Enumeration and Identification of Dominant Lactic Acid Bacteria in Indonesian "*Tempoyak*" During Low Temperature Fermentation

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ABSTRACT

Tempoyak is an Indonesian indigenous fermented food made from durian *(Durio zibethinus Murray)* flesh and produced by traditional methods through spontaneous fermentation for 4 weeks. The spontaneous fermentation was controlled by adding salt. The aim of this research was to enumerate and to identify dominant lactic acid bacteria in *tempoyak* fermented at 20±2°C with salt addition of 2 and 4%. The population of lactic acid bacteria increased during the first week; however, bacterial count decreased after the first week untui the end of the fermentation process. A number of 141 isolates was obtained and consisted of 100 (71%) and 41 isolates (29%) belonging to homofermentative and heterofermentative groups of lactic acid bacteria, respectively. *Furthermore,* six genera were involved in *tempoyak* fermentation, including Oenococcus, Leuconostoc, Enterococcus, Lactococcus, Pediococcus and Lactobacillus. *Leuconostoc* sp. was observed at the beginning of fermentation, whereas *Lactococcus* sp, and *Lactobacillus* sp. dominated the population in the middle of the fermentation. At the end of the fermentation, *Lactobacillus* sp. and *Pediococcus* sp. played dominant roles.

Key words : lactic acid bacteria, *tempoyak*, fermentation, at 20±2°C

1. Introduction

Indonesia has a long history in production of indigenous fermented foods. There are a lot of different kinds of fermented foods produced traditionally such as *tempe*, *tape*, soy sauce and *tempoyak*. Those fermented foods are usually produced by traditional methods using spontaneous fermentation without addition any microbial starter culture. However, the spontaneous fermentation is controlled by salt addition.

Tempoyak is one of the Indonesian fermented foods made from *durian* (*Durio zibethinus* Murray) flesh and characterized with the following properties: yellow-white color, pasta-like texture, creamy and specific aroma. Originally, the fermentation of durian flesh into *tempoyak* was intended to lengthen food shelf life as well as to diversify the food choice. Fresh durian can be stored for 4 to 6 days in room temperature (28 – 30°C). Nowadays, there are increasing interests to explore the functionality of fermented foods. Fermentation of foods is also important to improve the safety and quality of foods, to serve probiotic and functional foods.

Tempoyak is prepared by adding salt (2 – 15%) [1] to fresh durian flesh, placed in a jar and then mixed thoroughly. The jar is then closed tightly and incubated for 3 – 5 days at the room temperature (28 – 30°C). During spontaneous fermentation organic acids are produced as metabolic products of microbial growth. Salt addition is very critical in selecting the kinds of microorganism growing during the spontaneous fermentation. Lactic acid bacteria were usually predominant bacteria which grow in carbohydrate-based fermentations.

Generally, species of *Lactobacillus* sp., *Leuconostoc* sp., *Pediococcus* sp., and *Streptococcus* sp. grow in *tempoyak* [1]. Species of *Lactobacillus plantarum, Lab. brevis, Lab. mali, Lab. fermentum* were isolated from Malaysian *tempoyak* [2], whereas *Lab. plantarum, Lab. casei, Lab. corynebacterium* are species of lactic acid bacteria isolated from Indonesian *tempoyak* [3]. Other species such as *Leuconostoc durianis* (from Malaysian *tempoyak*) [4], *Leuconostoc mesenteroides, Pediococcus acidilactici, Weissella mesenteroides* [5] were also isolated from *tempoyak*.

Generally, spontaneous fermentation allowed the growth of most lactic acid bacteria species due to salt addition and temperature of fermentation. According to Steinkraus (1996) [6], fermentation occurred slowly at low temperature (±7.5°C). On the other hand, an optimal fermentation process would be achieved if the temperature is kept at 18°C. At this temperature *Lactobacillus brevis* and *Lactobacillus plantarum* grew well. The fermentation would be faster at higher temperature (32°C) and homofermentative lactic acid bacteria would predominant the bacterial population in such conditions.

In this experiment, fermentation of *tempoyak* was carried out at 20±2°C. This temperature will reduce the risk of contamination during fermentation. The species *Lactobacillus brevis* and *Lactobacillus plantarum*, both are obligate heterofermentative and facultative heterofermentative, respectively [7], could grow well at this temperature [6]. In addition to organic acids, heterofermentative lactic acid bacteria produced ethanol, acetic acid, propionic acid, hydrogen peroxide, carbon dioxide and bacteriocin. Those metabolites are responsible for preservation and sensory properties. Moreover, *Lactobacillus plantarum* is a potential species known for its antimicrobial activity against pathogenic bacteria [8-10] and probiotic properties [11,12].

The purpose of the salt addition to the fermentation process was to control the microbial growth during the spontaneous fermentation. When the concentration of salt was low, the variety the growing bacteria would vary in response to the reduced metabolic stress. Heterofermentative lactic acid bacteria dominated the microbial population. However, homofermentative lactic acid bacteria would be dominant when the salt concentration was high [6]. The aim of this experiment was to enumerate and identify the dominant lactic acid bacteria in Indonesian *tempoyak* during fermentation at 20±2°C with salt 2 and 4 % salt added.

2. Materials and Methods

2.1 *Tempoyak* preparation

The durian fruit used in this experiment was a local cultivar and obtained from The Tebing Tinggi district, South Sumatera Province. *Tempoyak* was prepared by a traditional method of fermentation. The Durian flesh was mixed with different levels of refined salt (2 and 4% w/w) and was placed in screw capped glass jars. The mixture was incubated at low temperature (20 ± 2 °C) for four weeks. This experiment was replicated five times. The sampling was carried out every week (0, 1, 2, 3, 4) by observing pH values, total acidity content, total lactic acid bacteria count, isolation and identification of the dominant lactic acid bacteria.

2.2. Enumeration of total lactic acid bacteria

Numbers of lactic acid bacteria (LAB) were determinated by MRS-1% $CaCO_3$ agar media. Five grams of the samples (*tempoyak*) suspended in 45 ml of sterile distilled water and then subjected to

serial dilutions. Appropriate dilutions were platted on MRS agar and incubated at 37°C for 24 – 48 hours.

2.3. Isolation and identification of dominant lactic acid bacteria

Six colonies which were recognized as lactic acid bacteria (LAB) (colonies with clear zones) were picked from the plates randomly. The samples were purified by streaking onto MRS agar and observed under the microscope for their purities. They were then stored in slice agar of MRS as well as in cryo vials containing 20% glycerol and stored in the freezer at -10°C. Preliminary tests, including cell morphology, Gram staining, catalase test and lactic acid production were carried out to confirm them as LAB. Isolates of LAB were further analyzed for their following functional properties, including cell configuration, ability to produce CO_2 from glucose and to grow in 10 and 45°C, pH 4.4 and 6.5, and NaCl 6.5% and 18%. The data were then used to determine their genera.

2.4. pH values

The pH measurement was done by using a pH meter (Eutech, Singapore) calibrated with standard buffers of pH 4 and 7.

2.5. Analysis of total acid content

Total acid content of *tempoyak* was measured by titration methods and calculated as % lactic acid ($CH_3CH(OH)COOH$) (AOAC, 2005). For this, the *tempoyak* (5 g) was suspended in distilled water up to 100 ml. Ten ml of the suspension were pipetted into an Erlenmeyer flask. Two-three drops of 1% phenolphthalein indicator were added to the suspension. The sample was titrated with basic standard solution (0.1 N NaOH) until its color turned pink.

3. Results

3.1. Population of lactic acid bacteria

The population of lactic acid bacteria (LAB) in *tempoyak* during fermentation at 20±2°C by addition of 2 and 4 % of salt measured every week for four weeks. The changes in lactic acid bacteria population during spontaneous fermentation of *tempoyak* is shown in Fig. 1. The population of LAB in both of *tempoyak* (2 and 4% salt) increased on the first week with a significant decrease during the later stages of fermentation until the 4^{th} week. The changes of pH during the *tempoyak* fermentation were shown in Fig. 2. LAB growing in *tempoyak* was able to ferment simple sugars of the durian flesh and converted them into lactic acid and acetic acid. Total acid content is also shown (Fig. 3).

3.2. Isolation and identification of dominant lactic acid bacteria

It has been collected 141 isolates consisting of 73 isolates (tempoyak with 2% salt) and 68 isolates (tempoyak with 4 % of salt). A number of 100 isolates belonged to homofermentative lactic acid bacteria (71%) and 41 isolates to heterofermentative lactic acid bacteria (29%). Further identification showed that the isolates could be grouped into these following genera: *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, *Enterococcus* and *Oenococcus* (Table 1). Profile of lactic acid bacteria isolated from *tempoyak* fermentation at 20±2°C can be shown in Table 2.

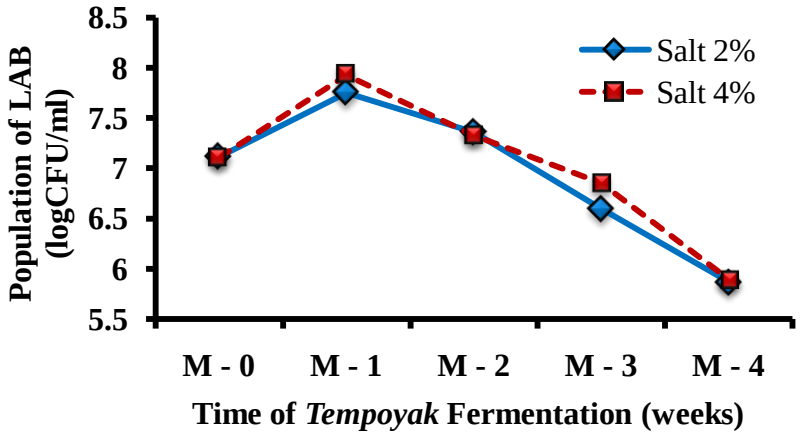

Fig 1. Colony count of lactic acid bacteria during the fermentation of *tempoyak* at 20±2°C

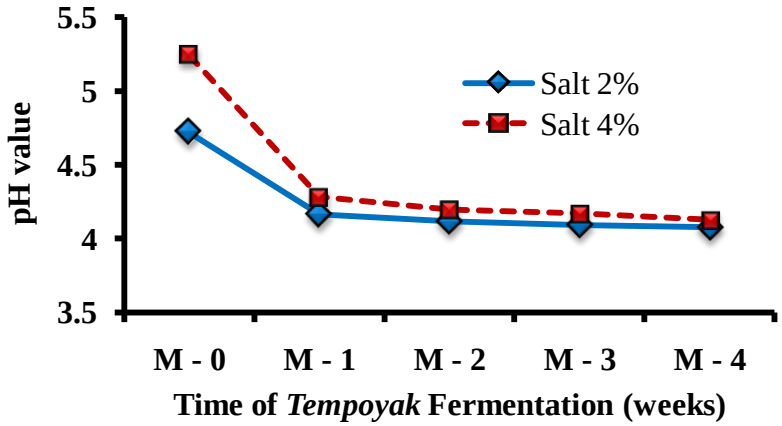

Fig 2. Changes of the pH during the fermentation of *tempoyak* at 20±2°C

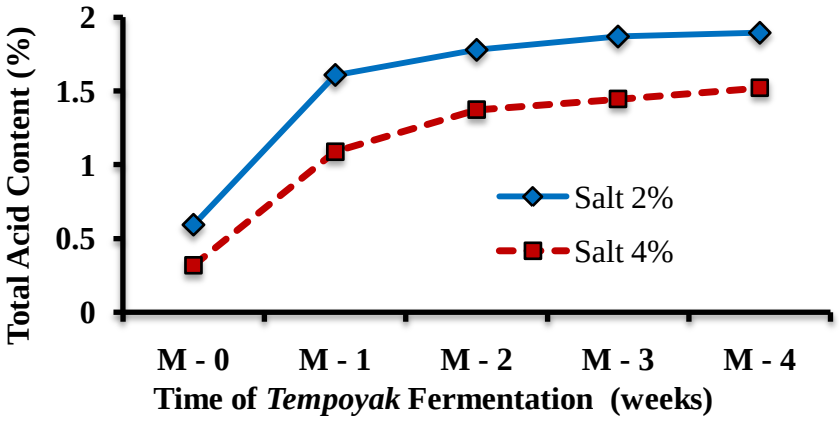

Fig 3. Changes in total acid content during the fermentation of *tempoyak* at 20±2°C

Table 1. Phenotypical characteristics of LAB isolated from *tempoyak* fermentation at 20±2°C

Characteristics		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
		hetero		homo		homo	hetero	homo	
Gram		+	+	+	+	+	+	+	+
Catalase		-	-	-	-	-	-	-	-
Cell Morphology		coccus	coccus	coccus	coccus	coccus	rods	rods	rods
Cell Configuration		1,2, chains	1,2, chains	1,2, chains	1,2, chains	tertads	1,2, chains	1,2, chains	1,2, chains
Acid from glucose		+	+	+	+	+	+	+	+
Gas from glucose		+	+	-	-	-	+	-	-
Tempera ture of growth	10°C	+	+	+	+	+	+	+	+
	30°C	+	+	+	+	+	+	+	+
	45°C	+	-	+	-	-	-	+	-
pH of growth	4.4	+	+	+	+	+	+	+	+
	6.5	+	+	+	+	+	+	+	+
NaCl of growth	6.5 %	+	+	+	+	+	+	+	+
	18 %	-	-	-	-	-	-	-	-
Number of isolate		5	25	10	34	5	11	19	32

SUSPECTED SPECIES :

1. *Oenococcus* sp.
2. *Leuconostoc* sp.
3. *Enterococcus* sp.
4. *Lactococcus* sp
5. *Pediococcus* sp.
6. *Lactobacillus* sp. (heterof.)
7. *Lactobacillus* sp. (homof.)
8. *Lactobacillus* sp. (homof.)

Table 2. Profile of lactic acid bacteria isolated from *tempoyak* fermentation at 20±2°C

Time of fermentation	2 % of salt		4 % of salt	
	Spesies BAL	%	Spesies BAL	%
Week 0	*Leuconostoc* sp. *Lactococcus* sp.	33,4 % 25.0 %	*Leuconostoc* sp. *Lactococcus* sp.	47,4 % 31,6 %
Week 1	*Lactococcus* sp. *Lactobacillus* sp. (heterof.)	25,0 % 25,0 %	*Lactococcus* sp. *Leuconostoc* sp.	55,6 % 44,4 %
Week 2	*Lactococcus* sp. *Lactobacillus* sp.1 (homof)	33,3 % 33,3 %	*Lactococcus* sp. *Enterococcus* sp	50,0 % 25,0 %
Week 3	*Lactobacillus* sp.2 (homof) *Lactobacillus* sp.1 (homof)	30,8 % 30,8 %	*Lactobacillus* sp.2 (homof) *Lactobacillus* sp.1 (homof)	45,2 % 15,4 %
Week 4	*Lactobacillus* sp.1 (homof) *Lactobacillus* sp.2 (homof)	47,3 % 26,2 %	*Lactobacillus* sp.1 (homof) *Pediococcus* sp.	40,0 % 25,0 %

4. DISCUSSION

4.1. Population of lactic acid bacteria during the fermentation of *tempoyak.*

An increase of the lactic acid bacteria (LAB) population was observed during the first of the fermentation of *tempoyak*. Afterwards, the population decreased. The salt levels showed no significant influences on the bacterial growth. Changes in the bacterial population seemed to be affected only by the pH changes in a range where no LAB could tolerate low pH.

On the other hand, the total acid content increased due to accumulation of lactic acid produced by LAB during fermentation. Homofermentative lactic acid bacteria appeared as dominant species at the end phase of the *tempoyak* fermentation (Fig. 4) and produced mainly lactic acid (85%) with minor amounts of other organic acid. Therefore, the total acid content during the *tempoyak* fermentation increased until the end of the experiment.

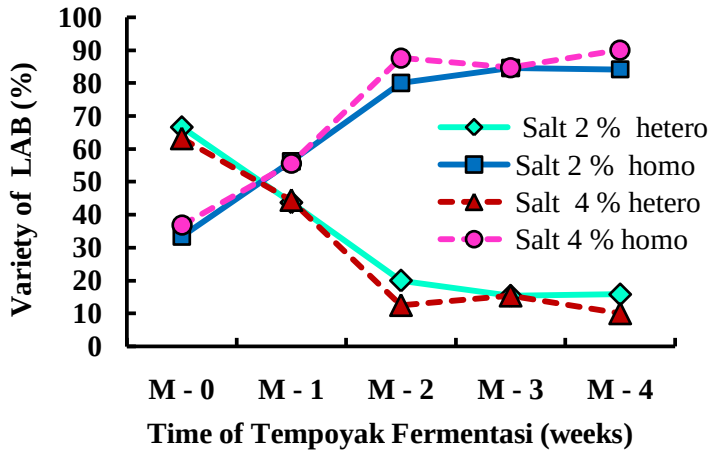

Fig 4. Percentage of heterofermentative and homofermentative lactic acid bacteria during the *tempoyak* fermentation at 20±2°C

4.2. Profile of lactic acid bacteria during the fermentation *Tempoyak*

The genus *Oenococcus* belongs to heterofermentative cocci of the lactic acid bacteria (LAB) and has similar characteristics to the *Leuconostoc* and *Weisella* genera. Nevertheless, *Oenococcus* genus has the ability to grow at higher temperatures (45°C) while the others failed [13]. In addition *Weissella* has specific morphological characteristics, namely the coccoid form [13, 14]. In general, *Oenococcus* sp. was characterized as Gram-positive, catalase negative, heterofermentative, cocci, with growth at 10 and 45°C and tolerant to 6.5 % NaCl. Thus phenotypically, there were 5 isolates belonging to *Oenococcus* during the fermentation of *tempoyak* at 20±2°C.

The genus *Oenococcus* can be identified only at the beginning of fermentation. The natural habitat of *Oenococcus* is in fruit and fruit pulp, such as grapes [15]. Moreover, *Oenococcus* has ability to metabolize malic acid found in grapes to form lactic acid through malolactic fermentation. In general, *Oenococcus* are facultative anaerobic, relatively tolerant to alcohol and are very important in wine making [16, 17]. Endo and Okada (2006) [18] isolated LAB samples having non-acidophilic properties, i.e. *Oenococcus kitaharae* sp.nov. Nevertheless, the occurrence of *Oenococcus* sp. in the

tempoyak fermentation was possibly related to the natural microflora in the durian flesh that would be fermented since durian flesh contains alcohol between 0.37% and 0.73% [19]. In addition, Yuliani (2005) [20] reported that there are high concentrations of malic acid, beside lactic acid and acetic acid in the *tempoyak.*

Isolates having the following characteristics: Gram-positive, heterofermentative, cocci, catalase negative, no growth at 45°C, producing D-lactate and tolerant to 6.5% NaCl could be classified as *Leuconostoc* sp. [13, 14, 21]. *Leuconostoc mesenteroides* could ferment various sugars, including glucose, galactose and lactose [22, 23]. Twenty-five of the isolates meet the above cited characteristics were classified as *Leuconostoc* sp.

Homofermentative, cocci LAB were referred to the genus *Lactococcus*, *Streptococcus* or *Enterococcus*. Furthermore, LAB growing at temperatures of 10 and 45°C, were identified as *Enterococcus* sp., whereas LAB growing at 10°C but no growth occurred at 45°C, were belonging to *Lactococcus* sp. On the other hand, LAB growing at 45°C were belonging to *Streptococcus* sp. [13, 14]. There were 10 and 34 isolates classified as *Enterococcus* sp. and *Lactococcus* sp., respectively.

Cocci-homofermentative LAB with tetrad configuration was classified as *Pediococcus* sp. There were 5 LAB isolates from the *tempoyak* fermentation belonging to *Pediococcus* sp. due to their ability to form tetrad configuration. Moreover, the 5 isolates could not grow at 45°C.

Rod-shaped LAB generally belonged to the genus *Lactobacillus*, which could be divided into 2 groups, namely heterofermentative and homofermentative groups. In this study, there were 11 isolates heterofermentative *Lactobacillus* isolate was not able to grow at 45°C. Homofermentative *Lactobacillus* bacteria are able to grow at 45°C which was positively checked for 19 isolates, while 32 isolates were belonging to the homofermentative *Lactobacillus* group not able to grow at 45°C.

At the beginning of the *tempoyak* fermentation, (week 0), the growth of LAB was dominated by heterofermentative *Leuconostoc* sp. There is no difference in dominant bacteria between *tempoyak* with the addition 2 and 4% of salt. *Leuconostoc* sp., particularly *Leuconostoc mesenteroides*, was a non-acidophilic lactic acid bacterium. According to Holzapfel (2002) [24], *Leuconostoc mesenteroides* usually dominates the early stages of the most spontaneous fermentations and is commonly associated with fermented plant food product [25]. The pH value of *tempoyak* at that time was still above 5 and it is known that this species has a wider temperature and salinity range in which it is able to grow compare to other LAB.

The observation of the *tempoyak* fermentation with 2 % salt addition at week 1 showed that the dominant species were *Lactococcus* sp. and heterofermentative *Lactobacillus* sp. This *Lactobacillus* sp. was not able to grow at 45°C. According to Nair and Surendran (2005) [26], Ali (2011) [27], this lactobacillus group was indicated as *Lactobacillus brevis*. Significant changes of the dominant LAB growing after one week fermentation were observed. It was due to the reduced pH (pH <4.5) that affected the s growth of *Leuconostoc* sp. especially *Leuconostoc* mesenteroides, a dominant species at the beginning of *tempoyak* fermentation. The pH range of *Lactobacillus brevis* was between 4.4 to 5.4, with the optimum pH was 5.0. [28]. At the first week of fermentation of tempoyak with 4% salt addition, it appeared that the dominant LAB were *Leuconostoc mesenteroides* and *Lactococcus* sp. Higher concentration of salt (4%) resulted higher pH and, as a consequence, lower total acid content.

The dominant species of LAB in week 2 of fermentation with 2% salt addition was still *Lactococcus* sp. as well as homofermentative *Lactobacillus* sp. Further observations indicated that these homofermentative *Lactobacillus* isolated could be identified as *Lactobacillus plantarum* with the following characteristics: no growth at 45 °C and in general able to ferment arabinose, lactose, raffinosa, ribose, trehalose, sucrose, cellobiose, mellibiose and maltose [27, 29-31]. Interestingly, during the fermentation with 4 % salt addition, *Lactococcus* sp. was also the dominant genus, however together with *Enterococcus* sp. Further observations indicated that this *Enterococcus* sp. was identified as *Enterococcus faecium*, since these cocci-forming LAB were able to grow at temperatures of 10 and 45 °C and were also capable of fermenting mannitol and arabinose [27, 32]. In these conditions, the above dominant species were homofermentative lactic acid bacteria. Changes in dominant species of LAB were due to pH change and the presence of oxygen.

On week 3 of the *tempoyak* fermentation, both with 2 and 4% salt addition, the dominant species of LAB were homofermentative *Lactobacillus* sp., including *L. plantarum* and *L. acidophilus.* The latter species were able to grow at 45°C [30].

Lactobacillus acidophilus and *Lactobacillus plantarum* remained dominant in the bacterial population during week 4 of tempoyak fermentation with 2% salt addition, whereas *Lactobacillus plantarum* and *Pediococcus pentosaceous* were the dominant LAB in fermentations with 4% salt addition. *P. pentosaceous* could grow at 45°C. *P. pentosaceous* was often difficult to distinguish it from *P. acidilactici. P. pentosaceous* was able to ferment maltose, in contrast to *Pediococcus acidilactici* [33, 34]. Moreover, according to Pal, et al. (2005) [33], *P. pentosaceous* could ferment maltose, trehalose, galactose, xylose and cellobiose.

In general, starting at week 3 the *tempoyak* fermentation became more stable. This means that no significant changes of the dominant species of LAB occurred. In addition the pH value and total acidity content stabilized. The presence of *L. plantarum* the dominant species resulted in a positive effect because of its ability to grow at a relatively low pH and limited glucose conditions [35].

5. CONCLUSION

A number of 141 isolates of lactic acid bacteria (LAB) were isolated from *tempoyak* fermentation with 2 and 4% salt addition at 20±2°C. Further identification revealed that the 6 following genera were involved in *tempoyak* fermentation, i.e. *Oenococcus*, *Leuconostoc*, *Enterococcus*, *Lactococcus*, *Pediococcus* and *Lactobacillus*. *Leuconostoc* sp. played an important role during early stages of the fermentation, whereas *Lactobacillus* sp. (heterof) and *Lactobacillus* sp.2 (homof) dominated the bacterial population in the middle stage. At the end, important species were *Lactobacillus* sp.1 (homof) and *Pediococcus* sp.

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