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STAPHYLOCOCCI AND MICROCOCCI FROM PORK, CURING BRINES AND BACON

J.T. PATTERSON

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J.T. Patterson,

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

Introduction

In Wiltshire curing brines there are present large number of bacteria. Ingram (1958) has obtained counts of 10^8 /ml. using phase contrast equipment and viable counts of 10^7 /ml. have been obtained by the same worker. These bacteria are mostly fastidious halophiles, whose function is largely unknown. However other groups of bacteria play a part in bacon curing, and the Micrococcaceae is one of the most important of these. Garrard and Lochhead (1939) found micrococci predominating in pre-curing contamination, and considered them able to withstand the curing process. Jepsen (1947) studied the bacterial flora of pork, and from all stages of slaughter floor treatment, isolated 95 strains of Micrococcus, Sarcina and Staphylococcus out of a total of 306 isolates. Kitchell (1958) singled this group out for special study, and found affinities between the micrococci of raw meat and those of the cured product, bacon. Ingram, Kitchell and Ingram (1958) found large numbers of Gram positive cocci, seemingly micrococci, in Wiltshire curing brines. Jespersen and Riemann (1958) also found large numbers of cocci in curing brines, and also on the sides after curing. Pohja (1960), Pohja and Gyllenberg (1962), have studied micrococci extensively, especially in relation to fermented meat products, and Baird-Parker (1962, 1963) has evolved a new system of classification for micrococci and staphylococci from bacon, human and pig skin. Micrococci and staphylococci, particularly S. aureus, are important when found on manufactured products such as sausages, prepared meat products and particularly in vacuum packaged bacon (Eddy and Ingram, 1962; Thatcher, Robinson and Erdman, 1962). They are also concerned in the spoilage of this product (Cavett, 1962; Kitchell, 1962; Tonge, Baird-Parker and Cavett, 1964). In view of the importance of this group

of bacteria in bacon curing in relation to bacon spoilage and factory hygiene; to their suspected role in nitrate reduction in the curing brines and the cured meat; flavour improvement in fermented meat products, and public health significance on manufactured products, it was felt that further study was necessary, and some work carried out in this connection will now be described.

Source, isolation and selection of strains.

All the strains used, of which there were 164, were obtained from the same factory, which used only the Wiltshire curing method. In Northern Ireland, the sides of meat, after injection with a high salt content brine (an almost saturated solution) are immersed in curing brines of 25-27% salt content for 4-5 days at 4-5°C, then removed, drained and allowed to mature for a similar period at the same temperature. One group of 47 strains, all coagulase negative, was from sides of meat which had come from the chilling room and were being butchered before immersion in the curing brine. A further group of 57 strains also coagulase negative was isolated from Wiltshire curing brines, and a third group of 60 strains, 3 of which were coagulase positive, was isolated from maturing bacon. These strains were isolated on nutrient agar, or nutrient agar with 4% of salt, at 25°C, and were all catalase positive, aerobic, Gram positive cocci. Strains which did not reduce nitrate were tested for the presence of catalase production on nutrient agar with 1% of glucose added (Felton, Evans and Niven, 1953) to eliminate Pediococcus strains. All were catalase positive on this medium.

Classification of the isolates.

The strains were classified by the methods of Shaw, Stitt and Cowan (1951) and Baird-Parker (1963). Details are given in Table 1.

Table 1. Classification of the isolates by the methods of Shaw, Stitt and Cowan (1951) and Baird-Parker (1963).

Source of isolates	No. of isolates	Strains falling into Shaw sub-groups				
		1	2	3	4	5
Fresh sides	47	0	28	17	0	2
Curing brines	57	0	16	39	0	2
Bacon sides	60 [#]	3	24	32	0	0

		Strains falling into Baird-Parker sub-groups												
		<u>Staphylococcus</u>						<u>Micrococcus</u>						
		<u>I</u>	<u>II</u>	<u>III</u>	<u>IV</u>	<u>V</u>	<u>VI</u>	1	2	3	4	5	6	7
Fresh sides	46 ^{##}	0	0	0	2	1	4	6	11	5	1	9	5	2
Brines	57	0	0	0	0	1	1	0	5	4	6	37	1	2
Bacon sides	60 [#]	3	0	1	1	0	4	4	3	2	12	26	3	0

[#] 1 strain not classifiable.

^{**} 1 strain not available for classification.

Neither classification was found completely satisfactory. That of Shaw et.al. (1951) because of the uncritical nature of some of the tests used to distinguish the sub-groups, and the manner in which sub-groups 2 and 3, into which most of the isolates fell, overlaps in many characteristics. Baird-Parker's classification, in our hands, was rather unsatisfactory because of the difficulty in distinguishing fermentative (Staphylococcus) strains from oxidative (Micrococcus) strains. Using these classifications with the strains, however, does indicate that the micrococcal flora on the sides going into cure is quite different from that in the brines, and the flora on the bacon sides appears to receive a contribution from both sources. The question now arises: what contribution do these bacteria make to the curing process? Some experiments in this connection will now be discussed.

1. Tests of ability to grow at 0° and 4°C.

If bacteria are to be active in the curing cycle, the ability to grow at the temperature of the curing and maturing cellars (4-5°C) is obviously important. Table 2 gives details of an experiment to see if the strains had this ability. Tables 2 and 3 are from work already published, (Patterson, 1963), where experimental details are given.

Table 2. The abilities of staphylococci and micrococci from a bacon factory to grow at low temperatures.

Source of strains	No. of strains tested	Percentage of strains growing at	
		0°	4°
Fresh sides	47	26	79
Curing brines	57	82	100
Bacon sides	60	68	85

Obviously many of the strains could grow slowly (incubation times of 3-4 weeks were given) at these temperatures, but whether they would be active in, e.g. nitrate reduction, lipolysis, proteolysis etc. is another matter.

2. Nitrate reduction at high salt concentration.

If the strains were active in nitrate reduction in the curing brine, they would have to withstand high salt concentrations. The ability to grow and reduce nitrate in various circumstances is given in Table 3.

Table 3. Growth and nitrate reduction in nitrate peptone water,
and the influence of salt concentration on these
abilities in nitrate-nutrient broth.

Source of strains	No. of strains tested	Reaction	Percentages of strains giving specified reactions in							
			Nitrate peptone water	NO ₃ -nutrient broth with NaCl at (%)						
				8	12	16	20	24	28	30
Fresh sides	47	Growth.	100	96	79	68	62	2	0	0
		Nitrate reduction.	66	----- Not tested -----						
Curing brines	57	Growth.	100	100	100	100	96	77	5	0
		Nitrate reduction.	84	86	86	84	79	44	0	0
Bacon sides	60	Growth.	100	98	90	82	82	63	0	0
		Nitrate reduction.	83	82	73	65	64	47	0	0

It can be seen that nitrate reduction fell off more quickly than growth with rising salt concentration until at 24%, very few of the strains from fresh sides, and only about half of those from the other sources were actively reducing. It is doubtful if these strains would be active in high salt curing brines, and at the temperature of the curing cellar, although with low salt brines they possibly would be active. Further work has shown (Patterson, 1963) that, even if the strains were not active at high salt concentration, many have the ability to remain viable at these concentrations, at 2-3°C. Details of an experiment on survival of a few of the isolates from fresh sides are given in Table 4.

Table 4. Survival of certain isolates after 30 min. in salt nutrient broth, pH 6.2, and in Seitz-filtered immersion brine*, at room temperature.

Strain No.	Initial colony count per ml. **	Colony counts per ml. ** after 30 min. in			
		0% NaCl	8% NaCl	30% NaCl	Seitz-filtered brine
1	50.0	60.0	49.0	50.5	48.0
2	40.5	72.5	36.3	28.8	52.0
3	47.3	39.0	67.5	44.5	42.0
4	98.6	130.0	100.3	142.5	100.0
5	157.5	97.5	92.5	217.5	250.0
6	24.8	7.0	6.3	6.8	7.3
7	58.5	2.5	5.0	< 2.5	5.0
8	69.8	58.5	57.3	40.0	36.8
9	130.5	54.5	95.0	92.5	55.0

* pH 7.7, NaCl 25.7%.

** x 10⁶.

From these results it appears that there is an initial kill off with certain strains, but none with others.

3. Tolerance to nitrate and nitrite.

If these bacteria are to be active in curing brines they must also be able to withstand concentrations of nitrate of the order of 1-2%, and of nitrite at least 1000 ppm., as well as high salt concentration. Tarr (1941a,b) has shown that bacterial spoilage of fish flesh was delayed by the incorporation of nitrite salts, but that bacterial inhibition was only obtained with low concentrations of nitrite at pH values below 7.0. Sodium nitrite in 0.02% concentration at pH 5.7 or 6.0 markedly inhibited species of Achromobacter, Flavobacterium, Pseudomonas, Micrococcus, Escherichia and Aerobacter. At pH 7 or above, nitrite did not inhibit growth significantly, but between pH 5.7 and 6.5 growth of all but two cultures (both Micrococcus spp.) was inhibited more or less severely or completely. He felt that this effect was inhibitory rather than bactericidal. Jensen (1954) recorded that a

tenth molar NaNO_2 solution at:

pH 5	=	2.5%	undissociated HONO	
" 6	=	0.25%	"	"
" 7	=	0.025%	"	" i.e. the undissociated HONO

increased by a factor of 10 with each unit of pH decrease. Eddy and Ingram (1956) working with a Bacillus sp. from canned bacon found that the addition of 1% potassium nitrate to the medium markedly increased tolerance to nitrite at pH 5.5, and that this organism was much less tolerant to nitrite under anaerobic conditions. Other workers such as Henry, Goret and Joubert (1954), Eddy (1958) felt that the redox potential of the medium was important, possibly as important as pH in controlling nitrite sensitivity.

In the nitrite sensitivity tests carried out, sodium nitrite was incorporated into a basal liquid medium containing 1% of peptone and 1% Lab-Lemco, together with a little agar (0.04-0.08%) to aid the detection of growth in the medium. To obtain different pH values it was necessary to buffer this medium, using 0.1 M phosphate buffer (Mackie and McCartney, 1960). Levels of from 0.04% to 1.0% of sodium nitrite were incorporated at pH values from 7.2 - 7.5 and the 60 strains from bacon tested, giving an incubation period of 10 days at 30°C. All strains grew at all levels of nitrite. However at the 1.0% nitrite level (which of course is much higher than that found in curing brines) at pH 5.8, 6.2 and 6.6 no strains grew at any pH even after 13 days. From the pH 5.8 tubes 15 strains, from the 6.2 tubes 39 strains, from the 6.6 tubes 15 strains were sub-inoculated on to nutrient agar, but none showed growth. At this level of nitrite the effect was therefore toxic rather than inhibitory. Other experiments with strains from fresh sides and curing brines confirmed this increase in toxicity with lowered pH and also increased toxicity under anaerobic conditions, but failed to show any definite lessening of toxicity due to the addition of 1% potassium nitrite. It does seem likely, however, that the normal level of nitrate and nitrite in a Wiltshire curing brine (0.5-1%, 0.05-0.1% respectively, at pH 6.0-6.5) a large number of these bacteria would still be able to

grow. For example, 18 strains from brines which grew in 24% salt broth at pH 7.1 also grew in 24% salt broth at pH 6.1 and 6.35 in the presence of 0.1% sodium nitrite.

4. Amino-acid metabolism.

(i) Decarboxylation

It was felt that representative strains from the isolates should be tested for the presence of decarboxylases. If certain sub-groups possessed these enzymes and others did not, this might help in classification. Also some indication would be obtained of the ability of the isolates to attack amino-acids at low pH values (and possibly therefore assisting in protein breakdown). To reduce the amount of work needed to test a number of strains, at different pH values, it was decided to grow about 10 strains representing each Shaw sub-group harvest and wash the cells and bulk strains of each sub-group together, and allow these to react with a mixture of certain amino-acids at different pH levels. The cells were grown in a medium supplemented with the amino-acids under test, at pH 6.1, viz. lysine, ornithine, glutamic acid, histidine, arginine, tyrosine, aspartic acid, all in the DL-form, and with 2% of glucose added. Gale (1952) stated "Only such amino-acids are attacked as have at least one chemically active (polar) group in the molecule other than the terminal -COOH and the $\alpha\text{-NH}_2$ groups. Thus decarboxylases have been described for arginine, ornithine, histidine, tyrosine, glutamic acid, aspartic acid and possibly tryptophan, but for no monamino-monocarboxylic acids". These decarboxylases are formed only when growth takes place in an acid environment. Sub-group 4 strains failed to grow in this medium and were grown in a nutrient broth with 2% glucose; these gave a pH of ca. 6.9 after growth in the medium. The amino-acids (all DL-) were made up in 0.05 M concentrations in 20 ml. 0.2 M citrate/phosphate buffer, except tyrosine, which was used as a saturated solution at room temperature. Arginine was not included because of its special metabolism, at this stage. After mixing, the

acids were adjusted to pH levels of 2.5, 3.5, 4.5, 5.5 and 6.5. The determination was carried out on a Warburg respirometer, and the reaction vessels were prepared as follows:-

<u>Main Compartment</u>	<u>Sidearm</u>
1.0 ml. cell suspension.	0.8 ml. of amino-acid mixture
0.2 " pyridoxal-5-phosphate, 100 µg/ml. in water.	at required pH (2.5-6.5) or
1.0 " 0.2 M citrate/phosphate buffer at required pH (2.5-6.5).	0.8 ml. of water (endogenous control).

Cells were available as follows:-

<u>Shaw sub-group</u>	<u>No. of strains tested</u>	<u>Dry wt. (mg/ml.)</u>
1	8	15
2	10 (brine isolates)	10
3	10 (" ")	7
4	5 (3 from brines and bacon, and N.C.T.C. 7523, 7520)	10
5	2 (1 from fresh sides, and N.C.T.C. 7011)	39

Readings were taken at 15 min. intervals up to 1 hr., then at 2 and 3 hr., the temperature in the waterbath being 30°C.

The results of this experiment showed that none of the sub-groups gave a Q_{CO_2} value (ul. CO_2 released/mg. dry wt./hr.) of more than 3, at any pH level. On the other hand E. coli (N.C.T.C. 86), prepared in the same way, at 8.5 mg/ml. dry wt. gave Q_{CO_2} values of ca. 20, 100, 45, 33, 17 at pH 2.5-6.5, showing that this strain could decarboxylate one or several of the amino-acids at each pH level. From the results it was concluded that the strains were showing little decarboxylase activity with these amino-acids, under these conditions.

(ii) Deamination

Deamination of amino-acids by two strains of Staphylococcus aureus has been studied by Hills (1940), who found L-arginine quite strongly attacked, and also small but definite attack on DL-serine, DL-threonine, DL-glutamine and "possibly other substrates". Elek (1959) noted that

alanine, glycine, proline, histidine, DL-glutamic acid, L-aspartic acid, DL-methionine, L-leucine, L-tyrosine, L-tryptophan, L-cysteine, and asparagine could all be attacked, some weakly, by S. aureus or S. albus. There seems to be little published work however about the ability of coagulase negative staphylococci or micrococci to deaminate amino-acids. It was decided to investigate this possibility with the amino-acids used in the decarboxylation experiment. The method used was that of Pelczar, Hansen and Konetzka (1958), with standard Conway microdiffusion units. Details of the strains examined, which were grown in nutrient broth at pH 7.2-7.6, are given below:

Shaw sub-group	No. of strains	Period of insubation	Dry weight mg/ml.
1	3	5-6 hr. at 30°C	20-29
2	6 and N.C.T.C.7292	5-7 " " "	10-20
3	7 " " 7617	5-7 " " "	9-16
4	4 " " 7520	5-8 " " "	12-15
5	3 " " 7011,8512	5-9 " " "	10-19

Only those strains which gave a Q_{NH_3} of 1 or more are given in Table 5. Many strains gave values of less than 1, particularly at pH 8, with glutamic acid, histidine, ornithine and aspartic acid.

Table 5. Deamination of certain amino-acids.

Strain No.	Shaw sub- group	Q_{NH_3} values (μ l/mg/hr) at 30°C						
		pH 6		pH 8				
		histidine	ornithine	lysine	glutamic	histidine	ornithine	aspar
20(Bacon))	1	1	2			2	2	
34("))		1	2			1	2	
NCTC 7447)						1	1	
22(Brine))	2				1			
25("))					1			
10(Brine))	3				1			
12("))					1			
NCTC 7617)					2	3	2	
32(fresh)	5					3		1
side))								
38(Brine))				1		2		
39("))				3		2	2	
NCTC 7011)						1		

With regard to attack on arginine, many of the isolates had this ability. When tested with the medium of Thornley (1960), 55% of the isolates from fresh sides, 68% from brines, and 67% from bacon gave an alkaline or slightly alkaline reaction in both open and closed tubes. The mode of attack is not yet clear, but further work has indicated that at high pH (pH 9) the arginase system, and at low pH (pH 6) the arginine dihydrolase system may be active, in certain strains.

5. Heat resistance.

In Northern Ireland, a number of the bacon factories as well as producing sides of Wiltshire-cured bacon also cure and cook hams, both for the local and export markets. These hams may be pumped and cured by the normal Wiltshire method, or by the more modern technique of arterial pumping. They are injected or pumped to about $7\frac{1}{2}\%$ of their weight with a solution of: NaCl (about 18%), polyphosphates, nitrate and nitrite, then cured in immersion brine. This procedure varies from factory to factory, but in any case the final cooking given is more of a mild "pasteurization" than a full cooking. The centre of the ham probably does not reach more than 70°C for a short period. The injection brine is freshly made up and if properly prepared should have less than 100 organisms present per ml. However, some micrococci probably do find their way into the ham, via the injection brine in which they can survive, or from the immersion brine. Ingram (1952) found in bacon gammons, which are very similar to hams though more heavily injected, large numbers of micrococci, which he thought were largely psychrophilic halophiles derived mainly from the injection brine. In view of the possible significance in ham spoilage of these organisms which can survive in the injection and immersion brines, it was thought necessary to study their heat resistance to see whether they would be likely to survive the heating process.

The test strains included those found resistant to high salt

concentrations at 2-3°C, and consisted of:-

4 Shaw sub-group 1 strain(s)				
16	"	"	2	"
21	"	"	3	"
1	"	"	4	"
2	"	"	5	"

These cultures were subinoculated in broth at least 4 times so that they were growing strongly. The method for testing heat resistance was based on that of Briggs (1953). One drop of broth culture of each strain was dropped, without touching the sides of the tube, into each of 9 test tubes each containing 5 ml. of nutrient broth, pH ca. 7.2. These were heated in a waterbath at 60°C, and three tubes removed after 30, 60 and 90 min., rapidly cooled and then incubated for 7 days at 30°C to see if any cells had survived. None survived for 90 min. and only N.C.T.C. 7564, the type strain of Staphylococcus lactis (Shaw et.al. 1951) survived 60 min. One strain from fresh sides and one coagulase positive strain from bacon survived 30 min. It was now decided to try a suspension medium more comparable to factory practice. A modification of the "micro-ham" of Buttiaux and Beerens (1955), in which glucose and tomato extract was omitted, and 1% lard added, together with 0.5 ml. of a 1% sodium nitrite to each tube. 32 strains were tested in the same way as with the nutrient broth except that heating for 90 min. was omitted. Only N.C.T.C. 7564 survived for 30 and 60 min. From these results it was concluded that the heat resistance of these isolates was less than 30 min. at 60°C, and therefore should not constitute a problem in properly cooked hams.

Conclusions

1. The micrococcal flora of bacon is composed of that carried in on the sides, and also receives a contribution from that of the curing brine.
2. When classified, the isolates fell largely into Shaw sub-groups 2 and 3, and into Baird-Parker sub-groups Micrococcus 4 and 5.
3. Many of the isolates were able to survive in high salt concentrations and grow slowly at 4°C. There appears to be no reason why micrococci on the sides going into cure cannot survive the curing cycle.
4. Although many of the isolates showed tolerance to nitrate and nitrite the latter was inhibitory or toxic at high concentration, particularly at low pH. There was no apparent protective effect by nitrate against the inhibitory effect of nitrite.
5. There were indications that the isolates might not be active in reducing nitrate to nitrite in normal Wiltshire bacon curing brines, as nitrite production from nitrate fell off more quickly than growth at high salt concentrations.
6. There was little evidence of decarboxylase activity with the strains and amino-acids tested, and deaminase activity was generally slight. Arginine was attacked by many of the isolates.
7. The heat resistance of those strains tested appeared to be less than 30 min. at 60°C.

S U M M A R Y

164 strains of staphylococci and micrococci from fresh sides, curing brines and bacon have been examined. When classified these isolates fell largely into Shaw sub-groups 2 and 3, and into Baird-Parker Micrococcus subgroups 4 and 5. Many of the isolates were able to survive high salt concentrations, and survival was very good at 2-3°C. The isolates were tolerant to nitrate and nitrite, although nitrite was toxic in high concentration particularly at low pH. Nitrite production from nitrate fell off more quickly than growth at high salt concentrations. Although arginine was attacked by many strains, there was little decarboxylase or deaminase activity with the other amino-acids tested. The heat resistance of the strains tested was generally less than 30 min. at 60°C.

RÉSUMÉ

164 souches de Staphylococcus et Micrococcus isolées dans des carcasses fraîches, des saumures de salaison et du bacon ont été étudiées. Les bactéries se sont classifiées pour la plupart dans les sous-groupes 2 et 3 de Shaw et dans les sous-groupes de Micrococcus 4 et 5 de Baird-Parker. Beaucoup des bactéries pouvaient survivre dans de hautes concentrations de sel, et la survie était spécialement bonne à 2-3°C. Les bactéries étaient tolérantes vis-à-vis du nitrate et du nitrite, bien qu'une haute concentration de nitrite fût toxique, surtout à un pH faible. La production de nitrite du nitrate diminuait plus rapidement que la croissance en présence d'une haute concentration de sel. Bien que l'arginine fût attaquée par beaucoup de souches, on a constaté peu d'activité de décarboxylase ou déaminase avec les autres acides aminés examinés. La thermorésistance des souches étudiées était généralement moins de 30 minutes à 60°C.

ZUSAMMENFASSUNG

164 Staphylokokken- und Mikrokokken-Stämme aus frischen Schweinehälften, Pökellaken und Speck wurden untersucht. Bei der Klassifikation fielen die isolierten Keime weitgehend in den Untergruppen 2 und 3 von Shaw und in den Mikrokokken-Untergruppen 4 und 5 von Baird-Parker. Viele Keime konnten bei hohen Kochsalzkonzentrationen lebensfähig bleiben, und das Überleben war besonders gut bei 2-3 C. Die Keime waren nitrat- und nitrit-tolerant, obgleich das Nitrit in hoher Konzentration, und zwar besonders bei einem niedrigen pH-Wert, toxisch wäre. Bei hohen Salzkonzentrationen verminderte sich die Bildung von Nitrit aus Nitrat schneller als die Vermehrung. Obgleich viele Stämme das Arginin angriffen, konnte man nur wenige Decarboxylase- oder Deaminase-Aktivität mit den anderen untersuchten Aminosäuren beobachten. Die Hitzefestigkeit der untersuchten Stämme betrug allgemein weniger als 30 Minuten bei 60°C.

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