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Lactic acid bacteria during fish fermentation (rusip)

Abstract

Rusip is one of typical lactic acid fish fermented food originating from Bangka Belitung. To develop this product, the data of lactic acid bacteria involved during fermentation is necessary. This research was aimed at preliminary identifying the lactic acid bacteria at selected day of "rusip" fermentation. A number of 29 isolates were chosen to be isolated and identified. The results showed that the lactic acid bacteria involved at day 1–15 were *Streptococcus* in the beginning and *Lactococcus* in the middle of fermentation, while *Leuconostoc* were present along with fermentation.

Keywords: rusip, lactic acid bacteria, fermentation, lactococcus, raw materials

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Neti Yuliana, Dyah Koesoemawardani, Susilawaty, Yessy Kurniati
Program Studi Teknologi Industri Pertanian, Fakultas Pertanian Universitas Lampung, Indonesia

Correspondence: Neti Yuliana, Program Studi Teknologi Industri Pertanian, Fakultas Pertanian Universitas Lampung, Jalan Sumantri Brojonegoro no1 Gedung Meneng Bandar lampung, Indonesia, Email neti.yuliana@fp.unila.ac.id

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Abbreviations: LAB, lactic acid bacteria; KB, spherical colonies; KM, widened colonies; KP, long colonies; KBS, large colonies

Introduction

Rusip is a traditional fermented fish products known from Bangka Belitung, and found in Lampung and West Kalimantan. Types of fish used as raw materials in the manufacture of rusip are generally small fish such as anchovy or bilis fish. Rusip fermentation is usually a spontaneous fermentation process involving lactic acid bacteria (LAB) with palm sugar as a source of carbohydrates.[1]

Genus of lactic acid bacteria involved in a food fermentation may vary depend on region, type of substrate and or fermentation stages. The lactic acid bacteria encountered in the end product of rusip with the addition of salt and roasted rice are Streptococcus and Lactobacillus, whereas in rusip with the addition of salt and brown sugar were Streptococcus and Leuconostoc.[2] These lactic acid bacteria were found in the final product of rusip originating from manufacturers in Bangka. Meanwhile, Kusmarwati et al.[3] found Pediococcus as bacteriocin producing lactic acid bacteria from commercial rusip in Bangka and West Kalimantan. The lactic acid bacteria profiles, however, that play a role during the fermentation stage are unknown. Thus, this study was aimed at identifying lactic acid bacteria of rusip at different fermentation stages. Data on the types of lactic acid bacteria that play a role during fermentation are needed to develop further product of rusip such as to improve the quality of the preparation of rusip with selected LAB starter.

Materials and methods

Material

Anchovy obtained from fish auction place in Lempasing, Salt and palm sugar obtained from Pasar Gintung Bandar Lampung. The chemicals used are aquades, phenolphthalein indicator, 0.1% peptone, 0.1 N NaOH, phosphate buffer, MRS De Man, Rogosa and Sharpe (MRS) agar, physiological salts, violet crystals, iodized salts, safranin, 95% alcohol, and 3% H_2O_2.

Fermentation of Rusip

Firstly, the anchovy (*Stolephorus* sp) was washed and drained. Salt was added to the drained anchovy as much as 25% (w/w) of the fish weight and was stirred until blended. After that, sugar palm was added as much as 10% (w/w) of the fish weight, and was then stirred until blended. These fish were put in a cleaned plastic jar, was closed tightly and incubated at room temperature to allow fermentation process.[4] The samples were withdrawn at certain period of time according to the research stages (3,5,10 and 15 fermentation days).

Evaluation of lactic acid bacteria (LAB)

Evaluation of LAB was done by identifying selected colonies. After grouping by shape and size, each isolate was purified on the MRS medium for a recurrent casting method using a 0.1% peptone diluents solution until a uniform colony was obtained based on the bacterial cell shape and colony color. The uniform colony was then purified over and over until a single colony was obtained for later identification. Identification was performed in two stages, first initial identification consisting of Gram staining, catalase test, spore test, and total lactic acid bacteria count. The next step after initial identification was perform biochemical tests on selected colonies to determine the genus of LAB following the Harrigan[5] procedure which included the production of CO_2 from glucose, the production of ammonia from arginine, and the production of dextran from sucrose and growth at a temperature of 10°C±2 (Table 1).

Table 1 Biochemical test results of lactic acid bacteria

	Lactococcus	*Sreptococcus*	*Pediococcus*	*Leuconostoc*
Gram	+	+	+	+
Catalase	-	-	-	-
Spore	-	-	-	-
Shape	Coccus	Coccus	Tetracoccus	Coccus

Table continued..

	Lactococcus	*Sreptococcus*	*Pediococcus*	*Leuconostoc*
Growth at 10°C	+	-	D	+
Growth at 45°C	-	D	D	-
CO_2 production from glucose	-	-	+	+
Growth at salt media (6,5%)	+	+	D	D
Production of ammonia from arginine	-	+	-	-
Dekstran production from sucorse	-	-	-	+

Results

Isolation of lactic acid bacteria

During 5 (five) sampling periods (0,3,5,10 and 15 day), there were 29 isolates found. Based on the shape of colonies, these isolates consisted of: spherical colonies (KB), widened colonies (KM), and long colonies (KP), whereas based on colony size, these consisted of: small colonies (KK), medium colonies (KSD) and large colonies (KBS).

Initial identification

Preliminary tests showed that all of 29 isolates were Gram-positive, coccus with single and chain formation, and did not produce spores. The isolates were negative catalase indicated by the absence of air bubbles after culture testing with H_2O_2.

Biochemical test

The results of testing the biochemical properties to determine the genus of lactic acid bacteria of 29 isolates can be seen in Table 2 and the spread of LAB at each fermentation periode is presented in Table 3 while the determination scheme can be seen in Figure 1.

Table 2 Biochemical Test

Testing						
No	**Code of Isolate**	**Production of CO_2 from Gibson media**	**Production of dexstran**	**Ammonia production from arginine**	**Growth at 10°C±2**	**Possibility of LAB genus.**
1	$KM_1$0	+	+	+		*Leuconostoc*
2	KBS2	+	+	+		*Leuconostoc*
3	KSD2	+	+	+		*Leuconostoc*
4	$KK_1$0	+	+	+		*Leuconostoc*
5	$KK_1$15	+	+	+		*Leuconostoc*
6	$KSD_2$15	+	+	+		*Leuconostoc*
7	KK3	+	+	+		*Leuconostoc*
8	KM5	+	+	+		*Leuconostoc*
9	KM10	+	+	+		*Leuconostoc*
10	$KSD_1$15	+	+	+		*Leuconostoc*
11	$KB_1$0	-	-	+		*Streptococcus*
12	$KM_2$0	-	-	+		*Streptococcus*
13	$KB_3$0	-	-	+		*Streptococcus*
14	$KP_2$0	-	-	+		*Streptococcus*
15	$KP_3$0	-	-	+		*Streptococcus*
16	$KM_3$0	-	-	+		*Streptococcus*
17	$KK_1$3	-	-	+		*Streptococcus*
18	$KK_2$3	-	-	+		*Streptococcus*

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Table continued..

Testing No	Code of Isolate	Production of CO_2 from Gibson media	Production of dexstran	Ammonia production from arginine	Growth at 10°C±2	Possibility of LAB genus.
20	KB5	-	-	+		*Streptococcus*
21	$KP_2$5	-	-	+		*Streptococcus*
22	$KK_3$15	-	-	+		*Streptococcus*
23	KK0	-	-	-	+	*Lactococcus*
24	$KK_2$0	-	-	-	+	*Lactococcus*
25	KSD5	-	-	-	+	*Lactococcus*
26	$KP_1$5	-	-	-	+	*Lactococcus*
27	$KSD_1$5	-	-	-	+	*Lactococcus*
28	$KSD_1$0	-	-	-	+	*Lactococcus*
29	$KK_2$15	-	-	-	+	*Lactococcus*

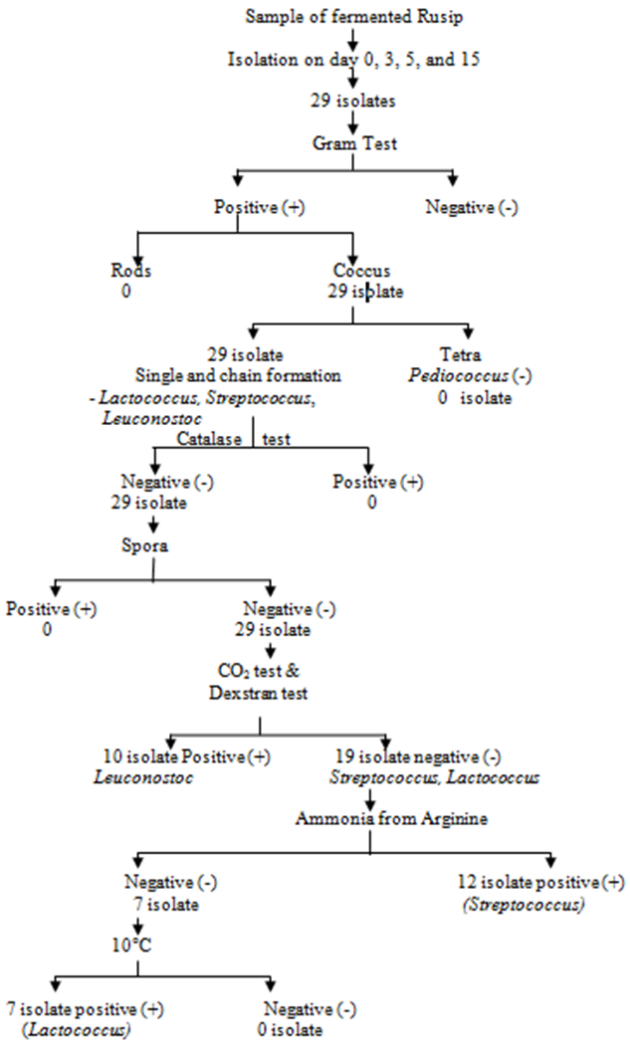

Figure 1 Identification scheme of lactic acid bacteria on fish fermentation (Rusip).

Table 3 Total isolates of fermented rusip

Day of fermentation	Genus of lactic acid bacteria	Total isolates
0	6 Isolate of *Streptococcus* 1 Isolate of *Leuconostoc* 2 Isolate of *Lactococcus*	9
3	3 Isolate of *Streptococcus* 2 Isolate of *Leuconostoc*	5
5	2 Isolate of *Streptococcus* 2 Isolate of *Leuconostoc* 3 Isolate of *Lactococcus*	7
10	2 Isolate of *Leuconostoc* 1 Isolate of *Lactococcus*	3
15	1 Isolate of *Streptococcus* 3 Isolate of *Leuconostoc* 1 Isolate of *Lactococcus*	5

Discussion

Preliminary tests of 29 isolates showed that all isolates were coccus with single or chain formation, therefore, the biochemical tests were focused to the assessment of the genus with coccus-shaped: Streptoccous, Lactococcus and Leuconostoc except Pediococcus which had morphologically tetrad-shaped.

Testing of CO_2 from glucose production on Gibsont Semi Solid media on 29 isolates showed 10 isolates producing CO_2 as indicated by the breakup of Gibsont Semi Solid media. Isolates that showed positive results were KM10, KBS2, KSD2, KK10, KK115, KSD215, KK3, KM5, KM10, and KSD115 which could be classified as lactic acid bacteria of Leuconostoc genus. According to Harrigan,[5] lactic acid bacteria that produce CO_2 on Gibson Semi Solid media are only Leuconostoc. In addition, this test showed that Leuconostoc was belonged to hetero fermentative lactic acid bacteria as it produced CO_2. The discovery of Leuconostoc on fish fermentation (rusip) at the beginning to the end of fermentation indicated that this bacterium was the dominant bacteria during rusip fermentation. Dessi[2] also found this bacteria in fermented rusip using brown sugar as a carbon source. The presence of these bacteria in rusip containing 10% palm sugar is not surprising because Leuconostoc is a lactic acid bacteria that is known as osmofilic bacteria, a group of bacteria that grow on high sugar content[6]. In addition, several studies have shown that Leuconostoc is a genus of lactic acid bacteria that play important role in the fermentation of foodstuffs. Many outhors reported that the Leuconostoc sp is a LAB genus that plays a role in several fermented foods such as fermented pickles (sauerkraut) and idli batter[7] fermented cassava,[8] and fermented fruit.[9] Leuconostoc is also a LAB reportedly present in fermented fish originally from Indonesia.[10]

To confirm that the isolates of KM10, KBS2, KSD2, KK10, KK115, KSD215, KK3, KM5, KM10 and KSD115, were Leuconostoc, the dextran forming test was performed because this test was specific only to Leuconostoc. The positive dextran formation test is characterized by the formation of mucoid colonies on the medium of Sucrose.[5] The test showed that the isolates of KM10, KBS2, KSD2, KK10, KK115, KSD215, KK3, KM5, KM10, and KSD115 showed positive results, thus it can be ascertained that the isolates were Leuconostoc. Holt et al.[11] stated that the genus of Leuconostoc are Gram positive, negative catalase, spherical in pairs or chain, not spore, not motile, and anaerobic facultative. Leuconostoc ferment carbohydrates with metabolic results in the form of lactic acid, acetic acid, ethanol, CO_2 and mannitol. The growth is rather slow and forms small colonies, and in sucrose-containing media forms mucoid colonies and does not hydrolyze arginine.[5]

Isolates that had negative results on both the CO_2 formation test and dextran formation were subjected to the ammonia formation test of the Arginine Broth Streptococci medium. Ammonia production test from Arginine showed that the 12 isolates gave positive result that were KB10, KM20, KB30, KP20, KP30, KM30, KK13, KK23, KSD3, KB5, KP25, KK315. Positive results were demonstrated by the formation of an orange ring after the Nessler Reagent was added to the Arginine Broth Streptococci medium.[5] This medium is specific only to Streptococcus, so it can be assumed that the isolates of KB10, KM20, KB30, KP20, KP30, KM30, KK13, KK23, KSD3, KB5, KP25, and KK315 were Streptococcus. According to Holt et al.,[11] the lactic acid bacterium of the genus Streptococcus is Gram positive, negative catalase, does not form spores and is anaerobic facultative, can grow at 25–45°C but the optimal temperature for its growth is 37°C. The presence of Streptococcus in fish and fermentation is also reported by Rahayu,[10] a LAB that dominates on peda, pindang, terasi, and wadi.

Observation of LAB from the fermentation time appeared that Streptococcus dominated in the early days of fermentation (on days 0 and days 3) and decreases with fermentation time. At the beginning of Streptococcus fermentation grows rapidly, the growth of these bacteria continues to dominate until mid-stage fermentation. In this study, the pH of fish in the early days was close to neutral (6 to 7). This pH value was favourable for the growth of Streptococus.[12,13] Presence of Streptococcus in the early stages of the fermentation was also reported in plaa-som process, a traditional fermented fish product of Thailand[14] as well as in "burong bangus,[15] a fermented fishery product from Philippine,".

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Isolates that showed negative results on the Arginin Broth Streptococci medium (KK0, KK20, KSD5, KP15, KSD15, KSD10 and KK215) were then tested for thier growth on MRS broth at 10°C. This test to support whether the isolates showed negative results on agar medium, Semi Solid Gibson media and Arginin Broth Streptococci Media. According to Harrigan,[5] Lactococcus can grow at 10°C, so only this test can be used as a reference to determine whether the isolates are Lactococcus or not. Lactococcus lactic acid bacteria are Gram positive, negative catalase, not spores, optimum temperature of 30°C, can grow at 10°C, fermenting carbohydrates with lactic acid end products.[11] The test results show that the possible isolates of KK0, KK20, KSD5, KP15, KSD15, KSD10, and KK215 are Lactococcus. Based on the spread of these bacteria, it appears that Lactococcus was dominant genus in mid-fermented rusip. Presence of this bacteria in rusip was suprising as Lactococcus was not found in other fermented fish products such as pekasam, peda, pindang, as well as other fermented fish, such as shrimp paste and wadi as reported by Rahayu[10]. This may be due to a unique mixed media medium of rusip fermentation that used palm sugar as a carbon source instead of rice or even without carbohydrate sources addition as in other fish fermentation. In addition, Lactococcus was mostly isolated from cane juice[16], dairy products[17] as well as vegetables and fermented fruits.[18,19]

During fermentation, the lactic acid bacteria use palm sugar as a source of energy with the result of lactic acid. This sugar metabolism results in an increase in total acid and a decrease in pH, thus supporting the growth of lactic acid bacteria that are more acid-resistant such as Lactococcus and Leuconostoc. The pH profile of fermented rusip showed that pH change began to occur on the 3rd day of fermentation (pH 6.3) and was highly significant at day 10 of fermentation (pH 5.9) and 15 (pH 5.5). This finding is in line with the results reported by previous study.[20,21] However, The pH of rusip obtained in this study was higher than previous work reported.[22]

Conclusion

Isolation of lactic acid bacteria during 15 days rusip fermentation obtained 29 isolates with characteristics of coccus, Gram positive (+), negative catalase (-), and negative spores (-). These 29 isolates of lactic acid bacteria consisted of 10 isolates of Leuconostoc, 12 isolates of Streptococcus, and 7 isolates of Lactococcus. Thus, it can be concluded that the lactic acid bacteria contributing during rusip fermentation (rusip) was Leuconostoc, Streptococcus, and Lactococcus. Based on the fermentation periods, presence of these bacteria was vary. The genus Streptococcus was more common in early fermentation, whereas the genus of Lactococcus was more common in mid-fermentation and at the end of fermentation, the most dominant was Leuconostoc.

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Conflict of interest

The authors declared that no conflict of interest exists.

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