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EFFECT OF DIFFERENT STORAGE CONDITIONS ON THE MICROBIOLOGICAL CHARACTERISTICS OF INSECT

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ABSTRACT

When introducing a novelty food, its safety needs to be monitored. One of the safety aspects of human health is microbial contamination. In this work, microbiological parameters of long-term stored edible insect material – mealworm (*Tenebrio molitor*), lesser mealworm (*Alphitobius diaperinus*), field cricket (*Gryllus assimilis*), and migratory locust (*Locusta migratoria*) were evaluated. The monitored indicators (colony forming units, enterobacteria, lactic acid bacteria, yeasts and moulds) were evaluated using common microbiological methods. All samples of stored insect were determined as safe for human consumption, except for the lesser mealworm sample from 2016, in which case the limit was exceeded. Sample of adult field cricket seems to be suitable for long-term storage, as it contained the lowest amount of microorganism. Sample of dried *Gryllus assimilis* from 2014 had the lowest microbial contamination. Further results suggest that, for long-term storage, the most suitable way of preparation is killing with boiling water, drying at 103 °C for 12 hours and subsequent hermetic packaging.

Keywords: edible insect; microbiota; microbiological safety; colony forming units; enterobacteria

INTRODUCTION

Microorganisms are part of every being, including edible insect. They are on the insect exoskeleton and inside the insect body. This microbiota can be dangerous for human health, thus it is necessary to pay attention to this problem when preparing human foodstuffs using edible insect, which belongs among novelty food **(van Huis et al., 2013; EFSA, 2015)**. Microorganisms produce enzymes with lipolytic and proteolytic abilities. They cause decomposition of fats and proteins in commodities, thus change the nutritional value of food **(Adams et Moss, 2002)**. According to **the Commission Regulation (EC) no. 2073/2005** the main source of human foodborne diseases is microbial danger. According to this regulation „Foodstuffs should not contain micro-organisms or their toxins or metabolites in quantities that pose an unacceptable risk to human health."

Microbiota in the insect digestive system is vital for its metabolism. It reflects the lifestyle of insect in the wild or its breeding conditions. In the culinary treatment, edible insect is often processed with intestinal contents, and it is therefore necessary to let the insects starve before further processing. Besides the microbiota found in the intestine, another microbiota is found on the insect's exoskeleton. Even this can be potentially dangerous for humans. This is the major safety risk in eating edible insects **(EFSA, 2015)**. However, microbiological limits have not yet been established for edible insects. Insects have the same allergen (chitin) as crustaceans, and from the culinary perspective, they are often compared with them. Because of this it could be possible to apply microbiological limits according to the food safety criteria defined in the **Commission Regulation (EC) no. 2073/2005** for the production of cooked crustaceans and molluscan shellfish. The limits in this category are set for microorganisms, their toxins and metabolites of *Salmonella* (absence in 25 g). It is also possible to use microbiological limits specified by **ČSN 56 9609**, which sets the M limit for total microorganism count 10^5 CFU.g^{-1}, 5.10^5 CFU.g^{-1} for *Escherichia coli* and absence of *Salmonella* spp. in 25 g.

There are very few specific scientific studies regarding the microbiological safety of edible insect bred in a controlled environment. Available literature declares a high number of bacteria $10^5 – 10^7$ CFU.g^{-1} **(van Huis et al., 2013; Grabowski et al., 2008)**. The most common bacteria in edible insect are: *Staphylococcus* spp., *Streptococcus* spp., *Bacillus* spp., *Proteus* spp., *Pseudomonas* spp., *Escherichia* spp., *Micrococcus* spp., *Lactobacillus* spp. and *Acetobacter* spp. **(EFSA, 2015)**.

Causal agent of alimentary diseases related to edible insect consumption can also be microscopic fungi (yeasts and moulds). Reason is the production of secondary

metabolites, which may be toxic to both animals and humans. Breeding environment has a very high impact on the concentration of the microscopic fibrous fungi, which are carried by insects. Another factor influencing the concentration of the micromycetes in processed material is further manipulation, processing and storage.

Scientific hypothesis

Scientific hypothesis is: Microbial load of long-term stored materials from selected edible insect species is safe for human consumption with a focus on the treatment. Drying and freezing and long-term storage of edible insect materials will ensure the microbial safety of this commodity for human consumption.

The aim of this work was to detect and compare the microbiological characteristic of long-term stored material produced using selected edible insect species, and based on the results choose the most suitable species for long-term storage.

MATERIAL AND METHODOLOGY

Material

Insect samples for the determination of microbiological parameters were obtained in cooperation with the Mendel University in Brno. The following species were analysed:

1. mealworm (*Tenebrio molitor* – TM) – larva, year of breeding 2012, 2015 and 2016
2. lesser mealworm (*Alphitobius diaperinus* - AD) – larva, year of breeding 2016
3. field cricket (*Gryllus assimilis* – GA) - nymph, year of breeding 2012 and 2016
4. field cricket (*Gryllus assimilis* – GA) – adult, year of breeding 2014
5. migratory locust (*Locusta migratoria* – LM) – adult, year of breeding 2012, 2015 and 2016

Live insect was put into sterile bag that was placed in a freezer box. Here the insect was killed and the microbiological characteristics were determined for the freshly killed samples (year of breeding 2016). This procedure was also used for 2012 samples, which were left in the freezer box for a long time. The rest of the samples were killed with boiling water, dried at 103°C for 12 hours, homogenized and subsequently stored at room temperature until analysis (January 2017).

Methods

Growth media and preparation of the dilution solution

To evaluate microbiological parameters, different types of growth media were used. To evaluate total count of mesophilic microorganism PCA (Plate Count Agar) produced by Hi Media Laboratories Pvt. Ltd., India was used. Dehydrated medium (20.5 g) was dissolved in 1000 mL of distilled water, stirred and sterilized by the procedure below.

Enterobacteria were evaluated using the VRBA (Violet Red Bile Agar) growth medium produced by Hi Media Laboratories Pvt. Ltd., India. Dehydrated medium (38.5 g) was dissolved in 1000 mL of distilled water stirred and sterilized by the procedure below.

Lactic bacteria (especially *Lactobacillus* spp.) were determined using MRS Agar (De Man Rogosa Sharpe Agar) from Oxoid Ltd., Great Britain. According to the instructions, 55.15 g of dehydrated soil and 15 g of agar were dissolved in 1000 mL of distilled water. Prepared growth medium was then stirred and sterilized by the procedure below.

Growth medium CHYGA (Chloramphenicol Yeast Glucose Agar) produced by Oxoid Ltd., Great Britain, was used to determine yeasts and moulds. Dehydrated medium (40 g) was dissolved in 1000 mL of distilled water. Prepared growth medium was then stirred and sterilized by the procedure below.

Sterilization of all growth media was done in autoclave at 121 °C for 20 minutes. After cooling, sterile growth media were put into sterile Petri dishes and after solidifying stored in the fridge upside down.

To prepare 1000 mL of dilution solution PPS (Physiological Peptone Solution) 1 g of peptone (Hi Media Laboratories Pvt. Ltd., India) and 8.5 g NaCl (PENTA, Ing. Petr Švarc, Czech Republic) were used. The weighed components were dissolved in 1000 mL of distilled water and the solution was sterilized in an autoclave at 121 °C for 20 minutes.

Sample processing

Samples were processed in January 2017. Weighing was different because of the different size and weight do specific insect. During subsequent homogenization, the insect was put into the homogenization bag with 50 mL of sterile PPS solution. The homogenization itself took 2 minutes using Stomacher homogenizer (Seward, Great Britain) and decimal dilution with PPS solution were then prepared. Homogenized sample represented dilution 10^0, and the dilution was done until 10^{-5}. Streak-plate inoculation was done out of each dilution, using 0.1 mL of inoculum.

Microbiological analysis

The following tests on microbiological analysis were performed:

1) the total number of aerobic mesophilic microorganisms (TNM) on Plate count agar (HiMedia, Bombai, India) at 30 °C for 48 h **(ISO Standard No. 4833-2; 2013)**;

2) the number of enterobacteria on violet red bile glucose agar (HiMedia) at 37 °C for 24 h **(ISO Standard No. 4832; 2006)**;

3) mesophilic lactic acid bacteria (LAB) on MRS agar (Oxoid, Basingstoke, UK) adjusted to pH 5.7 at 30 °C for 72 h **(ISO Standard No. 15214; 1998)**;

4) the number of yeasts and moulds on Chloramphenicol Yeast Glucose Agar (Oxoid, Basingstoke, UK) at 25 °C for 5 days **(ISO Standard No. 21527-1; 2008)**.

Results expression

Colony forming units, which grew in the Petri dishes on the growth media, were counted after the cultivation period. The following formulas (1, 2) were used **(Suchánková, 2016)**:

$$N = \frac{\frac{\sum c}{n} \times V_2}{V_1 \times d \times m} \quad (1)$$

$$N = \frac{\frac{\sum c}{n} \times V_2}{V_1 \times d \times Ks} \quad (2)$$

where:

N - total microorganisms [CFU.g^{-1}; CFU.mL^{-1}],
∑c - colony forming units (of each group of microorganisms) in all dishes used for counting,
d - diluent factor for each dilution,
V_1 - inoculum volume (pipetted sample or (suspension)) for each plate [mL],
V_2 - PPS volume used for sample homogenization [mL],
n - amount of dishes used for calculation,
m - sample weighing [g],
Ks - number of insect species used for microbiological analysis.

For the lowest dilution the formulas (3) and (4) were used:

$$N < \frac{1 \times V_2}{V_1 \times d_n \times m} \quad (3)$$

$$N < \frac{1 \times V_2}{V_1 \times d_n \times ks} \quad (4)$$

where:

d_n - diluent factor for the lowest dilution used for calculation.

Statisic analysis

The data were analysed using Excel 2013 (Microsoft Corporation, USA) and STATISTICA Cz version 12 (StatSoft, Inc., USA). Results were expressed by average and standard deviation. Each measurement was performed 3 times. Comparison of the results was performed using a Kruskal-Walllis test ($\alpha = 0.05$).

RESULTS AND DISCUSSION

Results of microbiological analysis for evaluated insects are shown in **T**Chyba! Nenašiel sa žiaden zdroj odkazov.. CFU did not exceed the limit for microbiological safety, except for the lesser mealworm (*Alphitobius diaperinus*), in whose case the limit was exceeded about 5x (2.3×10^6 CFU.g^{-1}). Selection of suitable thermal processing (in this case drying at 103 °C for 12 hours) leads to a reduction of the water activity **(Adams et Moss, 2002)**, thus reducing the amount of micro-organisms to a safe level. In the case of the CFU, a statistically significant difference (p <0.05) was found for mealworm (*Tenebrio molitor*), between both ways of heat treatment. Drying seems to be more effective as there was 10^5 × reduction (from 10^8 to 10^3), than freezing (10 × reduction). *Gryllus assimilis* and *Locusta migratoria* samples against freshly killed insects. Again, a statistically significant difference (p <0.05) was found between samples of freshly killed and dried insect. For these samples, there was at least 10^2 × reduction of the total number of microorganisms.

Recent studies **(Klunder et al., 2012; Vandeweyer et al., 2015; Stoops et al., 2016; Ssepuuya, 2017)** show microbiological characteristics for insect freshly killed with boiling water. Using this way of killing, our samples exceeded the limits for microbiological safety. Evaluation of the microbiological safety of samples, that were heat-treated and subsequently stored in boxes to prevent contamination from the environment, was done by **Grabowski (2017)**. This study suggests that in case of superworm (*Zophobas atratus*), boiling for 10 minutes and drying for 24 hours at 80 °C lowered CPM below the harmful level for 5 days of storage. When the temperature was 60 °C, CPM did not drop below the limit and the foodstuff remained inappropriate for human consumption. The last heat-treatment **Grabowski (2017)** tested was cooking for 30 minutes with subsequent drying at 80 °C for 12 hours and then drying at 100 °C for another 12 hours. It is interesting, that after this treatment, the CPM dropped below the limit only on the first day of storage, and for the rest of days it exceeded the limit. *Staphylococcus* spp. was not detected in these samples, on the contrary to other samples. In two-spotted cricket *(Gryllus bimaculatus)* **Grabowski (2017)** detected CPM above the limit in all samples.

When evaluating the microbial quality of 55 insect products in the Netherlands, 59 % of them were found to exceed the sanitary limits for aerobic bacteria

Table 1 Microbiological characteristic of material from selected insect species - Total microorganism count (CFU.g^{-1}), Enterobacteria (CFU.g^{-1}).

Species	Breeding year	Live cycle stage	Total microorganism count (CFU.g^{-1} ±SD)	Enterobacteria (CFU.g^{-1} ±SD)
Freshly killed				
Tenebrio molitor	2016	Larvae	$2.2 \times 10^8 \pm 2.4 \times 10^7$	$1.9 \times 10^8 \pm 7.1 \times 10^6$
Gryllus assimilis	2016	Nymphs	$3.3 \times 10^6 \pm 3.1 \times 10^5$	$3.5 \times 10^4 \pm 1.2 \times 10^3$
Locusta migratoria	2016	Nymphs	$2.8 \times 10^5 \pm 1.2 \times 10^4$	$1.5 \times 10^5 \pm 1.2 \times 10^4$
Frozen				
Tenebrio molitor	2012	Larvae	$3.4 \times 10^7 \pm 3.7 \times 10^6$	$4.2 \times 10^6 \pm 1.9 \times 10^5$
Gryllus assimilis	2012	Nymphs	$4.7 \times 10^6 \pm 3.9 \times 10^5$	$2.6 \times 10^5 \pm 1.2 \times 10^4$
Locusta migratoria	2012	Nymphs	$1.9 \times 10^6 \pm 1.4 \times 10^5$	$6.0 \times 10^4 \pm 2.5 \times 10^3$
Dried				
Tenebrio molitor	2015	Larvae	$6.6 \times 10^3 \pm 4.4 \times 10^2$	<10
Tenebrio molitor	2016	Larvae	$5.4 \times 10^3 \pm 1.9 \times 10^2$	<10
Alphitobius diaperinus	2016	Larvae	$2.3 \times 10^6 \pm 1.9 \times 10^5$	$1.6 \times 10^6 \pm 1.2 \times 10^5$
Gryllus assimilis	2014	Adults	$<10^2$	<10
Gryllus assimilis	2016	Nymphs	$7.1 \times 10^3 \pm 3.7 \times 10^2$	<10
Locusta migratoria	2015	Adults	$7.3 \times 10^3 \pm 3.9 \times 10^2$	<10

Table 2 Microbiological characteristic of material from selected insect species - Lactic acid bacteria [CFU.g^{-1}], Yeasts and moulds [CFU.g^{-1}].

Species	Breeding year	Live cycle stage	Lactic acid bacteria	Yeasts and moulds
Freshly killed				
Tenebrio molitor	2016	Larvae	$7.2 \times 10^7 \pm 3.1 \times 10^6$	$8.9 \times 10^3 \pm 3.7 \times 10^2$
Gryllus assimilis	2016	Nymphs	$5.8 \times 10^6 \pm 2.4 \times 10^5$	$4.4 \times 10^5 \pm 2.5 \times 10^4$
Locusta migratoria	2016	Nymphs	$1.5 \times 10^4 \pm 1.2 \times 10^3$	$< 2.2 \times 10^2$
Frozen				
Tenebrio molitor	2012	Larvae	$2.4 \times 10^5 \pm 1.2 \times 10^4$	$3.3 \times 10^4 \pm 2.4 \times 10^3$
Gryllus assimilis	2012	Nymphs	$5.0 \times 10^5 \pm 3.1 \times 10^4$	$5.1 \times 10^5 \pm 3.9 \times 10^4$
Locusta migratoria	2012	Nymphs	$1.5 \times 10^4 \pm 1.2 \times 10^3$	$1.5 \times 10^4 \pm 1.2 \times 10^3$
Dried				
Tenebrio molitor	2015	Larvae	$3.8 \times 10^3 \pm 2.1 \times 10^2$	$1.7 \times 10^4 \pm 1.9 \times 10^3$
Tenebrio molitor	2016	Larvae	$2.6 \times 10^3 \pm 1.9 \times 10^2$	$1.5 \times 10^3 \pm 1.2 \times 10^2$
Alphitobius diaperinus	2016	Larvae	$2.8 \times 10^6 \pm 2.5 \times 10^5$	$2.6 \times 10^6 \pm 1.9 \times 10^5$
Gryllus assimilis	2014	Adults	$<10^2$	$7.0 \times 10^2 \pm 5.5 \times 10^1$
Gryllus assimilis	2016	Nymphs	$2.2 \times 10^3 \pm 1.2 \times 10^2$	$6.0 \times 10^3 \pm 3.2 \times 10^2$
Locusta migratoria	2015	Adults	$1.6 \times 10^4 \pm 1.4 \times 10^3$	$2.6 \times 10^3 \pm 2.1 \times 10^2$

(10^6 CFU.g^{-1}). Analysed samples were freeze-dried with no further culinary treatment or processing. This study also evaluated the content of *Bacillus cereus* spores and the presence of bacteria *Clostridium perfringens*, *Salmonella* and *Vibrio*. *Clostridium perfringens*, *Salmonella* spp. and *Vibrio* spp. were not detected in samples and *Bacillus cereus* spores content was lower than 10^2 CFU.g^{-1} **(EFSA, 2015)** in 93 % of samples.

Klunder et al. (2012) did a microbiological evaluation of mealworm larvae (*Tenebrio molitor*), after he put them under the boiling temperature for several minutes. He found out, that *Enterobacteriaceae* were killed, but the spores survived the process and can be active again under optimal growth condition. The requested elimination of spores was done only by subsequent roasting. The case was similar for the house cricket (*Acheta domesticus*), where double culinary treatment (cooking and subsequent roasting) deactivated both the spore-forming bacteria and the *Enterobacteriaceae* bacteria. Dangerous limit ($> 10^5$ CFU.g^{-1}) was not exceeded in the samples, that were only boiled or only fried (10^3 CFU.g^{-1}.), but bacteria can multiply due to inappropriate storage, and the foodstuff may become harmful for consumers **(Hanboonsog et Durst, 2014)**. Belgian study, dealing with frozen samples of mealworm larvae (*Tenebrio molitor*) and migratory locust (*Locusta migratoria*), detected $10^7 – 10^9$ CFU.g^{-1} of aerobic bacteria and 10^4 CFU.g^{-1} of aerobic spores **(EFSA, 2015)**.

Another microorganisms evaluated in this study were the enterobacteria, which were not detected in any sample except for the lesser mealworm (*Alphitobius diaperinus*). In lesser mealworm (*Alphitobius diaperinus*) the detected value was 1.6×10^6 CFU.g^{-1}. This value is higher than in other samples because of breeding conditions and specific species. Although there has been a general reduction in the number of enterobacteria after freezing, a certain number of these bacteria were detected after this treatment. However, after drying, the number of enterobacteria was reduced below the detection limit. There is a statistically significant difference (p <0.05) between samples just after killing and after drying.

The available literature provides similar results after heat-treatment. **Klunder et al. (2012)** detected less than 10 CFU.g^{-1} of *Enterobacteriaceae* in boiled samples of the mealworm *(Tenebrio molitor)* and house cricket *(Acheta domestica)*. Values presented by this work comply with the mentioned references. Due to killing the specimens with boiling water the samples were heat treated for a short time, which, in accordance with the literature, effectively eliminated the enterobacteria.

Lactic acid bacteria (LAB) are widespread in nature, important considering the foodstuff and biotechnological point of view **(Tančinová et al., 2008)**, and have a positive impact on human health. Bacteria *Lactobacillus* spp. produce enzymes, that allow to decompose more complex substances in the foodstuff into simpler substances. Therefore LAB have beneficial effects mainly on the intestinal microbiota - improve the peristalsis of the intestines and prevent the growth of harmful bacteria. They also affect the immune system, enable the production of vitamins and help in the absorption of iron, calcium and other substances **(Agerholm-Larsen et al., 2000; Adams et Moss, 2002)**.

Due to the widespread availability of lactic acid bacteria in the wild, a relatively high amount of these bacteria was detected in freshly killed insect. **Vandeweyer et al. (2015)** evaluated freshly killed insect and reported $2.5 \times 10^7 – 1.6 \times 10^8$ CFU.g^{-1} in mealworm larvae (*Tenebrio molitor*) and 2.0×10^7-7.9×10^7 CFU.g^{-1} in the house cricket (*Acheta domestica*). **Stoops et al. (2016)** detected $4.0 \times 10^7 – 3.2 \times 10^8$ CFU.g^{-1} of LAB in freshly killed migratory locust nymphs (*Locusta migratoria*) and $1.0 \times 10^7 – 4.0 \times 10^8$ CFU.g^{-1} in mealworm (*Tenebrio monitor*). LAB detected in this work were from $<10^2$ CFU.g^{-1} in the field cricket *(Gryllus assimilis)* (adults 2014) to 2.8×10^6 CFU.g^{-1} in the lesser mealworm (*Alphitobius diaperinus*). In this sample the content of LAB significantly higher than in the rest of the samples. It is supposed, that in the rest of the samples the water activity dropped and stayed low during the whole storage period, which prevent microbial changes and subsequent foodstuff spoiling. Content of LAB in long-time stored

samples varied from 1.8×10^3 CFU.g^{-1} in migratory locust to 5.0×10^5 CFU.g^{-1} in field cricket nymphs. This proves that the heat treatment outside the optimal temperature range reduces the amount of LAB. When comparing the number of LABs between *Tenebrio molitor* larvae and *Gryllus assimilis* nymphs, a statistically significant difference (p >0.05) was not detected in the frozen samples, neither between the dried samples. On the other hand, there was a statistically significant difference (p <0.05) in freshly killed insects.

Yeasts and microscopic fibrous fungi, which produce mycotoxins with common influence on the human health, are also the indicators of foodstuff spoiling and environmental pollution **(Tančinová et al., 2008)**. Content of yeasts and moulds in the samples varied between 7×10^2 CFU.g^{-1} in the field cricket *(Gryllus assimilis)* (adult 2014) and 2.6×10^6 CFU.g^{-1} in the lesser mealworm (*Alphitobius diaperinus*). This sample contained a much higher amount of yeasts and moulds than other samples. Despite the heat treatment used for samples analysed in our work, which should eliminate yeasts and moulds, the subsequent storage took place in room temperature that is suitable for their growth. Results show that, yeasts and microscopic fibrous fungi are resistant to low temperatures and can prosper even in these conditions – their numbers grew. Other authors declare the content of yeasts and microscopic fibrous fungi $10^5 – 10^6$ CFU.g^{-1}. This work proves that drying is an effective way of reducing the numbers of yeasts and moulds in edible insects. In general, samples dried under laboratory conditions contained *Aspergillus* spp. *and Penicillium* spp. **(EFSA, 2015)**. Furthermore, *Fusarium* spp., *Chaetomium* spp., *Mucor* spp., *Mucorales* spp., *Alternaria* spp., *Drechslera* spp. and *Phoma* spp. were detected in insects **(Spiegel, 2013)**.

Toxins produced by microscopic fibrous fungi *Aspergillus* spp., *Penicillium* spp. and *Fusarium* spp. can be produced not only in the digestive track of insect, but can also come from the breeding substrate. Mycotoxins are considered toxic **(Tančinováet al., 2014)**, thus the concentration of these toxins may influence the mortality of bred specimens **(EFSA, 2015)**.

Mentioned facts lead to the conclusion, that the most suitable for long-term storage is the treatment chosen by us - killing with boiling water at 100 °C and then drying for 12 hours at 103 °C and storing in hermetically sealed containers. Microbial characteristics of individual insects in the freshly killed state are reported in the available literature. The amount of microorganisms in edible insects after various treatments (e.g. freshly killed, frozen, dried) depends not only on the species, feed or breeding conditions, but also on the specific conditions of its processing and storage. However, the conditions for the processing and storage of edible insect products are not yet legislatively established.

CONCLUSION

This work evaluated microbiological characteristic of selected edible insect species with regards to different storage period, and it was declared as safe for human consumption. The only exception was lesser mealworm (*Alphitobius diaperinus*), whose samples exceeded the limit. Regarding the microbiological characteristics during long-term storage, the best sample seems to be the adult field cricket (*Gryllus assimilis*) stored in 2014, which had the lowest microbial contamination.

Mentioned studies and research suggest that it is not good to consume insect in the freshly killed state, and heat treatment along with proper storage conditions is necessary. It is one of possible steps to eliminate alimentary diseases originating from edible insect. Our own results and references lead to the conclusion that, for long-term storage, the most appropriate procedure is to kill the insect with boiling water, dry it at 103 °C for 12 hours and subsequently hermetically pack it.

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