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MICROBIAL DIVERSITY OF *BETULA* TREES: POLLEN, CATKINS, LEAVES RELATIVELY OF FLOWERING

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Abstract

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Quantitative microbiological analysis by dilution plating method of pollen and additional male and female catkins, leaves of *Betula verrucosa* Ehrh. and its two cultivars: *'Purpurea'* and *'Youngii'* relatively of flowering period of *Betula* has been realized with the aim to provide new knowledge of the microbiological quality of anemophilous pollen for processing and its further application. Qualitative microbiological analysis with MALDI-TOF MS Biotyper was used in the identification of aerobic, anaerobic mesophilic bacteria and coliforms. Mixed microbiota was determined, consisting of aerobic (4.68–4.89 log cfu/g) and anaerobic (3.30–3.48 log cfu/g) mesophilic bacteria, lactobacilli (0–3.48 log cfu/g), coliform bacteria (0–4.57 log cfu/g), fungi and yeast (3.78–3.95 log cfu/g) on the pollen grains, that indicates acceptable quality in comparison with the microbiological quality parameters for bee pollen. *Pantoea agglomerans* was found associated with pollen of *Betula verrucosa* Ehrh. Recommendations on the collection of anemophilous pollen were established.

Keywords: *Betula verrucosa* Ehrh., microbiota, pollen contamination, microbiological quality, flowering

INTRODUCTION

Very little is known about contamination of plant pollen with microorganisms. But still more this question revealed about entomophilous pollen than anemophilous (Bogdanov, 2004, 2005; González *et al.*, 2005; Kačániová and Fikselová, 2007; Campos *et al.*, 2008; Brovarskij and Brindza 2010; Brindza *et al.*, 2010; Kačániová *et al.*, 2011; Hani *et al.*, 2012; Feás *et al.*, 2012; Estevinho *et al.*, 2012; Kačániová *et al.*, 2014). These knowledges can be interesting from the point of view – microorganisms as an adjuvant factor of initiation of allergic immune responses; microbiological quality of pollen for its consumption as a source of nutrients and medicinal substances; the nature of the mutual relations of microorganisms/pollen grains.

Gram-negative and Gram-positive bacteria, as well as endotoxin, mould were revealed on the surface of the pollen grains of anemophilous plants (Śpiewak *et al.*, 1996a; Heydenreich *et al.*, 2011; Mittag *et al.*, 2013). Mould is the source of allergens in itself (Heydenreich *et al.*, 2011). Endotoxin is a known airborne immunotoxicant for humans and animals (Śpiewak *et al.*, 1996). Besides, microbiological contamination of pollen can become the cause of contamination of allergenic extracts which are used for the treatment of allergic diseases in allergen-specific immunotherapy (Mittag *et al.*, 2013).

Anemophilous pollen also contains valuable chemicals for the human body (Nôžková *et al.*, 2014). For example, the protein content in the pollen of *Betula verrucosa* Ehrh. from Ukraine and Slovakia is

22.70 ± 2.28% at norm for dry bee pollen – 10–40% (Shevtsova *et al.*, 2013). Therefore, it is important to take into account the microbiological quality and of anemophilous pollen.

Microorganisms inhabit most plants (Després *et al.*, 2011). Bacteria and fungi, so-called endophyte, occur inside plant tissues of various plant organs, such as roots, stems, ovules and seeds without causing a disease. The presence of endophytic bacteria in a tissue for extended periods of time suggests a possible symbiotic-like interaction (Madmony *et al.*, 2005). Taking into account the nutrient content of pollen, it is a reliable and desired food source for a variety of microorganisms (González *et al.*, 2005; Madmony *et al.*, 2005). Also, an association microorganisms-pollen can protect microorganisms from environmental stresses (Madmony *et al.*, 2005). Furthermore, pollen grains can also be the transport for microorganisms, including economically important phytopathogenic bacteria. Pathogenic bacteria, to survive in the environment, associate with seeds, insects, other animals, the water cycle and also humans (De La Fuente and Burdman, 2011). Pollen grains also can be among them. And pollen and microorganisms can be spread in the composition of bioaerosols in the atmosphere. Often microorganisms attached to other particles in the atmosphere (Després *et al.*, 2011).

From the aforesaid follows that the microbiota of pollen is an important factor of its quality, which determines the possibility of its further use. Due to the lack of information in the scientific literature about the microbiological quality of anemophilous pollen some of questions remain unanswered. For example, what is a microbiota of anemophilous pollen? Are the microbiological characteristics of pollen and other plant organs coincide? At what stage the pollution of pollen is take place? Whether microbiological quality criteria of entomophilous pollen can be applied to anemophilous?

To these goals, the qualitative and quantitative microbiological analysis of the species *Betula*, growing in the Botanical Garden of the Slovak Agricultural University in Nitra, Slovakia was realized.

MATERIALS AND METHODS

Plant and Pollen Materials Description

The plant material was collected in March-April 2015 in the Botanical Garden (BG) of the Slovak Agricultural University in Nitra. Topographic data of the BG are: altitude 144 m, east longitude 18°06' and north latitude 48°19'. The BG is located in the administrative area Chrenova, which is the part of Nitra city. Substrate complex consists of alluvial soil with a high content of clay fractions, which formed the river Nitra.

Several cultivars of *Betula verrucosa* Ehrh. (syn. *B. pendula* Roth.) grow on the territory of the BG. For analysis *Betula verrucosa* Ehrh. (BV1), *Betula verrucosa* Ehrh. '*Purpurea*' (BV2); *Betula verrucosa* Ehrh. '*Youngii*' (BV3) were chosen. The trees were identified by prof. T. Baranec from the Department of Botany at the Slovak Agricultural University by means of botanical criteria. The main species of research was *Betula verrucosa* Ehrh. and its pollen like the most important and the most aggressive spring allergen in Slovakia and Europe as a whole (Šustrová, 2009; Laffársová, 2015). Male catkins before, during and after flowering, pollen, leaves and young female catkins during flowering were collected. The collected plant materials are shown in Tab. I.

I: *The plant material from Betula trees for microbiological analysis*

Part of tree	Species	Time of collection relative flowering
Male catkins	BV1	before flowering March
Male catkins	BV1	before flowering April
Male catkins	BV1	after flowering
Male catkins	BV3	before flowering April
Female catkins	BV1	during flowering
Leaves	BV1	during flowering
Pollen	BV1	April
Pollen	BV2	April
Pollen	BV3	April

All plant material was collected by adhering sterile conditions at each stage. All plant parts were placed in a cooler and conserved until analysis at +4 °C. Pollen was dried at room temperature overnight and collected the next day, keeping sterile conditions. Microbiological analysis of plant materials was carried out as to collect the material.

Air Samples

Air samples around the trees were studied for comparison the qualitative composition of the microbiota of trees and air. The atmosphere is not a living habitat for microbes, but they can spread through it, and therefore the atmosphere could act as a conveyor of pathogenic microbes. "Koch-type" sedimentation method was used for sampling the air (Tóth *et al.*, 2013). Open Petri dishes with meat peptone agar medium (Imuna, Slovak Republic) and malt extract agar base (Biomark Laboratories, India) were placed around the studied trees and exposed for 10 and 15 minutes. Air sampling was performed three times on different days. Values of actual allergens concentrations in the air for the study region – Nitra during the flowering period of *Betula* were registered according to "Current pollen news of the Slovak Republic" at http://www.alergia.sk.

Quantitative Determination of Microbiota

The concentration of microorganisms in the plant samples was determined by dilution plating. One gram of each sample was suspended in 99 mL of sterile saline solution. After vigorous shaking during 30 minutes at room temperature, 10-fold serial

dilutions were made up to 10^{-3}. The 1000 µL aliquots of each dilution were spread on sterile Petri dishes duplicate of media appropriate for determination of mesophilic aerobic bacteria, mesophilic anaerobic bacteria, *Lactobacillus, Enterococcus*, total coliforms and 100 µL – for fungi and yeast. The serial dilutions were inoculated on nutrient media. The plates with mesophilic aerobic and anaerobic bacteria were incubated on meat peptone agar medium (Imuna, Slovak Republic) for 48–72 hours at 25 °C in appropriate conditions; *Lactobacillus* were incubated on MRS Agar Modified (HiMedia, India) for 48–72 hours at 37 °C; *Enterococcus* – on Slanetz-Bartley medium (HiMedia, India) for 48–72 hours at 37 °C; coliform bacteria – on violet red bile with lactose agar (Pronadisa, Spain) for 24–48 hours at 37 °C and fungi on Sabouraud medium (Biomark Laboratories, India) for 5–7 days at 25 °C. The data are reported as log cfu/g.

Qualitative Determination of Microbiota

Then MALDI-TOF was evaluated in the identification of all groups of microorganisms, but only representatives of aerobic, anaerobic mesophilic bacteria and coliforms were identified by MALDI databases. Fungi were isolated and determined using the "Dictionary of the Fungi" (Kirk *et al*., 2001). All the preparatory stages for the analysis of the samples were carried out according to the manufacturer's recommendations of MALDI Biotyper (Bruker Daltonik, Germany). The culture of bacteria was pick out from Petri dishes and transferred it to 300 µL of distilled water in Eppendorf tubes. Nine hundred microliters of ethanol was added and the tubes were centrifuged for 2 minutes at 14,000 npm. The supernatant was removed, and the same spin was repeated on the pellet. All remaining ethanol was removed, and the pellet was allowed to dry. Ten microliters of 70% formic acid was mixed with the pellet by pipetting and vortexing. Then 10 µL of acetonitrile was added. Tubes were centrifuged for 2 minutes at 14,000 npm and 1 µL of the supernatant was applied to the MALDI target in duplicate. After drying, 1 µL of α-Cyano-4-hydroxycinnamic acid matrix was applied and allowed to dry. MALDI-TOF was performed on the Microflex LT (Bruker Daltonics) instrument using Flex Control 3.4 software and Biotyper Realtime Classification 3.1 with BC specific software. Criteria for successful identification as proposed by the manufacturer were a confidence score of ≥ 2.0 for species level and ≥ 1.7 for genus level.

Statistical Analysis

Results were evaluated by standard techniques using MS Excel and Statistica 8. The criterion of the least significant difference (LSD) has been applied to reveal reliable distinctions between the mean values for each microbiological marker. Significant differences were defined with a significance cut off value of 0.05.

RESULTS AND DISCUSSION

Microbial content of *Betula* trees samples is presented in Tab. II.

As the statistical analysis showed there are significant differences in the quantitative and qualitative composition of the microbiota between the parts of the plants of one tree, and the same parts of the trees of different cultivars. The total content of mesophilic aerobic bacteria of the investigated samples is 2.60 (leaves BV1) – 5.52 (male catkins BV1 before flowering) log cfu/g. The male catkins and pollen differ from the female catkins and leaves according to this indicator, which is typical for *B. verrucosa* Ehrh. Differences between cultivars have observed for male catkins before flowering for *B. verrucosa* Ehrh. (5.52 log cfu/g) and *B. verrucosa* Ehrh. 'Youngii' (4.09 log cfu/g). The leaves are contaminated least (2.60 log cfu/g). *Micrococcus luteus* on female catkins BV1 and *Rhodococcus pyridinivorans* and *Rhodococcus rhodochrous* on male catkins BV1 after flowering were identified using MALDI-TOF within this group of microorganisms.

The level of contamination by anaerobic bacteria in comparison with the aerobic bacteria is not high – 0.00 (male catkins BV1, BV3 before flowering) – 3.48 (pollen BV1, BV2) log cfu/g. Pollen samples of three *Betula* subvarieties are contaminated in a greater degree than the other studied parts of the plants. *Staphylococcus warneri* on male catkins BV1 before flowering was identified using MALDI-TOF within this group of microorganisms.

The amount of lactobacilli varies from 0.00 (female catkins BV1 and pollen BV2) to 4.60 (male catkins BV1 before flowering, March) log cfu/g. *Betula verrucosa* Ehrh. *'Purpurea'* pollen and *Betula verrucosa* Ehrh. female catkins significantly different from other parts of plants on this parameter.

Enterococci are detected only on leaves samples of *B. verrucosa* Ehrh. (2.00 log cfu/g).

The amount of total coliforms varies from 0.00 (female catkins BV1 and pollen BV2) to 4.57 (pollen BV1) log cfu/g. The highest values are characteristic for the two species of pollen. *Betula verrucosa* Ehrh. *'Purpurea'* pollen and *Betula verrucosa* Ehrh. female catkins are also not contaminated by coliform bacteria, as well as lactobacilli. *Pantoea agglomerans* on male catkins BV3 and BV1 before, during and after flowering as well on pollen samples BV1 and BV3 was identified using MALDI-TOF within this group of microorganisms.

Colonies of microscopic fungi and yeasts are the most numerical on samples of male catkins regardless of the flowering period. Pollen is contaminated smaller by them, but significant differences between the amount of colonies on the catkins and on pollen have not been identified. But differences are characteristic for the leaves and female catkins during the flowering of *B. verrucosa* Ehrh. They are contaminated with fungi and yeasts least of all. Microscopic fungi were identified at genus level, namely: *Penicillium, Alternaria* and

II: *Microbial enumeration of Betula trees samples collected in Slovakia in 2015, log cfu/g*

Plant part	Group of microorganisms	
	Total mesophilic aerobic counts	**Total mesophilic anaerobic counts**
Male catkins BV1 (bf March)	4.53[a]*	3.30[a]
Male catkins BV1 (bf April)	5.52[a]	0.00[ab]
Male catkins BV1 (af)	4.93[a]	3.00[a]
Male catkins BV3 (bf April)	4.09[ab]	0.00[ab]
Female catkins BV1 (df)	3.60[b]	2.00[a]
Leaves BV1 (df)	2.60[c]	2.30[a]
Pollen BV1	4.89[a]	3.48[a]
Pollen BV2	4.68[a]	3.48[a]
Pollen BV3	4.81[a]	3.30[a]
	Total lactobacillus counts	***Enterococcus* counts**
Male catkins BV1 (bf March)	4.60[a]	0.00
Male catkins BV1 (bf April)	3.60[a]	0.00
Male catkins BV1 (af)	3.85[a]	0.00
Male catkins BV3 (bf April)	4.28[a]	0.00
Female catkins BV1 (df)	0.00[b]	0.00
Leaves BV1 (df)	2.78[a]	2.00
Pollen BV1	3.30[a]	0.00
Pollen BV2	0.00[b]	0.00
Pollen BV3	3.48[a]	0.00
	Total coliforms	**Microscopic fungi and yeast**
Male catkins BV1 (bf March)	2.00[b]	4.96[a]
Male catkins BV1 (bf April)	3.00[a]	4.08[a]
Male catkins BV1 (af)	3.30[a]	4.55[a]
Male catkins BV3 (bf April)	2.30[a]	4.04[a]
Female catkins BV1 (df)	0.00[bc]	3.30[b]
Leaves BV1 (df)	2.30[a]	2.30[b]
Pollen BV1	4.57[a]	3.95[a]
Pollen BV2	0.00[bc]	3.95[a]
Pollen BV3	4.37[a]	3.78[a]

*[ab] Means within a column with the same letters are not significantly different according to Tukey's multiple range test ($P \leq 0.05$); bf – before flowering; df – during flowering; af – after flowering

unidentified genus *Mycelium sterillium* without creation fruiting bodies. *Penicillium* were present on male catkins BV1 before flowering in March and April, *Alternaria* – on male catkins BV1 before flowering in April and after releasing pollen as on pollen samples from BV1 and BV3, *Mycelium sterillium* – on male catkins BV1 after flowering. Yeasts were revealed on all samples.

In whole male catkins less contaminated with enterococci and mesophilic anaerobic bacteria at various stages relatively of flowering. There are zero values at least on one indicator. But male catkins contaminated by fungi and yeasts most of all samples. Leaves of *Betula verrucosa* Ehrh. most contaminated with studied microbial groups. *Betula verrucosa* Ehrh. '*Purpurea*' (BV2) pollen differ among the pollen samples. Pollens of *B. verrucosa* Ehrh. (BV1) and *B. verrucosa* Ehrh. '*Youngii*' (BV3) are not significantly different in microbial contamination. As to microbiota of female catkins of *Betula verrucosa* Ehrh., it is similar to the microbiota of pollen of *B. verrucosa* Ehrh. '*Purpurea*'.

With regard to air colonies of *Penicillium, Cladosporium, Alternaria, Mucor, Mycelium sterillium* without creation fruiting bodies were found in the samples near the trees at the BG and additionally *Bacillus licheniformis* was identified using MALDI-TOF.

According to data of aerobiological station of Nitra region of the Slovak Republic during the study period (plants sampling, flowering of *Betula*, air sampling) *Cladosporium* spores often dominated (values of average weekly concentrations of spores per 1 m^3 of air were in the range 13–141) in the air of Nitra region, and concentration levels of *Alternaria* (0–8/m^3), *Epicoccum* (0–3/m^3), *Stemphylium* (1–3/m^3), *Helminthosporium* (0/m^3) spores were low or very low.

Since all samples were collected from trees that grow relatively close to each other, in the same climatic, weather, edaphic conditions, the influence of the factor "habitat" has the same effect on the results.

The purpose of this study is to provide new understanding the microbiological quality of anemophilous pollen with allergenic potential, about the reasons for its contamination. A mixed microbiota, consisting of aerobic and anaerobic mesophilic bacteria, lactobacillus, coliform bacteria and fungi, is present on plant parts of *Betula* trees and its pollen with allergenic potential. It was found that the microbiota of pollen were not significantly different from the microbiota of male catkins or differs slightly before flowering of *Betula*. Much more microbiota of pollen does not coincide with the microbiota of leaves and female catkins. The result is logical: the pollen contained in the anthers, which form the inflorescence of catkins. Catkins were collected long before flowering or before flowering, when they emit pollen sporadically and begin to flower. Pollen also has been collected before it's released into the natural environment, that is, when the anthers are almost to dehisce. There is data that pollen is sterile inside the anther due to the content of antimicrobial substances (flavonoids, phenolic acids, other phytochemicals and antimicrobial peptides) (Hani *et al*., 2012; Nôžková *et al*., 2014). But catkins contact with the environment and plant materials, which are likely the factors of pollen contamination. Perhaps, the anthers are slightly dehisce before the mass flowering and pollen grains become attractive for microorganisms by variety of nutrients. At this phase, contamination of pollen grains by microorganisms occurs before they are will be released into the environment or collected manually. Leaves and emerging female catkins have been collected during flowering, that is, the availability of pollen, which is more attractive for microorganisms. Alternatively microorganisms may be airborne contaminants that alight on the pollen during collection and processing (Madmony *et al*., 2005). Microbial content from the air in the BG and fungi on plant parts confirm this in our case.

It was also found that microbiota of pollen of *Betula verrucosa* Ehrh. and its two cultivars qualitatively and quantitatively are the same. Sample of *Betula verrucosa* Ehrh. '*Purpurea*' (BV2) pollen was differed by lack of lactobacillus and coliform bacteria, which can be explained by long-term storage of the sample before analysis compared to BV1 and BV3.

Leading experts on the bee pollen from certain countries – Portugal, Switzerland, Brazil, Poland, Uruguay proposed bee pollen standards as a basis for world wide bee pollen standards (Campos *et al*., 2008). The microbial quality of bee pollen for using it as a source of nutrients and medicinal substances should be as follows: *Salmonella* – absent/10 g; *Staphylococcus aureus* – absent/1 g; *Enterobacteriaceae* – max 100/g; *Escherichia coli* – absent/1 g; total aerobic plate count < 100 000/g; mould and yeast < 50 000/g; Alfatoxin B1 – max 2 mg/kg; Alfatoxin B1+B2+G1+G2 – max 4 mg/kg. If the microbiological quality criteria of entomophilous pollen apply to the anemophilous, it's giving that under the maintenance of aerobic bacteria, *Enterococcus*, mould and yeast pollen has good microbial quality. However, according to content of coliforms, which include *E. coli*, only pollen of *Betula verrucosa* Ehrh. '*Purpurea*' correspond to quality. Normal content of bacteria from *Enterobacteriaceae* is of great importance in the case of pollen. Enterobacterial cell membrane contains an antigenic, pyrogenic and thermostable molecule, the endotoxin or lipopolysaccharide. The bacterial endotoxin is released during bacterial multiplication or death and triggers a series of important biological events that lead to an inflammatory response and bone resorption. Either raw or dried pollen may be considered as a potentially hazardous medicinal plant product. [10]

The presence of *Lactobacillus* on pollen and plant parts is a good indicator. In general, there are more *Lactobacillus* on male catkins than on pollen and leaves, although no significant differences between them. They have not been identified on female catkins.

Acceptable microbiological aspects testify favorable growing conditions for trees and the proper handling with the plant material, particularly pollen. Worldwide, pollen processing includes harvesting, drying, cleaning, packaging, storage and further application (Hani *et al*., 2012). Need to be careful on each of them.

Concern to the identified microorganism on pollen grains – *Pantoea agglomerans*, the similar result are known in the literature. Thus, Śpiewak *et al.* (1996) showed that Gram-negative bacteria *Pantoea agglomerans* (synonyms: *Erwinia herbicola, Enterobacter agglomerans*) are present on pollen grains. *Pantoea agglomerans* is from *Enterobacteriaceae* family. It possess adjuvant activity by greatly enhancing dendritic cells maturation and induction of Th1-, Th2- and Th17-mediated allergic inflammation (Heydenreich *et al*., 2011). *Penicillium* and *Alternaria* are known anemophilous pollen concomitants (Brindza *et al*., 2010; Hani *et al*., 2012; Kačániová *et al*., 2014; Śpiewak *et al*., 1996; Dziadzio, 2001; Rodríguez-Carrasco *et al*., 2013). These results suggest that, regardless of the country or region of origin for the pollen of different species general or a similar composition of the microbiota is characteristic. Therefore, the microbiological quality criteria may be and should be not only at national level but also at international.

An interesting indicators and objects, isolated from *Betula* male catkins, are *Rhodococcus pyridinivorans* and *Rhodococcus rhodochrous*, family *Nocardiaceae*. *R. pyridinivorans* is a gram positive, aerobic, rod shaped, non-motile, and non-sporulating mesophiles (Yoon *et al*., 2000). *R. rhodochrous* is aerobic, gram positive and nonmotile bacteria (Warhurst and Fewson, 1994), can cause pathogenesis in plants and mammals (Larkin *et al*., 2010). These species are commonly present in contaminated soil or

water with a variety of xenobiotics, specifically toxic aromatic compounds. These compounds are produced by both anthropogenic and natural activities and accumulate in the environment. *R. pyridinivorans,* as *R. rhodochrous,* contribute to bioremediation (Yoon *et al*., 2000; Zhao *et al*., 2004; Shinha *et al*., 2009). So, presence of these species can be like bioindicators.

Staphylococcus warneri is a part of the skin flora on humans. *S. warneri* rarely causes disease, but may occasionally cause infection in patients whose immune system is compromised (Kini *et al*., 2010). *Micrococcus luteus* can be found both on human skin and soil, dust. It caused infections in people with compromised immune systems (Mukamolova *et al*., 2002). *Bacillus licheniformis,* that was identified in air sample, Gilliam *et al.* (1979) isolated from almond pollen.

CONCLUSION

A mixed microbiota, consisting of aerobic and anaerobic mesophilic bacteria, lactobacillus, coliform bacteria and fungi, is present on plant parts of *Betula* trees and its pollen. It was found that the microbiota of pollen were not significantly different from the microbiota of male catkins or differs slightly before flowering of *Betula*. Much more microbiota of pollen does not coincide with the microbiota of leaves and female catkins. Probably, contamination of pollen grains by microorganisms in the environment is possible and occurs before mass flowering, when the anthers are slightly dehisce. The microbiological quality criteria of pollen is exhibitive for both entomophilous and anemophilous pollen. The environment, weather conditions, human factor are the most likely causes of contamination of anemophilous pollen before its releasing from the anthers. The "human factor" involves collecting, drying, packaging and storage of plant material. The correct conditions of pollen collecting are the most important step. Birch pollen should be collected after elongation, yellowing and swelling of male catkins, when no or barely noticeable amount of pollen grains on the hands remain, if touch the catkins. Catkins can be collected by arms, pincers or scissors. Laboratory gloves should be used. Hands and tools should be disinfected. As the container new kraft paper envelopes or sterile glass containers are best to use. Then catkins from glass containers need to be lay out on kraft paper with a thin layer or leave it in kraft envelope for a day at the room temperature. Then pollen need to be poured and shake off in a sterile glass container better with a not very narrow neck. Also separator can be used.

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