

## Halophilic Archaea Cause the ‘Red Cheese Spoilage’ of the Boiled White Cheese – Fulfillment of Koch’s Postulates

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### Abstract

*Boiled white cheese is seasonally produced by renneting a mix of sheep and goat milk, pressing and cutting the formed curd into pieces which are boiled in brine containing ~ 25% salt (NaCl). Cheese pieces are usually kept in the cooled brine, without refrigeration, for up to one year in tin cans. Producers and consumers have been complaining about spoilage of the boiled white cheese in which cheese pieces and brine show a pink to red discoloration accompanied by unwanted changes in flavor. (“The red cheese spoilage”). Boiled white cheese samples with red color deviations were collected and tested for the halophilic Archaea. Salt concentration in the brine ranged from 16 to 29% (w/v), with an average of 22.7%, and the pH ranged from 4.2 to 6.3 with the average of 5.2. Counts of halophilic Archaea were determined by applying the surface or spread plate method using a halophilic agar medium containing 25% (w/v) NaCl. Red, pink or orange colonies observed after incubation in plastic bags at 37°C for up to 4 wks were counted and were found to belong to the halophilic Archaea. The counts ranged from  $1.5 \times 10^5$  to  $5.1 \times 10^7$  cfu/ml, with an average of  $6.1 \times 10^6$  cfu/ml. The halophilic Archaea belonged to *Halobacterium*, *Haloferax*, *Haloarcula* and *Halococcus* of the family Halobacteriaceae. These findings and the ability of these Archaea experimentally to cause red cheese spoilage in freshly produced boiled white cheese after inoculation from active pure species cultures and storage at room temperature and to re-isolate the corresponding halophilic Archaea from the experimentally spoiled cheese could confirm that halophilic Archaea are responsible for the “red cheese spoilage” of the boiled white cheese, and thus fulfilling Koch’s postulates for red cheese spoilage halophilic Archaea.*

**Key words:** Boiled white cheese, Red cheese spoilage, Halophilic Archaea, Halobacteriaceae, Koch’s postulate

### 1. Introduction

Addition of excess salt, sodium chloride, to food is one of the oldest methods of food preservation and has been used for centuries. Although excessive salt may have negative effect on the sensorial quality of food, particularly flavor, salting is still used as a traditional preserving method of some fish, meat and dairy products (Potter and Hotchkiss, 1995; Saderson, et al., 1988).

White brined cheese is one of the most important dairy products manufactured traditionally in the Mediterranean region. The range includes Feta in Greece, Domiti in Egypt, Halloumi in Cyprus and Lebanon and the boiled white cheese in Jordan and some neighboring countries (Caric, 1987; Tamime and Robinson, 1991). Boiled white cheese is available commercially in the form of hard white cheese pieces (ca.  $4 \times 8 \times 2$  cm) that are usually completely immersed in brine (~25% NaCl) and stored in firmly closed tin cans. This type of cheese is produced seasonally by renneting sheep milk or a mix of sheep and goat milk, usually without pasteurization of the milk and without the addition of starter cultures. Production steps include boiling of pieces of white soft cheese in brine solution containing approximately 20% salt after which cheese pieces are then removed and cooled (Humeid and Tukan, 1986; Humeid et al., 1990). During cooling cheese pieces are usually filled into the tin cans and topped with the cooled brine. Such product could be kept at ambient temperature for up to one year (Yamani et al., 1987).

The producers and the consumers of the boiled white cheese have been complaining about a unique form of spoilage to their products known as the “red cheese spoilage”. In this spoilage, the cheese and the brine show a pink to red discoloration accompanied by unwanted changes in the flavor of the cheese. Our examination of samples of such cheese by routine tests, i.e., gram staining and determination of the aerobic plate count and the counts of lactic acid bacteria and yeasts and molds gave negative results.

The Archaea are widely distributed in nature, and have been recently recognized to form with Bacteria and the Eukaryota the three domains of life (Woese et al., 1990). Some well known genera of the Archaea, like those belonging

to the family *Halobacteriacea*, which grow only in the presence of high concentrations of salt, are extremophilic prokaryotic organisms. Members of the halophilic Archaea may cause spoilage of various protein-rich products preserved by salting, like salted fish, viscera and hides (Larsen, 1967; Tindall, 1992). In extreme cases these Archaea reveal themselves as a red-colored slimy mass with most offensive odor (Sanderson, 1988).

Boiled white cheese is rich in protein and is usually kept in brine with high salt concentration and stored for several months without refrigeration. These and the negative results of routine microbiological testing and the red discoloration of the spoiled cheese called us forth to conduct this work to investigate the role of the halophilic Archaea in the “red cheese spoilage” of the boiled white cheese.

## **2. Materials and Methods**

### **2.1. Samples**

We collected 41 boiled white cheese samples that showed different degrees of reddish discoloration, deviating from the normal white color were collected from the local markets and homes in Amman, Jordan. Each sample consisted of two to four cheese pieces and brine solution. Samples were transferred under aseptic conditions into sterile glass jars using sterile equipment and were taken to the laboratory and examined within 4 hours of the procurement.

### **2.2. Chemical Analysis**

Chemical analyses of collected samples were performed after the microbiological examination to avoid samples contamination.

The pH of the brine solutions was determined by immersing the electrode of a pH-meter (Hanna Instruments HI-8519) directly into the brine samples. The titration method against silver nitrate as described in the AOAC (1990) was followed for salt (NaCl) determination.

### **2.3. Microbiological Examination**

#### **2.3.1. Direct microscopic examination**

Smears from cheese brine samples and isolates from the discolored surface of cheese samples and from the red, pink or orange colonies grown on a halophilic agar (HA) were made in 25% sterile NaCl solution, left to dry on a glass slide, flooded with 2% acetic acid for 5 minutes before gram staining (Dussault, 1955). Microscopic examination was then performed.

#### **2.3.2. Enumeration of halophilic Archaea**

The surface or spread plate method was followed (Swanson et al., 1992). Ten-fold serial dilutions of the brine samples were made using a sterile 25% (w/v) NaCl (Riedel-de Haën) solution. Using a sterile L-shape glass rod, 0.1 ml of each dilution was spread over the surface of halophilic agar (HA) which contained 25% (w/v) NaCl (HA25). The plates were then left to dry and then placed in plastic bags to avoid medium dehydration. Plates were then incubated at 37°C for four wks after which red, pink, or orange bacterial colonies were counted (Larsen, 1984; Tindall, 1992).

HA25 halophilic agar was prepared by dissolving 250 g NaCl, 20 g MgSO<sub>4</sub>·7 H<sub>2</sub>O, 2 g KCl, 3 g trisodium citrate, 0.036 g FeCl<sub>2</sub>·6 H<sub>2</sub>O, and 0.00036 g MnCl<sub>2</sub>·4 H<sub>2</sub>O in 500 ml distilled water and then completing the volume to 600 ml with distilled water before adjusting the pH to 7 and autoclaving at 115°C for 15 min (part A of HA 25). Part B of HA25 was prepared by dissolving 5 g yeast extract (Oxoid), 5 g peptone (Oxoid), 5 g glucose (Merck) and 15 g agar (Oxoid) in 350 ml distilled water and then completing the volume to 400 ml with distilled water before adjusting the pH to 7 and autoclaving at 115°C for 15 min. After sterilization, 10 ml of a sterile 2% CaCl<sub>2</sub> (w/v) were added to part A, after which, parts A and B were carefully mixed and poured into petri dishes.

#### **2.3.3. Purification and Maintenance of the Isolates**

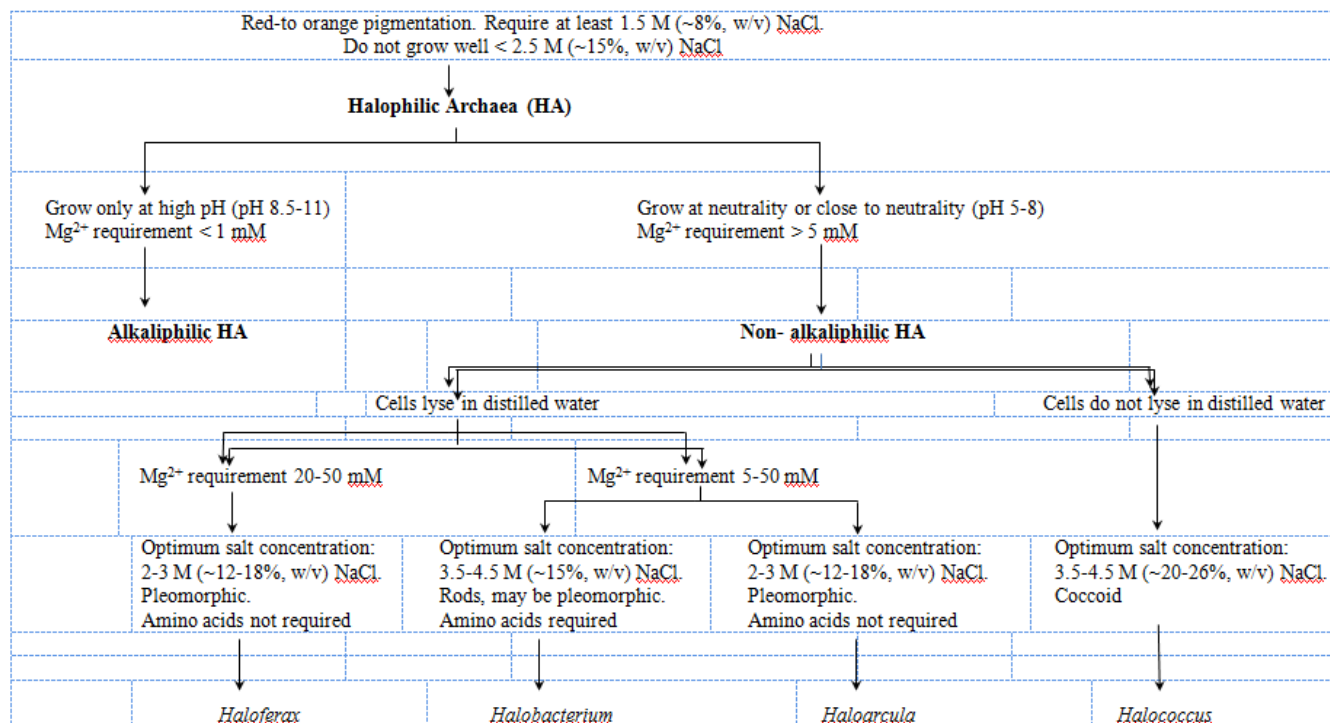
Red, pink or orange bacterial colonies were selected from the plates used in the enumeration and were tested by a modified gram stain method (Dussault, 1955). Pure cultures were obtained by sub-culturing two to three times on HA25 agar. Slants of HA25 agar were used for maintenance of the cultures, which were stored at 7°C and sub-cultured at 2-3 months intervals.

#### **2.3.4. Identification**

Tests used in the identification are those outlined in Bergey's Manual of Systematic Bacteriology (Grant et al., 2001) and The Prokaryotes (Oren, 2006). The tests included gram stain method (Dussault, 1955), oxidation / fermentation (Ventosa, 1982), catalase (Quesada et al., 1987), oxidase (Kovacs, 1956), anaerobic growth (Ventosa, 1982; Sanderson, 1988), motility (Ventosa et al., 1982), nitrate reduction (Sanderson, 1988; Ventosa, 1982), indole production (Ventosa, 1982), production of H<sub>2</sub>S (Ventosa, 1982; Quesada et al., 1987; Rodriguez et al., 1985), optimum concentration and

range of salt tolerance (Rodriguez et al., 1985), utilization of sugars (Leifson, 1963), hydrolysis of casein (Ventosa et al., 1982), gelatin (Ventosa et al., 1982; Sanderson, et al., 1988), starch (Rodriguez et al., 1985; Ventosa et al., 1982) and Tween hydrolysis (Quesada et al., 1987). Figure 1 shows the procedure we used for the identification of the isolates to the genus level. This procedure and the identification to the species levels were based on *Bergey's Manual of Systematic Bacteriology* and *The Prokaryotes*.

**Figure 1: Scheme used for the identification of the halophilic archaea isolated from brine samples of the white boiled cheese (Grant et al., 2001).**



## 2.4. Inoculation of boiled white cheese with halophilic Archaea

### 2.4.1. Manufacture of boiled white cheese

The method of boiled white cheese production as described by Yamani et al., (1998) was followed. Sheep's milk was warmed to 35°C and coagulated by rennet [(Rennimax 1/150, commercial *Mucormiehei* rennet powder) obtained from Tulip Co., origin of Spain, Amman, Jordan]. The formed curd was strained through cheese cloth and pressed for 2 hours. Produced cheese was then cut into small pieces (ca 4 × 8 × 2 cm). The pieces were sprinkled with salt and left overnight in the refrigerator and then boiled by dipping into boiling brine (20%, w/v NaCl) and heating at boiling temperature until the pieces became soft and float to the surface of the brine; boiling took about 15 min. Cheese pieces were then taken out of the brine, placed over a flat surface, reshaped by slight pressing, left to cool, and kept in 500 ml glass jars in the brine in which the cheese pieces were boiled.

### 2.4.2. Halophilic Archaea cultures preparation and inoculation of the cheese

Pure cultures of *Halobacterium salinarium*, *Haloarcula hispanica*, *Haloferax denitrificans* and *Halococcus* that were isolated from previous steps were inoculated in HA25 broth and incubating at 37°C for several days. The halophilic broth had the same composition as the halophilic agar, with the exception of the excluding of agar. Halophilic counts of the cultures were made when the cultures showed acceptable growth as judged by appearance of turbidity in the broth. Each culture was inoculated into 2 cheese jars with a range of 10<sup>3</sup> - 10<sup>4</sup> cfu/ml of the brine. Two jars were left without inoculation, to be used as control.

## 3. Results and discussion

Boiled white brined cheese is an interesting microbial ecosystem. The high salt content of the cheese and the brine make this type of cheese a suitable habitat for the growth of only salt tolerant microorganisms. The long shelf-life of the product (up to one year at room temperature) allows interactions and probably successions of the salt tolerant microorganisms that could affect the quality of the cheese.

Routine testing of the cheese and brine samples showing “red cheese spoilage”, including aerobic plate count, and counts of lactic acid bacteria (LAB), coliforms and yeasts and molds, gave negative results. To the best of our knowledge, no LAB or *Enterobacteriaceae* are able to grow at the high salt concentrations of the brine of the boiled white cheese.

Deviations from the normal color and smell in all cheese samples were observed. Cheese samples and the brine showed red discoloration (Figure 2) accompanied with offensive smell, denoting the typical “red cheese spoilage” of the boiled white cheese.

### 3.1. Chemical analysis

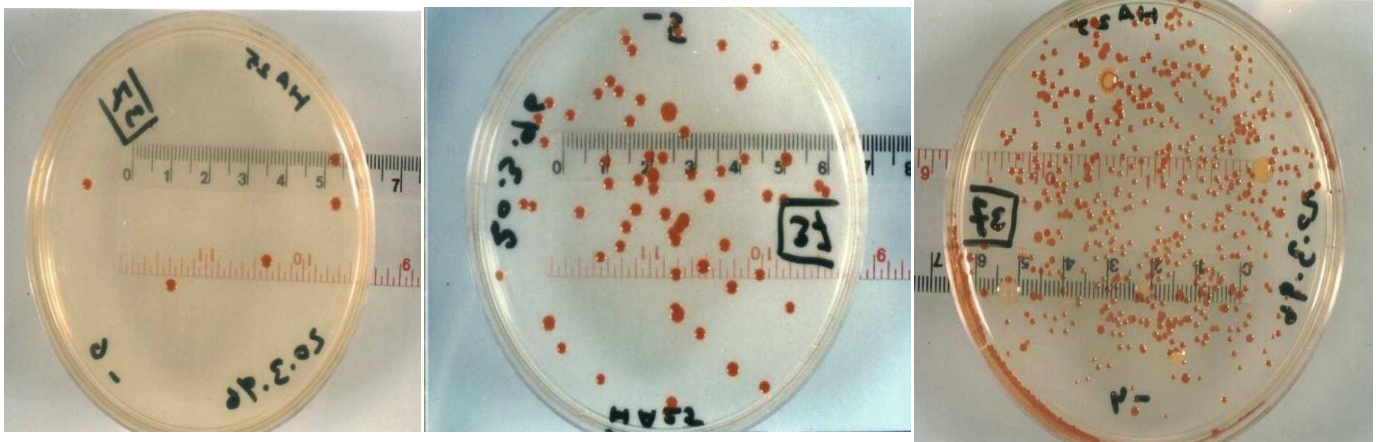
Noticeable differences were observed in the pH of the samples, which ranged between 3.4 and 6.3, with an average of 4.9. More than 50% of the samples (25) had a pH > 5 and only 6 samples had a pH value of less than 4.

Salt content (NaCl%, w/v) of the brine samples ranged from 9.4 to 36.7%, with an average of 23.1%. The majority of the samples (35, 75%) had a salt content of more than 20% and only one sample had a salt content less than 10%.

### 3.2. Microbiological examination

Counts and types of microorganisms that were reported in the cheese samples represented the microbiota at the time of sampling where salt concentration is the limiting growth factor that allowed only salt tolerant microorganisms to grow. Most of the brine samples harbored high numbers of such microorganisms, which were able to grow on the surface of HA25 forming colonies with different shades of red and orange coloration, suggesting halophilic Archaea (Figure 3). The elevated numbers of salt tolerant Archaea in the brine samples reflects the suitability of the brine pH and the availability of sufficient nutrients for halophilic Archaea needed for growth. Counts of the halophilic Archaea ranged from  $<10^3$  to  $7.5 \times 10^7$ cfu/g. The counts were  $>10^4$ cfu /g in 32 samples;  $>10^5$ cfu/g in 26 and  $>10^6$ cfu/g in 13 samples. Only Gram-negative microbial cells were detected on HA25 plates. Apparently, no relationship could be established between the pH and the salt content of the samples and their corresponding counts of the halophilic Archaea. The growth of the halophilic Archaea could explain the spoilage of the boiled white cheese in which the cheese and the brine were discolored in red, and which is similar to the spoilage of salted meat, fish and hides (Larsen, 1986). Moreover, the halophilic Archaea are known for their proteolytic activity (Larsen, 1984; Grant et al., 2001) that explains the unwanted changes in the flavor of the spoiled boiled white cheese.

**Figure 3. Typical red colonies of the halophilic Archaea on agar plates of testing samples of boiled white cheese showing “red cheese spoilage”.**



Halophilic Archaea need special culture media (Baross and Lenovich, 1992) and long incubation period (4 to 6 wks) (Grant et al., 2001; Tindall, 1992) during which the petri dishes should be placed in plastic bags to avoid medium dehydration (Tindall, 1992). Furthermore, many of the halophiles would lyse in water, so they could be examined microscopically only after modification of the gram stain method, in which fixation is done using diluted acetic acid (Grant et al., 2001; Tindall, 1992). This may explain why previous routine microbiological examinations were not able to give the true picture of the microbial flora of the boiled white cheese.

The Archaea are widely accepted, beside the Bacteria, and the Eucarya (Eucaryota), as one of the three domains of life as proposed by Woese et al., (1990). Currently, 14 genera belong to the halophilic Archaea within the family *Halobacteriaceae* of the order *Halobacteriales* (Grant et al., 2001);

Some are alkaliphilic, namely species of *Natronobacterium*, *Natronococcus*, *Natronomonas* and *Natronorurum*, which were excluded in this study because they grow only at high pH (8.5-11) (Grant et al., 2001).

Accordingly and after consulting the respective identification tables in *Bergey's Manual of Systematic Bacteriology* (Grant et al., 2001) and *The Prokaryotes* (Oren, 2006), we could identify the pigmented Archaea which were isolated from HA25 plates as probably belonging to *Halobacterium*, *Haloferax*, *Haloarcula* and *Halococcus*. Table 1 shows characteristics of the halophilic Archaea species isolated from samples of the boiled white cheese.

**Figure 1:** Scheme used for the identification of the halophilic archaea isolated from brine samples of the white boiled cheese (Grant et al., 2001).

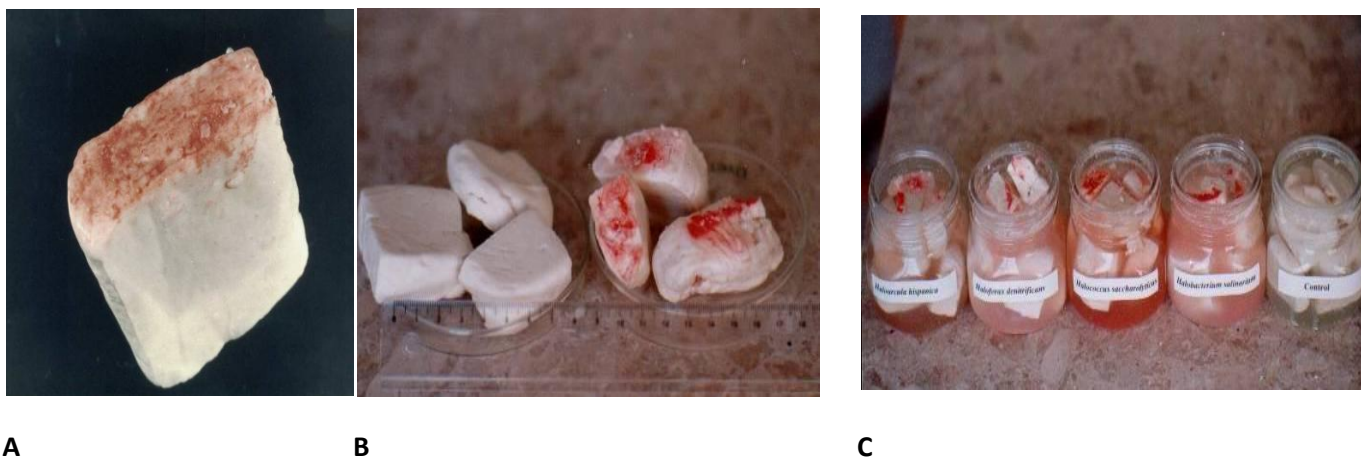
	<i>Halobacterium salinarium</i>	<i>Halobacterium rubrum</i>	<i>Haloarcula hispanica</i>	<i>Haloarcula marisortui</i>	<i>Haloferax dentrificans</i>	<i>Haloferax gibbonsii</i>	<i>Haloferax mediterraneus</i>	<i>Haloferax volcanii</i>	<i>Halococcus morrhuae</i>	<i>Halococcus sturkumensis</i>	<i>Halococcus saccharovorans</i>
Colony Color	Red	Red	Cream	Red	Cream	Red	Cream	Red	Red	Red/ Cream	Red
NaCl (%)											
Minimum	15	15	10	10	10	15	15	10	10	10	10
Optimum	20 – 25	20–25	15–20	15–20	15–25	15–25	15–25	15–25	15–25	20–25	15–20
Maximum	35	35	30	30	35	35	35	25	35	35	35
Lysis in distilled water	+	+	+	+	+	+	+	+	-	-	-
Mg <sup>2+</sup> (mM range)	0–40	0–40	5–40	5–20	10–40	10–40	20–40	10–40	5–40	5–40	5–40
Motility	+	+	+	+	+	+	+	+	+	-	-
Anaerobic growth	+	-	+	+	-	+	+	+	+	-	+
H <sub>2</sub> S production	-	-	-	+	+	+	-	-	-	-	+
Oxidation/Fermentation (O/F)	F	F	F	F	O	F	F	F	F	+	F
Nitrate reduction	+	-	+	+	+	+	-	-	-	+	-
Acid from											
Lactose	+	-	-	-	-	-	-	-	-	-	-
Glucose	-	-	-	-	-	-	-	-	-	-	+
Galactose	-	-	-	-	-	-	-	-	-	+	-
Ribose	+	+	+	+	+	+	+	+	+	-	+
Maltose	-	-	-	+	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-	-	-	+	-
Arabinose	-	-	-	+	-	+	+	+	+	-	+
Fructose	-	-	-	+	-	+	+	-	+	-	+
Glycerol	-	-	-	-	-	-	-	-	-	+	-
Mannose	-	-	-	-	+	-	-	-	-	-	-
Hydrolysis of											
Casein	-	+	+	-	+	-	+	-	-	+	-
Gelatin	+	-	+	-	-	+	+	-	+	-	+
Tween	-	+	+	-	+	-	+	-	-	+	+
Starch	-	-	+	+	-	+	+	-	-	-	-

### 3.3. Fulfillment of Koch's postulates for the "red cheese spoilage" halophilic Archaea

In 1890, Koch proposed formally criteria (known hitherto as Koch's postulates), which have been used for decades for establishing infectious disease causation (Evans, 1976). These guidelines were modified to overcome limitations of application in viral infections, chronic diseases and cancer (Rivers, 1937; Evans, 1976; Gilmour, 1990). However, the application of the postulates has been proved to be valid, especially in cases where the cause and effect are well defined, with no complicating interferences (Marshall et al., 1985; Osterhaus et al., 2004). This was the case in this study.

We could observe and isolate in pure cultures halophilic Archaea from every cheese sample showing signs of the "red cheese spoilage" (Figure 2). Furthermore, we were able to induce the spoilage in freshly produced boiled white cheese by inoculating the cheese with selected pure cultures of isolates obtained from spoiled cheese samples (Figure 3), and we were able to re-isolate the corresponding halophilic Archaea from the experimentally spoiled cheese. Outcome of these activities match the criteria of Koch's postulates.

Figure 2: Typical "red cheese spoilage" of the boiled white cheese; A, and B, (petri dish to the right). C, "red cheese spoilage" induced experimentally using four species of halophilic Archaea isolated from samples of spoiled cheese. Normal appearance of cheese in B (left petri dish) and C (right jar, control.)



By this fulfillment of the Koch's postulates, the authors could conclude that halophilic Archaea belonging to the genera *Halobacterium*, *Haloferax*, *Haloarcula* and *Halococcus* of the family *Halobacteriaceae* are responsible for the "red cheese spoilage" of the boiled white cheese produced traditionally in Jordan.

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