Most Commonly Found Bacteria That Cause Dental Plaque Inflammation in German Shepherd Dogs

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Abstract

The purpose of this study was to investigate which bacteria cause plaque inflammation in dogs. To meet this goal, samples were taken from dogs of the German Shepherd breed, brought and examined by a veterinarian, who were found to have problems with dental plaque. The examination covers a time interval of three years between January 2018 to January 2021. The samples were taken in veterinary clinics in the Pelagonija region. A total of 48 samples were taken from different dogs with an average age of 4 to 7 years and of different sexes. Five swabs were taken from each sample, which were cultivated on petri dishes in selective nutrient medium and cultured at different temperatures. 114 isolates were were identified by means of various biochemical tests isolated from such petri dishes after cultivation, by biochemical tests. From the obtained results it can be concluded that most often the appearance of dental plaque occurs in animals aged 4.2 years to 6.7 years. You may also notice that the appearance of dental plaque in dogs does not depend on the sex of the dog. It was found a good growth on the selective nutrient substrates, such as Baird Parker Agar, Bile eseulin azide, which are confirmed by the additional tests performed (Oxidase reaction, Catalysis activity, Indole test, Hydrolysis of hyporate, Acetoin formation, Acetylmethylcarbinol, Voges-Proscauer reaction) and Test methyl-Roth (MR-test). The most common bacteria that cause dental plaque problems in German Shepherd dogs in 2018 according to the results are the following generic *Staphylococcus* 25%, *Streptococcus* 22% and *Streptococcus Enterococcus* 18%, *Veillonella* 13%, *Bacteriodes* 10%, *Prevotella* 7%, *Lactobacillus* 5%. While the bacteria present and causing dental plaque problems in 2019 are *Staphylococcus* 29%, *Streptococcus* 25%, *Enterococcus* 19%, *Veillonella* 10%, *Bacterioides* 8%, *Prevotella* 6%, *and Lactobacillus* 3%. And according to our results in 2020 the most common bacteria that cause dental plaque injury are: *Staphylococcus* 33%, *Streptococcus* 27%, *Enterococcus* 16%, *Veillonella* 11%, *Bacterioides* 6%, *Prevotella* 4%, *and Lactobacillus* 3%. As a conclusion of the results it can be concluded that the most common genera that cause plaque injuries in dogs of German Shepherd breed in Pelagonija region are the genera: *Staphylococcus, Streptococcus* and *Enterococcus* the other genera occur but in a much smaller percentage. Inflammation of the dental plaque in this breed of dog has been found to be caused by more than three bacteria that can act together.

Key words: German Shepherd Dogs, Dental Plaque, Bacterial causes

Introduction

In healthy gingiva, the cocci represent nearly two-thirds of the bacteria, followed by non-motile small rods (Pieri et al., 2012). The bacteria present are mostly gram-positive and there is no significant representation of more virulent bacterial types. Identified bacteria that are present in ini-

tial supra-gingival plaque in ten young dogs and found the genera *Streptococcus*, *Staphylococcus* and *Enterococcus* as the main components (personal data) (Pieri et al., 2012). At the stage of gingivitis, the gram positive rods (non-motile) gradually increase in comparison with cocci, and the number of gram negative bacteria also increase. This change continues to go on until the periodontium involvement phase (periodontitis), when the more pathogenic gram-negative microorganisms become the majority, so that the spirochetes represent almost half of the bacteria, while grampositives are underrepresented (Niezengard et al., 1997; Riggio et al., 2011; Perchyonok, 2018). The main bacteria involved in the formation of dental plaque are *Streptococcus spp* (Gibbons, 1972; Tanzer et al,. 1974; Duchin and Houte, 1978; Slee and O'Connor, 1983; Slee et al., 1983; Corner et al., 1988; Murray et al., 1992; Harvey and Emily, 1993; Lang et al., 1997; Rosan and Lamont, 2000; Loesche and Grossman, 2001; Katsura et al., 2001; Drummond et al., 2004; Swerts et al., 2005; Marsh, 2006; Daboor et al., 2015.), *Actinomyces spp.* (Slee and O'Connor, 1983; Katsura et

al., 2001) and *Lactobacillus spp.* (Drummond et al., 2004; Roza et al., 2004). They colonise initially the adhered film of the enamel and then start to multiply and aggregate. It was found that surface receptors of the cocci and gram-positive rods allow adherence of gram-negative bacteria and, over time, they present the greatest biodiversity and pathogenic potential (Lang et al., 1997). In dogs, the most important bacteria of this group are *Veillonella, Bacterioides, Prevotella* (Domingues et al., 1999), *Fusobacterium* (Murray et al., 1992; Braga et al., 2005) and *Porphyromonas* (Domingues et al., 1999; Katsura et al., 2001; Braga et al., 2005; Senhorinho et al., 2011).

Material and Methods

Samples

The samples were taken from a German Shepherd dogs examined by a veterinarian who a found to have a problem with dental plaque. The swabs are taken for a period of three years (from January 2018 to January 2021). Five swabs were taken from each dog (Table 1)

Table 1. Samples taken from German Shepherd dogs examined by a veterinarian who have a problem with dental plaque (year, age and sex)

Sample 1			Sample 2			Sample3			Sample 4			Sample 5			Sample 6		
year	age	sex	year	age	sex	year	age	sex	year	age	sex	year	age	sex	year	age	sex
2018	5,1	M	2018	6	M	2018	4,8	F	2018	6,8	M	2018	4,1	M	2018	3,8	F
Sample 7			Sample 8			Sample 9			Sample 10			Sample 11			Sample 12		
year	age	sex	year	age	sex	year	age	sex	year	age	sex	year	age	sex	year	age	sex
2018	6,2	F	2018	5	M	2018	3,6	M	2018	6,4	M	2018	7	F	2018	7,3	M
Sample 13			Sample 14			Sample15			Sample 16			Sample 17			Sample 18		
year	age	sex	year	age	sex	year	age	sex	year	age	sex	year	age	sex	year	age	sex
2018	7,5	F	2018	4,4	F	2018	5,3	F	2018	6,6	F	2018	7,8	M	2019	5,8	M
Sample 19			Sample 20			Sample 21			Sample 22			Sample 23			Sample 24		
year	age	sex	year	age	sex	year	age	sex	year	age	sex	year	age	sex	year	age	sex
2019	6,3	F	2019	4,9	M	2019	5,6	M	2019	4	F	2019	3,9	M	2019	3,3	M
Sample 25			Sample 26			Sample 27			Sample 28			Sample 29			Sample 30		
year	age	sex	year	age	sex	year	age	sex	year	age	sex	year	age	sex	year	age	sex
2019	3,6	M	2019	5,2	F	2019	6,7	M	2019	4,7	M	2019	4,9	M	2019	3,5	F
Sample 31			Sample 32			Sample33			Sample 34			Sample 35			Sample 36		
year	age	sex	year	age	sex	year	age	sex	year	age	sex	year	age	sex	year	age	sex
2019	6,3	F	2019	3,9	F	2020	4,8	F	2020	5,5	M	2020	3,3	M	2020	4,4	F
Sample 37			Sample 38			Sample39			Sample 40			Sample 41			Sample 42		
year	age	sex	year	age	sex	year	age	sex	year	age	sex	year	age	sex	year	age	sex
2020	5,4	M	2020	7,5	M	2020	5,3	M	2020	6,4	F	2020	3,6	M	2020	6,6	F
Sample 43			Sample 44			Sample 45			Sample 46			Sample 47			Sample 48		
year	age	sex	year	age	sex	year	age	sex	year	age	sex	year	age	sex	year	age	sex
2020	5,1	F	2020	4,9	M	2020	6,7	M	2020	5,7	M	2020	3,9	M	2020	6,8	F

*M–Male, F–Female

Growth Media for Bacteria isolation

The five swabs taken from each dog were transferred to five nutrient selective media and cultured at different temperatures. We used the following nutrients:

-Baird Parker Agar (BPA)-11705, incubate at 34–38 °C for 24–48 hours;

-Bile eseulin azide (BEA)-06105 incubate at 37 °C for 24–48 hours or 5% CO_2 until isolated colonies appear;

-Veillonella Agar Base (VAB)-M416 incubate at 35–37 °C for 24–48 hours;

-Bacteroides Amikacin Vancomycin Agar (BAVA) incubate at 37 °C for 36–48 hours;

-MRS agar (De Man, Rogosa and Sharpe Agar, Merck Darmstadt, Germany). The plates were then incubated at 42 °C for 24–48 hours, Laked blood with canamycin and vancomycin agar) LKV at 35–37 °C. for up to 48 hours incubated in 5–10% CO_2).

Isolation of Bacteria from Dental Plaque

A total of 210 swabs were taken in the three years, of which 114 were isolated (Table 2).

Identification of Bacteria
Morphological Characteristics

The microorganisms were gram stained and observed under light microscope (40X). The shapes of bacterial cell were bacilli, coccid, single, paired, chain and dense clusters.

Biochemical Properties
The oxidase reaction

The culture is grown on solid nutrient medium to obtain individual colonies. Prepare a solution of diethyl (or tetramethyl) para-phenylenediamine HCl in distilled water: 10 mg in 1 ml of water. The solution is stored for 2–3 days in a dark glass container and in a dark room. A thin swab is made from the colony, which will be examined on a strip of filter paper placed on a glass slide. Para-phenylenediamine solution is instilled into the swab.

Catalysis activity

Development of a culture of solid nutrient medium. Preparation of 30% aqueous solution of hydrogen peroxide. Place a drop of dissolved hydrogen peroxide on a glass slide. Transfer with a mixture of culture material grown on a solid nutrient medium in a drop of hydrogen peroxide and its suspension.

Indol test
Preparation of the substrate

Components	Quantity
Bacto-peptone	10 g
NaCl	5 g
Distilled sterile water	100 ml
pH 7.2–7.4	

Table 2. Isolates obtained from swabs taken from dogs of German Shepherd breed that have a problem with dental plaque

№	Strains	№	Strains	№	Strains	№	Strains	№	Strains	№	Strains	№	Strains
1	01-A18	17	17-A18	33	33-A18	49	49-B19	66	66-B19	82	82-B19	99	99-C20
2	02-A18	18	18-A18	34	34-A18	50	50-B19	67	67-B19	83	83-B19	100	100-C20
3	03-A18	19	19-A18	35	35-A18	51	51-B19	68	68-B19	84	84-B19	101	101-C20
4	04-A18	20	20-A18	36	36-A18	52	52-B19	69	69-B19	85	85-C20	102	102-C20
5	05-A18	21	21-A18	37	37-A18	53	53-B19	70	70-B19	86	86-C20	103	103-C20
6	06-A18	22	22-A18	38	38-A18	54	54-B19	71	71-B19	87	87-C20	104	104-C20
7	07-A18	23	23-A18	39	39-A18	55	55-B19	72	72-B19	88	88-C20	105	105-C20
8	08-A18	24	24-A18	40	40-B19	56	56-B19	73	73-B19	89	89-C20	106	106-C20
9	09-A18	25	25-A18	41	41-B19	57	57-B19	74	74-B19	90	90-C20	107	107-C20
10	10-A18	26	26-A18	42	42-B19	58	58-B19	75	75-B19	91	91-C20	108	108-C20
11	11-A18	27	27-A18	43	43-B19	59	59-B19	76	76-B19	92	92-C20	109	109-C20
12	12-A18	28	28-A18	44	44-B19	60	60-B19	77	77-B19	93	93-C20	110	110-C20
13	13-A18	29	29-A18	45	45-B19	61	61-B19	78	78-B19	94	94-C20	111	111-C20
14	14-A18	30	30-A18	46	46-B19	62	62-B19	79	79-B19	95	95-C20	112	112-C20
15	15-A18	31	31-A18	47	47-B19	63	63-B19	80	80-B19	96	96-C20	113	113-C20
16	16-A18	32	32-A18	48	48-B19	64	64-B19	81	81-B19	97	97-C20	114	114-C20

*A 18 – strains isolation in 2018 year, B 19 – strains isolation in 2019year, C 20 – strains isolation in 2020years

Distribute the medium in narrow tubes and sterilize at 1 atm, 20 min.

- Inoculation of the medium with the examined culture and cultivation at an appropriate temperature of 24 to 48 hours.

- Regent preparation: Reagent of Kovach.

Solution A

Components	Quantity
p-dimethylaminobenzalde-hyde	5 g
Isoamyl alcohol	75 ml

The components are dissolved by gentle heating in a water bath and then stored in the refrigerator.

Solution B:_concentrated hydrochloric acid (HCl)

The Kovach reagent is prepared by mixing 3 volumes of solution A with 1 volume of solution B. Store in the dark and can be used for 2 months. Kovach reagent can be purchased ready-made from manufacturing companies.

0.2 ml of Kovach reagent is added to the culture and left for 24 or 48 hours, after which the test tubes are shaken well.

Hydrolysis of the hyporate
Procedure:

1. Preparation of a well-developed culture on a suitable solid medium in the form of light agar.

2. Prepare the solution on the substrate: Prepare 1% sodium hyporate (sodium hypurate – 0.25 g, distilled water – 25 ml). The solution should be sterilized by filtration through a bacterial filter (0.22 μm).

3. Preparation of quinhydrin reagent: ninhydrin – 3.5 g, acetone-butanol mixture (1 : 1) – 100 ml.

4. Prepare a moderately thick cell suspension from the developed culture in 2 drops of substrate solution (1% hyporat solution), pipetted into narrow tubes. Incubate the contents for 1 hour at 35–37 °C, then add 2 drops of ninhydrin reagent. This is followed by re-incubation for 15 minutes.

5. Interpretation of the results: Positive reaction – appearance of purple color within 15 minutes; adverse reaction – no staining within 15 minutes.

Acetoin formation (acetylmethylcarbinol, Voges-Proscauer reaction)

Procedure:
1. Preparation of WP environment:

Components	Quantity
Bacto peptone	7 g
K_2HPO_4	5 g
Glucose	5 g
Distilled water	1000 ml
pH 7.5	

The substrate is distributed in 2–3 ml tubes and sterilized at 0.5 atm, 20 min.

2. Preparation of reagents:

Reagent A: α-naphthol – 5 g, absolute ethanol – 100 ml. The reagent should be straw-colored, not darker.

Reagent B: CON – 40 g, distilled water – 100 ml.

3. Tubes with VP medium are inoculated with the examined cultures. Culture developed on agar medium is used for inoculum.

4. Inoculated test tubes are cultured for 4 days at an appropriate culture temperature.

5. At the end of the culture, add 0.6 ml of reagent A and 0.2 ml of reagent B to each test tube per milliliter of culture.

6. Interpretation: Positive reaction – appearance of red color; adverse reaction – no color or appearance of slightly pink.

Methyl-Roth test (MR – test)

Procedure:
1. Preparation of MR – HR environment

Components	Quantity
Bacto peptone	7 g
K_2HPO_4	5 g
Glucose	5 g
Distilled water	1000 ml
pH 7.5	

The substrate is distributed in 2–3 ml tubes and sterilized at 0.5 atm, 20 min.

2. Preparation of MR reagent: 0.25 g of methyl rot is dissolved in 100 ml of 75% ethanol. Tubes with MR – VP medium are inoculated with the

examined cultures. Culture developed on agar medium is used for inoculum.

3. Inoculated tubes are cultured for 4 days at a temperature appropriate to the culture.

4. Finally in culture, add one drop of methyl Roth solution to each test tube.

5. Interpretation: Positive reaction (MP-positive) – appearance of red color; Negative reaction – yellow appearance.

Results and Discussion

The results of our examination cover a period of three years. The study included only one breed of dog, the German Shepherd, which is the most common in our region. The performed study deals with dogs that had inflammation of the dental plaque as one of the most common diseases in dogs. For this test, 240 swabs were taken from 48 samples, 5 sterile swabs were taken from each dog and cultured on petri dishes with selective nutrient media. The age of the dogs from which the samples were taken is presented in a Table 3 A, B and C.

From the results presented in Table 3 A, B and C, it can be seen that, the age of the dogs from which the samples were taken in 2018 is from 3, 6 years to 7, 8 years. In the samples taken in 2019, the age of the dogs is from 3.3 to 6.7 years. And the samples taken in 2020, the age of the dogs is from 3,3 to 7,5 years. The results show that, the most common inflammation of the dental plaque in dogs of German shepherd breed occurs at the age of 4.2–6.7 years.

From the samples that, we have taken in 2018 and 2019 for examination and which are presented in Fig. 2 A, B and C show that the appearance of dental plaque in dogs can occur in both male and female dogs, and in the samples taken in 2020, it was noted that, most of the samples were taken from male dogs. From these results it can be concluded that the inflammation of the dental plaque in dogs of German Shepherd breed does not depend on the sex of the dog.

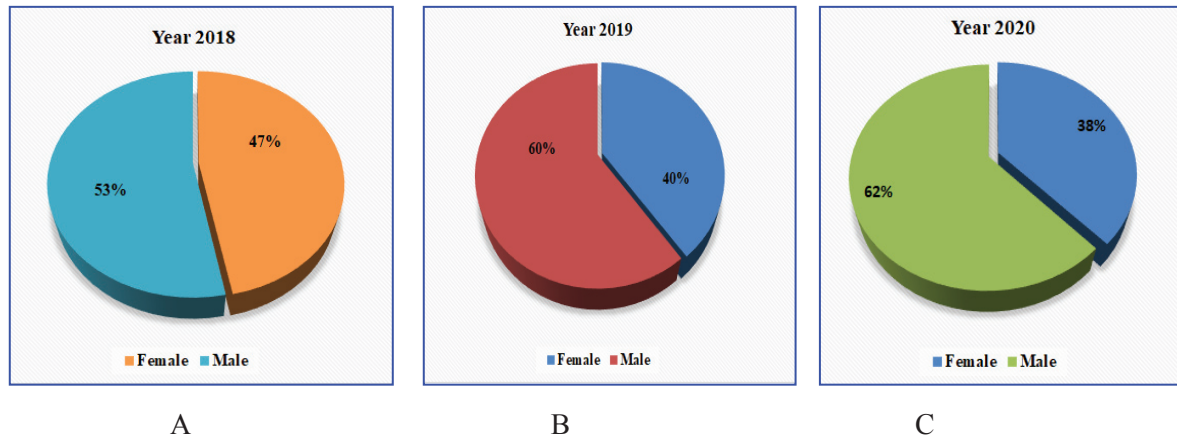**Table 3 A, B and C.** Age of dogs to the samples taken in 2018, 2019 and 2020
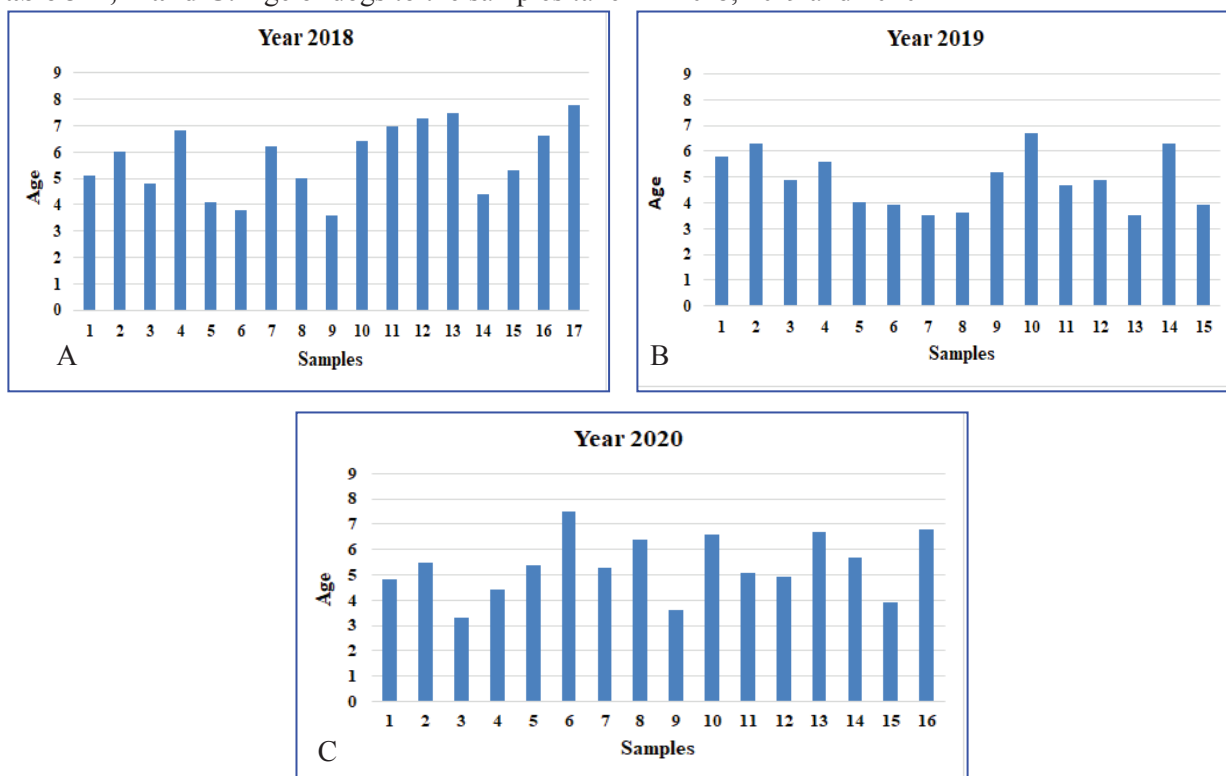
Fig. 1. A, B and C. Samples taken according to the sex of the animals for 2018, 2019 and 2020

Isolation and identification were performed on selective nutrients, five swabs were taken from each German Shepherd dog with inflammatory plaque, and further swabs were sown on five different substrates. The results on growth done on the five selective nutrient media are presented in (Table 4).

The results shown in Table 4 show that the swabs taken have good growth of the following selective nutrients: Baird Parker Agar, Bile eseulin azide and Veillonella. The results show that in one sample there may be more bacteria that cause inflammation of the dental plaque, which is confirmed by sowing swabs from one sample on different selective nutrient media (Table 4).

From the columns obtained on different selective media, 114 isolates were isolated using microbiological methods. The isolates were further tested using biochemical tests (Catalysis activity, Indole test, Hydrate hydrolysis, Acetoin formation (acetylmethylcarbinol, Voges-Proscauer reaction) and Methyl-Roth test.

According to our research over a period of three years and the results obtained, the most common bacterial causes of dental plaque inflammation in dogs of the Germanic Shepherd breed in the Pelagonija region are presented in Figure 2 A, B and C.

The results obtained from the examination of the samples we took in 2018, showed that the most common bacteria that, cause inflammation in dogsare, regardless to the sex of the dogs and the following genera of bacteria are identified: *Stapylococcus, Streptococcus, Enterococcus, Bacterioides, Prevotella* and *Lactobacillus*. More than 50% of the occurrence of dental plaque inflammation in 2018 of dogs is caused by two genera of bacteria, namely *Staphylococcus* 25% and *Streptococcus* 22%. From the samples taken for tests in 2019, it can be seen that in the bacteria that cause inflammation of the dental plaque in dogs, that there is a slight increase in the percentage of all types of bacteria. The samples taken for testing in 2020 show almost the same results as in the previous two years, with the exception of some genera where there is an increase. From results obtained in the three years we have covered, it can be concluded that the most common causes of dental plaque inflammation in dogs of the German Shepherd breed in the Pelagonija region are the genus *Staplylococus, Streptococcus Enterococcus*. Isolated and identified genus from us such as *Bacterioids, Prevotella* and *Lactobacillus,* although in a much smaller percentage, are still important in diagnosing and treating a dog with inflamed plaques. The results obtained from our examinations coincide with other examinations made by other scientists, for example (Gibbons, 1972; Tanzer et al., 1974;

Table 4. Growth on five selective nutrient media

Samples						Samples						Samples					
	Growth						Growth						Growth				
	BPA	BEA	VAB	BAVA	MRS		BPA	BEA	VAB	BAVA	MRS		BPA	BEA	VAB	BAVA	MRS
1	+	+	-	-	+	2	-	+	-	+	-	3	+	+	-	-	+
4	-	+	-	-	-	5	+	-	+	-	+	6	-	-	+	+	+
7	+	-	-	+	-	8	-	+	-	-	-	9	+	-	+	-	-
10	-	+	+	-	-	11	+	+	-	+	+	12	-	-	+	+	+
13	+	-	-	-	+	14	-	-	+	-	-	15	-	+	-	+	+
16	+	+	+	-	-	17	+	-	+	+	-	18	+	-	-	-	+
19	-	-	+	-	-	20	-	+	+	-	-	21	-	-	+	+	-
22	+	-	-	+	-	23	+	-	-	+	+	24	-	+	+	+	-
25	-	+	-	-	-	26	-	-	+	+	+	27	+	-	-	+	+
28	-	-	+	-	+	29	+	+	+	-	-	30	-	-	+	+	-
31	+	-	-	+	-	32	-	+	-	+	-	33	+	+	+	-	-
34	-	+	-	-	+	35	+	-	-	+	+	36	-	+	-	+	+
37	+	-	+	-	-	38	+	-	-	-	+	39	+	-	+	+	-
40	+	+	-	+	-	41	-	+	+	+	-	42	-	+	+	-	-
43	-	-	+	-	-	45	+	-	+	+	+	45	+	+	+	-	-
46	+	-	+	+	-	47	-	-	+	+	+	48	+	-	-	+	+

BPA-*Baird Parker Agar*   **BEA**-*Bile eseulin azide*   **VAB**-*Veillonella Agar Base*   **BAVA**-*Bacteroides Amikacin Vancomycin Agar* **MRS**
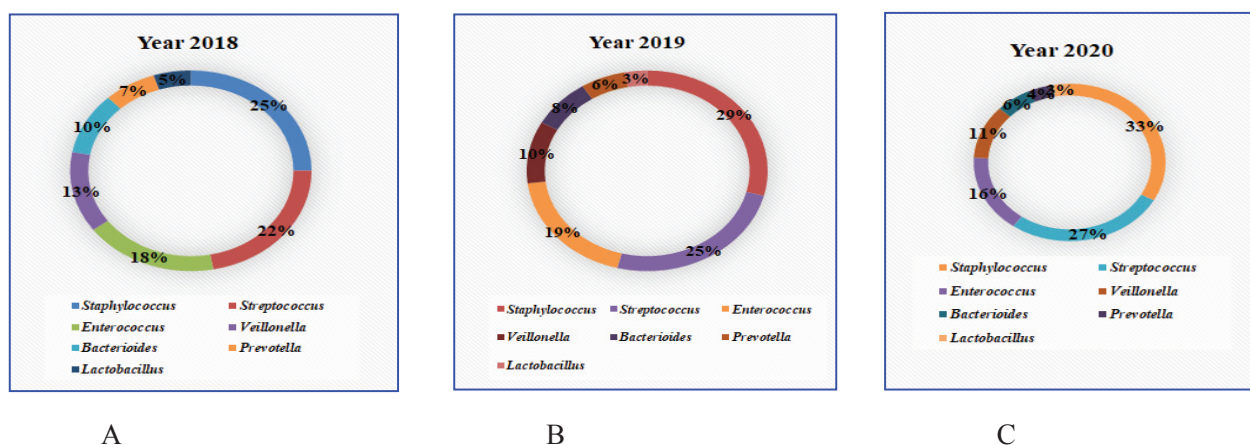
Fig. 2. A, B and C. Bacteria that cause inflammation of the dental plaque in dogs of German Shepherd breed for a period of three years in the Pelagonija region

Duchin and Houte, 1978; Slee and O'Connor, 1983; Slee et al., 1983; Corner et al., 1988; Murray et al., 1992; Harvey and Emily, 1993; Lang et al., 1997; Domingues et al., 1999; Rosan and Lamont, 2000; Loesche and Grossman, 2001; Katsura et al., 2001; Drummond et al., 2004; Roza, 2004; Swerts et al., 2005; Marsh, 2006; Daboor et al., 2015).

Conclusion

It was found that: (I) Dental plaque inflammation in the growth of German Shepherd dogs most commonly occurs between the ages of 4.2 and 7.2 years. (II) Inflammation of dental plaque does not depend on the sex of the dog. (III) The bacteria that cause inflammation can be anaero-

bic and aerobic. (IV) In the period of three years the inflammation causes dental plaque in dogs are one and the same bacteria. (V) In our region the most common genera that cause dental plaque inflammation in the German shepherd dog breed are *Staphylococcus, Streptococcus and Enterococcus*.

Recommendations:

• Dental plaque in dogs should be washed regularly with a special paste for pets. The use of other toothpastes can cause poisoning in dogs due to fluoride, which is poisonous to dogs.

• Feed your dog dry food, as liquid food can remain in the teeth and be a factor in the growth of bacteria.

• In samples taken for testing, the presence of three different bacteria was detected in one sample, this can help treat dogs that have plaque inflammation

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