A C T A A L I M E N T A R I A P O L O N I C A

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CHARACTERISTICS OF MIXED-STRAIN STARTERS OF *STREPTOCOCCUS CREMORIS* AND *LEUCONOSTOC CREMORIS*

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Key words: *Streptococcus cremoris, Leuconostoc cremoris*, mixed populations, butter starters

Growth parameters of *Str. cremoris* 329 and *Leuc. cremoris* 1388 in milk medium, and the mutual effect of these strains on growth and metabolic activity in mixed populations have been reported in the paper.

INTRODUCTION

For the manufacture of several fermented dairy products, multistrain starters composed of lactic acid producers (*Str. lactis* or *Str. cremoris*) and aroma producers (*Str. diacetilactis, Leuc. cremoris* or other species of *Leuconostocs*) are used. When considering strains responsible for production of aroma compounds, Galesloot and Hassing [8] classified starters into three groups:

type B — including *Leuconostoc strains* only,
type D — including *Str. diacetilactis* strains only,
type BD — including *Leuconostoc* as well as *Str. diacetilactis* strains.

The proper composition of butter starters is very significant since — as it has already been shown [13] that lactic bacteria themselves are often responsible for the flavour defect described as "green" or "yogurt — like". The cause of this defect is attributed to acetaldehyde. Harvey [10] stated that all the strains *Str. lactis, Str. cremoris* and *Str. diacetilactis* examined by him, grown in milk, produced various amounts of acetaldehyde i.e. from 0.4 up to 13.0 mg/l.

At the same time Badings and Galesloot [1] observed that *Leuc. cremoris* is able to utilize acetaldehyde in multi-strain starters. Besides, Collins and Speckman [6] pointed out that acetaldehyde stimulates the growth of *Leuconostoc* strains.

All these findings are very significant, for the proper ratios of diacetyl to acetaldehyde levels affect the flavour of butter starters. The ratio of diacetyl to acetaldehyde from 13:1 to 5.5:1 in a starter brings about its "harsh" flavour, whereas "green" on "yogurt-like" flavour appears when the ratio drops to less than 3.2:1. Only starters which produce diacetyl and acetaldehyde in ratios from 4.4:1 to 3.2:1 are found to have the desirable flavour [13]. On the basis of the given data it is now quite a common trend to apply B or BD type starters.
Numerous observations speak in favour of them [5, 8, 18, 20]:

1. D type starters in production conditions very often lose aroma-producing bacteria which is caused by the attack of bacteriophages. Such phenomenon was never observed in B type starters.

2. In spite of rather high diacetyl content in butter produced with the use of D type starters, such butter is liable to rapidly occuring oxidation processes.

3. The application of *Str. diacetilactis* strains ensures high production of diacetyl in young starters, but later on reductase of diacetyl converts diacetyl into acetoin, and thus reduces irreversibly the amount of this flavour component of butter.

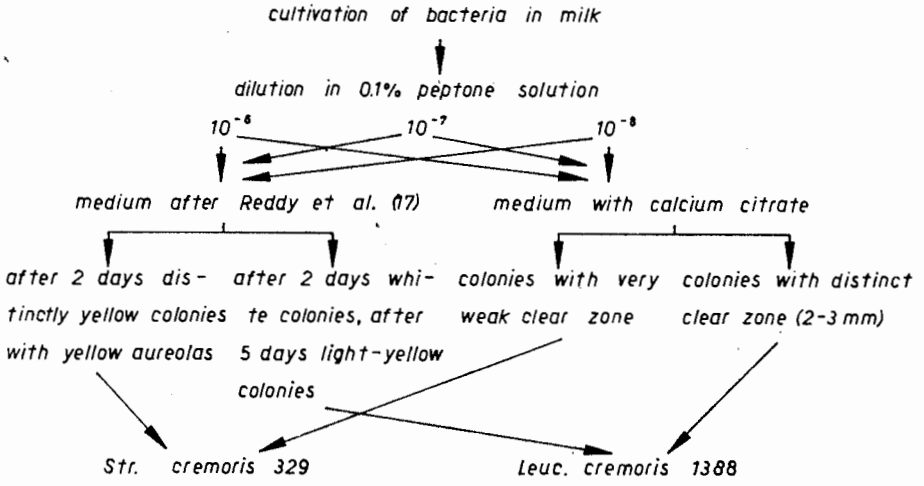
4. The addition of *Leuconostoc* strains to a starter prevents the "harsh" flavour of a product, which is due to utilization of the excess of acetaldehyde.

METHODS

Organisms: two strains of lactic acid bacteria, i.e. *Str. cremoris* 329 coming from Dairy Culture Collection in Olsztyn and *Leuc. cremoris* 1388 obtained from the National Collection of Dairy Organisms in Great Britain have been selected for the investigations. The cultures were transfered every 7-8 days into 10% reconstituted skim milk, incubated at 28°C and stored at 4°C.

Cultures: cultivations were carried out in 1000 ml of 10% reconstituted skim milk which was 3 times pasteurized at °C for 30 min at intervals of 24 hours. 5% inoculum coming from the early stationary growth phase of *Str. cremoris* 329 or *Leuc. cremoris* 1388 was used. The inocula used in order to obtain mixed populations were the mixtures of monocultures of the strains whose ratios were the following: S:L (v)v = 1:1, 1:5, 1:10. During the cultivation, the number of bacteria was determined by the plate method using differentiating medium after Reddy et al. [17], and the medium with calcium citrate: yeast extract — 0.5%, lactose — 1.0%, saccharose — 1.0%, liquid meat broth — 30%, enzymatic hydrolysate of casein — 1.0%, calcium citrate — 1.0%, agar — 1.5%. For

distinguishing component strains grown in mixed cultures the following scheme was applied:



Acid productivity: acid productivity was determined by titration every 2 hours of cultivation. Specific acidifying ability was estimated in the middle of the logarithmic growth phase of the cultures: for *Str. cremoris* 329 after 10 hr, and for *Leuc. cremoris* 1388 after 14 hr of cultivation. Index of specific acidifying activity was calculated from the formula:

$$\text{spec. acidifying activity} = \frac{\text{mM of lactic acid in 1 ml}}{\text{CFU in 1 ml}}$$

Aroma producing activity: acetoin and diacetyl contents were determined according to Brandl [2], whereas diacetyl production by means of distillation method [21]. Acetaldehyde content was evaluated using a combined method of distillation and paper chromatography. Acetaldehyde present in the distillate was bound in the reaction with 2,4-dinitrophenylhydrazine into non-volatile hydrazones which were seperated by means of paper chromatography. The paper was developed using the method of Lynne et al. [15]. After development the spots which had been identified as hydrazones of acetaldehyde, were eluted with ethanol and evaluated quantitatively at 358 nm. [15, 19, 20].

RESULTS AND DISCUSSION

On the basis of the investigations it has been found out that both selected strains had proper growth parameters in milk. The generation time of *Str. cremoris* 329 was 1.33 hr. After 4 hours cultivation the strain started its logarithmic growth phase which would end after

Table Growth parameters of *Str. cremoris* 329 and *Leuc. cremoris* 1388 grown in single and mixed populations

Strain	Parameter	Single populations	Mixed populations (S : L in inoculum) v/v		
			1 : 1	1 : 5	1 : 10
Streptococcus cremoris 329	T (hr)	1.33	1.65	1.46	1.38
	u_{max} (h^{-1})	0.52	0.42	0.47	0.50
	CFU/ml (stationary phase)	$6.5 \cdot 10^9$	5.5×10^9	4.6×10^9	4.3×10^9
Leuconostoc cremoris 1388	T /hr)	2.40	2.88	2.66	2.30
	u_{max} (h^{-1})	0.28	0.24	0.26	0.30
	CFU/ml	2.3×10^9	1.8×10^9	3.0×10^9	4.0×10^9

16 hr. In the stationary phase the number of cells was 6.5×10^9 CFU/ml. *Leuc. cremoris* 1388 had longer generation time — 2.40 hr, and its logarithmic phase used to begin after 5-6 hr cultivation and lasted for 19-22 hr. Maximum yield of *Leuc. cremoris* 1388 in milk was about 2.3×10^9 CFU/ml, and so it was almost 3 times poorer than that of *Str. cremoris* 329 (Table).

The control of acidifying activity of the strains showed that *Str. cremoris* 329 produced in the stationary phase about 1.00-1.15% of lactic

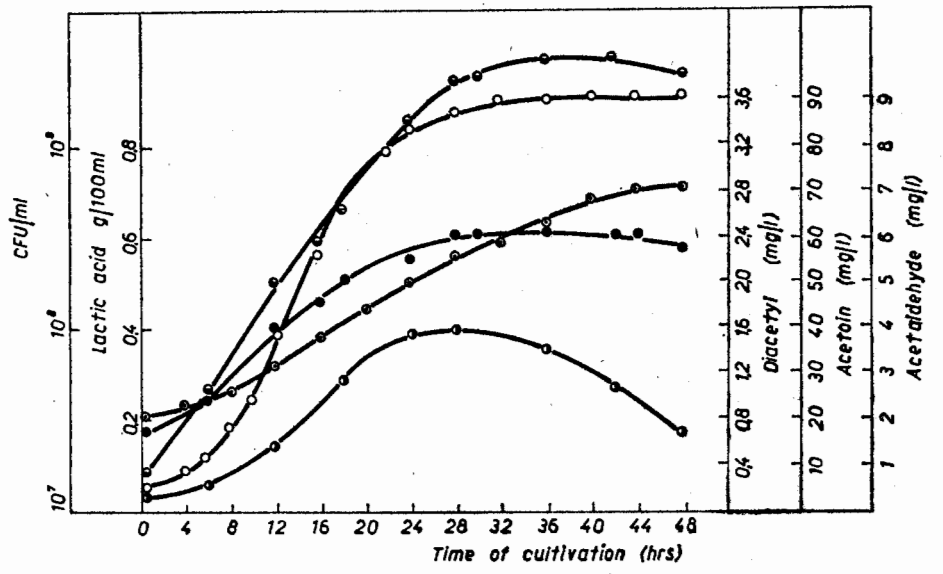


Fig. 1. Growth of *Leuconostoc cremoris* 1388 in milk. Effect of time of cultivation on viable count of cells (CFU/ml, ○) and on lactic acid (g/100 ml, ⊙), acetaldehyde (mg/l, ◑), diacetyl (mg/l, ●), acetoin (mg/l, ◒) producing ability of cells

acid. Under the same culturing conditions *Leuc. cremoris* 1388 accumulated markedly less lactic acid — about 0.6-0.7%. The obtained level of lactic acid production is typical [3].

Specific acidifying activity of *Str. cremoris* 329 was 1.51×10^{-10} and the value was almost twice as big as that of *Leuc. cremoris* 1388 (0.78×10^{-10} mM of lactic acid/CFU).

It has also been found that both of the examined strains were acetaldehyde producers. However, *Str. cremoris* 329 produced significantly higher amount of that compound. During the whole cultivation period (48 hr) the increasing production of acetaldehyde up to the level of 7.9 mg/l was observed. *Leuc. cremoris* 1388 produced the most acetaldehyde (about 4 mg/l) at the end of logarithmic phase. In the next incubation hours the content of that compound decreased markedly, reaching after 48 hr the level of 1.7 mg/l, i.e. more than two times lower than its maximum amount (Fig. 1 and 2).

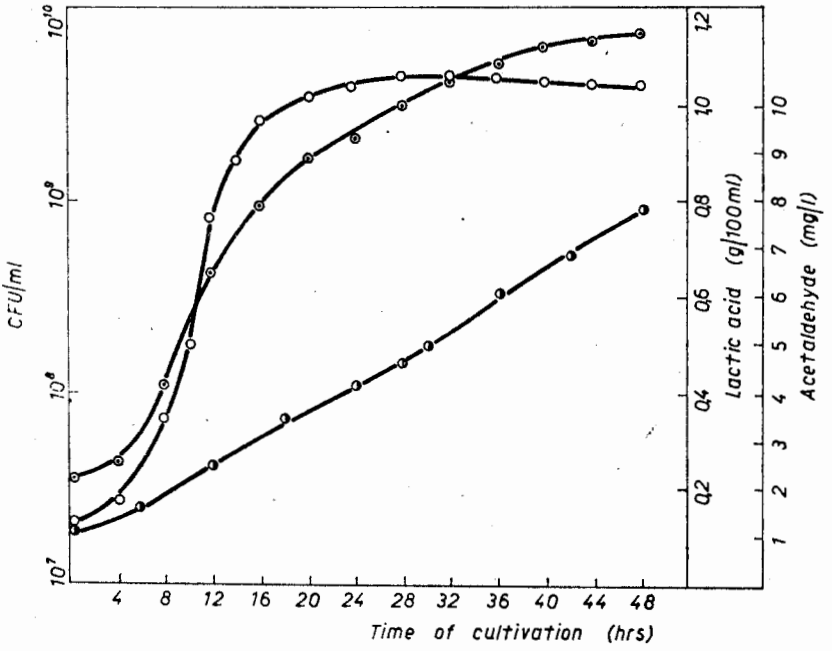


Fig. 2. Growth of *Streptococcus cremoris* 329 in milk. Effect of time of cultivation on viable count of cells (CFU/ml, ○) and on lactic acid (g/100 ml, ⊙), acetaldehyde (mg/l, ◑) producing ability of cells

Harvey [10] reports that among 7 examined *Str. cremoris* strains, most of them produced from 0.5 to 5.0 mg/l of acetaldehyde after 16 hr cultivation in milk. Only one strain *Str. cremoris* K, produced more aldehyde, i.e. from 5 to 9 mg/l. Similar results were obtained by Keenan et all [12] for two *Str. cremoris* strains. After 12-14 hr incubation they

produced 4.6-7.9 mg/l. Moreover, these authors observed that during the next hours of cultivation partial utilization of the accumulated acetaldehyde took place. On the other hand according to Cogan [3] not all *Leuconostoc cremoris* strains are able to produce acetaldehyde, e.g. *Leus. cremoris* CAF-1 did not develop detectable amounts of acetaldehyde, whereas *Leuc. cremoris* FR8-1 accumulated it during the whole cultivation time, reaching the yield of 6.5 mg/l after 60 hr.

Aroma compounds, i.e. acetoin and diacetyl were produced only by *Leuc. cremoris* 1388. The biggest amount of diacetyl — 2.46 mg/l was produced by the bacteria in the early stage of stationary phase (28 hr cultivation). The elongation of time up to 48 hr did not cause the decrease of diacetyl amount, and thus *Leuc. cremoris* 1388 did not exhibit the activity of reductase of diacetyl. This is in agreement with the results of Seitze et all [18], who reported the lack of activity of that enzyme for all 5 examined *Leuc. cremoris* strains. Production of acetoin was attributed to the stationary growth phase. The biggest amount of that compound (99.6 mg/l) was produced by *Leuc. cremoris* 1388 after 36-38 hr cultivation.

The recorded level of aroma compounds allowed to include *Leuc. cremoris* 1388 to strains possesing mean aroma-producing ability.

Most *Leuconostoc strains* have weaker aroma-producing activity than *Str. diacetilactis* bacteria, which accumulate from 1 to 10 mg of diacetyl per liter, and 230 to 500 mg/l of acetoin [4, 7, 12, 16, 18]. For paired cultures, similarly as in monocultures, the control of growth dynamics of the strains, as well as the control of milk acididying activity, aroma compounds production and accumulation of acetaldehyde was carried out. It has been found out that by introducing into milk ten times bigger volume of *Leuc. cremoris* 1388 culture than *Str. cremoris* 329, a mixed population is obtained with leveled off ratios of component strains.

In such populations 40% growth stimulation of *Leuc. cremoris* 1388 was observed, as well as shortening of generation time from 2.4 hr to 2.3 hr (Table 1). The increase of Str. cremoris 329 contribution brings about the growth of acidifying activity of the population from 0.87 do 1.05 g of lactic acid per 100 ml of milk, as well as the lowering of diacetyl production from 1.88 to 0.95 mg/l and acetoine production from 75.6 to 42.1 mg/l.

However, all the model starters had lower acidifying activity (from 9 to 24%) than strongly acidifying *Str. cremoris* 329 strain, but on the other hand, that activity was higher by 32 to 18% than for *Leuc. cremoris* 1388.

Acetoin and diacetyl production was lower by 23 to 60% in comparison with the level of those compounds produced by *Leuc. cremoris* 1388. Mixed populations, irrespective of their strain proportion, produced maximum amounts of diacetyl and acetoine in the early stationary phase

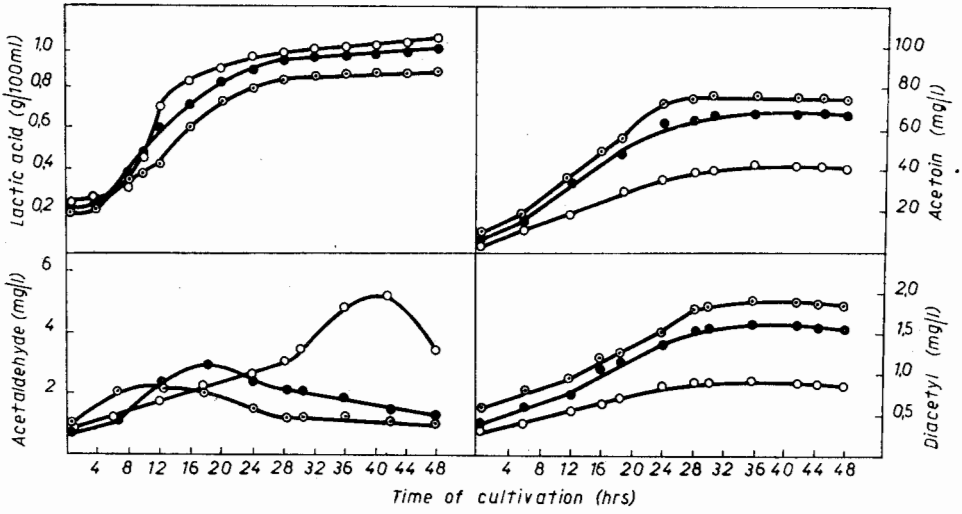


Fig. 3. Activity of mixed populations of *Streptococcus cremoris* 329 and *Leuconostoc cremoris* 1388. Effect of ratio of strains in inoculum (S:L — 1:1; ○; S:L — 1:5, ●; S:L — 1:10, ⊙) on lactic acid, acetaldehyde, acetoin and diacetyl production

(Fig. 3). Walsh and Cogan [20] found out that FR8 and 21 starters (type B) produced 85 mg/l of acetoin, i.e. the amount close to that obtained by us, but as they maintained it was much less than the production of BD and D type starters. The latter starters produced about 400-520 mg of acetoin per liter and about 12 mg/l of diacetyl.

As the contribution of *Leuc. cremoris* 1388 in mixed system grew bigger, the amount of acetaldehyde decreased and the maximum production of that compound was shifted to the earlier hoours of cultivation. In the population having the equalized amounts of component strains, the maximum level of acetaldehyde — 2.35 mg/l — was obtained in the logarithmic growth phase. Moreover, it has been shown that the elongation of cultivation lowered the amount of accumulated acetaldehyde by 30 to 50% (Fig. 3).

Lindsay et all [13] investigated the production of acetaldehyde and diacetyl by starters consisting of *Str. lactis* and *Leuc. citrovorum* (*cremoris*), and found out that after 12 hr cultivation they produced 0.8 mg of diacetyl and 3.4 mg of acetaldehyde per liter. Such a level of acetaldehyde and diacetyl resulted in a starter with "green" flavour. However, the additional 4 hours of cultivation significantly improved the starter flavour. During that period the activity of *Leuc. citrovorum* was strong enough to lower the acetaldehyde content to 0.38 mg/l and to increase the content of diacetyl up to 1.5 mg/l.

But, according to these authors, the starter containing the addition of *Str. diacetilactis* proved to be the best one, since it produced 0.66 mg of acetaldehyde and 2.16 mg of diacetyl per 1 liter. Its flavour, however,

grew much worse when *Leuc. citrovorum* contribution was decreased, for it brought about the increase of acetaldehyde content up to 6.72 mg/l and drop of diacetyl to 1.5 mg/l. Similar observations were made by Habaj et al. [9].

The ability of utilizing acetaldehyde by *Leuc. cremoris* strains found its application also in removing the excess of that component from ripe butter starters in order to improve their flavour [11].

In the present investigations a model starter, possessing the equalized amounts of component strains, produced about 1.9 mg of diacetyl and 1.2 mg of acetaldehyde per liter. Lindsay et all [14] stated that a good butter starter with distinctive flavour should contain about 3.0 mg of diacetyl per liter and the appropriate amount of acetaldehyde. Thus, in our investigations we have observed too low level of diacetyl at the proper level of aldehyde. But the addition of *Str. diacetilactis* into a model starter proposed by us, should bring about relatively high production of diacetyl and thus it should enable to obtain a starter with proper organoleptic characteristics.

CONCLUSIONS

1. By inoculating milk with 10 times bigger volume of *Leuc. cremoris* 1388 than *Str. cremoris* 329 it was possible to obtain mixed populations with the equalized ratios of component strains.
2. In such model starters 40% growth stimulation and the shortening of generation time of *Leuc. cremoris* 1388 were observed.
3. Acidifying activity of mixed populations was lower by 9 to 24% than of strongly acidifying *Str. cremoris* 329 strain, but at the same time it was higher by 18 to 32% than that of *Leuc. cremoris* 1388. Production of diacetyl and acetoin was lower by 27-62% and by 23-58% respectively in comparison with production of these compounds by *Leuc. cremoris* 1388.
4. In mixed populations *Leuc. cremoris* 1388 utilized the accumulated acetaldehyde lowering its level from 30 to 50%.
5. By introducing *Leuc. cremoris* 1388 into the composition of BD type starters in the way described in the paper, it may be possible to improve the quality of a product.

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CHARAKTERYSTYKA POPULACJI SKOJARZONYCH SZCZEPÓW MAŚLARSKICH *STREPTOCOCCUS CREMORIS* I *LEUCONOSTOC CREMORIS*

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Streszczenie

W hodowli w mleku scharakteryzowano monokultury *Str. cremoris* 329 i *Leuc. cremoris* 1388 oraz wzajemny wpływ tych szczepów na wzrost i aktywność metaboliczną w populacjach skojarzonych. Stwierdzono, że wprowadzenie do mleka 10-krotnie większej objętości hodowli *Leuc. cremoris* 1388 niż *Str. cremoris* 329 daje w efekcie populację skojarzoną o wyrównanych proporcjach szczepów składowych. W takich hodowlach obserwowano 40% stymulację wzrostu *Leuc. cremoris* 1388 oraz skrócenie jego okresu generacji. Zwiększenie udziału *Str. cremoris* 329 powoduje obniżenie produkcji dwuacetylu i acetoiny o 44-50% oraz zwiększenie aktywności kwaszącej o ok. 18%. Wykazano również, że *Leuc. cremoris* 1388 w populacjach skojarzonych wykorzystywał nagromadzony aldehyd octowy, obniżając jego poziom od 30 do 50%.