expressed as percentage of TN. To determine the acid degree value (ADV), 10 g of sample were homogenized and placed into a butyrometer for fat extraction with 10 ml of BDI reagent (30g triton X-100 and 70g of sodium tetraphosphate for a 1 L distilled water solution) and the mixture was incubated in a boiling water bath (100^{o}C) for 20 min and centrifuged (3000g) for 1min. Enough aqueous methanol was added to bring the fat into the graduated portion of the butyrometer's neck before the mixture was centrifuged again for another 1 min. The separated fat fraction was then transferred to an erlen meyer flask and weighed to be later dissolved in 5 ml fat solvent (4:1 petroleum ether and n-propanol). 5 drops of 1% phenolphthalein were added and titration followed with 0.02 N alcoholic KOH. Acid Degree Value (ADV) was calculated according to the following formula where N = normality of KOH solution:

$$ADV = \frac{(\text{ml KOH for sample} - \text{ml KOH for blank}) \times \text{N} \times 100}{\text{Weight of fat}}$$

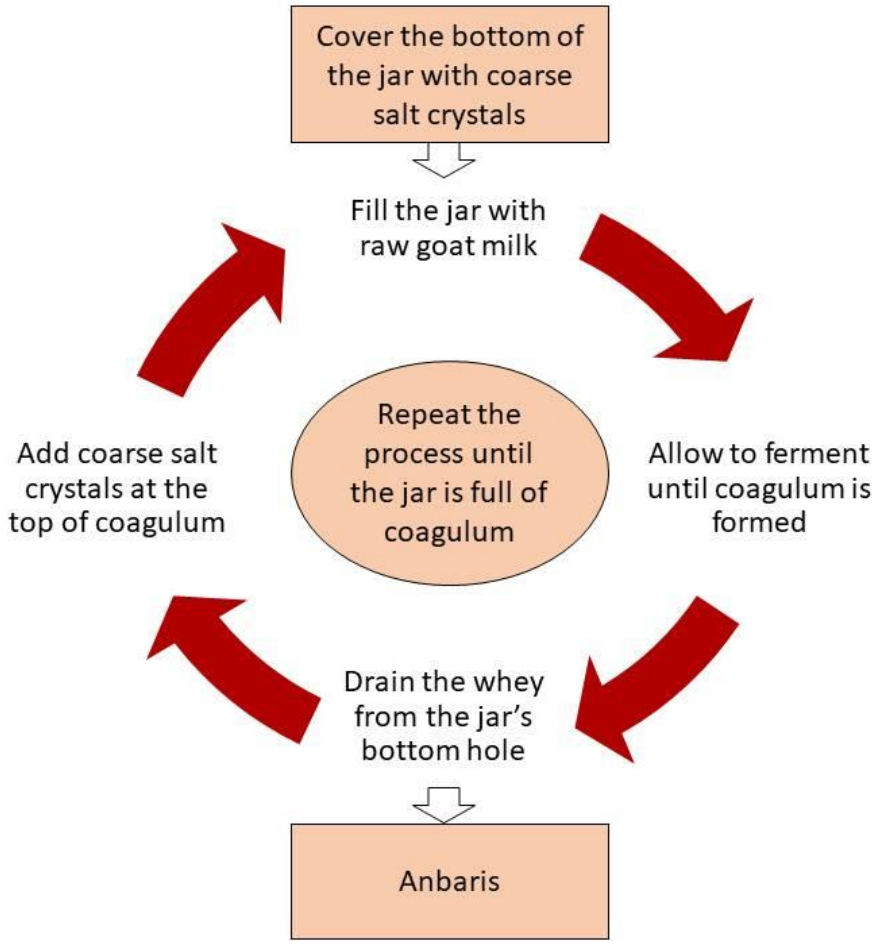

Figure S1 Artisanal manufacturing procedure of Anbaris

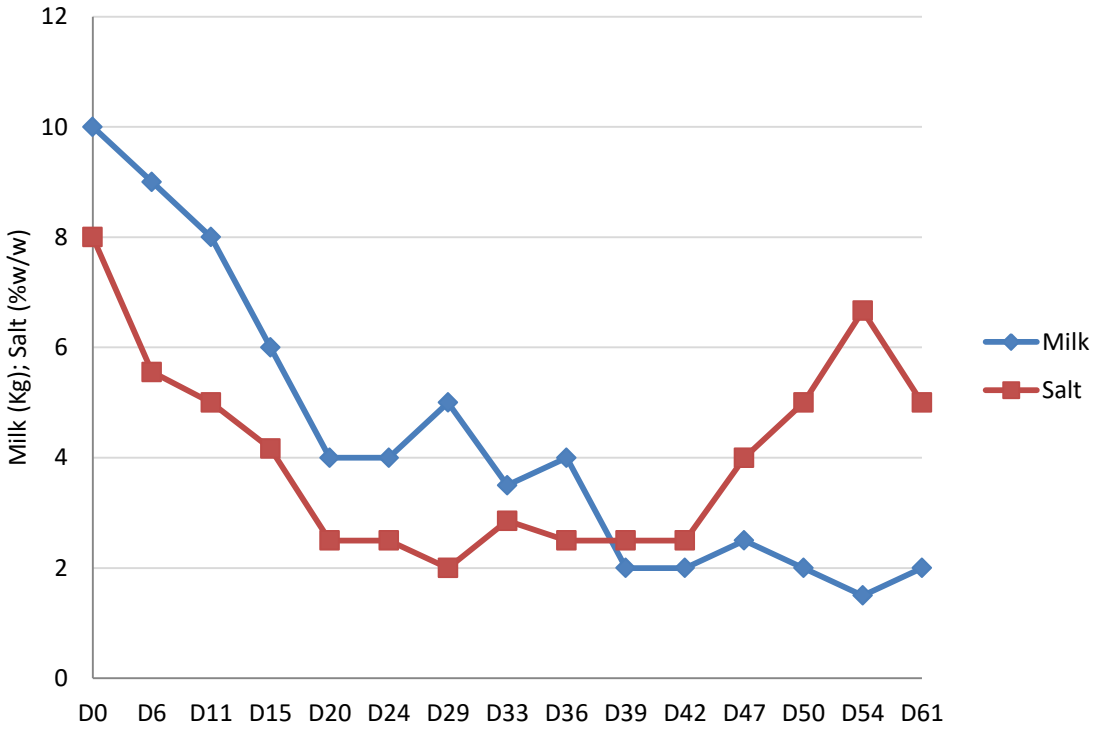

Figure S2 Milk (Kg) and Salt (g/100g) additions made during Anbaris manufacturing

Note: These percentages do not represent the percentage of salt homogenized in milk but rather the salt quantities added relatively to milk quantities. Salt is added at the bottom of the jar at D0, and at the top of the coagulum once the latter is formed. Salt additions are made before those of milk, allowing salt to slowly diffuse into the new coagulum that will be formed. For measured quantities of salt in the product refer to Table S2.

Table S1 Microbial counts recorded throughout the manufacturing process

Day	MA	MLB	MLS	Yeasts	Enterobact.	Coliforms
D0‡	6.37^{a} ±0.13	6.47^{a} ±0.28	6.03^{a} ±0.16	1.71^{a} ±0.16	4.27^{ab} ±0.28	3.97^{ab} ±0.35
D6	8.74^{c} ±0.37	8.22^{bc} ±0.14	7.82^{b} ±0.14	5.72^{b} ±0.10	4.47^{b} ±0.24	4.16^{ab} ±0.38
D15	8.58^{c} ±0.16	8.11^{bc} ±0.47	7.85^{b} ±0.25	5.88^{b} ±0.18	3.70^{ab} ±0.17	4.34^{b} ±0.14
D29	8.82^{c} ±0.24	8.13^{bc} ±0.16	7.53^{b} ±0.11	5.65^{b} ±0.03	3.65^{ab} ±0.27	3.73^{ab} ±0.44
D47	8.93^{c} ±0.44	8.60^{c} ±0.25	7.95^{b} ±0.21	5.83^{b} ±0.21	3.81^{ab} ±0.42	3.58^{ab} ±0.21
D61	8.26^{bc} ±0.27	7.77^{bc} ±0.37	7.71^{b} ±0.28	5.98^{b} ±0.14	4.19^{ab} ±0.21	3.88^{ab} ±0.13
D75	7.52^{b} ±0.28	7.42^{ab} ±0.35	5.78^{a} ±0.23	5.47^{b} ±0.20	3.21^{a} ±0.33	3.17^{a} ±0.15
Pr>F	0.000	0.004	<0.0001	<0.0001	0.032	0.054
Sig.	Yes	Yes	Yes	Yes	No	No

‡ log cfu/ ml for D0 (milk) and log cfu/ g for the other samples (Anbaris)
Data are means ± standard deviation of two repetitions
MA Mesophilic Aerobic flora
MLB presumptive Mesophilic Lactobacilli
MLS presumptive Mesophilic Lactococci and Streptococci
Enterobact. Enterobacteriaceae
Sig. Significance level 99%

Table S2 Chemical Parameters measured throughout the manufacturing process

Day	pH	TA g/100g lactic acid	Salt g/100g	Moisture g/100g	Protein g/100g	Fat g/100g	ADV meq.KOH/100g fat	WSN %TN	12%TCA-SN %TN
D0	6.52^{c} ±0.01	0.21^{a} ±0.01	-	88.09^{e} ±0.18	4.23^{a} ±0.11	4.61^{a} ±0.13	-	-	-
D6	3.54^{b} ±0.01	1.91^{b} ±0.14	3.81^{d} ±0.07	55.23^{b} ±0.43	16.66^{cd} ±1.05	22.75^{c} ±0.35	0.46^{a} ±0.04	10.24^{a} ±0.59	8.66^{abc} ±0.37
D15	3.47^{a} ±0.02	1.87^{b} ±0.07	2.17^{b} ±0.02	65.00^{d} ±0.57	13.20^{b} ±0.58	17.38^{b} ±0.18	0.49^{a} ±0.04	10.34^{a} ±0.46	6.21^{a} ±1.62
D29	3.50^{ab} ±0.03	1.97^{b} ±0.11	1.53^{a} ±0.03	61.47^{c} ±1.36	14.75^{bc} ±0.05	18.63^{b} ±0.88	0.52^{ab} ±0.06	12.18^{ab} ±0.58	8.26^{ab} ±0.81
D47	3.46^{a} ±0.01	1.95^{b} ±0.02	1.62^{a} ±0.07	55.31^{b} ±0.55	15.49^{cd} ±0.26	23.85^{c} ±1.63	0.70^{c} ±0.03	12.92^{abc} ±0.98	10.57^{bc} ±0.92
D61	3.51^{ab} ±0.01	2.03^{b} ±0.23	2.56^{c} ±0.02	51.89^{a} ±0.95	17.00^{d} ±0.21	24.75^{c} ±1.06	0.65^{bc} ±0.02	13.44^{bc} ±0.66	10.60^{bc} ±0.71
D75	3.49^{a} ±0.02	1.95^{b} ±0.08	4.25^{e} ±0.03	50.54^{a} ±0.04	17.51^{d} ±0.70	25.63^{c} ±0.53	0.80^{c} ±0.05	15.30^{c} ±1.13	11.95^{c} ±0.49
Pr>F	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.001	0.004	0.007
Sig.	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

Data are means ± standard deviation of two repetitions except for pH, TA and salt content, analysed in triplicate
TA Titrable Acidity
ADV Acid Degree Value
WSN Water Soluble Nitrogen
12% TCA-SN Nitrogen soluble in 12% Trichloro Acetic Acid
Sig. Significance level 99%