11th EUROPEAN MEETING OF MEAT RESEARCH WORKERS

- B E L G R A D E -

1 9 6 5

A-4

LIPOLYTIC ACTIVITIES OF STAPHYLOCOCCI AND MICROCOCCI

J.T. PATTERSON

- N. IRELAND -

LIPOLYTIC ACTIVITIES OF STAPHYLOCOCCI AND MICROCOCCI

J.T. Patterson,

Ministry of Agriculture for Northern Ireland
and The Queen's University of Belfast.

Introduction.

The importance of the lipolytic activities of micrococci in dry sausages and curing brines has been examined by Pohja and Niinivaara (1964) and Nurmi and Niinivaara (1964) and these workers have also surveyed the literature dealing with this subject. Kitchell (1962) reviewed the literature dealing with micrococci and coagulase negative staphylococci in cured meat and meat products and observed "the factors governing the development of "rancidity" in fat are complex. All that can be said of the role of micrococci is that a number of those isolated from meat products possess enzymes capable of bringing about relevant reactions." Although there does not appear to be much known about the lipolytic activities of this group of bacteria on bacon, several workers have indicated that they have some importance in the spoilage of this food. Jones (1949) has pointed out that when conditions are suitable, e.g. a long period of maturation, or a rise in temperature or humidity, sliminess can develop on bacon sides, largely due to micrococci, though Proteus, Bacillus, and other species may play a part. Slime formation may have a beneficial effect on flavour formation, but if allowed to develop too far, can result in taint of the meat. Ingram (1952) in an investigation of bacon gammons found a flora, mainly micrococci, which had an optimum temperature of 25°C, could tolerate high concentrations of salt and reduce nitrate. The type of spoilage was a gradually developing cheesiness and rancidity. More recent work has shown that micrococci play a large part in the spoilage of vacuum packed bacon. Of nearly 600 isolations made from sliced Wiltshire bacon by Kitchell (1962), 41% were micrococci or coagulase negative staphylococci. After

vacuum packaging and storage at 5°, 20° and 30°C until spoiled, the proportions of these types were found to be between 23 and 43%, the highest temperature of storage having the highest proportion. The remainder of the flora was comprised mainly of lactic acid bacteria. Tonge, Baird-Parker and Cavett (1964) also found that micrococci played a large part in the spoilage of vacuum packed bacon of both high and low salt concentration (in this case the bacon was cured with fresh brines). Proteolysis, lipolysis and reduction of nitrate and nitrite were attributed to coagulase negative staphylococci. Micrococci were not so active in proteolysis or lipolysis and tended rather to delay spoilage by catalase negative bacteria.

It was decided, in the light of these findings, to have a closer look at the lipolytic activities of a group of micrococci and staphylococci isolated from bacon sides cured by the Wiltshire method.

Source of the strains.

Sixty strains were isolated from bacon sides in the maturing cellar, 50 from sides 5 days out of cure, and 10 from bacon which had been 10 days in the maturing cellar. Primary isolation was on nutrient agar (40 strains) or on nutrient agar with 4% (w/v) of added salt (20 strains), the plates being incubated for 4 days at 25°C. Three of the isolates were coagulase positive. Two additional strains were used in some of the work, viz. N.C.T.C. 7291 (*Staphylococcus saprophyticus*), and N.C.T.C. 7447 (*S. aureus*).

Classification of the isolates.

The classification schemes used were those of Shaw, Stitt and Cowan (1951) and Baird-Parker (1963). One strain proved impossible to classify with certainty. Details are given in Table 1.

Table 1. Classification of the isolates.

No. of isolates	Strains falling into Shaw sub-groups				
	1	2	3	4	5
59	3	24	32	Nil	Nil

	Strains falling into Baird-Parker sub-groups												
	Staphylococcus						Micrococcus						
	I	II	III	IV	V	VI	1	2	3	4	5	6	7
59	3	Nil	1	1	Nil	4	4	3	2	12	26	3	Nil

Media and methods.

The following base medium was used throughout the study:-

	gm.
Peptone (Oxoid L37)	10
Yeast extract (Oxoid)	3
NaCl	5
Agar (Oxoid No.3)	12
Dist. water	1 litre

For experiments where the medium was used at or near neutrality the pH was adjusted to 7.0 and the medium autoclaved for 15 min. at 15 lb. 10 g. of the selected fat was added to 90 ml. of a 2% (w/v) Na citrate solution, the mixture heated to ca. 60°C and emulsified twice or more through a Pentacreme Home Cream Maker 2003, and sterilized by steaming for 30 min. on each of 3 successive days. 10 ml. of this emulsion were added to each 100 ml. of melted base medium just before the plates were poured. Poured plates were dried overnight at 37°C, and streaked with 3 strains on each plate, from cultures grown, generally overnight, in nutrient broth at 30°C. The plates were incubated for 6 days at 30°C, though examined after 2 or 3 days, or for 21 days at 4°C. Other experimental details are outlined in the relevant sections.

Experimental.

1. Hydrolysis of various fats.

The following fats were incorporated into the base medium at the 1% level:- butter, lard, tributyrin, triolein, palm kernel oil. The hydrolysis of tributyrin was determined by measuring the diameter of the clear zone, if any, around a single colony (Nurmi and Niinivaara, 1964):

- = no change around the colony.
(+) = slight clearing around the colony.
\+ = distinct clearing around the colony, 6-9 mm in diameter.
++ = marked clearing around the colony, 10 mm or greater in diameter.

Attack on the other fats was noted by the method of Berry (1933), in which the plate was flooded with a saturated solution of $CuSO_4$ for 5 min., and then any blue or bluish-green colour noted:

- = no change around the colony.
(+) = slight colouration around the colony.
\+ = distinct colouration around the colony.
++ = marked colouration around the colony, extending several mm. into the medium.

The results of this first experiment are given in Table 2.

Table 2. Hydrolysis of various fats by bacon isolates.

No. of strains tested	Extent of attack	No. of strains giving the specified reactions when these substrates were added to the base medium				
		Tributyrin	Lard	Butter	Triolein	Palm Kernel
60	No growth	2	0	0	0	0
	-	1	49	48	39	36
	(+)	37	9	11	8	20
	+	15	1	0	11	3
	++	5	1	1	2	1

50

From these results it can be seen that 57 (95%) of the isolates gave some hydrolysis of tributyrin, whereas the other fats were attacked by far fewer strains, viz. lard 18%, butter 20%, triolein 35% and palm kernel by 40% of the strains. These results do not agree very well with those of Nurmi and Niinivaara (1964) who found 32 out of 91 strains of Micrococcus (35%) attacked pork fat at pH 7 and 71 out of 108 (66%) attacked tributyrin at this pH. For S. aureus comparable figures were 6 out of 8 strains (75%) for pork fat, and 11 out of 11 (100%) for tributyrin. These workers felt that the hydrolysis of tributyrin was a useful indication of the attack on pork fat. Pohja and Niinivaara (1964) however, also pointed out that strains which cause lipolysis of tributyrin do not always cause lipolysis of animal fats, and it is therefore necessary to examine the lipolytic capacity of the isolates again, using animal fat in the medium. In this case they found that of 76 micrococci attacking tributyrin, 27 also attacked lard.

From the 60 strains thus tested, 19 were selected as being active in tributyrin hydrolysis. Fifteen gave at least a + reaction, and 4 at least (+). To these was added N.C.T.C. 7291, and also the three coagulase positive isolates and N.C.T.C. 7447, all at least +. This gave a total of 24 strains, 20 of which were coagulase negative, and 4 coagulase positive. The classification of these strains is given in Table 3.

Table 3. Classification of strains used in experiments on fat lipolysis.

No. of strains	Strains falling into Shaw sub-groups				
	1	2	3	4	5
24	4	8	12	0	0

No. of strains	Strains falling into Baird-Parker sub-groups												
	Staphylococcus						Micrococcus						
	I	II	III	IV	V	VI	1	2	3	4	5	6	7
24	4	0	0	1	0	1	0	2	1	3	12	0	0

2. Effect of adding varying levels of salt (NaCl) to the medium at pH 6 and 7, using tributyrin as substrate.

The various levels of salt were added to the base medium which was then resterilized at 10 lb. pressure for 10 min. before adding 1% of tributyrin. The medium at pH 6 was made up in 0.1 M citrate phosphate buffer (as in Mackie and McCartney, 1960). A pH check on the poured plates showed a variation of from 6.0 at 1% of salt to 5.6 at 6%. The results are summarized in Table 4. The plates were incubated for 6 days at 30°C.

Table 4. Hydrolysis of TBA* by selected strains at pH 6 & 7, with various levels of salt added.

No. of strains tested	Extent of clearing	Levels of salt added to base medium, at pH 7						
		0	1	2	3	4	5	6
20, coagulase negative.	-	0	0	0	1	1	0	0
	(+)	2	5	4	3	6	9	11
	+	9	9	12	14	11	10	7
	++	9	6	4	2	2	1	2
4, coagulase positive.	(+)	0	0	0	0	0	2	1
	+	2	2	2	2	2	0	1
	++	2	2	2	2	2	2	2

		Levels of salt added to base medium, at pH 6					
		1	2	3	4	5	6
20, coagulase negative.	-	0	1	1	1	1	2
	(+)	3	3	7	5	6	13
	+	13	15	11	13	12	3
	++	4	1	1	1	1	2
4, coagulase positive.	(+)	0	0	1	1	2	2
	+	2	2	2	2	0	0
	++	2	2	1	1	2	2

*TBA = tributyrin agar.

From these results it is clear that these strains can still attack the tributyrin at fairly high levels of added salt, at pH 6 as well as at pH 7.

3. Hydrolysis of TBA, with added salt, at 4°C.

It was thought of interest to see the effect of incubation at 4°C which is approximately maturation room temperature. These bacteria grow rather slowly at this temperature, if at all, so that a long incubation time (21 days) was required to give readable results. These are recorded in Table 5.

Table 5. Hydrolysis of TBA, at pH 7 after 21 days at 4°C.

No. of strains tested	Extent of clearing	Level of added salt in the base medium					
		1	2	3	4	5	6
20, coagulase negative.	No growth	5	5	4	6	4	5
	-	1	1	2	0	2	1
	(+)	14	14	14	14	14	14
4, coagulase positive.		All failed to grow.					

The addition of salt does not appear to affect lipolysis to any great extent, and it is significant that lipolysis still occurred with 70% of the coagulase negative strains at a temperature as low as 4°C.

4. Hydrolysis of TBA, with added salt, nitrate and nitrite.

When curing bacon sides by the Wiltshire method, nitrate and nitrite are used as well as salt, and are to be found in the cured product. To test whether the addition of these, at the levels which could be present in bacon would affect lipolysis, the following were added to the base media at pH 6 and 7.

Salt	0, 1, 2, 3, 4, 5	(as % w/v)
Potassium nitrate	2	(" " ")
Sodium nitrite		100 parts per million.

The results obtained after incubating the plates at 30°C for 6 days are summarized in Table 6 to show only the 0 and 5% salt levels. It can be seen that lipolysis was observed at both pH values in the presence of the various additions.

Table 6. Hydrolysis of TBA, at pH 6 & 7, with additions of 0 & 5% salt, 2% potassium nitrate and 100 ppm. sodium nitrite.

No. of strains tested	Extent of clearing	No. of strains giving the specified reactions with:-			
		No added salt		5% added salt	
	pH	6	7	6	7
18, coagulase negative.	-	1	1	3	0
	(+)	12	8	15	10
	+	5	9	0	8
4, coagulase positive.	(+)	1	1	3	1
	+	2	1	1	0
	++	1	2	0	3

5. Lipolysis of lard at pH 6 and 7, after 6 days at 30°C and 21 days at 4°C.

Although the hydrolysis of tributyrin may be indicative of an organism's ability to attack other fats it would be more valuable if it could be demonstrated that pig fat was attacked under conditions resembling those found in the maturing cellar. The results observed in this experiment are recorded in Table 7. In case the use of 0.1 M phosphate buffer had given false positive results, the experiment was repeated at pH using 0.1 M citric acid/sodium citrate buffer. This time only 6 of the coagulase negative strains gave slight lipolysis; 2 of the coagulase positive strains gave good lipolysis, and 2 gave slight. It seems likely that some of the slight positives recorded in Table 7 were false positives due to the presence of phosphate in the medium.

Table 7. Lipolysis of pig fat at pH 6 & 7, at 4° & 30°C.

No. of strains tested	Extent of lipolysis	No. of strains giving the specified reactions after 6 days at 30° or 21 days at 4°C.			
		pH 6		7	
		4°	30°	4°	30°
20, coagulase negative.	No growth	13	1	2	1
	-	7	4	5	19
	(+)	0	15	0*	0
	+	0	0	0	0
	++	0	0	0	0
4, coagulase positive.	No growth	4	0	4	0
	-	0	0	0	3
	(+)	0	4	0	0
	+	0	0	0	1

*13 strains showed greenish blue colour under colony.

6. Lipase adaption.

To see if the lipase, or lipases, of these strains were adaptive, the following procedure was adopted. Six strains from bacon were chosen, 5 of which gave slight tributyrin hydrolysis, the other none, but all of which were lipase negative on lard. The strains were grown at 30°C in the presence of lard or tributyrin (0.2% w/v) in broth similar in composition to the base medium, at pH 7. Each was subcultured 3 times into fresh broths over a total period of 6 days before streaking on to plates containing tributyrin or lard at pH 6 and 7. One strain previously negative on TBA gave slight clearing, and 2 strains previously negative on lard gave slight lipolysis at pH 7. However this aspect of the problem requires further investigation.

7. Attempt to develop a tube method for the detection of lipase activity.

In our hands, neither the method of Berry (1933), nor that of Jones and Richards (1952) gave really clear-cut results, especially with those strains which only appeared to give slight lipolysis.

The tributyrin results were easily read, but an accurate method for other fats would be of great value. An attempt was made to develop a tube method, where the colour developed could be read on a spectrophotometer. When a known lipase positive S. aureus was grown in 5 ml. of a nutrient broth with some fat added, for several days, and then 0.1 ml. saturated $CuSO_4$ added, a blue-green colour developed. The cells were spun down and the percentage transmission plotted against wavelength on a Unicam SP600 spectrophotometer, using a distilled water blank. A peak transmission was obtained at 505 mμ for tributyrin and at 510 mμ for lard at pH 7. At pH 6 the peak was at 530 mμ for both lard and tributyrin. All 60 strains have been tested by this method, but so far little correlation has been obtained between this and the plate method. Various factors would need to be studied, and possibly indicators other than $CuSO_4$.

Discussion and conclusions.

Most of the strains studied had the ability to hydrolyse tributyrin, but not many had the ability to attack lard, or at least this ability was not detected by the methods used. This suggests that the use of tributyrin to indicate attack on other fats may not be valid. Sierra (1964) has also pointed out that the hydrolysis of tributyrin alone may have led to false conclusions in assessing the lipolytic activity of proteolytic microorganisms. He showed that subtilisin could quite readily hydrolyse tributyrin, and possibly other proteolytic enzymes could bring about this reaction. However those strains which attacked lard, also attacked tributyrin. It seems probable that some of these strains could cause lipolysis of bacon fat in the maturing cellar, particularly if maturation was prolonged, or storage conditions suitable. In packaged bacon, where storage conditions are quite often not good, lipolysis would almost certainly occur if the bacon was not consumed quickly, or properly stored.

With regard to classification, of the 11 strains found causing lipolysis of lard in the first experiment (Table 2), 2 strains were coagulase positive, and 5 of the others (56%) belonged to Baird-Parker's Micrococcus sub-group 5. This sub-group is, according to Baird-Parker (1962, 1963), the predominant Micrococcus sub-group found on bacon. Of the 15 coagulase negative strains recorded as giving lipolysis of lard (Table 7), at pH 6, 10 (67%) were also of this sub-group. It looks as if the characteristic bacon types are mainly responsible for this type of spoilage, and only strict attention to hygiene at all stages seems likely to keep spoilage by these organisms to the minimum.

SUMMARY

The lipolytic activities of 60 strains of staphylococci and micrococci isolated from bacon have been studied, using tributyrin, lard, butter, triolein and palm kernel as substrates. 95% of the strains caused lipolysis of tributyrin, 18% of lard, 20% of butter, 35% of triolein and 40% of palm kernel. 20 coagulase negative and 4 coagulase positive strains, all of which hydrolysed tributyrin have been examined in more detail with regard to the effect of pH and temperature, and of additions of salt, nitrate and nitrite to the base medium. Hydrolysis of tributyrin occurred with many of these strains in the presence of 5% salt, 2% potassium nitrate and 100 ppm. sodium nitrite, at pH 6 and 7. 7% of the coagulase negative strains were also active at 4°C.

However, far fewer of the strains were detected as attacking lard when tested under similar circumstances, and the suitability of using tributyrin as an indicator of lipolysis is discussed. Of 15 coagulase negative strains causing lipolysis of lard at pH 6, 67% were Micrococcus sub-group 5. This characteristic bacon type appears to be mainly responsible for this type of spoilage.

ZUSAMMENFASSUNG

Unter Verwendung von Tributyrin, Schweineschmalz, Butter, Triolein und Palmkern als Substrate wurden die lipolytischen Aktivitäten von 60 aus Speck isolierten Staphylokokken- und Mikrokokken-Stämme untersucht. 95% der Stämme verursachten Lipolyse von Tributyrin, 18% von Schweineschmalz, 20% von Butter, 35% von Triolein und 40% von Palmkern. 20 koagulasenegative und 4 koagulasepositive Stamme, welche alle das Tributyrin hydrolisierten, wurden mit Bezug auf den Einfluss von pH-Wert und Temperatur und von Zusätze von Kochsalz, Nitrat und Nitrit zu dem Grundnährboden ausführlicher untersucht. Viele Stämme hydrolisierten das Tributyrin bei Anwesenheit von 5% Kochsalz, 2% Kaliumnitrat und 100 p.p.m. Natriumnitrit bei einem pH-Wert von 6 und 7. 7% der koagulasenegativen Stämme waren auch bei 4°C aktiv.

Man konnte aber feststellen, dass viel wenigere, unter gleichen Umständen untersuchten Stamme das Schmalz angriffen, und die Verwendung von Tributyrin als geeigneter Indikator von Lipolyse wird besprochen. Aus 15 koagulasenegativen Stämmen, welche die Lipolyse von Schmalz bei pH 6 verursachten, gehorten 67% zur Mikrokokkus-Untergruppe 5. Es scheint also, dass diese für Speck charakteristische Keimart für diese Art Faulnis hauptsächlich verantwortlich ist.

RÉSUMÉ

On a étudié les activités lipolytiques de 60 souches de Staphylococcus et Micrococcus isolées dans du bacon, en employant comme substrats de la tributyrine, la graisse de porc, du beurre, de la trioléine et l'amande de palmier. 95% des souches ont causé la lipolyse de la tributyrine, 18% de la graisse de porc, 20% du beurre, 35% de la trioléine, et 40% de l'amande de palmier. 20 souches coagulase-négatives et 4 souches coagulase-positives, qui ont toutes hydrolysé la tributyrine, ont été examinées en plus grand détail en ce qui concerne l'effet du pH et de la température, ainsi que des additions de sel, nitrate et nitrite au milieu basique. Beaucoup des souches ont causé l'hydrolyse de la tributyrine en présence de 5% sel, 2% nitrate de potasse et 100 p.p.m. nitrite de soude, à un pH de 6 et 7. 7% des souches coagulase-négatives étaient également actives à 4°C.

Cependant, beaucoup moins des souches ont attaqué la graisse de porc, quand examinées dans de pareilles conditions, et on discute l'emploi de la tributyrine comme indicateur approprié de la lipolyse. 67% des 15 souches coagulase-négatives, qui ont produit la lipolyse de la graisse de porc à pH 6, appartenaient au sous-groupe Micrococcus 5. Cette espèce caractéristique du bacon semble être principalement responsable de ce type de putrefaction.

.....

References.

BAIRD-PARKER, A.C. (1962). The occurrence and enumeration, according to a new classification, of micrococci and staphylococci in bacon and on human and pig skin. J. appl. Bact. 25, 352.

BAIRD-PARKER, A.C. (1963). A classification of micrococci and staphylococci based on physiological and biochemical tests. J. gen. Microbiol. 30, 409.

BERRY, J.A. (1933). Detection of microbial lipase by copper soap formation. J. Bact. 25, 433.

INGRAM, M. (1952). Internal bacterial taints ('Bone taint' or 'Souring') of cured pork legs. J. Hyg., Camb. 50, 165.

JONES, O. (1949). Bacon and ham manufacture. Food 18, 380.

JONES, A. and RICHARDS, T. (1952). Night blue and victoria blue as indicators in lipolysis media. Proc. Soc. appl. Bact. 15, 82.

KITCHELL, A.G. (1962). Micrococci and coagulase negative staphylococci in cured meats and meat products. J. appl. Bact. 25, 416.

MACKIE and McCARTNEY'S HANDBOOK OF BACTERIOLOGY (1960). Ed. R. Cruickshank. Edinburgh: E. & S. Livingstone Ltd.

NURMI, E. and NIINIVAARA, F.P. (1964). Lipolytic changes of fats in dry sausage. 10th European Meeting of Meat Research Workers, Roskilde.

POHJA, M.S. and NIINIVAARA, F.P. (1964). Eigenschaften und Bedentung der in Rohwurst und in Pökellake vorkommenden Fett abbauender Bakterien. Fleischwirtschaft 16, 435.

SHAW, C., STITT, J.M. and COWAN, S.T. (1951). Staphylococci and their classification. J. gen. Microbiol. 5, 1010.

SIERRA, G. (1964). Hydrolysis of trighycerides by a bacterial proteolytic enzyme. Can. J. Microbiol. 10, 926.

TONGE, R.J., BAIRD-PARKER, A.C. and CAVETT, J.J. (1964). Chemical and microbiological changes during storage of vacuum-packed sliced bacon. J. appl. Bact. 27, 252.