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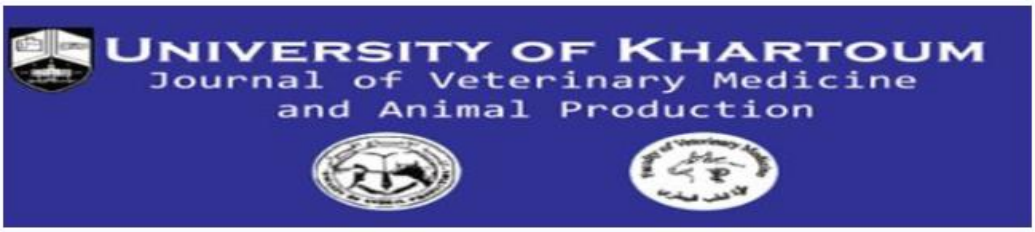

Effect of fattening on the Development of Abscess Disease in Sheep and Growth of *Staphylococcus aureus* subsp. *Anaerobius*

Abdulkhalig Babiker[1*], Khalid Rodwan[2], Kamal H. Eltom[3], and Sulieman M. El Sanousi[4]

[1]*Department of Pathology, Faculty of Veterinary Medicine, University of Khartoum, 13314, Shambat, Sudan*

[2]*Department of Pathology, Parasitology and Microbiology, College of Veterinary Medicine, Sudan University of Science and Technology, 13321, Hilat-Kuku, Sudan*

[3]*Unit of Animal Health and Safety of Animal Products, Institute for Studies and Promotion of Animal Exports, University of Khartoum, 13314, Shambat, Sudan*

[4]*Department of Microbiology, Faculty of Veterinary Medicine, University of Khartoum, 13314, Shambat, Sudan*

*Corresponding author: abdulkhalig2004@gmail.com

Abstract

The present study was designed to investigate factors related to fattened sheep that affect the growth of *Staphylococcus aureus* subsp. *anaerobius* (Sasa) and enhance its pathogenicity. The pH of sweat, cholesterol levels in sweat and sera of fattened and un-fattened sheep were measured and the effect of sera from fattened sheep on the growth of Sasa was studied. Fattened and un-fattened lambs were subjected to experimental infection using a strain of Sasa. The pH of the sweat of un-fattened group ranged between 5.0 and 6.0, while in fattened group it ranged from 6.2 to 7.6. Serum cholesterol level was significantly higher in fattened group (74.06 ± 5.56 mg/ dl) than in un-fattened group (43.00 ± 6.66 mg/ dl). Serum from fattened sheep promoted the growth of Sasa more than serum from un-fattened sheep. In experimental inoculation of sheep with this bacteria, abscesses developed in parotid lymph nodes and livers of all fattened animals (N = 6) and in 50% (N=3) of un-fattened animals and the size of the abscess positively co-related with the weight of the animal. The study concluded that changes in the blood and skin chemistry of fattened sheep enhance the growth of *S. aureus* subsp. *anaerobius* and thus its ability of infection leading to abscess formation.

Key Words: *Sheep abscess, Staphylococcus aureus anaerobius, fattening, cholesterol.*

الخلاصة

هدفت هذه الدراسة إلى تقصي العوامل المتعلقة بالأغنام المسمنة التي تؤثر على نمو المكورات العنقودية الذهبية نويع اللاهوائية وتعزز قدرتها الإسقامية. قيس في الدراسة الرقم الهيدروجيني للعرق، وقيست مستويات الكوليسترول في العرق وفي مصل الدم في كل من الأغنام المسمنة وغير المسمنة، ودرس تأثير مصل دم الأغنام المسمنة على نمو البكتيريا موضوع الدراسة، وأخضعت الحملان المسمنة وغير المسمنة لعدوى تجريبية بالبكتيريا العنقودية الذهبية نويع اللاهوائية. تراوح الرقم الهيدروجيني للعرق في المجموعة غير المسمنة بين 5.0 و6.0، بينما تراوح في المجموعة المسمنة من 6.2 إلى 7.6. وكان مستوى الكوليسترول في الدم أعلى بشكل

معنوي في المجموعة المسمنة (74.06 ± 5.56 مجم/ دل) منه في المجموعة غير المسمنة (43.00 ± 6.66 مجم/ دل). وعزز مصل دم الأغنام المسمنة نمو المكورات العنقودية الذهبية نويع اللاهوائية أكثر مما فعل مصل دم الأغنام غير المسمنة. وفي العدوي التجريبية بهذه البكتيريا، ظهرت دمامل في العقد اللمفية النكفية وفي الكبد في جميع الحيوانات المسمنة (100%، ع = 6) وفي 50% (ع = 3) من الحيوانات غير المسمنة. وقد ارتبط حجم الدمل بوزن الحيوان إيجابياً. خلصت الدراسة إلى أن التغيرات في كيمياء جلد ودم الأغنام المسمنة تعزز نمو المكورات العنقودية الذهبية نويع اللاهوائية وبالتالي قدرتها على الإصابة مما يؤدي إلى تكون الدمامل.

الكلمات المفتاحية: *دمامل الأغنام، المكورات العنقودية الذهبية اللاهوائية، التسمين، الكوليسترول.*

Introduction

Abscess Disease (Morel's disease) is a contagious lymphadenitis/ cellulitis disease of sheep and goats caused by *Staphylococcus aureus* subsp. *anaerobius* (shortly, *S. aureus anaerobius* or Sasa). This disease was firstly recognized in France by Morel (1911) and later was reported by several other French scientists. Further reports of the disease came from Spain (Blanco-Loizelier, 1958; de la Fuente and Suarez, 1985), Kenya (Shirlaw and Ashford, 1962), Iran (Afnan and Hedjazi, 1978), Hungary (Bajmocy et al., 1984), Sudan (El Sanousi et al., 1989) and Saudi Arabia (Alhendi et al., 1993). Recent outbreaks of the disease were reported from Denmark (Møler et al., 2000), the Sudan (Musa et al., 2007) and Poland (Szalus-Jordanow et al., 2011). The main pathological character of the disease is the formation of abscesses close to or within the superficial lymph nodes (parotid, pre-scapular, popliteal, etc.). Abscess disease commonly affects young sheep and it rarely affects the adult ones (de la Fuente and Suarez, 1985). The disease is endemic in nature with high morbidity and frequent relapses, but no mortality is directly attributable to it (Bajmocy et al., 1984). An early observation made by Aynaud (1928) was that the disease is mainly seen in animals of very good health that were kept for fattening. Later observations and reports in the Sudan showed higher incidence of the disease in feedlot systems compared to naturally grazing (pastoral) animals (Rodwan et al., 2013). However, this association between fattening and incidence of abscess disease was not fully interpreted. The present study shows that fattening triggers changes in the blood chemistry and skin micro-environment towards enhancing the growth of *S. aureus anaerobius* and thus formation of abscesses.

Materials and Methods

Investigations on fattened and un-fattened animals

Animals: Male sheep, of Hamari ecotype, were used all through this study. They were purchased from the local market and kept for adaptation period of 7 days during which they received de-worming drugs and antibiotics. The animals were divided into two groups: the first group (A) was provided a ration especially composed for fattening while the other group (B) received normal ration.

Collection of sweat samples: Sweat samples from animals of each group (A and B) were collected as follows: sterile nylon bag was used to stimulate sweating by covering the face of the animal allowing only the nostrils for respiration. A piece of cotton was used to collect the sweat after removal of the nylon bag. The cotton piece was soaked in chloroform, held for 2 h, squeezed and removed. The chloroform plus the traces of the sweat were then kept in the freezer before being analysed.

Sweat analysis: The pH of the sweat was determined immediately after removal of the nylon bag by use of pH indicator strips (Merck, Darmstadt,

Germany). Thin Layer Chromatography (TLC) Technique according to Egon (1969) was used to detect the lipid contents of the sweat. Cholesterol, palmitic, stearic and linoleic acids were used as standards. Lipids were visualised with iodine vapour (TLC Research Specialities, California, CA, USA) using ready plates with silica gel on aluminium cards (OC-Karten-SI, Riedel-de Häen, Germany).

Serum cholesterol level: A commercial kit (Randox Laboratories, New York, NY, USA) was used for the determination of cholesterol in the sera of both groups.

Skin swab samples: Twenty skin swab samples (10 from each group) were collected from sheep at feedlot. The swabs were rinsed with sterile normal saline before being applied on the skin at the sites of superficial lymph nodes (parotid and mandibular). The swabs were inoculated on the same day of collection onto blood agar plates and incubated in candle jar at 37 ºC for 24 h. Bacterial isolates were identified using standard biochemical tests.

Bacterial investigations

Bacterial strains: The following bacterial isolates were used in this study:

(1) *Staphylococcus aureus* subsp. *anaerobius* strain 11 SARD (highly haemolytic strain isolated from sheep with abscess disease in Al-Kadaro Veterinary Quarantine, Khartoum North). This strain was used in all experiments; (2) *S. aureus* strain 146 (isolated from lymph node abscess of sheep obtained at meat inspection in Omdurman abattoir) was used in experimental infection; (3) *S. epidermidis* (isolated from the skin of fattened sheep in this study) was used for comparison with *S. aureus anaerobius* SARD 11 in growth experiements. Identification of isolates was made using conventional bacteriological methods according to Barrow and Feltham (1993).

Effect of pH on the growth of *S. aureus* subsp. *anaerobius*: Reinforced Clostridium Medium (RCM) supplemented with 4% sterile horse serum was used to carry out this experiment. The pH of RCM was adjusted to different pH ranges (5, 5.5, 6, 6.5, 7, 7.5 and 8) before the addition of sterile horse serum. Five bottles of culture medium for each pH value were used for culturing the organism; the growth was noticed by turbidity and followed every 12 h up to 108 h.

Effect of sera of fattened and un-fattened sheep on the growth of *S. aureus* subsp. *anaerobius*: Sera from both fattened and un-fattened groups were collected aseptically. Blood agar plates were prepared with different pH values (5, 5.5, 6, 6.5,7, 7.5 and 8). Each plate was divided into two halves; each half was coated with one drop (equivalent to 20 µl) of either fattened sheep serum (fs) or un-fattened sheep serum (ufs). One drop of predetermined serially diluted culture of the organism was streaked on each half of the plate. The plates were then incubated anaerobically at 37 ºC for 48 h after which the number of colonies on each half of the plate was counted. Another set of blood agar plates (pH was adjusted to 7.5) were coated the same as above with fs and ufs, but two organisms were inoculated: Sasa strain 11 SARD and *S. epidermidis*; then, the plates were incubated at 37 ºC for 48 h (anaerbically) and at 37 ºC for 24 h (aerobically), respectively.

Effect of cholesterol concentration on the growth of *S. aureus* subsp. *anaerobius*: Aliquots of brain heart infusion broth (BHI) was supplemented with different concentrations of Cholesterol (2, 1, 0.5 and 0.25%), before being inoculated with the test organisms (Sasa and *S. epidermidis*).

Experimental infection with *S. aureus* subsp. *anaerobius* of sheep by different routes

To study the possible routes of infection with Sasa in sheep causing abscess disease, 8 animals were subjected to experimental infection using different inocula through three different routes. The inocula used were:

Bacterial culture: Inocula for experimental infection were made from 48 h cultures in 10% horse serum broth of Sasa strain 11 SARD (bc11) and *S. aureus* strain 146 (bc146).

Haemolysin: In this test, 100 ml of 48 h BHI broth culture of Sasa strain 11 SARD was centrifuged at 3000 rpm for 15 min at 10 ℃. The supernatant (containing haemolysin toxin) was concentrated 6 times using dialysis tube and filtered through 45 μm filter to assure a cell free culture filtrate containing haemolysin (CFCFH). About 1 ml of CFCFH was used for inoculation.

Whole pus (wp): Two grams of pus were aseptically collected from sheep abscess caused by Sasa. The pus was diluted with 3 ml sterile normal saline. About 0.5 ml of the diluted pus was used as inoculum.

Calcium chloride ($CaCl_2$): To induce necrosis and anaerobic conditions, 0.1 ml of 10% $CaCl_2$ was used.

Inoculation procedure: Animals were inoculated as follows: by subcutaneous route (s/c) one animal was inoculated with wp, one animal with bc11, two animals with bc146 + CFCFH + $CalCl_2$; by scarification one animal was inoculated with wp and another one with bc11; only one animal was inoculated with bc146 by intra- venous route (iv). Table 3 illustrates these inocula and routes. Scarification was done as follows: the area on left side of neck of the animals was shaved and disinfected, superficial minute abrasions were made with a sharp sterile blade, and the pus or culture (centrifuged broth culture) was applied on the abrasions using piece of sterile cotton as a bandage for two and half hours. The animals were examined daily for the development of abscesses.

Pathogenicity of *S. aureus* subsp. *anaerobius* in fattened and un-fattened sheep

In this study, 2 groups of sheep (12 each) were used. Group A was fed a fattening ration (52% high carbohydrate contents' sorghum, locally known as "*Feterita*", 47% cottonseed cake and 1% salt); group B fed only roughages (sorghum straws) for maintenance. Animals were followed up for any health problems and abnormalities; weights were taken weekly; initial weights, daily feed intake and final weight were recorded. Inoculation was done after 48 days of fattening and when the weight of fattened animals reached 35 – 44 kg; of un-fattened reached 25 – 30 kg. For inoculation, each group was divided into two subgroups: A to A1 and A2; B to B1 and B2. Groups A1 and B1 were inoculated with 48 hours' culture of Sasa in 10% serum broth containing 5.0 x 10^8 cfu/ ml; groups A2 and B2 were inoculated with 5.8 x10^8 cfu/ ml of *S. aureus* strain 146. Animals were inoculated by slight intradermal to subcutaneous route at the right side of the neck near the parotid lymph node. Inoculation sites were examined daily and clinical parameters were observed and measured for any health abnormalities. Animals were slaughtered 3 weeks post infection. The whole carcass, superficial lymph nodes, visceral lymph nodes, liver, lungs and heart were examined carefully for any abscesses. Head lymph nodes were firstly exposed and then examined for increase in size and abscess formation. Pus samples at the inoculation site and internal abscesses

were taken for bacteriological examination.

In conclusion, results of this study show that changes in the skin and blood chemistry of fattened sheep enhances the growth of *S. aureus anaerobius* and hence colonization and formation of abscess.

Results

Sweat analysis

Sweat samples from all fattened animals were found to contain traces of cholesterol in TLC analysis, while those from most un-fattened ones were negative for cholesterol. The pH of sweat from all un-fattened animals was found to fall in the acidic range (5.0 – 6.0) while from fattened ones it was shifting towards neutral and alkaline range (6.2 – 7.6), as shown in Table 1.

Table 1: pH of sweat obtained from fattened and un-fattened sheep

	No. of animals	
pH	**Fattened**	**Un-fattened**
5.0	-	12
5.3	-	8
5.6	-	9
5.8	-	6
6.0	-	5
6.2	5	-
6.3	8	-
6.6	7	-
7.0	10	-
7.6	10	-

Serum cholesterol

Serum cholesterol level was significantly higher ($p < 0.05$) in fattened sheep (74.06 ± 5.56 mg/ dl) when compared to un-fattened sheep (43.00 ± 6.66 mg/ dl).

Effect of Cholesterol on the growth of *S. aureus* subsp. *anaerobius*

Upon addition of traces of cholesterol to BHI growth inhibition was observed for *S. epidermidis*, but not for Sasa. Blood agar plates coated with sera from fattened (high cholesterol serum level) promoted the growth of Sasa more than serum from un-fattened sheep (low cholesterol serum level) by more than 1 log. The contrast occurred to *S. epidermidis*: its growth was promoted more with serum from un-fattened sheep than with serum from fattened sheep (Fig 1).

Effect of the pH on the growth *S. aureus* subsp. *anaerobius*

No growth was seen at pH below 6 for Sasa when incubated up to 108 h. Moderate growth was obtained at pH 7.0 and pH 7.5 after incubation for 72 and 48 h, respectively. Good growth was observed at pH 7.5 and pH 8.0 after incubation for 84 h, while confluent growth was observed at pH 8.0 after incubation for 96 h.

Microflora of the skin of sheep

Most of the swabs samples yielded mixed cultures of *Bacillus* spp., *S. epidermidis* and *S. aureus*. Out of 20 swabs samples, *Bacillus* spp. was isolated from 19 (95%); *S. epidermidis* form 14 (70%) and *S. aureus* from 8 swabs (40%) (Table 2).

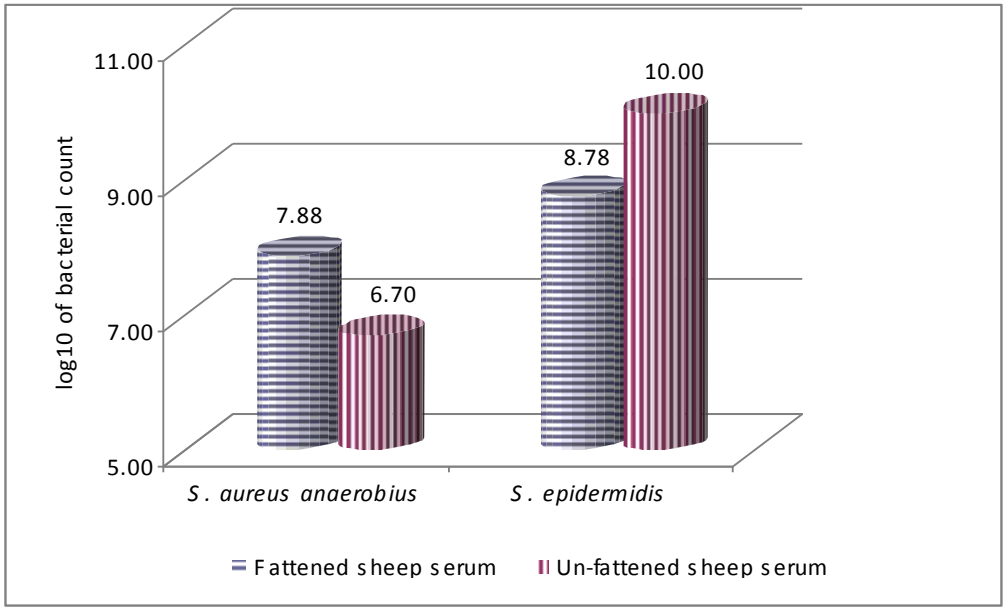

Fig. 1: Comparison between log 10 of viable count (cfu/ml) of *Staphylococcus aureus* subsp. *anaerobius* and *S. epidermidis* on blood agar plates coated with sterile sera obtained from fattened and un-fattened sheep

Table 2: Bacteria isolated from skin swabs taken from sheep at feedlot over the parotid and mandibular lymph nodes

Bacteria	Number of swab samples
Bacillus spp., *S. Epidermidis*	9
Bacillus spp., *S. epidermidis*, *S. Aureus*	5
Bacillus spp., *S. Aureus*	3
Bacillus spp.	2

Inoculation through different routes

Animals inoculated with whole pus developed abscesses in the pre-scapular and parotid lymph nodes, which opened at day 16 in the animal inoculated by s/c route, while that inoculated by scarification developed abscess in the mediastinal lymph node with inflamed left and right pre-scapular lymph nodes. Pre-scapular lymph nodes were hyperaemic in animals inoculated by scarification with broth culture. Abscesses of internal organs appeared in all inoculated animals: micro-abscess in livers of all animals and lung abscesses in pus inoculated animals (subcutaneous or scarification routes). Abscesses developed in the hearts of animals inoculated subcutaneously with either pus or the culture of *S. aureus* strain 146. The animal inoculated by iv route looked healthy post infection, unlike other inoculated animals; only few micro- abscesses were seen in the liver, but the organism could not be re-isolated form these abscesses (Table 3).

Pathogenicity of *S. aureus* subsp. *anaerobius* in fattened animals

Animals inoculated with *S. aureus* strain 146 developed no abscesses, but only micro- abscesses in the liver of 33% (N=2) of fattened sheep were recorded. External abscesses developed in the parotid lymph nodes of all fattened animals (in addition to subcutaneous abscesses in 2 animals) and of 50% (N=3) of non-fattened animals inoculated with Sasa strain 11

SARD. Two out of the three non-fattened animals that developed abscesses were in good body condition. The right pre-scapular lymph nodes in animals were inflamed; the left ones were less inflamed. The sizes of the abscesses in fattened sheep reached up to 6.0 – 6.5 cm in diameter. Most abscesses opened by day 8 post inoculation. Internal organs involved were mainly the liver, lung and heart. All fattened animals and 50% of un-fattened animals inoculated with Sasa developed micro-abscess in the liver; 50% of fattened animals developed abscesses in the hearts (one in the apex and two in the ventricles); 33% of fattened animals developed abscesses in the lung and mediastinal lymph node (Table 4).

Table 3: Post-mortem results of fattened sheep inoculated with *S. aureus* subsp. *anaerobius* and *S. aureus* using different routes of inoculation

Route of inoculation	Inoculum	Organs			
		Pre-scapular lymph node	Lung	Liver	Heart
s/c	Whole pus	Inflamed	Abscess	Micro-abscess	Abscess
	b. c. 146 + CFCFH + $CaCl_2$	-	Congested	Abscess	Abscess
Scarification	Whole pus	Inflamed	Abscess	Abscess	-
	Sasa 11 Culture	Congested (L & R)	Congested	Abscess	-
i/v	Sasa 11 Culture	-	-	Abscess	-

b. c. 146: culture of *S. aureus* strain 146; CFCFH: cell free culture filtrate containing haemolysin; Sasa 11: *S. aureus* subspecies *anaerobius* strain SARD 11, whole pus: pus from abscess caused by *S. aureus* subspecies *anaerobius* strain SARD 11

Table 4: Abscesses developed in sheep experimentally inoculated with *S. aureus* subsp. *anaerobius* and *S. aureus*

Organs	*S. aureus* subsp. *anaerobius* strain SARD 11		*S. aureus* strain 146	
	Fattened animals (N=6)	Un-fattened Animals (N=6)	Fattened animals (N=6)	Un-fattened Animals (N=6)
Lymph nodes	6	3	0	0
Lungs	2	0	0	0
Liver	6	6	3	0
Heart	2	0	0	0

Co-relation between abscess size and the body weight of fattened animal

Sizes of the abscesses that developed in fattened animals inoculated with Sasa were proportional to the body weight (Fig. 2).

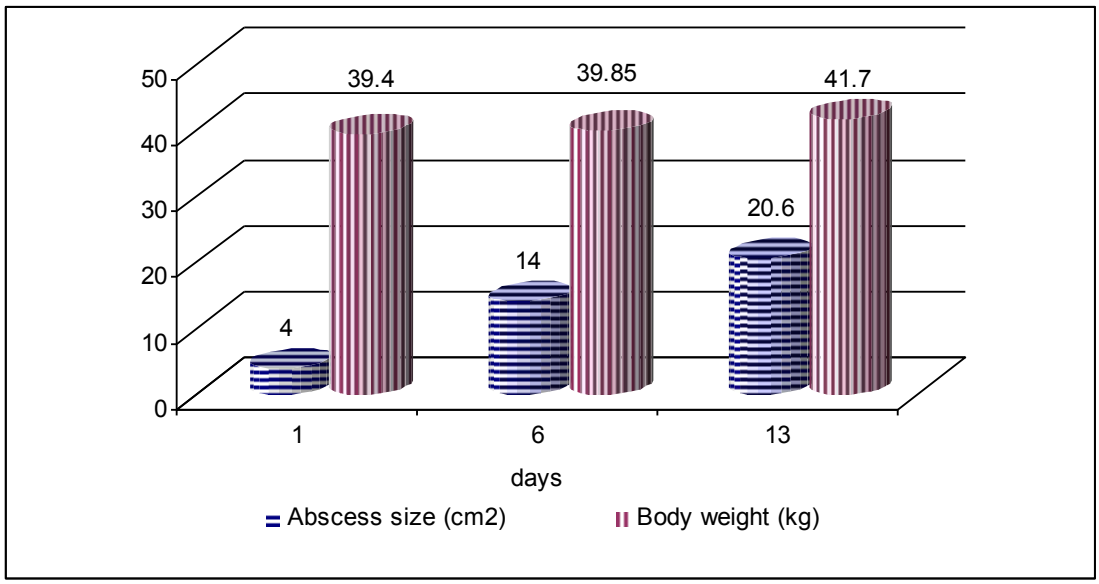

Fig. 2: Correlation between abscess size and the body weight of the animal in fattened sheep inoculated with *Staphylococcus aureus* subsp. *anaerobius* strain 11 SARD

Discussion

Morel's disease represents one of the major disease constraints of exports of sheep – the major export animal in the Sudan (Freigoun *et al*., 2009). During the 1990s many shipments of sheep were rejected from Saudi Arabia on the grounds of appearance of this disease in some of the shipped animals (Aklilu, 2002; Musa et al., 2012); the due economic losses amounted for some millions US Dollars (Musa et al., 2012). One of the main observations on this disease was the high incidence rate among sheep kept for fattening – a normal practice in sheep export industry. The same observation was reported earlier (Aynaud, 1928). Based on these observations the present investigation aimed at investigating the relationship between fattening and the incidence of Morel's disease in sheep. The approach adopted in this investigation was based on a simple hypothesis. As the main symptom of the disease is the appearance of subcutaneous abscesses either in superficial lymph nodes or adjacent to them, it was proposed that the organism gets through the cracked skin into the sub- cutis from which it is drained to the regional lymph nodes, where it causes the abscess. Accordingly, it was proposed that as the organism should come into contact with the skin so as to get access to the subcutis, some favourable conditions should prevail in the skin of fattened sheep. In this study, *S. aureus anaerobius* was found to grow better at neutral to alkaline pH (7.5 – 8.0), but not at pH below 6.0. As the organism requires neutral to alkaline pH for growth, and its growth is not supported by acidic pH, shift in the pH of the skin should occur for its favour. So, the pH of the sweat and serum and sweat cholesterol levels were measured in fattened sheep. The pH of the sweat of un-fattened animals ranged between 5 and 6 with the majority (87.5%) below 6, while that of fattened animals ranged from 6.2 to 7.5, with the majority above 6.8 (20% with pH 6.8 and 50% with pH 7.0 – 7.5). These findings indicate a shift of the pH of

the skin of fattened sheep from acidity towards alkalinity (Table 1). Sweat form fattened sheep was found to contain traces of cholesterol. The growth of *S. epidermidis* was suppressed by cholesterol while the growth of *S. aureus anaerobius* was enhanced *in-vitro* (Fig. 1). *S. epidermidis* was found in 70% of the skin swabs of sampled sheep. Suppression of *S. epidermidis*, if occurs *in-vivo*, might give a chance for other skin microflora to dominate. In this study, serum from fattened sheep supported the growth of *S. aureus anaerobius* more than did serum from un-fattened sheep. As the growth of *S. aureus anaerobius* was not at least suppressed, if not enhanced by serum from fattened sheep, it is more likely that the micro- environment of the skin of fattened sheep favours the growth of *S. aureus anaerobius*. Moreover, sera from fattened sheep were found to have significantly higher ($p < 0.05$) cholesterol levels than sera from non-fattened sheep. Support for these findings can be made by the findings of Shine and others who demonstrated that the growth of *S. aureus* was promoted with cholesterol (Shine et al., 1993). However, other factors in the serum of fattened sheep may also contribute to the growth promotion of *S. aureus anaerobius* Abolishing of the inhibitory effect of fatty acids (linoliec and myristic) on *S. aureus* by cholesterol *in vitro* was shown before (Naidoo, 1981).

In experimental infection of fattened and un- fattened sheep with *S. aureus* strain 146 none of the animals developed external abscesses, suggesting that this organism despite being isolated from lymph node abscesses at meat inspection (sub-clinical abscess), it is not likely to cause the clinical (classical) Morel's disease. In contrast, experimental infection with *S. aureus anaerobius* strain 11 SARD, external abscesses resembling those of the natural infection developed in the parotid lymph nodes of all fattened animals (with formation of subcutaneous abscesses in 2 animals). In non-fattened animals three animals (50%) developed external abscess, but two of them were in good body condition. These results confirm the observation that fattening renders sheep to be more susceptible to development of the abscesses caused by *S. aureus anaerobius* (Aynaud, 1928). The sizes of the abscesses in fattened sheep reached up to 6.0 – 6.5 cm in diameter, the size increased with the increase in the body weight during the fattening process. The main internal organ involved in abscess formation after experimental infection was the liver. Liver micro- abscesses were found in all fattened animals and 50% of un-fattened animals inoculated with *S. aureus anaerobius* strain 11 SARD as well as in 33% of un-fattened animals inoculated with *S. aureus* strain 146. The other internal organs involved in abscess disease were heart (50% of fattened animals) and the lung (33% of fattened animals). Involvement of these internal organs suggests a haematogenous dissemination of the organism, but there seem predilection sites for the organism.

In conclusion, results of this study showed that some changes in the blood chemistry as well as in the skin do occur in fattened sheep that enhance the growth of *S. aureus* subsp. *anaerobius*, and thus its ability to colonize the skin of sheep and consequently its ability of infection leading to abscess formation.

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