Article

Effect of the Addition of Milk Protein Preparations on Selected Quality Parameters and Nutritional Characteristics of Kefir

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Abstract: The aim of this study was to evaluate the effect of the addition of various milk protein powder preparations (MPC-80, WPC-70, sweet whey, non-demineralized or demineralized whey, whey permeate, rennet casein, buttermilk) to selected quality parameters and digestibility of milk proteins in kefir. Kefir samples analyzed in the study were prepared under laboratory conditions with three industrial bacterial starter cultures. They were examined microbiologically (lactobacilli, lactococci, yeast) and for pH, total protein content, hardness, adhesiveness, water-holding capacity (WHC), and protein digestibility (using two in vitro methods along with the determination of the content of available lysine and glycine). The counts of lactococci and lactobacilli were estimated at levels above 7 and 6 log(CFU/mL), respectively. Yeast was not found in the kefir samples. The pH value of the samples was 4.4–5.2. The pH, hardness, adhesiveness, and WHC were dependent on the type of kefir starter culture and the type of milk protein powder added. Each protein preparation added increased the amount of available lysine and glycine. However, when converted for 1 g of kefir protein, the changes in the content of available lysine and glycine were variable and depended on both the milk protein powder and starter culture used.

Keywords: fermented milks; kefir starter culture; hardness; adhesiveness; milk proteins digestibility; milk protein; whey protein; casein; buttermilk

Citation: Ziarno, M.; Hasalliu, R.; Cwalina, A. Effect of the Addition of Milk Protein Preparations on Selected Quality Parameters and Nutritional Characteristics of Kefir. *Appl. Sci.* **2021**, *11*, 966. <https://doi.org/10.3390/app11030966>

Academic Editor: Maria Kanellaki

Received: 6 January 2021

Accepted: 19 January 2021

Published: 21 January 2021

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1. Introduction

Kefir is fermented milk, traditionally made using cow's milk or goat's milk, originated from parts of Eastern Europe and Southwest Asia, which is usually produced using kefir grains or starter cultures containing the microflora that are necessary for carrying out lactic and alcoholic fermentation characteristic of kefir formation (i.e., lactobacilli, lactococci, yeasts, or sometimes acetic acid bacteria) [1–3]. Kefir regulations vary worldwide. Traditional kefir, made by adding kefir grains to milk, contains yeast (lactose fermenting yeasts such as *Kluyveromyces marxianus*, and non-lactose-fermenting yeasts such as *Saccharomyces unisporus*, *Saccharomyces cerevisiae*, and *Saccharomyces exiguus*) and lactic acid bacteria (mainly lactobacilli, but also *Leuconostoc*, *Lactococcus*, and *Acetobacter* growing in a strong specific relationship). Modern kefir-like fermented milks, obtained by applying kefir starter cultures, does not always contain yeast (or it contains only selected yeast groups, for example that ferment lactose such as *Kluyveromyces marxianus*, or do not ferment lactose, such as *Saccharomyces*), and it can contain only lactic acid bacteria. The technology used for the production of fermented milk involves the use of various additives for processing milk which improves the textural, sensory, and nutritional properties of the final products. In addition to the use of skimmed milk powder (SMP) or concentration of the processing milk in a vacuum evaporator, other forms of milk proteins, such as milk protein concentrates (MPCs) or whey protein concentrates (WPCs) are used to increase the solid content of the

milk base. Each of these additives produces a different effect on the quality and nutritional characteristics of fermented milk. For example, WPC has a higher biological value than SMP and stimulates the growth of several strains of lactic acid bacteria [4].

Kefir contains essential amino acids and easily digestible proteins, which when digested help the body to maintain its functions [5]. In recent decades, several innovative kefir drinks have been introduced, including those enriched with ingredients such as milk proteins which render the drink with specific and valuable properties [6–8]. The nutritional characteristics of kefir are related to the bioavailability of its ingredients. Bioavailability refers to the degree to which a nutrient has been released and absorbed from the digestive system and used by the human body. Digestibility in turn is related to bioavailability which refers to the susceptibility of products or nutrients to the activity of digestive enzymes. The digestibility of a food product is determined by its ingredients, including proteins, carbohydrates, and fats, or the food as a whole [9,10]. It is assessed by calculating the coefficients of the digestive process of a human or animal organism—digestion *in vivo*—or by testing the level of the enzymatic hydrolysis of meals under laboratory conditions—digestion *in vitro* [9–14].

This study aimed to evaluate the effects of the addition of various forms of milk proteins, including MPC, WPC, demineralized or non-demineralized whey, sweet whey, whey permeate, rennet casein, and buttermilk powders, on selected quality parameters and the digestibility of milk proteins in kefir. In addition, the study compared two methods applied for determining the digestibility of milk proteins using *in vitro* enzymatic digestion.

2. Materials and Methods

2.1. Materials

Kefir Starter Cultures. Three industrial bacterial starter cultures were used in this study: Kefir A—“Zakwaska do kefiru” (GAMA-TECH Leszek Marek Krześniak, Warsaw, Poland), mesophilic kefir culture containing *Lactococcus lactis*, *Streptococcus thermophilus*, *Leuconostoc mesenteroides*, and *Lactobacillus acidophilus* (or) *Bifidobacterium lactis*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, and *L. lactis* subsp. *lactis*; Kefir B—“VIVO” (GAMA-TECH Leszek Marek Krześniak, Warsaw, Poland), mesophilic kefir culture containing *L. lactis*, *S. thermophilus*, *L. mesenteroides*, and *L. acidophilus* (or) *B. lactis*, *L. delbrueckii* subsp. *bulgaricus*, *S. thermophilus*, and *L. lactis* subsp. *lactis*; Kefir C—Choozit Kefilmild 01 (DuPont Danisco, Copenhagen, Denmark), mesophilic kefir culture containing *L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris*, *L. lactis* subsp. *lactis* biovar. *diacetylactis*, *L. mesenteroides* subsp. *mesenteroides*, *S. thermophilus*, and *L. acidophilus*.

Milk Protein Powders. Eight commercial milk protein powders were used in this study: (1) MPC-80 (Hoogwegt Poland, Warsaw, Poland), MPC powder with a declared milk protein content of 80%; (2) WPC-70 (Mlekovita, Wysokie Mazowieckie, Poland), demineralized WPC powder with a declared whey protein content of 70%; (3) demineralized whey powder (AgroComplex, Wyszaków, Poland) preparation with a declared protein content of 80%; (4) non-demineralized whey powder (Promar, Zawiercie, Polska) preparation with a declared protein content of 65%; (5) sweet whey powder 6% (AgroComplex, Wyszaków, Poland), rennet whey powder preparation with a declared protein content of 6%; (6) whey permeate powder (Arla Foods Ingredients Group P/S, Viby, Denmark) preparation with a declared protein content of 3%; (7) rennet casein powder (Promar, Zawiercie, Polska) preparation with a declared protein content of 90%; and (8) buttermilk powder (OSM Łowicz, Łowicz, Poland) with a declared protein content of 11%.

2.2. Preparation of Kefir Samples

In the experiment, pasteurized cow milk “Łowickie” (OSM Łowicz, Łowicz, Poland) with a fat content of 2.0% was used. Milk protein preparations were added at an amount of 6 wt.% to a 1000-g portion of milk at 25 °C. The powders were thoroughly mixed in milk for 15 min with a laboratory homogenizing mixer (Silverson L4R, Silverson Machines Ltd., Chesham, England) and left for approximately 20 min at 25 °C to hydrate the preparations

and to allow swelling of the proteins contained in them. Then, the milk samples were poured into sterile glass jars (170 g each), sealed, and fermented at 25 °C for 24 h. After fermentation, the pH of the samples was measured to ensure the correctness of the process, and the samples were stored at 4 °C for 1 day before all analyses were performed. The experiment was performed twice.

2.3. Microbiological Analysis

Microbiological analysis was performed by the traditional plate method [15], according to the following standards: ISO 15214:1998 [16] and ISO 6611:2004 [17]. The number of each type of bacteria was determined with three repetitions. The following microbiological media were used for the analyses: MRS (de Man, Rogosa, and Sharpe) agar (Merck, Darmstadt, Germany), for determining the number of lactobacilli cells; M17 agar (Merck, Darmstadt, Germany), for determining the number of lactococci cells; and YGC (Yeast Extract Glucose Chloramphenicol) agar (Merck, Darmstadt, Germany), for determining the number of yeasts. All media were incubated at 30 °C for 72 h. The Petri dishes with the MRS agar were inserted in the anaerobic jars (Merck, Darmstadt, Germany), those containing the M17 agar were grown aerobically, and Petri dishes with the YGC agar were incubated aerobically for up to 5 days at 25 °C. The results are expressed as mean values of colony-forming unit per mL of kefir sample (CFU/mL) in two parallel replicates, and then as decadic logarithm.

2.4. Determination of the pH Value

The pH value was measured in well-mixed kefir samples using a CPO-505 digital pH-meter (Elmetron, Zabrze, Poland) calibrated at 25 °C with temperature compensation [18]. The measurements were made in three repetitions for each type of sample.

2.5. Texture Analysis

Hardness and adhesiveness of the kefir samples were analyzed using the method described by Ziarno and Zaręba [18]. A Brookfield CT3 10 K Texture Analyzer (Brookfield Engineering Laboratories Inc., Middleborough, MA, USA) was used for the analyses. During the analysis, the samples were not removed from the containers. A TA4/1000 probe (cylinder, 38.1 mm D, and 20 mm L) was used to determine the texture profile of samples (in triplicate) in 170-mL cups at 4 °C. The samples were compressed by 30 mm, with a trigger force of 4.5 g. The speed of the probe was fixed at 1 mm/s during the pretest compression and relaxation of the samples. In accordance with the manufacturer's instructions, hardness was defined as the force necessary to attain a given deformation (the maximal peak value recorded after the first immersion into the sample), while adhesiveness was expressed as the work necessary to break probe from the sample area (a product of the force necessary for emerging the probe from the sample and time).

2.6. Determination of Water-Holding Capacity (WHC)

The WHC of the kefir samples was determined using the method described by Ziarno and Zaręba [18]. Briefly, 15.00 g of the kefir samples was centrifuged at 3250 g for 10 min at 4 °C. Then, the supernatant was collected and weighed with an accuracy of two decimal places. WHC was calculated as follows: $WHC (\%) = (m_1/m_2) \times 100$, where m_1 is the mass of precipitate after centrifugation (g) and m_2 is the mass of the kefir sample (g). All the experiments were repeated three times.

2.7. Determination of Protein Content

Protein content was determined according to the standard ISO 89684:2016, Milk and milk products—Determination of nitrogen content—Part 4: Determination of protein and non-protein nitrogen content and true protein content calculation (reference method) [19].

2.8. Determination of Protein Digestibility

Protein digestibility was determined using two methods. The first method was described by Heś et al. [11]. The principle of this method is to determine the content of free lysine which in turn is used to assess the digestibility of protein [11]. Available lysine was determined using the method of Hall et al. [20]. The content of available lysine was expressed as mg per 100 g of protein. The analysis was carried out in three repetitions for each kefir sample.

The second method was described by Pokorska-Lis et al. [12,21]. Briefly, the principle of the method consists in subjecting the test samples to a two-step enzymatic hydrolysis process (first pepsin digestion in a hydrochloric acid environment and then trypsin digestion in a slightly alkaline environment) followed by spectrophotometric determination of the concentration of available glycine (reaction with ninhydrin) against a standard curve. The colored product formed from the reaction with ninhydrin was determined spectrophotometrically at a wavelength of 570 nm. The concentration of available glycine was calculated based on the standard curve [12,22]. The content of available glycine was expressed as mg per 100 g of product. The analysis was carried out in three repetitions for each kefir sample.

2.9. Statistical Analysis of Results

The results were statistically analyzed in Statgraphics XVII program (Statgraphics Technologies, Inc., The Plains, VA, USA), using multivariate analysis of variance and Tukey's test ($\alpha = 0.05$).

3. Results

3.1. Starter Microflora Population

The number of *Lactococcus* spp. in the kefir samples ranged from 7.8 to 9.2 log(CFU/mL) (Table 1). The type of kefir starter culture and milk protein powder used had a statistically significant effect on the *Lactococcus* spp. population ($p = 0.001$ in both cases). On the other hand, this population was not influenced by the final pH of kefirs ($p = 0.3761$). The number of lactobacilli in the kefir samples ranged from 6.8 to 8.2 log(CFU/mL) (Table 1). As observed for lactococci, the number of lactobacilli depended on the type of starter culture and milk powder used as an additive ($p = 0.001$ in both cases). The effect of the final pH of kefir samples on the number of these bacteria in kefir was also not found to be statistically significant ($p = 0.3669$). However, it is worth noting that the lactobacilli count was generally lower than the number of lactococcal cells. The presence of yeast was not observed at a dilution of 10^{-3} in any of the samples analyzed.

Table 1. Value of pH and lactic acid bacteria count in kefir samples (mean and standard deviation calculated from six repetitions).

Kefir Samples with the Addition of Milk Powder	pH	Lactococci Population (log(CFU/mL))	Lactobacilli Population (log(CFU/mL))
Kefir A			
control	4.4 ± 0.2 ^f	8.8 ± 0.0 ^{a,b}	7.8 ± 0.0 ^{a,b}
MPC-80	5.1 ± 0.1 ^a	9.2 ± 0.0 ^a	8.2 ± 0.0 ^a
WPC-70	5.1 ± 0.1 ^a	8.2 ± 0.1 ^d	7.2 ± 0.1 ^d
rennet casein	5.1 ± 0.1 ^a	8.9 ± 0.1 ^a	7.9 ± 0.1 ^a
sweet whey powder	4.7 ± 0.2 ^d	8.8 ± 0.1 ^{a,b}	7.8 ± 0.1 ^{a,b}
whey permeate powder	4.7 ± 0.2 ^{d,e}	8.8 ± 0.1 ^{a,b}	7.8 ± 0.1 ^{a,b}
whey powder (demineralized)	4.7 ± 0.2 ^e	8.8 ± 0.0 ^{a,b}	7.8 ± 0.0 ^{a,b}
whey powder (non-demineralized)	4.6 ± 0.2 ^e	8.6 ± 0.1 ^b	7.6 ± 0.1 ^{b,c}
buttermilk powder	4.6 ± 0.2 ^{e,f}	8.9 ± 0.2 ^a	7.9 ± 0.2 ^a

Table 1. Cont.

Kefir Samples with the Addition of Milk Powder	pH	Lactococci Population (log(CFU/mL))	Lactobacilli Population (log(CFU/mL))
Kefir B			
control	4.7 ± 0.2 ^e	8.7 ± 0.1 ^{a,b,c}	7.7 ± 0.1 ^{b,c}
MPC-80	5.1 ± 0.1 ^{a,b}	7.8 ± 0.3 ^e	6.8 ± 0.3 ^e
WPC-70	5.1 ± 0.1 ^{a,b}	8.3 ± 0.2 ^{d,e}	7.3 ± 0.2 ^d
rennet casein	5.2 ± 0.1 ^a	8.8 ± 0.4 ^{a,b}	7.8 ± 0.4 ^{a,b}
sweet whey powder	4.8 ± 0.2 ^c	8.4 ± 0.1 ^{c,d}	7.4 ± 0.1 ^c
whey permeate powder	4.7 ± 0.2 ^d	9.1 ± 0.0 ^a	8.1 ± 0.0 ^a
whey powder (demineralized)	4.8 ± 0.2 ^c	9.1 ± 0.0 ^a	8.1 ± 0.0 ^a
whey powder (non-demineralized)	4.8 ± 0.2 ^c	8.8 ± 0.0 ^{a,b}	7.8 ± 0.0 ^{a,b}
buttermilk powder	4.8 ± 0.2 ^c	8.5 ± 0.1 ^c	7.5 ± 0.1 ^b
Kefir C			
control	4.3 ± 0.2 ^g	9.0 ± 0.1 ^a	8.0 ± 0.1 ^a
MPC-80	4.9 ± 0.2 ^b	8.9 ± 0.1 ^a	7.9 ± 0.1 ^a
WPC-70	5.1 ± 0.1 ^a	9.2 ± 0.0 ^a	8.2 ± 0.0 ^a
rennet casein	4.8 ± 0.2 ^d	8.4 ± 0.6 ^{c,d}	7.4 ± 0.6 ^c
sweet whey powder	4.5 ± 0.2 ^f	9.2 ± 0.1 ^a	8.1 ± 0.1 ^a
whey permeate powder	4.5 ± 0.2 ^f	9.1 ± 0.0 ^a	8.1 ± 0.0 ^a
whey powder (demineralized)	4.4 ± 0.2 ^f	9.1 ± 0.0 ^a	8.1 ± 0.0 ^a
whey powder (non-demineralized)	4.5 ± 0.2 ^f	9.1 ± 0.0 ^a	8.1 ± 0.0 ^a
buttermilk powder	4.4 ± 0.2 ^f	9.1 ± 0.0 ^a	8.1 ± 0.0 ^a

^{a–g} the same letter indices within the same column do not mean statistically significant differences at the significance level of 0.05.

3.2. Acidity of Kefir Samples

The pH of milk significantly decreased ($p < 0.05$) after 24 h of fermentation with all kefir starters, although not all kefir samples were characterized by similar pH values. Statistical analysis showed that the pH value of the kefir samples significantly depended on the type of starter culture and milk protein powder used ($p = 0.001$ in both cases). In general, kefir C samples had lower pH values (average 4.6) than kefir A (average 4.8) and kefir B (average 4.9), and the samples prepared with WPC-70 (average 5.1), MPC-80 (average 5.1), or rennet casein powder (average 5.0) had a higher pH than the other kefir samples (average 4.6).

3.3. Texture Analysis of Kefir Samples

The hardness of kefirs prepared with milk protein powders was significantly different from that of the control sample (Table 2) and was statistically significantly influenced by the type of powder added as well as starter culture used for milk fermentation ($p = 0.001$ for both factors). In general, the lowest mean values of hardness were observed for kefir B samples, while the highest values were noted for kefir C samples. The significant influence of the starter culture used for milk fermentation on the hardness of the obtained kefir samples proved that with the addition of different milk protein powders, the hardness of kefirs differs. For kefir A, the highest hardness was noted in the samples prepared with buttermilk powder (1.98 N), while the samples prepared with sweet whey powder, whey permeate powder, and demineralized or non-demineralized whey powder had a lower hardness than the control samples (0.64, 0.73, 0.87, 1.07, and 1.35 N, respectively). However, it should be noted that different cultures resulted in different kefir texture properties on their own. This can be seen, for example, analyzing the results of the control samples. Kefir A control samples were characterized by significantly higher hardness (average 1.35 ± 0.04 N) than the control kefir samples B and C (average 0.65 ± 0.02 N and 0.96 ± 0.03 N, respectively).

Table 2. Physicochemical parameters of the kefir samples tested (mean and standard deviation calculated from six repetitions).

Kefir Samples with the Addition of Milk Powder	Protein Content (%)	WHC (%)	Hardness (N)	Adhesiveness (mJ)
Kefir A				
control	3.1 ± 0.1 ^f	63.9 ± 0.4 ^l	1.35 ± 0.04 ^f	7.60 ± 0.38 ^c
MPC-80	7.7 ± 0.1 ^b	92.9 ± 0.5 ^f	1.54 ± 0.05 ^d	6.40 ± 0.32 ^d
WPC-70	7.1 ± 0.1 ^c	78.9 ± 0.4 ^j	1.72 ± 0.05 ^c	6.20 ± 0.31 ^d
rennet casein	8.3 ± 0.2 ^a	87.1 ± 0.5 ^h	1.38 ± 0.04 ^f	5.70 ± 0.29 ^e
sweet whey powder	3.3 ± 0.1 ^f	95.6 ± 0.5 ^e	0.64 ± 0.02 ⁱ	1.50 ± 0.08 ⁱ
whey permeate powder	3.2 ± 0.2 ^f	93.0 ± 0.5 ^f	0.73 ± 0.02 ^j	1.00 ± 0.05 ⁱ
whey powder (demineralized)	7.7 ± 0.1 ^b	96.2 ± 0.5 ^d	0.87 ± 0.03 ^h	1.70 ± 0.09 ^h
whey powder (non-demineralized)	6.8 ± 0.1 ^d	95.7 ± 0.5 ^{d,e}	1.07 ± 0.03 ^g	1.90 ± 0.10 ^g
buttermilk powder	3.6 ± 0.1 ^e	98.2 ± 0.5 ^b	1.98 ± 0.06 ^b	7.90 ± 0.40 ^c
Kefir B				
control	3.1 ± 0.1 ^f	66.1 ± 0.4 ^l	0.65 ± 0.02 ^{aj}	1.60 ± 0.08 ^h
MPC-80	7.7 ± 0.1 ^b	93.6 ± 0.5 ^f	1.76 ± 0.05 ^c	10.70 ± 0.54 ^a
WPC-70	7.1 ± 0.1 ^c	76.9 ± 0.4 ^j	0.64 ± 0.02 ^j	0.50 ± 0.03 ^j
rennet casein	8.3 ± 0.1 ^a	86.9 ± 0.5 ^h	1.46 ± 0.04 ^e	7.60 ± 0.38 ^c
sweet whey powder	3.2 ± 0.2 ^f	96.0 ± 0.5 ^d	0.61 ± 0.02 ^j	2.20 ± 0.11 ^g
whey permeate powder	3.2 ± 0.2 ^f	89.4 ± 0.5 ^g	0.60 ± 0.02 ^j	2.20 ± 0.11 ^g
whey powder (demineralized)	7.7 ± 0.1 ^b	96.7 ± 0.5 ^d	0.88 ± 0.03 ^{h,i}	3.60 ± 0.18 ^f
whey powder (non-demineralized)	6.8 ± 0.1 ^d	97.4 ± 0.5 ^c	0.91 ± 0.03 ^h	3.10 ± 0.16 ^f
buttermilk powder	3.6 ± 0.1 ^e	99.0 ± 0.5 ^a	1.43 ± 0.04 ^e	8.70 ± 0.44 ^b
Kefir C				
control	3.1 ± 0.1 ^f	60.6 ± 0.3 ^l	0.96 ± 0.03 ^h	3.00 ± 0.15 ^f
MPC-80	7.7 ± 0.2 ^b	95.3 ± 0.5 ^e	1.62 ± 0.05 ^a	7.20 ± 0.36 ^{c,d}
WPC-70	7.1 ± 0.1 ^c	77.0 ± 0.4 ^j	2.44 ± 0.07 ^d	3.00 ± 0.15 ^f
rennet casein	8.3 ± 0.2 ^a	76.6 ± 0.4 ^k	1.32 ± 0.04 ^f	8.20 ± 0.41 ^b
sweet whey powder	3.3 ± 0.1 ^f	92.9 ± 0.5 ^f	0.81 ± 0.02 ⁱ	1.60 ± 0.08 ^h
whey permeate powder	3.2 ± 0.2 ^f	83.6 ± 0.5 ⁱ	0.81 ± 0.02 ⁱ	1.10 ± 0.06 ⁱ
whey powder (demineralized)	7.7 ± 0.1 ^b	93.2 ± 0.5 ^f	1.18 ± 0.04 ^g	1.90 ± 0.10 ^g
whey powder (non-demineralized)	6.8 ± 0.1 ^d	94.8 ± 0.5 ^e	1.34 ± 0.04 ^f	2.70 ± 0.14 ^g
buttermilk powder	3.6 ± 0.1 ^e	97.9 ± 0.5 ^c	2.16 ± 0.06 ^a	7.20 ± 0.36 ^{c,d}

^{a–l} the same letter indices within the same column do not mean statistically significant differences at the significance level of 0.05.

In the case of kefir B, the highest hardness was found for samples prepared with MPC-80 powder (1.17 N), while the lowest for control samples (0.65 N) and samples prepared with WPC-70 powder (0.64 N), sweet whey powder (0.61 N), and whey permeate powder (0.60 N). For kefir C, the highest hardness values were recorded for the samples prepared with WPC-70 (2.44 N) and buttermilk powder (2.16 N), and the lowest for samples containing sweet whey powder (0.81 N) or whey permeate powder (0.81 N). It follows that in each case, kefir prepared with sweet whey powder or whey permeate powder had the lowest hardness values, which were comparable to the control samples or even lower than them. The hardness value did not depend on the pH of the kefir samples ($p = 0.4745$). Of course, it should be noted that this could be more accurately determined by adjusting the acidity of the same samples to different pH values. This needs to be explored further in future.

The adhesiveness of kefirs prepared with milk protein powders differed statistically significantly from each other and depended both on the type of the added milk protein powder and starter culture used ($p = 0.001$ in both cases). It should be noted that different cultures resulted in different adhesiveness value of kefirs. This can be seen, for example, analyzing the results of the control samples. Kefir A control samples were characterized by significantly higher adhesiveness (average 7.60 ± 0.38 mJ) than the control kefir samples B and C (average 1.60 ± 0.08 mJ and 3.00 ± 0.15 mJ, respectively). Further analysis of samples containing the addition of various milk protein preparations allows to notice other dependencies, considering the total effect of the use of different stack cultures and the addition of different milk proteins. Kefir A and B samples had on average higher

adhesiveness (mean 4.43 and 4.47 mJ, respectively) than kefir C samples (mean 3.99 mJ). However, in the case of kefir A, only samples prepared with buttermilk powder had a statistically significantly higher adhesiveness (mean 7.900 mJ) than the control samples (mean 7.600 mJ), while the remaining samples had lower values than the control (in a range of 1.000–6.400 mJ) (Table 2). In the case of kefir B, only samples containing rennet casein powder had lower adhesiveness values (mean 0.500 mJ) than the control samples (mean 1.600 mJ), while the remaining samples had higher values than the control (in a range of 2.200–10.700 mJ). In turn, among kefir C samples, those prepared with WPC-70, sweet whey powder, whey permeate powder, and demineralized or non-demineralized whey powder had lower or the same adhesiveness values as the control samples (mean 3.000 mJ). On the other hand, kefir C samples containing rennet casein powder had the highest values of adhesiveness. It was observed that the adhesiveness value did not depend on the pH of the kefir samples ($p = 0.5049$).

3.4. Water-Holding Capacity of Kefir Samples

Total protein content influenced the WHC of kefir samples. All samples containing milk protein powders showed a higher WHC than the control kefir samples. More interestingly, the samples that had buttermilk powder had the highest WHC value (98.2%, 99.0%, and 97.9% for kefir A, B, and C samples, respectively). Statistical analysis showed that WHC significantly depends not only on the type of milk protein powder but also on the starter culture used ($p = 0.001$ for each of these factors). In general, we noted higher WHC values for kefir A (mean 89.1%) and B samples (mean 89.1%) than kefir C samples (85.8%). We also checked whether the WHC value of the analyzed kefir samples depended on pH, but our statistical analysis showed no such relationship ($p = 0.3669$).

3.5. Protein Content in Kefir Samples

We analyzed whether the hardness of kefir samples as well as their adhesiveness could be influenced by the total protein content but found no statistically significant correlations. However, we observed that the total protein content was influenced by the type of powdered milk protein preparation ($p = 0.0001$), although no significant influence was noted for the type of starter culture used ($p = 0.6522$). The highest amount of protein was contained in kefir samples prepared with rennet casein powder (mean 8.3%, Table 2) which is rich in casein proteins. Milk protein powders rich in whey protein (WPC-70, sweet whey powder, whey permeate powder, demineralized or non-demineralized whey powder) increased the level of total protein in kefir samples to 3.2–7.7%, depending on the type of powder used. In addition, in our experiments, we used two powders containing milk protein in general: buttermilk powder and MPC-80. In kefir samples prepared with these powders, we noted an increase in the total milk protein content to an average of 3.6% and 7.7%, respectively.

3.6. Protein Digestibility in Kefir Samples

Table 3 shows the content of available lysine in kefir samples prepared with the tested milk protein powders and fermented with various kefir starters (the results were expressed in terms of 1 g of protein and 100 g of kefir). In all samples, the content of available lysine was at least equal to or greater than that found in the control samples. It can be concluded that the content of available lysine in 1 g of protein differed statistically significantly depending on the type of milk protein preparation added and the kefir starter culture used. Based on the type of starter culture used, the kefir samples were divided into three homogeneous groups. Statistically, the highest content of available lysine was found in the kefirs obtained using starter culture C.

Table 3. Digestibility test results of the tested kefir (mean and standard deviation calculated from six repetitions).

Kefir Samples with the Addition of Milk Powder	The Concentration of Available Lysine (mg)		The Concentration Of Available Glycine (mg)	
	In 100 g of Protein	In 100 g of Product	In 100 g of Protein	In 100 g of Product
Kefir A				
control	124 ± 5 ^g	384 ± 16 ^j	28 ± 1 ^a	85 ± 4 ^{d,e}
MPC-80	136 ± 1 ^f	1045 ± 19 ^e	12 ± 1 ^d	90 ± 4 ^{d,e}
WPC-70	171 ± 19 ^b	1214 ± 15 ^c	13 ± 1 ^d	90 ± 5 ^{d,e}
rennet casein	137 ± 2 ^f	1139 ± 19 ^d	13 ± 2 ^d	110 ± 5 ^c
sweet whey powder	155 ± 15 ^d	512 ± 19 ⁱ	27 ± 2 ^{a,b}	91 ± 5 ^{d,e}
whey permeate powder	133 ± 2 ^f	425 ± 17 ^j	26 ± 3 ^b	84 ± 1 ^e
whey powder (demineralized)	121 ± 1 ^g	929 ± 14 ^f	11 ± 1 ^d	84 ± 4 ^e
whey powder (non-demineralized)	123 ± 2 ^g	834 ± 16 ^g	27 ± 1 ^{a,b}	180 ± 9 ^{a,b}
buttermilk powder	173 ± 2 ^b	622 ± 18 ^h	27 ± 2 ^{a,b}	96 ± 5 ^d
Kefir B				
control	126 ± 1 ^g	391 ± 10 ^j	27 ± 1 ^{a,b}	83 ± 4 ^e
MPC-80	163 ± 14 ^c	1251 ± 12 ^c	12 ± 1 ^d	89 ± 4 ^{d,e}
WPC-70	121 ± 1 ^g	857 ± 14 ^g	11 ± 1 ^d	78 ± 4 ^e
rennet casein	133 ± 3 ^f	1111 ± 24 ^d	13 ± 1 ^d	112 ± 6 ^c
sweet whey powder	145 ± 5 ^e	480 ± 15 ^{i,j}	29 ± 2 ^a	96 ± 5 ^d
whey permeate powder	131 ± 7 ^{f,g}	419 ± 23 ^j	25 ± 2 ^{b,c}	79 ± 4 ^e
whey powder (demineralized)	136 ± 5 ^f	1049 ± 17 ^e	11 ± 1 ^d	83 ± 4 ^e
whey powder (non-demineralized)	133 ± 3 ^f	907 ± 20 ^f	26 ± 2 ^b	176 ± 9 ^b
buttermilk powder	137 ± 2 ^f	494 ± 16 ^{i,j}	24 ± 2 ^c	86 ± 4 ^{d,e}
Kefir C				
control	130 ± 1 ^{f,g}	402 ± 14 ^j	26 ± 2 ^b	80 ± 4 ^e
MPC-80	184 ± 9 ^a	1420 ± 17 ^a	10 ± 1 ^d	80 ± 4 ^e
WPC-70	185 ± 10 ^a	1311 ± 14 ^b	12 ± 1 ^d	83 ± 4 ^e
rennet casein	162 ± 7 ^c	1349 ± 19 ^b	13 ± 1 ^d	105 ± 5 ^{c,d}
sweet whey powder	151 ± 5 ^d	500 ± 15 ⁱ	30 ± 2 ^a	98 ± 5 ^d
whey permeate powder	141 ± 1 ^{e,f}	450 ± 12 ^j	25 ± 2 ^{b,c}	79 ± 4 ^e
whey powder (demineralized)	123 ± 3 ^g	950 ± 22 ^f	11 ± 1 ^d	83 ± 4 ^e
whey powder (non-demineralized)	133 ± 3 ^f	907 ± 20 ^f	28 ± 2 ^a	193 ± 10 ^a
buttermilk powder	143 ± 3 ^e	516 ± 10 ⁱ	24 ± 2 ^c	88 ± 4 ^d

^{a-j} the same letter indices within the same column do not mean statistically significant differences at the significance level of 0.05.

Considering the type of milk protein preparation added, the highest content of available lysine was recorded in the kefir obtained from milk with WPC-70 or MPC-80 fermented with starter C (184 ± 9 and 185 ± 10 mg/g of protein, respectively), as well as in the samples obtained from milk with WPC-70 or buttermilk powder fermented with starter A (171 ± 19 and 173 ± 2 mg/g of protein, respectively). If the content of available lysine was expressed as the amount in 100 g of kefir samples, each added milk protein preparation increased the content, although in some cases this increase was found to be statistically insignificant. No relationship was found between the amount of available lysine and the total protein content. It can therefore be concluded that the available lysine concentration is influenced by the additive and type of milk protein preparation, as well as the proteolytic activity of the starter used for fermentation.

Available glycine content is an expression of protein digestibility, which was determined in this study by using the second method (Table 3). The digestibility of proteins in kefir samples enriched with various preparations of milk proteins, determined using this method, differed significantly from the results obtained with the first method, described above. It should be noted that the content of available glycine was statistically significantly influenced by the type of milk protein powder used ($p = 0.0001$), but not by the type of kefir starter used ($p = 0.1198$) or the pH of the kefir samples ($p = 0.7791$). The highest available glycine content in 100 g of kefir samples was found in the samples prepared with whey powder (non-demineralized) (180 ± 9, 176 ± 9, and 193 ± 10 mg/100 g of the product, respectively) as well as the samples prepared with rennet casein (110 ± 5, 112 ± 6, and 105 ± 5 mg/100 g of the product, respectively). In other cases, the content of available

glycine was comparable to that in control samples or was about 10% higher. Expressing the content of available glycine in 1 g of protein showed that the addition of milk protein preparations did not increase or reduce the content compared to the control samples. Only for kefir C obtained from milk with the addition of sweet whey powder was the content found to be significantly higher than the control samples.

4. Discussion

In our experiments, the counts of both *Lactococcus* spp. and lactobacilli were detected at levels above 7 and 6 log(CFU/mL) in the kefir samples, respectively (Table 1). The obtained results confirm those shown by other researchers [3,23–26]. For instance, Montanuci et al. [27] reported that the addition of inulin did not affect the counts of *Lactococcus* spp. in the kefir samples. Magra et al. [28] reported that the number of *Lactococcus* spp. in kefir samples reduced due to the high acidity of kefir. In our study, we did not observe the lactococci population to be dependent on the pH of kefirs, at least at the stage of milk fermentation. As mentioned in the Introduction section, many researchers have shown that, compared to SMP, WPC significantly stimulates the growth of several strains of lactic acid bacteria [4]. However, we did not notice such an effect in the analyzed kefir samples for lactococci and lactobacilli.

Usually, fermentation is carried out until the kefir reaches a pH of 4.4–4.6, which takes from 16 to 24 h [2,26,29,30]. García Fontán et al. [23] described the 24-h fermentation process as the most effective for kefir production in the context of pH changes. In our experiments, we fermented milk for 24 h to analyze how the addition of different milk protein powders could affect the pH changes (Table 1). The typical pH of commercial kefirs ranges from 4.3 to 4.4 [2], which are comparable to the values measured in our research. However, it should be noted that our kefir samples contained added milk protein powders, and as we know, milk proteins have some buffering capacity and thus reduce the observed pH reduction rate [4,29,31]. This is probably the reason for the higher pH values (in the range of 4.4–5.2) observed in the samples prepared with milk protein powders compared to the control sample (mean pH 4.5).

The acid gel formed during the fermentation of milk with lactic acid bacteria is a three-dimensional protein matrix in which some other components of the milk base, including polysaccharides produced by the microflora of starter cultures, are trapped. Therefore, when analyzing the texture parameters of kefir prepared using lactic acid bacteria starters, it could be expected that the nature and relative proportions of different proteins play a significant role in determining the texture of the final product, which has already been shown by many researchers [32,33]. However, studies on the textural properties of kefir prepared with milk proteins are not numerous. The protein content in milk dedicated to the production of kefir can be increased by adding WPC or MPC, or possibly retentate, and caseinate. The use of such additives will have different effects on the protein composition of the dairy base, as the ratio of protein to total dry weight and that of whey proteins to caseins will change, which has a significant impact on the texture of the product. The effect of milk base, starter cultures, and process parameters on texture of fermented milk, based on the example of yogurt, were discussed in detail in reference [33]. The exact influence of these proportions on the texture of kefirs is not easy to estimate, given the additional influence of the starter culture. Very few studies have examined the effects of the ratio of whey protein to casein on the texture of fermented milk beverages without changing the proportion of total protein in the dry matter of processing milk [33]. In our research, we added the same amount of milk protein powders to the processing milk, but these powders were characterized by different protein content and different composition of milk proteins, due to which the kefir samples obtained differed in the total protein content and the proportion of proteins from whey to casein as well as the proportion of total whey proteins. Milk powders or milk protein concentrate powders addition, which are usually applied in fermented milks production, gives the proportion of proteins to total solid content and the proportion of whey protein to casein content close to that of native milk.

In contrast, whey protein powders and whey protein concentrates are an important source of whey proteins, with a variable content of other dairy ingredients (e.g., mineral salts) depending on the degree of purification of the preparations (e.g., demineralized whey powders contain about half as much ash than non-demineralized whey powders whey powders and salt content can alter the results). Moreover, it must be remembered that the effect of the addition of a milk base on milk proteins is strongly dependent on the heat treatment of milk and the added milk powder [33]. For example, rennet casein powder contains denatured casein proteins, so the role of these proteins in acid clot formation is not significant. This is probably why the hardness as well as the adhesiveness of the kefir samples prepared with milk protein powders depended on the type of milk protein powder added. These factors, individually and in combination with the effects of using different starter cultures, led to a difficult interpretation of the results obtained by us. We have also shown that the tested texture parameters were strongly influenced by the type of kefir starter culture used. It should be assumed that the decisive factor in this case is the qualitative and quantitative microorganism composition of the starter culture. Many scientists have proven in their research that the microflora can produce exopolysaccharides (EPS), which significantly affects the texture of fermented milk [34].

The WHC of kefir refers to its tendency to retain water or its resistance to phase separation. However, it should be remembered that the centrifugation test used in our experiments may give results irrelevant for syneresis under normal storage conditions. This is because the mechanical stability of the protein network is investigated more under the influence of G forces than under normal storage conditions [33]. Many authors [34–37] reported that the higher the milk protein content, the higher the WHC. We also showed in this study that the addition of milk powders increased the WHC value of kefir. Wachter-Rodarte et al. [34] showed that the use of microflora influenced the structural parameters and balanced the effect of dry matter enrichment on the WHC value. However, the type of protein added to the milk plays an important role. Increasing the concentration of casein enhances the interaction between its molecules. In this case, the casein chains become shorter, the pore size in the gel becomes smaller, and the gel density increases. In addition, an increased concentration of whey proteins added leads to an increase in the WHC value [35,36,38,39]. This was also confirmed by Guzman-Gonzalez et al. [40,41] with the addition of WPC to yogurt. Furthermore, Aguilera and Kessler [35] and Harwalkar and Kalab [36] confirmed that yogurt pH has a significant effect on WHC.

The available lysine content is a measure of the nutritional value of the milk protein [42]. The content of lysine in food and its availability are extremely important because it is an essential amino acid that is often found in relatively small amounts in diet [43–45]. Irigoyen et al. [24] determined the content of available lysine in kefir samples obtained in laboratory conditions at a level of 41 mg/100 g of product; for comparison, in 100 g of commercial yogurt, there was an average of 113 mg of available lysine (similar to the content achieved in our experiments). The decrease in the available lysine concentration in milk may be due to thermal treatment (e.g., pasteurization) [42]. Lysine is susceptible to modifications occurring during the processing or long-term storage of food samples. For example, it can react with lactose or other substances and form nutritionally inaccessible (nonreactive) derivatives [44,45]. Hence, the addition of milk protein preparations seems an interesting solution to increase the level of available lysine. Our experiments showed that this can be achieved in kefir with the use of WPC-80 or WPC-70. It is also important to select an appropriate starter culture for the fermentation of milk into kefir because the enzymatic activity of the microflora present in the starter determines the amount of lysine in the final product [46,47]. Furthermore, Simsek et al. [48] showed that fermentation had no significant effect on kefir proteins obtained from inulin-enriched goat milk. Using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, researchers found that raw milk, heated milk, and kefir showed comparable protein profiles.

We can, however, suggest that the effects of fermentation on the quality of milk proteins depend on the method used for determining their digestibility. Kopf-Bolan et al. [49]

claimed that fermentation improves digestibility in products due to partial degradation of casein proteins [49]. It should be noted that these authors used colloidal Coomassie blue staining and identified proteins by liquid chromatography–mass spectrometry after trypsin digestion. Our experiments on protein digestibility using two assay methods showed different results. From the results of the analytical method used, we can conclude that the starter culture has a significant effect on the availability of selected amino acids (available lysine and glycine), and thus the digestibility of milk proteins, as well as that milk protein preparations have a different influence on the quality of protein in kefir.

5. Conclusions

The rules of kefir production vary worldwide, while the recipe and technological parameters significantly affect the quality features of the final product. The quality features are one of factors that influence customer's acceptance on product, including fermented milks such as kefir. Our study showed that various milk protein preparations can be successfully used as additives for milk intended for kefir fermentation. Although the influence of these additives on the quality parameters tested by us was significant, it was not clear. The addition of various forms of milk proteins, including MPC, WPC, demineralized or non-demineralized whey, sweet whey, whey permeate, rennet casein, and buttermilk powders, had an impact on the population of lactic acid bacteria in the obtained kefirs, and therefore on the final pH of the kefirs. However, the type of starter culture used to obtain these kefirs also influenced the microflora, as well as the final total protein content in kefirs resulting from the addition of milk protein preparations with different amounts of proteins. Depending on their type, and the starter culture used, milk protein preparations increased the WHC of kefirs, as well as changed their hardness and adhesiveness. In most cases, the investigated quality parameters of kefirs differed depending on both the type of milk protein powder added and the starter culture used. This means that when using the additives of milk protein preparations tested by us in industrial production, the starter culture should be carefully selected to obtain kefir with the desired and expected quality parameters, including hardness.

Enrichment of milk with milk protein preparations resulted in a change in the digestibility of proteins in kefir. However, proper selection of the method is important to investigate the digestibility of proteins in the final kefir. We compared two methods applied for determining the digestibility of milk proteins using *in vitro* enzymatic digestion. The principle of the first method is to determine the content of free lysine which in turn is used to assess the digestibility of protein. The principle of the second method consists in subjecting the test samples to a two-step enzymatic hydrolysis process followed by the determination of the concentration of available glycine. An increase in the amount of available lysine as well as available glycine was observed. However, kefirs prepared with different additives in the milk protein preparations exhibited different levels of these amino acids. In the case of the availability of lysine, we observed differences between kefirs obtained using different starter cultures. Testing the digestibility of kefir proteins by determining the available glycine *in vitro* showed different results than those observed with the determination of the available lysine *in vitro*. It turned out that the digestibility of milk proteins tested using the enzymatic digestion method *in vitro* indicated the effect of the addition of milk protein preparations and the type of starter culture used on the protein quality of kefirs.

Author Contributions: Conceptualization, M.Z. and R.H.; methodology, M.Z. and A.C.; formal analysis, M.Z., R.H., and A.C.; investigation, M.Z., R.H., and A.C.; data curation, M.Z.; writing—original draft preparation, M.Z., R.H., and A.C.; writing—review and editing, M.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by a grant from Warsaw University of Life Sciences—WULS—SGGW.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data sharing not applicable.

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

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