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469

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THE MICROBIOLOGY OF BRINES USED IN CURING OF "BRESAOLA"

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Much interest has been recently shown in the microbiology of brines and several studies have been made on their microbial flora.
It is believed that the quality of finished products depends not only from the action of the salts present in the brines but also and perhaps particularly from that of the bacteria present originating from the environment, meat, water, the use of ingredients, and from many studies it seems that bacteria play a decisive role in curing processes.
Although there is a reasonable concordance in the opinions of Authors concerning the interpretation of the chemical reaction taking place during curing, results of investigations on the composition of the microflora and on its effects are less concordant.
Considerable progress in the microbiology of brines has made by Buttiaux (1957), Deibel and Niven (1957), Henry et Alii (1957), Buttiaux and Moriamez (1957), Riemann (1957) with regards to ham: According to Buttiaux the "active flora", that is the flora which acts in the denitrification and acidification processes, comprises micrococci and vibrios. The micrococci, halotolerant, seem to play a secondary role while the vibrios, some of which can utilize saccharose (Vibrio costicolus), also alophylic, osmophylic, denitrifier seem to be the principal agent responsible for the production of good quality ham (Henry et Alii, 1954), Buttiaux, 1957).
Anglosaxon Microbiologists (Ingram, 1957), Deibel and Niven, 1957)) in their important contributions in this field have found that in bacon and ham brines the microbial flora comprises prevalently micrococci and lactobacilli.
This difference may be due to the fact that in Anglosaxon countries nitrite brines are used whereas in Latin countries nitrate ones.
These considerations toghether with scant knowledge of microbial floras of brines used in Italy, have induced us to undertaken the present investigation in which we have examined the brines used in the preparation of "Bresaola", a kind of salted meat typical of Valtellina, which is prepared according to a traditional process which has been used for at least two centuries.
The characteristics of this product and the methods of preparation have been fully described by Calcinardi (1963). Suffice it to say here that the meat used is bovine leg from a not less than three years old animal. The meat is dry salted threating it with a mixture of NaCl (2.5 to 3.5 Kg per quintal), potassium nitrate (20 to 25 gr per quintal) grinded pepper (100 gr per quintal). The meat is then placed in vats of either oakwood or, in more modern factories, of plastics (polyester reinforced with glas fibers) and left so that the saline solution is made from the juice of the meat itself.

Salting is carried out at variyng temperatures (from 2 to 6° up to as much as 12°C.)) for a period of 20 days, after which the meats are taken out from the brine and left to ripening for 40 to 50 days at about 15°C.

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Five brine samples have been examined, taken from different bresaola factories; three were from a factory of industrial type and were of three different ages (4, 12, 20 days), the other two samples were from two different artisan factories and were respectively of 4,30 hours and 15 days.

Furthermore to have a more complete picture, four samples of meat which had been in brines for 3, 8, 10, 15 days were also taken from the same factories.

Bacteriological examinations were carried out with brine samples taken towards the centre of the vats at a depht of about 10 cm taking aseptic precautions. Samples were of about 1 litre and bacteriological examinations were carried within 4 hours from sampling, during which period the brines were kept at low temperature (not more than 10°).

The meat samples were examined for the superficial flora: the samples were taken from salting vats (whole of the bresaola) and placed in sterile glas vessels with hermetic closure, the sampling of the material to study the superficial flora were carried out agcording to the method of Caserio et Alii (1963).

For the quantitative and qualitative studies of the flora quantities of both brines and buffer washings of meat surfaces were diluted tenfold in salt tryptone solution (NaCl 6 gr, Tryptone 0,1 gr in 100 ml of distilled water) from which variyng from case to case either for incorporation or for superficial spreading, samples were seeded in tryptose agar for total counts, in V.F. agar for anaerobic and facultative aerobic counts (with plates closed according to Beerens, 1968) and on potato agar with tetracyclin for yeasts and moulds.

Achromobacter, Aeromonas and Vibrio were isolated on saccharose bromochresolpurple agar with or without the addition of antibiotics to prevent the growth of micrococci which have an antagonistic action agains vibrios (Buttiaux, 1957) and of molds, very numerous in these brines.

For inhibiting micrococci penicillin was used (2 I.U. per ml); to inhibit the yeasts and moulds a polyhene antibiotic was used (antibiotic 583, Giolitti, 1954) which has the advantage with respect to others such as Actidione of strongly inhibiting both yeast-like and filamentous fungi and yet is completely inert towards schyzomicetes even at high concentrations (we have used 25 mcg per ml).

Micrococci and Staphylococci were counted using Chapman's medium (mannitol salt agar with phenolred), Streptococci D on Barnes's medium which allways gave excellent results, Lactobacilli on thome's medium of Rogosa, Mitchell and Wiseman (1958), Pseudomonas on KingB medium; Enterobacteria on desoxycholate agar, sulphyte-reducing anaerobes (spores) on V.F. sulphyte agar with previous heating of the

brine to 80° for 10 minutes.
The Gram reaction and Rhodes stain (for flagella) were carried out according to the description of the methods given by Buttiaux, Beerens, Tacquet (1963).

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Results are gathered in table 1.
In addition to counts, identification of microbial species found in examined brines was carried out.
Among the Micrococcaceae the following have been identified: M. roseus, M. caseolyticus, M. flavus, M. conglomeratus, S. epidermidis (using the indications of Murray, Breed and Smith, Bergey's Manual, 1957).
Among the Enterobacteriaceae, E? coli (only in young brines), and more often Aerobacter, Citrobacter and Proteus rettgeri. Among the Streptococci D allways very numerous, S. faecalis (both varieties: zymogenes and liquefaciens), S. faecium, S. durans and S. bovis.
None of the lactobacilli strains could be classified according to Sharpe (1962) insofar that they possessx different characters from those described by this Author, both Streptobacterium and Betabacterium were found. The characteristics of strains are referred in table 2.
Among the Pseudomonaceae, P. fluorescens, Xantomonas, Aeromonas, Halobacterium and very many other germs which seem to be considered as belonging to Achromobacter have been identified, they are capable of producing particularly in solid media abundant quantities of a polisaccharide, the chemical structure of it has been investigated by Massacra (1964).
Among the Vibrio, V. costicolus and halotolerant vibrios capable of utilizing arabinose but not saccharose have been identified.
Some Bacillus species have also been identified (B. subtilis and B. licheniformis), the presence of which was not rare in the examined brines.
With regards to Clostridia we have limited ourselves to seeking only Cl. perfringens, which has never been found.
Identification of yeasts and moulds was not attempted, we had limited ourselves to counts.
It was also thought useful to carry out some chemical examinations of the brines; pH (Electrometrically), the amount of nitrates and nitrites (Giolitti, 1960), NaCl concentration, lactic acid concentration (according to Snell and Snell, 1954), total nitrogen by microkjeldhal (performing the mineralisation according to Beet, 1954), soluble nitrogen by the same method with previous sodium tungstate precipitation, free aminoacids by two directional chromatography (first solvent: phenol NH_3, second solvent: butanol, acetic acid and water). The results of these tests are given in table 3.

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The preparation of the brines we have examined is carried out empi=

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rically and without particular hygienic care, we consider this to be responsible at least in part for the great variety of the microbial flora present in them.

Of this flora, part can be considered as "active" and part as saprophitic. Although the products obtained with the brines we have examined have been recognized by specialists as being of good taste, it is to be supposed that such a vaste microbial flora could jeopardize good production or at least give poor results from the hygienic point of view.

Most of the factorie are small artisan ones and in these (and also in larger concerns) the attention of the producers is directed more to the choice of good quality meats or generally speaking suitable for bresaola production rather than towards the realization of scrupolous hygiene during manufacturing.

It is interesting to observe that the microbial flora found in bresaola brines is somehow different from that of brines used in Anglo-saxon Countries (bacon) or in France (Ham).

In the bacon's brines mostly micrococci and lactobacilli occur, but it should be remembered that the brines are nitrite ones; in France Buttiaux (1957) has shown the presence of micrococci, allways abundant but playing a secondary role in curing, vibrios, allways abundant in all brines and to divide in two groups, saccharose + and saccharose - (saccharose + vibrios described by Henry, Goret and Joubert,(1954), are responsible for the production of good quality hams) and lastly Achromobacter, frequent but not constant and allways in small number and which do not take a significant part in denitrification processes (although Buttiaux (1957) did find one Achromobacter which possesses characteristics useful for participation in curing).

Furthermore the same Author found: Enterobacteriaceae: E. coli almost constantly present but not very abundant, rarely Paracolobactrum; Pseudomonas rare in fresh brines but in old ones can be found in mud deposits; Streptococci D, frequently found and excusively represented by S. faecium, towards which Barnes and Ingram had already drawn attention in 1955.

In the brines we have examined our findings were:

1) presence of numerour Achromobacter with very uniform characters especially with regards to their denitrifying ability.

Almost all the strains examined had the following characteristics: Rods of variable form, often curved, in short chains, sometimes coccoid, gram variable, non motile, capsulated, producer on solid media of abundant polysaccharidic substances.

They are: Catalase +, Oxidase + (Kowacs, 1956), reduce nitrates to nitrites and nitrites are further on decomposed; indole variable; H_2S -; urea -; Gelatin liquefied, milk coagulated and peptonized, glicides not utilized, Hugh and Leifson medium alcalinized, growt from 10° to 30°, not at 37°; halotolerant.

These Achromobacter strains are different from the one described by Buttiaux which was motile (perithrychous flagella) glucose +, saccharose +, halotolerant. They were more numerous in brines than on the meat surfaces.

2) presence of numerous micrococci in both brines and meats, pigmented and not pigmented, aerobic, not fermenting mannitol; Gaffkya has not been found.

3) presence of lactobacilli, numerous in brines, but even more nu-

merous on meat surfaces, these lactobacilli at the pH of the brines (5.7 to 5.0) are capable of utilizing nitrites (Coretti, 1954; Cantoni, 1964).

4) Presence of numerous Streptococci D, which could be identified as S. faecalis (var. Zymogenes and liquefaciens), S. faecium, S. durans S. bovis.

5) presence of Enterobacteria: E? coli was found rarely (Mc Kenzie test) and only in young brines. Lactose + Aeromonas strains have been counted for convenience toghether with enterobacteria. It was observed that enterobacteria were rather more numerous on meat surfaces that on brines.

6) presence of many Aeromonas constant in all brines, weakly utilizing nitrates or nitrites.

7) Vibrios were not allways found and in any case allways in very small number even when media contained penicillin and antibiotic 583. It cannot excluded that the good growth of Achromobacter and Aeromonas on such media may have rendered the isolation of Vibrios difficult, but had these been present in considerable numbers they would not excaped observation. It can be concluded that under our conditions the environment is not favourable for the growth of these bacteria.

8) Yeasts and moulds were numerous and this we think is due to the environmental conditions.

9) Limited numbers of Pseudomonas fluorescens, Xantomonas and Halobacterium werd identified and also in limited numbers sulphite reducing Clostridia.

With regards to chemical aspects of these brines (Massacra, 1964) the pH is allways low and right from the first hours it remains constant around 5 or little above for the all of the curing period. Nitrates are rapidly reduced to nitrites and these are further on metabolized. Different microbial species carry out this reduction and one of us (Cantoni, 1964) has shown that in the reduction of nitrates to nitrîtes in these brines micrococci, vibrios and to a lesser extent yeasts and Aeromonas but especially the Achromobacter play a part; these last germs are able to break down successively the nitrites, whereas the vibrios were weak nitrites reducer.

The temperature at which the brines are held has a considerable influence on the intensity and rapidity of nitrates and nitrites reduction (Cantoni, 1964).

The concentration of NaCl with the exception of very fresh brines ranges from 7.9 per cent to 5.8 per cent in relation to the manner of preparing brines and of the season (the NaCl concentration is higher during warm seasons).

The concentration of lactic acid is fairly constant, around 1 per cent, and it should be recalled that saccharose is not added to the brines.

The quantity of total nitrogen and soluble nitrogen is high, free aminoacids found were; glutammic acid, alanine, serine, leucine, alpha-aminobutirric acid.

From these results it can be deduced that in bresaola brines the active flora consists probably of Achromobacter, lactobacilli and

micrococci, the presence of which is high nnmerically and constant. Streptococci D are also numerous, we think the reason of their pre= sence in the bresaola brines needs to be further investigated in order to ascertain whether they have to be considered as contami= nants or as plaiyng a role in curing.

Attention must also be drawn on the considerable number of Ente= robacteria present on meat surfaces, though a part of the total count could be represented by lactose + Aeromonas.

The considerable numbers of yeasts and moulds, higher in brines from artisan undertakings, could be due in part to the fact that the curing of the bresaole take place in vats of oakwood and that only recently these are being substituted (and for the moment only in larger factories) with plastics vats. It is also probable that the environmental conditions help to mantain high the number of yeasts and moulds.

At least it seem unlikely that these organisms take part in curing, though it cannot excluded that they may influence the qualitative composition of the brine flora.

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RESUME'

On a examiné la microflore des saumures de "Bresaola", une salaison typique de la Valtélline, dans lequelles des groupes de bactéries sont constamment présents: les Achromobacter, les Micrococci, les Lactobacilles, les Aeromonas et les Streptococques D.
Les Vibrio sont très rares et il est extrèmement difficile de les déceler même sur les milieux séléctifs salés a 6 pour cent et avec de la pénicilline et un antibiotique antifongique.
Selon notre avis les Achromobacter, Lactobacillus et Micrococci devraient avoir une importance dans le processus de saumurage et peuvent être considérés comme flore active. Le rôle des Streptocoques D, toujours très nombreux est encore à étudier.
Les lévures et les moisissures sont toujours présentes et nombreuses surtout dans les produits artisans.

SUMMARY

The microflora of Bresaola brines (a typical product of the Valtellina) has been examined. Several groups of Bacteria were found in them: Achromobacter, Micrococci, Lactobacilli, Aeromonas, Streptococci D, Enterobacteria. The Vibrios are rare and it is very difficult to isolate them even using antibiotics to inhibit the antagonistic flora (agar containing 6% NaCl, saccharose, penicillin and an antifungal antibiotic).
We believe that the active flora should be represented by Achromobacter, Lactobacilli and Micrococci. Streptococci D, allways present and numerous need further investigations to ascertains their rôle.
Yeasts and fungi are numerous mainly in brines of artisan type.
Several other species of bacteria have been identified.

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The characteristics of this product and the methods of preparation have been fully described by Calcinardi (1963). Suffice it to say here that the meat used is bovine leg from a not less than three years old animal. The meat is dry salted threating it with a mixture of NaCl (2.5 to 3.5 Kg per quintal), potassium nitrate (20 to 25 gr per quintal) grinded pepper (100 gr per quintal). The meat is then placed in vats of either oakwood or, in more modern factories, of plastics (polyester reinforced with glas fibers) and left so that the saline solution is made from the juice of the meat itself.

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480

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Results are gathered in table 1.
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1) presence of numerour Achromobacter with very uniform characters especially with regards to their denitrifying ability.

Almost all the strains examined had the following characteristics: Rods of variable form, often curved, in short chains, sometimes coccoidw, gram variable, non motile, capsulated, producer on solid media of abundant polysaccharidic substances.

They are: Catalase +, Oxidase + (Kowacs, 1956), reduce nitrates to nitrites and nitrites are further on decomposed; indole variable; H_2S -; urea -; Gelatin liquefied, milk coagulated and peptonized, glicides not utilized, Hugh and Leifson medium alcalinized, growt from 10° to 30°, not at 37°; halotolerant.

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merous on meat surfaces, these lactobacilli at the pH of the brines (5.7 to 5.0) are capable of utilizing nitrites (Coretti, 1954; Cantoni, 1964). 483

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With regards to chemical aspects of these brines (Massacra, 1964) the pH is allways low and right from the first hours it remains constant around 5 or little above for the all of the curing period. Nitrates are rapidly reduced to nitrites and these are further on metabolized. Different microbial species carry out this reduction and one of us (Cantoni, 1964) has shown that in the reduction of nitrates to nitrîtes in these brines micrococci, vibrios and to a lesser extent yeasts and Aeromonas but especially the Achromobacter play a part; these last germs are able to break down successively the nitrites, whereas the vibrios were weak nitrites reducer.

The temperature at which the brines are held has a considerable influence on the intensity and rapidity of nitrates and nitrites reduction (Cantoni, 1964).

The concentration of NaCl with the exception of very fresh brines ranges from 7.9 per cent to 5.8 per cent in relation to the manner of preparing brines and of the season (the NaCl concentration is higher during warm seasons).

The concentration of lactic acid is fairly constant, around 1 per cent, and it should be recalled that saccharose is not added to the brines.

The quantity of total nitrogen and soluble nitrogen is high, free aminoacids found were; glutammic acid, alanine, serine, leucine, alpha-aminobutirric acid.

From these results it can be deduced that in bresaola brines the active flora consists probably of Achromobacter, lactobacilli and

micrococci, the presence of which is high numerically and constant. Streptococci D are also numerous, we think the reason of their pre= sence in the bresaola brines needs to be further investigated in order to ascertain whether they have to be considered as contaminants or as plaiyng a role in curing. 484

Attention must also be drawn on the considerable number of Ente= robacteria present on meat surfaces, though a part of the total count could be represented by lactose + Aeromonas.

The considerable numbers of yeasts and moulds, higher in brines from artisan undertakings, could be due in part to the fact that the curing of the bresaole take place in vats of oakwood and that only recently these are being substituted (and for the moment only in larger factories) with plastics vats. It is also probable that the environmental conditions help to mantain high the number of yeasts and moulds.

At least it seem unlikely that these organisms take part in curing, though it cannot excluded that they may influence the qualitative composition of the brine flora.

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RESUME'

On a examiné la microflore des saumures de "Bresaola", une salaison typique de la Valtélline, dans lequelles des groupes de bactéries sont constamment présents: les Achromobacter, les Micrococci, les Lactobacilles, les Aeromonas et les Streptococques D.
Les Vibrio sont très rares et il est extrèmement difficile de les déceler même sur les milieux séléctifs salés a 6 pour cent et avec de la pénicilline et un antibiotique antifongique.
Selon notre avis les Achromobacter, Lactobacillus et Micrococci devraient avoir une importance dans le processus de saumurage et peuvent être considérés comme flore active. Le rôle des Streptococques D, toujours très nombreux est encore à étudier.
Les lévures et les moisissures sont toujours présentes et nombreuses surtout dans les produits artisans.

SUMMARY

The microflora of Bresaola brines (a typical product of the Valtellina) has been examined. Several groups of Bacteria were found in them: Achromobacter, Micrococci, Lactobacilli, Aeromonas, Streptococci D, Enterobacteria. The Vibrios are rare and it is very difficult to isolate them even using antibiotics to inhibit the antagonistic flora (agar containing 6% NaCl, saccharose, penicillin and an antifungal antibiotic).
We believe that the active flora should be represented by Achromobacter, Lactobacilli and Micrococci. Streptococci D, allways present and numerous need further investigations to ascertains their rôle.
Yeasts and fungi are numerous mainly in brines of artisan type.
Several other species of bacteria have been identified.

TABLE 1

	BRINES					MEAT			
	4?30 H	4 days	12 days	15 days	20 days	3 days	8days	10 days	15 days
TOTAL COUNT	144.10^5	52.10^5	39.10^5	11.10^6	187.10^6	80.10^5	136.10^5	196.10^5	120.10^5
MICROCOCCI	28.10^4	318.10^4	91.10^4	145.10^5	215.10^5	256.10^4	66.10^5	106.10^5	87.10^5
LACTOBACILLI	50.10^3	80.10^4	60.10^4	20.10^4	77.10^5	n. d.	80.10^4	50.10^5	104.10^5
AEROMONAS AND ACHROMOBACTER	21.10^4	47.10^4	13.10^4	85.10^4	45.10^4	17.10^3	21.10^3	12.10^3	24.10^3
STREPTOCOCCI D	65.10^3	348.10^4	48.10^4	172.10^4	385.10^4	35.10^3	152.10^3	228.10^3	148.10^4
ENTEROBACTERIA	24.10^2	45.10^2	41.10^3	48.10^3	75.10^3	95.10^4	41.10^4	88.10^4	128.10^4
YEASTS and MOULDS	83.10^4	25.10^4	20.10^4	153.10^4	84.10^4	44.10^2	164.10^2	264.10^2	760.10^2
VIBRIO	2	1	1	2	0	0	0	0	0
CLOSTRIDIA (spores)	3	0	1	3	15	1	13	0	0

n.d. = not determined

n N

487

TABLE 2

Characters of Lactobacilli isolated from brines

Number of strains	Gram	Hydrogen peroxyde prod	gas production	NH_3 from arginine	Growth at 15°	Growth at 30°	Growth at 45°	Esculin	Arabinose	Lactose	Melibiose	Raffinose	Rhamnose	Sacchafose	Xylose	Fructose	Maltose	Mannitol	Dextrose
7	+	+	−	+	+	+	−	−	+	+	+	−	−	+	−	−	+	−	+
5	+	+	−	+	+	+	−	−	−	+	+	−	−	+	−	−	−	−	+
1	÷	+	−	+	+	+	−	−	−	+	−	−	−	−	−	−	−	−	+
1	+	+	−	+	+	+	+	+	+	−	−	−	−	+	−	−	−	−	+
3	+	+	+	+	+	+	−	+	+	+	+	+	−	+	−	∵	+	−	+
4	+	+	+	+	+	+	−	−	−	+	+	−	−	+	−	+	+	−	+
4	+	+	+	+	+	+	−	+	+	+	+	+	−	−	+	+	+	−	+
3	+	+	+	+	+	+	−	+	+	+	+	−	−	+	−	+	+	−	+
2	+	+	+	+	+	+	−	+	+	+	+	+	−	+	−	+	+	−	+
1	+	+	+	+	+	+	−	+	−	−	+	−	−	+	−	+	+	−	+

TABLE 3

Brine	Time	pH	Nitrates mg %	Nitrites mg%	NaCl g%	Lactic acid g %	Total N g %	Soluble N g %	Glut. ac.	Ser.	Alan.	Leuc.	∝-Amino butyric acid
1	4,30h	5,4	710	-	31	-	4,34	-					
2	4 d.	5,0	174	3,5	6,7	0,99	10,7	2,7	+	+	+	+	+
3	12 d.	5,0	92	-	5,8	1,1	11,0	3,7	+	+	+	+	+
4	15 d.	5,1	71,5	10,3	7,9	n.d.	9,18	n.d.	+	+	+	+	+
5	20 d.	5,1	77,0	5,3	5,9	1,05	12,2	4,95	+	+	+	+	+

n.d. = not determined

+ = present