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# The Change of Bacterial Spectrum after Storage of *X. campestris* pv. *campestris* Inoculated Cabbage Heads (*Brassica oleracea* var. *capitata* L.)

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**Abstract:** Changes in the bacterial spectrum of cabbage heads after storage under commonly used storage conditions were examined in this study. Cabbage seeds (*Brassica oleracea* var. *capitata* L.) were artificially inoculated with *X. campestris* pv. *campestris* (Xcc), a serious pathogen of cruciferous plants causing black rot. Isolation of bacterial cultures from Xcc-inoculated and non-inoculated cabbage heads were carried out in two time points—at the day of harvest and after four months of storage. According to our previous research and literature reports, the most frequent genera of bacteria were chosen for PCR testing, i.e., *Bacillus cereus* group, *Bacillus subtilis* group, *Pseudomonas* sp., and *X. campestris* pv. *campestris*. A few of the obtained bacterial cultures were negative for the four above-mentioned species. In those, other bacteria were identified by 16S rRNA sequencing. In both Xcc-inoculated and non-inoculated cabbage heads, changes of the bacterial spectrum over time were observed. The severity of Xcc infection of heads increased after four months of storage. *Bacillus* species represented the most frequently occurring bacterial genus. The presence of the *Bacillus subtilis* group increased significantly after storage in non-inoculated cabbage heads. The minor part of the other genera identified by sequencing in the first sampling were not detected in the stored cabbage heads. This was associated with a possible antagonistic behavior of *Pseudomonas* sp. and *Bacillus* sp.

**Keywords:** black rot; sequencing; post-harvest; storage; vegetable; *Pseudomonas*; cabbage; *Bacillus*

## 1. Introduction

Bacterial soft rots are very important post-harvest diseases of many vegetable crops and cause great losses in stored vegetables [1,2]. Cabbage (*Brassica oleracea* var. *capitata*), an economically important crop, is susceptible to bacterial soft rot, caused by *Pectobacterium carotovorum* subsp. *carotovorum* [*Erwinia carotovora*] [3]. Besides *Erwinia* species, major causal agents of bacterial soft rots of cruciferous vegetables are various species of *Pseudomonas, Bacillus, Lactobacillus* and *Xanthomonas campestris* pv. *campestris* [1,4]. Generally, soft rot occurs in stored fleshy vegetables and it is spread worldwide. The estimated losses caused by bacterial soft rot in stored cabbage vary between 15–30% of the harvested yield. The disease symptoms can be observed in the field, storage, or during transport and

marketing [5]. Latent infection in harvested vegetables is not unusual and may cause serious damage upon storage, especially in poor transport and storage conditions with higher temperatures and high humidity [6]. *Xanthomonas campestris* pv. *campestris* (Xcc), the causal agent of black rot, is one of the most serious pathogens of cabbage and related cruciferous vegetables worldwide, particularly under warm and wet conditions [4]. Primary infection source of Xcc is plant debris, soil or seeds [7–10]. Secondary transmission of the pathogen from infected plants proceeds through cruciferous weeds, insects and agrotechnical procedures in the field supported by rain or sprinkler irrigation [9,11–13]. Pathogen enters the plant through hydathodes and wounds, and colonizes its vascular system, causing V-shaped chlorotic or necrotic lesions on leaf margins often with dark-colored veins. However, the symptoms are variable among the cruciferous species [14,15]. Heavily infected leaves of cabbage fall off and soft rot may occur caused by secondary invasion of *Pseudomonas* species or *Erwinia* species [15]. There is no effective chemical control for the bacteria causing bacterial soft rot [16]. Hot water treatment is recognized as a simple control technique [17]. However, it is associated with higher energy costs [18] and a low suppression effect was reported by some authors [19]. Chlorination using sodium or calcium hypochlorite can be applied as a preventive treatment, but its effectiveness is also limited and can damage the vegetables [20]. The use of proper packing material (e.g., alum, newspaper wrap) can reduce mechanical damage particularly during transport, thus decreasing the incidence and spread of bacterial soft rot pathogens [3]. According to several authors, the antagonistic behavior of *Pseudomonas* and *Bacillus* species against other bacterial genera has been presumed [21–23]. Antagonistic effects of *Bacillus* sp. against Xcc was reported by Wulff [24].

Many studies focused on the survival of Xcc in plant debris, in the field or on seeds [7–13,25–27]. The black rot disease quickly develops in warm, humid conditions and can spread rapidly by rain dispersal and irrigation [28]. Unveiling the persistence of Xcc in 1–2 °C, the usual storage temperature, is challenging. Currently, it has not been reported. Hence, the main aim of this study was (i) to describe the persistence of Xcc in Xcc-inoculated cabbage heads during storage; (ii) to compare the spectrum of the most frequent bacterial genera of cultivable bacteria on cabbage heads inoculated and non-inoculated with Xcc, and (iii) to measure the relative frequency of *Bacillus* and *Pseudomonas* species, as related to the presence of Xcc on inoculated cabbage heads.

## 2. Material and Methods

### *2.1. Plant Material and Sampling*

The experiment was set up using head cabbage (*Brassica oleracea* var. *capitata*) cv. Avak (Moravoseed, Mikulov, Czech Republic) which is suitable for immediate consumption as well as for storage (Moravoseed, Mikulov, Czech Republic). Avak is an open-pollinated cultivar, very popular in the Czech Republic. It was selected based on our previous experience of showing the black rot symptoms in conditions suitable for Xcc reproduction. Approximately 0.2 g cabbage seeds were artificially inoculated with Xcc (isolate HRIW 3811, UK). The inoculation was performed by soaking seeds in a bacterial suspension containing approximately $10^8$ CFU/mL for two hours at room temperature, followed by drying seeds on the filter paper in a ventilation hood [29], as proposed previously [30]. In total, 60 seedlings, 30 Xcc-inoculated and 30 non-inoculated plants, were grown under controlled conditions in the growth chamber. At the stage of four true leaves, 20 plants from both groups were randomly selected. In total, 40 plants were transferred to the field (Lednice, Czech Republic, GPS 48.7999189N, 16.8033931E) on June 2017. The plants were cultivated using standard practices, the plants were optimally fertilized and irrigated. The isolation distance between Xcc-inoculated and non-inoculated plants was 30 m, separated by cereals. The field was weeded manually. After three months of cultivation, cabbage heads were harvested by pulling and the roots were cut out in the laboratory with a sterile knife. The average head weight was 2.2 kg in both treatments.

The cabbage heads were stored in the storage hall at 1–2 °C and high humidity (95% RH) controlled by an air conditioner, which is recommended for the storage of cabbage. The cabbage heads were

stored in perforated plastic boxes. The treatments were put in different places in the storage hall to avoid cross-contamination. The sampling before storage was done in October 2017, at the day of harvest, and after four months of storage in February 2018. At both time points, 10 Xcc-inoculated and 10 non-inoculated cabbage heads were analyzed.

The weather conditions during the experiment were recorded by the weather station located in Faculty of Horticulture (Lednice, Czech Republic, GPS 48.791470, 16.801696). The station measures temperature three times per day (7 a.m., 2 p.m., 9 p.m.), relative humidity, hours of sunlight, soil temperature and amount of precipitation.

### 2.2. Isolation of Bacteria From Cabbage Heads

The presence of bacteria was evaluated in three different head parts: cabbage stalk, inner leaves and superficial leaves (Figure S1). From each part, three pieces of plant tissue (4 × 4 mm) were disinfected in 2% sodium hypochlorite solution and washed twice in sterile distilled water according to Eichmeier et al. [31]. Tissue samples were placed on two different media, both supplemented with 0.05 g/L cycloheximide (Biosynth, Staad, Switzerland) to avoid fungal contamination. One piece of tissue per dish was used. For the description of Xcc persistence in cabbage heads through storage and its possible elimination by naturally present antagonists, the semi-selective medium PXCAB (Phyto Xano Camp Agar Base, HIMEDIA®, Mumbai, India) was used. The original medium was developed by Chang [32] for the isolation of *Xanthomonas campestris* which was then modified by lowering the pH of the medium using additional potassium dihydrogen phosphate. Bacterial composition and its changes after storage were evaluated on a non-selective medium MPA (Meat-Peptone Agar, Sigma-Aldrich, St. Louis, USA). All samples were cultivated at 25 °C in the dark and the growth of bacterial cultures was observed once a day. All cultures obtained from Petri dishes with MPA were re-cultivated to separate the present genera and identify them through PCR using genus-specific primers or through Sanger sequencing of the PCR amplicons.

### 2.3. Detection of the Bacterial Group, PCR, and Sequencing

The samples were tested by PCR using four primer pairs targeting the *motB* gene sequence of *Bacillus cereus* group, the *gyrA* gene of *Bacillus subtilis* group, the 16S rRNA gene of *Pseudomonas* sp., and the *hrpF* gene of *Xanthomonas campestris* pv. *campestris* (Table 1).

**Table 1.** Specific primer pairs used for identification of the four targeted bacterial genera.

| Genus | Primer Pair | Sequence 5′-3′ | Product Size | Gene | AT * | Ref. |
|---|---|---|---|---|---|---|
| *Bacillus cereus* group | BCFomp1 | ATCGCCTCGTTGGATGACGA | 575 pb | *motB* | 55 °C | [33] |
| | BCRomp1 | CTGCATATCCTACCGCAGCTA | | | | |
| *Bacillus subtilis* group | gyrA-f | CAGTCAGGAAATGCGTACGTCCTT | 1024 pb | *gyrA* | 54 °C | [34] |
| | gyrA-r | CAAGGTAATGCTCCAGGCATTGCT | | | | |
| *Pseudomonas* sp. | Pseudomonas_F | CTACGGGAGGCAGCAGTGG | 150 pb | 16S rRNA | 62 °C | [35] |
| | Pseudomonas_R | TCGGTAACGTCAAAACAGCAAAGT | | | | |
| *Xanthomonas campestris* pv. *campestris* | DLH 120 | CCGTAGCACTTAGTGCAATG | 618 pb | *hrpF* | 63 °C | [36] |
| | DLH 125 | GCATTTCCATCGGTCACGATTG | | | | |

* AT—annealing temperature.

In cases where none of the four targeted species was detected, DNA was extracted using 5 mg of bacterial culture according to Roothie and Umesha [37]. The identity of isolates was determined by Sanger sequencing of the V3-V4 region of 16S rRNA [38], as described by Eichmeier et al. [31]. CLC Genomics Workbench 6.0 (CLC Bio, Aarhus, Denmark) was used to analyze the obtained sequences. The genus of bacteria was determined with similarities higher than 90% in GenBank/NCBI, as proposed by Klindworth et al. [38].

### 2.4. Statistical Analysis

PAST 3.03 [39] was used for the statistical analysis. Bray-Curtis distance was used as a metric of the similarity of bacterial species composition between samples. The effect of the sampling date, the Xcc inoculation and the head part were tested by one-way and two-way PERMANOVA using Bray-Curtis distances ($n$ = 99,999). In all cases, differences at $p < 0.05$ were considered as statistically significant.

The relation of bacteria presence/absence to (i) the sampling time point, (ii) inoculation with Xcc and (iii) the cabbage head part (cabbage stalk, inner leaves and superficial leaves) was analyzed by multivariable analysis, using Canoco 5 (Biometris, Wageningen University and Research centre, Wageningen, The Netherlands; University of South Bohemia in České Budějovice, České Budějovice, Czech Republic). Since the effect of the cabbage head part was not significant, this factor was excluded from subsequent analyses. Canonical correspondence was chosen as a statistical method [40]. Significance level was calculated by Monte-Carlo permutation test calculated by 999 permutations. The analyzed data comprised results from testing all three cabbage head parts, thus in each plant the bacteria genera were detected from 0 to 3 times.

## 3. Results

### 3.1. Persistence of Xcc on Cabbage Heads after Storage

In case of plants from Xcc-inoculated seeds, the presence of Xcc was confirmed in five heads (50%) in the first sampling point. Four of these heads showed typical symptoms of the black rot, V-shaped lesions on superficial leaves or black veins. Xcc was also detected in one head from the non-inoculated control, although all control heads were symptomless with respect to the typical symptoms caused by Xcc. After four months of storage, the number of heads with Xcc increased to seven (70%) within Xcc-inoculated plants and almost all of them showed the symptoms. Xcc was also detected on three heads (30%) after storage (Table 2, Figure 1). Symptoms on the Xcc-inoculated and non-inoculated cabbage heads after storage are shown in Figures S2 and S3.

**Table 2.** Number of heads with positive *X. campestris* pv. *campestris* (Xcc) detection.

| | Before Storage | After Storage |
|---|---|---|
| **Xcc inoculated** | 5 | 7 |
| **Non-inoculated** | 1 | 3 |

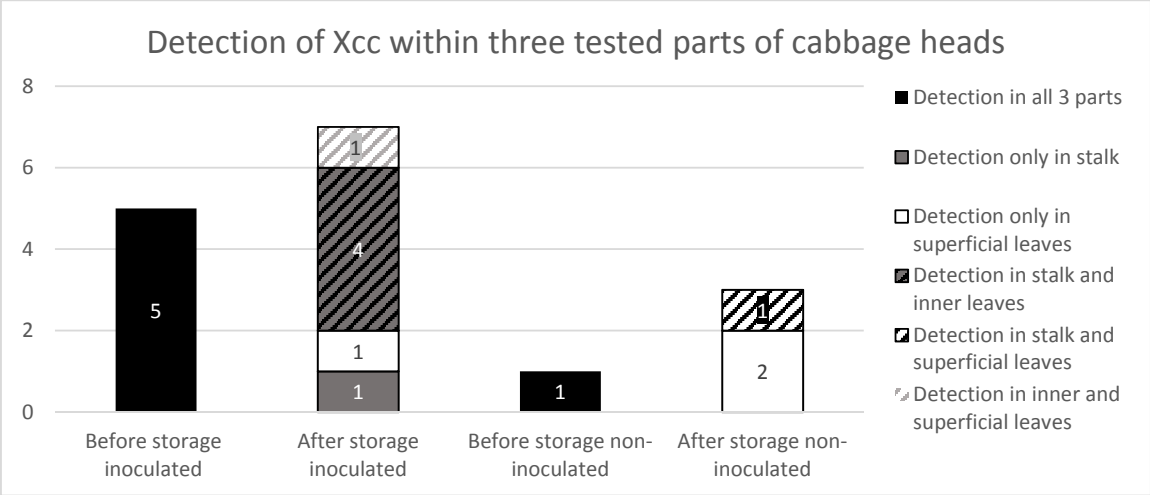


**Figure 1.** Detection of Xcc within three parts of inoculated and non-inoculated cabbage heads at harvest and after storage.

*X. campestris* pv. *campestris* was detected in all three parts of five Xcc-inoculated cabbage heads. However, in the stored cabbage heads the Xcc was not detected on all three parts of the seven Xcc-positive cabbage heads. After storage, Xcc was present mostly on stalks and inner leaves. Similarly, Xcc was detected on all three parts of one non-inoculated cabbage head before storage, while after storage was Xcc present on inner leaves of two heads on superficial leaves of one non-inoculated head (Table 2, Figure 1).

### 3.2. Changes of Bacterial Spectra Present in Cabbage Heads Inoculated and Non-Inoculated with Xcc

At harvest, *Pseudomonas* and *Bacillus* species were the genera with the highest occurrence. *Pseudomonas* sp. was present on all ten Xcc-inoculated heads and ten non-inoculated heads; while *Bacillus cereus* group was detected on eight non-inoculated heads. The minor bacterial genera, not detected using the PCR assay, were identified by sequencing as *Acinetobacter, Chryseobacterium*, and *Enterobacter*.

Two bacterial cultures from the sampling at the time of harvest of Xcc-inoculated heads and four from non-inoculated heads could not be identified, as they were with the most similar to undetermined bacteria in GenBank/NCBI (Figure 2).

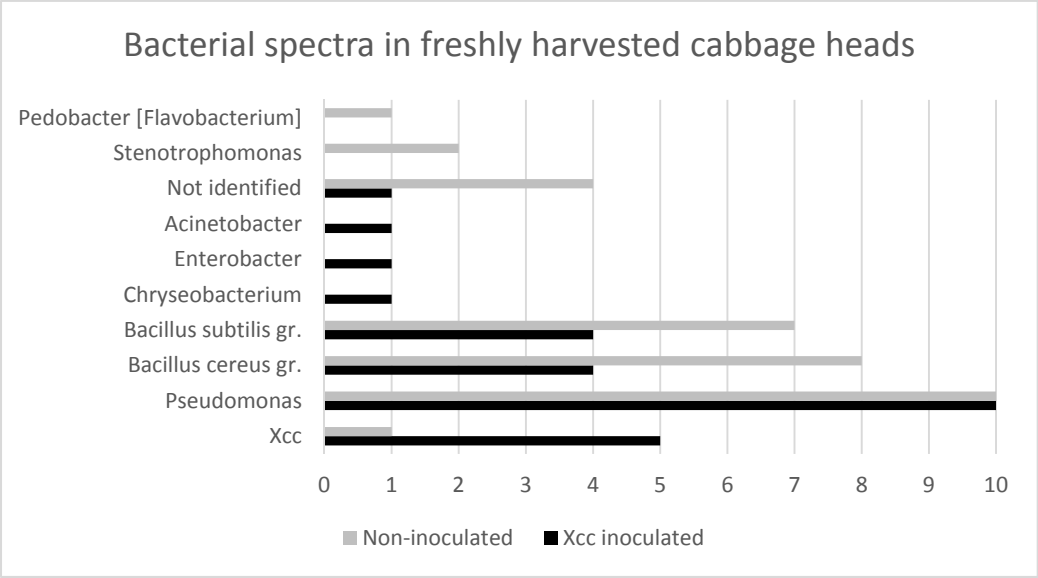


**Figure 2.** The number of cabbage heads from the sampling at the day of harvest of Xcc-inoculated and non-inoculated cabbage heads with positive detection of the bacterial genera.

After storage, no other bacterial genera besides four tested ones were present on cabbage heads. The *Bacillus cereus* group was detected only in one Xcc-inoculated cabbage head, however, its presence markedly decreased (Figure 3).

### 3.3. Effect of Bacillus and Pseudomonas Species to Xcc Incidence

The result of CCA (canonical correspondence analysis) indicates, that the occurrence of Xcc was the most frequent within Xcc-inoculated cabbage heads and even more prevalent in the stored heads (Figure 4). In contrast, the *Bacillus subtilis* group was present more often in non-inoculated heads. *Pseudomonas* species and the *Bacillus cereus* group were more often detected on freshly harvested heads. The analysis indicates a negative correlation between the presence of Xcc and the *Bacillus subtilis* group. The mutual appearance of these genera within the three parts of cabbage heads was the least frequent. no correlation was found between *Pseudomonas* and Xcc or factors determining the relationship were not included in the analysis. Analyzed data and results summary: DF = 2; total variation is 0.90033; pseudo-F = 13.7; $p = 0.001$.

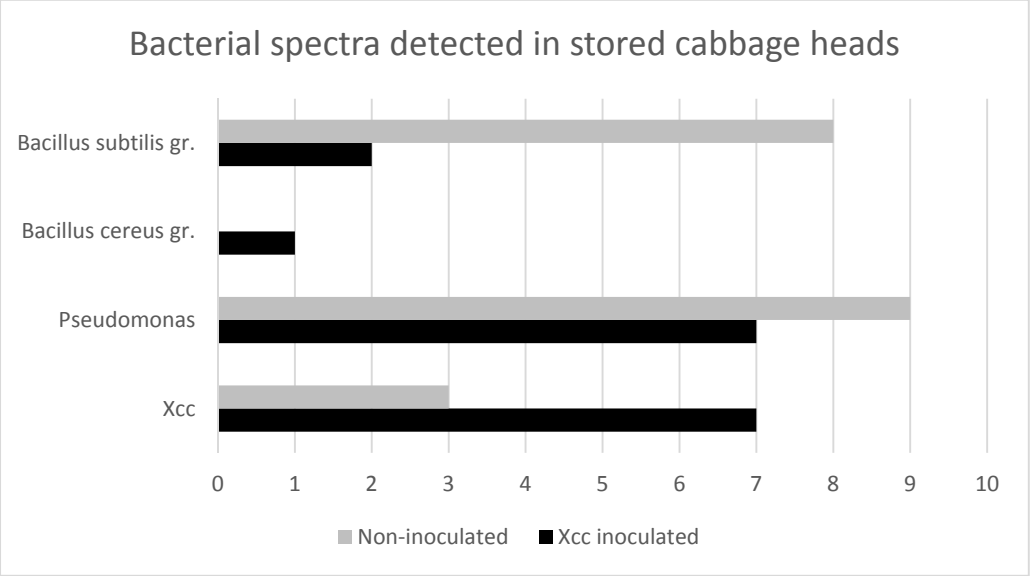


**Figure 3.** The number of cabbage heads from the sampling after storage of Xcc-inoculated and non-inoculated cabbage heads with positive detection of the bacterial genera.

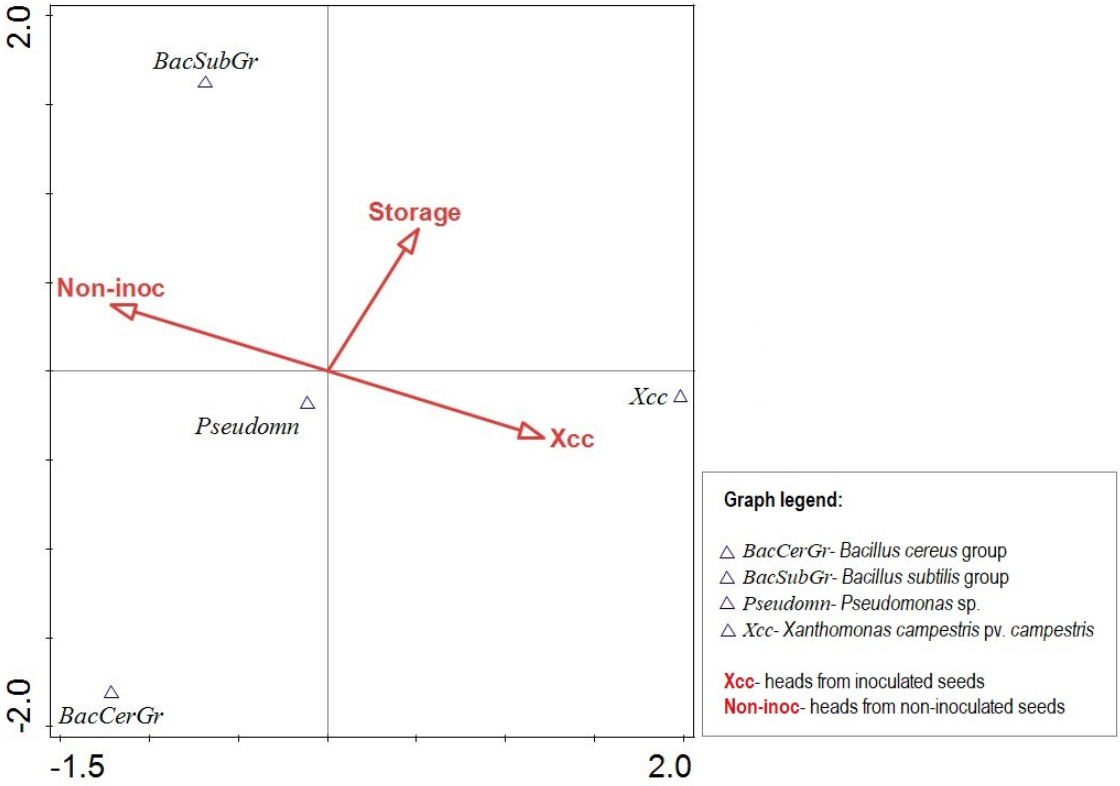


**Figure 4.** CCA ordination diagram shows the relationships between the presence of the bacterial genera and two factors including the time point of sampling (at the harvest and after storage) and the artificial inoculation with Xcc.

PERMANOVA indicated that the presence of bacteria is significantly determined by the date of sampling ($p = 0.00009$) and inoculation with Xcc ($p = 0.00005$). Sampling different head parts did not affect the composition of the bacterial spectrum significantly ($p = 0.7577$). Two-way PERMANOVA showed that the sampling date and inoculation with Xcc were significant (both $p = 0.00001$), while their interaction was not.

## 4. Discussion

### *4.1. Persistence of Xcc on Cabbage Heads during Storage and Potential Antagonistic Effect of Bacillus Species*

The optimal growth temperature for *X. campestris* pv. *campestris* varies between 25–30 °C and spreading of the pathogen is strongly facilitated by humid and wet conditions (e.g., heavy rains, overhead irrigation) [4,41,42]. During summer months (June, July, and August) of 2017 in Lednice, 58% of the days reached maximum daily temperatures above 28 °C and average relative humidity from May to September was low (38%). Such weather conditions were not favorable to the development of black rot symptoms even on plants produced from artificially Xcc-inoculated seeds [28].

Despite the fact that the growth-limiting temperature for Xcc was estimated as 5 °C, the increase of Xcc occurrence after storage was observed in this study [42]. On Xcc-inoculated cabbage heads, Xcc presence was higher on cabbage stalks and inner leaves, while on non-inoculated heads the infection on superficial leaves was more frequent and no infection was detected on inner leaves. These findings suggest that inoculation with Xcc was successful, while some cabbage heads from non-inoculated seeds were naturally infected in the field and Xcc symptoms developed from the inside during the post-harvest period.

The negative correlation between the presence of *X. campestris* pv. *campestris* and *Bacillus subtilis* group indicates antagonistic potential of the latter microorganism. Such a behavior was reported for some isolates of naturally occurring epiphytic non-pathogenic bacteria (*Bacillus* species and *Pseudomonas fluorescens*) against *X. axonopodis* pv. *phaseoli* [43] or *X. campestris* pv. *campestris* [24]. However, to support this statement, detailed research focused on species identification of *Bacillus* genera is necessary. Besides, various species of *Pseudomonas* and *Bacillus* are also causal agents of soft rot [5].

### *4.2. Changes of Bacterial Spectra Presented in Cabbage Heads Inoculated and Non-Inoculated by Xcc*

We showed significant differences in the bacterial spectrum of cabbage heads, in relation to the time point of sampling (before and after storage) and seed inoculation with Xcc. Plants after harvest are generally more vulnerable to infections due to loss of their defense mechanisms against microorganisms [44]. An increase of microbial populations was confirmed by King and Bolin in stored lettuce [45]. *Pseudomonas* species appeared more on cabbage heads at harvest, however, the bacterial spectrum changed over time in favor of this genus. The decline of other bacterial genera after four months of cold storage was also observed in our previous study [21] and it could be explained by strong antagonistic behavior of *Pseudomonas* sp. [46] and *Bacillus* species against other bacteria [47,48].

*Bacillus* species (i.e., *Bacillus subtilis*, *Bacillus cereus*) are widespread in the environment and can be found in soil, foods of plant origin, e.g., grains, vegetables, raw and cooked rice, further in egg white, milk, and meat [49–52]. Spores of *Bacillus* species can grow and germinate at refrigeration temperatures on different vegetable substrates providing optimal pH for bacterial growth [53,54]. In this study, the presence of the *B. subtilis* group in control heads was similar in both samplings and detection of the *B. cereus* group decreased markedly after storage. However, in our previous studies, the increase of the latter genus during storage was observed [21]. Species belonging to *Bacillus* genera are inconsistently reported as food poisoning microorganisms frequently present in cooked and slowly cooled-down food [55,56], while on the other hand some are considered as potential bio-control agents against postharvest pathogens of vegetables and fruits [48,57].

## 5. Conclusions

The results showed that Xcc, a causal agent of black rot and bacterial soft rot, can survive at 1–2 °C after four months on Xcc-inoculated cabbage heads. *Bacillus* and *Pseudomonas* were the most frequent bacterial genera on cabbage heads. The storage of cabbage heads markedly decreased the diversity of bacterial genera. *Pseudomonas* species have no considerable effect on Xcc persistence on cabbage heads.

The *B. subtilis* group showed an opposite pattern of occurrence on cabbage heads, as compared to Xcc, which will be the subject of further research.

**Supplementary Materials:** The following are available online at http://www.mdpi.com/2073-4395/10/3/443/s1; Figure S1: Definition of cabbage stalks, inner leaves and superficial leaves; Figure S2: Symptoms on Xcc-inoculated cabbage heads after storage; Figure S3: Symptoms on non-inoculated cabbage heads after storage.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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