Effect of additives and ripening parameters on growth and toxin production of Clostridium botulinum

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It has been stated previously that under precisely controlled ripening of raw sausages addition of nitrate and or nitrite can be lowered in amount or can be ommitted entirely. This causes a change - though not unpleasant - in flavor and makes ripening conditions more critical. On the other hand the human health hazard i.e. potential risk of Clostridium botulinum remained to be elucidated.

For that reason raw sausages were produced

a/ with KNO_3 /600 ppm/

b/ with $NaNO_2$ /150 ppm/

c/ without nitrate and nitrite,

10^3 spores of Clostridium botulinum were injected into the core and the sausages were ripened. Samples were taken at weekly intervals and extracted with phosphate buffer. Mice were i.p. injected with the supernatant of the extract after centrifuging at 7000 r.p.m. for 30 mins. Mice injected with antitoxin + supernatant and with heated supernatant served as controls.

The mouse-test proved that the spores germinated, grew and produced toxin during the first ripening period in sausages containing no curing salt. The same phenomenon has been noticed with sausages containing nitrate or nitrite very rarely. After some weeks of ripening, when a_w value became lower, no further toxin production could be detected and the toxin previously formed was inactivated.

Inactivation of toxin was found also when pure toxin instead of spores was injected into core, in other words neither spore-injected, nor toxin-injected samples caused death with mice. This means that no toxin can be detected in the raw sausage by the end of a longer ripenig period even if no nitrate or nitrite is used. The mechanism of toxin inactivation is under investigation.

Einfluss von Zusatzstoffen und Reifungsparametern auf das Wachstum und Toxinbildung von Clostridium botulinum

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Aufgrund unserer vorherigen Experimenten konnte festgestellt werden, dass der Nitrit/Nitrat-Zusatz bei bestimmten Rohwursttypen und bei Einhaltung gewisser Reifungsparameter stark zu vermindern sogar zu vermeiden ist. Die Verminderung oder das Auslassen der Nitrit/Nitrat-Menge hat eine gewisse aber nicht unangenehme Aenderung im Geschmack und eventuell auch eine geringere Produktionssicherheit zur Folge. Es besteht jedoch das Problem des Gesundheitsrisikos und zwar die eventuelle Zunahme der Clostridium-Gefahr in Würsten ohne Nitrit/Nitrat Zugabe.

Zur Klärung der Frage wurden Rohwürste a/mit KNO_3 b/mit $NaNO_2$ c/ohne KNO_3 und $NaNO_2$ hergestellt und in den Kern wurden 10^3 Clostridium botulinum B Sporen injektiert. Die Reifung verlief wie üblich und den Würsten wurden wöchentlich Proben entnommen. Nach Extrahieren der Probe mit Phosphat-Puffer und Zentrifugieren bei 7000 U/min für 30 Min. wurden mit dem Überstand Mäuse geimpft. Als Kontroll dienten Mäuse die mit Antitoxin + Überstand und mit gehitztem Überstand geimpft wurden. Am Anfang der Reifung zeigten die Sporen Auskeimung, Wachstum und Toxinproduktion. Ausnahmsweise kann dieser Vorgang auch bei Würsten vorkommen die mit Nitrit/Nitrat hergestellt sind. In späteren Perioden der Reifung, bei niedrigeren A_W - Werten konnte keine Auskeimung und Toxinbildung festgestellt werden, sogar wurde das vorher gebildete Toxin inaktiviert.

Diese Toxininaktivierung war auch bei den mit reinem Toxin injektierten Würsten zu beobachten. Das bedeutet also, dass -anhand entsprechender Reifungsparameter- in der schnittfesten Rohwurst kein Botulotoxin nachweisbar ist, auch wenn die Wurst ganz ohne Pökelsalz hergestellt worden war. Experimente bezüglich des Mechanismus der Toxininaktivierung sind im Gange.

Edellinen sivu tyhjä

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L'influence des additifs et de la maturation sur la multiplication de Clostridium botulinum et la productivité des toxines

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Au cours des expériences précédentes nous avons constaté que dans le cas de certains types de saucissons secs l'addition du nitrite et du nitrate peut être diminuée ou abandonnée respectant les paramètres de maturation avec rigueur. L'abandon du nitrite ou du nitrate peut provoquer un changement du goût tolérable mais dans certains cas le décroissement de la sécurité de fabrication.
Toutefois, la question de l'assurance sanitaire, c.à.d. l'augmentation éventuelle de menace de Clostridium reste problématique.
Pour éclaircir cette question nous avons injecté le spore B du Clostridium botulinum en quantité de 10^3 dans le centre des saucissons secs, préparés avec ou sans additifs.
Les saucissons secs ont été soumis à la maturation habituelle; nous avons prélevé les échantillons par semaines. Après avoir extrait l'échantillon par un tampon de phosphate nous avons inoculé les souris avec le surnageant. Ce surnageant a été obtenu par une centrifugation de 30 minutes /7000 rev/minute/.
Les souris inoculées avec antitoxine + surnageant ainsi que celles inoculées avec des extraits de traitement thermique servaient comme contrôles. On pouvait constaté qu'au début de la maturation les spores germaient et multipliaient, produisants de toxines dans les saucissons secs préparées sans additifs /nitrite ou nitrate/. Dans des cas exceptionnels cette phénomène peut se produire aussi avec les saucissons secs préparées avec nitrite ou nitrate. Dans la phase suivante de la maturation - évidemment à cause de la diminution de l'activité de l'eau - la germination, la multiplication et la production de toxine diminue considérablement et même les toxines déjá produites commencent à s'inactiver.
L'inactivation de la toxine a été aussi démontrée dans le cas où l'on a injecté la toxine pure dans le saucisson sec.
Si une maturation plus longe correspond strictement aux prescriptions technologiques, le produit fini ne contient plus la toxine botulinum dans le cas de défaut du nitrite et du nitrate.

Actuellement des expériences, concernant le mécanisme de l'inactivation de toxine sont en cours.

Влияние посолочных ингредиентов и параметров созревания на развитие и токсинобразование Clostridium botulinum

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Результаты своих работ, проводенных заранее показали, что есть возможность для снижения или для исключения нитрата и нитрита из производства некоторых сырокопченных колбас при соблюдении определённых параметров созревания. При этом в какой-то степени изменяется вкус, но это ещё не является неприятным, и иногда снижается стабильность технологии. Но может возникать вопрос об опасности на здаровье health risk /, точнее о возможности увеличения Clostridium риска при производстве без применения посолочных ингредиентов.

Для выяснения данного вопроса в центр батона сырокопченных колбас вприскивали C.botulinum в количестве 10^3, ф именно в батоны изготовленные без применения нитрата и нитрита и в батоны с применением их. Подвергали их созреванию по действующей технологии и пробы отбирали один раз в неделю /для проб использовали целый батон/. Пробу экстрагировали в фосфатной буфере, потом центрифугировали при 7000 об/мин в течении 30 минут и сверхуплавающейся частью вприскивали мыши, контролем использовали те мыши, в которые вприскивали антитоксин, то есть экстракт подвергнутый термической обработке. В результате проведенных исследований было установленно, что в начале созревания в батонах без нитрата и нитрита наблюдается рост спор, размножение и токсинобразование. В исключительных случаях наблюдали это и у тех проб, которые не содержали нитрат и нитрит. На позднейшей стадии созревания - из-за снижения свободной влаги /относительная влажность в продукте/ не наблюдается рост, размножение и токсинобразование, даже замечается инактивация раньше образовавшихся токсинов.

Инактивацию токсина удалось доказать и в том случае, когда в колбасу вприскивали чистый токсин. При соблюдении определённых параметров во время созревания из готового сырокопченного продукта не удалось выделить токсин Botulinum , когда колбасу изготовили без применения нитрата и нитрита. Опыты, направленные на изучение механизма инактивации токсина в ностаящее время продолжаются.

Influence of additives and ripening parameters on growth and toxin production of Clostridium botulinum

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Introduction

It has been a common effort in recent 10 years to decrease the amount of nitrite and nitrate in meat products or to omit them entirely. Well-known reason of this effort is the potential health-hazard exhibited by eventual formation of nitrosamines from curing additives and secondary amines.

The main lines in nitrosamine-research can be summed up as follows:

1./ Study of technological and sensoric problems caused by decrease of curing additives.

2./ Study of microbiological-hygienic problems caused by decrease of curing additives.

3./ Detection of nitrosamines in meat products prepared with lowered amount of curing additives. Screening, monitoring service, analytical studies.

4./ Possibility of lowering the nitrosamine-level in foods by partial or total replacement of nitrite-nitrate.

5./ Animal feeding tests with nitrite-cured, eventually nitrosamine containing foods. Chronic toxicity test with nitrite.

6./ Detection of nitrosamines in animal stomachs and in model "stomachs" "fed" with potential precursors.

In this paper the microbiological problems arisen from the decrease of curing additives will be dealt with.

As known, nitrite inhibits many micro-organisms of food origin most important of which is the inhibition of toxin production by Clostridium botulinum.

Since the use of nitrite and sometimes nitrate is therefore of vital importance, the consequences of their decrease must not be overlooked. In order to avoid a raise in health-risk, the eventual decrease of inhibiting effect should be checked and be compensated some other way.

In our previous experiments /Incze, Farkas, Zukal, 1973; Farkas, Incze and Zukal, 1973; Incze Farkas, Mihályiné, Zukal, 1974/ the effect of lowered nitrite concentration on Cl. sporogenes spores has been studied in heat treated meat products. The lower inhibiting effect has been successfully compensated by other chemical and/or physical methods.

Just a few of the numerous authors dealing also with microbiological effect of nitrite in heat treated meat products could be mentioned here: Christiansen et al. /1973/ studied the effect of curing salts on Cl. botulinum in comminuted meat, Hustad et al. /1973/carried out similar studies in Wieners and Christiansen et al. /1975/ in summer style sausage.

Another group of meat products consists of the non heat treated ones where the microbiological consequences of the decrease of curing salts should be cleared as well.

Skjelkvale and Tjaberg /1974/ found practically no difference between the microflora of salami sausages prepared with or without nitrite resp. In their work presence and number of micrococci, lactic acid bacteria, aerobic sporeformers, pseudomonas and coliform bacteria were determined, nevertheless the health risk aspect has not been dealt with.

Collins-Thompson et al. /1974/ and Christiansen et al. /1974/studied the effect of curing salts on Cl. botulinum in bacon and recently Ivey et al. /1978/ the combined effect of nitrite and sorbate on Cl. botulinum in bacon. Bacon is nonetheless consumed mostly after frying and there are other reasons too, why from bacon to dry

sausage no direct extrapolation can be made.

In order to get some idea on the potential enhancement of health risk in dry sausage when lowering the amount of curing salts the following experiments have been carried out:

Dry sausage batches were produced

a/ by traditional method /600 ~~ug~~ mg/kg KNO_3/

b/ with nitrite curing salt /cca 150 ~~ug~~ mg/kg $NaNO_2$/

c/ without nitrite and nitrate

Materials and methods

Under factory conditions raw sausage was produced with nitrate, nitrite or without them /see above/ and spores of Cl. botulinum type B were injected into the core. Concentration of spore suspension was adjusted to 10^4/ml, 0,1 ml of which was injected. This meant a final number of 10^3 spores in product. The sausages were ripened in a usual way and samples were taken at weekly intervals /every time a new sausage unit/. The samples were homogenized and extracted with gel-phosphate buffer /pH 6,2/ and centrifuged at 7000 r.p.m. for 30 mins according to the standard procedure.

Mice were injected i.p. with the supernatant, animals injected with heated supernatant as well as with antitoxin plus supernatant served as controls.

In preliminary experiments sausages were treated with Cl. botulinum toxin. 0,1 ml of the 10fold dilution of concentrated toxin was injected in the sausage and samples were taken, extracted and used for mouse test as described previously /1000fold dilution of concn. toxin killed mice within one day/.

Results and discussion

No toxin has been found in sausages immediately and 3 days after inoculation /Table 1./. This means that no toxin was administered unintentionally when injected Cl. botulinum spores and no toxin formation took place in 3 days either. After a ripening period of one week the extract of sausage with no added nitrite and nitrate caused death of mice in both series of experiments. Extracts of nitrite- or nitrate containing sausages caused also death but not with all mice and only in one series of experiment. After two weeks no lethal effect has been found with these latter samples, while toxin was still detected in extract of sausage with no added nitrite or nitrate. After a ripening period of 3 and more weeks no lethal effect was found with any samples.

While trying to explain this phenomenon we think that a_w-value in fresh product is fairly high /0,96-0,97/, thus it may promote germination and outgrowth of spores and toxin production by vegetative cells of Cl. botulinum, even if temperature is below suboptimal level. If curing salt is also present, then combination of low temperature, suboptimal a_w-value and curing additive - even nitrate - is sufficient for inhibition of toxin formation. In lack of curing additive water activity should be lowered - this happens during ripening - in order to inhibit toxin formation by Clostridium botulinum.

It was rather interesting to learn that botulinum toxin previously formed "disappeared" somehow, no toxin could namely be detected at the end of ripening period. Whether this was caused by inactivation or by other mechanism is under investigation.

When toxin was injected into the core of sausages mice test proved positive only after 1 or 2 days /Table 2./ and never after 1 week or later no matter nitrite-nitrate containing or nitrite-nitrate free samples have been tested. Preliminary investigations aimed at explanation of this mechanism may suggest that increase of salt concentration during ripening is probably not the cause of this "inactivation".

Summing up the results briefly we might conclude that no increase of health risk should be calculated with in case of dry sausage produced without nitrite and nitrate if ripening is carried out under strictly maintained circumstances.

Main criteria of this ripening are low temperature and correct way of drying. Thus Cl. botulinum has no chance to grow or its initial growth is blocked and in the ripened product no toxin can be detected. In this respect long ripened dry sausage should be considered a special product compared to other meat products:

while a_w-value is constant with most of those products, it steadily decreases with dry sausages and this fact causes a great difference in terms of safety.

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Table 1. Toxin formation in samples inoculated with 10^3 Cl. botulinum spores

Sampling after	Lethality rate of mice					
	KNO_3		$NaNO_2$		no added nitrite and nitrate	
	1.ser.	2.ser.	1.ser.	2.ser.	1.ser.	2.ser.
inoculation	-	-	-	-	-	-
3 days	-	-	-	-	-	-
1 week	-	66 %	-	66 %	100 %	100 %
2 weeks	-	-	-	-	100 %	100 %
3 weeks	-	-	-	-	-	-
4 weeks	-	-	-	-	-	-
13 weeks	-	-	-	-	-	-

Table 2. Change in toxicitiy with samples injected with pure toxin[x]

Sampling after	Lethality rate of mice	
	KNO_3	no added nitrite and nitrate
1 day	100 %	100 %
1 week	-	-
2 weeks	-	-
5 weeks	-	-

[x] The 0,1 ml toxin injected was a 10fold dilution of concn. toxin, 1000fold dilution of which was still lethal to mice.